Journal of Industrial Microbiology & Biotechnology (2002) 28, 88–96

www.nature.com/jim

Metabolite repression inhibits degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by *Stenotrophomonas maltophilia* VUN 10,003

AL Juhasz[1,a], GA Stanley[2] and ML Britz[1,b]

[1]*Centre for Bioprocessing and Food Technology, Werribee Campus (W008), Victoria University of Technology, P.O. Box 14428 Melbourne City MC, Melbourne 8001, Australia;* [2]*School of Life Sciences and Technology, Werribee Campus (W008), Victoria University of Technology, P.O. Box 14428 Melbourne City MC, Melbourne 8001, Australia*

Large inocula of *Stenotrophomonas maltophilia* VUN 10,003 were used to investigate bacterial degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene. Although strain VUN 10,003 was capable of degrading 10–15 mg l^{-1} of the five-ring compounds in the presence of pyrene after 63 days, further addition of pyrene after degradation of the five-ring polycyclic aromatic hydrocarbons (PAHs) ceased did not stimulate significant decreases in the concentration of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene. However, pyrene was degraded to undetectable levels 21 days after its addition. The amount of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene degraded by strain VUN 10,003 was not affected by the initial concentration of the compounds when tested at 25–100 mg l^{-1}, by the accumulation of by-products from pyrene catabolism or a loss of ability by the cells to catabolise benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene. Metabolite or by-product repression was suspected to be responsible for the inhibition: By-products from the degradation of the five-ring compounds inhibited their further degradation.
Journal of Industrial Microbiology & Biotechnology (2002) **28,** 88–96 DOI: 10.1038/sj/jim/7000216

Keywords: benzo[*a*]pyrene; biodegradation; dibenz[*a*,*h*]anthracene; inhibition; metabolites; *Stenotrophomonas maltophilia*

Introduction

Bioremediation of soils contaminated by polycyclic aromatic hydrocarbons (PAHs) has been effective in removing low molecular weight PAH compounds, but PAH bioremediation continues to be unreliable at removing higher molecular weight PAHs [7,31]. The major reasons for the recalcitrance of high molecular weight PAHs to biodegradation include the lack of microflora in the soil (indigenous or inoculated) that contain the appropriate catabolic pathways, the low bioavailability of the compounds and a deficit of essential nutrients in the soil [6]. Other factors may also play a role depending on the microbial, chemical and physical environment of the site. For example, the rate of high molecular weight PAH biodegradation may be affected by the presence of low molecular weight PAHs or other organic compounds through preferential utilisation of the more easily degradable substrates [23,27]. Alternatively, degradation of high molecular weight PAHs may be inhibited by the presence of other more water-soluble PAHs via competition at active sites of oxygenases or through the accumulation of toxic products following cometabolism of other PAHs [4,29].

In an effort to overcome some of these shortfalls in PAH bioremediation, a major focus of research has been on the isolation of high molecular weight PAH-degrading microorganisms with the ability to degrade mixed PAHs simultaneously [21,24]. Isolated microorganisms that have demonstrated ability to degrade high molecular weight PAHs could be useful for *in situ* or *ex situ* remediation of soils containing these compounds that would otherwise remain undegraded [8].

We isolated a strain of *Burkholderia cepacia* (strain VUN 10,003), reclassified as *Stenotrophomonas maltophilia* using 16SrRNA gene analysis [15]), with the ability to degrade benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene [17]. When liquid cultures were inoculated at high cell numbers, strain VUN 10,003 was able to degrade 10–15 mg l^{-1} of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene after 63 days. However, a characteristic of the kinetics of degradation was a 21- to 28-day lag period before degradation commenced and a rapid decline in the degradation rate of the five-ring PAHs after approximately 56 days. This cessation was not due to a low viable population. Possible mechanisms behind the cessation of five-ring PAH degradation include: the need for a threshold concentration of substrate to stimulate PAH catabolism, catabolic repression due to the accumulation of high molecular weight PAH degradation by-products or the more general loss of metabolic activity due to production of microbial inhibitors. This study investigates if one or more of these mechanisms play a role in reducing the rate of five-ring PAH degradation by *S. maltophilia* VUN 10,003.

Materials and methods

Microorganisms

S. maltophilia VUN 10,003 was isolated from soil obtained from an abandoned factory site located near Port Melbourne, Australia [18]. Strain VUN 10,003 could grow on fluorene, phenanthrene and pyrene (100 mg l^{-1}) when supplied as sole carbon and energy

Correspondence: Dr Albert L. Juhasz, CSIRO Land and Water, Private Mail Bag 2, Glen Osmond, SA 5064, Australia
[a]Current address: CSIRO Land and Water, Private Mail Bag 2, Glen Osmond, SA, 5064, Australia.
[b]Current address: Department of Food Science and Agribusiness, The University of Melbourne, Sneydes Road, Werribee, Melbourne 3030, Australia.
Received 30 January 2001; accepted 10 October 2001

source [18]. Resting cell experiments performed with high cell densities of strain VUN 10,003 demonstrated that benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene and coronene could also be degraded when supplied alone [17].

Media and growth conditions

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) at the highest available purity. Stock solutions of pyrene (25 mg ml^{-1}), benzo[*a*]pyrene (5 mg ml^{-1}) and dibenz[*a*,*h*]anthracene (5 mg ml^{-1}) were prepared in dimethylformamide (DMF). A basal salts medium (BSM) [16] was supplemented with the respective PAH stock solutions to achieve a final concentration of 250 mg l^{-1} for pyrene and 25, 50 or 100 mg l^{-1} for benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene. Unless otherwise stated, cultures were incubated at 30°C and 175 rpm in the dark. Inocula for PAH degradation studies were prepared as described previously [19].

Benzo[*a*]pyrene mineralisation

Benzo[*a*]pyrene mineralisation by *S. maltophilia* VUN 10,003 was monitored with replicate (triplicate) biometer flasks (Bellco Glass, Vineland, NJ). Aliquots of high cell density suspensions (20 ml, 2.5×10^8 cells ml^{-1}), resuspended in BSM, were inoculated into flasks containing 50 mg l^{-1} unlabelled benzo[*a*] pyrene supplemented with 1.0 μCi of [7-^{14}C]benzo[*a*]pyrene (26.6 mCi $mmol^{-1}$). Cultures were incubated at 30°C and 175 rpm in the dark for 70 days. Uninoculated BSM–benzo[*a*]pyrene medium and mercuric chloride-killed cells served as the controls. Mineralisation of benzo[*a*]pyrene was determined by monitoring the evolution of $^{14}CO_2$ over the incubation period. Mass balances were calculated at the end of the incubation period by determining the distribution of ^{14}C in the culture medium, cell pellet and gaseous phase.

Degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene in the presence of pyrene

High cell density suspensions of strain VUN 10,003 (10 ml; 2.5×10^8 cells ml^{-1}) were added to serum bottles and PAHs were added at concentrations of 250 mg l^{-1} for pyrene and 50 mg l^{-1} for benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene. Incubations were performed in triplicate for each set of culture conditions (pyrene and benzo[*a*]pyrene or pyrene and dibenz[*a*,*h*]anthracene) and sample period. After 63 days, a further 250 mg l^{-1} of pyrene was added to the cultures and incubation continued. Samples were removed for the analysis of PAH concentration and microbial numbers at weekly or fortnightly intervals over the 126 day incubation period. Control cultures included PAHs added to BSM without cells or mercuric chloride-killed cells.

Degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene in the presence of pyrene metabolites

The influence of pyrene metabolites on the degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene was determined by inoculating high cell densities (10 ml; 2.5×10^8 cells ml^{-1}) of strain VUN 10,003 into media containing pyrene metabolites and one of the five-ring compounds. Initially, VUN 10,003 was inoculated into BSM containing 250 mg l^{-1} of pyrene. After the complete degradation of pyrene (7–10 days), the cells were removed by centrifugation (10,000 rpm for 10 min at 4°C). The spent BSM was then supplemented with pyrene (250 mg l^{-1}) and benzo[*a*]pyrene or pyrene (250 mg l^{-1}) and dibenz[*a*,*h*]anthracene (50 mg l^{-1}), plus nitrate and phosphate (400 mg l^{-1} of K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$) (designated "pyrene-spent medium"). A fresh high cell density inoculum of strain VUN 10,003 (2.5×10^8 cells ml^{-1}) was added to the pyrene-spent medium. Cultures were incubated and triplicate samples were removed for analysis at weekly intervals for an incubation period of a further 63 days.

Degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene in the presence of five-ring PAH metabolites

The influence of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene metabolites on the degradation of five-ring PAHs was evaluated by inoculating strain VUN 10,003 into media containing the respective PAH metabolites. The experimental medium was prepared by growing parallel sets in BSM using pyrene and benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene as the carbon sources using high initial cell densities. When the degradation of the five-ring PAHs ceased (after 63 days), one set of cultures was treated to prepare a culture broth containing mostly polar products of five-ring PAH catabolism, the other set of cultures was treated to produce a culture broth containing dichloromethane (DCM)-extractable products of five-ring PAH catabolism. These culture broths formed the media for subsequent incubations.

Firstly, cells and undegraded benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene were removed from the respective cultures by centrifugation (5000 rpm for 10 min at 4°C). The culture supernatants, presumably containing mostly polar by-products of five-ring catabolism, were then used as the culture medium for further experiments. Supernatants were distributed into serum bottles and fresh benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene were added at a concentration equal to that detected before centrifugation. Pyrene (250 mg l^{-1}) was also added to the culture supernatants, which were then inoculated at high cell densities with fresh VUN 10,003 grown in BSM-pyrene. Cultures were incubated for a further 63 days and samples were removed at weekly or fortnightly intervals. This experiment was designed to examine the degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by a fresh inoculum of strain VUN 10,003 in the presence of mostly polar by-products of their metabolism.

Secondly, from a parallel set of cultures, nonpolar and some polar by-products of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene catabolism were extracted using DCM from 63-day-old cultures containing pyrene and benzo[*a*]pyrene or pyrene and dibenz[*a*,*h*]anthracene. The extracts were concentrated, solvent exchanged with DMF (100 μl) and added to 10 ml of fresh BSM containing pyrene (250 mg l^{-1}) and benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene (50 mg l^{-1}). This medium was then inoculated with fresh pyrene-grown VUN 10,003. Cultures were incubated for a further 63 days and samples were removed at weekly or fortnightly intervals. This second experiment was designed to examine the degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by fresh inocula of strain VUN 10,003 in the presence of mostly nonpolar by-products of their catabolism.

Determination of microbial growth

Microbial numbers in liquid cultures were determined aerobically in nutrient broth using a three-tube microtitre tray most probable

number (MPN) method. The viable count was estimated from the results using statistical tables [28]. Growth at the expense of PAHs was also established by an increase in protein concentration. Cells were collected from culture samples (10 ml) by centrifugation (5000 rpm for 10 min) and washed twice in Ringer's solution. Cell pellets were resuspended in 1.0 ml 4.6 M NaOH and boiled for 10 min to lyse the cells. Protein concentrations were measured by the method of Lowry *et al* [22].

Extraction of polar and nonpolar metabolites

Benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene cultures (10 ml) were extracted with two equal volumes of DCM after incubation with strain VUN 10,003 for 63 days. To enhance the recovery of acidic metabolites, supernatants were acidified to pH 2.5 [13] with 10 M HCl and extracted with two additional volumes of DCM. The extracts were pooled, dried with anhydrous Na_2SO_4 and evaporated *in vacuo* at 35°C (Eyela Rotary evaporator with SB-650 water bath, Tokyo, Japan) to approximately 1.5 ml. The extracts were transferred to brown glass sample vials and further concentrated under a gentle stream of nitrogen (Techne, Cambridge, UK, sample concentrator). Extracts were stored at −20°C.

Analytical procedures

PAH extraction methods and gas chromatographic operation conditions have been described elsewhere [18]. Briefly, PAHs were extracted from culture fluid (10 ml) with DCM (1.0 ml). Benzo[*b*]fluorene (1 mg ml^{-1}) was used as an internal standard. The efficiency of the extraction procedure was greater than 98% for all PAHs. Gas chromatographic analysis of DCM extracts and PAH standards were performed on a Varian Star 3400 gas chromatogram equipped with a flame ionisation detector (GC-FID), using a BPX-5 capillary column (25 m×0.22 mm, SGE, Melbourne, Australia). The oven temperature was programmed at 200°C for 1 min followed by a linear increase of 10°C min^{-1} to 320°C, holding at 320°C for 10 min. Injector and detector temperatures were maintained at 300°C.

Pyrene, benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene metabolites were separated and visualised by thin-layer chromatography (TLC) using silica gel 60 plates (Merck, Frankfurt, Germany) and a three-phase solvent system as described by Guerin and Jones [11]. After solvent development, PAH metabolites were visualised by observing the plates on a UV light box (302 nm) (LKB 2011 mactovue transilluminator, Bromma, Sweden).

$^{14}CO_2$ from [7-^{14}C]benzo[*a*]pyrene mineralisation experiments was collected in 0.1 M NaOH (5.0 ml). At various time intervals, the NaOH was removed from the side arm flask and replaced with fresh NaOH. At the final sample point (70 days), 10 M HCl (0.5 ml) was added to the culture medium to release dissolved CO_2. Aliquots (2×1.0 ml) of NaOH were added to Cytoscint scintillation cocktail (9.0 ml, ICN) and beta emissions measured using a Wallac 1410 liquid scintillation counter (Pharmacia, Freiburg, Germany). To determine the distribution of residual ^{14}C-label, culture fluid was centrifuged at 15,000 rpm for 10 min at 4°C. Culture supernatants were assayed for radioactivity by combining aliquots (2×1.0 ml) of the supernatant with Cytoscint scintillation cocktail (9.0 ml). PAH and cell pellets were extracted with DCM (10 ml) and aliquots (2×1.0 ml) of the DCM extracts were added to Insta Gel scintillation cocktail (9.0 ml) and the beta emissions measured. To determine the amount of ^{14}C incorporated into cellular material, the cell debris after extraction with DCM, was suspended in 5.0 ml dH_2O. Aliquots of the cell debris (2×1.0 ml) were combined with Cytoscint scintillation cocktail (9.0 ml) and the radioactivity assayed.

