Biodegradation **12:** 393–400, 2001.

Fluoranthene degradation in *Pseudomonas alcaligenes* PA-10

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Accepted 31 October 2001

Key words: fluoranthene, indole, PAH, *Pseudomonas alcaligenes*

Abstract

Pseudomonas alcaligenes strain PA-10 degrades the four-ring polycyclic aromatic hydrocarbon fluoranthene, co-metabolically. HPLC analysis of the growth medium identified four intermediates, 9-fluorenone-1-carboxylic acid; 9-hydroxy-1-fluorene carboxylic acid; 9-fluorenone and 9-fluorenol, formed during fluoranthene degradation. Pre-exposure of PA-10 to 9-fluorenone-1-carboxylic acid and 9-hydroxy-1-fluorene-carboxylic acid resulted in increases in fluoranthene removal, while pre-exposure to 9-fluorenone and 9-fluorenol resulted in a decrease in fluoranthene degradation. The rate of indole transformation was similarly affected by pre-exposure to these metabolic intermediates, indicating a link between fluoranthene degradation and indigo formation in this strain.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic pollutants which can enter the environment in a number of ways, both natural and anthropogenic. They may be released during the use and processing of petroleum products and during the incomplete combustion of fossil fuels (Cerniglia & Heitkamp 1989). Industrial effluents from processes such as waste incineration and coal gasification and liquefaction also contain high levels of PAHs as does the wood preservative creosote. Natural sources of these compounds include forest and prairie fires (Cerniglia 1992). The presence of PAHs in the environment is a cause for concern as many have been shown to have mutagenic, toxigenic, and in the case of some of the larger compounds, carcinogenic (Mortlemans et al. 1986; Bos et al. 1987; Cerniglia 1993; Rummel et al. 1999). As a result, there is much interest in microorganisms which can degrade PAHs and in the mechanisms through which this degradation occurs.

The pathways and genes involved in the metabolism of naphthalene as well as the three-ring PAHs phenanthrene and anthracene, have been well-documented (Cerniglia 1984; Yen & Serdar 1988; Juhasz & Naidu 2000) but considerably less is known about the degradation of the higher molecular weight PAHs. These compounds tend to be very stable and hydrophobic and more resistant to microbial attack (Cerniglia 1992). However, in the last decade there have been considerable advances in this area with several studies reporting the isolation of strains capable of degrading PAHs containing 4 rings, such as pyrene and fluoranthene (Heitkamp et al. 1988a; Boldrin et al. 1993; Sepic et al. 1998). In some cases intermediates have been identified and metabolic pathways have been proposed (Heitkamp et al. 1988b; Weissenfels et al. 1991).

Because fluoranthene contains a five-member ring, it is structurally similar to other chemicals of concern (carbazoles, dibenzothiophenes, dibenzofluorans and dibenzodioxins) and as such is also a useful model compound for biodegradation studies. *Sphingomonas paucimobilis* can utilise fluoranthene as a sole carbon source and has the ability to degrade a range of other high molecular weight PAHs, including chrysene, pyrene and benz[a]anthracene (Ye et al. 1996). Fluoranthene and pyrene degradation have also been observed in *Mycobacterium* sp. strain PYR-1. Several intermediates have been identified and pathways have been proposed for the metabolism of both

compounds in this strain (Kelley et al. 1993; Heitkamp et al. 1998).

Despite these recent advances, there is still a lack of information available concerning mechanisms involved in regulating catabolic pathways involved in the degradation of PAHs containing more than 3 rings. In this study, we describe the formation of 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 9-fluorenone, and 9-fluorenol as intermediates in the fluoranthene catabolic pathway in *P. alcaligenes* strain PA-10, and we demonstrate that these intermediates may modulate both fluoranthene degradation and indigo formation in this strain.

Materials and methods

Bacterial strains

Pseudomonas alcaligenes was isolated from a bioreactor treating PAH contaminated waste, following enrichment on media containing PAHs. It was subsequently identified using the API 20NE system. It can utilise, naphthalene and phenanthrene as sole sources of carbon and energy, while it can co-metabolise fluoranthene, fluorene and anthracene when grown in the presence of yeast extract and tryptone (Gordon 2001).

