Genetic characterization of caffeine degradation by bacteria and its potential applications

Ryan M. Summers,† Sujit K. Mohanty,‡ Sridhar Gopishetty and Mani Subramanian

1Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, AL 35487, USA.
2Department of Chemical and Biochemical Engineering and 3Center for Biocatalysis and Bioprocessing, The University of Iowa, Coralville, IA 52241, USA.

Summary

The ability of bacteria to grow on caffeine as sole carbon and nitrogen source has been known for over 40 years. Extensive research into this subject has revealed two distinct pathways, N-demethylation and C-8 oxidation, for bacterial caffeine degradation. However, the enzymological and genetic basis for bacterial caffeine degradation has only recently been discovered. This review article discusses the recent discoveries of the genes responsible for both N-demethylation and C-8 oxidation. All of the genes for the N-demethylation pathway, encoding enzymes in the Rieske oxygenase family, reside on 13.2-kb genomic DNA fragment found in Pseudomonas putida CBB5. A nearly identical DNA fragment, with homologous genes in similar orientation, is found in Pseudomonas sp. CES. Similarly, genes for C-8 oxidation of caffeine have been located on a 25.2-kb genomic DNA fragment of Pseudomonas sp. CBB1. The C-8 oxidation genes encode enzymes similar to those found in the uric acid metabolic pathway of Klebsiella pneumoniae. Various biotechnological applications of these genes responsible for bacterial caffeine degradation, including bio-decaffeination, remediation of caffeine-contaminated environments, production of chemical and fuels and development of diagnostic tests have also been demonstrated.

Introduction

Caffeine (1,3,7-trimethylxanthine) and related methylxanthines are natural purine alkaloids found in many plants around the world (Ashihara and Crozier, 1999). These compounds are hypothesized to serve as natural insecticides, and have been shown to protect the plants from insects and other predators (Nathanson, 1984; Hollingsworth et al., 2002). Other possible reasons for biosynthesis of caffeine include inhibition of plant matter (Waller, 1989) and improved pollination (Wright et al., 2013).

Methylxanthines are often consumed by humans in foods and beverages, including chocolate, coffee and tea. Coffee is a major worldwide agricultural commodity, with millions of metric tons produced and distributed globally each year (Summers et al., 2014). In addition to the food industry, caffeine and related methylxanthines are also used in pharmaceuticals as stimulants, diuretics, bronchodilators, vasodilators and in the treatment and/or prevention of axial myopia, glaucoma and macular degeneration (Stavric, 1988a,b,c; Trier et al., 1999; Dash and Gummadi, 2006b; Daly, 2007).

Although bacterial caffeine degradation has been studied since the 1970s, very little was known concerning the enzymes and genes responsible for caffeine degradation until recently. Several excellent reviews have summarized the bacterial caffeine catabolic pathways (Mazzafera, 2004; Dash and Gummadi, 2006a,b; Gummadi et al., 2012), including recent developments (Gopishetty et al., 2012). In this review, we focus on the discoveries made since the publication of these reviews, with specific emphasis on the genes responsible for caffeine degradation in bacteria.

Caffeine metabolism

To date, over 35 bacterial strains that are capable of degrading caffeine have been isolated and reported (Table 1). While there is some diversity among the types
of bacteria isolated, the majority are *Pseudomonas*, primarily *Pseudomonas putida*. Caffeine-degrading bacteria are geographically dispersed, and have been found in coffee fields (Yamaoka-Yano and Mazzafera, 1998), wastewater streams (Ogunseitan, 1996) and garden soil (Blecher and Lingens, 1977; Yu *et al*., 2008; 2009; 2014).

Metabolic studies with these caffeine-degrading bacterial isolates have revealed only two catabolic pathways: N-demethylation and C-8 oxidation. The N-demethylation pathway appears to be the most common, as it has been observed in over 80% of reported isolates where metabolism has been characterized. In both pathways, bacteria break caffeine down to carbon dioxide and ammonia to harvest energy and cellular building blocks.