Results

Benzo[a]pyrene mineralisation

Previous research demonstrated the potential of *S. maltophilia* VUN 10,003 to mineralise pyrene [14]. In addition, resting cell experiments showed the ability of VUN 10,003 to remove benzo[*a*]pyrene from a basal salts culture medium [17,18].

[7-^{14}C]Benzo[*a*]pyrene mineralisation experiments demonstrated that less than 0.15% of the total radioactivity was recovered as $^{14}CO_2$ after 70 days. The majority of the ^{14}C (83%) was recovered from the organic phase as undegraded substrate and/or nonpolar extractable metabolites. The remainder of the labelled carbon was distributed between the aqueous phase (5%) and the cell debris (12%). Abiotic benzo[*a*]pyrene degradation was minimal. Labelled carbon from the uninoculated cultures or those containing killed inocula was detected in small amounts in the aqueous phase (0.5–0.6%), cell debris (0.5%) and as $^{14}CO_2$ (<0.1%): 91% of the total radioactivity was recovered from the inoculated flasks with reference to controls.

As benzo[*a*]pyrene disappearance by *S. maltophilia* VUN 10,003 could be attributed to transformation of the compound and

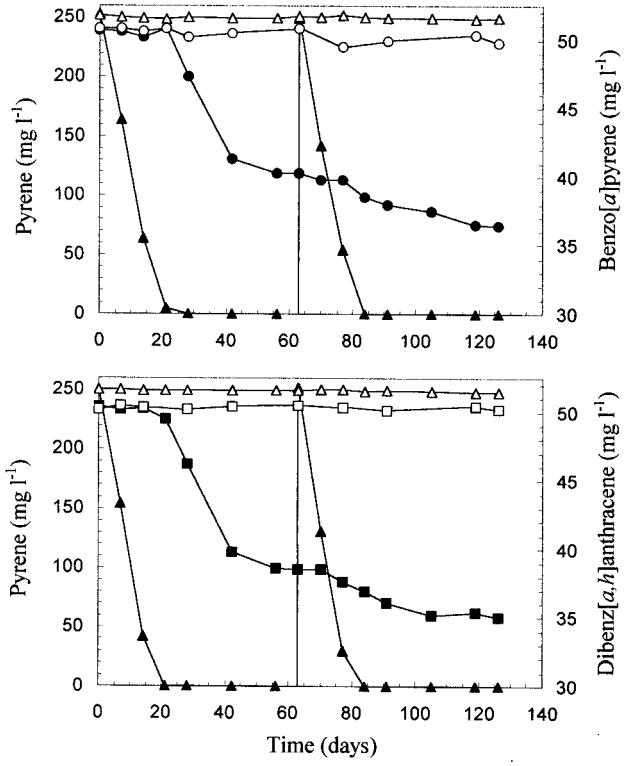

Figure 1 Degradation of benzo[*a*]pyrene (●) or dibenz[*a*,*h*]anthracene (■) in the presence of pyrene (▲) using pyrene-grown *S. maltophilia* VUN 10,003. Fresh pyrene was resupplied to cultures after 63 days. Mercuric chloride-killed controls for pyrene (△), benzo[*a*]pyrene (○) and dibenz[*a*,*h*]anthracene (□) are also shown.

not to mineralisation, further experiments utilised unlabelled benzo[*a*]pyrene and monitored the loss of the compound.

The effect of pyrene spiking on benzo[a]pyrene and dibenz[a,h]anthracene degradation

When inoculated into BSM containing pyrene (250 mg l^{-1}) and benzo[*a*]pyrene or dibenz[*a,h*]anthracene (50 mg l^{-1}), strain VUN 10,003 rapidly degraded pyrene in the presence of the five-ring compounds (Figure 1). Over 95% of the initial pyrene was degraded after 21 days. A lag period of 21 days was observed before the commencement of benzo[*a*]pyrene or dibenz[*a,h*]anthracene degradation, which was similar to the degradation lag periods observed in a previous study [17]. The extended lag period was attributed to the time required for the induction of five-ring PAH catabolism. The energy source during this time was probably from stored carbohydrate reserves [3]. Although degradation of the five-ring compounds was slow relative to pyrene, significant decreases in the concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene (9.5–11.6 mg l^{-1}) were observed up to 56 days but their degradation decreased after 56 days incubation. Therefore, more pyrene (250 mg l^{-1}) was added to the benzo[*a*]pyrene and dibenz[*a,h*]anthracene cultures on day 63 in order to stimulate the rate of five-ring PAH degradation. The added pyrene was again rapidly degraded, such that this substrate was undetectable in all cultures on day 84. Small decreases in the concentration of benzo[*a*]pyrene (3.8 mg l^{-1}) and dibenz[*a,h*]anthracene (3.5 mg l^{-1}) were observed during the 63-day period after pyrene spiking. Numbers of viable cells decreased over the 126-day incubation period for all benzo[*a*]-pyrene and dibenz[*a,h*]anthracene incubations from initial cell counts of 2.5×10^8 cells ml^{-1} to $5.0–7.5\times10^7$ cells ml^{-1}.

Effects of substrate concentration on degradation of benzo[a]pyrene and dibenz[a,h]anthracene

Benzo[*a*]pyrene or dibenz[*a,h*]anthracene added individually to BSM were degraded by high cell populations when the initial concentration of these PAHs was in the range of 25–100 mg l^{-1}. The degradation rate of these PAHs was independent of their initial concentration (Figure 2). The degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene at all concentrations was preceded by a lag period of approximately 21 days. Although the percentage of the five-ring compounds degraded over 56 days decreased with increasing initial concentration, the amounts degraded were similar: the degradation of benzo[*a*]pyrene resulted in decreases of 14.3, 12.8 and 11.3 mg l^{-1} from cultures with initial concentrations of 25, 50 and 100 mg l^{-1}, respectively (Figure 2). A similar result was observed for dibenz[*a,h*]anthracene (Figure 2): degradation resulted in decreases of 16.3, 15.8 and 14.3 mg l^{-1} for cultures with initial dibenz[*a,h*]anthracene concentrations of 25, 50 and 100 mg l^{-1}, respectively. Microbial numbers decreased slightly over 56 days for all benzo[*a*]pyrene or dibenz[*a,h*]anthracene incubations from around 2.5×10^8 cells ml^{-1} to $5.0–7.5\times10^7$ cells ml^{-1}.

