PLP Catabolism: Identification of the 2-(Acetamidomethylene)succinate Hydrolase Gene in *Mesorhizobium loti* MAFF303099[†]

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Received March 11, 2008; Revised Manuscript Received April 1, 2008

ABSTRACT: The function of the mlr6787 gene from *Mesorhizobium loti* MAFF303099 has been identified. This gene encodes 2-(acetamidomethylene)succinate hydrolase, an enzyme involved in the catabolism of pyridoxal 5′-phosphate (vitamin B_6). This enzyme was overexpressed in *Escherichia coli*, purified to homogeneity, and characterized. 2-(Acetamidomethylene)succinate hydrolase catalyzes the hydrolysis of 2-(acetamidomethylene)succinate to yield succinic semialdehyde, acetic acid, carbon dioxide, and ammonia. The k_{cat} and K_M for this reaction were 0.6 s^{-1} and 143 μM, respectively. The enzyme was shown to utilize the *E* isomer of 2-(acetamidomethylene)succinate.

Only a few bacteria that are capable of surviving on vitamin B_6 as a sole source of carbon and nitrogen (*1*) have been identified, and two catabolic routes for vitamin B_6 degradation have been identified (*2*) (Figure 1). In the first pathway, prevalent in *Pseudomonas* sp. MA-1, pyridoxine (**1**), vitamin B_6, is degraded to succinic semialdehyde (**9**), while in the second pathway seen in *Pseudomonas* sp. IA and *Arthrobacter* Cr-7, it is degraded to 2-(hydroxymethyl)-4-oxobutanoate (**14**). Each of the catabolic intermediates has been isolated and characterized, and the enzymes which produce them have been identified over the past few decades (*2*). Recently, six genes in *Mesorhizobium loti* MAFF303099 involved in PLP[1] catabolism have been identified. The genes that were identified encoded pyridoxine-4-oxidase (mlr6785) (*3*), 4-pyridoxolactonase (mlr6805) (*4*), pyridoxal-4-dehydrogenase (mlr6807) (*5*), 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (mlr6788) (*6*), 4-pyridoxic acid dehydrogenase (mlr6793) (*7*), and the 3-hydroxy-2-methylpyridine-4,5-dicarboxylate decarboxylase (mlr6791) (*8*). The gene encoding 2-(acetamidomethylene)succinate hydrolase, which catalyzes the last step in the degradation of vitamin B_6 (*9*), has not yet been identified. The genes that have been identified to date are clustered on the chromosome. Inspection of the nearby genes revealed a putative hydrolase (mlr6787) which is currently annotated as a "putative DHNA-CoA thioesterase" proposed to be involved in menaquinone biosynthesis (*10*). Here we report the purification and characterization of the mlr6787 gene product and show that it catalyzes the hydrolysis of 2-(acetamidomethylene)succinate (**8**) to form succinic semialdehyde (**9**), acetic acid, ammonia, and carbon dioxide. The enzyme was shown to utilize the *E* isomer of 2-(acetamidomethylene)succinate.

EXPERIMENTAL PROCEDURES

Materials. A dehydrated form of LB broth was purchased from EMB Chemicals (Gibbstown, NJ). Ampicillin and IPTG were obtained from Laboratory Scientific Inc. (Livingston, NJ). 4-Pyridoxic acid, NAD, FAD, NADH, NADPH, glutamate dehydrogenase, α-ketoglutarate, L-alanine, phosphorus pentoxide, diethyl maleate, sodium hydride, and succinic semialdehyde were purchased from Sigma (St. Louis, MO). Triethylamine was obtained from Fisher (Fairlawn, NJ). Trifluoroacetic acid, methanol (HPLC grade), ethanol, chloroform, potassium hydroxide, sodium chloride, imidazole, 2-mercaptoethanol, sodium hydroxide, ethyl formate, diethyl succinate, ether, acetamide, and anhydrous cupper sulfate were purchased from Acros Organics (Morris Plains, NJ). Sodium dihydrogen phosphate monohydrate, concentrated sulfuric acid, and anhydrous potassium carbonate were from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Deuterium oxide (D_2O) and methanol-d_4 were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Microcon YM-10 centrifugal filter devices (10000 MWCO) and the Amicon Ultra centrifugal filter device (10000 MWCO) were obtained from Millipore (Billerica, MA). The Supelcosil LC-18-T column for HPLC analysis was from Supelco (Bellefonte, PA). *Escherichia coli* strain MachI and the Gateway system were from Invitrogen (Carlsbad, CA). The Nucleospin purification kit, Phusion DNA polymerase, *E. coli* BL21(DE3), and the Ni-NTA superflow resin were obtained from Macherey-Nagel (Easton, PA), New England Biolabs (Ipswich, MA), Novagen (San Diego, CA), and Qiagen (Valencia, CA), respectively.

Molecular Cloning. Standard methods were used for DNA manipulations (*11, 12*). Plasmid DNA was purified with the Qiagen Miniprep kit, and DNA fragments were

[†] This research was supported by a grant from the National Institutes of Health (GM069618).

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; IPTG, isopropyl β-D-thiogalactopyranoside; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; TFA, trifluoroacetic acid; LB, Luria-Bertani; FAD, flavin adenine dinucleotide; DNP, 2,4-dinitrophenylhydrazine; NOESY, nuclear Overhauser enhancement spectroscopy.

