Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes

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Bacteria with ability to degrade polyaromatic hydrocarbons (PAHs), isolated from wastewater and soil samples, were investigated for their taxonomic, physiological and genetic diversity. Eighteen isolates able to metabolize naphthalene or phenanthrene as sole carbon source were taxonomically affiliated to different subclasses of the *Proteobacteria* (*Sphingomonas* spp., *Acidovorax* spp., *Comamonas* spp. and *Pseudomonas* spp.) and to phyla of Gram-positive bacteria with low and high DNA G+C content (*Paenibacillus* sp. and *Rhodococcus* spp., respectively). Representatives of the genera *Pseudomonas* and *Sphingomonas* formed a remarkably high fraction of these isolates; 9 out of 18 strains belonged to these groups. Tests for enzyme activities showed that the majority of the isolates growing with PAHs as sole sources of carbon and energy had an active catechol 2,3-dioxygenase (C23O). C23O specific activities were very diverse, ranging from 0.1 to 650 mU (mg protein)⁻¹. *Pseudomonas* and *Sphingomonas* strains showed considerably higher activities than the other isolates. All PAH degraders were examined for the presence of an initial PAH dioxygenase and C23O, which catalyse key steps of PAH degradation, by PCR amplification of gene fragments and subsequent hybridization. PCR primers and internal oligonucleotide probes were developed for the specific detection of the genes of *Pseudomonas* and *Sphingomonas* strains.

Keywords: PAH degradation, initial PAH dioxygenase, catechol 2,3-dioxygenase, PCR-based approach, DNA probes

INTRODUCTION

Some fungi and bacteria are capable of oxidizing polyaromatic hydrocarbons (PAHs). There are three types of microbial PAH degradation: complete mineralization, co-metabolic transformation and non-specific oxidation (Cerniglia, 1984). To effectively decontaminate polluted sites it is desirable that there is complete mineralization to prevent accumulation of metabolites. Complete mineralization of different PAHs has been described for some bacterial strains belonging to different phylogenetic groups (Cerniglia & Heitkamp, 1990; Cerniglia, 1992). The biochemical pathways of

bacterial degradation of low-molecular-mass PAHs such as naphthalene, phenanthrene and anthracene have been elucidated in detail (Cerniglia, 1992). The genus *Pseudomonas* has been the subject of much research as regards its ability to degrade PAHs (Foght & Westlake, 1988; Kiyohara *et al.*, 1994; Johnsen *et al.*, 1996).

The first step in the aerobic degradation of polyaromatic compounds is dependent on the presence of a multi-component enzyme system, the initial PAH dioxygenase (EC 1.14.12.12), catalysing the hydroxylation of the substrate to the corresponding *cis*-dihydrodiol (Ensley *et al.*, 1982; Simon *et al.*, 1993; Williams & Sayers, 1994). Extradiol cleavage and subsequent degradation with the stepwise removal of aromatic rings completes the upper pathway finally leading to catechol, one of the central intermediates of PAH degradation. *Meta*-cleavage of catechol catalysed by catechol 2,3-dioxygenase (C23O;

Abbreviations: C23O, catechol 2,3-dioxygenase; FAME, fatty acid methyl ester; PAH, polyaromatic hydrocarbon.

EC 1.13.11.2) seems to be the most common pathway in the subsequent steps of PAH degradation (lower pathway) (Houghton & Shanley, 1994). The four published nucleotide sequences of genes encoding the initial PAH dioxygenase originated from three different *Pseudomonas putida* strains and one *Pseudomonas aeruginosa* strain (Simon *et al.*, 1993; Takizawa *et al.*, 1994) and are approximately 87–93% similar. C23O gene sequences for bacteria of the genus *Pseudomonas* have also been determined (Keil *et al.*, 1985; Ghosal *et al.*, 1987; Bartilson & Shingler, 1989; Benjamin *et al.*, 1991; Harayama *et al.*, 1991; Ng *et al.*, 1994; Carrington *et al.*, 1994; Herrmann *et al.*, 1995), as well as sequences from degraders of other different genera, such as *Ralstonia* (Kabisch & Fortnagel, 1990; Moon *et al.*, 1995; Kukor & Olsen, 1996), *Sphingomonas* (Yrjälä *et al.*, 1994; Kim & Zylstra, 1995) *Bacillus* and *Rhodococcus* (Candidus *et al.*, 1994). Protein and nucleotide sequences of the C23O of most of the examined *Pseudomonas* and *Sphingomonas* strains are relatively similar to each other, forming two distinct sequence clusters. Sequences of other bacteria show lower similarity to these two clusters.

On account of the key position of the initial dioxygenase and the C23O during complete PAH mineralization, these genes seem to be suitable targets to detect the presence of PAH degradation potential at the DNA level. Genotypic detection could be achieved by PCR-based approaches and hybridization with gene probes. These molecular methods are advantageous when considering the time required for cultivation-dependent isolation of pure cultures. Specific probes and PCR primers could be designed based on existing DNA sequences encoding these enzymes. This approach has already been followed on a limited number of sequences for C23O by Joshi & Walia (1996) and Wikström *et al.* (1996). The aim of the present investigation was to isolate bacterial PAH degraders from aquatic and terrestrial sites, to determine their taxonomic affiliation and to characterize them physiologically and genetically with respect to their ability to degrade PAHs.