Chemicals

Fluoranthene, 9-hydroxy-1-fluorene carboxylic acid, 9-fluorenone-1-carboxylic acid, 9-fluorenol, 9-fluorenone, indole and dimethyl-formamide were purchased from Sigma-Aldrich Chemie, Steinham, Germany. All solvents and bacterial media were supplied by Merck KgaA, Darmstadt, Germany. All chemicals used were of the highest purity commercially available.

Growth in PAH-containing media

Starter cultures of strain PA-10 were grown in LB broth and a 1% inoculum was used to inoculate the fluoranthene-containing medium. PA-10 was grown in basal salts medium (BSM) containing 8.71 g K_2HPO_4, 1.98 g $(NH_4)_2SO_4$, 0.095 g $MgCl_2$ and 0.006 g $FeSO_4$ per litre (pH 7.O). The medium was supplemented with 500 mg l^{-1} each of yeast extract and tryptone. Stock solutions of fluoranthene, 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene carboxylic acid, 9-fluorenone and 9-fluorenol were prepared in dichloromethane and were added to the BSM post autoclaving, at a concentration of 5 mg/litre. All cultures were incubated, shaking, at 30 °C

Fluoranthene degradation and identification of intermediates

Degradation of fluoranthene was established by measuring changes in the fluoranthene concentration in the flasks and by detecting the formation and disappearance of metabolites every day over a 7-day period. Cells were grown in 50 ml PAH-containing medium in 250 ml Ehrlenmeyer flasks. For every time point determination, the entire contents of each flask was extracted for high-performance liquid chromatography (HPLC) analysis at 24 hour intervals. Abiotic controls involving the use of uninoculated flasks were included, and each experiment was performed in triplicate.

Extraction of samples for HPLC analysis

The contents of each flask were extracted twice with an equal volume of ethyl acetate, acidified to pH 2.5 with 1N HCl, and extracted twice more with ethyl acetate. The neutral and acidic extracts were combined and then H_2O was removed over anhydrous sodium sulphate. Following filtration, the ethyl acetate was evaporated on a rotary evaporator at 40 °C. Residual ethyl acetate was removed under a stream of N_2 gas, and the samples were redissolved in 3 ml of acetonitrile. HPLC analysis was performed using a Spectra-Physics isocratic HPLC system, equipped with a UV detector (set at 254 nm). Separation was achieved on a SUPELCOSIL^{TM} ABZ^{+}Plus column (15 cm $\times$ 4.6 mm, 5 μm particle size) fitted with a 20 μl holding loop. The mobile phase consisted of 55% acetonitrile and 45% 25 mM phosphate buffer, pH 5.0. Standard solutions of fluoranthene, 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 9-fluorenone and 9-fluorenone were prepared in acetonitrile at a concentration of 1 mg ml^{-1} and were run under the same conditions as the samples. The concentrations of fluoranthene and the potential intermediates were determined using the internal standard programme on the integrator. The HPLC machine was calibrated with standards solutions of known concentration. These solutions and the samples contained the same concentrations of the internal standard being used. Fluorene was the internal standard used during measurement of fluoranthene concentration and fluoranthene was used as the internal standard for the analysis of the four potential metabolites. The linear range extended from 0.002 mg l^{-1} to 15 mg l^{-1}, and all

experimentally determined concentrations were within this range. The limit of quantitation was determined to be 0.003 mg l^{-1}. A method bias was not detected.

Biotransformation experiments

PA-10 was grown in the presence of the four potential intermediates as previously described and the entire contents of each flask was extracted after 72 hours, in the case of 9-fluorenone-1-carboxylic acid, and after 24 hours, in the case of the other 3 compounds. The concentration remaining in the medium was determined for each intermediate as described above. Again each experiment was performed in triplicate

Pre-exposure studies

PA-10 was grown in the presence of the four potential intermediates as described above. When the cells had reached the mid-exponential phase of growth, (O.D. 600 nm of 0.6) which occurred typically 19 hours post inoculation; the medium was filtered through glass wool, in order to remove any undissolved compound. The cells were then harvested by centrifugation, washed twice and resuspended in BSM. The cell suspension was used to inoculate 50 ml of yeast extract tryptone medium containing BSM and fluoranthene to an $O.D_{600\ nm}$ of 0.06. The entire flask contents were extracted and the fluoranthene concentration determined after 0, 2, 4, 6 and 8 hours incubation. Control flasks, containing PA-10 grown in the absence of the four compounds were also included in the experiment. All determinations were performed in triplicate.

