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Characterization of a broad-specificity non-haem iron *N*-demethylase from *Pseudomonas putida* CBB5 capable of utilizing several purine alkaloids as sole carbon and nitrogen source

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N-Demethylation of many xenobiotics and naturally occurring purine alkaloids such as caffeine and theobromine is primarily catalysed in higher organisms, ranging from fungi to mammals, by the well-studied membrane-associated cytochrome P450s. In contrast, there is no well-characterized enzyme for *N*-demethylation of purine alkaloids from bacteria, despite several reports on their utilization as sole source of carbon and nitrogen. Here, we provide what we believe to be the first detailed characterization of a purified *N*-demethylase from *Pseudomonas putida* CBB5. The soluble *N*-demethylase holoenzyme is composed of two components, a reductase component with cytochrome *c* reductase activity (Ccr) and a two-subunit *N*-demethylase component (Ndm). Ndm, with a native molecular mass of 240 kDa, is composed of NdmA (40 kDa) and NdmB (35 kDa). Ccr transfers reducing equivalents from NAD(P)H to Ndm, which catalyses an oxygen-dependent *N*-demethylation of methylxanthines to xanthine, formaldehyde and water. Paraxanthine and 7-methylxanthine were determined to be the best substrates, with apparent K_m and k_{cat} values of 50.4 ± 6.8 μM and 16.2 ± 0.6 min^{-1}, and 63.8 ± 7.5 μM and 94.8 ± 3.0 min^{-1}, respectively. Ndm also displayed activity towards caffeine, theobromine, theophylline and 3-methylxanthine, all of which are growth substrates for this organism. Ndm was deduced to be a Rieske [2Fe–2S]-domain-containing non-haem iron oxygenase based on (i) its distinct absorption spectrum and (ii) significant identity of the N-terminal sequences of NdmA and NdmB with the gene product of an uncharacterized caffeine demethylase in *P. putida* IF-3 and a hypothetical protein in *Janthinobacterium* sp. Marseille, both predicted to be Rieske non-haem iron oxygenases.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and related methylxanthines are naturally occurring purine alkaloids that are present in many plant species. As a major human dietary component, caffeine can be found in common food and beverage products such as coffee, tea, colas and chocolates. Caffeine has been used in pharmaceutical preparations as a neurological, cardiac and respiratory stimulant. It is also used as an analgesic enhancer in cold, cough and headache medicines (Daly, 2007). Other pharmaceutical applications of methylxanthines include use as a diuretic and a bronchodilator, and for relief of bronchial spasms and control of asthma (Daly, 2007). 1-Methylxanthine, 1-methyluric acid and uric acid have also been studied for their antioxidant properties (Lee, 2000). Because of their wide use in food and pharmaceutical industries, caffeine and related methylxanthines enter the environment via liquid effluents and solid wastes from processing facilities, decomposition of plant matter in coffee and tea fields, and domestic wastes. This can have adverse environmental effects, as methylxanthines act as a soil sterilant by inhibiting seed germination (Smyth, 1992). Caffeine has been suggested as an anthropogenic marker for wastewater pollution of drinking water (Buerge *et al.*, 2003; Ogunseitan, 1996; Seiler *et al.*, 1999).

Some bacteria utilize caffeine as sole growth substrate and metabolize it via oxidation at the C-8 position to form 1,3,7-trimethyluric acid (Madyastha & Sridhar, 1998; Mohapatra *et al.*, 2006; Yu *et al.*, 2008). This reaction is catalysed by a number of different enzymes in several bacteria. An 85 kDa flavin-containing caffeine oxidase was purified from a mixed culture of *Rhodococcus* sp. and *Klebsiella* sp. (Madyastha *et al.*, 1999). A 65 kDa caffeine oxidase was also purified from *Alcaligenes* sp. strain CF8 (Mohapatra *et al.*, 2006). A heterotrimeric, non-NAD(P)$^+$-dependent caffeine dehydrogenase from *Pseudomonas* sp. strain CBB1 was recently

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purified by our group. This enzyme stoichiometrically and hydrolytically oxidized caffeine to trimethyluric acid (Yu *et al.*, 2008).

Several bacterial strains capable of utilizing caffeine as sole growth substrate metabolize caffeine via *N*-demethylation. The majority of these belong to the genus *Pseudomonas* (Asano *et al.*, 1993; Blecher & Lingens, 1977; Dash & Gummadi, 2008; Sideso *et al.*, 2001; Woolfolk, 1975; Yu *et al.*, 2009); however, a strain of *Serratia marcescens* was also found to *N*-demethylate caffeine (Mazzafera *et al.*, 1996). *N*-Demethylation of caffeine in bacteria results in production of either theobromine (3,7-dimethylxanthine) or paraxanthine (1,7-dimethylxanthine). Paraxanthine and theobromine are further *N*-demethylated to 7-methylxanthine and xanthine. Xanthine is subsequently oxidized to uric acid by xanthine oxidase/dehydrogenase, and the uric acid enters the normal purine catabolic pathway to form CO_2 and NH_4^+ (Dash & Gummadi, 2006). Theophylline, the major caffeine metabolite in filamentous fungi (Hakil *et al.*, 1998), has not been reported as a bacterial metabolite of caffeine.

We recently reported a new caffeine-degrading bacterium, *Pseudomonas putida* CBB5, capable of utilizing a number of natural purine alkaloids as sole source of carbon and nitrogen (Yu *et al.*, 2009). This organism metabolizes caffeine via sequential *N*-demethylation to paraxanthine and theobromine, 7-methylxanthine and xanthine. A previously unknown pathway for *N*-demethylation of theophylline to 1- and 3-methylxanthines, which were further *N*-demethylated to xanthine, was also discovered in strain CBB5. While the caffeine and theophylline degradation pathways were co-expressed in the presence of caffeine, theophylline and related methylxanthines, the intermediate metabolites of the two pathways do not overlap until xanthine (Yu *et al.*, 2009). In spite of several reports of *N*-demethylation in the metabolism of purine alkaloids by bacteria, there is to our knowledge no report of a well-characterized enzyme that enables the utilization of these substrates for growth. Here, we describe the first purification and biochemical characterization of a soluble, broad-specificity methylxanthine *N*-demethylase from *P. putida* CBB5. This enzyme is composed of a reductase component (Ccr) and a two-subunit *N*-demethylase component (Ndm). Ndm activity is dependent on NAD(P)H oxidation, catalysed by Ccr. Furthermore, Ndm is predicted to be a Rieske [2Fe–2S]-domain-containing non-haem iron oxygenase based on analysis of the N-terminal amino acid sequences of its subunits and its distinct UV/visible absorption spectrum. When coupled with Ccr, Ndm exhibited broad-based activity towards caffeine, paraxanthine, theobromine, theophylline, 7-methylxanthine and 3-methylxanthine, converting all of these to xanthine.

