Delineation of the Caffeine C-8 Oxidation Pathway in *Pseudomonas* sp. Strain CBB1 via Characterization of a New Trimethyluric Acid Monooxygenase and Genes Involved in Trimethyluric Acid Metabolism

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The molecular basis of the ability of bacteria to live on caffeine via the C-8 oxidation pathway is unknown. The first step of this pathway, caffeine to trimethyluric acid (TMU), has been attributed to poorly characterized caffeine oxidases and a novel quinone-dependent caffeine dehydrogenase. Here, we report the detailed characterization of the second enzyme, a novel NADH-dependent trimethyluric acid monooxygenase (*TmuM*), a flavoprotein that catalyzes the conversion of TMU to 1,3,7-trimethyl-5-hydroxyisourate (TM-HIU). This product spontaneously decomposes to racemic 3,6,8-trimethylallantoin (TMA). *TmuM* prefers trimethyluric acids and, to a lesser extent, dimethyluric acids as substrates, but it exhibits no activity on uric acid. Homology models of *TmuM* against uric acid oxidase *HpxO* (which catalyzes uric acid to 5-hydroxyisourate) reveal a much bigger and hydrophobic cavity to accommodate the larger substrates. Genes involved in the caffeine C-8 oxidation pathway are located in a 25.2-kb genomic DNA fragment of CBB1, including *cdhABC* (coding for caffeine dehydrogenase) and *tmuM* (coding for *TmuM*). Comparison of this gene cluster to the uric acid-metabolizing gene cluster and pathway of *Klebsiella pneumoniae* revealed two major open reading frames coding for the conversion of TM-HIU to S-(+)—trimethylallantoin [S-(+)-TMA]. The first one, designated *tmuH*, codes for a putative TM-HIU hydrolase, which catalyzes the conversion of TM-HIU to 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU). The second one, designated *tmuD*, codes for a putative TM-OHCU decarboxylase which catalyzes the conversion of TM-OHCU to S-(+)-TMA. Based on a combination of enzymology and gene-analysis, a new degradative pathway for caffeine has been proposed via TMU, TM-HIU, TM-OHCU to S-(+)-TMA.

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid, is one of the most widely used psychoactive substances in the world, with an estimated global consumption of 120,000 tons per year (13). The average consumption of caffeine in humans ranges from 80 to 400 mg per person per day (14). The main mode of entry of caffeine and other natural xanthines into the human system and subsequently into the environment is through coffee, tea, caffeine-containing cola drinks, and chocolates. Caffeine is also a large environmental contaminant introduced to the environment from agro-industrial wastes, such as husk and pulp water, which are released by coffee and tea manufacturers (36). The implications of caffeine consumption on human health and environment have resulted in a great deal of scientific and public interest in caffeine and its biodegradation (1, 2, 15; http://www.uiowa.edu/~biocat/).

Humans metabolize caffeine mainly via N-dealkylation catalyzed by the hepatic cytochrome P450s 1A2 and 2E1 (2). Caffeine transformation by N-dealkylation has also been documented in microorganisms such as bacteria, fungi, and yeast (4, 6). Although N-dealkylase activity has been detected in these microorganisms as early as the 1970s (3, 41), the metabolic pathway, enzymology, and molecular genetics of N-dealkylation, including *ndm* genes, were elucidated only recently in *Pseudomonas putida* CB85 (37, 38, 43). Highly specific *N₁-, N₃-, and N₇*-N-dealkylases convert caffeine and other methylxanthines to xanthine and formaldehyde. This organism consumes both of these compounds.

The alternate pathway of caffeine transformation via C-8 oxidation is much less characterized. Only two early studies (28, 29) have reported the presence of caffeine oxidases involved in C-8 oxidation. However, these enzymes and the corresponding reactions catalyzed by them are poorly characterized. Recently, our group reported the purification and characterization of a novel caffeine dehydrogenase (*Cdh*) from *Pseudomonas* sp. strain CB85, a bacterium capable of growth on caffeine as the sole source of carbon and nitrogen. This heterotrophic caffeine dehydrogenase oxidized caffeine at the C-8 position to form 1,3,7-trimethyluric acid (TMU) with coenzyme Q$_0$ as the preferred electron acceptor (42). The enzyme was specific for caffeine, much less active on theobromine but had no activity on xanthine (42). The enzymology and genes involved in further transformation of TMU have not been elucidated. Madyastha and Sridhar (27) reported that TMU was further degraded to 3,6,8-trimethylallantoin (TMA) by resting cells of a mixed culture of *Rhodococcus* and *Klebsiella*. However, details of this reaction are lacking.

In the present study, we report the enzymology and molecular characterization of TMU conversion to 1,3,7-trimethyl-5-hydroxyisourate (TM-HIU), catalyzed by a novel NADH-dependent oxidase. Here, we report the detailed characterization of the second enzyme, a novel NADH-dependent trimethyluric acid monooxygenase (*TmuM*), a flavoprotein that catalyzes the conversion of TMU to 1,3,7-trimethyl-5-hydroxyisourate (TM-HIU). This product spontaneously decomposes to racemic 3,6,8-trimethylallantoin (TMA). *TmuM* prefers trimethyluric acids and, to a lesser extent, dimethyluric acids as substrates, but it exhibits no activity on uric acid. Homology models of *TmuM* against uric acid oxidase *HpxO* (which catalyzes uric acid to 5-hydroxyisourate) reveal a much bigger and hydrophobic cavity to accommodate the larger substrates. Genes involved in the caffeine C-8 oxidation pathway are located in a 25.2-kb genomic DNA fragment of CBB1, including *cdhABC* (coding for caffeine dehydrogenase) and *tmuM* (coding for *TmuM*). Comparison of this gene cluster to the uric acid-metabolizing gene cluster and pathway of *Klebsiella pneumoniae* revealed two major open reading frames coding for the conversion of TM-HIU to S-(+)—trimethylallantoin [S-(+)-TMA]. The first one, designated *tmuH*, codes for a putative TM-HIU hydrolase, which catalyzes the conversion of TM-HIU to 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU). The second one, designated *tmuD*, codes for a putative TM-OHCU decarboxylase which catalyzes the conversion of TM-OHCU to S-(+)-TMA. Based on a combination of enzymology and gene-analysis, a new degradative pathway for caffeine has been proposed via TMU, TM-HIU, TM-OHCU to S-(+)-TMA.
trimethyluric acid monoxygenase (TmuM), a flavoprotein. TM-H1U is unstable and is readily converted nonenzymatically to racemic TMA. TmuM has no activity on uric acid. Analysis of a 25.2-kb gene cluster containing genes encoding Cdh (cdhABC) and TmuM (tmuM) identified two other open reading frames (ORFs), which are proposed to be responsible for the enzymatic conversion of TM-H1U to S(-)-TMA via 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU). A pathway for caffeine transformation in CBB1 has been proposed that is based on a similar analysis of the uric acid degradation pathway in Klebsiella pneumoniae (8). This is the first report of enzymatic and genetic characterization of caffeine transformation via C-8 oxidation.

