Alternative Route for Glyoxylate Consumption during Growth on Two-Carbon Compounds by *Methylobacterium extorquens* AM1[▽]

Yoko Okubo,¹ Song Yang,¹ Ludmila Chistoserdova,¹ and Mary E. Lidstrom^{1,2*}

Department of Chemical Engineering¹ and Department of Microbiology,² University of Washington, Seattle, Washington 98195-2180

Received 29 August 2009/Accepted 17 January 2010

Methylobacterium extorquens AM1 is a facultative methylotroph capable of growth on both single-carbon and multicarbon compounds. Mutants defective in a pathway involved in converting acetyl-coenzyme A (CoA) to glyoxylate (the ethylmalonyl-CoA pathway) are unable to grow on both C₁ and C₂ compounds, showing that both modes of growth have this pathway in common. However, growth on C₂ compounds via the ethylmalonyl-CoA pathway should require glyoxylate consumption via malate synthase, but a mutant lacking malyl-CoA/β-methylmalyl-CoA lyase activity (MclA1) that is assumed to be responsible for malate synthase activity still grows on C₂ compounds. Since glyoxylate is toxic to this bacterium, it seemed likely that a system is in place to keep it from accumulating. In this study, we have addressed this question and have shown by microarray analysis, mutant analysis, metabolite measurements, and ¹³C-labeling experiments that *M. extorquens* AM1 contains an additional malyl-CoA/β-methylmalyl-CoA lyase (MclA2) that appears to take part in glyoxylate metabolism during growth on C₂ compounds. In addition, an alternative pathway appears to be responsible for consuming part of the glyoxylate, converting it to glycine, methylene-H₄F, and serine. Mutants lacking either pathway have a partial defect for growth on ethylamine, while mutants lacking both pathways are unable to grow appreciably on ethylamine. Our results suggest that the malate synthase reaction is a bottleneck for growth on C₂ compounds by this bacterium, which is partially alleviated by this alternative route for glyoxylate consumption. This strategy of multiple enzymes/pathways for the consumption of a toxic intermediate reflects the metabolic versatility of this facultative methylotroph and is a model for other metabolic networks involving high flux through toxic intermediates.

Methylobacterium extorquens AM1 grows on one-carbon (C₁) compounds using the serine cycle for assimilation (25). This metabolism requires the conversion of acetyl-coenzyme A (CoA) to glyoxylate, which occurs via a novel pathway in which acetyl-CoA is converted to methylsuccinyl-CoA via acetoacetyl-CoA, β-hydroxybutyryl-CoA, and ethylmalonyl-CoA (30–33). Recently, the steps involved in the conversion of methylsuccinyl-CoA to glyoxylate have been elucidated, and the pathway has been termed the ethylmalonyl-CoA (EMC) pathway (1, 19, 20, 40). Careful labeling measurements coupled to measurements of intermediates has confirmed that, during the growth of *M. extorquens* AM1 on methanol, methylsuccinyl-CoA is converted to glyoxylate and propionyl-CoA via mesaconyl-CoA and β-methylmalyl-CoA (40).

This finding has raised questions regarding how *M. extorquens* AM1 grows on two-carbon (C₂) compounds. The pathway involved in the conversion of acetyl-CoA to glyoxylate is known to operate during growth on both C₁ and C₂ compounds, as mutants in genes involved in this conversion are unable to grow on either C₁ or C₂ compounds, and in both cases they are rescued by glyoxylate (11, 15–17, 44). If glyoxylate is produced as an end product of this pathway during C₂ growth, then it must be converted to an intermediate of central metabolism, which has been proposed to involve a malate synthase activity (2, 14–17) (Fig. 1). In *M. extorquens* AM1, the apparent malate synthase activity is carried out in two steps,

first by converting acetyl-CoA and glyoxylate to malyl-CoA by malyl-CoA lyase and then by converting malyl-CoA to malate by malyl-CoA hydrolase (Fig. 1) (14). However, a mutant (PCT57) defective in malyl-CoA lyase (MclA1) (22), which contains no detectable malate synthase activity during growth on methanol, is able to grow on C₂ compounds (43).

Clearly, the finding that glyoxylate is generated as a direct product of the EMC pathway presents a conundrum. Apparently acetyl-CoA is converted to glyoxylate via this pathway, but *M. extorquens* AM1 lacking malate synthase is able to grow on C₂ compounds. Another apparent conundrum involving the malyl-CoA lyase (*mclA1*) mutant is that the EMC pathway requires an enzyme that carries out β-methylmalyl-CoA cleavage, a reaction that homologs of MclA1 are known to carry out (38). The MclA1 enzyme has been purified from *M. extorquens* AM1 and shown to have activity with glyoxylate and propionyl-CoA (27), which would produce β-methylmalyl-CoA. These results have led to the suggestion that MclA homologs actually are malyl-CoA/β-methylmalyl-CoA lyases (38). Since the *mclA1* mutant does not contain detectable malyl-CoA lyase activity, and by inference has correspondingly low β-methylmalyl-CoA lyase activity, it was not clear how *M. extorquens* AM1 could convert acetyl-CoA to propionyl-CoA and glyoxylate via the EMC pathway in the *mclA1* mutant.

The purpose of this study was to solve these conundrums and determine how mutants of *M. extorquens* AM1 grow on C₂ compounds in the absence of malyl-CoA/β-methylmalyl-CoA lyase or malate synthase activity. Our results show (i) that the known homolog of MclA1 (MclA2) appears to be capable of supporting both β-methylmalyl-CoA cleavage and condensation between glyoxylate and acetyl-CoA in the *mclA1* mutant,

* Corresponding author. Mailing address: Office of Research, University of Washington, Box 351202, Seattle, WA 98195. Phone: (206) 616-5282. Fax: (206) 616-5721. E-mail: lidstrom@u.washington.edu.