METHODS

Chemicals. Caffeine, theophylline, theobromine, paraxanthine, 7-methylxanthine, 3-methylxanthine, xanthine, ammonium acetate, acetic acid, 2,4-pentanedione, spinach ferredoxin reductase and spinach ferredoxin were purchased from Sigma-Aldrich. Reductase and ferredoxin of *Pseudomonas* sp. 9816 naphthalene dioxygenase were kindly provided by Dr David T. Gibson, University of Iowa. Soytone was obtained from Becton, Dickinson. HPLC-grade methanol (J. T. Baker) was used in chromatographic studies.

Culture conditions. CBB5 was grown in M9 mineral salts medium (Sambrook *et al.*, 1989) containing 2.5 g caffeine l^{-1} and 4 g soytone l^{-1}, at 30 °C with shaking at 200 r.p.m. Cell density was monitored by measuring the optical density at 600 nm (OD_{600}). Upon reaching an OD_{600} of 2.1, cells were harvested by centrifugation (10 000 ***g*** for 10 min at 4 °C) and washed twice in 50 mM potassium phosphate (KP_i) buffer (pH 7.5). Pelleted cells were suspended in 50 mM KP_i buffer with 5 % (v/v) glycerol and 1 mM DTT (KPGD buffer) to a final volume of 2 ml for every 1 g cells (wet wt) and stored at −80 °C.

Cell extract preparation. About 16.6 g frozen cells suspended in 35 ml KPGD buffer were thawed and DNase I was added to a final concentration of 10 μg ml^{-1}. The cells were broken by passage through a chilled French press cell twice at 138 MPa. Unbroken cells and debris were removed from the lysate by centrifugation (16 000 ***g*** for 20 min at 4 °C), and the supernatant was designated the cell extract.

Enzyme purification. All purification procedures were performed at 4 °C using an automated fast protein liquid chromatography system (ÄKTA FPLC, Amersham Pharmacia Biotech). After each chromatographic step, eluate fractions were assayed for *N*-demethylase (Ndm) and cytochrome *c* reductase (Ccr) activities as described below. Fractions with activities were concentrated using Amicon ultrafiltration units with MWCO 10 000 (Millipore). Purity of the enzyme was determined under native and denaturing conditions by PAGE on 4–15 % Tris/HCl gels (Bio-Rad) and 10 % Bistris gels with MOPS running buffer containing SDS (Invitrogen), respectively.

Cell extract was loaded onto a 160 ml (bed volume) DEAE Sepharose column (GE Healthcare) pre-equilibrated in KPGD buffer. After washing unbound proteins from the column with 160 ml KPGD buffer, the bound proteins were eluted from the column with a 540 ml linear gradient of KCl (0 to 0.4 M) in KPGD buffer. Fractions with Ndm activity were pooled and concentrated.

A 4.0 M ammonium sulfate solution was added to the Ndm-activity-containing fractions from DEAE Sepharose to a final concentration of 0.25 M with constant stirring. After 20 min, the mixture was centrifuged (16 000 ***g*** for 20 min), and the supernatant was loaded onto a 30 ml (bed volume) Phenyl Sepharose High Performance column (Amersham) pre-equilibrated in KPGD buffer containing 0.25 M ammonium sulfate. Unbound proteins were eluted from the column with 60 ml KPGD buffer containing 0.25 M ammonium sulfate. Bound proteins were eluted with a 30 ml reverse linear gradient of ammonium sulfate (0.25 to 0 M in KPGD buffer) at a flow rate of 1 ml min^{-1}. The column was then washed with 45 ml KPGD buffer, followed by 60 ml deionized water containing 1 mM DTT and 5 % (v/v) glycerol.

Phenyl Sepharose eluates with *N*-demethylase activity were pooled, concentrated to 2 ml, and loaded onto a 5 ml (bed volume) Q Sepharose column (GE Healthcare) pre-equilibrated in KPGD buffer containing 0.2 M KCl. After washing unbound proteins from the column with 10 ml equilibration buffer, bound proteins were eluted from the column using a 120 ml linear gradient of KCl (0.2 to 0.4 M) in KPGD buffer. Ndm was eluted from the Q Sepharose column as a single peak around 0.29 M KCl.

Enzyme activity assays. NADH : cytochrome *c* oxidoreductase (cytochrome *c* reductase, Ccr) activity was determined as described

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by Ueda *et al.* (1972). A typical 1 ml reaction contained 300 μM NADH, 87 μM bovine cytochrome *c* (type III; Sigma), and 1–20 μg CBB5 protein (depending on purity) in 50 mM KPi buffer. The activity was determined by monitoring the increase in absorbance at 550 nm due to reduction of cytochrome *c* at 30 °C. An absorption coefficient of 21 000 M^{-1} cm^{-1} for reduced minus oxidized cytochrome *c* was used for quantifying the activity. One unit of activity was defined as 1 μmol cytochrome *c* reduced min^{-1}.