MATERIALS AND METHODS

Chemicals. Caffeine, 1,3,7-trimethyluric acid (TMU), 3,7-dimethyluric acid, 1,3-dimethyluric acid, 1-methyluric acid, and uric acid were all purchased from Sigma-Aldrich (St. Louis, MO). Yeast nitrogen base without amino acids and ammonium sulfate (YNB) was obtained from ForMedium (Norfolk, United Kingdom). High-pressure liquid chromatography (HPLC)-grade methanol, chloroform, hexane, and ethanol were obtained from Sigma-Aldrich.

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are listed in Table S1 in the supplemental material. Media and growth conditions. Strain CBB1 was grown in M9 mineral salts medium (34) with 2.5 g of caffeine liter−1 and 4 g of YNB liter−1 at 30°C with shaking at 200 rpm. Cell growth was monitored by measuring the optical density at 600 nm (OD600). Cells were harvested at the late log phase by centrifugation (13,800 × g for 10 min at 4°C) as described by Yu et al. (42). Cell pellets were washed twice with 50 mM potassium phosphate (pH 7.5) buffer. Proteins were eluted from the column with the same buffer containing 0.5 M ammonium sulfate. Bound proteins were eluted with a reverse linear gradient of ammonium sulfate (0.5 to 0 M in KP, buffer) at a flow rate of 1 ml min−1. Fractions exhibiting TMU oxidation activity were pooled, concentrated, and exchanged into 50 mM KP buffer (pH 7.5). PCR amplification of the gene catalyzing TMU oxidation from CBB1 genome. The forward degenerate primer tmuM-degF1 was designed based on the lowest degeneracy of amino acids (KIGADVT) in the N-terminal sequence of the purified protein (see Table S2 and Fig. S1 in the supplemental material). A BLASTp search of the N-terminal amino acid sequence showed significant homology with five proteins (GenBank accession nos. ACF60813, EAA44903, and CAJ95547), including a FAD-binding monoxygenase (GenBank accession no. EDZ74364) and salicylate 1-monoxygenase (GenBank accession no. AAA62959). A CLUSTAL W2 (25) alignment of these five homologous proteins identified a conserved region near the 3’ end of this multiple sequence alignment (see Fig. S1 in the supplemental material). Reverse degenerate primer tmuM-degR1 was designed from this conserved region (see Table S2 and Fig. S1 in the supplemental material). PCR using tmuM-degF1 and tmuM-degR1 was conducted using PfuUltra DNA polymerase HF (Agilent Technologies, La Jolla, CA) with fosmid DNA pMVS848 as a template. Fosmid library construction and isolation of fosmid clone E. coli EPI300(pMVS848) are described in the supplemental material. The PCR conditions were 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplified PCR product was cloned into vector pSC-B-amp/kan (Agilent Technologies), and three clones were chosen for DNA sequencing. The specific primers tmuM-up1, tmuM-up2, tmuM-down1, and tmuM-down2 (see Table S2 in the supplemental material) were designed from the DNA sequence of this amplified PCR product and used to sequence the flanking region of the PCR product with fosmid DNA pMVS848 as a template. Protein expression and purification. Primers tmuM-F-NdeI and tmuM-R-NotI (see Table S2 in the supplemental material) were used with the fosmid DNA pMVS848 as the template to amplify the entire coding gene sequence of TMU-oxidizing enzyme. The forward primer was also designed by removing the wild-type stop codon so that the histidine tag contained in the pET32a vector would be fused to the expressed protein. PCRs were carried out with PfuUltra DNA polymerase. The amplification product was cloned into pSC-B-amp/kan to generate pSC-tmuM. The restriction fragment containing the tmuM gene obtained from NdeI-NotI digestion of pSC-tmuM was ligated into the dephosphorylated vector, pET32a (Novagen), previously digested by NdeI and NotI. DNA sequencing confirmed that no mutation was introduced in the gene encoding the TMU-oxidizing enzyme in plasmid pET32a. The resulting plasmid pMVS848a (see Table S1 in the supplemental material) was electroporated into E. coli BL21(DE3) cells. The expression of recombinant Histagged TMU-oxidizing enzyme in E. coli BL21(DE3) and the two-step purification using metal affinity and ion-exchange chromatography are described in the supplemental material. Protein quantitation, the determination of native and subunit molecular weights, the N-terminal sequence, the bound flavin content, the kinetic parameters, the oxygen re-
A typical TMU oxidation activity assay was carried out at 30°C in a 1-ml reaction mixture containing 0.5 mM NADH, 0.5 mM TMU, and appropriate amounts of crude cell extract, fractions from FPLC, or purified protein, in 50 mM KP buffer (pH 7.5). The reaction was initiated by the addition of TMU. NADH oxidation was monitored at 340 nm using a UV-Visible spectrophotometer (Shimadzu UV-2450). An extinction coefficient of 6,200 M⁻¹ cm⁻¹ for reduced minus oxidized NADH was used in calculating the enzyme activity. One unit of activity was defined as the amount of protein required to oxidize 1 μmol of NADH per min under defined reaction conditions. The TMU oxidation activity was also determined by measuring the disappearance of trimethyluric acid from the reaction mixture by HPLC analysis. Periodically, 10 μl from the reaction mixture was mixed with equal volume of acetonitrile for quantifying trimethyluric acid.