During N-demethylation, the caffeine molecule is sequentially N-demethylated to form xanthine (Fig. 1A). Each of the three methyl groups is removed with incorporation of molecular oxygen to produce one formaldehyde and one water molecule per reaction. Theobromine (3,7-dimethylxanthine) is the major metabolite formed from the first step in the pathway, with small amounts of paraxanthine (1,7-dimethylxanthine) also reported in some strains (Yamaoka-Yano and Mazzafera, 1999; Yu *et al*., 2009; 2014). The second step of the pathway is the N-demethylation of theobromine or the N-demethylation of paraxanthine to form 7-methylxanthine. 7-Methylxanthine is further N-demethylated to form xanthine. Finally, xanthine is converted to uric acid, which enters normal purine catabolic pathway.

*Theophylline* (1,3-dimethylxanthine) has not been reported as a metabolite of caffeine in bacteria. However, it is the first major metabolite of caffeine in fungi (Hakil *et al*., 1998), and is further degraded via N-demethylation to 3-methylxanthine and xanthine. Although the bacterium *P. putida* CBB5 does not produce theophylline from caffeine, it has been reported to degrade theophylline by N-demethylation (Yu *et al*., 2009). Both 3-methylxanthine (major product) and 1-methylxanthine (minor product) are formed from theophylline in CBB5 and are further N-demethylated to form xanthine, as in the caffeine catabolic pathway.

Some of the metabolites formed during the bacterial N-demethylation of caffeine also undergo C-8 oxidation to form their corresponding uric acids (Blecher and Lingens, 1977; Yamaoka-Yano and Mazzafera, 1999). In most strains, this involves the formation of 3,7-dimethyluric acid, 1,7-dimethyluric acid and 7-methyluric acid from theobromine, paraxanthine and 7-methylxanthine respectively. These methyluric acids are not formed during caffeine N-demethylation in *P. putida* CBB5. However, CBB5 converts approximately 25% of the entire theophylline metabolite pool to 1,3-dimethyluric acid, 1-methyluric acid and 3-methyluric acid. There is no evidence that these methyluric acids are further metabolized (Yu *et al*., 2009).
C-8 oxidation involves the oxidation of caffeine to form 1,3,7-trimethyluric acid (TMU), which is further degraded by a pathway homologous to the uric acid metabolic pathway (Fig. 2). This pathway has been observed in both mixed cultures (Madyastha and Sridhar, 1998) and pure bacterial isolates (Mohapatra et al., 2006; Yu et al., 2008; Mohanty et al., 2012). 1,3,7-trimethyluric acid is further metabolized to sequentially form 1,3,7-trimethyl-5-hydroxyisouric acid (TM-HIU), 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU) and 3,6,8-trimethylallantoin (TMA) (Mohanty et al., 2012). Further degradation of TMA has not yet been fully characterized. However, it is believed that only S-(+)-TMA is formed enzymatically and its degradation proceeds through trimethylallantoinic acid (TMAA) before being mineralized to glyoxylic acid, dimethylurea and monomethylurea (Madyastha and Sridhar, 1998; Mohanty, 2013). These latter compounds are then assumed to enter the central metabolic cycles of the bacterial cell.

Genetic basis for caffeine degradation

Although bacterial degradation of caffeine has been studied for over 40 years, very little was discovered concerning the enzymes involved in the metabolism. Similarly, the genetics of bacterial caffeine metabolism were unknown. Recent work has revealed the nature of enzymes involved in both N-demethylation and C-8 oxidation pathways (Asano et al., 1994; Madyastha et al., 1999; Yamaoka-Yano and Mazzaferra, 1999; Yu et al., 2008; 2009; Summers et al., 2011; Mohanty, et al., 2012). The gene sequences for these enzymes have also been elucidated, reported and deposited in the GenBank database (Mohanty et al., 2012; Summers et al., 2012; 2013). Currently, the only known bacterial genes responsible for caffeine metabolism are those that are directly responsible for catabolism. Other genes encoding proteins for caffeine uptake, chemotaxis and regulation of caffeine-degrading enzymes have not yet been reported.

N-demethylation

Many of the earliest works on caffeine N-demethylase enzymes indicated that they are labile in nature and quickly lost activity during purification (Hohnloser et al., 1980; Glück and Lingens, 1988; Sideso et al., 2001; Beltran et al., 2006). Glück and Lingens (1988) reported partial purification of a 7-methylxanthine demethylase from P. putida WS that was not active towards any other
methylxanthine. This enzyme lost activity within 12 h and could not be completely purified. Asano and colleagues (1994) observed two distinct N-demethylase fractions eluting from an ion-exchange chromatography column loaded with cell extracts of *P. putida* No. 352. One protein fraction was active towards caffeine, resulting in formation of theobromine. The second fraction converted theobromine to 7-methylxanthine. These results indicate that caffeine is N-demethylated by at least three methylxanthine-specific enzymes.