10.1021/bi8004158 CCC: $40.75
Published on Web 05/14/2008

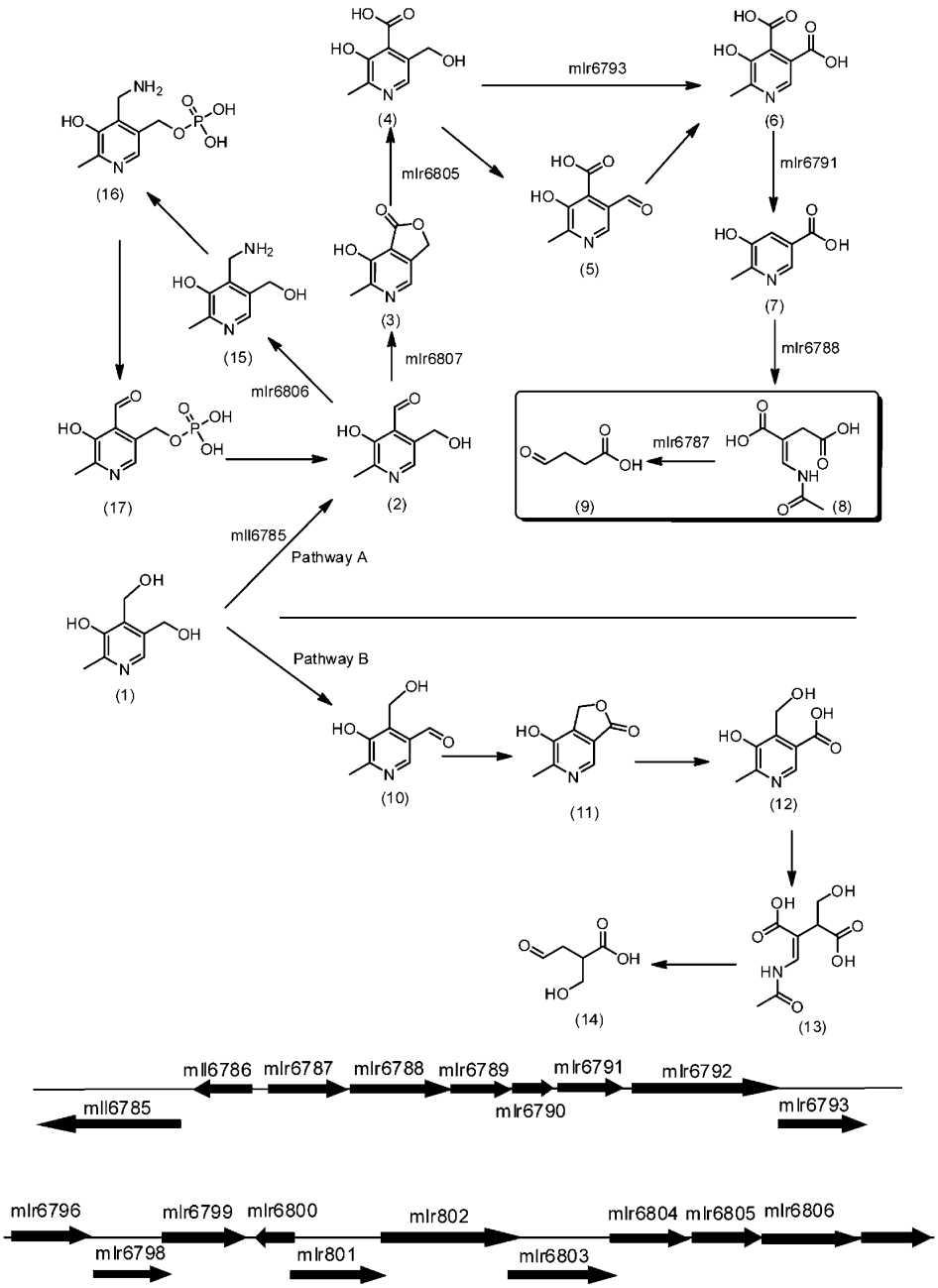

FIGURE 1: Vitamin B_6 catabolic pathways. Pathway A is observed in *Pseudomonas* sp. MA-1 (*27*), while pathway B is observed in *Pseudomonas* IA and in *Arthrobacter* Cr-7. A minor variation of pathway A is seen in *M. loti* MAFF303099 where the formation of **5** is not seen. The reaction shown in the box is catalyzed by (*E*)-2-(acetamidomethylene)succinate hydrolase. The bottom figure shows the gene organization in *M. loti*. The genes that have been shown to participate in PLP degradation are identified in catabolic pathway A: (**1**) pyridoxine, (**2**) pyridoxal, (**3**) 4-pyridoxolactone, (**4**) 4-pyridoxic acid, (**5**) 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylate, (**6**) 3-hydroxy-2-methylpyridine-4,5-dicarboxylate, (**7**) 3-hydroxy-2-methylpyridine-5-carboxylate, (**8**) 2-(acetamidomethylene)succinate, (**9**) succinic semialdehyde, (**10**) isopyridoxal, (**11**) 5-pyridoxolactone, (**12**) 3-hydroxy-4-(hydroxymethyl)-2-methylpyridine-5-carboxylate, (**13**) 2-(acetamidomethylene)-3-(hydroxymethyl)succinate, (**14**) 2-(hydroxymethyl)-4-oxobutanoate, (**15**) pyridoxamine, (**16**) pyridoxamine 5-phosphate, and (**17**) pyridoxal 5-phosphate.

purified from an agarose gel with the Nucleospin purification kit. *E. coli* strain MachI was used as a recipient for transformations during plasmid construction and for plasmid propagation. Phusion DNA polymerase was used for PCR following the manufacturer's recommendations. The pENTR-TEV-D-TOPO and Gateway system were used following the manufacturer's instructions with slight modifications.

Cloning of M. loti Ml5331. The *M. loti* mlr6787 gene was amplified from genomic DNA by PCR with the following primer pair: 5′-CAC CAT GGA CAT GGC GGC AGA CAT AGC TTC C-3′ and 5′-TCA GGC GTC TAT GAA GTT GGT GAT GG-3′. The PCR product was purified and used in a topoisomerase-mediated reaction with pENTR-TEV-D-TOPO following the manufacturer's instructions. Clones were screened by PCR and verified by sequencing. A correct clone was used in an LR reaction with the pDESTF1 plasmid, which is a Gateway-adapted vector based on the pET system. The pDESTF1 plasmid encodes an N-terminal six-His tag and is under the control of the T7*lac* promoter. Again, clones were screened by restriction digestion. A correct clone was named pMl5331.XF1.

Overexpression and Purification. The pMl5331.XF1 plasmid was used to transform *E. coli* BL21(DE3). A starter culture was prepared by growing a single colony of transformed cells in 10 mL of LB medium containing 100 μg/mL ampicillin at 37 °C with overnight agitation. One liter of LB medium (20 g/L), containing 100 μg/mL ampicillin, was inoculated with this starter culture. The cells were grown at 37 °C with shaking until the culture reached an OD_{590} of 0.6, at which point they were induced by adding IPTG to a final concentration of 0.8 mM, the temperature was lowered to 15 °C, and the cells were allowed to grow for a further 12 h. The cells were then harvested by centrifugation at 10000*g* for 8 min at 4 °C.

Cells from 1 L of culture were resuspended in 20 mL of binding buffer [50 mM NaH_2PO_4, 150 mM NaCl, and 10 mM imidazole (pH 7.7)], and approximately 2 mg of lysozyme was added. The cells were then lysed by sonication (Misonix Sonicator 3000; pulse on time, 1.0 s; pulse off time, 1.0 s; output level, 0.8; 30 cycles) five times on ice. The cell debris was removed by centrifugation at 39000*g* for 40 min at 4 °C. The clarified supernatant was loaded onto a 5 mL Ni-NTA affinity column pre-equilibrated with binding buffer kept at 4 °C. The Ni-NTA affinity column was then washed with 100 mL of wash buffer [50 mM NaH_2PO_4, 150 mM NaCl, and 20 mM imidazole (pH 7.7)]. The protein was eluted from the column with elution buffer [50 mM NaH_2PO_4, 150 mM NaCl, and 200 mM imidazole (pH 7.7)] at 4 °C. The fractions containing protein were pooled and concentrated using YM-10 Amicon ultracentrifugal filters at 5000*g* to a final volume of 500 μL. The concentrated sample was desalted into 100 mM phosphate buffer (pH 8.0) using an Econo-Pac 10DG disposable chromatography column. SDS−PAGE showed 95% pure protein. The yield of the purified protein was 25 mg/L. The protein concentration was measured by the Bradford assay (*13*).

