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Removal of caffeine in sewage by *Pseudomonas putida*: Implications for water pollution index

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A strain of *Pseudomonas putida* (biotype A) capable of growing on caffeine (1,3,7-trimethylxanthine) was isolated from a domestic wastewater processing operation. It used caffeine as the sole carbon source with a mean growth rate constant (k) of 0.049 h^{-1} (approximately 20 h per generation), whereas k for glucose utilization under similar incubation conditions was 0.31 (3.3 h per generation). The isolate contained at least two plasmids, and the increased expression of a 40 kDa protein was attributable to growth on caffeine. Degradation byproducts of caffeine metabolism by the bacterial isolate included other xanthine derivatives. The slow bacterial catabolism of caffeine in sewage has implications for the effectiveness of wastewater purification, re-use and disposal.

Key words: Caffeine, *Pseudomonas putida*, sewage, water pollution.

The contamination of natural water resources by faecal materials occurs through sewage disposal and wildlife defaecation. It is important to detect faecal contaminants in water because of the strong link of such contamination to the presence of enteric pathogens which endanger aquatic food resources, potability of water and the use of water for recreational activities such as swimming. The most widely applied method for the detection of faecal pollution of water is the coliform count (Eaton *et al.* 1995). However, this method has several shortcomings: Coliform organisms may originate from sources other than faeces; there is a long time delay between water sampling and confirmation of faecal pollution; it is not possible to distinguish human from animal sources of faecal pollution; there is a tenuous indication of enteric viruses, and chlorinated sewage effluents often show low coliform counts even though the water quality is poor (Singley *et al.* 1974). Due to these shortcomings, many attempts have been made to find alternatives to and supplementary methods for the coliform count, including chemical indicators of faecal pollution. Chemical indicators that have been studied include uric acid, cholesterol, and coprostanol (Singley *et al.* 1974). None of these have become widely used because of the inability to distinguish between human and animal sources of faecal pollution (Singley *et al.* 1974). This report presents initial investigations into a proposal to use caffeine as a universal chemical indicator of human urine and faecal materials in water systems.

Caffeine is ubiquitous in the human diet and it is probably the most widely consumed drug in the world (Barone & Roberts 1984; James 1991; Battig & Welzl 1993). Consumption of caffeine occurs in beverages, condiments, colanuts, analgesics, anti-congestants, and diet pills (Willaman & Schubert 1961; Marks & Kelly 1973; Suzuki & Takahashi 1975; Graham 1978; Daly 1993). The global average consumption of caffeine is 70 mg per person per day, but can be more than 400 mg in certain countries (James 1991). It has been estimated that between 0.5% and 7% of caffeine ingested by humans is excreted unchanged in urine (Arnaud 1993), and that caffeine is the predominant com-

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Table 1. Population densities of caffeine-degrading bacteria in water samples.

Incubation Period (Days)	Water Source	Number of Bacterial Colonies (log_{10} c.f.u./ml)	
		Glucose*	Caffeine*
0	Raw Wastewater	6.19	< 1
0	Primary Effluent	6.29	0.48
0	Secondary Effluent	4.38	< 1
0	Creek	4.49	< 1
1	Raw Wastewater	7.34	< 1
1	Primary Effluent	6.25	2.54
1	Secondary Effluent	4.48	3.69
1	Creek	4.80	< 1
3	Raw Wastewater	6.66	< 1
3	Primary Effluent	8.26	5.38
3	Secondary Effluent	7.79	7.17
3	Creek	4.46	< 1

*Minimal medium agar plates with 0.02 M glucose or 0.02 M caffeine provided as sole carbon source.

pound in the organic base fraction of raw and treated sewage in municipal wastewater samples (Garrison *et al.* 1977).

Due to the increasing need to understand ecological effects of sewage spills and to recycle domestic wastewater, it is important to understand the fate of caffeine in water treatment facilities where not all organic compounds are removed prior to chlorination and re-distribution. As an initial step toward elucidating the environmental fate of caffeine excreted by humans, this study isolated and partially characterized the bacterial species *Pseudomonas putida* (biotype A) involved in caffeine metabolism in domestic sewage.

Materials and Methods

Wastewater Samples

Wastewater samples were collected from the Irvine Ranch Water District (Irvine, CA), a facility that employs aerobic treatment of wastewater from 46,309 sewage connections (approximately 150,000 people) at a flow rate of 4.16×10^7 l/day. Total processing time for water is 6 hours. The treated effluent is chlorinated (6 mg/l) prior to redistribution. Raw wastewater samples (3 × 1 l) were collected from the influent port, and effluent samples (3 × 1 l) were collected from both primary and secondary treatment tanks. Freshwater samples (3 × 1 l) were also collected from an adjacent creek (San Diego creek) that occasionally receives treated effluent from the facility, and drains directly into the Pacific Ocean through the upper Newport Bay ecological preserve.