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FIG. 1. Enzymes and genes involved in the ethylmalonyl-CoA pathway. The colors of gene names denote a change in gene expression from microarray results comparing wild-type cells grown on ethylamine to those grown on succinate: dark red, >3-fold increase; light red, 1.5- to 3-fold increase; black, no significant change (1.49-fold increase to 1.49-fold decrease); light green, 1.5- to 3-fold decrease; dark green, >3-fold decrease. Parentheses denote a predicted function not confirmed by the mutant phenotype. See Table 1 for enzyme names.

and (ii) that an alternative route for glyoxylate consumption occurs in this bacterium, in which it is converted to intermediates of central metabolism via a part of the serine cycle coupled with the glycine cleavage system.

MATERIALS AND METHODS

Cultivation. *M. extorquens* AM1 (39) was grown at 28°C in batch culture using a mineral salts medium described previously (42). Ethylamine was used at 20 mM. Antibiotic concentrations (in $\mu\text{g ml}^{-1}$) were the following: tetracycline, 10; kanamycin, 50; rifampin, 50.

Microarray analysis. Cultures for microarray analysis were grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.8, harvested, and used for RNA isolation. RNA isolation, purification, and digestion with DNase I was carried out as previously described (42). cDNA production and labeling, hybridization, and array scanning were carried out by MOgene (St. Louis, MO), using the DNase I-digested RNA and custom Agilent 60-mer microarrays. RNA from ethylamine-grown cells was compared to RNA from succinate-grown cells, and four replicates were carried out. Microarray data are available at the NCBI Gene Expression Omnibus (18) and are accessible through GEO Series accession number GSE20365 at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20365>. The complete genome sequence of *M. extorquens* AM1 is available in GenBank (50). Data were analyzed as previously described (42) using standard deviation instead of *P* value.

Enzyme activities. Two hundred-milliliter cultures for enzyme activities were grown to an OD_{600} of 0.6 to 1.3, washed, and resuspended in 3 ml of 0.2 M Tris-HCl (pH 8.0) buffer. Enzyme activities were determined in crude extracts obtained by passing the cell suspension through a French pressure cell at 1.2×10^8 Pa, followed by centrifugation for 10 min at approximately $21,000 \times g$, ultracentrifugation for 1 h at $90,000 \times g$, and the concentration of the soluble fraction by an Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, MA). Measurements were done at 30°C in a total volume of 0.9 ml. Malate synthase was measured in the direction of malate synthesis as described previously (24).

Mutant generation. An insertion mutation was generated in *gcvP* by inserting the kanamycin (Km) resistance gene using the pAYC61 suicide vector, essentially as previously described (6). To generate mutants in *mclA2* and *sga*, the respective genes were replaced in the chromosome with the Km resistance gene using pCM184 (36), and unmarked versions of these mutants were generated by expressing *cre* recombinase as previously described (36). The double *mclA1 mclA2*, *sga mclA1*, and *sga mclA2* mutants were generated by introducing the *mclA1* mutation into the unmarked *mclA2* and *sga* mutant, using the previously described donor construct (12) and by introducing the *mclA2* mutation into the unmarked *sga* mutant, respectively. All mutants were confirmed by diagnostic PCR.

Metabolite determinations. (i) **Extracellular sample preparation.** The samples for the determination of extracellular metabolites were removed from each flask when the cells had reached an OD_{600} of 0.5 ± 0.15 . Ten ml of the cell culture was filtered using a Millipore membrane (0.22 μm) and then lyophilized at approximately -45°C in a FreeZone 4.5-liter Benchtop Freeze Dry System lyophilizer (Labconco, MO). One ml of double-distilled H_2O (ddH_2O) containing 150 μl of 6N HCl was added to the dried culture medium and then was applied to a C_{18} solid-phase extraction (SPE) column (1 ml; Restek, Bellefonte, PA) to remove the salts. The eluate from the SPE column was collected into a 2-ml glass vial and dried in a vacuum centrifuge (Centrivap Concentrator System; Labconco, MO). The complete dried sample that was analyzed by gas chromatography (GC) $\times$ GC-time-of-flight mass spectrometry (TOFMS) was further derivatized in two steps (26). First, keto groups were methoximated by adding 50 μl methoxyamine solution (25 mg ml^{-1} methoxyamine hydrochloride in pyridine) and incubation at 60°C for 30 min. In the second step, trimethylsilylation was performed by adding 50 μl trimethylsilyl (TMS) reagent (*N,O*-bis(trimethylsilyl)-trifluoroacetamide-trimethylchlorosilane 99:1) and heating at 60°C for 60 min.

(ii) **Intracellular sample preparation.** Six ml of the cell culture at an OD of 0.5 ± 0.15 was carefully and rapidly pipetted into the center of 25 ml of the quenching solution [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered (70 mM, pH 6.8) aqueous 60% methanol (vol/vol) solution (-40°C)]. The quenched biomass was precipitated in a refrigerated centrifuge (6 min, 10,000 rpm, -20°C ; Dupont Sorvall RC5B, Waltham, MA). The supernatant was removed, and the cell pellets were resuspended in 5 ml of the same methanol solution and again centrifuged for 6 min at 10,000 rpm.