Recently, the five enzymes (NdmABCDE) that catalyse the entire caffeine N-demethylation pathway in *P. putida* CBB5 were purified and characterized (Summers et al., 2011, 2013). These same enzymes are also responsible for metabolism of theobromine. The second fraction converted theobromine to 7-methylxanthine. These results indicate that caffeine is N-demethylated by at least three methylxanthine-specific enzymes.

Both NdmA and NdmB are entirely dependent upon NdmD (Fig. 1A), which is a partner reductase that transfers electrons from nicotinamide adenine dinucleotide (NADH) to power the reaction (Summers et al., 2011). NdmD is a redox-dense protein, containing two [2Fe-2S] clusters, one FMN binding domain and one NADH binding domain. When NdmD is expressed with NdmC and NdmE, the three proteins form a large protein complex that catalyses the N7-demethylation of 7-methylxanthine to xanthine (Summers et al., 2013).

Among the Rieske oxygenase family, NdmCDE protein complex is very unique (Summers et al., 2013). The reductase, NdmD, is the only reported Rieske reductase that contains an extra [2Fe-2S] cluster. NdmC is also a non-heme iron monooxygenase but does not contain a Rieske [2Fe-2S] cluster, as do NdmA and NdmB. NdmE is a glutathione-S-transferase-like protein that is postulated to have a structural role in aligning the extra Rieske cluster found on NdmD with the NdmC subunit to catalyse the last N-demethylation step.

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the Rieske domain of the oxygenase is split off and fused to the reductase. Also, this was the first reported instance in which a glutathione-S-transferase-like protein is absolutely required for solubility and activity of a Rieske oxygenase. Interestingly, a BLAST search using the ndmCDE genes as queries identified an additional 18 organisms with homologous ndmCDE clusters coding for enzymes of unknown function (Summers et al., 2013). The ndmCDE genes clustered most closely with homologues from Janthinobacter sp. Marseille (accession no. NC_009659.1), Klebsiella pneumoniae subsp. pneumoniae WGLW2 (accession no. NZ_JH930420.1) and Pseudomonas sp. TJI-51 (accession no. NZ_AEWE01000207.1). Other bacteria containing ndmCDE homologues predominantly belong to the Sinorhizobium and Mesorhizobium genera. Although the functions of these homologues are unknown, the widespread dissemination of ndmCDE homologues indicates that similar caffeine-degrading enzymes may be found in bacteria other than those in the Pseudomonas genera.

Enzymes and genes homologous to NdmABCDE were also found in caffeine-grown Pseudomonas sp. CES cells (Yu et al., 2014). The sequences of these homologous enzymes revealed high similarity to the enzymes (80–90% identity) and genes (72–77% identity) in P. putida CBB5. The similarity between the caffeine-degrading enzymes in P. putida CBB5 and Pseudomonas sp. CES indicate that these N-demethylase enzymes may be conserved among many bacteria that metabolize caffeine via N-demethylation. The ndmABCDE genes in P. putida CBB5 and Pseudomonas sp. CES are encoded on the genomic DNA. In contrast, Dash and Gummadi (2006a) reported that N-demethylation in Pseudomonas sp. NCIM 5235 is encoded on a 12 kb plasmid, although the gene sequences have not yet been reported. Other caffeine N-demethylase genes may yet be discovered in the future, which will only increase our understanding of how bacteria respond to many other N-methylated compounds in the environment.

Flanking the ndmABCDE genes on the CBB5 chromosome are two genes homologous to those known to catalyse the conversion of formaldehyde to formic acid, frmA and frmB (Fig. 1B). Formaldehyde production during N-demethylation has been detected many times (Blecher and Lingens, 1977; Glück and Lingens, 1988; Summers et al., 2011; 2012; 2013). Thus, the genetic basis of caffeine N-demethylation, including the cellular utilization of formaldehyde produced as a by-product has been substantiated.

