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Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism

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Abstract Six bacterial strains capable of using, as sole carbon and energy source, at least one of the following polycyclic aromatic hydrocarbons (PAH), naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene, were isolated. The interactions between these PAH during their biodegradation were studied in experiments involving PAH pairs, one PAH at least being used as a carbon source. All individual strains were found capable of cometabolic degradation of PAH in a range varying among strains. Inhibition phenomena, sometimes drastic, were often observed but synergistic interactions were also detected. Naphthalene was toxic to all strains not isolated on this compound. Strain associations were found efficient in relieving inhibition phenomena, including the toxic effect of naphthalene. Accumulation of water-soluble metabolites was consistently observed during PAH degradation.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous compounds that originate from natural and anthropogenic pyrolysis of organic matter such as forest fires, automobile exhaust, coal-refining processes and the oil industry. Because of their genotoxicity (Kramers and van der Heijden 1990), these xenobiotics are of environmental concern and their biodegradation is a field in which quite interesting progress is now being observed (Cerniglia 1992, 1993; Wilson and Jones 1993) such as the isolation of bacteria capable of utilizing the four-ring-aromatics fluoranthene and pyrene as the sole carbon and energy sources (Boldrin et al. 1993; Kelley and Cerniglia 1991; Mueller et al. 1990; Walter et al. 1991; Weissenfels et al. 1990). Because PAH, like other classes of hydrocarbon compounds, constitute a series of homologous compounds, interactions between these compounds are likely to constitute important features of their biodegradation process. Cometabolism, a phenomenon well documented in the case of hydrocarbons (Horvath 1972; Perry 1979), has already been reported in the case of PAH (Walter et al. 1991; Weissenfels et al. 1991).

In the present study, six bacterial strains were isolated. Each of these strains was capable of using, as sole carbon and energy source, at least one of the following PAH: naphthalene, phenanthrene, anthracene, fluorene, fluoranthene and pyrene. The interactions between these PAH during their biodegradation by individual strains were studied, as well as the cooperative effects resulting from the use of defined mixed cultures.

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Materials and methods

Chemicals

HPLC-grade acetonitrile was obtained from SDS (Peypin, France). PAH were purchased from Fluka (Buchs, Switzerland). Stock solutions of PAH were prepared by dissolving them in diethyl ether until saturation.

Culture media

Trypticase soy agar was obtained from Biomerieux (Charbonnières les Bains, France). The vitamin-supplemented mineral salt medium (MSM) contained (l^{-1}) 0.68 g KH_2PO_4, 4.5 g $Na_2HPO_4 \cdot 12H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g NH_4NO_3, 1 mg $FeSO_4 \cdot 7H_2O$ and 1 ml trace element solution. This solution contained (mg/l) 100 $MnSO_4 \cdot H_2O$, 25 $CuCl_2$, 25$(NH_4)_6Mo_7O_{24} \cdot H_2O$, 25 Na_2

$B_4O_7 \cdot 10\,H_2O$, 25 $Co(NO_3)_2 \cdot 6\,H_2O$, 25 $ZnCl_2$ and 10 NH_4VO_3. This medium was sterilized by autoclaving (30 min at 121° C). After cooling, 1 ml/l vitamin solution sterilized by filtration on a 0.22-μm membrane was added. This solution contained (mg/l) 200 D-biotin, 100 pyridoxine hydrochloride, 50 riboflavin, 50 niacin, 50 folic acid, 50 sodium D-pantothenate, 50 *p*-aminobenzoic acid, 15 thiamine hydrochloride and 1.5 cyanocobalamin.

Enrichment, isolation and identification of microorganisms

Several microorganisms were isolated in our laboratory for their ability to mineralise PAH as sole carbon and energy source. Two enrichments methods were employed.

In the first one, enrichments of PAH-degrading microorganisms were conducted in 500-ml percolators each containing a soil from a defined site mixed with sand to facilitate percolation. The circulating fluid (MSM) was aerated and recycled. After 1 month of cultivation at room temperature, an aliquot of the circulating fluid was used to inoculate 20 ml MSM (5% v/v) in 50-ml tightly closed conical flasks. Crystals of an individual PAH were added as sole carbon and energy source. PAH used were naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene. Cultures were incubated on a rotary shaker at 30° C in the dark. Several transfers, in the same conditions, allowed enrichment and selection of strains. Well-grown cultures were purified by plating on trypticase soy agar medium. Then, each type of colony was removed and subcultured individually in MSM with the enrichment PAH.

In the other method employed, 5 g soil was directly suspended in sterilized 50-ml conical flasks containing 20 ml MSM. Then the procedure was the same as above.

The identifications of strains were conducted by Institut Pasteur, Laboratoire des Identifications (Paris, France).

Pure strains were maintained in liquid MSM with their isolation PAH as carbon source. They were periodically checked for purity by plating on trypticase soy agar medium.

Growth experiments in conical flasks

Growth on different PAH of the isolated strains was investigated in 50-ml conical flasks. PAH crystals were added into liquid MSM (20 ml) after autoclaving. Naphthalene was also supplied in the vapour phase by placing crystals into a central well of modified flasks. A 1-ml sample of a culture grown on the isolation PAH was used as inoculum. Flasks incubated simultaneously without a carbon source were used as controls. Flasks were incubated on a rotary shaker in the dark at 30° C. Growth at the expense of a PAH was established by observing biomass increase and medium coloration visually.