Bacterial Analysis

To enumerate colony-forming units (c.f.u.) of total aerobic bacteria, 1 ml samples of water were serially diluted in phosphate-buffered saline (PBS), containing (g/l): NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.15; and KH_2PO_4, 0.2. The pH was adjusted to 7.0 (Ogunseitan & Olson 1993). Diluted samples were spread on agar plates of a chemically-defined, minimal medium containing (g/l); $NaNO_3$, 4.0; KH_2PO_4, 1.5; Na_2HPO_4, 0.5; $FeCl_3.6H_2O$, 0.0005; $CaCl_2.2H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2, supplemented with either glucose, 2.0 or caffeine, 2.0, (Sigma, St. Louis, MO) with the initial pH adjusted to 7.0. Inoculated plates were routinely incubated at 25 ± 2°C for 7 days prior to colony enumeration, subculture and purification.

To enrich environmental water samples for bacteria capable of degrading caffeine, 100 ml of wastewater or creekwater was incubated in triplicate with caffeine at 1 mg/ml at 25°C on a rotary shaker at 100 rev. min^{-1}.

To follow the rate of cell proliferation and caffeine disappearance in axenic cultures of bacteria isolated from the environmental water samples, culture media (or ten-fold dilutions in unamended minimal medium) were subjected to spectrophotometric scanning in the ultraviolet/visible light range, or to peak absorbance measurements at 600 nm for bacterial cell density and at 273.5 nm for caffeine concentration. Kinetic parameters of bacterial growth were calculated solely from data based on exponential growth phase.

The specific identification of bacterial isolates was done by gas chromatographic fatty acid fingerprinting based on a reference database containing profiles for more than 7,000 bacterial strains (Microcheck, Inc. Northfield, VT, U.S.A.). In order to determine the genomic organization in caffeine-degrading bacteria, plasmid profiles for isolated bacteria were generated using the method of Anderson and McKay (1983) for genomic DNA extraction. Extracted DNA was resolved electrophoretically and visualized by ethidium bromide staining (Sambrook *et al.* 1989). To determine if growth on caffeine induces specific proteins in the caffeine-degrading bacteria, proteins were extracted from bacteria grown on caffeine, glucose, or equimolar concentrations of caffeine and glucose as the sole carbon source. The extracted protein molecules were resolved electrophoretically and stained with silver nitrate according to standard protocols (Sambrook *et al.* 1989).

Analysis of Caffeine Metabolism

Thin-layer chromatography (TLC) was used to analyse the occurrence of caffeine and metabolites in wastewater, creekwater, urine and bacterial culture supernatants. For TLC analysis, aqueous samples were extracted twice with an equal volume of chloroform: methanol (9:1 v/v), and the organic phase was evaporated to dryness using a centrifugal evaporator under vacuum. The pellet was resuspended in 5 µl chloroform:methanol (9:1 v/v), and loaded on 200 µm layer, octadecylsilane-bonded, reverse phase TLC plates ($KC_{18}F$, Whatman). The eluting solvent was chloroform: ethanol (9:1 v/v). Xanthine derivatives were visualized by spraying air-dried TLC plates with a solution of iron (III) chloride-iodine-tartaric acid (Hurst *et al.* 1984), whereas purines and pyrimidines were visualized by spraying plates with 0.01% fluorescein in ethanol (Krebs *et al.* 1969).

Results and Discussion

Very few bacteria in the environmental water samples formed colonies on growth medium containing caffeine as the only carbon source (Table 1). When caffeine was added directly to the water samples, the population density of caffeine utilizers increased in the effluent of both primary and secondarily processed wastewater. The increased bacterial population density occurred simultaneously with caf-