Overexpression and Purification of Succinic Semialdehyde Dehydrogenase. The gene product of the MtbH37*Rv gabD1* (Rv0234c) gene from *Mycobacterium tuberculosis* is reported to be succinic semialdehyde dehydrogenase (*14*). The MtbH37*Rv gabD1* (Rv0234c) gene in the pET28b vector was overexpressed and purified in a similar manner as described above. Kanamycin was used as the antibiotic to a final concentration of 40 μg/mL. After the protein had been loaded onto the Ni-NTA affinity column pre-equilibrated with binding buffer kept at 4 °C, the column was washed with 300 mL of wash buffer containing 50 mM NaH_2PO_4, 150 mM NaCl, and 40 mM imidazole (pH 7.7). The purified protein was 80% pure as determined by SDS−PAGE. A Bradford assay (*13*) was used to measure the protein concentration, and the yield of the purified protein was 2 mg/L.

HPLC Analysis. HPLC analysis of the enzymatic reaction mixture was performed on a Hewlett-Packard 1100 instrument using a Supelcosil LC-18-T (15 cm × 4.6 mm, 3.0 μm) column. Two different methods were used. In method A, solution A contained water, solution B contained 100 mM sodium phosphate buffer (pH 6.6), and solution C contained methanol. The following linear gradient was used: 0 to 10% solution A and 100 to 90% solution B from 0 to 5 min; 10 to 48% solution A, 90 to 40% solution B, and 0 to 12% solution C from 5 to 12 min; 48 to 50% solution A, 40 to 30% solution B, and 12 to 20% solution C from 12 to 14 min; 50 to 30% solution A, 30 to 10% solution B, and 20 to 60% solution C from 14 to 18 min; 30 to 0% solution A, 10 to 100% solution B, and 60 to 0% solution C from 18 to 20 min; and 0% solution A, 100% solution B, and 0% solution C from 20 to 25 min (*8*). The flow rate was maintained at 1 mL/min, and the following compounds were readily separated (retention time and wavelength in parentheses): 2-(acetamidomethylene)succinate (**8**) (2.1 min, 254 nm), 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**) (2.8 min, 320 nm), 3-hydroxy-2-methylpyridine-5-carboxylate (**7**) (5.5 min, 320 nm), NAD (12.9 min, 254 nm), NADH (14.3 min, 254 nm), and FAD (19.3 min, 254 nm). In method B, solution A contained water with 0.1% TFA and solution B contained methanol with 0.1% TFA. The following linear gradient mixing solution A with solution B was used: 100% solution A from 0 to 2 min, 100 to 60% solution A from 2 to 7 min, 60 to 0% solution A from 7 to 10 min, 0 to 100% solution A from 10 to 11 min, and 100% solution A from 10 to 15 min. The flow rate was 1 mL/min, and by this method, the following compounds were readily separated (retention time and wavelength in parentheses): 2-(acetamidomethylene)-succinate (**8**) (4.3 min, 261 nm), DNP (11.3 min, 261 nm), and DNP hydrazone of succinic semialdehyde (**29**) (11.65 min, 261 nm).

*Synthesis of 3-Hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**).* The synthesis of 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**) began with the known protocols for making *N*-formylalanine (**19**) from L-alanine (**18**) (*15*) (Figure 2a), which was then converted to 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**) according to literature procedures (*16*): mp 270−272 °C; ^{1}H NMR (D_2O, 300 MHz) δ 2.60 (s, 3H), 8.21 (s, 1H); ^{13}C NMR (D_2O, 75 MHz) δ 173.7, 171.2, 155.3, 146.1, 135.7, 130.1, 110.0, 16.0.

*Synthesis of 2-(Acetamidomethylene)succinic Acid (**8**).* 2-(Acetamidomethylene)succinic acid (**8**) was synthesized starting from diethyl succinate (**23**) according to the scheme shown in Figure 2b. α-Formyl diethyl succinate (**24**) was prepared from diethyl succinate (**23**) and ethyl formate in accordance with a known literature procedure (*17*). Acetamidomethylene diethyl succinate (**25** and **26**) was prepared from acetamide and α-formyl diethyl succinate (**24**) from a known literature procedure (*18*). After workup, following the literature protocol, a yellow-colored oil was produced. TLC in 30% ethyl acetate and hexane showed three well-separated spots with r_f values of 0.48, 0.37, and 0.15. The three compounds were isolated by flash chromatography. The spot with an r_f value of 0.48 was found to be unreacted starting material, while the other two spots corresponded to the *E* and *Z* geometric isomers (**25** and **26**, respectively), both as pale yellow solids. The assignment of *E* and *Z* isomers was made on the basis of their two-dimensional NOESY spectra (Figure 1 of the Supporting Information). The spot with an r_f value of 0.37 was identified as the *Z* form (**25**), and the one with an r_f value of 0.15 was identified as the *E* form (**26**). For the *Z* form (**25**): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.17 (t, J = 7.2 Hz, 3H), 1.19 (t, J = 7.2 Hz, 3H), 2.07 (s, 3H), 3.10 (s, 2H), 4.05 (q, J = 7.2 Hz, 2H), 4.12 (q, J = 7.2 Hz, 2H), 7.37 (d, J = 11.4 Hz, 1H), 10.40 (d, J = 11.4 Hz, 1H). For the *E* form (**26**): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.23 (t, J = 7.2 Hz, 3H), 1.25 (t, J = 7.2 Hz, 3H), 2.12 (s, 3H), 3.34 (s, 2H), 4.11 (q, J = 7.2 Hz, 2H), 4.17 (q, J = 7.2 Hz, 2H), 8.11 (d, J = 11.4 Hz, 1H), 8.9 (d,

