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ORIGINAL PAPER

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Insights into the genetic diversity of initial dioxygenases from PAH-degrading bacteria

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Abstract Alpha subunit genes of initial polyaromatic hydrocarbon (PAH) dioxygenases were used as targets for the PCR detection of PAH-degrading strains of the genera *Pseudomonas*, *Comamonas* and *Rhodococcus* which were obtained from activated sludge or soil samples. Sequence analysis of PCR products from several *Pseudomonas* strains showed that alpha subunits (*nahAc* allele) of this genus are highly conserved. PCR primers for the specific detection of alpha subunit genes of initial PAH dioxygenases from *Pseudomonas* strains were not suitable for detecting the corresponding genes from the genera *Comamonas* and *Rhodococcus*. Southern analysis using a heterologous gene probe derived from the *P. putida* OUS82 PAH dioxygenase alpha subunit identified segments of the PAH-degradation gene cluster from *C. testosteroni* strain H. Parts of this gene cluster containing three subunits of the initial PAH dioxygenase were isolated. These three subunits [ferredoxin (*pahAb*), alpha (*pahAc*) and beta (*pahAd*) subunit] were amplified by PCR as one fragment and expressed in *Escherichia coli* DH5α, resulting in an active initial dioxygenase with the ability to transform indole and phenanthrene. The DNA sequence alignment of alpha subunits from *C. testosteroni* H and various PAH-degrading bacteria permitted the design of new primers and oligonucleotide probes which are useful for the detection of the initial PAH dioxygenases from strains of *Pseudomonas*, *Comamonas* and *Rhodococcus*.

Introduction

Bacteria from different genera are able to catabolize polyaromatic hydrocarbons (PAH; Cerniglia 1992; Pothuluri and Cerniglia 1994). The genetics of the PAH degradation pathway have been investigated in detail for strains belonging to the genus *Pseudomonas* (Williams and Sayers 1994; Johnsen et al. 1996). It is divided into an upper pathway, leading to central intermediates such as catechol or protocatechuate (Yen and Serdar 1988; Kiyohara et al. 1994), and a lower pathway resulting in the formation of tricarboxylic acid cycle intermediates (Houghton and Shanley 1994). The initial PAH dioxygenases of the upper pathway are the key enzymes for various bacteria and enable them to attack aromatic ring structures. They are multimeric and comprise several subunits: a reductase subunit, a ferredoxin subunit, a large (alpha) and a small (beta) iron sulfur protein (Ensley et al. 1982; Ensley and Haigler 1990; Mason and Cammack 1992; Parales et al. 1999). These enzymes could be suitable targets for genetic screening strategies to detect bacteria with PAH-catabolizing potential (Herrick et al. 1993; Fleming et al. 1998; Hamann et al. 1999; Meyer et al. 1999; Wilson et al. 1999).

It was possible to detect the PAH dioxygenases of different *Pseudomonas* species catabolizing PAH, using PCR and an oligonucleotide probe-based screening approach with the alpha subunits of the terminal PAH dioxygenases as target (Meyer et al. 1999). However, in that study it was not possible to detect the genes of PAH dioxygenases from other genera, such as *Comamonas* and *Rhodococcus*, catabolizing naphthalene and phenanthrene. Zylstra et al. (1997) showed that *Comamonas* has a distinctive initial PAH dioxygenase, compared to the highly conserved *Pseudomonas* naphthalene dioxygenase genes (*nahAaAbAcAd*; Williams and Sayers 1994). Taking these results into account, we used a heterologous gene probe from *P. putida* OUS82 (Kiyohara et al. 1994) to identify and isolate the corresponding genes from *Comamonas* strains by Southern hybridization, since this probe might be less specific than the PCR primers for *Pseudomonas* PAH dioxygenase. In this study, it was expected that new sequence information would result in the design of specific primers for the alpha subunit genes of initial PAH dioxygenases from strains belonging to the genera *Comamonas* and subse-

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quently *Rhodococcus*. This could provide the ability to prove the presence of one of the three types of initial PAH dioxygenases using these primers. Therefore it might be a useful tool for the characterization of a PAH-catabolizing microbial community and shed light on the great diversity of initial PAH dioxygenases from PAH-catabolizing bacteria in the environment.