The intracellular metabolites were extracted by using a previously reported protocol (52). D6-salicylic acid was added as the internal standard to correct for variation due to sample extraction and injection. One ml of boiling ethanol solution (75% [vol/vol] ethanol-water) was added to a given cell pellet and incubated at 100°C for 5 min. The extracted cell suspension was cooled on ice for 3 min, and the cell debris was removed by centrifugation at 5,000 rpm for 5 min. The cell-free metabolite extract was centrifuged again at 14,000 rpm for 8 min. The supernatant was transferred into a 2-ml glass vial and dried in a vacuum

centrifuge (CentriVap Concentrator System; Labconco, MO) to complete dryness. The dried sample was derivatized further for GC × GC-TOFMS analysis in two steps as described above.

(iii) **GC × GC-TOFMS measurement.** GC × GC-TOFMS experiments were performed using a LECO Pegasus III time-of-flight mass spectrometer with the 4D upgrade (LECO Corp., St. Joseph, MI) as described previously (52). Differences between samples were assessed by a laboratory-written software methodology, referred to as the signal ratio method (S-ratio method) (41).

(iv) **¹³C labeling.** The incubation of cells with uniformly labeled glyoxylate (1,2-¹³C; 99%; Cambridge Isotope Laboratories, Andover, MA) was carried out at 28°C in a 25-ml glass tube containing 9.5 μl of 0.532 M labeled glyoxylate. Five ml of an ethylamine-grown culture was rapidly pipetted into the tube and vortexed for about 1 s. The final labeled glyoxylate concentration was 1 mM. After various incubation times, the quenching solution noted above was added, and amino acids and organic acids were extracted as described above and further analyzed by liquid chromatography (LC)-MS/MS.

(v) **LC-MS/MS measurement.** LC-MS/MS experiments were carried out on a Waters (Milford, MA) LC-MS system consisting of a 1,525-μ binary high-pressure liquid chromatography (HPLC) pump with a 2777C autosampler coupled to a Quattro Micro API triple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom). The mass spectrometer was operated in both the positive and negative electrospray ionization modes and scanned using multiple reaction monitoring (MRM) to determine mass (M) isotopomer distribution patterns. The MRM pairs (parent→daughter ions) of ¹²C and ¹³C metabolites are the following: glycine (M + 0, 76→30; M + 1, 77→30 and 77→31; M + 2, 78→31), serine (M + 0, 106→60; M + 1, 107→60 and 107→61; M + 2, 108→61 and 108→62; M + 3, 109→62), and malate (M + 0, 133→115; M + 1, 134→116; M + 2, 135→117; M + 3, 136→118; M + 4, 137→119). LC solvents for the pentafluorophenylpropyl-bonded silica column (Luna PFPP; 150 mm by 2 mm; 3 μm; Phenomenex, Torrance, CA) were the following: mobile phase A consisted of 0.1% formic acid in water, while mobile phase B was acetonitrile. The following linear gradient was used: 100% A for 8 min, 100 to 70% A for 7 min, 70 to 50% A for 1 min, 50% A for 5 min, 50 to 0% A for 1 min, 0% A for 4 min, 0 to 100% A for 1 min, and 100% A for 8 min. The total run time was 35 min at 0.20 ml/min. The mass isotopomer distributions were corrected for the natural isotope contribution by using a matrix-based method (21, 53) and calculated as the relative abundances of the different possible mass isotopomers of a metabolite.

Flux calculations. Fluxes through the EMC pathway and to glyoxylate required to support a specific doubling time were calculated by assuming cells are 47% carbon (dry weight) (23) and 50% protein (dry weight) (48), and that 40% of the carbon flux through the EMC pathway ends up as glyoxylate, 60% as propionyl-CoA based on the proportions shown in Fig. 1.

RESULTS

Microarray comparison of ethylamine- and succinate-grown cells. To provide global information on gene expression during growth on C₂ compounds, the microarray analysis of *M. extorquens* AM1 was carried out as described previously (42), comparing ethylamine-grown cells to succinate-grown cells (Table 1, Fig. 1). All of the known *mau* genes required for methylamine dehydrogenase, which carries out ethylamine oxidation during growth on ethylamine (6), were highly induced (8- to 52-fold). The genes involved in the portion of the EMC pathway for converting acetyl-CoA to β-hydroxybutyryl-CoA (*phaA*, *phaB*) showed little change. The genes involved in the portion of the EMC pathway for converting β-hydroxybutyryl-CoA to glyoxylate and propionyl-CoA were induced 2.1- to 2.5-fold (*croR*, *ccr*, *ecm*, *msd*), except for *mcd*, encoding the mesaconyl-CoA hydratase, for which expression was decreased slightly (1.5-fold decrease). The genes for converting propionyl-CoA to R-methylmalonyl-CoA (*pccA*, *pccB*) did not show a significant change, while three of the genes for converting R-methylmalonyl-CoA to succinyl-CoA were induced 1.6- to 2.4-fold (*mcmA*, *mcmB*, *meaD*). The genes for converting succinyl-CoA to malate (*sdhA*, *sdhC*, *sdhD*, *fum*) all showed either no significant change or decreased expression compared to

those converting to succinate. In keeping with its apparent nonessential role in C₂ growth, *mclA1* (formerly termed *mclA* [12]) showed slightly decreased expression (1.5-fold decrease), while a second Mcl homolog, termed *mclA2*, was induced 2.4-fold. In addition, a cluster of genes (MexAM1_META1p1550 to MexAM1_META1p1553) predicted to be involved in nitrogen metabolism via glutamate synthase was highly induced (4.5 to 18-fold; data not shown), as might be expected for growth on an amine. An additional gene of interest also was induced, predicted to encode acetyl-CoA synthase (MexAM1_META1p2531), which showed a 3.3-fold increase. Two others that were induced were predicted to be involved in acetaldehyde oxidation, MexAM1_META1p3652 (14-fold increase), which had homology to aldehyde dehydrogenases, and MexAM1_META1p3924 (3-fold increase), which had homology to alcohol oxidases (Table 1, Fig. 1). These results are in keeping with previous results showing that multiple acetaldehyde-oxidizing enzymes are present in this bacterium (51) and are consistent with an ethylamine utilization route via acetaldehyde, acetate, and then acetyl-CoA (Fig. 1).