METHODS

Bacterial strains. The following reference strains were used in this study: *P. putida* OUS82 (Kiyohara *et al.*, 1994) and *P. putida* NCIB 9816 (Simon *et al.*, 1993); both are reported to possess an initial dioxygenase. *P. putida* F1 is reported to possess a toluene dioxygenase but not PAH dioxygenase (Zylstra & Gibson, 1989). *Escherichia coli* SURE (Promega), lacking any PAH degradation ability, and *P. putida* KT2440 (= DSM 6125), described as a strain lacking C23O (Franklin *et al.*, 1981), were used as negative controls for amplification of gene fragments of the initial dioxygenase and C23O, respectively. Further strains described as PAH degraders were also examined: *Pseudomonas stutzeri* AN11 (kindly provided by R. Rosselló-Mora; Max Planck Institute for Marine Microbiology, Bremen, Germany); *Sphingomonas yanoikuyae* DSM 6900 (Gibson *et al.*, 1973) and *Sphingomonas paucimobilis* BA2 (Kästner *et al.*, 1994).

Collection of environmental samples and isolation of PAH-degrading bacteria. Environmental samples for enrichment of PAH-degrading strains were collected in sterilized jars from aquatic and terrestrial sites (Table 1). The samples were transported and stored on ice until further treatment.

Soil samples were prepared as described by Kästner *et al.* (1994) within a day of collection. Basal medium (25 ml) supplemented with either 0.05% (w/v) crystalline naphthalene or phenanthrene was inoculated with an aliquot of suitable dilutions of the aquatic samples or the soil extracts. The enrichment cultures were incubated aerobically at 25 °C on a rotary incubator at 180 r.p.m. Following visible growth, aliquots of the cultures were transferred to fresh basal medium, also containing 0.05% of the PAH. Naphthalene- and phenanthrene-degrading strains were isolated from these enriched cultures by repeated streaking on R2A agar (Oxoid) until individual colonies were obtained.

Identification of PAH-degrading isolates. Cell morphology was determined by phase-contrast microscopy. Oxidase tests were performed as described by the manufacturer (Merck) and Gram-staining was performed using a modified Hucker method (Doetsch, 1981). Typing with rRNA-targeted oligonucleotides was performed for basic phylogenetic affiliation. Fluorescently labelled group- and genus-specific probes were used for hybridization of fixed cells of the investigated strains (Amann *et al.*, 1990). The following rRNA-targeted oligonucleotides were used: EUB, a probe specific for the *Bacteria* (Stahl *et al.*, 1989); ALF1b, BET42a and GAM42a, specific for members of the alpha-, beta- and gamma-subclasses of the *Proteobacteria*, respectively (Manz *et al.*, 1992); and HGC69a, specific for Gram-positive bacteria with a high DNA G+C content (Roller *et al.*, 1994). Oligonucleotides PS, PPU (Schleifer *et al.*, 1992), COM1424 and ACI208 (Amann *et al.*, 1996) are specific for most genuine pseudomonads, distinct *P. putida* strains, and the genera *Comamonas* and *Acidovorax*, respectively.

Further examination of the isolates was done by physiological tests as reported previously (Kämpfer *et al.*, 1991). Fatty acid methyl ester (FAME) patterns were used as a further marker for identification. Preparation of FAME extracts, gas chromatographic analysis and numerical identification by the MIDI system (Sherlock 2.11) were performed as described previously (Kämpfer *et al.*, 1996).

Determination of naphthalene and phenanthrene mineralization potential. The potential of the isolated bacteria for mineralization of PAHs was examined by gas chromatographically quantifying remaining PAH and simultaneously monitoring bacterial growth in batch cultures. For each tested strain, five airtight flasks were each filled with 10 ml basal medium containing either 0.05% naphthalene or phenanthrene and were inoculated with approximately 10⁷ cells. Cell number was estimated by measuring the optical density at 600 nm. Remaining PAH in the cultures was extracted by vigorously shaking (30 min) with 5 ml n-hexane for gas chromatographic analysis. The gas chromatograph HP 6890 was equipped with a flame-ionization detector and a 25 m capillary column (Ultra 2, cross-linked 5% phenyl methyl siloxan; Hewlett Packard). Separation procedures were as follows: carrier gas, H₂; rate of flow, 0.5 ml min⁻¹; split ratio, 28 ml min⁻¹; injector temperature, 250 °C; detector temperature, 300 °C; column temperature programme, 50–275 °C at 15 °C min⁻¹, holding at 275 °C for 1 min, 275–325 °C at 20 °C min⁻¹. Recovery of PAH from the medium

Table 1. Taxonomic affiliation, capacity for degradation of naphthalene and phenanthrene, and specific activity of C23O of environmental isolates from various sampling sites