**C-8 oxidation**

To date, three enzymes catalyzing the C-8 oxidation of caffeine have been purified and characterized. An 85-kDa caffeine oxidase was purified from a mixed culture of Klebsiella sp. and Rhodococcus sp. (Madyastha et al., 1999). Mohapatra and colleagues (2006) discovered a 65-kDa caffeine oxidase in Alcaligenes sp. Both caffeine oxidase enzymes displayed low activity towards theobromine, theophylline and a few other theobromine analogues. A heterotrimeric caffeine dehydrogenase (Cdh) enzyme was discovered in Pseudomonas sp. CBB1 (Yu et al., 2008), which catalysed C-8 oxidation of caffeine to form TMU (Fig. 2). This 158-kDa protein was a novel quinone-dependent oxidoreductase (EC 1.17.5.2) that exhibited no activity with NAD(P)+. The second step of the C-8 oxidation pathway, conversion of TMU to TM-HIU, was catalysed by a 43-kDa NADH-dependent trimethyluric acid monooxygenase (TmuM) also isolated from CBB1 (Mohanty et al., 2012). TM-HIU generated by TmuM was found to be unstable and spontaneously degraded to racemic TMA. However, the two step enzymatic transformation of TM-HIU is expected to yield S-+(+)-TMA via TM-OHCU in biological systems, analogous to the uric acid metabolic pathway (Ramazzina et al., 2006).

In Pseudomonas sp. CBB1, several genes, including genes encoding Cdh and TmuM, have been identified in a 25.2-kb caffeine gene cluster of the CBB1 genome (Fig. 2 inset). A BLASTX-based sequence analysis of Cdh genes (cdhABC) revealed significant homology with other heterotrimeric enzymes such as xanthine dehydrogenase, hydratase/alcohol dehydrogenase, aldehyde oxidase and carbon monoxide dehydrogenase. The high similarity between cdhABC and their homologues further facilitated in associating these genes with their respective cofactor-binding subunits (Mohanty et al., 2012). Similarly, the tmuM gene showed significant homology with FAD (Flavin adenine dinucleotide)-containing aromatic-ring hydroxylases. In particular, tmuM showed a high degree of similarity with hpxO gene (encoding HpxO, a FAD-dependant uric acid oxidase) from K. pneumoniae. Further, a protein homology model of TmuM based on HpxO led to a better understanding of the changes in the active site pocket of this enzyme and its specificity towards methyluric acids (Mohanty et al., 2012).

Two other genes in this cluster, tmuH and tmuD (Fig. 2 inset), are proposed to encode putative enzymes (TM-HIU hydroxylase and TM-OHCU decarboxylase, respectively) of the C-8 oxidation pathway (Mohanty et al., 2012) based on their homology to enzymes of the uric acid metabolic pathway in K. pneumoniae (French and Ealick, 2010; 2011; Mohanty, 2013). The presence of these putative enzymes in CBB1 suggests enzymatic formation of S-+(+)-TMA from TM-HIU via TM-OHCU in the C-8 oxidation pathway (Mohanty et al., 2012). Klebsiella pneumoniae also contains an allantoin racemase to covert (R)-allantoin, formed by spontaneous degradation
of hydroxyisouric acid, to (S)-allantoin (French et al., 2011). Mohanty and colleagues (2012) proposed a similar conversion of (R)-(-)-TMA, formed by spontaneous degradation of TM-HIU, to (S)-(-)-TMA by a TMA racemase. However, none of the genes in the 25.2-kb CBB1 genetic cluster showed any homology to allantoin racemase (Mohanty, 2013). Thus, there yet remain undiscovered genes encoding enzymes active in the caffeine C-8 oxidation pathway.

Further degradation of S-(+)-TMA to glyoxylic acid, dimethylurea and monomethyl urea, as suggested by Madayastha and Sridhar (1998), is poorly understood. Mohanty and colleagues (2012) have identified an open reading frame (orf1) in the 25.2-kb caffeine gene-cluster in the CBB1 genome, which encodes a putative trimethylallantoine homologous to allantoinases that hydrolyse allantoin to allantoic acid (Fig. 2). This suggests that S-(+)-TMA is further hydrolysed into TMAA in the C-8 oxidation pathway. Subsequent formation of glyoxylic acid, dimethylurea and monomethylurea, which then enter the central metabolic pathway for total mineralization, may occur by hydrolysis of non-peptide C-N bonds of TMAA (Mohanty, 2013).