Phenotypes of *mcl* mutants. A possible reason for the ability of the original malyl-CoA lyase mutant (PCT57) to grow on C₂ compounds is that it contained multiple mutations, one of which allowed C₂ growth, and/or that C₂-grown cells contained malate synthase activity, since activity in cells grown on C₂ compounds was not reported (43). We retested the phenotype of an *mclA1* insertion mutant that had been generated previously in our laboratory (12). This insertion mutant had no detectable malyl-CoA lyase activity in cells grown on succinate or succinate induced with methanol, and it was unable to grow on C₁ compounds (12). Although previous work on C₂ compounds in *M. extorquens* AM1 generally has involved ethanol as a substrate, in our hands, growth on both plates and in liquid culture is more robust with ethylamine, and our work has utilized that substrate. In either case, the doubling time is 10 to 12 h, as reported previously for ethanol (16). When *mclA1* mutants were streaked on ethylamine plates, colonies arose more slowly than they did with the wild type (Table 2). Growth curves in liquid medium confirmed a partial growth defect and showed that the *mclA1* mutant had a doubling time of about twice that of the wild type. No malate synthase activity was detected in this mutant grown on succinate, succinate induced with methanol, or ethylamine, although activity was present in the wild type under these conditions at levels of 16 to 35 nmol min⁻¹ mg protein⁻¹, which is similar to values previously reported (16, 46). In our hands, the detection limit of this assay was 1 to 2 nmol min⁻¹ mg protein⁻¹. As reported previously for PCT57 (43), the *mclA1* mutant was inhibited by glyoxylate at a lower concentration than that for the wild type. On plates, apparent second-site suppressor mutants grew up readily at the top of the streak, and care was necessary to grow liquid cultures that maintained the original phenotype. These apparent second-site suppressor mutants still were impaired for growth on C₂ compounds and grew on ethylamine with a doubling time between that of the original *mclA1* mutant and that of the wild type. Ethylamine-grown cultures of this more rapidly growing strain also did not contain detectable malate synthase activity.

As noted above, the *M. extorquens* AM1 genome (50) contains another gene with homology to *mclA1*, MexAM1_META1p4295,

TABLE 1. Microarray gene expression results for selected genes involved in C₁ and C₂ metabolism, comparing ethylamine-grown cells to succinate-grown cells

Gene category and no. ^a	Gene name	Description	Fold change ^b	Required for growth ^c			Reference(s)
				MeOH	MtNH ₂	EtNH ₂	
Methylamine dehydrogenase genes							
MexAM1_META1p2769	<i>mauF</i>	Essential for methylamine dehydrogenase activity	52		+	+	6
MexAM1_META1p2770	<i>mauB</i>	Methylamine dehydrogenase large subunit	42		+	+	6
MexAM1_META1p2771	<i>mauE</i>	Methylamine dehydrogenase small subunit maturation	32 (33%)		+	+	6
MexAM1_META1p2772	<i>mauD</i>	Methylamine dehydrogenase small subunit maturation	27 (39%)		+	+	6
MexAM1_META1p2773	<i>mauA</i>	Methylamine dehydrogenase small subunit	24 (31%)		+	+	6
MexAM1_META1p2774	<i>mauC</i>	Amicyanin	20 (37%)		+	+	6
MexAM1_META1p2775	<i>mauJ</i>	Essential for methylamine dehydrogenase activity	17 (40%)		+	+	6
MexAM1_META1p2776	<i>mauG</i>	Essential for methylamine dehydrogenase activity	9 (43%)		+	+	6
MexAM1_META1p2777	<i>mauI</i>	Essential for methylamine dehydrogenase activity	8		+	+	6
MexAM1_META1p2778	<i>mauM</i>	Methylamine utilization ferredoxin-type protein	11		+	+	6
MexAM1_META1p2779	<i>mauN</i>	Methylamine utilization ferredoxin-type protein	8		+	+	6
Ethylmalonyl-CoA pathway genes							
MexAM1_META1p3700	<i>phaA</i>	Beta-ketothiolase	NC	+	+	+	30–33
MexAM1_META1p3701	<i>phaB</i>	Acetoacetyl-CoA reductase	NC	+	+	+	30–33
MexAM1_META1p3675	<i>croR</i>	Crotonase, R-specific enoyl-CoA hydratase	2.4	+	+	+	30
MexAM1_META1p0178	<i>ccr</i>	Crotonyl-CoA reductase	2.1	+	+	+	11, 30
MexAM1_META1p0180	<i>ecm (meaA)</i>	Ethylmalonyl-CoA mutase	2.5	+	+	+	30, 45
MexAM1_META1p2223	<i>msd (ibd2)</i>	Methylsuccinyl-CoA dehydrogenase	2.2	+	+	+	20, 30
MexAM1_META1p4153	<i>mcd (meaC)</i>	Mesaconyl-CoA hydratase	−1.7	+	+	+	37
MexAM1_META1p4295	<i>mclA2</i>	Malyl-CoA lyase homolog	2.4	See Table 2	NR	See Table 2	
Genes for converting propionyl-CoA to succinyl-CoA							
MexAM1_META1p1433	<i>meaD</i>	Cobalamin adenosyltransferase (methylmalonyl-CoA mutase cofactor regeneration)	1.6	+	+	+	37
MexAM1_META1p5251	<i>mcmA</i>	Methylmalonyl-CoA mutase (MCM) alpha subunit	2.4	+	+	+	30
MexAM1_META1p2390	<i>mcmB</i>	Methylmalonyl-CoA mutase beta subunit	2.1	+	+	+	30
MexAM1_META1p0188	<i>meaB</i>	Essential for MCM reaction	NC	+	+	+	30
MexAM1_META1p3203	<i>pccA</i>	Propionyl-CoA carboxylase, alpha subunit	NC	+	+	+	30
MexAM1_META1p0172	<i>pccB</i>	Propionyl-CoA carboxylase, beta subunit	NC	+	+	+	11, 30
MexAM1_META1p0839	<i>epm</i>	Methylmalonyl-CoA epimerase	−1.5				30
Genes for converting succinyl CoA to malate							
MexAM1_META1p3124	<i>sct</i>	Succinyl-CoA transferase	NC	NM	NM	NM	50
MexAM1_META1p3859	<i>sdhC</i>	Succinate dehydrogenase subunit	−1.5	NM	NM	NM	50
MexAM1_META1p3860	<i>sdhD</i>	Succinate dehydrogenase subunit	−1.7	NM	NM	NM	50
MexAM1_META1p3861	<i>sdhA</i>	Succinate dehydrogenase alpha subunit	−2.0 (36%)	+	NR	NR	33, 49