Analysis of indigo formation from indole

To test for indole transformation, PA-10 was grown on LB plates containing 0.5 mM indole and the colonies were examined for the presence of the blue dye, indigo. To study the rate of formation of indigo from indole in liquid medium, the cells were grown in the presence of the four potential intermediates, harvested in mid-exponential phase, (O.D. 600 nm 0.6) washed and resuspended in BSM. The specific rate of indigo formation was determined according to the method described by O'Connor et al. (1997), with the exception that protein concentration was used, instead of cell dry weight, as a measure of biomass. Protein levels were determined using the BIORAD method (Bradford 1976). All determinations were performed in triplicate.

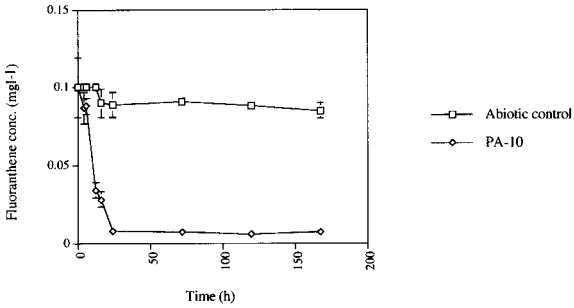

Figure 1. Change in fluoranthene concentration in the growth medium of *P. alcaligenes* strain PA-10 (—◇—) and an abiotic control (—□—).

Results and discussion

Degradation of flouranthene and identification of metabolic intermediates

Monitoring of the fluoranthene concentration in HPLC extracts indicated that, following a delay of approximately 8 hours, PA-10 lowered the concentration of fluoranthene present in the medium, with only 17% remaining after 24 hours and after 168 hours 95% of the fluoranthene had been removed (Figure 1). A similar lag phase of 6–12 hours has also been reported in *Mycobacterium* sp. strain PYR-1 (Kelley et al. 1991) and in *Mycobacterium* sp. strain KR20 (Rehmann et al. 2001), both of which can utilise fluoranthene as a sole source of carbon and energy. By monitoring the O.D 600 nm of the medium it appears that after 24 hours of growth, cells enter the stationary phase (with an O.D. 600 nm of 0.8) and given that fluoranthene is co-metabolised by PA-10 and does not support growth; this would explain the fact that complete removal of fluoranthene from the growth medium did not occur.

In order to elucidate the fluoranthene degradative pathway in operation in PA-10, extracts from the growth medium were analysed for potential intermediates using HPLC, with retention times being compared to those of authentic standards of 9-fluorenone-1-carboxylic acid (9F1CA), 9-hydroxy-1-fluorene-carboxylic acid (9H1FCA), 9-fluorenone and 9-fluorenol. These compounds had previously been identified as fluoranthene metabolites in both *Mycobacterium* sp. strain PYR-1 (Kelley et al. 1991, 1993) and *Pasteurella* sp. strain IFA (Sepic et al. 1998). Several peaks were observed in the combined neutral and acidic extracts of the medium following 24 hours incubation with fluoranthene. One metabolite with a retention time of 4.81 min (Figure 2a), which corres-