Growth and PAH interaction experiments in test-tubes

Saturating stock ether solutions of individual PAH were diluted and an appropriate precise volume of the diluted solutions was introduced into 30-ml tubes. The solvent was evaporated slowly. The amount of each PAH introduced was approximately 500 ppm in the final culture medium. The precise amount corresponding to the volume introduced was determined in two tubes. Except in the case of naphthalene, PAH were dissolved in acetonitrile in these two tubes and quantified by high-performance liquid chromatography (HPLC). Naphthalene was dissolved in cyclohexane and quantified by gas chromatography (GC). In the other tubes, 1.5 ml liquid MSM (pH 7 or pH 2) was added. A 5% (v/v) solution of a culture grown on the PAH employed as main growth substrate was used as inoculum. Interaction tests were conducted at pH 7. pH 2 tubes were used to control abiotic losses and also to calculate extraction yields. Tubes were closed by Teflon-lined butyl rubber caps and sealed. Test and control tubes were treated in triplicate. Incubation was conducted at 30° C in the dark with reciprocation shaking.

Analytical procedures for test-tube experiments

When naphthalene was employed as substrate or cosubstrate, the degradation of PAH in test-tubes was quantified by GC. After incubation, residual PAH were extracted with equal weighed volumes of cyclohexane during 1 day at 20° C. Extraction yields were evaluated by the quantity of PAH extracted in pH 2 controls. They were found to be close to 100%. The extract in cyclohexane was diluted and PAH were quantified on a 3400 gas chromatographic system equipped with an automatic sampler and a flame-ionisation detector (Varian, Walnut Creek, Calif., USA). The injected sample (1 μl) was analysed by a DB5 30 m × 0.32 mm (internal diameter) column (J&W Scientific, Folsom, Calif., USA) and the following oven programme: 50° C for 5 min then 7° C/min to 260° C and 1 min at 260° C. The extraction and dilution cyclohexane were quantified by weighing.

When naphthalene was not employed, PAH degradation was quantified by HPLC. Residual PAH were extracted from the culture fluid by an equal weighed volume of pentane (1 day of extraction at 30° C). Extraction yields (evaluated as above) were near 100%. A weighed aliquot of the pentane extract was removed and evaporated slowly. PAH were then dissolved in acetonitrile. An appropriate PAH was added as internal standard. Quantification was performed, after dilution, by UV detection at 254 nm after HPLC separation on a Supelcosil LC-PAH (25 × 4.6 mm) column (Supelco Inc., Bellefonte, Pa., USA) with isocratic elution (water/acetonitrile: 40/60 v/v) at 37° C. The instrumentation involved a model 510 isocratic pump (Waters, Milford, Mass., USA), an SP 8875 automatic sampler (Spectra-physics France, Les Ullis, France), an injection valve with a 20-μl sampling loop and a variable-wavelength detector (Spectromonitor 3100, LDC Analytical/Milton Roy, Riviera Beach, Fla., USA).

Dissolved organic carbon was determined on the supernatant of centrifuged cultures with DC 80 equipment (Xertex, Dorhman Division, Santa Clara, Calif., USA) after elimination of dissolved CO_2 by acidification and bubbling for 10 min with oxygen. The dissolved organic carbon was expressed as the percentage of carbon consumed after subtraction of a blank value. This value (10 ppm) was evaluated on MSM after evaporation of added pentane and elimination of dissolved CO_2. When substrate and cosubstrate degradations were too low (less than 40 ppm total degradation), dissolved organic carbon values were considered too small to be significant and were not reported.

Assays, routinely performed in triplicate, exhibited a satisfactory reproductibility with individual values within 10% of the amounts of PAH introduced.

Results

Isolation and characterization of PAH-degrading strains

Isolation and identification of PAH-degrading bacteria were performed as described in Materials and methods. Strains capable of growth on each of the PAH studied were isolated (Table 1).

These strains were tested for their ability to use different individual PAH as their sole carbon and energy source (Table 2). As the direct addition of naphthalene tended to cause toxic effects (Mueller et al. 1990; Weissenfels et al. 1991), the alternative method of

Table 1 Isolation of polycyclic-aromatic-hydrocarbon(PAH)-degrading microorganisms (*NAP* naphthalene, *FLU* fluorene, *PHE* phenanthrene, *ANT* anthracene, *FLT* fluoranthene, *PYR* pyrene)

Denomination	Identification	Isolation PAH	Source
S Nap Ru 1	*Rhodococcus* sp.[a]	NAP	Garden soil
S Nap Ka1	*Pseudomonas stutzeri*	NAP	Manufactured gas plant soil (Karlsruhe)
S Flu Na 1	*Rhodococcus* sp.[b]	FLU	Manufactured gas plant soil (Nantes)
S Phe Na 1	*Pseudomonas* sp.	PHE	Manufactured gas plant soil (Nantes)
S Ant Mu 3	Coryneform bacillus[c]	ANT	Manufactured gas plant soil (München)
S Flt Na 1	*Rhodococcus* sp.[b]	FLT	Manufactured gas plant soil (Nantes)
S Pyr Na 1	*Rhodococcus* sp.[b]	PYR	Manufactured gas plant soil (Nantes)

[a] Close to *Rhodococcus rhodochrous*
[b] Close to *Rhodococcus equi*
[c] Close to the genus *Aureobacterium*

vapour-phase supply was also used for this compound and yielded the same results. To avoid misleading growth responses resulting from PAH brought with the inoculum, several transfers were carried out on the carbon source used. Growth was said to be positive when biomass increase and medium coloration were still evident after at least two transfers. Positive growth responses were confirmed by further tests where PAH utilization was determined by HPLC or GC. In these conditions, the range of substrates utilized by the isolated strains was often found to be fairly limited. In the case of strains *S Flt Na 1* and *S Pyr Na 1*, we observed that growth on phenanthrene decreased gradually with the number of transfers. Phenanthrene could, in these two cases, be a poor inducer of its degradation pathway. Although similar, the two latter strains presented some differences: only *S Pyr Na 1*, for example, was able to attack dibenzothiophene.