Continued on following page

TABLE 1—Continued

Gene category and no. ^a	Gene name	Description	Fold change ^b	Required for growth ^c			Reference(s)
				MeOH	MtNH ₂	EtNH ₂	
MexAM1_META1p1338	<i>fum</i>	Fumarase	−11	+	NR	NR	33, 49 7
MexAM1_META1p1537	<i>mdh</i>	Malate dehydrogenase	1.9	E	NR	NR	
<i>Serine cycle</i>							
MexAM1_META1p2944	<i>gck</i>	Glycerate kinase	2.2	+	+		13
MexAM1_META1p1726	<i>sga</i>	Serine glyoxylate aminotransferase	1.5	+	+	See Table 2	9, 29
MexAM1_META1p1727	<i>hpr</i>	Hydroxypyruvate reductase	NC	+	+		8
MexAM1_META1p1733	<i>mclA1</i>	Malyl-CoA lyase	−1.5	+	+	See Table 2	4, 12
MexAM1_META1p3384	<i>glyA</i>	Serine hydroxymethyltransferase	NC	+	+	See Table 2	10
MexAM1_META1p1732	<i>ppc</i>	PEP carboxylase	2.0	+	+		4
MexAM1_META1p2984	<i>eno</i>	Enolase	NC	E	NR	NR	7
<i>Glycerate conversion</i>							
MexAM1_META1p1218	<i>pgm</i>	Phosphoglycerate mutase	NC	NM	NM	NM	50
<i>Serine synthesis</i>							
MexAM1_META1p0485	<i>serC</i>	Phosphoserine aminotransferase	NC	NM	NM	NM	50
MexAM1_META1p0486	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	1.7	NM	NM	NM	50
MexAM1_META1p2848	<i>serB</i>	Phosphoserine phosphatase	NC	*	NR	+**	28
<i>Glycine cleavage</i>							
MexAM1_META1p0620	<i>gcvP</i>	Glycine decarboxylase	NC	See Table 2	NR	See Table 2	
<i>Aldehyde and acetate conversions</i>							
MexAM1_META1p3652	<i>aldA</i>	Aldehyde dehydrogenase	14	NM	NM	NM	50
MexAM1_META1p3924	<i>alo</i>	Aldehyde oxidase	3.0	NM	NM	NM	50
MexAM1_META1p2531	<i>acsA</i>	Acetyl-CoA synthase	3.3	NM	NM	NM	50

^a Gene numbers reflect their order on the chromosome.^b Fold change in the ethylamine/succinate level; standard deviations were within 25% of the means, unless otherwise indicated in parentheses.^c *, partial growth defect; **, on ethanol; NR, not reported; NC, no significant change (change between 1.3- and 0.77-fold); E, presumed essential (mutants not obtained); NM, no mutant available; MeOH, methanol; MtNH₂, methylamine; EtNH₂, ethylamine.

which will be referred to as *mclA2*. The protein products of these homologs also have been denoted Mcl1a and Mcl1b, respectively (38). The translated product of *mclA2* classes in a separate phylogenetic branch from the products of *mclA1* and the *mcl* in *Rhodobacter sphaeroides* that is proposed to function

in the EMC pathway in this bacterium during growth on acetate (38). The role of MclA2 in C₁ or C₂ metabolism in *M. extorquens* AM1 is not known, but the MclA2 protein was induced in a proteomics study comparing methanol growth to succinate growth (35). In the microarray analysis in this paper,

TABLE 2. Growth characteristics of *M. extorquens* AM1 wild type and mutants on agar plates^a