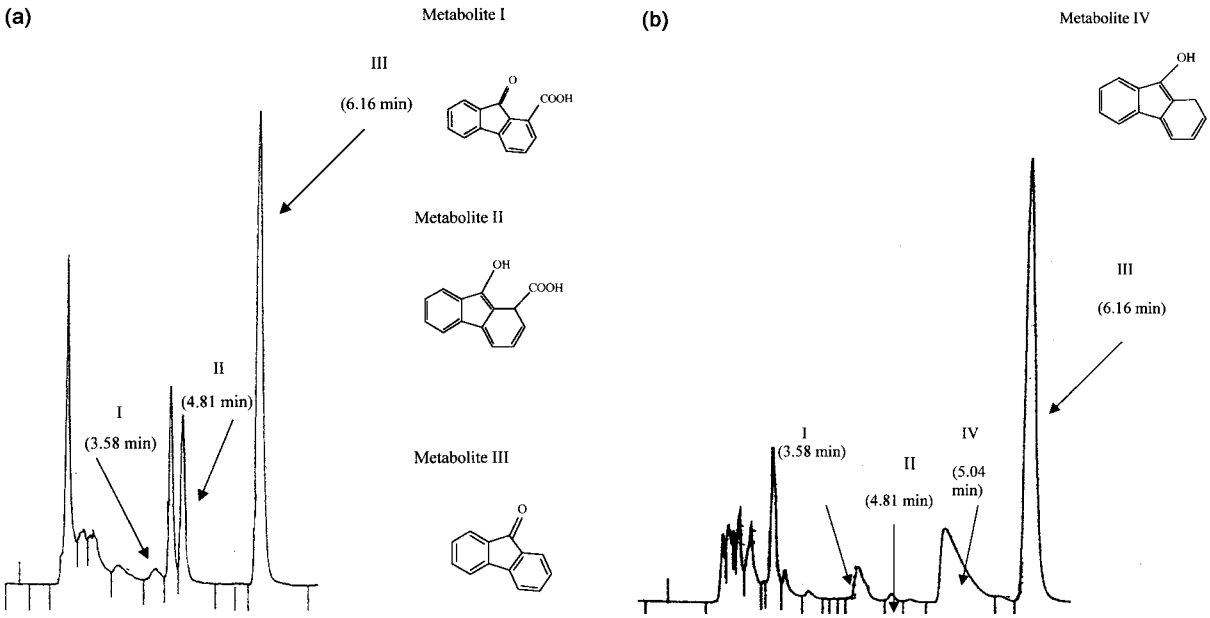

Figure 2. (a) HPLC elution profile of metabolites extracted from PA-10 growth medium after 72 hours incubation. (b) HPLC elution profile of metabolites extracted from PA-10 growth medium after 120 hours incubation with fluoranthene.

ponded to the standard 9H1FCA solution; was present in all extracts from 24 to 144 hours. The 9H1FCA peak diminished after 120 hours and could not be detected in the 168 hour extracts. In biotransformation studies when PA-10 was grown in the presence of 9H1FCA, after 24 hours the concentration of 9H1FCA had decreased by 50% (Figure 3a) and peaks with retention times corresponding to 9-fluorenone and 9- fluorenol were detected. These results indicate that 9H1FCA is rapidly metabolised in PA-10 to form 9-fluorenol and 9-fluorenone.

Another significant peak (Figure 2a) with a retention time (3.58 min) identical to that of authentic 9F1CA, was detected in all of the extracts from 24 to 168 hours, decreasing in size after 96 hours and to almost undetectable levels by 120 hours. Biotransformation studies on 9F1CA showed a decrease in concentration of 30% after 72 hours (Figure 3b), with trace amounts of 9-fluorenone and 9-fluorenol being detected together with other as yet unidentified peaks. 9H1FCA was not detected, but this may be due to its rapid transformation to 9-fluorenol. Thus it appears that 9F1CA which is formed during fluoranthene degradation by PA-10 is converted to 9-fluorenol. However, the fact that 9F1CA can be detected in all extracts from 24 to 168 hours indicates that it is not rapidly metabolised by PA-10.

Two other fluoranthene metabolites were identified in extracts of the growth medium. After 72 hours, a peak with a retention time of 6.16 min, corresponding to a 9-fluorenone standard was detected. This peak was still detectable after 144 hours but was not visible in the 168 hour extract. Biotransformation studies with 9-fluorenone showed a 25% decrease in concentration after 24 hours, confirming the strains ability to transform 9-fluorenone (Figure 3c). Finally a fourth metabolite with the same retention time as a 9-fluorenol standard (5.04 min) was also detected in the growth medium (Figure 2b). 9-fluorenol was detected in the 96 and 120 hour samples and was also formed during biotransformation studies with 9-fluorenone. After 24 hours incubation with PA-10, the concentration of 9-fluorenol in the medium decreased by 30% (Figure 3d), and a peak representing 9-fluorenone was also observed.