The methylxanthine *N*-demethylase activity assay contained, in 1 ml total volume, 0.5 mM methylxanthine, 1 mM NADH, 50 μM $Fe(NH_4)_2(SO_4)_2$, and an appropriate amount of Ndm (0.08–7.5 mg protein, depending on purity of the protein) in 50 mM KP_i buffer. Approximately 4 U of partially purified Ccr was added to the reaction mixture when assaying Ndm in Phenyl Sepharose and Q Sepharose eluate fractions (Ccr was not added to the enzyme reaction mixture when assaying Ndm activity in DEAE Sepharose eluate fractions because Ccr co-eluted with Ndm). The reaction mixture was incubated at 30 °C with 300 r.p.m. shaking on an incubating microplate shaker (VWR). Periodically, a small aliquot was sampled from the reaction mixture and mixed with an equal volume of acetonitrile for quantifying concentrations of methylxanthines and *N*-demethylated products by HPLC. One unit of *N*-demethylase activity was defined as the consumption of 1 μmol methylxanthine min^{-1}. When Ndm was coupled with the reductase and ferredoxin components of naphthalene dioxygenase of *Pseudomonas sp.* strain 9816 (Haigler & Gibson, 1990a, b), 30 and 100 μg of the respective proteins were used. Spinach ferredoxin reductase and ferredoxin were also substituted for Ccr as described by Subramanian *et al.* (1979).

Molecular mass estimation. The molecular masses of the Ndm subunits were estimated under denaturing conditions by PAGE on 10 % Bistris gels with MOPS running buffer containing SDS (Invitrogen). The native molecular mass of Ndm was determined by gel filtration chromatography using an 80 ml (V_c, geometrical volume) Sephacryl S-300 HR column (Amersham) equilibrated with 0.1 M KCl in 50 mM KP_i buffer at 1 ml min^{-1}. The void volume (V_0) of the column was determined by measuring the elution volume (V_e) of a 1 mg ml^{-1} solution of blue dextran 2000 (GE Healthcare). The column was calibrated with ferritin (440 kDa), catalase (232 kDa), adolase (158 kDa) and conalbumin (75 kDa). The V_e of each standard protein was measured, from which the respective K_{av} value was calculated according to the equation $K_{av}=(V_e-V_0)/(V_c-V_0)$. A standard curve of K_{av} values against the logarithmic molecular masses of the standard proteins was then used to determine the native molecular mass of Ndm.

Determination of pH optimum. To determine pH optimum, Ndm activity was measured as described above at various pH values within the range 6.0–8.0 by using 50 mM KPi buffer.

Determination of kinetic parameters. Apparent kinetic parameters of Ndm were determined by measuring the initial rate of disappearance (v_0) of paraxanthine or 7-methylxanthine in 50 mM KP_i buffer (pH 7.5) at 30 °C. The paraxanthine and 7-methylxanthine concentrations ([*S*]) used in these experiments ranged from 20 to 500 μM. Substrates were incubated with Ndm and Ccr under standard conditions for 15 min. At 1, 5, 10 and 15 min, samples were removed from the reaction mixtures to quantify substrate concentrations by HPLC. Plots of substrate concentrations against time were used to determine the initial rates of disappearance of substrates, which were linear over 15 min. The apparent kinetic parameters were determined from Michaelis–Menten plots of v_0 against [*S*] fitted with the equation $v_0=(k_{cat}[E_T][S])/(K_m+[S])$, where $[E_T]$ is the concentration of enzyme in the reaction. All the experiments were performed in triplicate and the data were analysed by using GraFit 5.0 software (Erithacus Software).

Determination of oxygen requirement. Oxygen consumption by Ndm during *N*-demethylation of paraxanthine was determined in a closed reaction vessel equipped with a Clark-type oxygen electrode (Digital model 10, Rank Brothers). The electrode was calibrated by using glucose oxidase (Sigma) and glucose for consumption of oxygen. Ndm activity assay was performed at 30 °C in a total volume of 1 ml air-saturated 50 mM KP_i buffer (pH 7.5) with 100 μM paraxanthine, 200 μM NADH, 50 μM $Fe(NH_4)_2(SO_4)_2$, 0.2 mg purified Ndm and 4 U partially purified Ccr. The reaction was initiated by adding Ndm and partially purified Ccr after equilibration of all other reaction components for 3.3 min. After 13 min, a 100 μl aliquot was withdrawn from the reaction, immediately mixed with an equal volume of acetonitrile to stop the enzyme reaction, and analysed for *N*-demethylation products by HPLC. Background oxygen consumption, possibly due to NADH oxidase enzyme activity in the partially purified Ccr, was quantified in control reactions containing all reaction components except paraxanthine.

Formaldehyde determination. Production of formaldehyde during *N*-demethylation of paraxanthine was determined by derivatizing formaldehyde with Nash reagent prepared by the method of Jones *et al.* (1999). Twenty microlitres of Nash reagent was added to 50 μl Ndm reaction sample plus 50 μl acetonitrile. This mixture was incubated at 51 °C for 12 min. After cooling to room temperature, 3,5-diacetyl-1,4-dihydrolutidine formed from formaldehyde and Nash reagent was analysed at 412 nm by an HPLC instrument equipped with a photodiode array detector. Standards were prepared with known concentrations of formaldehyde added to control Ndm enzyme reaction mixtures without paraxanthine.

Analytical procedures. Identification and quantification of methylxanthines and their metabolites were conducted with a Shimadzu LC-20AT HPLC system equipped with a SPD-M20A photodiode array detector and a Hypersil BDS C18 column (4.6 × 125 mm) as described previously (Yu *et al.*, 2009). For analysis of 3,5-diacetyl-1,4-dihydrolutidine, methanol/water/acetic acid (30 : 20 : 0.5, by vol.) was used as an isocratic mobile phase at a flow rate of 0.5 ml min^{-1}. Protein concentration was determined by the Bradford method (Bradford, 1976) using BSA as the standard with a dye reagent purchased from Bio-Rad. The N-terminal amino acid sequences of both Ndm subunits were determined at the Protein Facility, Iowa State University, Ames, Iowa, USA. Iron content in Ndm was determined by ICP-MS. An aliquot of purified Ndm was mixed with an equal volume of trace-metal-free, ultrapure concentrated nitric acid. The mixture was heated at 160 °C for 1 h to break down all organic materials. The acid digest was then diluted appropriately with ultrapure water for quantification of iron by a Thermo X-series II ICP-MS system at the Department of Geosciences, University of Iowa. The ICP-MS system was calibrated with high-purity iron standard solution. Bovine cytochrome *c* was used as positive control.