C-8 oxidation of N-demethylated metabolites

There are several reports concerning the C-8 oxidation of N-demethylated metabolites. Woolfolk (1985) discovered a xanthine dehydrogenase capable of oxidizing xanthine, 1-methylxanthine and 1,3-methylxanthine in P. putida 40. Yamaoka-Yano and Mazzafera (1999) reported that P. putida L contains a broad-specificity xanthine oxidase responsible for C-8 oxidation of theobromine, para-xanthine and 7-methylxanthine. The purified oxidase also oxidized xanthine, 3-methylxanthine and theophylline. Pseudomonas putida CBB5 also contains a broad-substrate xanthine dehydrogenase that was partially purified from cell extracts and found to be active towards theophylline, 1,3-methylxanthine, 3-methylxanthine and xanthine (Yu et al., 2009). In all cases, the methyluric acids formed from N-demethylated metabolites accumulate in the growth media and are not further degraded. In addition, none of the methyluric acids were utilized as sole carbon and nitrogen sources. Thus, the reason for their production is currently unknown, although it is likely that they are simply the result of a broad-specificity xanthine dehydrogenase that was partially purified from cell extracts of CBB5 (Yu et al., 2009). To our knowledge, these are the only three reports that describe the enzymes responsible for C-8 oxidation of N-demethylated metabolites in bacteria. Currently, it is unknown whether these enzymes are specific for N-demethylated metabolites or are simply the general xanthine dehydrogenase with broad specificity. No bacterial gene has been associated with the C-8 oxidation activity of N-demethylated caffeine metabolites to date.

Applications of bacterial caffeine degradation

An understanding of the genes involved in bacterial caffeine degradation may open up several new biotechnological applications. Some of these include biological decaffeination of coffee, tea and caffeinated plant matter, environmental remediation of soils and waters with high caffeine concentrations, synthesis of alkylxanthines and alkyl uric acids for use as chemicals or pharmaceuticals and development of a rapid diagnostic test to detect caffeine and related methylxanthines.

Bio-decaffeination

Bio-decaffeination of coffee and tea using whole microbial cells or enzymes has been discussed for a number of years (Kurtzman and Schwimmer, 1971; Sideso et al., 2001; Beltran et al., 2006; Gopishetty et al., 2012). Pseudomonas putida CBB5 can completely decaffeinate coffee and tea extracts, while Pseudomonas sp. CBB1 has also been used to decaffeinate tea extracts (Gopishetty et al., 2012). In terms of relative efficacy, strain CBB5 used the N-demethylation pathway to degrade a higher amount of caffeine in a shorter amount of time than did strain CBB1 through the C-8 oxidation pathway. An immobilized mixed culture of Klebsiella sp. and Rhodococcus sp. was also used to decaffeinate tea extract via C-8 oxidation under both batch and continuous processes (Summers et al., 2014). Overall, the N-demethylation pathway appears to be more efficient than C-8 oxidation for use in the microbial decaffeination of coffee. However, the use of bacterial cells for biodecaffeination of beverages may not be feasible due to the potential for release of endotoxins.

Alternately, use of purified caffeine-degrading enzymes (either soluble or immobilized) may provide a viable alternative (Beltran et al., 2006; Gopishetty et al., 2012) to eliminate endotoxin problems. The ndmABCDE genes could be cloned into Escherichia coli for large-scale recombinant enzyme production in order to carry out biodecaffeination of beverages. Another approach is to clone caffeine-degrading genes into Saccharomyces cerevisiae or another generally regarded as safe (GRAS) organism for whole-cell bio-decaffeination (Gopishetty et al., 2012), thus circumventing the endotoxin problem. Through this method, optimized genetic cassettes could be transformed into the GRAS organism, creating an enhanced caffeine-degrading strain.

A greater opportunity for microbial bio-decaffeination may be in the decaffeination of coffee and tea wastes. During processing of coffee, millions of metric tons of
waste are generated each year (Brand et al., 2000). This waste has a high nutritional content, including a carbohydrate content of 45–60% (dry weight) (Summers et al., 2014). Unfortunately, a caffeine concentration in the waste greater than 1% makes it unsuitable as animal feed or as a biofuel feedstock. Treatment of coffee waste with caffeine-degrading microorganisms (either wild type or recombinant) may transform the waste into a valuable by-product, rather than a waste stream.