The formation of 9H1FCA, 9F1CA, 9-fluorenol and 9-fluorenone during growth of PA-10 in the presence of fluoranthene, together with the order in which they appeared indicates that fluoranthene degradation in *P. alcaligenes* strain PA-10 may proceed through a pathway similar to that previously described for *Mycobacterium* sp. strain PYR-1 (Figure 4). In PYR-1 Kelley and coworkers have proposed that the initial step is catalysed by a dioxygenase and involves the incorporation of oxygen into the aromatic ring to form a

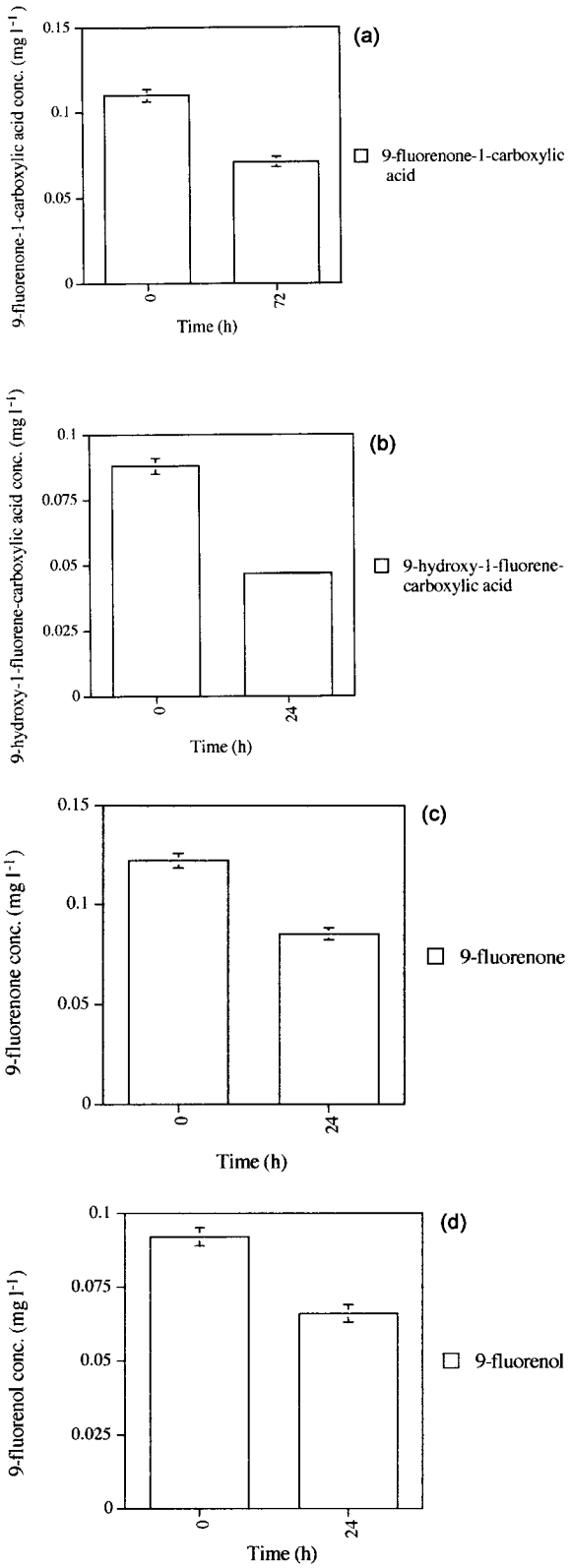

Figure 3. Biotransformations showing removal of fluoranthene degradative intermediates [(a) 9-fluorenone-1-carboxylic acid; (b) 9-hydroxy-1-fluorene-carboxylic acid; (c) 9-fluorenone; (d) 9-fluorenol] from PA-10 growth medium.