RESULTS

N-Demethylase activity of CBB5 cell extracts

NADH-dependent *N*-demethylase (Ndm) activity in cell extracts prepared from CBB5 grown on caffeine plus soytone was tested on caffeine, theophylline, paraxanthine and theobromine in order to determine which substrate was utilized most rapidly. A 0.5 mM paraxanthine solution was completely utilized by cell extract containing 7.2 mg protein ml^{-1} in about 20 min (Fig. 1). Degradation of theobromine, caffeine and theophylline was significantly slower. Hence, paraxanthine was chosen as the substrate to

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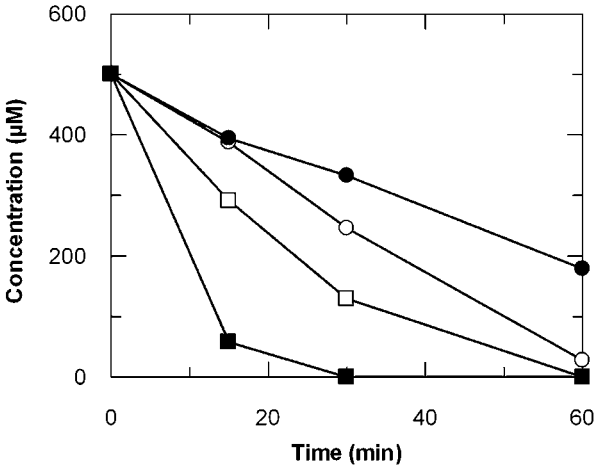

Fig. 1. Degradation of paraxanthine (■), theobromine (□), caffeine (○) and theophylline (●) by cell extracts prepared from *P. putida* CBB5 grown in soytone-supplemented M9-caffeine medium. All reactions contained 0.5 mM substrate, 0.5 mM NADH and 7.2 mg protein ml^{-1}. This experiment was repeated three times and similar patterns were observed in all replicates.

monitor the purification of the enzyme, and in subsequent enzyme activity assays.

Purification of Ndm

Ndm was purified from CBB5 cell extracts (Table 1). The three-step procedure resulted in a 26-fold purification of Ndm, relative to the activity in cell extracts. An NAD(P)H : cytochrome *c* oxidoreductase (Ccr) activity co-eluted with Ndm from the DEAE Sepharose column at 0.23 M KCl in KPGD buffer. NADH is the preferred substrate of Ccr; activity with NADPH was only 22 % of that with NADH. When this DEAE Sepharose eluate was loaded onto the Phenyl Sepharose column, Ccr eluted from the column at 0.05 M $(NH_4)_2SO_4$ and was gold in colour. Ndm was dark red in colour and eluted from the column with water, indicating that this component is more hydrophobic. Ndm eluted from Phenyl Sepharose did not contain any Ccr activity. Neither Ccr, Ndm, nor any other Phenyl Sepharose eluate had paraxanthine *N*-demethylase activity when assayed singly in the presence of NADH. When Ccr and Ndm fractions were combined in the presence of NADH, *N*-demethylation activity was negligible. However, exogenous addition of 50 μM Fe^{2+} to this reaction mixture resulted in *N*-demethylation activity of 25.6 mU mg^{-1}. This partially purified Ccr fraction was used for assay in the presence of Fe^{2+} during further purification of Ndm in a Q Sepharose column. The dark red colour was retained throughout purification. Specific activity of this purified Ndm, in the presence of a saturating amount of Ccr, was 55.4 mU mg^{-1}. 7-Methylxanthine and xanthine were detected as products.

Biochemical characterization of Ndm

Analysis of the Ndm fraction from Q Sepharose in PAGE gel under denaturing conditions revealed two bands (Fig. 2a), with apparent molecular masses of 40 kDa (NdmA) and 35 kDa (NdmB). When this solution was loaded onto a gel filtration chromatography column, the protein eluted as a single, symmetrical peak, and the native molecular mass was estimated to be about 240 kDa. The 40 kDa and 35 kDa proteins co-eluted from the gel filtration column, as determined by SDS-PAGE (data not shown). Other chromatographic methods could not further resolve these two proteins. These results suggest that the native Ndm enzyme is probably composed of the two subunits in a hexameric configuration. Ndm stored in KPGD buffer was stable for at least 5 days at 4 °C and for over one month at −80 °C without significant loss of activity (data not shown).

The N-terminal sequence, determined by Edman degradation, of NdmA was MEQAIINDEREYLRHFWHPVCTVTE, while that of NdmB was MKEQLKPLLEDKTYLRHFWH-PVCTL. A BLASTP (Altschul *et al.*, 1997) search of the GenBank database, using the NdmA and NdmB N-terminal amino acid sequences as queries, showed that both subunits were most similar to the gene product of the *P. putida* strain IF-3 caffeine demethylase gene (accession no. AAB15321) in patent US5550041 (Koide *et al.*, 1996), and a hypothetical protein in *Janthinobacterium* sp. Marseille (mma_0224, accession no. YP_001351914). The NdmA and NdmB N-terminal sequences were 84 and 48 %, respectively, identical to the first 25 residues of caffeine

Table 1. Purification of methylxanthine *N*-demethylase from *Pseudomonas putida* CBB5

Purification step	Total protein (mg)	Total activity (mU)*	Sp act (mU mg^{-1})	Purification (fold)	Yield (%)
Cell extract	2361	5053	2.1	1	100
DEAE Sepharose	480.2	1767	3.7	1.7	35
Phenyl Sepharose HP	37.5	962	25.6	12	19
Q Sepharose	7.0	388	55.4	26	7.7

*One unit of activity corresponds to 1 μmol paraxanthine consumed min^{-1}, in the presence of saturating amounts of Ccr and 50 μM Fe^{2+}, as described in Methods.