Sampling site	Isolate designation	Taxonomic affiliation	Degradation of PAHs in liquid medium*	Degradation of phenanthrene on solid medium†			Specific activity of C23O [mU (mg protein) ⁻¹ ‡]
				Plain	+ 0.05 % salicylate	+ 10 % TSB	
Municipal wastewater plant, Giessen, Germany	E	<i>Acidovorax</i> sp.	p	+	—	+	0.1 (p, 5d)
	F	<i>Pseudomonas</i> sp. (<i>P. putida</i>)§	n	o	+	+	20.1 (n, 3d); 12.6 (n, 6d)
	G	<i>Comamonas</i> sp. (<i>C. testosteroni</i>)§	n	+	+	—	0.6 (n, 5d)
	H	<i>Comamonas</i> sp. (<i>C. testosteroni</i>)§	n	—	+	+	0.3 (n, 4d); 0.5 (n, 5d)
	L7	<i>Pseudomonas</i> sp. (<i>P. mendocina</i>)§	n	—	+	—	0.6 (n, 3d)
	M7	<i>Comamonas</i> sp. (<i>C. terrigena</i>)§	n	—	o	—	0.9 (n, 3d)
	A10	<i>Pseudomonas</i> sp. (<i>P. fluorescens</i>)	n	—	+	+	140 (n, 3d)
	F9	<i>Pseudomonas</i> sp. (<i>P. fluorescens</i>)	n	—	+	—	380 (n, 3d)
	H9	<i>Pseudomonas</i> sp. (<i>P. fluorescens</i>)	n	—	+	+	270 (n, 3d)
	T9	<i>Pseudomonas</i> sp. (<i>P. fluorescens</i>)	n	—	+	¶	240 (n, 3d)
	E10	<i>Acidovorax</i> sp.	p	o	o	+	0.5 (p, 5d)
	V10	<i>Acidovorax</i> sp.	p	+	+	+	1.7 (p, 5d)
	G10	Actinomycete (<i>Rhodococcus erythropolis</i>)§	n	—	o	—	0 (n, 5d)
	Y9	<i>Sphingomonas</i> sp.	n, p	+	o	+	8.9 (p, 3d); 100 (p, 4d); 140 (p, 5d)
Waste disposal site, leakage water, Reiskirchen, Germany	E3	<i>Sphingomonas</i> sp.	n, p	+	+	+	270 (p, 4d); 440 (p, 5d)
Industrial wastewater plant, Frankfurt, Germany	B1	Actinomycete (<i>Rhodococcus rhodochrous</i>)§	n	o	+	—	0 (n, 5d)
Soil from parking place, Giessen, Germany	C4	<i>Pseudomonas</i> sp. (<i>P. fluorescens</i>)	n, p	o	+	+	240 (n, 3d)
Tar oil contaminated soil, Hulin, Czech Republic	3	<i>Paenibacillus</i> sp.	n, p	—	o	o	0 (n, 5d)

* p, Phenanthrene; n, naphthalene.

† TSB, trypticase soy broth; +, formation of colonies surrounded by clear zones; o, formation of colonies surrounded by a weak clear zone; —, no formation of colonies observed.

‡ Strains were grown in basal medium (Kästner *et al.*, 1994) supplemented with 0.05 % naphthalene (n) or phenanthrene (p) as sole carbon source for 3–6 d as shown in parentheses. One unit of specific activity is defined as the amount of enzyme that converts 1 μ mol substrate min⁻¹ (mg protein)⁻¹. The specific activities of the reference strain *P. putida* OUS82 were 380 (n, 4d) and 650 (p, 4d), and of *S. yanoikuyae* DSM 6900 0.85 (n, 5d) and 6.1 (p, 5d).

§ Strains could be identified at the species level by FAME (criterion for successful identification at the species level was a score >0.5) and physiological tests (criterion for successful identification at the species level was a Wilcoxon probability >0.9 with low scores of taxonomic distances and their standard errors and not more than five characters 'against').

|| Strains could be identified at the species level by physiological tests. (The authentic pseudomonads are very similar with respect to their fatty acid pattern. The approach with physiological tests was considered more reliable.)

¶ Isolate T9 showed clearing zones with 10 % brain heart infusion instead of TSB.

Table 2. Sequence and position of PCR primers and internal probes for amplification and detection of dioxygenase sequences

Primer target/designation	Position	Annealing temp. (°C)	Sequence (5' to 3')*	Reference
PAH dioxygenase				
ISPGRL1B	2505–2521†	60	AAAGATCTGTACGGCG	This study
ISPGRR11B	3385–3404†	60	TAAGCCCGGTAGAAACCACG	This study
C23O				
23OF	207–229‡	63	ATGGATD TDATGGGDTTCAAGGT§	Wikström <i>et al.</i> (1996)
23OR	906–928‡	63	ACDGT CADGAADCGDTCGTTGAG§	Wikström <i>et al.</i> (1996)
PSCA23St	15–34‡	57	ATGAAMAAAGGHGTWATGCG	This study
PSCA23End	908–928‡	57	DGTCADGAADCGDTCGTTGAG	This study
SPHCA23St	28–56¶	60	ATGGCWYTGACYGGTGTAMTTCC	This study
Probe target/designation	Position	Stringency of hybridization	Sequence (5' to 3')*	Reference
PAH dioxygenase				
ISPGRL2B	2809–2828†	45, 55#	TCTATCTTCTCGTCGCTCGC	This study
ISPGRR13A	2548–2564†	45, 50#	CGAGCGACTTCTTTCAA	This study
C23O				
PS1	595–613‡	50**	TCGTGSGCYTTGGTCGACA	This study
PS2	702–719‡	30**	GACCTGATCTCCATGACC	This study
SPH	689–709¶	20, 50**	CTGGCACGATGTTGGCCATG	This study
C23O	765–785‡	50**	GAAGAAGTAGATGGTCTTGCC	This study

* D = A + T + G, S = C + G, Y = C + T, W = A + T, M = A + C.