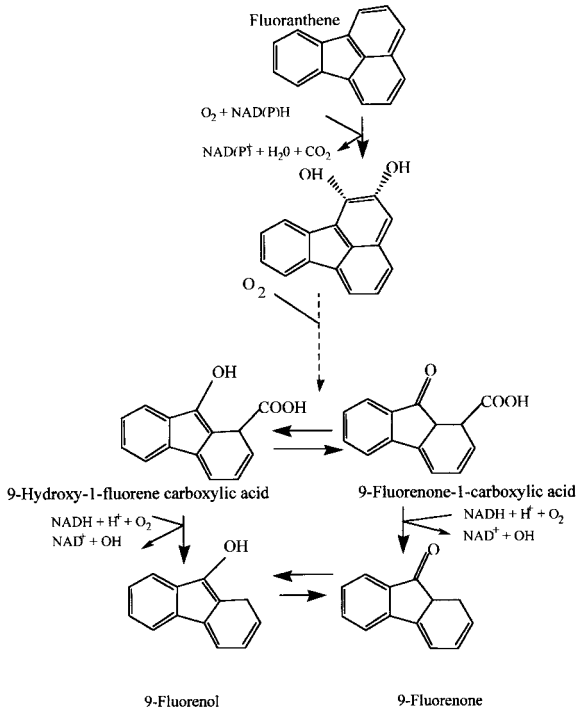

Figure 4. Proposed pathway for the degradation of fluoranthene by PA-10.

dihydroxylated intermediate. The formation of 9F1CA and 9H1FCA suggests that this intermediate undergoes meta-cleavage, followed by decarboxylation to form 9-fluorenone and 9-fluorenol. Both 9-fluorenone and 9-fluorenol have also been shown to be produced in the fluoranthene degrading *Pasteurella* sp. strain IFA (Sepic et al. 1998).

Modulation of fluoranthene degradation

While previous studies have shown that many aromatic catabolic pathways are frequently regulated by a pathway intermediate, little information is available concerning modulation of pathways involved in the degradation of high molecular weight PAHs. Fluoranthene degradation was assessed following prior exposure of PA-10 to compounds identified as intermediates of the fluoranthene degradative pathway (Figure 5, Table 1). In the controls no lag phase was observed and fluoranthene was degraded rapidly in the first two hours. After 8 hours incubation 87.5% of the fluoranthene had been removed from the medium. However following prior exposure to 9H1FCA, only 14% of the fluoranthene remained after two hours and this continued to undetectable levels by four hours. Prior exposure to 9F1CA also resulted in an increase

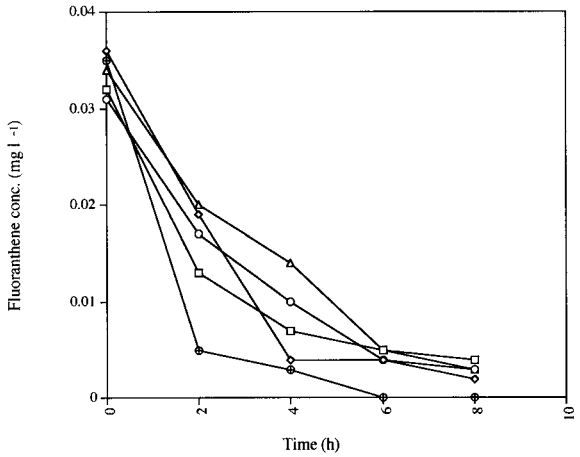

Figure 5. Decrease in fluoranthene concentration in the growth medium of PA-10, grown in the presence of 9-fluorenone-1-carboxylic acid (—◇—); 9-hydroxy-1-fluorene-carboxylic acid (—⊕—); 9-fluorenone (—△—); 9-fluorenol (—○—) and in the medium of uninduced PA-10 cells (—□—).

in fluoranthene removal, with 88.5% being removed after four hours, compared to 78.1% in the control. However prior exposure to either 9-fluorenol or 9-fluorenone resulted in a decreases in fluoranthene removal (Figure 5, Table 1). Following four hours of incubation, cells exposed to 9-fluorenol had degraded approximately 10% less fluoranthene than the control cells, while in the case of 9-fluorenone the difference was 20%. These results indicate that fluoranthene metabolism in PA-10 can be modulated by pre-exposure to any of the four catabolic intermediates. Thus, like the naphthalene degradative pathway which is induced by salicylate (Yen and Serdar 1988) the higher molecular weight fluoranthene degradative pathway in our strain also appears to be modulated by metabolic intermediates of the pathway. The reduced ability to degrade fluoranthene following pre-exposure to 9-fluorene and 9-fluorenol may result from feed back inhibition, possibly to prevent the accumulation of these compounds in the growth medium.