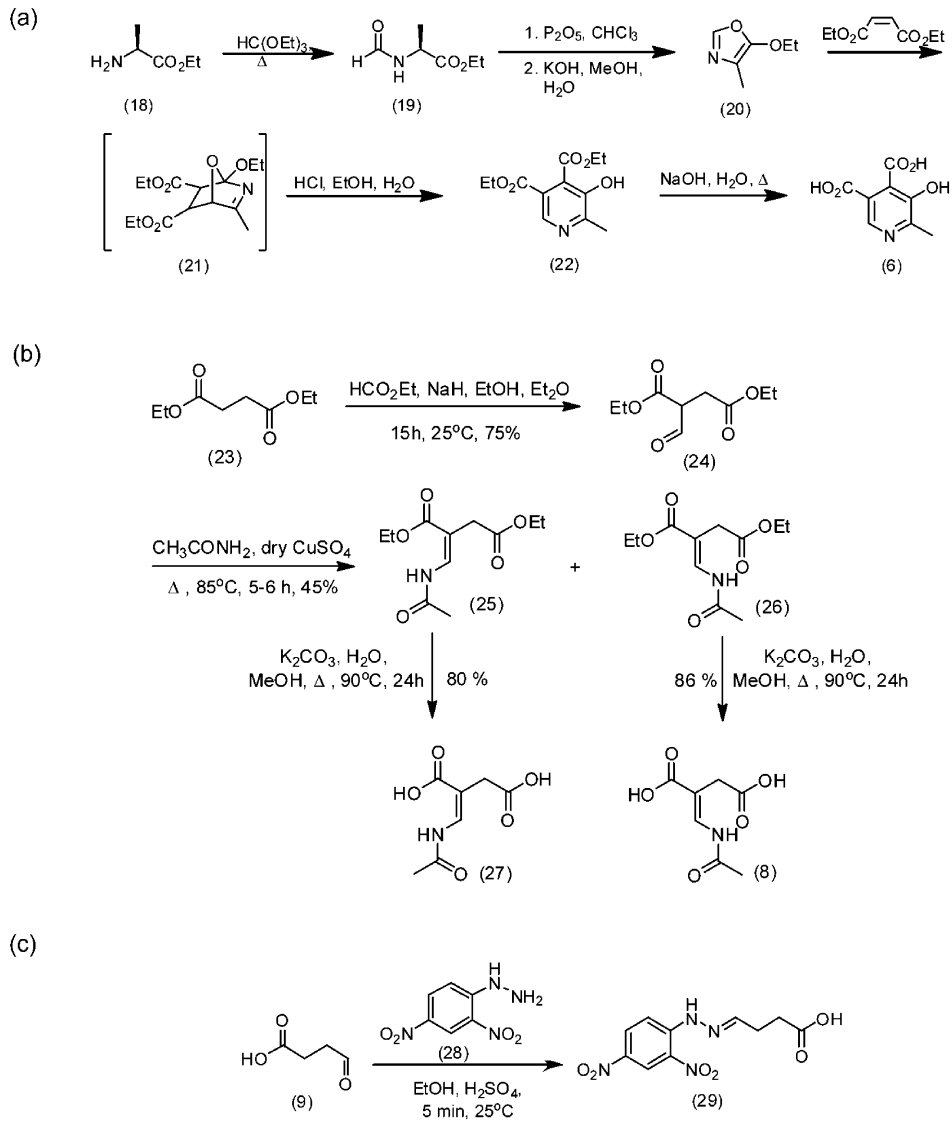

FIGURE 2: Synthetic scheme for the preparation of substrate and reference compounds (a) 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**), (b) (*E*)- and (*Z*)-2-(acetamidomethylene)succinate (**8** and **27**), and (c) DNP hydrazone of succinic semialdehyde (**29**).

J = 11.4 Hz, 1H). The (*E*)-2-(acetamidomethylene) diethyl succinate (**26**) (13 mg, 0.05 mmol) was dissolved in MeOH (75 μL) and H_2O (0.5 mL) and treated with K_2CO_3 (27 mg, 0.2 mmol). After being heated to 90 °C for 24 h, the solution was acidified with 4 M HCl, and the solvent was evaporated *in vacuo*. The residue was dissolved in EtOH and filtered to remove salt. Evaporation of the filtrate resulted in a precipitate which proved to be the (*E*)-2-(acetamidomethylene)succinic acid (**8**) (8 mg, 86%) as a white solid: mp 218−220 °C; ^{1}H NMR (D_2O, 300 MHz) δ 2.14 (s, 3H), 3.41 (s, 2H), 8.05 (s, 1H). The (*Z*)-2-(acetamidomethylene) diethyl succinate (**25**) after a similar treatment as mentioned above produced (*Z*)-2-(acetamidomethylene)succinic acid (**27**) in 80% yield as a white solid: mp 215−218 °C; ^{1}H NMR (D_2O, 300 MHz) δ 2.14 (s, 3H), 3.06 (s, 2H), 7.09 (s, 1H).

*Synthesis of DNP Hydrazone of Succinic Semialdehyde (**29**).* Succinic semialdehyde (**9**) (200 μL of a 15% solution in water) was added to 2.2 mL of ethanol, to which 63 mg of DNP in 600 μL of concentrated sulfuric acid was added. Formation of hydrazone was rapid, and the orange product was filtered and dried (Figure 2c). It was purified by flash chromatography with a 5% methanol/chloroform mixture: ^{1}H NMR (CD_3OD, 300 MHz) δ 2.48−2.64 (m, 4H), 7.7 (t, J = 3.9 Hz, 1H), 7.86 (dd, J = 1.2, 9.6 Hz, 1H), 8.21 (ddd, J = 1.5, 2.7, 9.6 Hz, 1H), 8.92 (dd, J = 1.5, 2.7 Hz, 1H).

*Enzymatic Synthesis of the 2-(Acetamidomethylene)succinate (**8**).* The biologically relevant isomer of 2-(acetamidomethylene)succinate (**8**) was synthesized enzymatically from 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**). A reaction mixture (10 mL) containing 20 μM freshly purified 3-hydroxy-2-methylpyridine-4,5-dicarboxylate decarboxylase (*8*), 10 μM 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (*6*), 2 mM 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**), 120 mM β-mercaptoethanol, 600 μM FAD, and 4 mM NADH in 100 mM sodium phosphate buffer (pH 8.0) was incubated overnight at room temperature. It was filtered using a YM-10 Amicon ultracentrifugal filter at 5000*g* to remove all protein, lyophilized, redissolved in a minimum volume of 100 mM sodium phosphate buffer (pH 8.0), and then purified by HPLC using method A. It was then rotary evaporated to remove methanol and lyophilized again. The lyophilized sample was then redissolved in a minimum volume of water containing 0.01% TFA and desalted by HPLC over multiple injections using method B.