**Incorporation of ^18^O_2 into trimethyluric acid.** An ^18^O_2 (Cambridge Isotope Laboratories, Inc., Andover, MA) incorporation experiment was conducted in a 1-ml (total volume) reaction mixture containing, 0.5 mM TMU, 0.5 mM NADH, and 10 μl of purified recombinant TMU-oxidizing enzyme. The reaction mixture was transferred into a modified 0.5 l round-bottom flask with two side arms and a quartz cuvette fused at the bottom. The enzyme was pipetted into one side arm. After the air in the flask was evacuated, argon was introduced. The enzyme in the side arm was tilted into the reaction mixture. NADH consumption was monitored at 340 nm at 30°C to confirm there is no oxidation under anaerobic conditions. ^18^O_2 was then introduced into the reaction vessel to initiate the reaction. The enzyme activity was monitored by the decrease of absorbance at 340 nm. After 30 min, an aliquot of the reaction mixture was mixed with an equal volume of acetonitrile to precipitate the protein. The supernatant was then analyzed by an HPLC system equipped with a Shimadzu LCMS-2010EV single-stage quadruple mass analyzer. A parallel control reaction was carried out with ^18^O_2 (air), and the product was analyzed.

**Liquid chromatography-mass spectrometry (LC-MS) analysis of time course of product formation.** The time course of TMU-oxidizing enzyme-catalyzed reaction with TMU was conducted with the recombinant enzyme. Aliquots sampled from the reaction mixture were mixed with equal volumes of acetonitrile to stop the reaction. Identification and quantification of trimethyluric acid, reaction intermediates, and end products (Rf 0.59) and the reaction mixture was transferred into a modified 0.5 l round-bottom flask with two side arms and a quartz cuvette fused at the bottom. The enzyme was pipetted into one side arm. After the air in the flask was evacuated, argon was introduced. The enzyme in the side arm was tilted into the reaction mixture. NADH consumption was monitored at 340 nm at 30°C to confirm there is no oxidation under anaerobic conditions. ^18^O_2 was then introduced into the reaction vessel to initiate the reaction. The enzyme activity was monitored by the decrease of absorbance at 340 nm. After 30 min, an aliquot of the reaction mixture was mixed with an equal volume of acetonitrile to precipitate the protein. The supernatant was then analyzed by an HPLC system equipped with a Shimadzu LCMS-2010EV single-stage quadruple mass analyzer. A parallel control reaction was carried out with ^18^O_2 (air), and the product was analyzed.

**Homology modeling.** A BLASTp search with TMU-oxidizing enzyme and HpxO (30) as queries against the PDB identified a flavin-containing hydroxylase involved in pyocyanin biosynthesis (PhzS) from *Pseudomonas aeruginosa* (16) as the best match, with 30% sequence identity to both. PhzS belongs to the family of flavin-dependent aromatic hydroxylases, of which the best characterized is p-hydroxybenzene hydroxylase (PHBH). Homology models for TMU-oxidizing enzyme and HpxO were generated by using the program Modeller 9.10 (9) and PhzS (PDB ID 3C96) as the template. The homology models (ten for each protein) were superimposed on PhzS and PHBH (PDB ID 1PBE) using PyMOL 1.5 (Schrödinger, LLC), and the FAD- and substrate-binding cavities were identified. The ligand-bound structure of VOIDOO (23) was used to calculate the solvent-accessible volumes of the active site cavities for each of the models and then averaged for each protein.

**RESULTS**

**Purification of TMU-oxidizing enzyme from cell extracts of strain CBB1.** The consumption of trimethyluric acid in crude cell extract was dependent on the presence of NADH (data not shown). While 0.2 mM TMU was completely utilized in 15 min by crude cell extract (200 μg/ml) in the presence of NADH, only marginal activity was observed without the cofactor. Activity was not observed with boiled cell extract. TMU-oxidizing enzyme was purified 55-fold, but with a low recovery of 0.2%. In the absence of TMU, no NADH oxidation was observed with the purified enzyme. The specific activity of the purified enzyme was 4128 mU per mg (Table 1, Fig. S2). The molecular mass of TMU-oxidizing enzyme as estimated by SDS-PAGE gel electrophoresis was ~43 kDa (see Fig. S2 in the supplemental material), and its N-terminal sequence was determined to be SRPLRVTTIIGAGIGGLSAAVALR (KIGADVYT).

**Cloning, expression, and purification of TMU-oxidizing enzyme from CBB1.** PCR using the primers tmuM-degFl and tmuM-degR1 successfully amplified a single 900-bp PCR product from the fosmid DNA pMVS848 (see Table S1 in the supplemen-
A BLASTX search using the DNA sequence of this 900-bp product showed that this amplified partial gene has significant homology to the FAD-binding monoxygenase family of proteins. Further sequencing of the flanking region of this partial gene revealed a complete ORF with 1,191 nucleotides (nt). The gene was designated tmuM, and it encoded a 396-amino-acid protein with a theoretical Mr of 42,619 and a pl of 6.12. N-terminal sequence derived from the tmuM gene matched the experimentally determined N terminus of the purified TMU-oxidizing enzyme from CBB1. The amplified gene was cloned into the pET32a plasmid with a His6 tag at the C-terminal end. The recombinant protein was overexpressed in a soluble form and purified to electrophoretic homogeneity using immobilized-metal affinity chromatography and Q-Sepharose chromatography (see Fig. S2 in the supplemental material). The specific activity of the recombinant TMU-oxidizing enzyme was 9,700 mU per mg, which is twice that of the purified enzyme from CBB1. Given the higher activity of the recombinant enzyme, this preparation was used for further characterization.

Biochemical characterization of TMU-oxidizing enzyme.