Indigo formation is linked to fluoranthene degradation

Many microorganisms which degrade aromatic hydrocarbons possess mono-and dioxygenases which have been shown to transform indole to indigo (Mermod et al. 1986). Microbial indigo formation has been used to clone genes encoding oxygenases that degrade both aromatic hydrocarbons and their acids (Ensley et al. 1983; Keil et al. 1987; Yen et al. 1992).

Table 1. Rate of indigo formation and percentage fluoranthene degraded by PA-10 cells grown in the presence and absence of four intermediates.

Inducer	Rate of indigo formation (ηmoles min^{-1} mg protein^{-1})	% Fluoranthene degraded after 4 hours incubation
9F1CA	0.301 ± 0.003	88.89 ± 0.001
9H1FCA	0.300 ± 0.008	91.40 ± 0.003
9-Fluorenone	0.0300 ± 0.004	58.88 ± 0.002
9-Fluorenol	0.022 ± 0.003	67.74 ± 0.002
Uninduced control	0.180 ± 0.002	78.12 ± 0.002
Salicylic acid	0.410 ± 0.006	ND

ND = not determined.

P. alcaligenes PA-10 tested positive for indigo production, forming dark blue colonies on LB plates containing 0.5 mM indole. In order to explore the possibility that indigo formation might be linked to fluoranthene degradation we monitored indigo formation in PA-10; in the presence of fluoranthene catabolic pathway intermediates (Table 1). The regulation of indole transformation to indigo followed a similar pattern to that of fluoranthene degradation. When cells were pre-exposed to either 9H1FCA or 9F1CA, the rate of indigo formation after four hours was 0.3 nmoles min^{-1} mg protein^{-1} which is almost two fold higher than 0.1 nmoles min^{-1} mg protein^{-1}, the rate observed in the control (Table 1). However when cells were pre-exposed to either 9-fluorenone or 9-fluorenol, there was a marked decrease in indigo formation, with levels of 0.03 and 0.02 nmoles min^{-1} mg protein^{-1}, being observed; which were 6 and 8 fold lower respectively than controls. The detection of significant levels of indigo formation, 0.18 nmoles min^{-1} mg protein^{-1} even in the absence of any inducer (Table 1) implies a basal level of expression of this indole transforming activity in PA-10.

Thus the fact that fluoranthene degradation and indigo formation are modulated in a similar manner by the same metabolic intermediates indicates a link between these two activities and suggests that an oxygenase-like activity may be involved in each process. Further proof of a link comes from the fact that salicylic acid was found to induce indigo formation in PA-10 to levels even higher than those of 9F1CA and 9H1FCA (0.410 nmoles min^{-1} mg protein^{-1}), (Table 1). Thus, given that the activity of an indole-

transforming enzyme has been reported to be induced by salicylic acid in the naphthalene degrading *Pseudomonas putida* strain RKJ (Bhushan et al. 2000), and that salicylic acid has also been identified as an inducer of the metabolism of several high molecular weight PAH, including fluoranthene (Mahaffey et al. 1988; Chen & Aitken 1999), we feel that an oxygenase type enzyme may be involved in fluoranthene metabolism in on our strain. In addition, the indole transforming activity of this enzyme has allowed the cloning of the gene encoding this oxygenase type activity in *P. alcaligenes* PA-10 (Gordon, 2001). The gene encodes a novel oxygenase which is essential for fluoranthene degradation since a chromosomal mutant of strain PA-10 where the gene has been disrupted fails to metabolise the PAH (Gordon 2001; Gordon & Dobson 2002). Preliminary evidence also suggests that transcription of this gene is modulated by intermediates of the fluoranthene degradative pathway. Work is currently underway to elucidate the mechanism(s) involved.

Conclusions

Fluoranthene is rapidly co-metabolised by *P. alcaligenes* PA-10, resulting in the formation of 9F1CA, 9H1FCA, 9-fluorenone and 9-fluorenol as intermediates in the catabolic pathway. These intermediates affect fluoranthene degradation and indigo formation in the strain. Indigo formation is linked to fluoranthene degradation in PA-10, with evidence suggesting that the same enzymatic activity is involved in both systems. This basal level of enzymatic activity can be modulated to higher levels by 9F1CA, 9H1FCA and salicylic acid and to lower levels by 9-fluorenol and 9-fluorenone.

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