Interactions during PAH degradation by individual strains

Interactions during PAH degradation by the individual strains isolated were investigated and cometabolism in particular. This point was very interesting to elucidate in view of the restricted PAH carbon source range of these strains.

Table 3 summarizes the experiments involved. Growth and interactions between PAH were evaluated by quantitative determination of the utilization of all PAH involved. Each strain was tested with a series of PAH pairs, one of them at least, in each case, being used as growth substrate. Growth tests, on the PAH used alone as carbon source, were conducted simultaneously for comparison. The possible influence of the nature of the growth substrate on cosubstrate degradation was investigated with two strains, *S Flt Na 1* and *S Pyr Na 1*. For each of the latter strains, two series of comparative interaction experiments were carried out employing two different growth substrates.

Table 2 Individual PAH utilized as growth substrates by the different strains studied (+ Growth, − no growth, +/− growth decreased gradually and was lost after a few transfers)

Strain	PAH used as growth substrate					
	NAP	FLU[a]	PHE[a]	ANT[a]	FLT[a]	PYR[a]
S Nap Ru 1	+ [a]	−	−	−	−	−
S Nap Ka 1	+ [a]	−	−	−	−	−
S Flu Na 1	− [a, b]	+	−	−	−	−
S Phe Na 1	− [a, b]	−	+	−	−	−
S Ant Mu 3	− [a, b]	−	−	+	+	+
S Flt Na 1	− [a, b]	−	+/−	−	+	+
S Pyr Na 1	− [a, b]	−	+/−	−	+	+

[a] PAH substrates were added as crystals directly in the liquid media
[b] Naphthalene was supplied in the vapour phase. Crystals of naphthalene were placed into a central well of modified conical flasks

Table 4 shows the cometabolism capacities of strains *S Nap Ru 1* and *S Nap Ka 1* growing on naphthalene. They were different for the two strains and limited.

Interactions between PAH during degradation by *S Flu Na 1* are presented in Fig. 1. Using growth on fluorene alone as a reference, all cosubstrates were found inhibitory and, except weakly for phenanthrene, not cometabolized. Inhibition was total in the case of naphthalene, none of the two PAH being degraded. In all the experiments, dissolved organic carbon determinations in the medium suggested that a portion of the degraded PAH accumulated as soluble metabolites.

Fig. 2 presents the cometabolism capacities, and other PAH interactions, in the case of strain *S Phe Na 1*. As in the case of strain *S Flu Na 1*, napthalene was toxic. In fact, this was observed for all the strains studied, except for those isolated on naphthalene. Fluorene, which was cometabolized, was also moderately inhibitory for phenanthrene utilization. No inhibition by other PAH could be observed and cometabolism occurred, especially with anthracene and fluoranthene.

Table 3 Strains tested with different PAH pairs: experimental grid. Growth experiments (substrate and cosubstrate identical) were conducted on 500 ppm of the PAH concerned

Substrate	Cosubstrate					
	NAP	FLU	PHE	ANT	FLT	PYR
NAP	*S Nap Ru 1* *S Nap Ka 1*	*S Nap Ru 1* *S Nap Ka 1*	*S Nap Ru 1* *S Nap Ka 1*	*S Nap Ru 1* *S Nap Ka 1*	*S Nap Ru 1* *S Nap Ka 1*	*S Nap Ru 1* *S Nap Ka 1*
FLU	*S Flu Na 1*	*S Flu Na 1*	*S Flu Na 1*	*S Flu Na 1*	*S Flu Na 1*	*S Flu Na 1*
PHE	*S Phe Na 1* *S Pyr Na 1*	*S Phe Na 1* *S Pyr Na 1*	*S Phe Na 1* *S Pyr Na 1* *S Flt Na 1*	*S Phe Na 1* *S Pyr Na 1*	*S Phe Na 1* *S Pyr Na 1*	*S Phe Na 1* *S Pyr Na 1*
ANT	*S Ant Mu 3*	*S Ant Mu 3*	*S Ant Mu 3*	*S Ant Mu 3*	*S Ant Mu 3*	*S Ant Mu 3*
FLT	*S Flt Na 1* *S Pyr Na 1*	*S Flt Na 1* *S Pyr Na 1*	*S Flt Na 1* *S Pyr Na 1*	*S Flt Na 1* *S Pyr Na 1*	*S Flt Na 1* *S Pyr Na 1* *S Ant Mu 3*	
PYR	*S Flt Na 1*	*S Flt Na 1*	*S Flt Na 1*	*S Flt Na 1*		*S Flt Na 1* *S Ant Mu 3*

The results of experimentation with strain *S Ant Mu 3* are presented in Fig. 3. This strain was capable of growth on pyrene, fluoranthene and anthracene, the latter being used as the main substrate in the experiments. Besides the toxicity of naphthalene, a strong inhibition by phenanthrene, which was cometabolized, could be observed. Fluorene also was cometabolized but without being inhibitory. When two growth substrates were brought together (anthracene + fluoranthene and anthracene + pyrene), a good simultaneous utilization of both PAH was observed.