Inhibition of benzo[a]pyrene and dibenz[a,h] anthracene degradation by pyrene degradation by-products

To assess whether by-products of pyrene catabolism inhibited the degradation of five-ring PAH compounds, pyrene-spent medium was prepared by growing strain VUN 10,003 in BSM pyrene

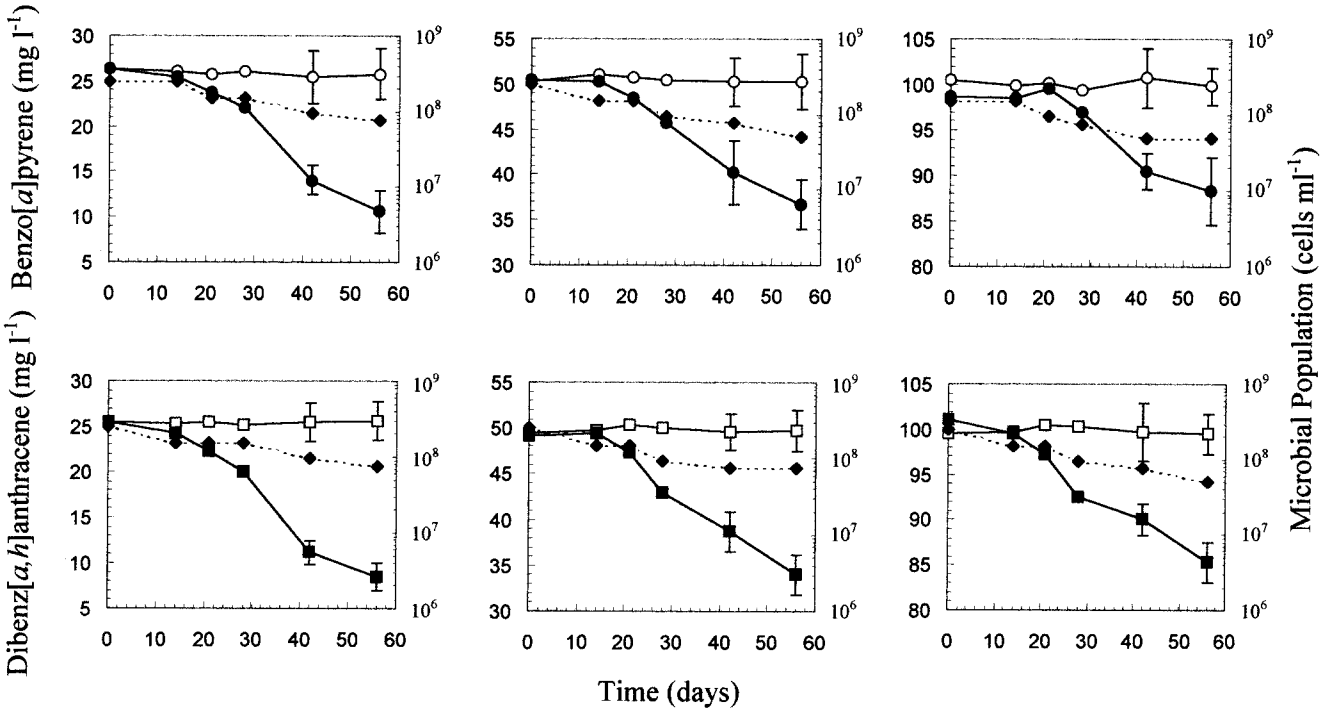

Figure 2 Relationship between initial concentration of benzo[*a*]pyrene (●) or dibenz[*a,h*]anthracene (■) and the kinetics of degradation by *S. maltophilia* VUN 10,003. Benzo[*a*]pyrene or dibenz[*a,h*]anthracene was added to BSM containing *S. maltophilia* VUN 10,003 at the following concentrations: 25, 50 and 100 mg l^{-1}. Microbial numbers (◆) in inoculated cultures as well as benzo[*a*]pyrene (○) and dibenz[*a,h*]anthracene (□) concentrations in mercuric chloride-killed controls are also shown.

Table 1 Chromatographic mobility (R*f* value) and colour of metabolites isolated from the degradation of pyrene, benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by *S. maltophilia* VUN 10,003 by preparative thin-layer chromatography

Metabolite No.	Metabolites purified from:					
	Pyrene		Benzo[*a*]pyrene		Dibenz[*a*,*h*]anthracene	
	R*f* value	Colour[a]	R*f* value	Colour[a]	R*f* value	Colour[a]
1	0.22	yellow	0.19	yellow	0.19	yellow
2	0.28	white	0.49	pale orange	0.55	pale lime
3	0.53	purple	0.66	orange/red	0.63	orange
4	0.71	yellow	0.70	pale orange	0.83	blue
5	0.81[b]	blue	0.74	yellow	0.89	orange
6	0.91[c]	blue	0.76	blue	0.91	orange/red
7	0.96	pale yellow	0.89	pale lime	0.97	blue
8	–	–	0.96	blue	0.99	yellow/green
9	–	–	0.97	orange	–	–

[a]The colour of metabolites was observed under ultraviolet light (302 nm).
[b]Pyrene metabolite number 5 was identified as 4-hydroxyperinaphthenone.
[c]Pyrene metabolite number 6 was identified as 4-phenanthroic acid.

(250 mg l^{-1}) medium. After the complete degradation of pyrene, cells were removed by centrifugation and the resulting supernatant was tested for the accumulation of pyrene catabolic by-products, which accumulated in the medium. Over 20 different metabolite bands were resolved by TLC after 5 days incubation. R_f values varied from 0.22, for the most polar compound, to 0.96 for ring oxidation products and nonpolar metabolites. Seven metabolites were isolated and purified (Table 1) and two were identified as 4-hydroxyperinaphthenone and 4-phenanthroic acid [14].

When inoculated into pyrene-spent medium containing added pyrene and benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene, strain VUN 10,003 degraded greater than 97% of the pyrene supplied initially after 21 days (Figure 3). Benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene degradation was preceded by a lag period of approximately 21 days, after which 27–28% (14.2 and 13.6 mg l^{-1}) of the five-ring compounds were degraded after 56 days, respectively (Figure 3). Microbial numbers decreased over the incubation period for all benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene incuba-