Strain	Growth on:					
	Succinate	Methanol		Ethylamine		Doubling time (h) on ethylamine
		Without glyox	With glyox	Without glyox	With glyox	
Wild-type	++++	+++	+++	++	++	12
<i>mclA1</i>	++++	—	½ +	+	½ +	25
<i>mclA2</i>	++++	+++	+++	++	++	15
<i>sgaA</i>	++++	—	—	+	½ +	47
<i>gcvP</i>	++++	+++	+++	+	+	16*
<i>mclA1 mclA2</i>	++++	—	—	—	—	NG
<i>mclA1 sga</i>	++++	—	—	½ +	½ +	ND
<i>mclA2 sga</i>	++++	—	—	½ +	½ +	ND

^a Glyox, 2 mM glyoxylate. +++++, colonies in 3 days; +++, colonies in 4 to 5 days; ++, colonies in 6 to 8 days; +, colonies in 10 to 20 days; ½+, small colonies in 10 to 14 days; —, no growth above the level of a control with no substrate. Two replicates were used for doubling times; values agreed within ±10%. NG, no growth; ND, not determined; *, maximum OD₆₀₀ = 0.5. All growth curves showed a lag phase similar to that of the wild type and reached a final OD₆₀₀ similar to that of the wild type. All mutants grew like the wild type on succinate in liquid culture.

Other mutants with partial defects for growth on C₂ compounds. It has been reported previously that mutants lacking serine hydroxymethyltransferase (*ghyA*) and phosphoserine phosphatase (*serB*) activities both had defects in growth on C₂ compounds (10, 28, 29). The phenotypes of these mutants suggest that interconversions of glycine and serine play a role in C₂ metabolism. A possible pathway for converting glyoxylate to three-carbon (C₃) and four-carbon (C₄) compounds via glycine and serine involving known enzymes and genes in *M. extorquens* AM1 is outlined in Fig. 2 (Table 1 lists the enzyme names). To further pursue this concept, a mutant was generated in a gene of the glycine cleavage system (*gcvP*), which interconverts glycine and methylene-H₄F plus CO₂, generating NADH. *M. extorquens* AM1 previously has been shown to generate C₁ units from glycine or glyoxylate (29). The *gcvP* mutant and existing mutants defective in serine-glyoxylate aminotransferase (*sga*) and hydroxypyruvate reductase (*hpr*) were tested for growth on C₁ and C₂ compounds. The *sga* and *hpr* mutants are unable to grow on methanol (9, 29), as these enzymes play a central role in the serine cycle. The *gcvP* mutant grew normally on methanol. Both the *gcvP* and *sga* mutants were found to have a partial defect for growth on ethylamine on plates similar to that observed for the *mclA1* mutant. Like the *mclA1* mutant, the *sga* mutant was inhibited by glyoxylate at a lower concentration than that of the wild type, but the *gcvP* mutant did not respond to glyoxylate supplementation (Table 2). In liquid culture, the *sga* mutant showed a strong growth rate defect on ethylamine (47-h doubling time), while the *gcvP* mutant showed a small growth rate defect (16-h doubling time) but did not grow past an OD₆₀₀ of 0.5, while the others all grew to an OD₆₀₀ of 1.2 to 1.5, like the wild type. As

Metabolite analysis. The *sga*, *mclA1*, and *gcvP* mutants were analyzed for metabolites, both in cell extracts (intracellular) and in the supernatant, and were compared to the wild type to assess possible metabolic imbalances occurring that might contribute to their impaired growth on ethylamine. *M. extorquens* AM1 has a number of possible routes for glyoxylate consumption, including conversion to glycolate and oxalate (Fig. 3), so glycolate and oxalate were measured, as well as a number of other targeted metabolites. In addition, an S-ratio analysis (41) was carried out to identify the major differences between the cultures (Fig. 4). The patterns in the supernatant and intracellular analyses were significantly different, showing that the metabolites in the supernatant were not simply a result of cell lysis. The most striking differences between the wild type and mutants involved amino acids. Several amino acids, including proline, alanine, leucine, valine, and threonine, were high in all or most of the three mutants, either intracellularly, in the supernatant, or both. In addition, intermediates of valine, isoleucine, or leucine synthesis (2-oxoisovaleric acid, 3-methyl-2-oxopentanoic acid, 4-methyl-2-oxopentanoic acid), respectively, were high in the supernatant mainly in the *sga* mutant but also, to some extent, in the other mutants. The level of glycine was very high in the *gcvP* mutant, both intracellularly and in the supernatant, which may explain the lack of growth to high cell densities, since glycine is known to be inhibitory to this bacterium (28). In addition, the *sga* and *gcvP* mutants showed slightly higher intracellular oxalic acid, while the *sga*

FIG. 4. Comparison of intracellular and supernatant metabolites in wild-type and mutant strains grown on ethylamine. Results are ratios of values from each mutant compared to wild-type values. Top, supernatant; bottom, intracellular. *, significant difference ($P < 0.05$) between mutants and the wild type. Error bars show the range for three biological replicates, each with three technical replicates. 3Mt2oxoPen, 3-methyl-2-oxopentanoic acid; 4Mt2oxoPen, 4-methyl-2-oxopentanoic acid; MtSuc, methylsuccinic acid; Pyru, pyruvate.

mutant showed higher mesaconic acid, the non-CoA derivative of a CoA intermediate of the EMC pathway. Other than the amino acids that also were elevated in the other mutants, the *mclA1* mutant showed relatively minor (2-fold or less) changes in the tested metabolites. The increase in amino acid pools may reflect higher ammonium transfer activity as a result of growth on an amine in combination with the lower growth rate.