With strain *S Flt Na 1*, two series of experiments were performed using two different main substrates: fluoranthene and pyrene. Results are given in Fig. 4. When fluoranthene was employed as substrate, the cosubstrates were inhibitory, while being cometabolized (fluorene) or not (anthracene). When pyrene was used as growth substrate, little change in pattern could be observed, except for a lesser degradation of fluorene. In the two series of experiments, mutual inhibition was noted when another growth substrate, phenanthrene, was supplied with the main substrate, fluoranthene or pyrene. Phenanthrene/fluoranthene and phenanthrene/pyrene behaved as antagonistic substrate pairs for strain *S Flt Na 1*.

Strain *S Pyr Na 1* also was tested with two main substrates, phenanthrene and fluoranthene, and the results are presented in Fig. 5. When phenanthrene was used as the main substrate, its poor degradation was enhanced in the presence of fluorene, which was cometabolized. Another point of interest is the strong antagonism between the two substrates phenanthrene and fluoranthene. We have termed "malefic association" the phenomenom where the association of two substrates results in blocking of their respective degradations. When fluoranthene was used as the main substrate, some difference in pattern could be observed as fluorene, in this case, inhibited the utilization of the main substrate. The malefic association of phenanthrene and fluoranthene was again observed. The difference in phenanthrene degradation between the experiments presented in Fig. 5, above and below the horizontal line, appears related to the already mentioned gradual growth decrease of *S Pyr Na 1* on phenanthrene with the number of transfers, the inoculum being grown on phenanthrene in the first series of tests and on fluoranthene in the second one.

Table 4 Cometabolism capacities of strains *S Nap Ru 1* and *S Nap Ka 1* growing on naphthalene. Experimental conditions are described in Materials and methods. Analyses were performed after 35 days of incubation for strain *S Nap Ru 1* and 40 days of incubation for strain *S Nap Ka 1*

Strain	Cosubstrate degradation (%)				
	FLU	PHE	ANT	FLT	PYR
S Nap Ru 1	10	24	15	6	1
S Nap Ka 1	8	13	0	13	8

Cooperation between strains in PAH degradation

In the experiments just presented, strong inhibitions were observed. We tried to discover whether adding a well-chosen strain in characteristic cases, such as naphthalene toxicity or PAH antagonism, could relieve the inhibition. Three cases were tested.

Growth of strain *S Phe Na 1* on phenanthrene was inhibited in the presence of naphthalene. The addition of strain *S Nap Ka 1* capable of growth on naphthalene was tested. The results are presented in Table 5. It can be observed that the inhibition of phenanthrene degradation by *S Phe Na 1* was relieved when *S Nap Ka 1* was added, very likely because of naphthalene consumption by the latter strain.

Another case studied, presented in Table 6, was that of phenanthrene and fluoranthene, which allowed growth of strain *S Flt Na 1* when employed individually but were antagonists when employed as a pair.

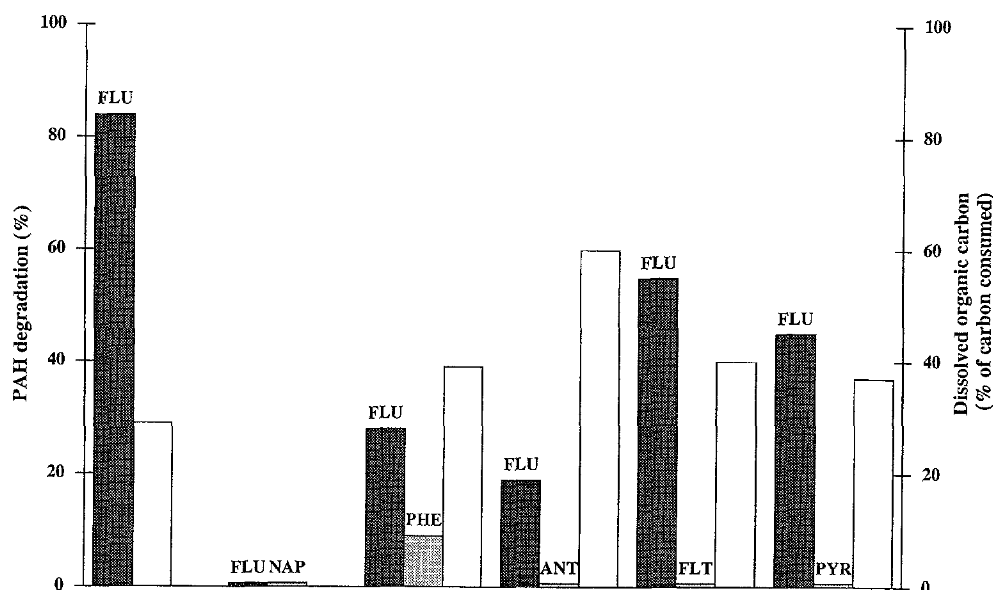

Fig. 1 Effect of interactions between polycyclic aromatic hydrocarbons (PAH) on their degradation by strain *S Flu Na 1*. Experimental conditions are described in Materials and methods. Analyses were performed after 60 days of incubation. ■ Degradation of PAH substrate, ▩ degradation of PAH cosubstrate, □ dissolved organic carbon. *PHE* phenanthrene, *NAP* naphthalene, *FLU* fluorene, *ANT* anthracene, *FLT* fluoranthene, *PYR* pyrene

When a strain growing on phenanthrene, *S Phe Na 1*, was added, the inhibition of *S Flt Na 1* by the pair phenanthrene/fluoranthene was relieved, probably following phenanthrene degradation by *S Phe Na 1*.