The recombinant TMU-oxidizing enzyme was similar to the enzyme purified from CBB1 in terms of the native (−45-kDa) and subunit (~43-kDa) molecular mass, and UV-visible absorption spectrum (see Fig. S2 in the supplemental material). Both the native and the recombinant enzymes were monomeric with UV-visible absorption spectrum maxima at 450, 380, and 280 nm, which is characteristic of a bound flavin cofactor. HPLC analysis of the cofactor released from the recombinant enzyme upon heat denaturation was identified as FAD with TmuM:FAD stoichiometry (1:1; data not shown). The apparent Km for TMU and the kcat of the recombinant enzyme in 50 mM KP, (pH 7.5) at 30°C were 10.2 ± 2.2 μM and 448.9 ± 21.7 min⁻¹, respectively (Table 2).

Characterization of the enzyme as TMuM.

When the enzyme reaction was carried out in a completely anaerobic environment, no TMU disappearance or NADH oxidation was observed. Upon the introduction of 18O₂ into the reaction chamber, activity was immediately observed and was monitored spectrophotometrically. The end product of the reaction was characterized by ESI-LC/MS analysis as TMA. TMA produced in the presence of atmospheric O₂ had an m/z of 201, which agreed with the (M + 1)⁺ ion of the compound (Fig. 1b). TMA produced in the presence of 18O₂ had an m/z of 203, indicating the incorporation of one atom of isotopic oxygen (Fig. 1a). Further characterization of TMA is described in detail in the product characterization and product identification sections below. Monitoring the oxygen consumption in a Clark-type oxygen electrode and simultaneously quantifying TMU disappearance by HPLC established the stoichiometry of the reaction. After 5 min of reaction, 190.5 nmol of O₂ was consumed, while 185 nmol of TMU was degraded. NADH to TMU stoichiometry was also established as 1:1 in a separate experiment by monitoring both NADH oxidation and TMU disappearance. Based on these results, the TMU-oxidizing enzyme was designated as TMU monoxygenase (TmuM).

Substrate specificity of TmuM.

The apparent kinetic parameters of the recombinant enzyme with various tri-, di-, and monomethyluric acids and uric acid are shown in Table 2. Initial activity assays with wild-type TmuM and recombinant TmuM-His₆ suggested that the enzyme was active on TMU and other methyluric acids, but not on uric acid (data not shown). The catalytic activity kcat (turnover number) of TmuM-His₆ for TMU was found to be 2.5, 3.8, and 15.4 times higher than that of 1,3- and 3,7-dimethyluric acid and 1-methyluric acid, respectively (Table 2). These results suggested that TmuM is the preferred substrate for TMuM, followed by 1,3-dimethyluric acid, 3,7-dimethyluric acid, and 1-methyluric acid. In contrast, the catalytic efficiency kcat/Km for 3,7-dimethyluric acid was two times higher than that of TMU due to an order of magnitude lower Km. 1-Methyluric acid was the least preferred substrate, although its Km was only an order of magnitude lower than that of TMU (Table 2).

Detailed characterization of the product of TmuM-catalyzed reaction.

The time course of TMU oxidation and product formation catalyzed by recombinant TmuM-His₆ was monitored by LC-MS (Fig. 1d). Initial HPLC analysis showed that TMU was completely consumed while NADH was converted to NAD⁺ (data not shown). Since no product peak could be detected when UV absorption was used as the detection method during HPLC analysis, the intermediates and product formation were subsequently monitored by ESI-MS (Fig. 1d). The ESI-LC/MS analysis data were monitored in the positive-ion mode at m/z = 211, 227, 245, or 201, based on the expected products formed from TMU transformation, which were hypothesized to be TM-HIU (molecular weight [MW], 226), TM-OHCU (MW, 244), or TMA (MW, 200),

<table>
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<th>Substrate</th>
<th>kcat (min⁻¹)</th>
<th>Km (μM)</th>
<th>kcat/Km (min⁻¹ μM⁻¹)</th>
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<tr>
<td>Trimethyluric acid</td>
<td>448.9 ± 21.7</td>
<td>10.2 ± 2.2</td>
<td>44.1 ± 2.1</td>
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<td>1,3-Dimethyluric acid</td>
<td>185.0 ± 16.4</td>
<td>126.5 ± 29.3</td>
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<td>3,7-Dimethyluric acid</td>
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<td>1.3 ± 0.6</td>
<td>89.4 ± 6.3</td>
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<td>1-Methyluric acid</td>
<td>29.1 ± 2.4</td>
<td>1.2 ± 0.5</td>
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<tr>
<td>Uric acid</td>
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<th>Yield (%)</th>
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<td>55</td>
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</table>

The data suggest that TmuM has significant activity on TMU as the substrate, with a preference for 1-methyluric acid. However, further analysis is required to confirm the identity of the intermediates and the final product formed from TMU oxidation.
respectively. Corresponding nonmethylated metabolites have been reported for the uric acid pathway (8, 31, 32). Selected ion monitoring (SIM) revealed the presence of substrate TMU, metabolite II, and metabolite IV with \( m/z = 211 \), \( m/z = 227 \) (Fig. 1c), and \( m/z = 201 \) (Fig. 1b), respectively. The observed molecular masses of 226 (with \( m/z = 227 \) for metabolite II and 200 (with \( m/z = 201 \) for metabolite IV matched to that of TM-HIU and TMA, respectively. Metabolite II at \( m/z = 227 \) could be detected initially but disappeared after 10 min (Fig. 1d). In contrast, TMA at \( m/z = 201 \) continued to accumulate until the end of the reaction (Fig. 1d). Based on these results, and the observation of 5-hydroxyuric acid in the uric acid degradation pathway (22), metabolite II (TM-HIU) was proposed as the true product of TMU monooxygenase reaction. Metabolite II (TM-HIU) was unstable under the reaction condition and spontaneously converted to TMA (possibly via metabolite III), as is the case in the uric acid degradation pathway (8, 31, 32). Metabolite III (TM-OHCU) could not be detected.