Materials and methods

Bacterial strains, plasmids, reagents and media

All strains and plasmids are listed in Table 1. All chemicals were reagent grade and were purchased from either Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany). Mineral salts medium (MM) with salicylate as inducer (0.05%; Meyer et al. 1999) was used for phenanthrene degradation tests and for testing the transformation ability of indole to indigo (Ensley et al. 1983) by wild-type *Comamonas* strains. Solid basal medium contained 1.5% Bacto-agar (Difco Laboratories, Detroit, USA). Luria Bertani broth (LB; Lech and Brent 1988) was used for growing *Escherichia coli* strains at 37 °C. Solid LB medium contained 1.5% Bacto-agar. Ampicillin (150 µg/ml) was added to select for pUC19, pGEM-T and their derivatives. *P. putida* F1, *P. putida* NCIB9816, *P. putida* OUS82, *P. stutzeri* AN11 and all other PAH-degrading bacterial strains were grown at either 30 °C or 26 °C.

PCR reactions and direct amplicon sequencing

About 10 ng of DNA were used for PCR reactions. The final PCR mix contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 20 pmol of each primer (synthesized by Metabion, Martinsried, Germany) and 1 U of *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). Reactions were performed in a total volume of 50 µl, using a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, USA). A general PCR program was 3 min at 94 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for each primer pair for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 3 min. To obtain PCR fragments of alpha subunits of initial dioxygenases from PAH-degrading *Pseudomonas* strains for direct sequencing, PCR was done using genomic DNA and the primer pair NAHAC1 A/PSE1rev (Table 2). The PCR fragments were extracted from agarose gels using a PCR purification and gel extraction kit (Qiagen, Hilden, Germany) and sequenced with the internal primer pair ASEQL1 A/ASEQR1 A (Table 2).

DNA isolation, manipulation and Southern hybridizations

DNA was isolated and purified using standard procedures (Wilson 1994). Restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany) or from Amersham-Pharmacia Biotech (Freiburg, Germany). Restriction digestions and ligations of DNA samples were performed, if not otherwise stated, by standard procedures (Sambrook et al. 1989). Agarose gel electrophoresis was performed in 20 mM Tris-20 mM acetate-0,5 mM EDTA buffer with Seakem LE-agarose (FMC Bioproducts, Rockland, USA). DNA fragments used as probes were labelled either by PCR-labelling with digoxigenin (DIG)-dUTP (dTTP:DIG-dUTP-ratio =3:1) or by the random-prime-labelling procedure with [α-^{32}P]dATP, klenow fragment and Rapid-Hyb-buffer as described by the manufacturers (Roche Diagnostics; Amersham Pharmacia Biotech). To identify the initial PAH dioxygenases from *Comamonas* strains, total genomic DNA were digested with *Pst*I and fragments were separated by agarose gel electrophoresis. Transfer of DNA from agarose gels to hybond-N+ nylon membranes (Amersham Pharmacia Biotech) was carried out using a fast capillary blot procedure. Heterologous hybridization was performed using a radioactively labelled PCR fragment (nahAc1) obtained by the primer pair ISPLE2 C/PSE1rev (Table 2) from the alpha subunit of *P. putida* OUS82 PAH dioxygenase. Southern hybridizations were per-