When employed as a pair, phenanthrene and fluoranthene formed a malefic association for strain *S Pyr Na 1*. As in the previous case, *S Phe Na 1* was added in order to consume phenanthrene. It was observed (data not shown) that the inhibition of *S Pyr Na 1* by the pair phenanthrene/fluoranthene was not relieved by *S Phe Na 1*, probably because degradation of phenanthrene by the latter strain was not complete in this experiment (94% of the initial amount).

Discussion

The PAH-degrading strains isolated in this study belong to various species, in agreement with other literature reports. The narrow range of growth substrates utilized by most of these strains is worth noting as well as the subtle growth responses obtained for a substrate like phenanthrene with strains *S Flt Na 1* and *S Pyr Na 1*. Strains with comparable restricted substrate patterns have been reported (Boldrin et al. 1993; Kiyohara et al. 1992) but cases of strains with a wider carbon source specificity are also known (Walter et al. 1991; Weissenfels et al. 1991).

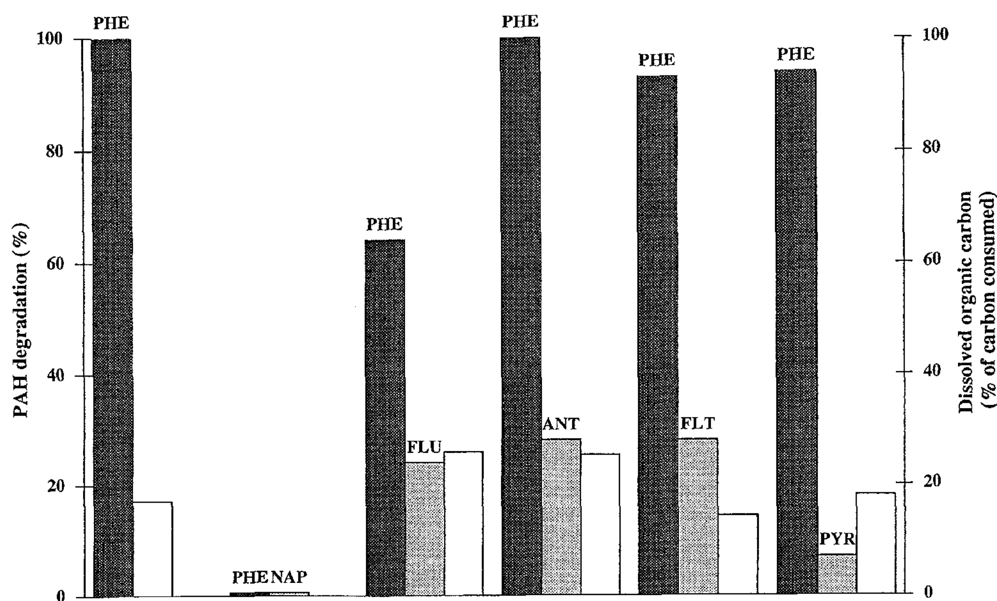

Fig. 2 Effect of interactions between PAH on their degradation by strain *S Phe Na 1*. Experimental conditions and symbols as in Fig. 1. Analyses were performed after 25 days of incubation

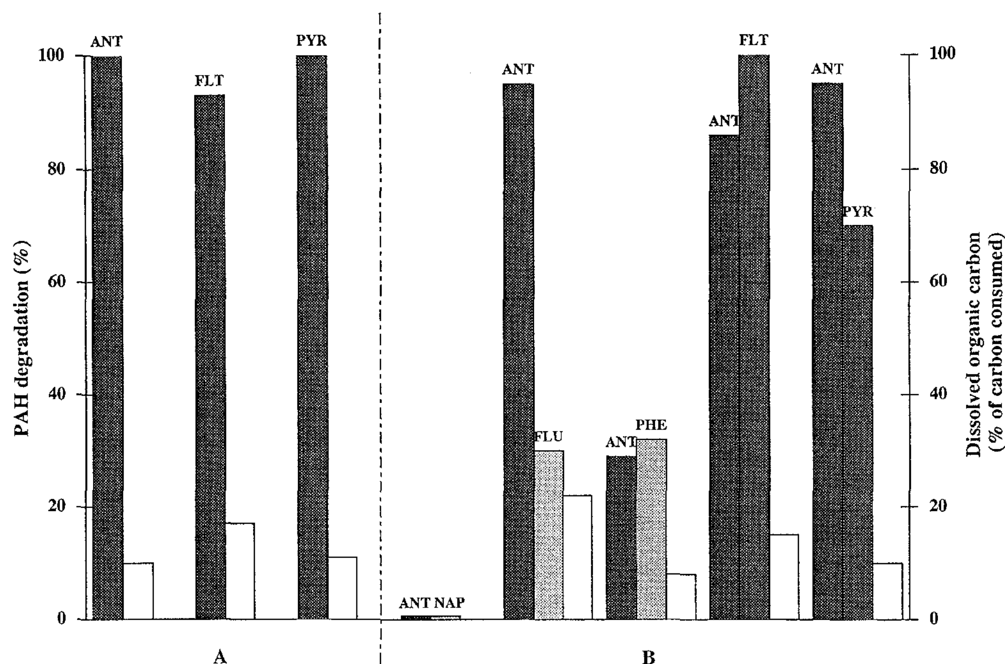

Fig. 3A, B Effect of interactions between PAH on their degradation by strain *S Ant Mu 3*. **A** Reference growth experiments on individual PAH. **B** Degradation of PAH in pairs involving anthracene as main substrate. Experimental conditions and symbols as in Fig. 1. Analyses were performed after 46 days of incubation