**Identification of metabolite IV as racemic TMA.** The structure of metabolite IV (TMA), purified from the TmuM reaction by using preparative-scale thin-layer chromatography, was established by spectroscopic analyses. HRESIMS showed that metabolite IV \( M^+ \) was \( m/z = 200.0907 \), indicating an empirical formula of \( C_7H_{12}N_4O_3 \) (calculated value, 200.0909). Thus, metabolite IV contained two hydrogen atoms more and one carbon atom less than TMU. The \( ^1H \) NMR spectrum of metabolite IV contained all signals of TMU, in addition to two extra signals (Table 3). These included signals for a new secondary amine proton at 5.77 ppm for \( H-8 \) (singlet, broad) and a doublet at 5.19 ppm (J = 7.77 Hz) for \( H-5 \) coupled to the \( H-1 \) signal of the secondary amine group. The \( ^13C \) NMR spectra of metabolite IV showed one less signal than TMU but contained all three methyl group signals at 40.7 ppm.

<table>
<thead>
<tr>
<th>H</th>
<th>Chemical shift (ppm)</th>
<th>Peak type</th>
<th>J coupling (Hz)</th>
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<td>H-1 (1H)</td>
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<td></td>
<td>C-7 (CO)</td>
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<td>Doublet</td>
<td>4.6</td>
<td>C-10 (CH₃)</td>
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<tr>
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<td></td>
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<td></td>
<td>C-11 (CH₃)</td>
<td>24.9</td>
</tr>
</tbody>
</table>
From these data it is clear that loss of the carbon atom in metabolite IV was not due to N-demethylation but occurs via ring rupture. The $^{13}$C NMR spectrum of metabolite IV also showed two carbonyl group signals at 171.5 ppm (C-2) and 158.3 ppm (C-7) and one altered carbonyl group signal at 156.1 ppm (C-4), which is farther downfield than the original TMU signal. In addition, the spectrum of metabolite IV contained signal for a new secondary carbon atom at 65.93 ppm (C-5). Signal assignments and connectivities were confirmed using COSY, HMBC, and HMQC spectral editing. COSY analysis correlated H-8 with H-11 and correlated H-1 with H-5. HMBC showed 3-bond correlations between H-11 and C-7, i.e., H-9 between C-2 and C-4, H-10 between C-5 and C-7, and H-5 between C-2 and C-10. Based on all of these data, metabolite IV was characterized as TMA. It should be pointed out that there is no commercial sample of TMA available.

The specific rotation ($[\alpha]_25^D$) of metabolite IV was close to zero ($0.003^\circ$, concentration of 0.2 g/100 ml, CHCl$_3$). This indicates that the stereochemistry of the C-5 atom (in metabolite IV) does not correspond to one specific enantiomer but rather is a racemic mixture of two enantiomers. The racemic nature of the product was further confirmed by chiral stationary-phase HPLC with a Chiralcel IA column. Pure metabolite IV, which eluted as a single peak at retention time 8.14 min with reversed-phase C$_{18}$ column, resolved into two separate peaks of equal peak area with retention times of 10.56 and 12.35 min, respectively (see Fig. S3a in the supplemental material). The ESI-LC-MS analysis confirmed the molecular mass of both peaks as 200 (see Fig. S3b in the supplemental material). These results further suggest that metabolite IV (TMA) isolated from the TmuM reaction is a racemic mixture of two enantiomers of TMA.

Identification of cdhA, cdhB, cdhC, tmuM, tmuH, and tmuD genes in CBB1. Previously, caffeine dehydrogenase (Cdh) purified from CBB1 was proposed to have an αβγ (α [90-kDa], β [32-kDa], and γ [20-kDa])-subunit structure (42). PCR based on the primers (see Table S2 in the supplemental material) designed from the N-terminal protein sequence of the large (90-kDa) and medium (40-kDa) subunits of caffeine dehydrogenase resulted in amplification of a 2,392-nt DNA fragment from CBB1 genome (see Table S2 in the supplemental material). This amplified fragment contained a 2,376-nt ORF, which encoded an 87-kDa product. Its deduced protein sequence was identical to the N-terminal protein sequence of Cdh large subunit. Therefore, this 2,376-nt ORF was designated cdhA (GenBank accession no. ADH15879, ADH15880, ADH15881, J743481, J743482, and J743483, respectively).

FIG 2 Physical map of genes for caffeine transformation in a 25.2-kb gene cluster in Pseudomonas sp. strain CBB1. Genes are denoted by arrows, and the extents and directions of transcription are indicated. The two genetic modules are denoted by the substrate upon which the gene products act: caffeine, black; and trimethyluric acid, dark gray. The sequence similarity and function assignment of the ORF-encoded proteins are indicated in the table.

### Table

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of codons</th>
<th>Proposed / confirmed Function</th>
<th>GenBank accession number: Homologous protein: % Identity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>tmuH</td>
<td>114</td>
<td>TM-HIU hydrolase</td>
<td>YP_002976942 Hydroxysoritate hydrolase 50% Q92UG5</td>
</tr>
<tr>
<td>tmuD</td>
<td>291</td>
<td>TM-OHCU deacarboxylase</td>
<td>YP_003695277 OHCU deacarboxylase 39% D4GPU8</td>
</tr>
<tr>
<td>tmuM</td>
<td>385</td>
<td>TMU monoxygenase</td>
<td>YP_003741647 FAD-binding monoxygenase 38% Q40412</td>
</tr>
<tr>
<td>cdhA</td>
<td>791</td>
<td>Cdh molybdopterin binding subunit</td>
<td>YP_00335893 Xanthine dehydrogenase 49% P19919</td>
</tr>
<tr>
<td>cdhB</td>
<td>297</td>
<td>Cdh FAD-binding subunit</td>
<td>ADV16272 Alcohol dehydrogenase 39% P19914</td>
</tr>
<tr>
<td>cdhC</td>
<td>167</td>
<td>Cdh [2Fe-2S]-binding subunit</td>
<td>YP_002521823 aldehyde oxide small 59% P19915</td>
</tr>
</tbody>
</table>

*% identity was determined by aligning the gene product of each orf with the homologous protein using ClustalW2 (25).