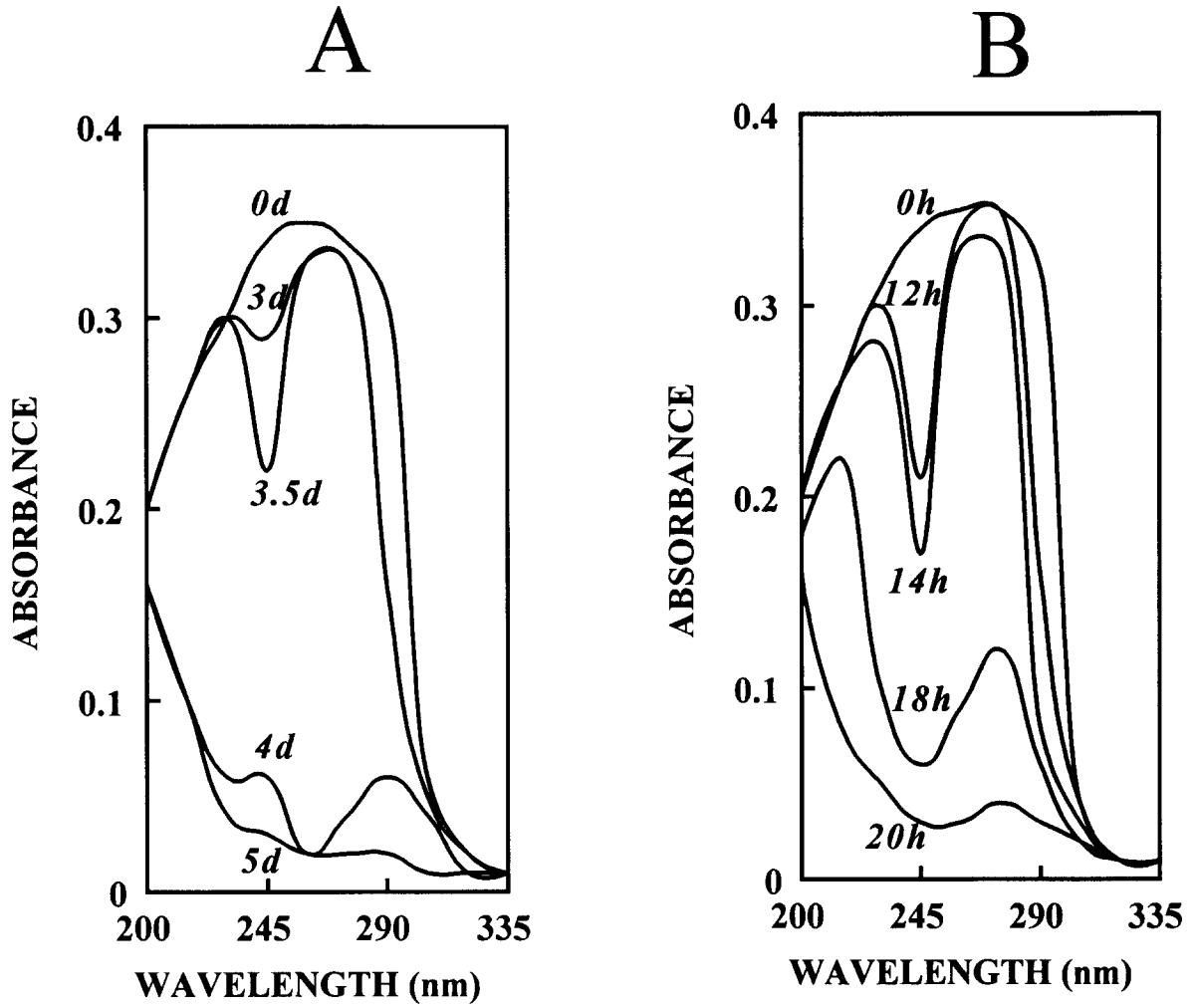

Figure 1. Spectrophotometric scans showing caffeine degradation in domestic wastewater collected after secondary aerobic treatment. Panel A shows progressive scans following initial amendment with 1 mg caffeine per ml of secondarily-treated wastewater. The flask became visibly turbid after 5 days and when additional caffeine was added (1 mg/ml), degradation proceeded much more rapidly as shown in panel B. No peaks in the range scanned were observed for unamended raw wastewater, primary and secondary effluents, or creekwater. Decrease in absorbency did not occur in experiments with sterilized water samples.

feine disappearance as monitored by spectrophotometric scan of filtered wastewater samples (Table 1 and Figure 1). Caffeine was not degraded in the creekwater even after 2 months' incubation. The only bacterial isolate capable of growing independently on caffeine as the only carbon source was identified through fatty acid analysis as *Pseudomonas putida* (biotype A). The *P. putida* strain contained at least two plasmids (Figure 2, panel A) and produced a protein molecule of approximately 40 kDa when grown on minimal medium supplemented with caffeine which was not produced on minimal medium supplemented with glucose (Figure 2, panel B). The mean growth rate constant (k) determined for the *P. putida* strain on caffeine was 0.049 (about 20 h/generation), whereas k for growth on glucose was 0.31 (3.3 h/generation) and k for growth on equimolar concentrations of both substrates was 0.22 (4.6 h/generation) (Figure 3, panel A). The growth rate data, considered with spectrophotometric data on remaining caffeine concentrations, suggest the simultaneous degradation of both carbonaceous substrates. There was an initial accumulation of a degradation product absorbing at 273 nm, that was rapidly degraded during late exponential growth (Figure 3).