A summary of the various interactions between PAH during degradation by a defined strain, observed in this study, is presented in Table 7. It must be noted here that only kinetic studies of PAH degradation could provide a detailed description of these interactions, the present determinations allowing the identification of general patterns. Various interactions of the types observed here have been reported in the case of monoaromatic hydrocarbons (Arvin et al. 1989; Chang et al. 1993).

Concerning PAH, several cases of cometabolic degradation, sometimes at extensive rates, have been reported (Mueller et al. 1990; Walter et al. 1991; Weissenfels et al. 1991). In the present study, all strains were found capable of the cometabolic degradation of PAH, although the range of compounds attacked varied markedly among strains. Thus, it appears that cometabolism is an important feature of the degradation of PAH, as it commonly occurs and widens the range of PAH attacked by a defined strain, a point that

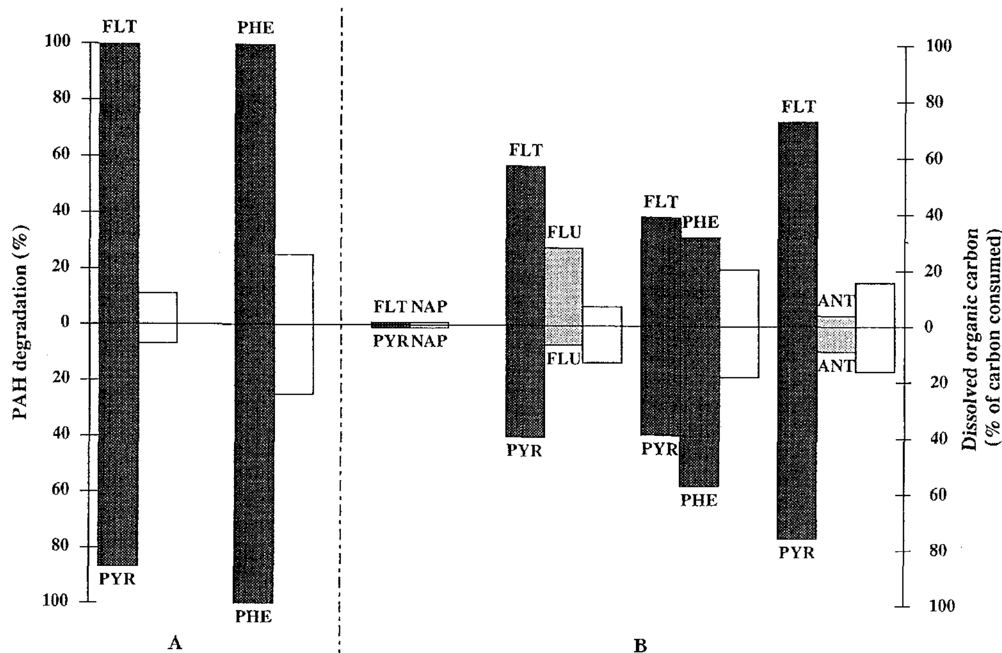

Fig. 4A, B Effect of interactions between PAH on their degradation by strain *S Flt Na 1*. **A** Reference growth experiments on individual PAH. **B** Degradation of PAH in pairs, involving as main substrate (above the horizontal line) fluoranthene and (below the horizontal line) pyrene. Experimental conditions and symbols as in Fig. 1. Analyses were performed after 30 days of incubation