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(C-9), 26.8 ppm (C-10), and 24.9 ppm (C-11) (Table 3). From these data it is clear that loss of the carbon atom in metabolite IV was not due to N-demethylation but occurs via ring rupture. The $^{13}$C NMR spectrum of metabolite IV also showed two carbonyl group signals at 171.5 ppm (C-2) and 158.3 ppm (C-7) and one altered carbonyl group signal at 156.1 ppm (C-4), which is farther downfield than the original TMU signal. In addition, the spectrum of metabolite IV contained signal for a new secondary carbon atom at 65.93 ppm (C-5). Signal assignments and connectivities were confirmed using COSY, HMBC, and HMQC spectral editing. COSY analysis correlated H-8 with H-11 and correlated H-1 with H-5. HMBC showed 3-bond correlations between H-11 and C-7, i.e., H-9 between C-2 and C-4, H-10 between C-5 and C-7, and H-5 between C-2 and C-10. Based on all of these data, metabolite IV was characterized as TMA. It should be pointed out that there is no commercial sample of TMA available.

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stream of cdhA (Fig. 2). While the first ORF encoded a 32.5-kDa product with its deduced protein sequence matching the N-terminal protein sequence of Cdh/H9252-subunit, the second ORF encoded an 18.4-kDa product, which matched the molecular weight of Cdh/42. Based on these results, the 888-nt ORF was designated cdhB (GenBank accession no. ADH15880), and the 528-nt ORF was designated cdhC (GenBank accession no. ADH15881). A BLASTX search of full-length cdhA, cdhB, and cdhC against the nonredundant protein sequence (nr) database identified the molybdopterin-binding subunit of a putative xanthine dehydrogenase (GenBank accession no. YP_003395893), the medium subunit of a bifunctional hydratase/alcohol dehydrogenase (GenBank accession no. ADV16272) involved in anaerobic transformation of cyclohexanol, and the small subunit of a putative aldehyde oxidase (GenBank accession no. ADH15881) as the best hits, with 49, 39, and 59% identities, respectively. Search against the Swiss-Prot database identified the large (GenBank accession no. P19919), medium (GenBank accession no. P19914), and small (GenBank accession no. P19915) subunits of carbon monoxide dehydrogenase as the best hits, with 49, 39, and 59% identities, respectively. Search against the Swiss-Prot database identified the large (GenBank accession no. P19919), medium (GenBank accession no. P19914), and small (GenBank accession no. P19915) subunits of carbon monoxide dehydrogenase as the best hits, with 49, 39, and 59% identities, respectively (Fig. 2). Xanthine dehydrogenase, hydratase/alcohol dehydrogenase, aldehyde oxidase, and carbon monoxide dehydrogenase have an αβγ- subunit structure like that of Cdh and belong to the molybdopterin-binding oxidoreductase family containing molybdopterins, FAD, and [2Fe-2S] clusters as cofactors (5, 19). The location of the tmuM gene encoding the enzyme TmuM was also identified and amplified from the fosmid DNA pMVS848 (see Table S1 in the supplemental material). A BLASTX search of tmuM against the nr database identified a FAD-binding monoxygenase from Erwinia billingiae Eb661 (Fig. 2) and HpxO (GenBank accession no. ACF60813) from Klebsiella pneumonia as the best hits, with sequence identities of 38 and 37%, respectively.

Identification of the genes for the first two reactions of the C-8 oxidation pathway for caffeine metabolism (Fig. 3) in fosmid DNA pMVS848 prompted us to sequence the flanking regions of cdhA, cdhC, and tmuM genes. A total of 25.2 kb of the DNA sequence was obtained from the fosmid DNA pMVS848. Computational analysis of this 25.2-kb genomic DNA sequence identified 21 complete ORFs, including the cdhA, cdhB, cdhC, and tmuM genes and two incomplete ORFs (Fig. 2). A BLASTX search of tmuH, the first complete ORF in the gene cluster (Fig. 2), against the nr database identified a putative hydroxyisourate (HIU) hydrolase from Rhizobium leguminosarum bv. Trifolii WSM1325 (GenBank accession no. YP_00297642) as the best match with 50% identity. When the same search was performed against the Swiss-Prot database, putative HIU hydrolase 2 from Sinorhizobium meliloti 1021 (GenBank accession no. Q92UG5) was identified as the best match with 44% identity. Similarly, a BLASTX search of tmuD, the second complete ORF (Fig. 2) in the gene cluster, against the nr database identified putative OHCU decarboxylase from Starkeya novella DSM 506 (GenBank accession no. YP_003695277) with 39% sequence identity. The best hit from the Swiss-Prot database was putative OHCU decarboxylase from Haloferax volcanii DS2 (GenBank accession no. D4GPU8) with 37% identity (Fig. 2). Thus, on the basis of these sequence simi-
larities, *tmuH* encoding a 114-amino-acid product and *tmuD* encoding a 291-amino-acid product were designated putative 1,3,7-trimethyl-5-hydroxyisourea hydrolase (TM-HIU hydrolase) and putative 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidomimidazole decarboxylase (TM-OHCU decarboxylase), respectively.

**DISCUSSION**

In this report, we have proposed a new pathway for the degradation of caffeine via C-8 oxidation in a caffeine-degrading bacterium, *Pseudomonas* sp. strain CBB1 (Fig. 3). The first reaction of the proposed pathway is the hydrolytic oxidation of caffeine to TMU, catalyzed by a novel caffeine dehydrogenase (Cdh) (42). Previously, Yu et al. had established by sequence homology that *cdhA*, the large (α [90-kDa]) subunit of Cdh, contained the molybdoenzyme cofactor (42). In the present study, total sequence analysis of *cdhB* and *cdhC* revealed that *cdhB* had significant homology with FAD binding medium subunits of similar enzymes such as bifunctional hydratase/alcohol dehydrogenase and carbon monoxide dehydrogenase. In addition, *cdhC* was homologous to the corresponding iron-sulfur containing small subunits of all of the heterotrimeric enzymes. This suggests that the β (40-kDa)-subunit of Cdh contains FAD cofactor and that the γ-subunit contains the iron-sulfur cluster. Cdh is similar to the known NAD⁺-dependent xanthine dehydrogenases in terms of catalysis, subunit structure (αβγ) and cofactor content. However, this enzyme is quinone dependent, does not use NAD(P)⁺, and has no activity with xanthine (42). This is the first report of complete sequence of Cdh.