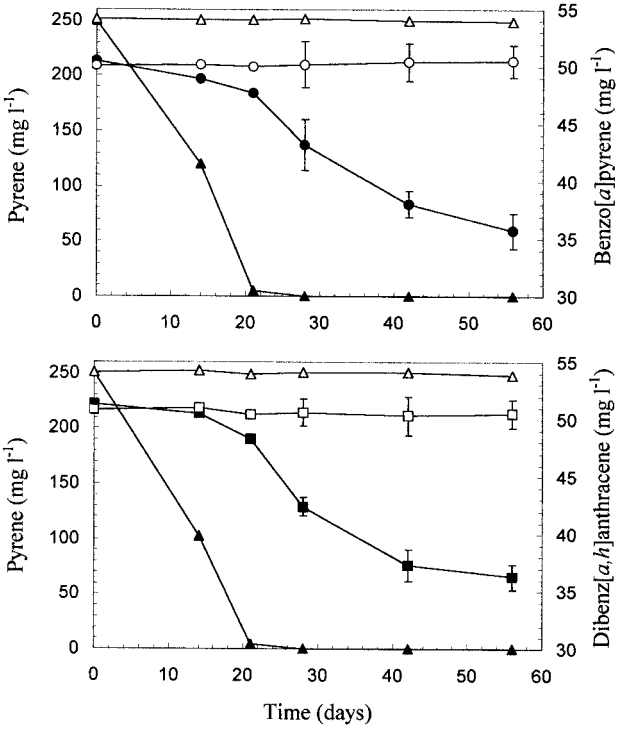

Figure 3 Effect of presumptive pyrene catabolism by-products on the degradation of benzo[*a*]pyrene (●) or dibenz[*a*,*h*]anthracene (■) in the presence of pyrene (▲). Pyrene-grown *S. maltophilia* VUN 10,003 was inoculated into pyrene-spent BSM, which had previously been supplied initially for the degradation of pyrene. The pyrene (△), benzo[*a*]pyrene (○) and dibenz[*a*,*h*]anthracene (□) concentrations in mercuric chloride-killed controls are also shown.

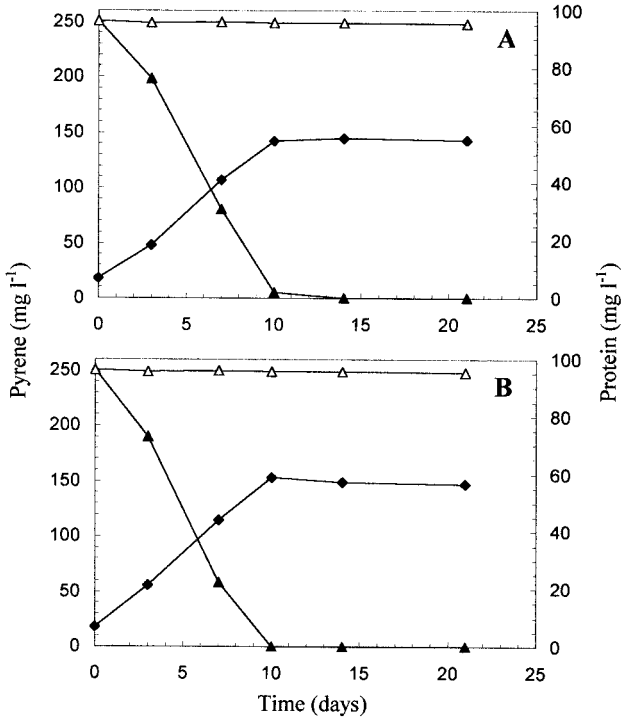

Figure 4 Time course for pyrene degradation by *S. maltophilia* VUN 10,003 previously exposed to benzo[*a*]pyrene (A) or dibenz[*a*,*h*]anthracene (B) for 63 days. Pyrene-containing medium was inoculated with 10% unwashed benzo[*a*]pyrene- or dibenz[*a*,*h*]anthracene-exposed cells. Pyrene (▲) and protein (◆) concentrations were determined as described in the Materials and methods. The pyrene (△) concentration in uninoculated control cultures is also shown.

tions (2.5×10^8 cells ml^{-1} to $5.0–7.5\times10^7$ cells ml^{-1}). By-products from pyrene catabolism did not inhibit the degradation of pyrene, benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene.

Five-ring PAH catabolic activity of strain VUN 10,003 incubated on benzo[a]pyrene or dibenz[a,h] anthracene for extended periods

After exposure to benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene for 63 days, cells were removed from the culture broth and inoculated into fresh BSM containing either pyrene alone or pyrene and a five-ring PAH. Strain VUN 10,003 degraded pyrene as a sole carbon and energy source or in combination with benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene (50 mg l^{-1}) (Figure 4). Pyrene (250 mg l^{-1}) alone was degraded to undetectable levels after 10–14 days and this was accompanied by an increase in protein (6.8 to 55.5–58.9 mg l^{-1}) (Figure 4). When benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene was supplemented with pyrene, greater than 90% of the pyrene was degraded after 28 days (Figure 5). A concurrent increase in protein concentration from 6.7 mg l^{-1} to 49.6–53.4 mg l^{-1}, was observed during this period. Degradation of the five-ring compounds resulted in a 10.0–11.3 mg l^{-1} decrease in their concentration over a 56-day period (Figure 5). The amount degraded was independent of which five-ring compound had been used to preincubate the inocula. Protein concentrations decreased slightly after day 28 resulting in final protein concentrations of 45.8–49.2 mg l^{-1}. Exposure to benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene for extended periods did not affect the ability of strain VUN 10,003 to degrade five-ring compounds.

Inhibition of benzo[a]pyrene and dibenz[a,h] anthracene degradation by their metabolites

The degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene, in the absence of pyrene, produced 15 distinct bands for each PAH. Nine metabolites, with R_f values ranging from 0.19 to 0.97 were purified from benzo[*a*]pyrene culture extracts (Table 1), while eight metabolites, with R_f values from 0.19 to 0.99 were purified from dibenz[*a*,*h*]anthracene culture extracts (Table 1). Although these metabolites could be visualised by TLC, attempts to identify the products (using GC-MS and NMR) proved unsuccessful.

An experiment was conducted to test whether five-ring PAH degradation by-products had an inhibitory effect on the further degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene. The addition of fresh cells in these experiments, as opposed to the 63-day-old cells, should not have an influence on the outcome since in previous experiments 63-day-old cells performed like fresh cells when inoculated into fresh medium (Figure 4).

In the first part of the experiment (before day 63), pyrene was rapidly degraded in the presence of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene (data not shown). The degradation rates were similar to those observed in the pyrene-spiking experiments (Figure 1); greater than 95% of the added pyrene was degraded