Table 1 Strains and plasmids. *n* Naphthalene, *p* phenanthrene and *t* toluene

Strain/plasmid	PAH-degradation and genetic markers	Reference
Pseudomonas fluorescens A10, F9, and H9	n, p	Meyer et al. 1999
P. fluorescens C4	n, p	Meyer et al. 1999
P. putida F	n, p	Meyer et al. 1999
P. putida N30	n, p	Meyer 1999
P. stutzeri AN11	n, p	R. Rosselló-Mora, MPI for Aquatic Microbiology, Bremen, Germany
P. putida NCIB9816	n, p	Ensley et al. 1982; Kurkela et al. 1988
P. putida OUS82	n, p	Kiyohara et al. 1994
P. putida F1	no PAH degradation; t	Zylstra and Gibson 1989
Comamonas testosteroni G and H	n, p	Meyer et al. 1999
C. terrigena M7	n, p	Meyer et al. 1999
Rhodococcus rhodochrus B1	n, p	Meyer et al. 1999
R. erythropolis G10	n, p	Meyer et al. 1999
Rhodococcus sp. N22 and N23	n, p	Meyer 1999
Escherichia coli JM109	[e14-(McrA–) recA1 endA1 gyrA96 thi-1 hsdR17 ($r_K^-m_K^+$) supE44 relA1 Δ(lac-proAB] [F′traD36 proAB lacIqZΔM15	Yanisch-Perron et al. 1985
E. coli DH5α	[F$^-$ ϕ80dlacZΔM15 Δ(lacZY-argF)U169 deoR recA1 endA1 hsdR17(r_K^- m_K^+) supE44 thi-1 gyrA96 relA1]	German Culture Collection [DSM(Z)], Braunschweig, Germany
E. coli DSM1576	no PAH degradation	DSM(Z), Braunschweig, Germany
Plasmids		
pUC19	3,662 bp, Amp^R, lacZfα	Yanisch-Perron et al. 1985
pGEM-T	3,003 bp, Amp^R, lacZfα	Promega, Madison, Wis., USA
pCORM1	Genomic plasmid clone (PstI)/7.8 kb, Amp^R	This study
pCDIOX1	PCR-pGEM-T subclone of pCORM1/5.5 kb, Amp^R	This study

Table 2 PCR primers and internal probes used for amplification, direct amplicon sequencing and detection of alpha subunits of initial PAH dioxygenases

Primer/ probe	Position	Temperature (°C)	Sequence (5′ to 3′)	Reference
Amplicon sequencing				
NAHAC1 A	5,584–5,609[a]	55	GTTTGCAGCTATCACGGCTGGGGCTT	Herrick et al. 1993, modified
PSE1rev	6,520–6,540[a]	55	CCGATAGAAGCCACGATAACT	this study
ASEQL1 A	5,857–5,876[a]	–[d]	ATCAAGGCCAACTGGAAGGC	this study
ASEQR1 A	6,300–6,319[a]	–[d]	CCAAGCGGCGCTTGAGATCC	this study
DNA probe nahAc1				
ISPLE2 C	5,935–5,970[a]	52	GGTCTTCTCGTCGTTCGCT	this study
PSE1rev	6,520–6,540[a]	52	CCGATAGAAGCCACGATAACT	this study
Amplification of initial PAH dioxygenase from *C. testosteroni* H[e]				
FERHIND1	366–385[b]	55	**AAGCTT**CGCACATGACTCAGAACTGG	this study
BETAXBA1	2,704–2,723[b]	55	**TCTAGA**CCCCAGGGTTCACAGGAAGG	this study
Differential PCR				
Pseudomonas PAH dioxygenases				
PSE1for	5,646–5,665[a]	55	AAAAGAGCTGTATGGCGAGT	this study
PSE1rev	6,520–6,540[a]	55	CCGATAGAAGCCACGATAACT	this study
Comamonas PAH dioxygenases				
COM1for	1,125–1,144[b]	55	AAAAGAGTTGTACGGCGATG[g]	this study
COM1rev	2,000–2,019[b]	55	ACGGTAGAATCCGCGATAGC[g]	this study
Rhodococcus PAH dioxygenases				
RHO1for	4,210–4,229[c]	55	CAAGGATGCGTATGGCAATC[g]	this study
RHO1rev	5,097–5,116[c]	55	TTCGAGGAAGTTGCGCTGGT[g]	this study
Oligonucleotide probes				
PSE/COM1α	1,570–1,589[b]	48[f]	TTGATGGCATTCGGCGGCGC	this study
RHO1α	4,660–4,679[c]	48[f]	GCGTCGGCTCACGCCGGAGC[g]	this study