† Numbered according to the published sequence of *P. putida* OUS82 PAH dioxygenase, (GenBank accession number D16629).

‡ Numbered according to the published sequence of *P. putida* plasmid NAH7 C23O (M17159).

§ Deoxyinosine nucleotides within the primers described by Wikström *et al.* (1996) were replaced by wobble nucleotides (A + T + G).

|| Modification of the primer 23OR.

¶ Numbered according to the published sequence of *S. yanoikuyae* B1 C23O (U23375).

Hybridization and washing temperatures (°C).

** Percentage formamide in hybridization buffer. Hybridization was performed at 35 °C.

was determined using a blank reference, i.e. sterile media with PAH.

Monitoring phenanthrene degradation on solid media. Solid basal medium (DSMZ no. 457), both plain and supplemented with 0.05% salicylate, 10% trypticase soy broth or 10% brain heart infusion was coated with a crystalline layer of phenanthrene by adding 250 µl of a 1% phenanthrene/acetone solution. Freshly grown bacterial colonies on R2A agar were transferred with an inoculation needle to the surface of the different phenanthrene plates after evaporation of acetone. The agar plates were incubated at 30 °C and checked for clearing zones and increasing biomass around the areas of inoculation every 24 h.

Determination of C23O activity. Bacterial strains were grown in 150 ml basal medium (Kästner *et al.*, 1994) supplemented with 0.05% phenanthrene or naphthalene to exponential phase of growth. Preparation of the crude enzyme lysate and the enzyme assay were performed as described by Hamzah & Al-Baharna (1994), using 20 mM potassium phosphate buffer (pH 6.8) for cell suspension and 0.05–5 mg of the crude lysate in 750 µl 20 mM phosphate buffer (pH 6.8) with 0.5 nmol catechol for the enzyme assay. Protein concentrations were

determined by the Biuret method, using BSA as a standard. C23O activity was determined by measuring the increase in absorbance at 375 nm due to the formation of 2-hydroxy-muconic semialdehyde (molar absorption coefficient $4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (Nozaki *et al.*, 1963). The absorption spectra from 230 to 450 nm were recorded over 3.5 min or, in the case of low enzyme activity over 14 min, at periodic intervals of 30 s or 2 min using an Ultrospec 4000 UV/VIS spectrophotometer (Pharmacia).

Design of PCR primers and internal oligonucleotide probes. Previously published gene sequences of initial PAH dioxygenase and C23O were aligned using the MacMolly Tetra 3.7 software (Sequence 17; Softgene). Four published sequences of the initial dioxygenase were aligned with sequences of three other dioxygenases; for C23O gene fragments, 12 completely known sequences of C23O genes were considered. Pairs of primers for PCR and internal oligonucleotide probes were selected from the conserved regions of these genes.

DNA extraction and PCR amplification. DNA was isolated and purified using standard procedures (Wilson, 1994).

About 10 ng of isolated DNA was used for PCR. The final PCR mix contained 67 mM Tris/HCl (pH 8.8), 16.6 mM

(NH_4)₂SO₄, 0.45% Triton X-100, 0.2 mg gelatin ml⁻¹, 120 μM of each dNTP (Promega), 2 mM MgCl₂, 100 pmol of each primer (synthesized by Gibco BRL) and 1 U *Taq* DNA polymerase (Pharmacia Biotech). Reactions were performed in a total volume of 50 μl using a Touch Down thermal cycler (Hybaid). PCR consisted of an initial denaturation step for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at the respective temperature for each pair of primers for 30 s (see Table 2), extension at 72 °C for 45 s, and a final extension step at 72 °C for 3 min. The PCR mixes (5 μl) were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV transillumination (254 nm).

Southern blots and hybridization of nucleic acids. The products of PCR amplification were transferred to a positively charged nylon membrane (Hybond-N+; Amersham) for hybridization with internal oligonucleotide probes using standard protocols (Sambrook *et al.*, 1989). Southern blots were hybridized with oligonucleotides either labelled with peroxidase or labelled with [γ -³²P]dATP. Peroxidase labelling reactions were performed as described by Urdea *et al.* (1988). The stringency conditions were adjusted by adding different amounts of formamide (Table 2) according to each oligonucleotide. Bound probes were detected by enhanced chemiluminescence (ECL; Amersham) as described by the manufacturer.

Radioactive labelling and hybridization with oligonucleotide probes using [γ -³²P]dATP, T4 polynucleotide kinase (New England Biolabs) and Rapid-Hyb-buffer (Amersham) were performed as described by the manufacturer. Stringency conditions were adjusted by increasing the temperature (standard: 45 °C) of hybridization and by post-hybridization washing procedures (standard salt concentration: $2 \times \text{SSC}$; 0.1% SDS; and standard washing temperature: 45 °C).

RESULTS AND DISCUSSION

Isolation and taxonomic affiliation of the isolated PAH degraders

The isolation of bacteria degrading PAH was most successful with the samples originating from the municipal wastewater treatment plant. Other methods tested for isolating PAH degraders were less successful. Pure cultures isolated by an overlay technique described by Bogardt & Hemmingsen (1992) showed no PAH degradation ability. The method described by Thiem & Fritsche (1995) using the surfactant Sapogenat T-300 as additive was not suitable for selective enrichment of PAH degraders since the detergent could be alternatively utilized as carbon and energy source. The minimal medium described by Kästner *et al.* (1994) was used because it allowed optimal growth of different PAH-degrading reference organisms in comparison to other minimal media tested (Weissenfels *et al.*, 1990; Keuth & Rehm, 1991).