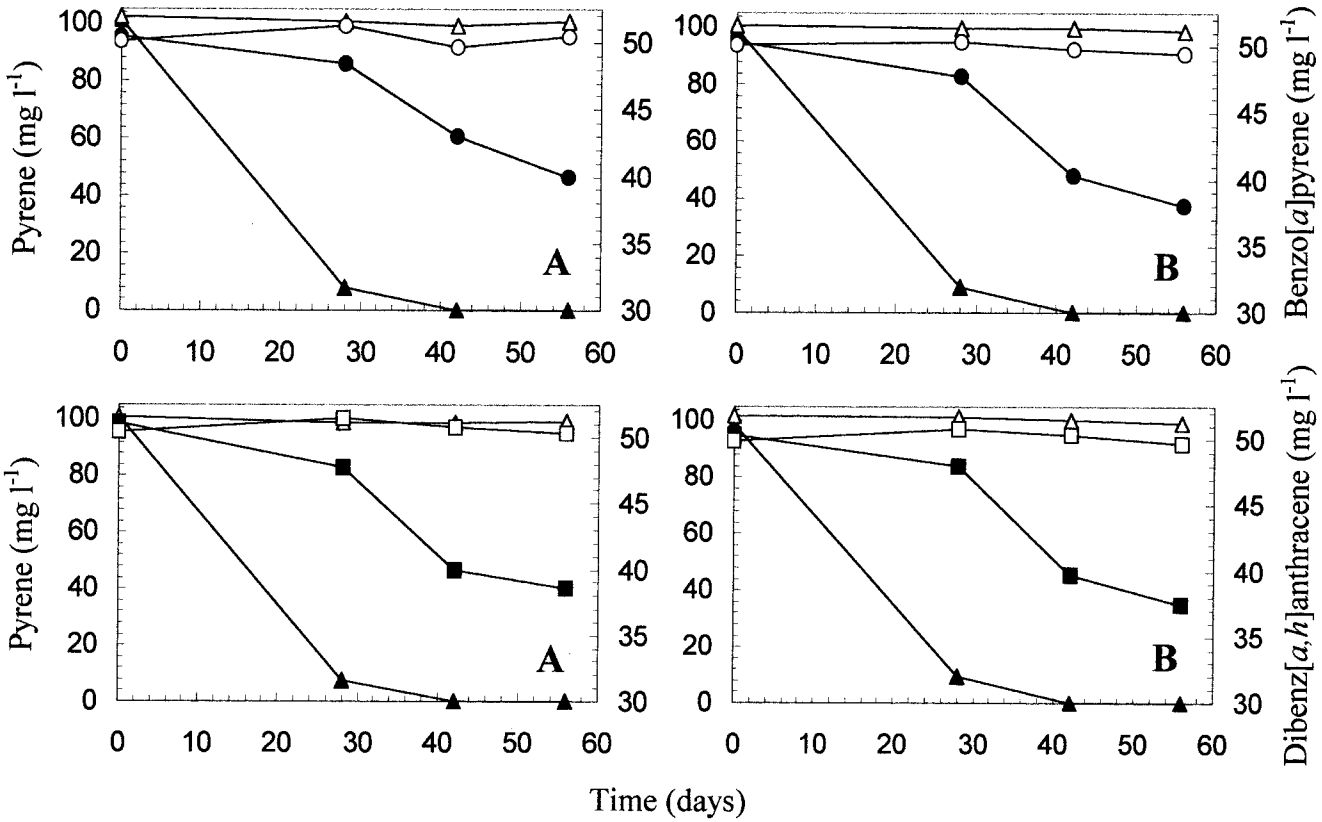

Figure 5 Degradation of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene in the presence of pyrene by *S. maltophilia* VUN 10,003 previously exposed to benzo[*a*]pyrene (A) or dibenz[*a*,*h*]anthracene (B) for 63 days. PAH-containing medium was inoculated with 10% unwashed benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene-exposed cells. Pyrene (△), benzo[*a*]pyrene (○) and dibenz[*a*,*h*]anthracene (□) concentrations in mercuric chloride-killed controls are also shown.

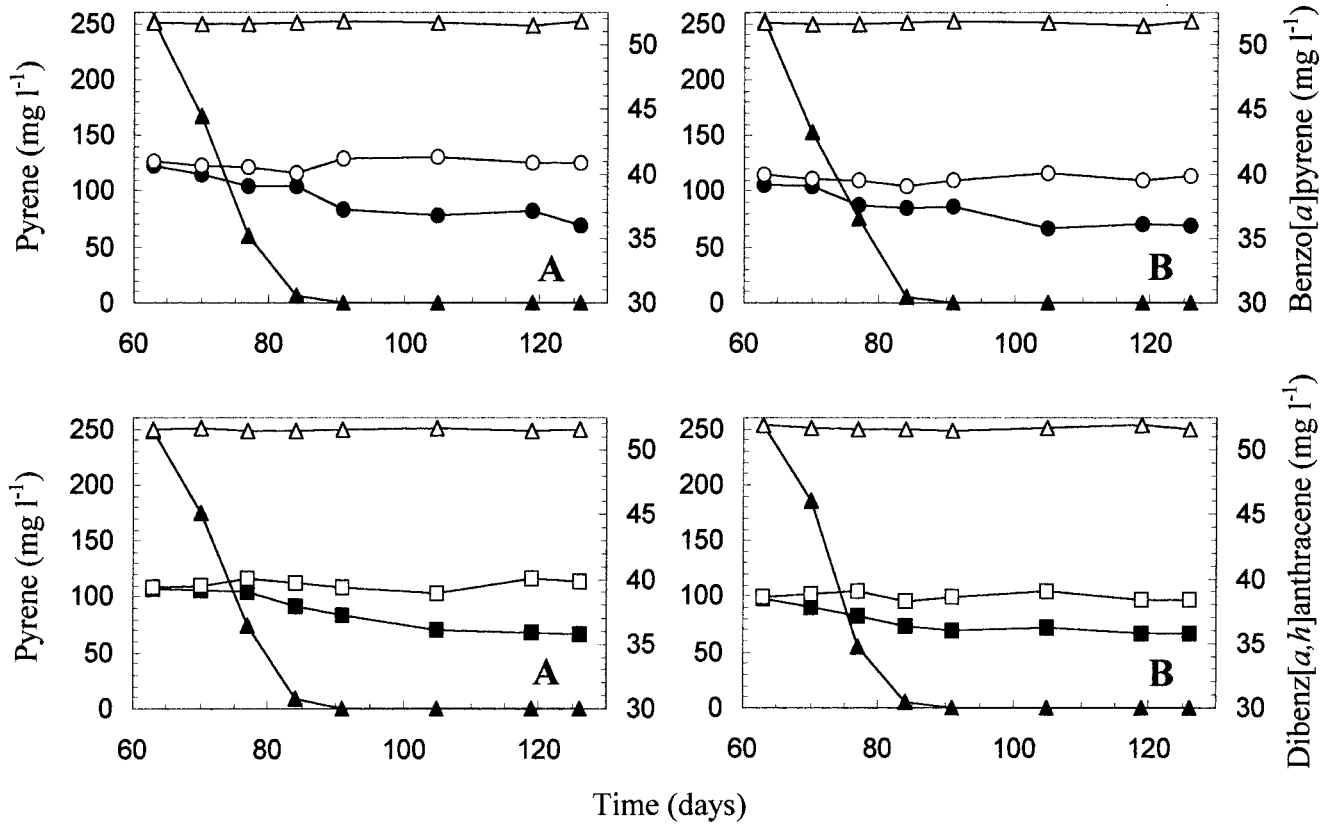

Figure 6 Effect of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolic by-products on the degradation of benzo[*a*]pyrene (●) or dibenz[*a,h*]anthracene (■) by *S. maltophilia* VUN 10,003 in the presence of pyrene (▲). Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to cultures containing pyrene-grown cells. After 63 days, two treatments were used to assess the effects of polar metabolic by-products (A) and total metabolic by-products (B). For polar metabolic by-products, cultures were centrifuged and the supernatants, containing presumptive five-ring by-products, were used as the medium for the remainder of the experiment. Pyrene was resupplied at a concentration of 250 mg l^{-1}; benzo[*a*]pyrene or dibenz[*a,h*]anthracene was added at a concentration equal to that prior to centrifugation and the medium was inoculated with freshly grown cells. For total metabolic by-products, cultures were extracted with DCM, then transferred to dimethylformamide (0.1 ml). Culture extracts were added to sterile BSM, pyrene was added at 250 mg l^{-1} and cultures were inoculated with freshly grown cells. Mercuric chloride-killed controls for pyrene (△), benzo[*a*]pyrene (○) and dibenz[*a,h*]anthracene (□) are also shown.

after 21 days. A lag period of 21 days was observed before the commencement of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. Degradation of the five-ring compounds resulted in concentration decreases of 9.5–12.0 mg l^{-1} for benzo[*a*]pyrene and 10.5–11.5 mg l^{-1} for dibenz[*a,h*]anthracene by day 63.