Several microorganisms have been investigated by other investigators for the ability to decaffeinate coffee, as an alternative to chlorinated solvent extraction (Kurtzman & Schwimmer 1971; Horman & Viani 1972; Katz 1984). Those organisms, including fungi (*Fusarium oxysporum, Penicillium roquefort*) and bacteria (*Bacillus coagulans, Pseudomonas aeruginosa*), produce broad-spectrum enzymes such as xanthine dehydrogenase and uricase capable of degrading methylated xanthines to methyl allantoin (Kurtzman & Schwimmer 1971). In addition, a strain of *P. putida* capable of growing on many *N*-methyl derivatives of xanthine was isolated through soil enrichment by Woolfolk (1975). The

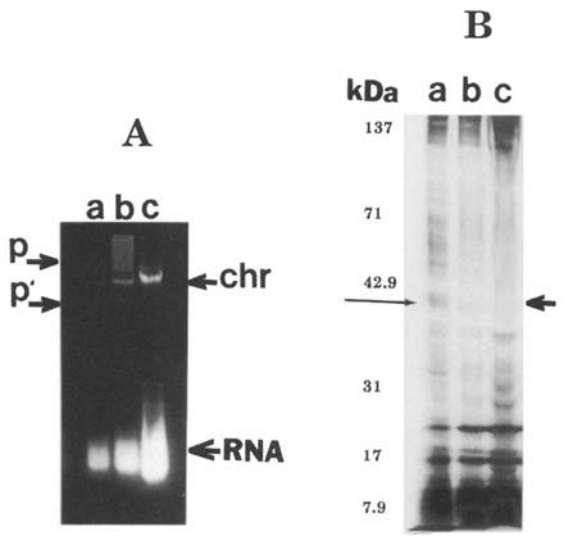

Figure 2. Molecular characteristics of a caffeine-degrading strain of *Pseudomonas putida* (biotype A) isolated from human wastewater.

Panel A: Picture of ethidium bromide-stained agarose gel showing the presence of at least two large plasmids (p and p') in the caffeine-degrading *P. putida* strain. Plasmids were found in the strain regardless of cultivation in minimal medium containing 0.02 M caffeine as the sole carbon source (lane a), or a combination of 0.01 M caffeine and 0.01 M glucose (lane b), or only 0.02 M glucose (lane c). **chr** denotes the position of chromosomal DNA.

Panel B: Picture of silver-stained denaturing-polyacrylamide gel showing proteins produced by cultures of caffeine-degrading *P. putida* grown in minimal medium with 0.02 M caffeine as the sole carbon source (lane a), equimolar concentrations (0.01 M each) of caffeine and glucose (lane b), or 0.02 M glucose alone (lane c). Arrow points to the most distinct protein molecule made only in the presence of caffeine.

postulated pathway of methylxanthine degradation by *P. putida* is through hydrolytic demethylation, yielding methanol and free xanthine, which is further degraded via conventional pathways of purine degradation. The initial demethylation pathway is likely to be the one followed by the wastewater *P. putida* strain reported here, based on the accumulation during axenic growth in minimal medium, of a UV-absorbing compound (Figure 3) identified by thin-layer chromatographic analysis as xanthine or its derivative (data not shown).

If the average national (U.S.A.) caffeine consumption of 210 mg/person.day is assumed for the population served by the Irvine Ranch Water District, then the daily load of caffeine into the treatment facility is 0.79 to 2.2 kg/day, flowing into the treatment process at a concentration of 0.02 to 0.05 mg/l. Based on the initial rate (the first 3 days, Figure 1) of wastewater caffeine degradation determined in this study (0.0029 mg/ml.day) and the length of time allowed for domestic wastewater treatment (6 hours), it is apparent that wastewater treatment processes dependent on bacterial metabolism are not capable of eliminating caffeine from the wastewater. Indeed, caffeine is the predominant undegraded basic organic compound in activated sludge-treated domestic wastewater effluent (Garrison *et al.* 1977). It is important to note that with extended incubation (5 days, Figure 1, panel A) or bacterial enrichment (Figure 1, panel B), the rate of caffeine degradation is sufficiently high to eliminate caffeine from the system, within the usual wastewater residence time in treatment processes. However, such enrichments are currently not practical for large municipal waste treatment operations. The accumulation of UV-absorbing products was not observed in wastewater (Figure 1), as it was in minimal medium (Figure 3), but the chromatographic resolution of post-secondary treatment effluent extracts clearly showed the presence of some xanthine derivatives. The chlorination of residual caffeine and caffeine degradation by-products warrants further investigation for ecological and public health reasons, especially because chlorinated caffeine (8-chlorocaffeine) has been shown to be a more efficient potentiating agent than caffeine, through enhancement of chromosomal aberrations produced by alkylating agents (Alderson & Khan 1967; Kihlman 1974 and 1977; Sturelid & Kihlman 1975; Mohr *et al.* 1993; Purves & Sullivan 1993).