Eighteen isolates degrading PAH were affiliated to different taxa (Table 1). Almost all the organisms could be identified at the species level. Strains of the alpha-, beta- and gamma-subclasses of *Proteobacteria* as well as Gram-positive bacteria with high and low DNA G+C content were isolated from the different sites. More Gram-negative organisms were isolated (15 out of 18

strains) and representatives of the genera *Pseudomonas* and *Sphingomonas* formed a remarkably high proportion of the isolates (Table 1).

Degradation of phenanthrene and naphthalene

Results of tests in liquid medium (Table 1) showed that representatives of the same genera possessed the same abilities to degrade phenanthrene and naphthalene. An exception was the *Pseudomonas fluorescens* strain C4, which degraded both PAHs whilst all other *Pseudomonas* isolates metabolized only naphthalene. It is remarkable that C4 was the only *Pseudomonas* strain isolated from soil and not from activated sludge. Isolates from the same environmental sample seemed to be more similar to each other regarding their PAH degradation potential compared with representatives of the same species but from another site.

The results obtained in liquid medium were confirmed by a test on solid medium; on this medium a *Comamonas* strain (G) also degraded phenanthrene and two other strains (F and B1) showed weak activity towards this carbon source, that is these isolates were generally able to utilize phenanthrene but in liquid medium it seemed to be unavailable. It is interesting that the addition of a further carbon source (salicylate, trypticase soy broth or brain heart infusion) induced the degradation of phenanthrene by most isolates. However, some contrary results were obtained, for example salicylate inhibited phenanthrene degradation by isolate E.

On the basis of these results it could be concluded that the degradation of PAHs is regulated in a complicated manner and that isolates of the same species were differently influenced by additional carbon sources.

Kinetics of phenanthrene and naphthalene degradation

Gas chromatographic measurement of phenanthrene and naphthalene concentrations in sterile reference flasks showed that abiotic processes only caused an insignificant decrease in phenanthrene in the medium used, whilst naphthalene, being very volatile, was abiotically reduced to amounts of less than 50% of the initial value during the course of 5 d. Phenanthrene degradation kinetics were similar when determined using isolates C4, E, E10, E3 or Y9. The phenanthrene content in the medium decreased constantly. After 5 d, approximately 60% of the initial amount was metabolized. The simultaneous increase in the optical density showed that the PAH was used as the source of carbon and energy.

The growth curves with phenanthrene of isolate E (*Acidovorax* sp.) and isolate C4 (*P. fluorescens*) are shown as examples in Fig. 1(a). The degradation of naphthalene was faster for most of the isolates. After 2 d, about 80% of the initial amount was degraded as is shown for *P. fluorescens* strain C4 (Fig. 1b). Optical density increased during the first 2 d and remained

Fig. 1. Growth (open symbols) and PAH degradation (filled symbols) of various environmental isolates in mineral medium with naphthalene (a) or phenanthrene (b) as sole source of carbon and energy. PAH removal was measured gas chromatographically and was corrected for abiotic losses using a sterile control. (a) ○, ●, isolate C4 (*P. fluorescens*); ◇, ◆, isolate E (*Acidovorax* sp.). (b) □, ■, isolate C4 (*P. fluorescens*); △, ▲, isolate B1 (*R. rhodochrous*).

stable until the source of carbon was finally consumed. Similar results were obtained for naphthalene degradation by other *Pseudomonas* (F, F9, T9, A10 and L7) and *Comamonas* (G, H and M7) isolates.

The more rapid microbial degradation of naphthalene compared to phenanthrene could be explained by its lower recalcitrance, which correlates positively with molecular mass and negatively with aqueous water solubility (Cerniglia, 1992). The *Rhodococcus rhodochrous* strain B1 seemed to be an exception (Fig. 1b). No significant degradation of naphthalene was recorded during the first 2 d. Only 60% of the initial amount was degraded within 5 d. The optical density increased only minimally. Similar results were obtained with the second *Rhodococcus* isolate (G10, *Rhodococcus erythropolis*). The PAH degradation pathway of these two Gram-positive *Rhodococcus* isolates seems to be regulated in a different manner to that in proteobacterial isolates.

Catechol dioxygenase activity

The enzymic conversion of catechol showed that C23Os were significantly induced in cells of the two reference strains (*P. putida* OUS82 and *S. yanoikuyae* DSM 6900)

and of the environmental isolates, excepting the Gram-positive bacteria (isolates 3, B1 and G10). None of the strains showed catechol 1,2-dioxygenase activity. Thus *meta*-cleavage of catechol was most common among the environmental isolates for the lower pathway of PAH degradation. The strains of the different genera varied extremely with respect to their specific enzymic activities (Table 1). Cells of *Pseudomonas* species possessed the highest specific activity on average, with values between 140 and 650 mU (mg protein)⁻¹. Exceptions were a *P. putida* strain (F) with a specific activity of about 20 mU (mg protein)⁻¹ and a *P. mendocina* strain (L7) with less than 1 mU (mg protein)⁻¹. These results showed that C23O specific activities in different species of the same genus could vary extremely. Specific activities of C23O in the three different *Sphingomonas* strains were also highly heterogeneous. Isolate E3 and Y9 possessed an enzyme displaying an activity as high as the *P. fluorescens* strains. All isolates affiliated to the beta-subclass of *Proteobacteria*, *Acidovorax* spp. and *Comamonas* spp. possessed very low specific C23O activities.