After fresh cells were inoculated into preincubated BSM (with benzo[*a*]pyrene or dibenz[*a,h*]anthracene) (resulting in BSM containing polar metabolites) with added pyrene and benzo[*a*]-pyrene or dibenz[*a,h*]anthracene, pyrene was again rapidly degraded: greater than 95% of added pyrene was degraded at day 84 (21 days after spiking) (Figure 6A). Small decreases in the concentrations of benzo[*a*]pyrene (4.6 mg l^{-1}) and dibenz[*a,h*]-anthracene (3.2 mg l^{-1}) were observed 63 days after pyrene spiking (Figure 6A).

Similar results were observed when fresh cells were inoculated into BSM containing extracts of nonpolar by-products from benzo[*a*]pyrene or dibenz[*a,h*]anthracene degradation (Figure 6B). Greater than 95% of pyrene was degraded at day 84 (21 days after respiking). At the end of the incubation period, small decreases in the concentrations of benzo[*a*]pyrene (2.5 mg l^{-1}) and dibenz[*a,h*]anthracene (2.7 mg l^{-1}) were also observed (Figure 6B). Microbial numbers decreased over the incubation period for all benzo[*a*]pyrene and dibenz[*a,h*]anthracene incubations, from 2.5×10^{8} cells ml^{-1} to $5.0–7.5\times10^{7}$ cells ml^{-1}. Accumulation in the medium of by-products from five-ring PAH catabolism appeared to account for the cessation of their degradation after 63 days incubation.

Discussion

The transformation of high molecular weight PAHs has been observed by a number of researches [10,12,20,24,25]; however, none addressed the reasons why the degradation of the five-ring compounds is limited. Although *S. maltophilia* VUN 10,003 was capable of degrading benzo[*a*]pyrene and dibenz[*a,h*]anthracene, their degradation ceased after approximately 10–15 mg l^{-1} of the PAHs were degraded [17], independent of the initial concentration of substrate supplied. It was presumed that the cessation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene was due to the loss of catabolic enzyme activity. Although strain VUN 10,003 was unable to utilise benzo[*a*]pyrene or dibenz[*a,h*]anthracene as a growth source [18], prior growth of the cells on pyrene appeared to induce a catabolic pathway that could at least partly degrade the five-ring

compounds. Strain VUN 10,003 was unable to mineralise benzo[*a*]pyrene, however, it degraded the compound resulting in accumulation of water soluble transformation products. Presumably, the activity of this catabolic pathway was not sustained by benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene when they were the only PAHs in the cultures or, alternatively, inhibitory intermediates or products formed, which limited further degradation.

To test this hypothesis, an experiment was conducted in which pyrene was spiked into cultures containing the five-ring PAHs when their degradation had ceased. The reasoning behind the pyrene addition was that supplementation of the medium with a growth-supporting substrate (PAH) may stimulate continued degradation of the five-ring PAHs. Nutrient supplementation stimulates degradation of high molecular weight PAHs. Kanaly *et al* observed that the addition of a diesel fuel (0.2% wt vol^{-1}) significantly enhanced mineralisation of benzo[*a*]pyrene by a bacterial consortium from soil [20]. The failure of pyrene spiking to restart benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene degradation suggested that other factors apart from enzyme induction may be responsible for inability of the strains to degrade benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene significantly when they are supplied as the sole carbon and energy sources.

One possibility was that the degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by strain VUN 10,003 may be affected by a minimum threshold PAH concentration, below which degradation does not proceed. Spain *et al* [26] observed a threshold concentration for *p*-nitrophenol, below which adaptation and subsequent degradation of the compound did not occur. The effect of organic compound concentration on their biodegradation by natural microbial communities was also investigated by Boethling and Alexander [2]. Little mineralisation of 2,4-dichlorophenoxyacetate, 1-naphthyl-*N*-methylcarbamate or 1-naphthol-*N*-methylcarbamate occurred when these compounds were present at initial concentrations of 2–3 $\mu g\ l^{-1}$ or less. However, when the compounds were supplied at higher concentrations, 60% or more of the compounds were converted to CO_2. This was not the case in cultures containing the five-ring PAHs and strain VUN 10,003 since the initial concentration of the five-ring compounds in the culture medium up to 100 mg l^{-1} did not affect the total amount of benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene degraded.

Metabolite or by-product repression was responsible for the inhibition of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene degradation. A similar phenomenon has been observed for the three-ring PAH fluorene [5]. Casellas *et al* observed that the addition of 9-fluorenone, a metabolic by product of fluorene metabolism, to cultures of *Arthrobacter* strain F101 resulted in inhibition of fluorene degradation [5]. Polar and nonpolar by-products produced from degradation of the five-ring compounds inhibited their further degradation. Interestingly, benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene degradation was not inhibited by the presence of pyrene metabolites present in spent pyrene medium. This indicates that the inhibition of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene degradation stems only from products specific to their degradation. The production and accumulation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene metabolites or by-products may be inhibitory, but not lethal, to cellular activity or the metabolites may repress PAH catabolic enzyme activity, either of which would prevent further degradation of the compounds. The concentration of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene metabolites appears to accumulate to high enough levels to suppress PAH degradation after 10–15 mg l^{-1} of the five-ring compounds have been degraded. Cells that were previously exposed to benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene and their metabolites were able to degrade the five-ring compounds in the presence of pyrene when transferred to fresh medium. This indicates that the cells were still metabolically active and capable of degrading benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene in the absence of the metabolites and therefore loss of plasmid/genetic ability did not occur.