Because caffeine was not degraded at all in San Diego creek water, its presence could be used as a chemical indicator of human faecal contamination of natural water sources. Further investigation of concentrations and limits of quantitative extraction and analysis of caffeine in natural water systems is warranted. Although the enzymatic and genetic determinants of caffeine metabolism in humans have been characterized (Berthou *et al.* 1991), nothing is currently known about the genetic basis for microbial caffeine degradation. The detection of genes specifying caffeine degradation by the isolated *Pseudomonas putida* or other strains in natural water systems may also indicate the occurrence of sewage spills.

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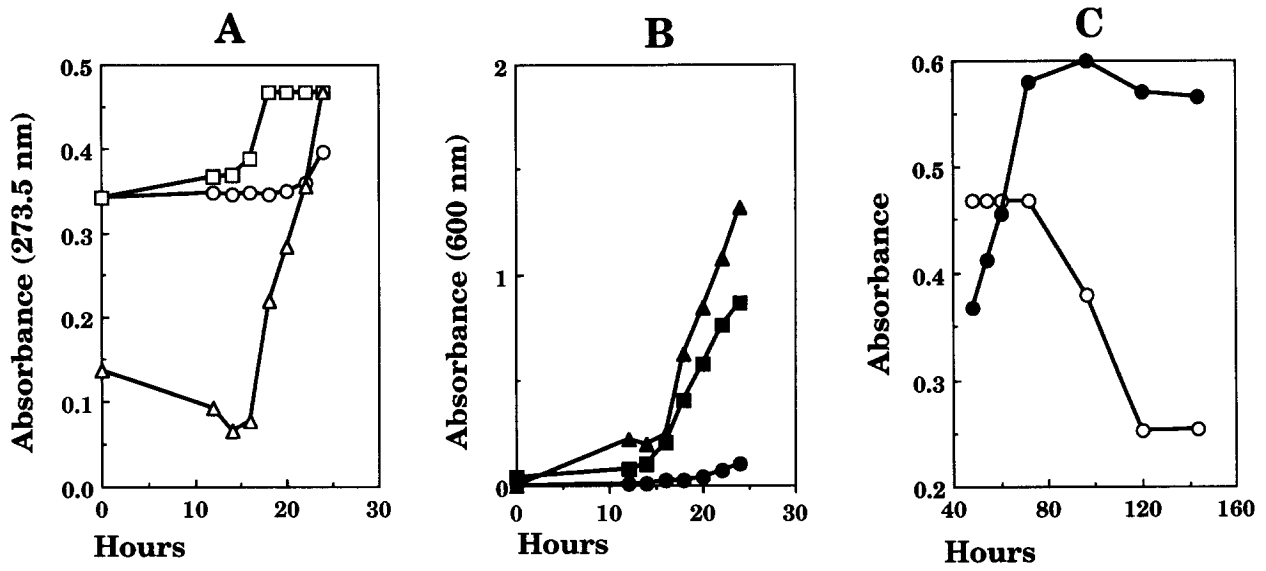

Figure 3. Spectrophotometric growth curve of a caffeine-degrading strain of *P. putida* isolated from human wastewater.

Panel A: Bacterial metabolism of caffeine monitored by absorbance at 273.5 nm of cultures containing 0.02 M caffeine (○); 0.01 M each of caffeine and glucose (□); or 0.02 M glucose (△).

Panel B: Bacterial proliferation measured by absorbance at 600 nm of cultures containing 0.02 M caffeine (●); 0.01 M each of caffeine and glucose (■); or 0.02 M glucose (▲). Absorbance readings of 0.5 at 600 nm corresponded to 83 ± 2 μg cell dry weight per ml.

Panel C: Data from a continuation of the same experiment represented in panels A and B, showing the reduction in absorbance at 273.5 nm following prolonged incubation. Absorbance at 600 nm (●); absorbance at 273.5 nm (○).

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