The specific activities for the *Sphingomonas* isolates E3 and Y9 increased during prolonged growth with phenanthrene as sole source of carbon. It is noticeable that growth with phenanthrene resulted in a higher specific activity for both reference strains than with naphthalene.

Information on C23O enzymic activities with respect to utilization of monoaromatic compounds already exists. Worsey *et al.* (1978) reported that cells of *P. putida* mt-2 grown in toluene had a specific C23O activity of 3300 mU (mg protein)⁻¹, whilst Brinkmann *et al.* (1994) determined a C23O activity for *P. putida* PaW1 (pWW0) of 1360 mU (mg protein)⁻¹.

The wide variety of specific activities of the C23Os examined suggests heterogeneity in enzyme structure or gene regulation of the different isolates.

Detection of genes encoding initial PAH dioxygenase by PCR and hybridization

DNA sequences of different bacterial multicomponent dioxygenases are divergent with regard to comparisons of initial naphthalene dioxygenases with benzene, toluene and biphenyl dioxygenases. When comparing the DNA sequences of *Pseudomonas* dioxygenase large iron-sulfur subunits, the similarity of calculated sequence pair distances of *P. putida* NCIB 9816 (GenBank accession no. M23914), *P. putida* G7 (M83949), *P. putida* OUS82 (D16629) and *P. aeruginosa* PaK1 (D84146) PAH dioxygenases with toluene dioxygenase of *P. putida* F1 (J04996), benzene dioxygenase of *P. putida* ML2 (L04642) and biphenyl dioxygenase of *P. putida* LB400 (M86348) is between 45% and 49% (Simon *et al.*, 1993; Kiyohara *et al.*, 1994).

Specific primer pairs for the large iron-sulfur subunit of the PAH dioxygenases were created based on the sequence alignment. By using primers ISPGRLE1B and

Fig. 2. Ethidium-bromide-stained 1.5% agarose gel (a) showing PCR amplification products obtained with genomic DNA extracted from various reference strains and environmental isolates using primer pairs ISPGRLE1B/ISPGRR11B and the corresponding Southern blot (b). Lanes: M1, lambda HindIII marker; A, isolate F (*P. putida*); B, isolate A10 (*P. fluorescens*); C, isolate C4 (*P. fluorescens*); D, isolate F9 (*P. fluorescens*); E, isolate H9 (*P. fluorescens*); F, isolate T9 (*P. fluorescens*); G, isolate L7 (*P. mendocina*); H, *P. stutzeri* AN11; I, *P. putida* OUS82; J, *P. putida* NCIB 9816; K, *P. putida* F1; L, *P. putida* KT2440; N, *E. coli* SURE; O, no DNA control.

ISPGRR11B (Table 2) amplification products of 0.9 kb could be generated for reference strains (positive controls *P. putida* OUS82 and NCIB 9816), all naphthalene- and phenanthrene-degrading isolates belonging to the genus *Pseudomonas*, F (*P. putida*), A10, C4, F9, H9, T9 (all *P. fluorescens*) and *P. stutzeri* AN11. Isolate L7 (*P. mendocina*) showed a weak but smaller-sized amplicon (0.83 kb; Fig. 2a). However, isolated strains not belonging to the genus *Pseudomonas*, but able to degrade naphthalene and/or phenanthrene, had no significant PCR amplicon pattern. In this context it should be mentioned that *E. coli* generated amplification products (± 2.0 and ± 1.1 kb), under low-stringency conditions but the nonspecific nature could be determined by sequencing.

To provide a second criterion for specifying the detection of PAH dioxygenase genes in bacterial communities, different oligonucleotide probes located in the internal region of the amplicons, were designed (Table 2). Hybridization signals even under high-stringency conditions (55 °C) were obtained for isolated *P. fluorescens* strains (isolates A10, C4, F9, H9 and T9), *P. putida* (isolate F) and the positive reference strains, *P. putida* NCIB 9816 and OUS82, using the radioactively

labelled oligonucleotide probes ISPGRLE2B and ISPGRR13A (Fig. 2b). Weak hybridization signals were obtained for *P. stutzeri* AN11 PCR amplicons under this stringency condition only for probe ISPGRLE2B. This result can be explained by the fact that there is a slight divergence in the DNA sequence between *P. stutzeri* AN11 and *P. fluorescens* and *P. putida* (results not shown). Isolate L7 (*P. mendocina*) showed no signal under various hybridization conditions, though a smaller weak PCR amplicon could be detected in ethidium-bromide-stained agarose gels (Fig. 2a). These results lead to the conclusion that *Pseudomonas* PAH dioxygenases are quite different at the DNA level without affecting the active site of the large iron-sulfur subunit of multicomponent PAH dioxygenases. It is necessary to gain more sequence information for different strains of the genus *Pseudomonas* to establish a universal *Pseudomonas* PAH dioxygenase PCR and hybridization detection system. With the present method, it is possible to detect a specific allele of PAH dioxygenase large iron-sulfur subunit from *P. putida*, *P. fluorescens* and *P. stutzeri*.