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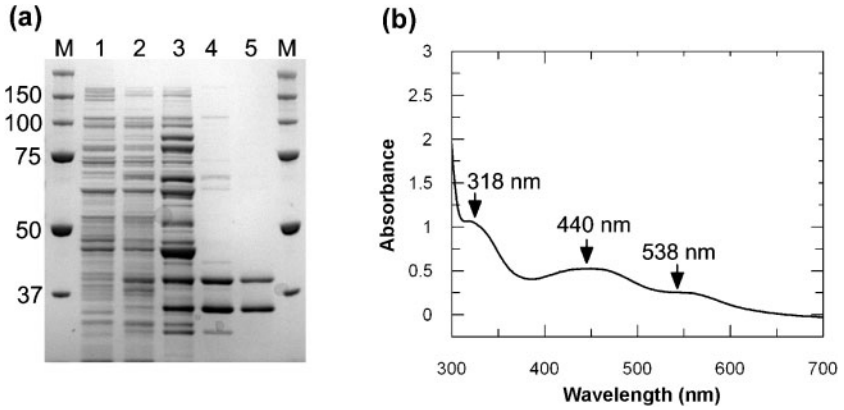

Fig. 2. (a) SDS-PAGE analysis of *P. putida* CBB5 *N*-demethylase, Ndm, during the purification process. Molecular masses of markers (in kDa) are shown on the left and right (lanes M). Ndm is composed of two subunits with apparent molecular masses of 40 kDa (NdmA) and 35 kDa (NdmB). Lane 1, cell extracts of CBB5 grown on soytone alone; lane 2, cell extracts of CBB5 grown on soytone plus caffeine; lane 3, DEAE Sepharose eluate; lane 4, Phenyl Sepharose eluate; lane 5, Q Sepharose eluate. (b) UV/visible absorption spectrum of Ndm (1.7 mg ml^{-1}) from Q Sepharose.

demethylase, and 52 and 64 %, respectively, identical to the first 25 residues of mma_0224 (Fig. 3a).

Sequence analyses of the caffeine demethylase gene product and mma_0224 predicted both proteins to be Rieske [2Fe–2S]-domain-containing non-haem iron oxygenases (Fig. 3b). The UV/visible absorption spectrum of oxidized Ndm had maxima at 318, 440 and 538 nm (Fig. 2b), characteristic of proteins with Rieske-type [2Fe–2S] clusters (Capyk *et al.*, 2009; Fee *et al.*, 1984; Subramanian *et al.*, 1979; Yu *et al.*, 2007). The purified Ndm preparation had an *R*-value (A_{280}/A_{440}) of 11.1 and contained 8.5 ± 0.1 mol iron per mol hexameric Ndm, with specific activity of 55 mU mg^{-1} when the enzyme reaction mixture contained 50 μM Fe^{2+}. Specific activity decreased to 10 mU mg^{-1} if 50 μM Fe^{2+} was not included in the enzyme reaction mixture. Pre-incubation of Ndm for 15 min with 1 mM Fe^{2+}, followed by desalting, increased the iron content to 20.1 ± 0.3 mol per mol hexameric Ndm. After this treatment, Ndm specific activities increased to 161 mU mg^{-1} and 127 mU mg^{-1} when 50 μM Fe^{2+} was present or absent, respectively, in the enzyme reaction mixture.

Stoichiometric analysis of Ndm reaction

Under anaerobic conditions, Ndm had no *N*-demethylation activity on paraxanthine. Exposure of the anaerobic enzyme reaction mixture to air immediately restored Ndm activity (data not shown), indicating that *N*-demethylation by Ndm could be oxygen dependent. Stoichiometric consumption of oxygen during *N*-demethylation of paraxanthine by Ndm was demonstrated by monitoring the reaction with a Clark-type oxygen electrode. After 13 min of reaction, 79.5 μM oxygen was consumed (Fig. 4) while 60.2 μM paraxanthine was *N*-demethylated. Approximately 39.9 μM 7-methylxanthine and 20.3 μM xanthine were present in the reaction products. These results

(a)

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NdmA        M-EQ-AIINDEREYLRHFWHPVCTVTE 25
Cdm         M-EQ-TINNNDREYLRHFWHPVCTVTE 25
NdmB        MKEQLKPLLEDKTYLRHFWHPVCTL-- 25
mma_0224    MS--FTALLADKSYLRHFWHPVCTLKE 25
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(b)

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mma_0224    61-RCAHRFAKLSKGCVKQGEAGDRLECPYHGWQYDKDGACRTIPACPELPIPKKARSDAFEC-120
Cdm         61-RCAHRSAKLSLGTIAN----DRLQCPYHGWQYDTEGACKLVPACPNSPIPNRAKVQRFDC 116

mma_0224   121-EVRYDIVWVRLDASFGVTEIPVFSDWEEEGMRVIVVDSYVWQTTAERRWENFTDFSHFAF-180
Cdm        117-EERYGLIWVRLDSSYACTEIPYFSAASDPKLRVVIQEPYWWNATAERRWENFTDFSHFAF-176

mma_0224   181-VHPGTLYDAAYARPPLAPVSRVGGEMRFSIEPPKEMTDQLPDDCPIGAFIYRCTMPYTIN-240
Cdm        177-IHPGTLFDPNNAEPPIVPMDRFNGQFRFVYDTPEDMA--VPDQAPIGSFSYTCSMPFAIN-234
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Fig. 3. (a) Multiple sequence alignment of the N-terminal protein sequences of NdmA and NdmB with the first 25 amino acid residues of caffeine demethylase (Cdm) of *P. putida* IF-3 and the hypothetical protein mma_0224 in *Janthinobacterium* sp. Marseille. (b) Consensus sequences of the Rieske [2Fe–2S] domain ($CXHX_{16}CX_2H$, highlighted in black) and the mononuclear ferrous iron domain [$(D/E)X_3DX_2HX_4H$, bold type] are identified in Cdm and mma_0224.

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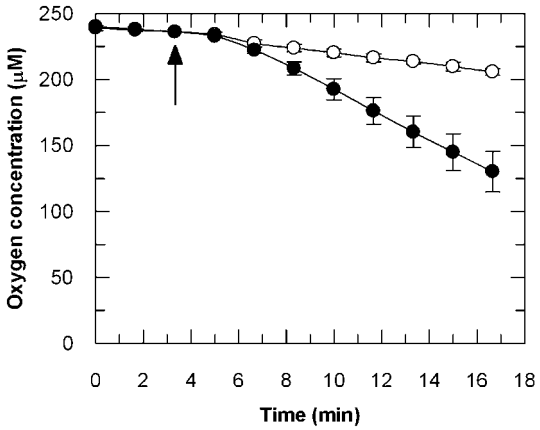

Fig. 4. Oxygen consumption by 0.2 mg purified Ndm with 4 U partially purified Ccr with (●) or without (○) 100 μM paraxanthine in the reaction mixture. The reactions were carried out in air-saturated buffer and equilibrated at 30 °C for 3.3 min before addition of Ndm plus partially purified Ccr to initiate the reactions (indicated by the arrow). At 13 min after initiation of the reactions, a 100 μl aliquot was withdrawn from the reaction mixture and immediately quantified for *N*-demethylation products by HPLC. The oxygen concentrations reported are means of four replicates with standard deviations.