¹³C-labeling analysis. The results described above suggested that some glyoxylate is converted to central metabolic intermediates via glycine and serine. If the pathway shown in Fig. 2 operates to carry out this conversion, then it should be possible to observe carbon flow from labeled glyoxylate into glycine and serine. ¹³C-labeled glyoxylate was used for these experiments, and all isotopomers were measured for these three compounds in samples taken at short time points (10 s to 5 min) after the addition of the labeled substrate. Major possible labeling patterns are shown in Fig. 5. Glyoxylate processed by the glycine/serine (Sga/Gcv/GlyA) route would be expected to generate

doubly labeled serine with unlabeled methylene H₄F, or triply labeled serine as methylene H₄F becomes labeled. Singly labeled serine could come from unlabeled glycine reacting with labeled methylene H₄F. The labeled serine then would generate the same label in malate via PEP as that shown in Fig. 3. Glyoxylate fluxing through the malyl-CoA route would produce doubly labeled malate, if acetyl-CoA is not made from glyoxylate, or quadruply labeled malate, in the unlikely scenario that acetyl-CoA can be made from glyoxylate. Malate that enters the tricarboxylic acid (TCA) cycle loses a label, and serine synthesized from malate via oxaloacetate, PEP, and the phosphoserine pathway would maintain the same label as that in the malate.

The results are consistent with both pathways operating to utilize glyoxylate (Fig. 6). More than 95% of the glycine pool becomes doubly labeled within 10 s (Fig. 6A), demonstrating the rapid conversion of glyoxylate to glycine under these conditions. For serine, 75% or more of the pool remains unlabeled

FIG. 5. Predicted labeling patterns from [^{13}C]glyoxylate. Unlabeled products are not shown.

throughout the experiment (Fig. 6B), suggesting that about 3/4 of it is synthesized from the phosphoserine pathway without incorporating label. However, by 90 s, about 25% of the pool is labeled. The doubly labeled compound appears first, fol-

lowed by the triply labeled compound, which is consistent with a flow of glyoxylate-derived carbon through the glycine/serine pathway and also with the labeling of the methylene H_4F pool within 30 s. A small amount of singly labeled serine appears later, which could be derived from the small unlabeled glycine pool reacting with labeled methylene H_4F . About one-third of the malate pool is quickly generated as doubly labeled, with 55 to 65% of the pool remaining unlabeled. Only a very small amount of the malate pool occurs in other labeled species (singly and quadruply labeled). These results suggest that more than half of the malate is being generated within the TCA cycle without incorporating label, but that the remainder is generated via glyoxylate. If all of the serine were generated from malate, the labeling pattern for serine would parallel that for malate. However, it does not. The doubly labeled malate stays high, but the doubly labeled serine drops and is replaced by triply labeled serine, which is the pattern predicted for the glycine/serine route. These results do not rule out a previously unknown, novel pathway for the synthesis of serine, but they are consistent with the proposal that a significant amount of the labeled serine is generated from glycine.

Two other conversion pathways are known for glyoxylate based on *in vitro* results, involving conversion to glycolate (3, 8, 17, 34) and oxalate (5). It was not possible to accurately determine the labeling of glycolate due to the presence of contaminating glycolate in the [^{13}C]glyoxylate preparation, but no labeled glycolate was observed above the background amount. It also was not possible to detect labeled oxalate, suggesting that if flux to oxalate occurs, it was below our detection limit. Since both glycolate and oxalate are detected in ethylamine-grown cells (52), it is likely that some flux to these compounds occurs.

DISCUSSION

The prediction that malate synthase activity is required for *M. extorquens* AM1 to grow on C_2 compounds via the EMC pathway stands in contrast to the literature evidence that a mutant lacking malate synthase activity still is able to grow on C_2 compounds (43). Possible explanations for this contradiction are the following: (i) the EMC pathway does not operate during growth on C_2 compounds, (ii) glyoxylate or a product

FIG. 6. Time course of ratios of isotopomers of glycine (top), serine (middle), and malate (bottom) after the incubation of ethylamine-grown cells with [^{13}C]glyoxylate in the presence of [^{12}C]ethylamine as a fraction of the total compound. M + 0, unlabeled; M + 1, singly labeled; M + 2, doubly labeled; M + 3, triply labeled; M + 4, quadruply labeled.

made from glyoxylate is excreted, (iii) malate synthase activity actually is present in the malyl-CoA lyase mutant grown on C₂ compounds, or (iv) an alternative glyoxylate consumption pathway exists.

Explanation i clearly is not correct, since a large body of evidence has shown that *M. extorquens* AM1 grows on C₂ compounds using the same pathway that is involved in converting acetyl-CoA to glyoxylate during growth on C₁ compounds (2, 11, 15–17, 30–33, 43). Most compelling is the fact that mutants in this pathway are defective for growth on both C₁ and C₂ compounds and are rescued for growth on both C₁ and C₂ compounds by the addition of glyoxylate (11, 15–17, 30–33, 44). The results presented here are consistent with the operation of the EMC pathway during growth on ethylamine. Most of the genes involved in the conversion of acetyl-CoA to glyoxylate and propionyl-CoA by the EMC pathway showed higher expression in cells grown on ethylamine than in cells grown on succinate, similarly to the result found with methanol-grown cells (42) (Fig. 1, Table 1).