Detection of genes encoding C23O by PCR and hybridization

Published nucleotide sequences of C23O were aligned and similarity distances were calculated pairwise. The C23O sequences of different *Pseudomonas* strains (*P. aeruginosa* J1104, GenBank accession no. X60740; *P. putida* CF600, M33263; *P. putida* P 35X, X77856; *P. putida* pDK1, M65205; *P. putida* strain H, X80765; *P. putida* pWW0, M64747; *P. putida* NAH, M17159; and *Pseudomonas* sp. IC, UO1825) displayed similar values between 75% and more than 95%. These clusters of highly similar sequences were further designated as the 'Pseudomonas group'. Sequences of two *Sphingomonas* strains have also been published. C23O DNA sequences of *Sphingomonas* sp. HV3 (L10655) and *S. yanoikuyae* B1 (U23375) are 76.5% similar to each other ('Sphingomonas group'). The similarities to the sequences of the 'Pseudomonas group' amounts to about 50%.

C23O sequences from bacteria belonging to other genera, such as *Ralstonia* (*R. pickettii*, formerly *Pseudomonas pickettii* (U20258), *R. eutropha*, formerly *Alcaligenes eutrophus* JMP (52415)), *Rhodococcus* (*R. rhodochrous* CTM, X69504), *R. rhodochrous* NCIB 13064 (L77225) and *Bacillus* (*B. stearothermophilus* FDTP-3, X67860) are rather heterogeneous. These sequences are not very similar to each other or to the 'Pseudomonas group' and 'Sphingomonas group'. For this reason it was not likely that all C23O genes could be detected by one universal pair of primers.

The application of the PCR primers 23OF/23OR (Wikström *et al.*, 1996) resulted in detection of C23O sequences of all reference strains and isolates belonging to the 'Pseudomonas group' and 'Sphingomonas group' (Table 3). However, the other isolates which have an active C23O detected by the enzymic test showed no

Table 3. Results of PCR amplification and hybridization of initial dioxygenase and C23O gene sequences of reference strains and environmental isolates

Bacterial strain	PCR product				Hybridization with probe					
	ISPGRL1B ISPGRR11B	23OF 23OR	PSCA23St PSCA23End	SPHCA23St 23OR	ISPGR LE2B	ISPGR RI3A	PS1	PS2	SPH	C23O
Reference strains										
<i>P. putida</i> OUS82	+	+	+	-	+	+	+	+	-	+
<i>P. putida</i> NCIB 9816	+	+	+	-	+	+	+	+	-	+
<i>P. stutzeri</i> AN11	+	+	+	-	+	+	+	+	-	+
<i>S. paucimobilis</i> BA2	-	+	-	+	NA	NA	-	-	+	*
<i>S. yanoikuyae</i> DSM 6900	-	+	-	+	NA	NA	-	-	+	+
<i>P. putida</i> KT2440	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>P. putida</i> F1	-	ND	+	-	NA	NA	-	+	-	+
<i>E. coli</i> SURE	-	-	-	-	NA	NA	NA	NA	NA	NA
Environmental isolates										
<i>Sphingomonas</i> sp. (isolate Y9)	-	+	-	+	NA	NA	-	-	+	+
<i>Sphingomonas</i> sp. (isolate E3)	-	+	-	+	NA	NA	-	-	+	+
<i>Acidovorax</i> sp. (isolate E)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>Acidovorax</i> sp. (isolate E10)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>Acidovorax</i> sp. (isolate V10)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>C. testosteroni</i> (isolate G)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>C. testosteroni</i> (isolate H)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>C. terrigena</i> (isolate M7)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>P. fluorescens</i> (isolate A10)	+	+	+	-	-	+	+	+	-	+
<i>P. fluorescens</i> (isolate F9)	+	+	+	-	+	+	+	+	-	+
<i>P. fluorescens</i> (isolate H9)	+	+	+	-	+	+	+	+	-	+
<i>P. fluorescens</i> (isolate T9)	+	+	+	-	+	+	+	+	-	+
<i>P. fluorescens</i> (isolate C4)	+	+	+	-	+	+	+	+	-	+
<i>P. mendocina</i> (isolate L7)	-	+	+	-	NA	NA	-	+	-	+
<i>P. putida</i> (isolate F)	+	+	+	-	+	+	+	+	-	+
<i>R. rhodochrous</i> (isolate B1)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>R. erythropolis</i> (isolate G10)	-	-	-	-	NA	NA	NA	NA	NA	NA
<i>Paenibacillus</i> sp. (isolate 3)	-	-	-	-	NA	NA	NA	NA	NA	NA

NA, No amplification product for hybridization test obtained, see columns 2-5.

ND, Not determined.

* Showed only a weak hybridization signal using relaxed conditions of hybridization (20% formamide).

amplification products, even using relaxed conditions for primer annealing (60 °C) (data not shown).