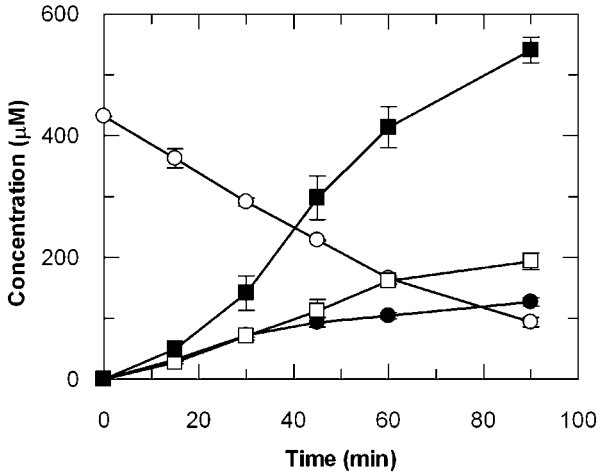

Fig. 5. Stoichiometric *N*-demethylation of paraxanthine (○), via 7-methylxanthine (●) to xanthine (□) by purified Ndm with concomitant production of formaldehyde (■). The concentrations reported are means of triplicates with standard deviations.

indicate that approximately 1 molecule of O_2 was consumed per methyl group removed from paraxanthine. These results also indicate that Ndm could remove both methyl groups, at the N-1 and N-7 positions, from paraxanthine.

N-Demethylation of paraxanthine by Ndm also resulted in production of formaldehyde. After 90 min of incubation, 338.5 ± 7.7 μM paraxanthine was *N*-demethylated by 7.4 units of Ndm and 540.6 ± 20.9 μM formaldehyde was produced (Fig. 5). 7-Methylxanthine (126.9 ± 6.4 μM) and xanthine (193.7 ± 14.0 μM) were the *N*-demethylated products. Tallying up all the products formed from paraxanthine, it was determined that approximately one molecule of formaldehyde was produced per methyl group removed.

Substrate preference of Ndm

Maximal Ndm activity was observed at pH 7.5 in 50 mM KP_i buffer but Ndm was active in the pH range 6–8 (see Supplementary Fig. S1, available with the online version of this paper). The optimal temperature for Ndm activity was determined to be 30 °C (data not shown). Therefore, kinetic parameters for Ndm, in the presence of saturating amounts of Ccr and 50 μM Fe^{2+}, were determined at 30 °C in 50 mM KP_i buffer pH 7.5. Apparent K_m and k_{cat} values for paraxanthine and 7-methylxanthine were 50.4 ± 6.8 μM and 16.2 ± 0.6 min^{-1}, and 63.8 ± 7.5 μM and 94.8 ± 3.0 min^{-1}, respectively (Supplementary Fig. S2). Ndm exhibited broad-based activity towards caffeine, theophylline, theobromine, 7-methylxanthine and 3-methylxanthine, all of which are growth substrates for CBB5. The relative activities in reference to paraxanthine are reported in Table 2. Production of xanthine from all of these methylxanthines was confirmed. Ndm was most active on 7-methylxanthine, followed by paraxanthine, theobromine, 3-methylxanthine, caffeine and theophylline. Ndm did not catalyse *O*-demethylation of vanillate or vanillin, even after prolonged incubation.

Reductase requirement of Ndm

No *N*-demethylase activity was observed when purified Ndm was assayed without the Ccr fraction. Ndm is predicted to be a Rieske [2Fe–2S]-domain-containing non-haem iron oxygenase, and oxygenases of this type are known to be promiscuous in terms of the partner reductases (Subramanian *et al.*, 1979; Yu *et al.*, 2007). However, Ndm did not function in the presence of the reductase and ferredoxin components of *Pseudomonas* sp. 9816 naphthalene dioxygenase, a well-characterized Rieske [2Fe–2S]-domain-containing non-haem iron oxygenase (Ensley & Gibson, 1983). Likewise, Ndm did not couple with ferredoxin reductase plus ferredoxin from spinach.

DISCUSSION

In this report, we describe the first purification and characterization of a broad-specificity, soluble *N*-demethylase from CBB5. This enzyme is composed of a reductase component (Ccr) and an oxygenase *N*-demethylase component (Ndm). The *N*-demethylase component (Ndm) itself is a two-subunit enzyme with broad substrate specificity. It can remove *N*-methyl groups from caffeine, all three natural

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Table 2. Substrate preference of Ndm towards methylxanthines, relative to paraxanthine

Substrate	Relative *N*-demethylase activity at 500 or 200 μM substrate		Products identified*
	500 μM	200 μM	
Paraxanthine	100.0 ± 3.1	100.0 ± 3.7	7-Methylxanthine, xanthine
7-Methylxanthine	455.7 ± 17.6	664.2 ± 61.1	Xanthine
Theobromine	59.8 ± 1.9	80.2 ± 7.8	3- and 7-methylxanthine, xanthine
3-Methylxanthine	55.2 ± 6.0	68.9 ± 2.6	Xanthine
Caffeine	30.8 ± 1.7	48.2 ± 4.8	Paraxanthine, theobromine, 7-methylxanthine, xanthine
Theophylline	17.6 ± 1.0	14.2 ± 1.6	1- and 3-methylxanthine, xanthine

*The same products were identified at both concentrations of substrates.

dimethylxanthines, 3-methylxanthine and 7-methylxanthine (Table 2) to produce xanthine. 7-Methylxanthine was the best substrate for Ndm, with a k_{cat}/K_m value almost sixfold higher than that for paraxanthine. No *O*-demethylation was observed when vanillate and vanillin were provided as the substrates for Ndm. Enzyme activity of Ndm was absolutely dependent on oxygen as a co-substrate. One molecule of O_2 was consumed for each *N*-methyl group removed from paraxanthine (Fig. 4). *N*-Methyl groups removed from methylxanthines were stoichiometrically oxidized to formaldehyde (Fig. 5). Formation of formaldehyde from bacterial *N*-demethylation of caffeine has been reported previously (Asano *et al.*, 1994; Blecher & Lingens, 1977) but the stoichiometry of the reaction was not established. Whether the formaldehyde produced is utilized by CBB5 needs to be determined.