The thrust of this work is on the second reaction, hydroxylation of TMU to 1,3,7-trimethyl-5-hydroxyisourea (TM-HIU; metabolite II) catalyzed by a novel NADH-dependent trimethyluric acid monoxygenase (TmuM), a flavoprotein. A time course of this reaction, analyzed by ESI-MS in the positive-ion mode, revealed transient accumulation of unstable TM-HIU and continuous accumulation of TMA (Fig. 1d). Therefore, the true oxidation product of TmuM catalyzed reaction was identified as TM-HIU (Fig. 3). One atom of molecular oxygen incorporated into TMU, forming TM-HIU, was detected in TMA (Fig. 1a). The second oxygen was incorporated into water. TM-HIU is unstable and spontaneously decomposes to racemic TMA during the course of the reaction (Fig. 1d and 3a). The racemic TMA was characterized by chiral-HPLC (see Fig. S3a in the supplemental material), NMR (Table 3), ¹⁸O₂ incorporation (Fig. 1a), and optical rotation (data not shown). Transient accumulation of the hypothesized second unstable metabolite in the C-8 caffeine oxidation pathway (Fig. 3b; metabolite III, TM-OHCU) with a m/z of 245) could not be detected during the course of the reaction. This may be due to not enough positive or negative ions generated under the reaction condition or due to rapid decarboxylation of TM-OHCU. This supposition is similar to the corresponding uric acid degradation pathway reaction (32), where the oxidation of uric acid by urate oxidase results in the transient accumulation of 5-hydroxyisourea (HIU) (30). HIU formed from uric acid by the traditional urate oxidase (not a monoxygenase) from soybean root nodules was also shown to be unstable, with a half-life of less than 30 min at neutral pH (20–22). This unstable compound spontaneously hydrolyzes to 2-oxo-4-hydroxy-4-carboxy-5-ureidomidazoline (OHCU), which gets further decarboxylated spontaneously to racemic allantoin, the stable end product of urate oxidase reaction (32). The instability of TM-HIU can be attributed to its structural similarity to HIU. An ¹⁸O₂ incorporation experiment with TmuM was conducted as an endpoint reaction (Fig. 1a and b) due to sampling difficulties from the enriched atmosphere. Thus, isotopic oxygen-labeled ¹⁸O-TM-HIU could not be detected at the end of this experiment. Nevertheless, the retention of isotopic oxygen in ¹⁸O-TMA with a m/z of 203 (Fig. 1a) conclusively established TmuM as a monoxygenase.

The gene encoding TmuM (*tmuM*) is 1,191 nt in length. Sequence analysis of *tmuM* revealed that this enzyme belongs to the family of FAD containing aromatic-ring hydroxylases, which includes FAD-dependent urate oxidase (HpxO; GenBank accession no. ACF60813) from Klebsiella pneumoniae (30), a flavin-dependent hydroxylase (PhzS; GenBank accession no. AAG07605) from Pseudomonas aeruginosa (16), salicylate 1-monoxygenase (GenBank accession no. AAZ62959), and other uncharacterized/putative FAD-binding monoxygenases (GenBank accession nos. EAA44903, CAJ95547, and EDZ47364). A multiple sequence alignment of these homologous proteins revealed that the primary sequence of TmuM contains conserved residues that include an FAD and NADH-binding domains (see Fig. S1 in the supplemental material). Phylogenetic analysis of these seven homologous flavoprotein monoxygenases (data not shown) revealed TmuM and HpxO clustered together in a clade, distinct from the other five flavin monoxygenases. TmuM and HpxO appear diverging from a common ancestor.

There are two types of enzymes that catalyze reactions similar to TmuM. The first one is the most prevalent urate oxidase (EC 1.7.3.3), which does not require any cofactor (20). The coproduction of this reaction, according to the enzyme nomenclature, is hydrogen peroxide. The second type of enzyme is NADH-dependent, FAD-containing urate oxidase. This enzyme follows a catalytic mechanism similar to flavin containing aromatic-ring hydroxylases (18). HpxO from K. pneumoniae (30) and TmuM are examples of the second type. TmuM is closely related to HpxO in terms of sequence similarity, phylogeny, cofactor content, NADH dependence and, likely, the catalytic mechanism. However, the enzymatic conversion of urate to HIU catalyzed by HpxO, although shown to be oxygen dependent (30), has not been characterized as a monoxygenase. It has been mis-assigned as urate oxidase. Hydrogen peroxide is not one of the products of this reaction. Based on the similarity of HpxO catalyzed reaction to TmuM, we feel that HpxO should be designated as uric acid monoxygenase. Both TmuM and HpxO belong to a unique class of (methyl) urate monoxygenases. However, TmuM is distinct from HpxO; it has no activity on uric acid. TmuM has the highest catalytic activity for trimethyluric acid compared to di- and monomethyluric acids (Table 2). A comparison of the active-site cavities of TmuM and HpxO based on homology models (Fig. 41) reveals a much bigger cavity for TmuM (244 ± 45 Å³) compared to the cavity for HpxO (121 × 10 Å³). Also, the GRAVY value based on the Kyte and Doolittle hydropathy scale (24) for the residues lining the putative active site reveals a much more hydrophobic cavity for TmuM (0.673) (Fig. 4IA) compared to HpxO (−0.218) (Fig. 4IB). These results are consistent with the enzyme data (Table 2) and provide an initial explanation of the specificity of TmuM to the larger TMU and 3,7-dimethyluric acid.