On account of the high homology of C23O sequences of different *Pseudomonas* strains on the one hand and *Sphingomonas* strains on the other, it was checked if it was possible to detect the C23O genes of these genera specifically by PCR. Two specific pairs of primers were developed for this purpose (Table 2). The primers PSCA23St and PSCA23End were designed to detect representatives of the '*Pseudomonas* group'. SPHCA23St was specifically designed to detect C23O sequences of the '*Sphingomonas* group' in combination with primer 23OR. The resulting amplicons have a length of approximately 900 bp and therefore make up nearly the complete gene. Table 3 shows the results of the PCR tests. Application of PSCA23St/PSCA23End

resulted in the expected 900 bp fragment for the reference strains *P. putida* OUS82 and *P. putida* NCIB 9816, as well as for all *Pseudomonas* isolates (Fig. 3a). *P. putida* F1 and *P. mendocina* (isolate L7) showed only weak amplification products. *P. putida* KT2440 lacking C23O and all other isolates yielded no PCR products. Using the primers SPHCA23St and 23OR, only the *Sphingomonas* isolates (Y9 and E3) and the respective reference strains (*S. paucimobilis* BA2 and *S. yanoikuyae* DSM 6900) showed an amplification product of the predicted size (Fig. 4a). The results clearly demonstrated that the PCR-based approach with these primers is suitable for specific detection of *Pseudomonas* and *Sphingomonas* C23O gene sequences.

Oligonucleotide probes were designed for further confirmation of the identity of the PCR amplicons, targeting

Fig. 3. Ethidium-bromide-stained 1.5% agarose gel (a) showing PCR amplification products obtained with genomic DNA extracted from various reference strains and environmental isolates using primer pair PSCA23St/PSCA23End and the corresponding Southern blot (b). Lanes: M1, lambda *EcoR* I/*Hind*III marker; A, isolate F (*P. putida*); B, isolate A10 (*P. fluorescens*); C, isolate C4 (*P. fluorescens*); D, isolate F9 (*P. fluorescens*); E, isolate H9 (*P. fluorescens*); F, isolate T9 (*P. fluorescens*); G, isolate L7 (*P. mendocina*); H, *P. stutzeri* AN11; I, *P. putida* OUS82; J, *P. putida* NCIB 9816; K, *P. putida* F1; L, *P. putida* KT2440; N, *E. coli* SURE, O, no DNA control; M2, 100 bp ladder.

internal regions (Table 2). The probe PS1 hybridized specifically with amplification products of the *Pseudomonas* strains with stringent hybridization conditions (50%, v/v, formamide). Only the weak PCR products of *P. putida* F1 and L7 did not hybridize with this probe, even at relaxed hybridization conditions (20%, v/v, formamide). However, hybridization signals were obtained for all *Pseudomonas* strains using the second *Pseudomonas*-specific oligonucleotide probe PS2 (Fig. 3b). The 'Sphingomonas group' probe SPH hybridized specifically with amplification products of the different *Sphingomonas* strains. Only the PCR amplicon of *S. paucimobilis* BA2 did not hybridize (Fig. 4b). When hybridized at a formamide concentration of 20%, the PCR product of *S. paucimobilis* BA2 also showed a weak hybridization signal. In comparison to these specific probes, a second general probe, C23O, hybridized with PCR products of all *Pseudomonas* strains as well as all *Sphingomonas* strains, except again *S. paucimobilis* BA2.

Based on the results of PCR and hybridization, it could be suggested that C23O genes of the different environmental *Pseudomonas* and *Sphingomonas* spp. isolates clustered with their respective sequence group. By

Fig. 4. Ethidium-bromide-stained 1.5% agarose gel (a) showing PCR amplification products obtained with genomic DNA extracted from various reference strains and environmental isolates using the primer pair SPHCA23St/23OR and the corresponding Southern blot (b). Lanes: M1, lambda *EcoR* I/*Hind*III marker; A, isolate E3 (*Sphingomonas* sp.); B, isolate Y9 (*Sphingomonas* sp.); C, *S. paucimobilis* BA2; D, *S. yanoikuyae* DSM 6900; E, isolate A10 (*P. fluorescens*); F, *P. putida* OUS82; G, *P. putida* NCIB 9816; H, *P. putida* F1; I, *P. putida* KT2440; J, *E. coli* SURE; K, no DNA control; M2, 100 bp ladder.

a general PCR step and subsequent hybridization' with the individual *Pseudomonas* and *Sphingomonas* gene probes PS1/PS2 or SPH, or alternatively, by a PCR-based approach with *Pseudomonas*- and *Sphingomonas*-specific primers, it is possible to specifically detect the presence of C23O sequences of these genera. Further sequence analysis should promote optimal probe design and it should, for example, be possible to develop a probe for sphingomonads also recording the *S. paucimobilis* BA2 gene.

Conclusions

The isolation of bacteria degrading PAH was most successful with the method of Kästner *et al.* (1994). Bacteria with PAH degradation abilities were present in the investigated habitats independent of the extent of PAH contamination. The majority of isolates were affiliated to the genera *Pseudomonas* and *Sphingomonas*, but it has to be considered that the selected

isolation strategy may have favoured *Pseudomonas* and *Sphingomonas* strains. The C23O test established high enzyme activities for most representatives of these two genera. The development of sets of primers and internal oligonucleotides allowed the specific detection of the genes of initial PAH dioxygenase and C23O for members of these two genera. However, there may still be a potential for false negative results wherein catabolic genes involved in the degradation of PAHs (that lack DNA sequence homology to the primers and/or probes) are not detected. A verification of the method with PAH-degrading pseudomonads and sphingomonads from a diversity of other sites is still necessary. It was not possible to detect further PAH degraders belonging to the genera *Comamonas* and *Acidovorax* using this approach because the catabolic gene sequences seem to be heterogeneous compared with *Pseudomonas* and *Sphingomonas* sequences. To use these as a detection system for PAH degradation potential at the DNA level, the development of an enlarged set of primers and probes, founded on new sequence information, is necessary.

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