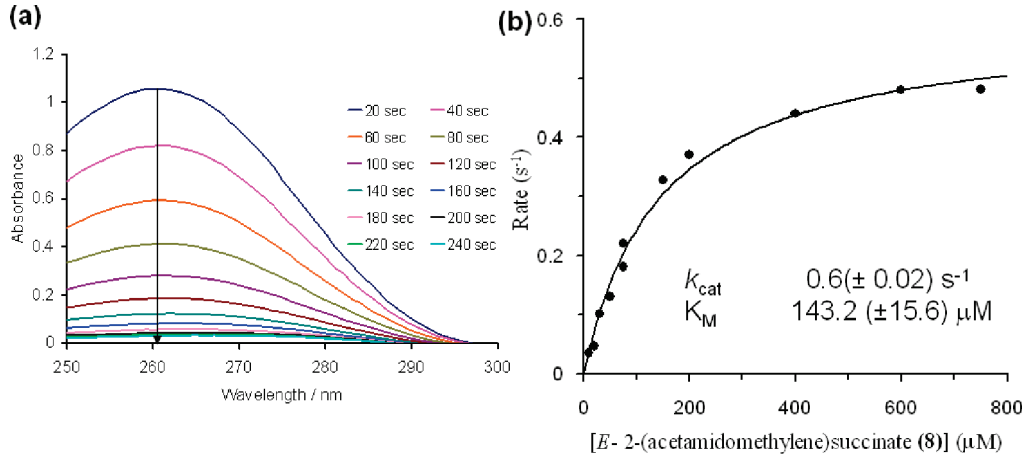

FIGURE 3: (a) UV−visible scan of an enzymatic reaction mixture (500 μL) containing 60 μM (*E*)-2-(acetamidomethylene)succinate (**8**) and 2 μM freshly purified (*E*)-2-(acetamidomethylene)succinate hydrolase in 100 mM sodium phosphate buffer (pH 8.0). (b) The steady state kinetic parameters for 2-(acetamidomethylene)succinate hydrolase with (*E*)-2-(acetamidomethylene)succinate as a substrate were determined by monitoring the absorbance at 261 nm (for substrate concentrations of 10, 20, 30, and 50 μM), 280 nm (for substrate concentrations of 75 and 150 μM), 290 nm (for substrate concentrations of 200, 400, and 600 μM), and 295 nm (for a substrate concentration of 750 μM) over time. The amount of substrate depleted was determined by dividing the change in the absorbance at a particular wavelength by the extinction coefficient at that wavelength.

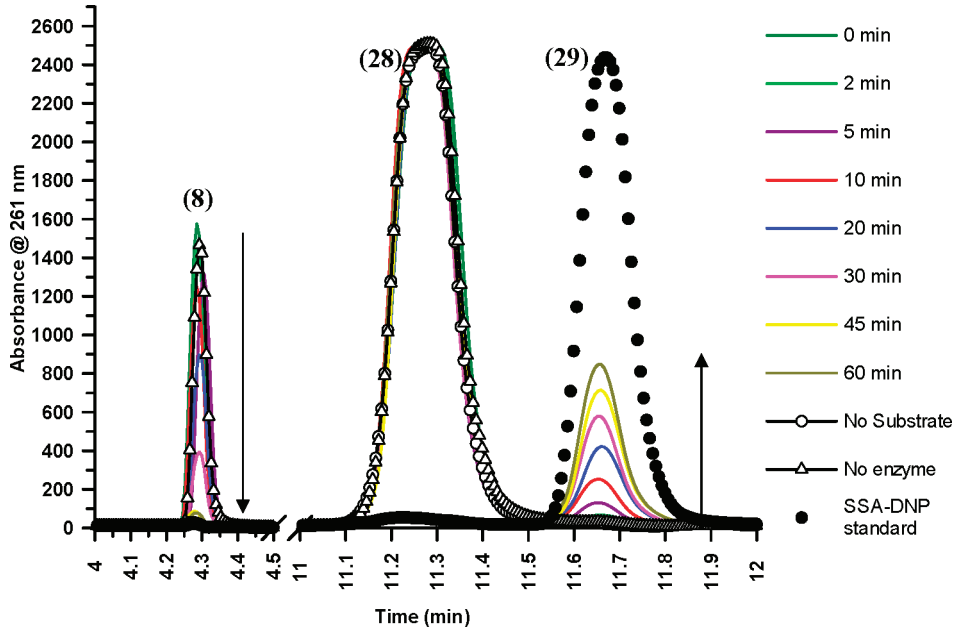

FIGURE 4: HPLC trace showing the formation of the DNP hydrazone of succinic semialdehyde (**29**) with time. Details appear in Experimental Procedures.

Methanol was removed by rotary evaporation, and TFA was removed *in vacuo*. Trace amounts of TFA and methanol were finally removed by lyophilization, leaving a white solid. The NMR of this sample was identical with that of the chemically synthesized (*E*)-2-(acetamidomethylene)succinic acid (**8**).

Reaction Time Course and Depletion of Substrate. A time course was determined by monitoring the disappearance of (*E*)-2-(acetamidomethylene)succinate (**8**) by UV−visible spectrophotometry as shown in Figure 3. UV−visible scans were taken every 20 s from 300 to 250 nm at a rate of 800 nm/s. The reaction mixture (500 μL) contained 60 μM (*E*)-2-(acetamidomethylene)succinate (**8**) and 2 μM freshly purified enzyme in 100 mM sodium phosphate buffer (pH 8.0).

*Reaction Time Course and Formation of DNP Hydrazone of Succinic Semialdehyde (**9**).* To a 3 mM solution of (*E*)-2-(acetamidomethylene)succinate (**8**) (500 μL) in 100 mM sodium phosphate buffer (pH 8.0) was added 1 μM freshly purified (*E*)-2-(acetamidomethylene)succinate hydrolase. At various time intervals, 40 μL of the reaction mixture was removed and quenched by addition to 50 μL of 8 mM DNP (prepared in 3 mM HCl) and extracted with ethyl acetate (2 × 300 μL). The extracted sample (100 μL) was analyzed by HPLC (Figure 4). Enzyme and substrate negative controls were run in a similar fashion in which buffer was substituted for the enzyme and the substrate, respectively. A standard of the DNP hydrazone of succinic semialdehyde (**29**) was also treated similarly.

Assay for Ammonia. The assay for ammonia was conducted by monitoring the disappearance of NADPH over time in a coupled assay with α-ketoglutarate and glutamate dehydrogenase (*19*). The assay mixture (500 μL) consisted of 10 units of glutamate dehydrogenase (unit defined as the amount of glutamate dehydrogenase that will reduce 1 μmol

of α-ketoglutarate to glutamate per minute at pH 8.3 and 30 °C), 10 mM α-ketoglutarate, 1 mM EDTA, 250 μM NADPH, 123 μM (*E*)-2-(acetamidomethylene)succinate (**8**), and 2.4 μM (*E*)-2-(acetamidomethylene)succinate hydrolase. The reduction of α-ketoglutarate to glutamate was not rate-limiting under these assay conditions. The reaction was started by the addition of (*E*)-2-(acetamidomethylene)succinate hydrolase and was monitored at 340 nm. All the solutions were made in 100 mM sodium phosphate buffer (pH 8.0).