The metabolites downstream of TMU were characterized by gene cluster analysis of the 25.2-kb genomic DNA segment of CBB1, which harbored all of the genes of the pathway (Fig. 2 and...
We undertook this method due to (i) the lack of availability of the metabolites TM-HIU, TM-OCHU, and racemic and S-(+)-TMA proposed in this pathway (Fig. 3) and (ii) the lack of published synthetic methods for these compounds. Homology and gene cluster-based analysis is a valid tool for pathway analysis and has also been used extensively in recent years for elucidation of uric acid degradation pathway in various microbial strains, including Bacillus subtilis (35), Klebsiella pneumoniae (8, 17), and Klebsiella oxytoca (31). Analysis of the hpx gene cluster in K. pneumoniae (8) identified seven genes involved in oxidation of hypoxanthine to allantoin via uric acid. Of particular relevance to the present work are three genes, hpxO, hpxT, and hpxQ, encoding urate oxidase (HpxO) (30), HIU hydrolase, and OHCU decarboxylase, respectively. To our knowledge, this is the first report on the three-step enzymatic conversion of uric acid to S-(+)-allantoin in bacteria based on gene cluster analysis. This led to the functional expression of hpxO, hpxT, and hpxQ in E. coli and the crystal structure elucidation of KpHIUH (hpxT) (11) and KpOHCU decarboxylase (hpxQ) (10). Even though the substrates of KpHIUH and KpOHCU are unstable, the catalytic activities of these enzymes were confirmed by the generation of substrates in situ. Rajmazina et al. (32) identified HIU hydrolase (MuraH) and OHCU decarboxylase (MuraD) from mouse by phylogenetic comparison of genomes and sequence analysis. This led to the cloning of these genes in E. coli and the establishment, by 13C NMR, of the conversion of HIU to S-(+)-allantoin via OHCU (32).

A similar gene homology and cluster-based analysis of the 25.2-kb genomic DNA segment of CBB1 revealed the presence of tmuH and tmuD upstream of tmuM (Fig. 2). The gene tmuH had 50 and 44% identities, respectively, to putative HIU hydrolases from Rhizobium leguminosarum bv. Trifolii and Sinorhizobium meliloti. Gene tmuD had 39 and 37% identity, respectively, with putative OHCU decarboxylases from Starkeya novella and Haloferax volcanii (Fig. 2). Comparison of the organization of tmuH and tmuD (Fig. 2) to the corresponding (characterized) genes hpxO, hpxT, and hpxQ of the uric acid pathway in the hpx gene cluster from K. pneumoniae (8) and the 22.872-kb purine utilization gene cluster from K. oxytoca (31) reveals several interesting features. Although hpxO from hpx gene cluster of Klebsiella pneumoniae (8) had 38% identity to tmuM, the hpxT and hpxQ genes from the same cluster had 29 and 19% sequence identities to tmuH and tmuD, respectively. A BLASTP analysis of tmuH and
hpxT revealed that both of the proteins belong to transhyretin-like superfamily (11). The organization of tmuM, tmuH, and tmuD in the caffeine gene cluster of CBB1 (Fig. 2) is similar to that of the corresponding hpxO, hpxT, and hpxQ genes in the hpx gene clusters in Klebsiella in terms of orientation, the direction of transcription, and gene location. The genes tmuH and tmuD are next to each other, with the same direction of transcription and separated from the oppositely transcribing gene tmuM by a single 1,188-nt ORF (Fig. 2). This is the same as the organization of the hpxT and hpxQ genes and their separation from the oppositely transcribing hpxO by hpxP (8, 31). Further, in the K. oxytoca gene cluster, hpxB, located ~8 kb downstream of hpxO, codes for an allantoinase (31). In the caffeine gene cluster (Fig. 2), orf1 with homology to allantoinases, along with other genes (orf2 and orf3, Fig. 2), are 14 kb downstream of tmuM. It is tempting to propose that these genes are involved in further downstream metabolism of S-(-)-TMA, but this needs further substantiation. The larger separation of orf1 from tmuM can be attributed to the recruitment of 3.765-kb cdhABC genes, coding for heterotrimetric caffeine dehydrogenase (Cdh) (Fig. 2). Based on this, tmuH is proposed to catalyze the N1-C6 hydrolysis and ring opening reaction of TM-HIU to TM-OHCU (Fig. 3), and tmuD is proposed to catalyze the stereoselective decarboxylation of TM-OHCU to S-(-)-TMA (Fig. 3). Another strong piece of evidence for the formation of S-(-)-TMA is that only S-(-)-allantoin seems to be formed in cells (39) capable of utilizing uric acid. These organisms rely on the enzymatic conversion of HIU to S-(-)-allantoin via OHCU (32). Our contention is that the putative TM-HIU hydrolase and a putative TM-OHCU decarboxylase in CBB1 are specific for methyl substituents in the respective substrates (Fig. 3). This is based on the preference of both Cdh and TmuM to trimethylated substrates with no activity on xanthine and uric acid. Hence, in the proposed conversion of TMU to S-(-)-TMA, all enzymes are designated with preference for (tri)methylated substrates (Fig. 3).

Further metabolism of S-(-)-TMA in CBB1 is under investigation. Organisms capable of utilizing uric acid as a source of carbon and nitrogen possess allantoinase and allantoin recemase (17, 33). Recently, Güzman et al. (17) reported the presence of another gene cluster in K. pneumoniae, which harbors genes responsible for further transformation of allantoin. In particular, the two genes hpxA and hpxB, which encode allantoin recemase and allantoinase, respectively, were identified. This led to the crystal structure determination of heteroligously expressed hpxA gene product KpHpxA (12). Thus, it is well established that (R)-allantoin is converted by allantoin recemase to (S)-enantiomer (12), which then gets further transformed to allantoate by allantoinase (33, 39, 40). Very likely, in CBB1, (R)-TMA is converted to (S)-TMA and then to trimethylallantoate. This compound could potentially be transformed to glyoxylic acid, dimethylurea, and monomethylurea (27), as in the uric acid pathway (40). Our initial analysis of DNA sequence downstream of Cdh genes has identified orf1 with homology to allantoinase. However, we are unable to locate a trimethylallantoic acid racemase. Any clarity on the biochemistry of formation and metabolism of S-(-)-TMA (Fig. 3) will come from functional expression of tmuH and tmuD, along with identification of the genes responsible for its transformation.

In summary, a new pathway has been proposed for transformation of caffeine via C-8 oxidation to S-(-)-TMA. The first two steps, i.e., conversion of caffeine to TMU by Cdh and TMU to TM-HIU by TmuM, are based on detailed enzymology and gene analysis. Subsequent transformation of TM-HIU to S-(-)-TMA is based on gene cluster analysis and comparison to uric acid transformation, which is based on gene cluster analysis as well. To our knowledge, this is the first report of biochemical and genetic analysis of caffeine transformation via C-8 oxidation.

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REFERENCES