**Environmental remediation**

There are many routes by which caffeine enters the environment, where it can exhibit toxic effects on the surrounding plants, insects and microbes (Nathanson, 1984; Waller, 1989). In coffee and tea fields, fallen leaves, stems and seeds decompose, releasing caffeine into the soil. Solid and liquid wastes from coffee and tea processing plants also contain high levels of caffeine, which enter soil and groundwater. The widespread use of caffeine in foods, beverages and pharmaceuticals lead to high levels of caffeine in human wastewater streams, as well. In fact, caffeine can be used as an anthropogenic marker for wastewater contamination in the environment (Buerge et al., 2003). In all of these cases, either wild-type or recombinant caffeine-degrading microorganisms may be of use in removing caffeine from contaminated environments.

**Chemical production**

While caffeine is a relatively inexpensive molecule, many of the metabolites formed by both N-demethylation and C-8 oxidation of caffeine and their analogues are high-value chemicals. Many of these chemicals have great potential in the pharmaceutical and cosmetic industries. Uric acid and methyluric acids are antioxidants (Nishida, 1991; Schlotte et al., 1998), and 8-oxomethylxanthines may be used in treatments for obesity, skin cosmetics and anti-dandruff products (Simic and Jovanovic, 1989). Methylxanthines have been used as diuretics, bronchodilators, antioxidants and asthma control (Lee, 2000; Daly, 2007).

Most methylxanthines and methyluric acids are difficult to synthesize chemically because selective alkylation of each nitrogen atom is difficult to achieve (Taylor et al., 1961; Shamim et al., 1989; Gopishetty et al., 2012). The recent discovery of genes encoding bacterial caffeine-degrading enzymes may help to facilitate synthesis of these high-value chemicals. The ndmA and ndmD genes catalyse specific N-demethylation of alkylxanthines, which leave a specific methyl group open for chemical derivatization. Caffeine dehydrogenase from *Pseudomonas* sp. CBB1 can oxidize caffeine to TMU, and displays some activity towards other methylxanthines, as well.

Currently, there is only one report of methylxanthine production from caffeine using engineered cells (Summers et al., 2014). The genes ndmA and ndmD were cloned into *E. coli*, resulting in a bacterial strain that was able to effectively convert caffeine to theobromine. A second *E. coli* strain was constructed to convert theobromine to 7-methylxanthine using ndmB and ndmD genes. This preliminary report demonstrated the feasibility of specific methylxanthine production from caffeine.

**Diagnostics**

Indiscriminate introduction of caffeine in food and beverages has led to growing concern among caffeine-sensitive consumers and the US Federal Drug Administration, giving rise to an ever-increasing demand for a suitable ‘in-home’ test to detect caffeine. Recently, a Cdh enzyme-based colorimetric test was developed. This test was rapid and sensitive enough to detect caffeine in beverages, including coffee, soft drinks and nursing mother’s milk, within minutes (Mohanty et al., 2014). Based on the type of dye (electron acceptor) used, the test developed a bright colour upon exposure to caffeine even at 1–5 ppm level (Fig. 3). The test could successfully detect caffeine in samples with a wide range of pH and variations, with milk and sugar, or with other active pharmaceutical ingredients. Thus, this test is now deemed to be highly suitable for further development into an ‘in-home’ type strip-based test (Mohanty et al., 2014).

The *N*-demethylase genes were also used to addic *E. coli* to caffeine (Quandt et al., 2013), resulting in a whole-cell caffeine biosensor. Genes ndmABCD and an ndmE homologue were cloned into an *E. coli* guanine auxotroph. In order to grow, the engineered cells were required to convert caffeine to xanthine, which was further converted to guanine. This engineered *E. coli* strain was then used to accurately determine the caffeine concentration of various beverages. While impractical for an at-home caffeine diagnostic test, this *E. coli* strain could find use detecting caffeine in a laboratory setting or in environment samples.

**Conclusion**

Research for over 40 years has uncovered two distinct caffeine metabolic pathways in bacteria: *N*-demethylation and C-8 oxidation. While there are a couple of reports on the enzymes involved in these processes, work was stalled due to lack of knowledge concerning the genetics of bacterial caffeine degradation. The recent discovery of bacterial genes responsible for metabolism of caffeine, by
both *N*-demethylation and C-8 oxidation routes, opens numerous potential biotechnological applications. These novel genes and enzymes may be of great use in home diagnostic tests, remediation of caffeine-contaminated environments and production of chemicals, pharmaceuticals, animal feed and biofuels.

**Conflict of interest**

None declared.

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