Assay for Succinic Semialdehyde. The enzymatic hydrolysis of (*E*)-2-(acetamidomethylene)succinate (**8**) was coupled with an assay for succinic semialdehyde dehydrogenase to detect the formation of succinic semialdehyde (**9**). Succinic semialdehyde dehydrogenase catalyzes the oxidation of succinic semialdehyde (**9**) to succinic acid using NAD. Succinic semialdehyde dehydrogenase was used in excess and was not rate-limiting. The enzymatic reaction mixture (500 μL) consisted of 2 mM NAD, 1 mM 2-mercaptoethanol, 135 μM (*E*)-2-(acetamidomethylene)succinate (**8**), 1.2 μM (*E*)-2-(acetamidomethylene)succinate hydrolase, and 17 μM succinic semialdehyde dehydrogenase. The (*E*)-2-(acetamidomethylene)succinate hydrolase was added last, and the reaction was monitored at 340 nm. All the solutions were made in 100 mM sodium phosphate buffer (pH 8.0).

Steady State Kinetic Parameters. The (*E*)-2-(acetamidomethylene)succinate (**8**) has a λ_{max} at 261 nm and an extinction coefficient at 261 nm of 18600 M^{-1} cm^{-1} (*9*). The extinction coefficient of this molecule was also determined at 280, 290, and 295 nm from the absorbance measurements at various concentrations of (*E*)-2-(acetamidomethylene)succinate (**8**). For lower concentrations of (*E*)-2-(acetamidomethylene)succinate (**8**), the rate of the reaction was determined from the change in absorbance at 261 nm over time. At higher substrate concentrations, the rate of change of the absorbance of (*E*)-2-(acetamidomethylene)succinate (**8**) was monitored at higher wavelengths (see the legend of Figure 4b for details). To a reaction mixture (500 μL) containing 250 nM enzyme were added varying concentrations of (*E*)-2-(acetamidomethylene)succinate (**8**). The rate of depletion of (*E*)-2-(acetamidomethylene)succinate (**8**) was monitored over 2 min. The K_M and k_{cat} for the enzyme were determined by fitting the initial rate (less than 10% conversion) of substrate depletion as a function of substrate concentration to the Michaelis−Menten equation by nonlinear regression using Grafit 5.0.11 (Erithacus Software Ltd., Surray, U.K.) (Figure 3). All solutions were made in 100 mM sodium phosphate buffer (pH 8.0).

RESULTS AND DISCUSSION

Protein Overexpression and Purification. (*E*)-2-(Acetamidomethylene)succinate hydrolase was overexpressed, and 25 mg of protein per liter of cells was obtained at 95% purity as determined by SDS−PAGE. In contrast, even though the succinic semialdehyde dehydrogenase was overexpressed well, the best preparation yielded only 2 mg of protein per liter of cells at 80% purity.

Substrate Identification. 2-Methyl-3-hydroxypyridine-5-carboxylic acid oxygenase catalyzes the oxidative pyridine ring opening reaction in vitamin B_6 catabolism (Figure 1) (*20*). This enzyme has been characterized in *Pseudomonas* sp. MA-1 (*21–26*). The product, 2-(acetamidomethylene)-succinate (**8**), could exist as two geometric isomers, *E* (**8**) and *Z* (**27**). The stereochemistry of the enzymatic product had not been previously determined, and the rate of isomer conversion was unknown.

During the chemical synthesis of 2-(acetamidomethylene)-succinate (**8**), we observed that treatment of acetamide with α-formyl diethyl succinate resulted in two compounds which were purified by flash chromatography and characterized by two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) as the *E* (**26**) and *Z* (**25**) isomers of acetamidomethylene diethyl succinate (Figure 1 of the Supporting Information). In the case of the *E* isomer (**26**), the amide hydrogen at 8.9 ppm has a cross-peak that correlates with the allylic hydrogen at 3.3 ppm, while no cross-peak is seen between the vinyl hydrogen at 8.1 ppm and the allylic hydrogen at 3.3 ppm. In case of the *Z* isomer (**25**), no cross-peak is observed between the amide hydrogen at 10.4 ppm and the allylic hydrogen at 3.1 ppm while the vinyl hydrogen at 7.37 ppm has a cross-peak that correlates with the allylic hydrogen at 3.1 ppm.

The biologically relevant isomer of 2-(acetamidomethylene)succinate was enzymatically synthesized starting from 3-hydroxy-2-methylpyridine-4,5-dicarboxylate (**6**) using 3-hydroxy-2-methylpyridine-4,5-dicarboxylate decarboxylase (*8*) and 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (*6*). NMR analysis demonstrated that the enzymatic product was the *E* isomer of 2-(acetamidomethylene)succinate (**8**).

Both the *E* and *Z* isomers of 2-(acetamidomethylene)succinate (**8** and **27**, respectively) were tested as substrates for the mlr6787 gene product. The *E* isomer (**8**) was readily hydrolyzed. In contrast, no hydrolysis was observed for the *Z* isomer (**27**), indicating that the enzyme was specific for the hydrolysis of *E*-isomer (**8**) (Figure 5). There is no detectable nonenzymatic isomerization of (*E*)-2-(acetamidomethylene)-succinate (**8**) over a period of 15 days in 100 mM sodium phosphate buffer (pH 8.0) in D_2O kept at room temperature.

Product Characterization. The 2,4-dinitrophenylhydrazone of sucinnic semialdehyde was chemically synthesized and found to elute at 11.65 min by HPLC, using method B. The reaction time course shown in Figure 4 indicates the depletion of the (*E*)-2-(acetamidomethylene)succinate (**8**) at 4.26 min and the formation of the DNP hydrazone of succinic semialdehyde (**29**) at 11.65 min over time, thereby showing that succinic semialdehyde (**9**) is the product of the enzymatic reaction. No hydrazone (**29**) was formed in the absence of either the enzyme or the substrate.

Further proof that succinic semialdehyde (**9**) is a product of the enzymatic hydrolysis of (*E*)-2-(acetamidomethylene)-succinate (**8**) comes from the coupled assay with succinic semialdehyde dehydrogenase (*14*) in which NADH formed as a result of the coupled enzymatic reaction can be monitored at 340 nm. An increase in absorbance at 340 nm was seen upon addition of succinic semialdehyde dehydrogenase to the reaction mixture containing NAD, (*E*)-2-(acetamidomethylene)succinate (**8**), and the (*E*)-2-(acetamidomethylene)succinate hydrolase. No NADH was formed in the absence of the succinic semialdehyde dehydrogenase or the (*E*)-2-(acetamidomethylene)succinate hydrolase (Figure 6a).