[a] Position according to sequence of *P. putida* OUS82 PAH dioxygenase (GenBank accession number AB004059)
[b] Position according to sequence of *C. testosteroni* H PAH dioxygenase (GenBank accession number AF252550)
[c] Position according to sequence of *Rhodococcus sp.* I24 PAH dioxygenase (GenBank accession number AF121905
[d] Primers were used in amplicon sequencing, temperature=60 °C
[e] Introduced restriction sites are highlighted by *gray boxes*
[f] Hybridization temperature for oligonucleotide probes
[g] Underlined nucleotides demonstrate mismatches to the corresponding primer/oligonucleotide PSE1for, PSE1rev and PSE/COM1α

formed following the Amersham protocol at 55 °C (hybridization and post-hybridization washing procedures). Stringency conditions were adjusted by increasing the standard salt concentration during the washing procedures (standard salt concentration: 2×SSC, 0.1% SDS) to 0.5×SSC,0.1% SDS.

Preparation of partial genomic DNA library from *C. testosteroni* strain H

The partial DNA library of *C. testosteroni* strain H was constructed using *Pst*I-digested genomic DNA. DNA fragments ranging over 2.5–5.5 kb were eluted from an 0.7% agarose gel, ligated into *Pst*I-digested pUC19 vector and transferred to *E. coli* JM109 (Himeno et al. 1984). Ampicillin-resistant colonies were picked, transferred to a nitrocellulose membrane (Stratagene, La Jolla, USA) and lyzed. Screening was performed by colony hybridization, using the DIG-labelled DNA probe nahAc1 from *P. putida* OUS82 PAH dioxygenase (Hanahan and Meselson 1983; Weis 1987).

Activity of initial PAH dioxygenase from *C. testosteroni* H

To test the activity of the initial PAH dioxygenase, the three open reading frames *pahAb*, *pahAc* and *pahAd* were amplified by PCR as one fragment with the primer pair FERHIND1/BETAXBA1 (Table 2). The resulting PCR product was ligated into a pGEM-T vector, following the instructions of the manufacturer (Promega). *E. coli* DH5α was transformed (Himeno et al. 1984) and plated onto LB plates containing 150 µg ampicillin/ml, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 49 µg X-gal/ml. Clones containing recombinant plasmids were identified by blue–white screening. These were picked and checked for the formation of indigo on [LB/ampicillin (150 µg/ml)/IPTG (1 mM)/indole (1 mM)] agar plates and the transformation of phenanthrene (Meyer et al. 1999).

Differential PCR screening and oligonucleotide probing

Total genomic DNA (10 ng) of strains belonging to the genera *Pseudomonas*, *Comamonas* and *Rhodococcus*, including the negative controls *P. putida* F1 and *E. coli* DSM1576 (Table 1) was tested by PCR for the presence of alpha subunit genes of initial PAH dioxygenases. The specific primer pairs for alpha subunits of *Pseudomonas* PAH dioxygenases PSE1 (PSE1for/PSE1rev), *Comamonas* PAH dioxygenases COM1 (COM1for/COM1rev) and *Rhodococcus* PAH dioxygenases RHO1 (RHO1for/RHO1rev) are listed in Table 2. These primers were designed by aligning the se-

quences of selected alpha subunits of initial PAH dioxygenases and aromatic dioxygenases using MegAlign (DNASTAR, Madison, Wis., USA). The following DNA sequences for the design of primers were taken from GenBank via the National Center for Biotechnology Information server: *P. putida* NCIB9816-4 (M83950), *P. putida* G7 (M83949), *P. putida* OUS82 (AB004059), *P. stutzeri* AN10 (AF039533), *P. aeruginosa* PaK1 (D84146), *C. testosteroni* strain H (AF252550; this work), *Pseudomonas* sp. U2 (AF036940), *Rhodococcus* sp. NCIMB12038 (AF082663), *Rhodococcus* sp. I24 (AF121905), *Burkholderia* RP007 (AF112137), *A. faecalis* AFK2 (AB024945), *P. putida* F1 (J04996), *P. putida* ML2 (L04642) and *bphA-Pseudomonas* sp. LB400 (M86348). Obtained PCR fragments were separated by agarose gel electrophoresis and transferred to a positively charged nylon membrane. Gene probes were obtained by labelling 100 ng of oligonucleotides using [γ-^{32}P]dATP and T4-polynucleotide kinase, following the instructions of the manufacturer (Amersham-Pharmacia Biotech). Southern hybridizations were done at 48 °C with Rapid-Hyb-buffer, using a specific probe for PCR products obtained from *Pseudomonas* or *Comamonas* PAH dioxygenases (PSE/COM1α; Table 2) and from *Rhodococcus* PAH dioxygenases (RHO1α; Table 2). Stringency was adjusted during post-hybridization washings with 0.5×SSC,0.1% SDS or 0.1× SSC,0.1% SDS.