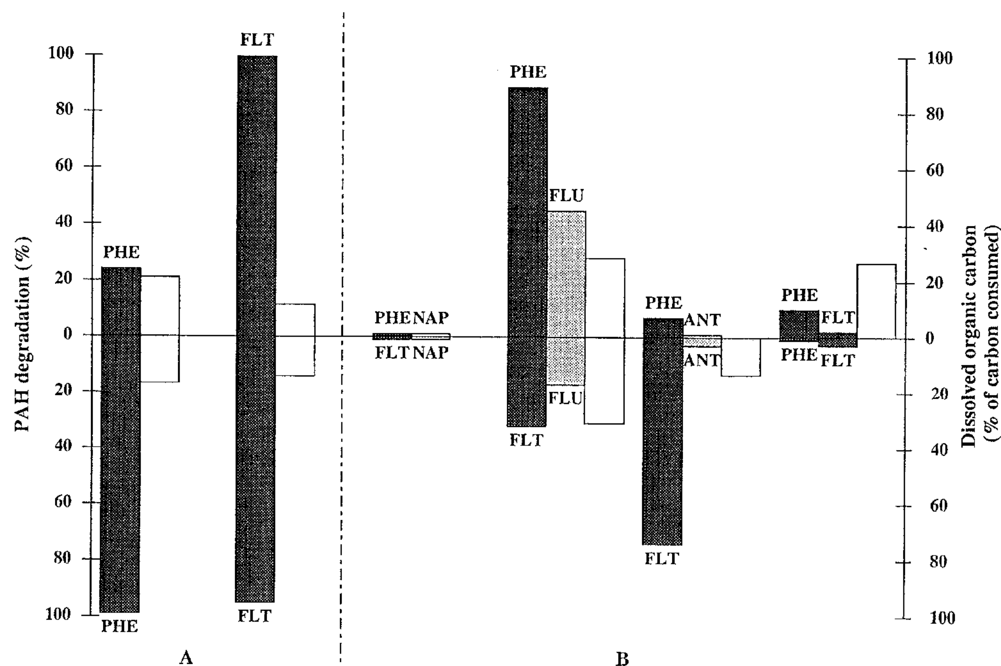

Fig. 5A, B Effect of interactions between PAH on their degradation by strain *S Pyr Na 1*. **A** Reference growth experiments on individual PAH. **B** Degradation of PAH in pairs, involving as main substrate (above the horizontal line) phenanthrene and (below the horizontal line) fluoranthene. Experimental conditions and symbols as in Fig. 1. Incubation times were respectively 60 days and 30 days for the series of experiments with phenanthrene and with fluoranthene as main substrates

can be important in the degradation of high-molecular-mass PAH.

Another very common situation observed in the present study, not yet reported in the literature, is the more or less extensive inhibition of the degradation of a defined PAH in the presence of a second one. Inhibition was most commonly observed when the added PAH was more water-soluble. The most conspicuous case, involving naphthalene, will be discussed below but fluorene and phenanthrene (solubilities about 2 ppm and 1 ppm respectively) were most frequently inhibitory, indicating a relationship between inhibitory capacity and solubility of the PAH. However, instances of inhibition by anthracene, a very poorly soluble compound (around 50 ppb), were also observed.

Inhibition took place whether cometabolism occurred or not (situations 2 and 5 in Table 7) and its high frequency of incidence has to be related to the fact that PAH, as homologous compounds, are susceptible to interactions at several levels. Competition at the active site of enzymes, in particular the initial oxygenase, has to be expected. When cometabolism occurred, a second possible cause of inhibition, accumulation of toxic dead-end products, ensued as recently well illustrated in the case of *o*-xylene and *p*-xylene in a *Pseudomonas stutzeri* strain (Barbieri et al. 1993). Interactions at the level of enzyme induction can also take place. They could be involved in the intriguing case of malefic association of phenanthrene and fluoranthene with strain *S Pyr Na 1* (situation 8 in Table 7), which would result here in the complete block of the induction of the initial oxygenase. Such a possibility is illustrated in comparable situations, for example in the degradation of tryptophan by tryptophan oxygenase, where different tryptophan analogues devoid of inducing activities could either enhance or block enzyme induction by tryptophan (Rosenfeld and Feigelson 1969).

Interactions at the level of enzyme induction may also be involved in another case observed in this study

Table 5 Association of strains *S Nap Ka 1* and *S Phe Na 1* for the degradation of naphthalene and phenanthrene. The growth capacities of the two strains and of a mixture of them on naphthalene or on phenanthrene, supplied as individual compounds, are shown in the first two columns. The last two columns present the respective degradations of the two PAH when supplied as a pair, by each of the three bacterial systems. All experiments were run simultaneously using a 5% (v/v) inoculum for each strain. Analyses were performed after 25 days of incubation

Strains used	PAH degradation (%) when tested			
	Individually		As a pair	
	NAP	PHE	NAP	PHE
S Phe Na 1	0	99	9	0
S Nap Ka 1	100	0	100	11
S Nap Ka 1 + *S Phe Na 1*	100	95	100	83

Table 6 Association of strains *S Phe Na 1* and *S Flt Na 1* for the degradation of phenanthrene and fluoranthene (*ND* not determined) Same presentation of the results as in Table 5. Analyses were performed after 35 days of incubation. Other conditions as in Table 5

Strains used	PAH degradation (%) when tested			
	Individually		As a pair	
	PHE	FLT	PHE	FLT
S Flt Na 1	100	100	39	32
S Phe Na 1	100	0	93	16
S Phe Na 1 + *S Flt Na 1*	ND	ND	100	96

Table 7 Characterization of the different PAH interactions observed (*C* PAH partially or completely consumed: reference value, *NC* PAH not consumed, *C* – PAH less consumed than the reference value, *C* + PAH more consumed than the reference value)

PAH degradation				PAH interaction	Example of strains and PAH involved
Individually		As a pair			
PAH 1	PAH 2	PAH 1	PAH 2		
C	NC	C	NC	No cometabolism (1)	*S Phe Na 1*, PHE + PYR
C	NC	C –	NC	No cometabolism and inhibition (2)	*S Flu Na 1*, FLU + ANT
C	NC	NC	NC	No cometabolism and toxicity (3)	*S Flt Na 1*, FLT + NAP
C	NC	C	C	Cometabolism (4)	*S Phe Na 1*, PHE + FLT
C	NC	C –	C	Cometabolism with inhibition (5)	*S Phe Na 1*, PHE + FLU
C	NC	C +	C	Cometabolism with synergy (6)	*S Pyr Na 1*, PHE + FLU
C	C	C –	C	Preferential substrate degradation (7)	*S Ant Mu 3*, PYR + ANT
C	C	C –	C –	Substrate antagonism (8)	*S Pyr Na 1*, PHE + FLT

where the degradation of phenanthrene by strain *S Pyr Na 1* was actually enhanced instead of being inhibited in the presence of the added PAH, fluorene (situation 6 in Table 7). As previously mentioned in Results, phenanthrene may be a rather poor inducer of its degradation system and the enhancement of its degradation by fluorene may result from a positive analogue effect on enzyme induction. Situations where non-growth-supporting analogues were inducers of enzymes required for the complete metabolism of growth-supporting substrates, can be found in the literature (Hegeman 1966; Rosenfeld and Feigelson 1969; Van Eyk and Bartels 1968).