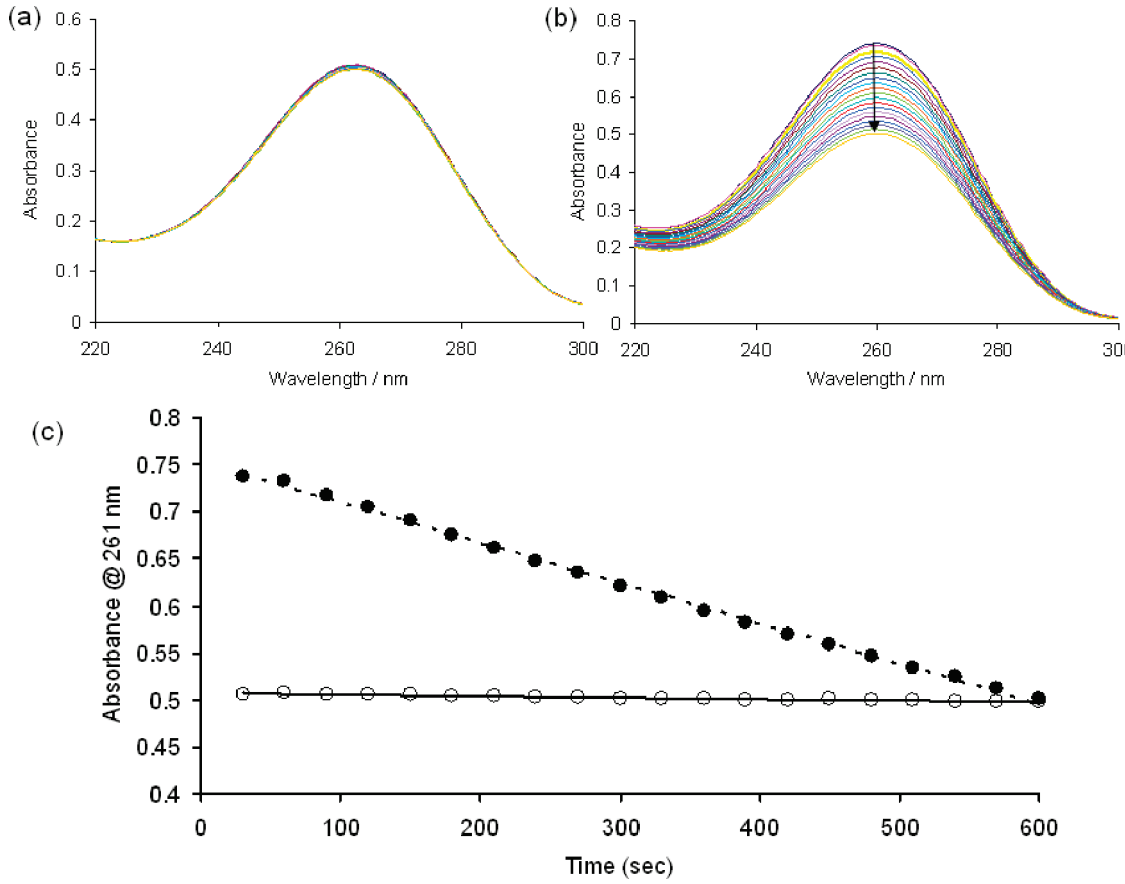

FIGURE 5: (a) Assay of the mlr6787 gene product with (*Z*)-2-(acetamidomethylene)succinate (**27**). The assay mixture consisted of 75 μM (*Z*)-2-(acetamidomethylene)succinate (**27**) and 250 nM freshly purified enzyme. (b) Assay of the mlr6787 gene product with (*E*)-2-(acetamidomethylene)succinate (**8**). The assay mixture consisted of 75 μM (*E*)-2-(acetamidomethylene)succinate (**8**) and 250 nM freshly purified enzyme. (c) Plot showing the decrease in absorbance at 261 nm over time for the *E* and *Z* substrate isomers: (—) (*Z*)-2-(acetamidomethylene)succinate (**27**) and (•••) (*E*)-2-(acetamidomethylene)succinate (**8**).

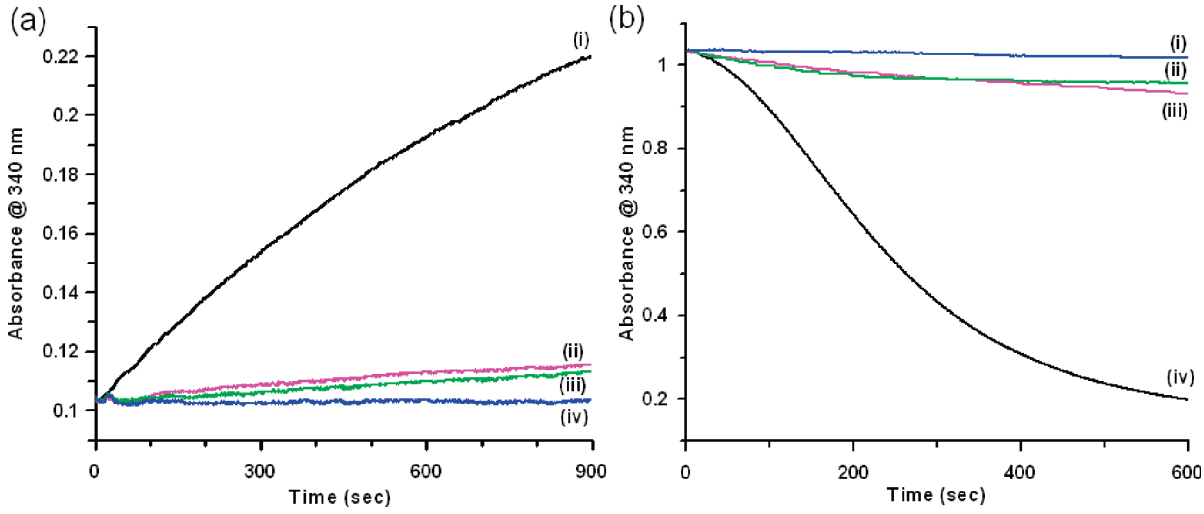

FIGURE 6: Enzymatic assays for the production of succinic semialdehyde and ammonia. (a) Trace (i) shows the formation of NADH (at 340 nm) for the enzymatic reaction mixture (500 μL) consisting of 2 mM NAD, 1 mM β-mercaptoethanol, 135 μM (*E*)-2-(acetamidomethylene)succinate (**8**), 1.2 μM (*E*)-2-(acetamidomethylene)succinate hydrolase, and 17 μM succinic semialdehyde dehydrogenase. Traces (ii)−(iv) show the formation of NADH for the reaction mixture which had all the components of trace (i) except (*E*)-2-(acetamidomethylene)succinate (**8**), (*E*)-2-(acetamidomethylene)succinate hydrolase, and succinic semialdehyde dehydrogenase, respectively. (b) Trace (iv) shows the formation of NADH (at 340 nm) for the enzymatic reaction mixture (500 μL) consisting of 10 units of glutamate dehydrogenase, 10 mM α-ketoglutarate, 1 mM EDTA, 250 μM NADPH, 123 μM (*E*)-2-(acetamidomethylene)succinate (**8**), and 2.4 μM (*E*)-2-(acetamidomethylene)succinate hydrolase. Traces (i)−(iii) show the formation of NADH for the reaction mixture (500 μL) which had all the components of trace (iv) except glutamate dehydrogenase, (*E*)-2-(acetamidomethylene)succinate (**8**), and (*E*)-2-(acetamidomethylene)succinate hydrolase, respectively.