The N-terminal protein sequences of both Ndm subunits were substantially identical to the gene product of an uncharacterized caffeine demethylase gene in *P. putida* IF-3 (Koide *et al.*, 1996) and a hypothetical protein in *Janthinobacterium* sp. Marseille. The physiological functions of these proteins have not been established; however, both of these proteins were predicted to be Rieske [2Fe–2S]-domain-containing non-haem iron oxygenases. The UV/visible absorption spectrum of Ndm was characteristic of a protein with a Rieske [2Fe–2S] cluster (Fig. 2b). Exogenous addition of 50 μM Fe^{2+} to the Ndm enzyme reaction mixture stimulated Ndm enzyme activity, similar to other other Rieske [2Fe–2S]-domain-containing non-haem iron oxygenases such as toluene, naphthalene and biphenyl dioxygenases (Ensley & Gibson, 1983; Subramanian *et al.*, 1979; Yu *et al.*, 2007). Generally, the mononuclear ferrous iron in Rieske oxygenases is not tightly bound and often dissociates during protein purification. However, it could be reconstituted by incubating the oxygenase with excess Fe^{2+}. In fact, after incubating Ndm alone with 1 mM Fe^{2+}, the iron content of Ndm increased from 8.5 to 20.1 mol per mol hexameric Ndm. Furthermore, Ndm specific activity also increased from 55 mU mg^{-1} to 161 mU mg^{-1}. Additional exogenous Fe^{2+} in the enzyme reaction mixture was not absolutely necessary for high Ndm activity. All of these data support the hypothesis that Ndm is a Rieske [2Fe–2S]-domain-containing, non-haem iron oxygenase.

The native molecular mass of Ndm was estimated to be 240 kDa by gel filtration chromatography. Both subunits of Ndm, NdmA and NdmB, were approximately 40 kDa in size (Fig. 2a), suggesting that Ndm is possibly a hexameric protein. Since 20.1 iron atoms were present per molecule of Ndm, it is very likely that NdmA and NdmB each contain three iron atoms, in agreement with the presence of a [2Fe–2S] cluster and a mononuclear ferrous iron in each subunit. This finding is intriguing because the mononuclear ferrous iron is known to be the catalytic centre of Rieske oxygenases (Ferraro *et al.*, 2005). Both NdmA and NdmB are proposed to contain a mononuclear ferrous iron, which may suggest there are two catalytic centres in native Ndm. However, this remains to be substantiated via cloning of NdmA and NdmB individually. Some Rieske oxygenases are known to be in hexameric $\alpha_3\beta_3$ configuration (Dong *et al.*, 2005; Friemann *et al.*, 2005; Kauppi *et al.*, 1998; Yu *et al.*, 2007) but only the α subunits contain the [2Fe–2S] cluster and the mononuclear ferrous iron, while the β subunits are mainly for structural purposes (Ferraro *et al.*, 2005). The 2-oxo-1,2-dihydroquinoline 8-monooxygenase of *P. putida* 86 was reported to be in an α_6 configuration, determined by gel filtration chromatography (Rosche *et al.*, 1995), but the crystal structure showed that it is actually a homotrimer (Martins *et al.*, 2005). The proposed presence of two catalytic centres in Ndm raises some important questions, such as (i) how reducing equivalents generated from the reductase component are distributed between these two catalytic centres, (ii) whether both catalytic centres are functional, and (iii) if they have different or overlapping substrate specificities which in combination account for Ndm's substrate preferences. Moreover, our present data could not completely exclude the possibility that NdmA and NdmB are individual Rieske oxygenases that co-purify or agglomerate together during purification and each of them could have

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different or overlapping substrate specificity. Heterologous expression of each subunit separately will answer some of these questions.

Ndm enzyme activity was dependent on the presence of a reductase component. This reductase component oxidized NAD(P)H and concomitantly reduced cytochrome *c in vitro*; hence, it was designated Ccr (cytochrome *c* reductase component). The reductases of several well-characterized Rieske non-haem iron oxygenases are also known to reduce cytochrome *c in vitro* (Subramanian *et al.*, 1979; Haigler & Gibson, 1990b; Yu *et al.*, 2007). Partially purified Ccr was used for the purification of the Ndm component, and the Ccr requirement for *N*-demethylation by Ndm was specific. Ferredoxins and/or reductases of either spinach or the *Pseudomonas* sp. 9816 naphthalene dioxygenase system (Haigler & Gibson, 1990a, b) did not support the *N*-demethylase reaction by Ndm. In contrast, the reductase components of toluene dioxygenase of *P. putida* (Subramanian *et al.*, 1979) and biphenyl dioxygenase of *Sphingobium yanoikuyae* B1 (Yu *et al.*, 2007) are easily substituted by spinach ferredoxin reductase and the reductase of naphthalene dioxygenase, respectively. A proposed reaction scheme for *N*-demethylation of paraxanthine (and other methylxanthines) by Ccr plus Ndm is presented in Fig. 6. This scheme is similar to those of other known microbial mono- and dioxygenases; however, none of the other oxygenases is known to catalyse *N*-demethylation. We hypothesize a specific Ccr in the transfer of electrons to Ndm, the oxygenase component exhibiting broad-based *N*-demethylation activity on purine alkaloids, thus enabling CBB5 to utilize these substrates. Molecular oxygen is incorporated into formaldehyde and water.