As also illustrated in Table 7, neither inhibition nor synergy was observed in a number of cases involving cometabolism (situation 4) or not (situation 1).

Among PAH, naphthalene presented specific characteristics. Strains isolated on this compound did not use other PAH as carbon sources, a common but not exclusive phenotype according to Kiyohara et al. (1992), and showed limited cometabolism capacities for these other PAH. Naphthalene was toxic to other strains, probably because of its high water-solubility (about 30 ppm). This was shown, for example, in the case of strain *S Phe Na 1*, which could not grow on phenanthrene in the presence of 500 ppm naphthalene but grew on phenanthrene when naphthalene was supplied in the vapour phase (data not shown). However, using naphthalene as sole carbon source, at non-toxic concentrations (vapour phase) never allowed growth of any of the strains isolated on three- or four-ring PAH. Concerning cometabolism capacities of the latter strains for naphthalene, because of naphthalene toxicity when supplied as crystals, no conclusions can be drawn from the experiments presented here. Evidence of naphthalene toxicity for PAH-degrading strains can be found in the literature (Mueller et al. 1990; Weissenfels et al. 1991), although Walter et al. (1991) reported the cometabolic degradation of naphthalene at a concentration of 250 ppm by a pyrene-degrading *Rhodococcus* unable to grow on naphthalene alone.

The results presented here point to the strong toxicity of naphthalene in the degradation of a mixture of PAH containing this compound. This point is important to consider with respect to the case of coal-tar-polluted industrial sites, which often contain naphthalene in association with other PAH (Wilson and Jones 1993). With the strains isolated here, prior degradation of naphthalene by a naphthalene-utilizing strain capable of standing high concentrations of this compound was required for further PAH degradation by specialized strains. Strain associations were also efficient in relieving specific cases of inhibition by particular PAH associations such as phenanthrene and fluoranthene for strain *S Flt Na 1*. Degradation of a PAH mixture thus appears as a cooperative process involving a consortium of strains with complementary capacities.

The accumulation of dissolved organic carbon, often around 20% of the degraded carbon, was consistently observed in these experiments. Although the presence of dissolved organic carbon is likely to result from a variety of sources including, in particular, products of cell lysis, the presence in these soluble compounds of unidentified PAH metabolites was suggested by UV spectrophotometric examination at 220 nm, showing, for example, characteristic peaks in experiments involving phenanthrene degradation (data not shown). For a long time for naphthalene and phenanthrene (as summarized by Gibson and Subramanian 1984), and recently for fluorene (Griffoll et al. 1992; Monna et al. 1993), fluoranthene (Kelley et al. 1993; Weissenfels et al. 1991) and pyrene (Walter et al. 1991), degradation pathways have been proposed. Although studies, aimed at elucidating these pathways, pointed out the presence of transient or dead-end specific PAH metabolites, little can be deduced about the amounts of these products in culture fluids. In particular, this is the

case of experiments where non-uniformly ^{14}C-labelled PAH were employed. Heitkamp and Cerniglia (1988) noted the production of significant levels of metabolites by a *Mycobacterium* sp. when degrading one of the following PAH: naphthalene, phenanthrene, fluoranthene or pyrene. On the other hand, Weissenfels et al. (1990) did not detect, by any of several methods, the accumulation of dead-end products by a *Pseudomonas vesicularis* growing on fluorene, a *Pseudomonas paucimobilis* growing on phenanthrene and an *Alcaligenes denitrificans* growing on fluoranthene.

Here, the production of dissolved organic carbon was observed in all instances of PAH degradation although its extent varied, being rather high in the case of fluorene and phenanthrene. The results thus suggest that, in our conditions, a relatively small and variable but significant portion of the degraded PAH accumulated as soluble metabolites in the medium. Metabolite accumulation did not appear to be related to oxygen availability. Indeed, changing the volume of the gas phase or, in the case of strain *S Phe Na 1*, the amount of phenanthrene introduced, did not affect the portion of substrate converted into metabolites (data not shown). PAH degradation by cometabolism increased, but not in all cases, the accumulation of dissolved organic carbon. In fact, from the data presented, it can be calculated that the portion of PAH such as fluorene and anthracene converted into metabolites was often clearly higher when they were degraded by cometabolism than when they were used as growth substrates. However, their global contribution to the dissolved organic carbon in these cometabolism experiments remained moderate because of the limited amount attacked when they were used as cosubstrates. Whether metabolite accumulation is mainly related to the breakdown of particular PAH or to the degradative capacities of the strains involved, and to what extent such accumulation can be influenced by cometabolism phenomena or by the concerted action of several strains in mixed cultures, are important questions that deserve further investigations.

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