Likewise, the utilization of propionyl-CoA during growth on C₂ compounds has been suggested to follow a standard conversion scheme via propionyl-CoA carboxylase and methylmalonyl-CoA mutase, succinate dehydrogenase, and fumarase (Fig. 1) (30–33, 40), and mutants in these genes also show defects in growth on both C₁ and C₂ compounds that are rescued by the addition of glyoxylate or glycolate (7, 11, 30). Of these genes, only those involved in generating active methylmalonyl-CoA mutase showed higher expression in cells grown on ethylamine than succinate. However, the slow growth observed on C₂ compounds does not require high enzyme levels to support the flux required. It can be calculated that the flux through this pathway required to support a 12-h doubling time is approximately 15 nmol min⁻¹ mg protein⁻¹, and these enzymes are present in succinate-grown cells at *in vitro* activities greater than this (11, 33). These results all are consistent with the previous mutant data showing that the EMC pathway must operate for the growth of *M. extorquens* AM1 on C₂ compounds.

For explanation ii, the excretion of glyoxylate or related compounds would drop the yield, but it could allow the cells to grow on the propionyl-CoA generated from β -methylmalyl-CoA. About 40% of the total carbon flux to biomass goes to glyoxylate via the EMC pathway, so the amount to be excreted would be significant. *M. extorquens* AM1 is known to have *in vitro* activities for interconverting glyoxylate and glycolate (3, 8, 17, 34) and interconverting glyoxylate and oxalyl-CoA, which then can be converted to oxalate (5), although not all of the genes involved in these activities have been identified. Measurements of intracellular and extracellular glyoxylate, glycolate, and oxalate showed that these compounds all were detectable in the wild type both intracellularly and extracellularly. However, the *mclA1* mutant did not show major differences in the levels of these compounds compared to those of the wild type, either intracellularly or extracellularly, suggesting that the excretion or accumulation of glyoxylate or products derived from glyoxylate does not explain the growth of the *mclA1* mutant on C₂ compounds. It is notable that the intracellular levels of glyoxylate in the *mclA1* mutant were not significantly different from those of the wild type, suggesting that an alternative glyoxylate consumption route exists. Although the me-

tabolite detection methods used here did not determine absolute concentrations, based on previous analyses (52) it can be estimated that the total amount of glyoxylate, glycolate, and oxalate detected in the supernatant of the *mclA1* mutant was at least two orders of magnitude lower than expected if the glyoxylate flux was directed to excretion.

Explanation iii, that the *mclA1* mutant contains malate synthase activity during growth on C₂ compounds, is at best only a partial explanation as, based on Mcl activity measurements, the low activity would not account for the utilization of all of the glyoxylate generated via the EMC pathway and would not support the 25-h doubling time of this mutant on ethylamine (approximately 7.5 nmol min⁻¹ mg protein⁻¹). However, we did show that the growth of the *mclA1* deletion mutant is only partially impaired on ethylamine, suggesting that MclA2 performs a similar function but at a reduced level. The existence of this isoenzyme thus provides a partial solution to the conundrum.

Explanation iv, the presence of an alternative glyoxylate consumption pathway, was shown to provide the final solution to the conundrum. This pathway involves *sga*, *glyA*, and *gcv*, and it appears to operate not only in mutants defective in the EMC pathway but also in the wild type during growth on C₂ compounds. Evidence for this pathway was obtained by observing mutant phenotypes and was consistent with labeling experiments involving both mutants and the wild type, as described above. Further confirmation of the importance of this glycine/serine pathway during growth on C₂ compounds was obtained from the finding that both *mclA1 sga* and *mclA2 sga* double mutants have (partially) impaired glyoxylate consumption routes and show significant growth defects on ethylamine, unlike each of the single mutants.

Once glyoxylate is converted to serine, it has multiple routes for incorporation, including conversion to protein and to other C₂, C₃, and C₄ compounds via the routes shown in Fig. 3. Strains containing mutations in some steps of these pathways are known to grow on C₂ compounds, including strains lacking *hpr* (8) and *gck* (3, 13), suggesting either that serine is routed into central metabolites via alternative pathways or that, *in vivo*, other enzymes are present that carry out this function at the low flux required to support the observed growth rate. A possible pathway involving these steps can be drawn for converting 2-glyoxylate to a malate (Fig. 2 and 3), using known enzymes and genes in *M. extorquens* AM1, although it is energetically expensive, requiring one NADH molecule and one ATP molecule per malate molecule, and even more if an energy-requiring transamination reaction is involved. However, glyoxylate is a reactive aldehyde and is inhibitory to *M. extorquens* AM1 above 2 mM in the external medium. Accumulation inside the cell is likely to be detrimental, and it is possible that the slow growth observed on C₂ compounds in the wild type is due in part to the tradeoff between the detriments of glyoxylate accumulation and the low yield involved in this pathway. Our results show that even in the mutants analyzed here, glyoxylate does not accumulate, pointing to a careful control system to keep it from building up.

In summary, our results demonstrate that during growth on ethylamine, *M. extorquens* AM1 uses an alternative route for glyoxylate consumption via glycine and serine to complement the expected malate synthase route. Neither pathway alone

supports wild-type growth, but the combination allows this bacterium to grow normally on C₂ compounds. This finding suggests that the two-step malate synthase reaction in *M. extorquens* AM1 creates a bottleneck for glyoxylate consumption, which the cell has overcome by shunting glyoxylate through a second pathway. Although the measured *in vitro* activity of malate synthase (16 nmol min⁻¹ mg protein⁻¹ for ethylamine-grown cells) should be just sufficient to support the growth rate on C₂ compounds, our results show that the *in vivo* activities must restrict flux through this route. The presence of alternative routes for the consumption of a toxic intermediate is a logical metabolic strategy and demonstrates the versatility and flexibility of the metabolic network in this facultative methylotroph. In addition, it represents a possible model for other metabolic networks involving high flux through a toxic intermediate.

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