Presently, it is not clear whether additional *N*-demethylases are present in CBB5. Some indirect experiments by others indicate the presence of specific, maybe multiple *N*-demethylases in caffeine-degrading bacteria. A recent study by Dash & Gummadi (2008) showed that caffeine and theobromine *N*-demethylases in *Pseudomonas* sp. NCIM 5235 were inducible in nature but caffeine *N*-demethylase activity in theobromine-grown cells was 10-fold lower than that of caffeine-grown cells. This implies different *N*-demethylases for caffeine and theobromine *N*-demethylation. Glück & Lingens (1988) partially purified a specific 7-methylxanthine *N*-demethylase from caffeine-degrading *P. putida* WS, which exhibited no activity on caffeine, theobromine or paraxanthine. Caffeine and theobromine also did not inhibit 7-methylxanthine *N*-demethylation by this enzyme. These results also imply multiple *N*-demethylases in *P. putida* WS for degradation of caffeine. Finally, multiple *N*-demethylases were also implicated in caffeine-degrading *P. putida* no. 352 (Asano *et al.*, 1994). Caffeine *N*-demethylase activity from this strain was partially purified and resolved into three distinct fractions. At the same time, a theobromine *N*-demethylase activity which produced 7-methylxanthine was reported to co-elute with one of the caffeine *N*-demethylase fractions. This activity was inhibited by Zn^{2+} while caffeine *N*-demethylase activities in all three fractions were not. The theobromine *N*-demethylase was subsequently purified to homogeneity and reported to have a native molecular mass of 250 kDa and a subunit molecular mass 41 kDa (homohexamer). This enzyme was brown in colour and displayed an absorbance maxima of 415 nm. Unfortunately, this *N*-demethylase is not well characterized. Thus, a case could be built that multiple *N*-demethylases may enable the utilization of purine alkaloids in some bacteria. Nevertheless, Ndm of CBB5 is distinct from all of the above *N*-demethylases in terms of substrate specificity, UV/visible absorption spectrum and subunit structure, as well as the requirement of a reductase component.

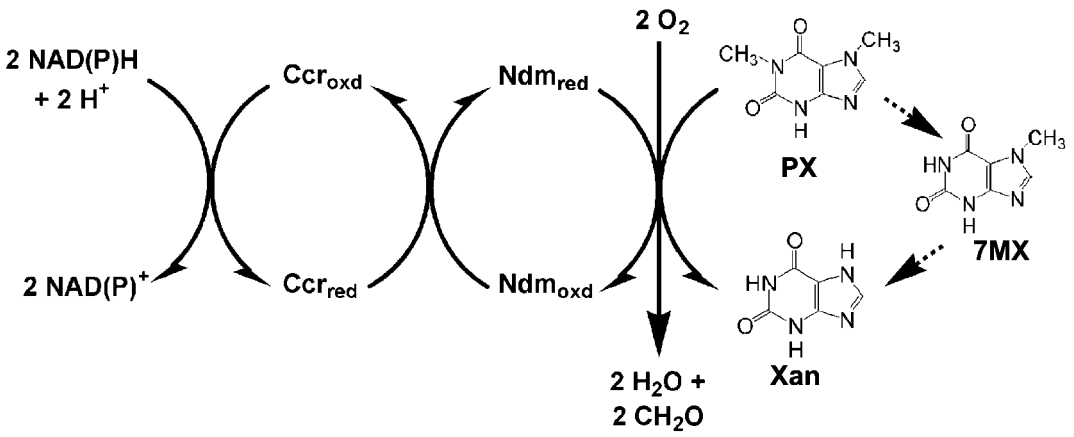

Fig. 6. Proposed reaction scheme for paraxanthine *N*-demethylation by the *P. putida* CBB5 two-component *N*-demethylase. Reducing equivalents are transferred from NAD(P)H to the two-subunit *N*-demethylase component (Ndm) by a specific Ccr. One mole of paraxanthine (PX) is *N*-demethylated to xanthine (Xan) through 7-methylxanthine (7MX) as an intermediate (dashed arrow).

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Although the caffeine demethylase gene in *P. putida* IF-3 (Koide *et al.*, 1996) was predicted to encode a Rieske non-haem iron oxygenase, the gene function was assigned solely based on reversion of a caffeine-negative phenotype of a mutated strain of *P. putida* IF-3. There are no direct enzymological data to substantiate this claim. Also, the physiological function of this enzyme with respect to the ability of bacteria to utilize purine alkaloids as sole source of carbon and nitrogen is unknown. Naphthalene dioxygenase of *Pseudomonas* sp. strain 9816 has been reported to *N*-demethylate *N*-methylaniline and *N*,*N*-dimethylaniline to aniline (Lee, 1995). This reaction is not well characterized and indicates only the promiscuity of naphthalene dioxygenase, not its physiological role in *N*-demethylation. This enzyme in fact enables the utilization of naphthalene by converting it to *cis*-dihydroxy-1,2-dihydronaphthalene (Ensley & Gibson, 1983). To our knowledge, the *P. putida* CBB5 methylxanthine Ndm provides the first concrete example of a Rieske non-haem iron oxygenase with broad-based *N*-demethylase activity on a number of purine alkaloids. *N*-Demethylation (or *N*-dealkylation) reactions in eukaryotes and prokaryotes are known to be catalysed only by cytochrome P450s (Abel *et al.*, 2003; Asha & Vidyavathi, 2009; Caubet *et al.*, 2004; Cha *et al.*, 2001; Guengerich, 2001; Tassaneeyakul *et al.*, 1994), various flavo-enzymes (Chang *et al.*, 2007; Kvalnes-Krick & Jorns, 1986; Meskys *et al.*, 2001; Nishiya & Imanaka, 1993; Phillips *et al.*, 1998; Shi *et al.*, 2004; Wagner, 1982) and a ketoglutarate-dependent non-haem iron oxygenase (Tsukada *et al.*, 2006). This study therefore broadens our understanding of the possible enzymic mechanism for *N*-demethylation and will aid the discovery of new microbial *N*-demethylases in the future.

In summary, *P. putida* CBB5 uses a broad-specificity, two-component *N*-demethylase system to initiate biotransformation of caffeine and related natural purine alkaloids. The *N*-demethylation of these compounds is catalysed by the terminal *N*-demethylase (Ndm), a two-subunit oxygenase. A specific Ccr component with cytochrome *c* reductase activity is involved in the transfer of electrons from NADH to the Ndm. The N-terminal protein sequences of Ndm subunits were significantly homologous to two proteins predicted to be Rieske [2Fe–2S]-domain-containing, non-haem iron oxygenases.

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