The lack of literature regarding the mechanisms and pathways involved in the degradation of the five-ring compounds makes it difficult to interpret which by-products of their catabolism limit the amount degraded to 10–15 mg l^{-1} for *S. maltophilia* VUN 10,003. In this study, benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene metabolites could be visualised by TLC; however, attempts to identify these products proved unsuccessful. The difficulties in identifying benzo[*a*]pyrene metabolites have been shared by a number of researchers. Although reductions in benzo[*a*]pyrene concentration were observed after incubation of the PAH with pure [1,9,12,18,24] and mixed cultures [20,30], few studies have isolated metabolic by-products of benzo[*a*]pyrene catabolism. *cis*-Dihydrodiols (either 4,5–, 7,8– or 9,10–) have been identified as initial transformation products of *Beijernickia* strain B-836 [9] and *Mycobacterium* strain RJGII-135 [24]. Furthermore, Schneider *et al* proposed that *Mycobacterium* strain RJGII-135 formed 4,5-chrysene-dicarboxylic acid as an *ortho* fission product of *cis*-4,5-benzo[*a*]pyrene while *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid resulted from *meta* fission of the hydroxylated compound [24]. At present, no literature exists on possible metabolic by-products of dibenz[*a*,*h*]anthracene catabolism, which makes it impossible to speculate as to which products may be responsible for the inhibition of its degradation by *S. maltophilia* VUN 10,003. As these pathways are further defined in this strain, the compounds responsible for inhibition will be identified, providing the basis for developing strategies to overcome this limiting trait.

Acknowledgements

The research was funded by PhD scholarships from Australian Research Award (Industry), in conjunction with Australian Defence Industries, the Victoria Education Foundation and Centre for Bioprocessing and Food Technology, Victoria University of Technology.

References

1 Barnsley EA. 1975. The bacterial degradation of fluoranthene and benzo[*a*]pyrene. *Can J Microbiol* 21: 1004–1008.
2 Boethling RS and M Alexander. 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. *Appl Environ Microbiol* 37: 1211–1216.
3 Boonchan S, ML Britz and GA Stanley. 1998. Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. *Biotechnol Bioeng* 59: 482–494.
4 Bouchez M, D Blanchet and J-P Vandecasteele. 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. *Appl Microbiol Biotechnol* 43: 156–164.
5 Casellas M, M Grifoll, J Sabate and AM Solanas. 1998. Isolation and characterization of a 9-fluorenone-degrading bacterial strain and its role in synergistic degradation of fluorene by a consortium. *Can J Microbiol* 44: 734–742.
6 Cerniglia CE. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3: 351–368.

7 Erickson DC, RC Loehr and EF Neuhauser. 1993. PAH loss during bioremediation of manufactured gas plant site soil. *Water Res* 27: 911–919.
8 Forsyth JV, YM Tsao and RD Bleam. 1995. Bioremediation: when is augmentation needed? In: Hinchee RE, Fredrickson J, Alleman, BC (eds), Bioaugmentation for site remediation. Battelle Press, OH, pp. 1–14.
9 Gibson DT, M Venkatanayarana, DM Jerina, H Yagi and H Yeh. 1975. Oxidation of the carcinogens benzo[*a*]pyrene and benzo[*a*]anthracene to dihydrodiols by a bacterium. *Science* 189: 295–297.
10 Grosser RJ, D Warshawsky and JR Vestal. 1991. Indigenous and enhanced mineralisation of pyrene, benzo[*a*]pyrene and carbazole in soil. *Appl Environ Microbiol* 57: 3462–3469.
11 Guerin WF and GE Jones. 1988. Mineralisation of phenanthrene by a *Mycobacterium* sp. *Appl Environ Microbiol* 54: 937–994.
12 Heitkamp MA and CE Cerniglia. 1989. Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. *Appl Environ Microbiol* 55: 1969–1973.
13 Heitkamp MA, JP Freeman, DW Miller and CE Cerniglia. 1988. Pyrene degradation by a *Mycobacterium* sp.: identification of oxidation and ring fission products. *Appl Environ Microbiol* 54: 2256–2265.
14 Juhasz AL. 1997. Pyrene degradation and metabolite formation by *Burkholderia cepacia* strain VUN 10,003. *Australas Biotechnol* 7: 350–354.
15 Juhasz AL. 1998. Microbial degradation of high molecular weight polycyclic aromatic hydrocarbons. PhD thesis, Victoria University of Technology, Melbourne, Australia.
16 Juhasz AL, ML Britz and GA Stanley. 1995. Isolation of polycyclic aromatic hydrocarbon degrading microorganisms. Third International Symposium: *In Situ* and On-Site Bioreclamation, San Diego, Battelle Press, OH.
17 Juhasz AL, ML Britz and GA Stanley. 1996. Degradation of high molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas cepacia*. *Biotechnol Lett* 18: 577–582.
18 Juhasz AL, ML Britz and GA Stanley. 1997. Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a*,*h*]anthracene by *Burkholderia cepacia*. *J Appl Microbiol* 83: 189–198.
19 Juhasz AL, ML Britz and GA Stanley. 1997. Degradation of benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene and coronene by *Burkholderia cepacia*. *Water Sci Technol* 36: 45–51.
20 Kanaly RA, R Bartha, K Watanabe and S Harayama. 2000. Rapid mineralisation of benzo[*a*]pyrene by a microbial consortium growing on diesel fuel. *Appl Environ Microbiol* 66: 4205–4211.
21 Kastner M, M Breuer-Jammali and B Mahro. 1994. Enumeration and characterisation of the soil microflora from hydrocarbon-contaminated soil sites able to mineralise polycyclic aromatic hydrocarbons (PAH). *Appl Microbiol Biotechnol* 41: 267–273.
22 Lowry OH, NJ Rosebrough, AL Farr and RJ Randall. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265–275.
23 Mueller JG, SE Lantz, BO Blattmann and PJ Chapman. 1991. Bench-scale evaluation of alternative treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials: slurry-phase bioremediation. *Environ Sci Technol* 25: 1056–1061.
24 Schneider J, R Grosser, K Jayasimhulu, W Xue and D Warshawsky. 1996. Degradation of pyrene, benz[*a*]anthracene and benzo[*a*]pyrene by *Mycobacterium sp*. strain RJGII-135, isolated from a former coal gasification site. *Appl Environ Microbiol* 62: 13–19.
25 Shiaris MP. 1989. Seasonal biotransformation of naphthalene, phenanthrene and benzo[*a*]pyrene in surficial estuarine sediments. *Appl Environ Microbiol* 55: 1391–1399.
26 Spain CJ, PH Pritchard and AW Bourquin. 1980. Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Appl Environ Microbiol* 40: 726–734.
27 Stringfellow WT and MD Aitken. 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading Pseudomonads. *Appl Environ Microbiol* 61: 357–362.
28 Taylor J. 1962. The estimation of numbers of bacteria by ten fold dilution series. *J Appl Bacteriol* 25: 54–61.
29 Tiehm A and C Fritzsche. 1995. Utilisation of solubilised and crystalline mixtures of polycyclic aromatic hydrocarbons by a *Mycobacterium* sp. *Appl Microbiol Biotechnol* 42: 964–968.
30 Trzesicka-Mlynarz D and OP Ward. 1995. Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures obtained from a PAH-contaminated soil. *Can J Microbiol* 41: 470–476.
31 Wilson SC and KC Jones. 1993. Bioremediation of soils contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environ Pollut* 88: 229–249.