A coupled assay with glutamate dehydrogenase in the presence of α-ketoglutarate was used for the detection of ammonia (*19*). In the presence of ammonia, α-imino glutarate is formed and subsequently reduced to glutamine by glutamate

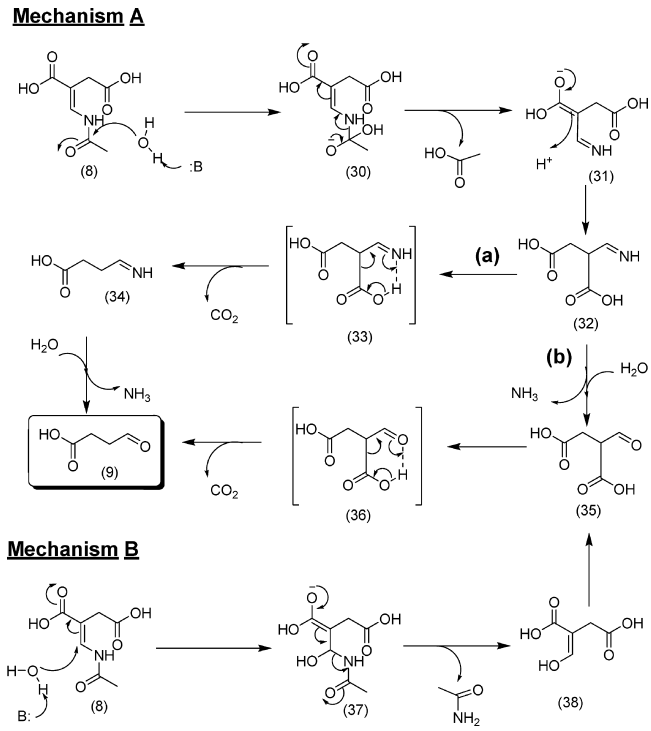

FIGURE 7: Mechanistic analysis of the hydrolysis of (*E*)-2-(acetamidomethylene)succinate (**8**).

dehydrogenase. This enzyme requires NADPH as the cofactor, which is oxidized to NADP during the reaction, resulting in a decrease in absorbance at 340 nm. In the event, the coupled assay with mlr6787 showed a decrease in absorbance at 340 nm. No decrease in absorbance at 340 nm was seen in the absence of either the glutamate dehydrogenase or the (*E*)-2-(acetamidomethylene)succinate hydrolase. This confirmed the production of ammonia during the enzymatic hydrolysis of (*E*)-2-(acetamidomethylene)succinate (**8**) (Figure 6b).

The previously reported hydrolysis of 2-(acetamidomethylene)succinate (**9**) in *Pseudomonas* MA-1 (*9*) and *M. loti* also yields the same products.

Steady State Kinetic Parameters. The (*E*)-2-(acetamidomethylene)succinate (**8**) has a λ_{max} at 261 nm, and the extinction coefficient at 261 nm has been reported to be 18600 M^{-1} cm^{-1} (*9*). A continuous assay at 261 nm at higher concentrations of the substrate was not possible as the initial absorbance was too high to obtain an accurate measurement. To avoid using a discontinuous assay, the extinction coefficients at longer wavelengths were measured. The extinction coefficients of (*E*)-2-(acetamidomethylene)succinate (**8**) at 280, 290, and 295 nm in 100 mM sodium phosphate buffer (pH 8.0) were determined to be 6900, 2000, and 700 M^{-1} cm^{-1}, respectively. Steady state kinetic parameters were obtained from the concentration dependence of the rate of depletion of (*E*)-2-(acetamidomethylene)succinate (**8**) at a constant concentration of 2-(acetamidomethylene)succinate hydrolase. The enzymatic reaction exhibited Michealis−Menten kinetics with K_M and k_{cat} values of 143 μM and 0.6 s^{-1}, respectively. The k_{cat}/K_M for HMPDdc was determined to be 4196 M^{-1} s^{-1} (Figure 3).

Mechanistic Analysis. Two mechanisms for the production of succinic semialdehyde are shown in Figure 7. In mechanism A (*9*), the amide bond of (*E*)-2-(acetamidomethylene)succinate (**8**) is first hydrolyzed to give acetic acid and the intermediate (**32**) which could then undergo a decarboxylation followed by deamination to generate succinic semialdehyde (**9**). The order of these two steps could also be reversed. Mechanism B predicts a conjugate addition of water to the (*E*)-2-(acetamidomethylene)succinate (**8**) to generate intermediate **37** which could then eliminate acetamide resulting in **38**. Facile tautomerization would give **35** which could then be decarboxylated to produce succinic semialdehyde (**9**). Since the enzymatic hydrolysis of (*E*)-2-(acetamidomethylene)succinate (**8**) generates ammonia rather than acetamide and acetamide is stable under our reaction conditions, the hydrolysis of (*E*)-2-(acetamidomethylene)-succinate (**8**) is likely to proceed via mechanism A.

Comparative Genomics Analysis. (*E*)-2-(Acetamidomethylene)succinate hydrolase catalyzes the last step in the degradative pathway of PLP, generating products that can be directly incorporated into primary metabolism. The identification of its gene completes the gene assignment of all the enzymes involved in PLP catabolism in *M. loti.* Surprisingly, when we search for an equivalent pathway in available sequenced bacterial genomes using the SEED database, we find that this pathway is unique to *M. loti*, suggesting that other PLP catabolic pathways still remain to be discovered.

ACKNOWLEDGMENT

We gratefully acknowledge the help of Dr. Yasunobu Ohkawa (National Institute of Agrobiological Sciences, Ibaraki, Japan) for providing us with the *M. loti* MAFF303090 strain, Dr. Cynthia Kinsland for preparation of the overexpression plasmid, Dr. Carl Nathan (Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY) for providing us with the MtbH37*Rv gabD1* (Rv0234c) plasmid, Dr. Ivan Kereszteś and Anthony Condo for their assistance with the NOESY experiment, and Dr. Jeremiah Hanes for insightful discussions.

SUPPORTING INFORMATION AVAILABLE

Two-dimensional NOESY spectrum of (*E*)-2-(acetamidomethylene)succinate (**8**) and (*Z*)-2-(acetamidomethylene)-succinate (**27**). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI8004158