DNA sequencing and analysis

All sequencing was carried out at the laboratory of Dr. Meixner (Freie Universität, Berlin, Germany) on an ABI373 dye terminator chemistry sequencer (PE Biosystems, Foster City, USA). Nucleotide sequences obtained by primer-walking were analyzed and assembled using SeqMan (DNASTAR). BLASTX, BLASTP (Altschul et al. 1990, 1997) and GenBank (Benson et al. 1999) via the National Center for Biotechnology Information server were used for analyzing sequence data and for the amino acid identity searches. Phylogenetic analysis including the calculation of bootstrap values were done using CLUSTALX (Heringa 1999), PHYLIP (Retief 2000) and TreeView (version 1.5; University of Glasgow (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Nucleotide sequence accession number

The DNA sequence of 4,165 bp carrying the 3′-end of *pahH*, the complete *pahAb*, *pahAc*, *pahAd*, and *pahB* genes and the 5′-end of *pahF* from *C. testosteroni* strain H has been submitted to GenBank under accession number AF252550.

Results

Highly conserved alpha subunits of initial PAH dioxygenases of *Pseudomonas*

Since the PCR screening of initial PAH dioxygenases from bacteria catabolizing naphthalene and phenanthrene only resulted in the detection (PCR fragments) of the corresponding genes from strains belonging to the genus *Pseudomonas*, we sequenced DNA fragments obtained by PCR using primers NAHAC1 A and ISPRI1C2 (Table 2). Sequence analysis of the corresponding amplicons showed a high degree of similarity to the dioxygenase *nahAc* allele of known *Pseudomonas* strains (Fig. 1). According to the sequence alignment, the calculated identity values of the *nahAc* alleles of *Pseudomonas* strains catabolizing naphthalene or phenanthrene range over 61–100%.

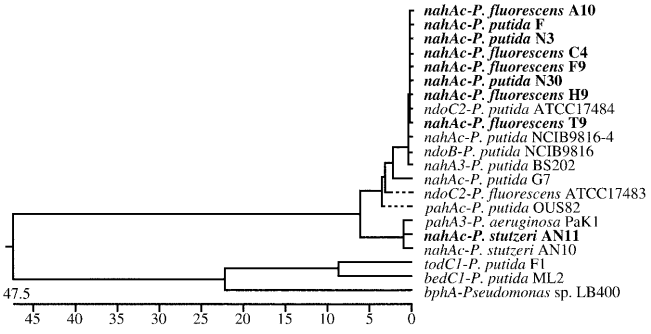

Fig. 1 Phylogenetic relationships of large alpha-subunits of initial aromatic dioxygenases from *Pseudomonas*. The DNA sequences were aligned using MegAlign (DNASTAR). *Pseudomonas* sequences (*boldface type*) resulting from direct amplicon sequencing. All other alpha subunits were taken from GenBank. The following accession numbers correspond to the listed strains (from top to bottom): *ndoC2 P. putida* ATCC17484 (AF004284), *nahAc P. putida* NCIB9816–4 (M83950), *ndoB P. putida* NCIB9816 (M23914), *nahA3 P. putida* BS202 (AF010471), *nahAc P. putida* G7 (M83949), *ndoC2 P. fluorescens* ATCC17483 (AF004283), *pahAc-P. putida* OUS82 (AB004059), *pahA3-P.aeruginosa* PaK1 (D84146), *nahAc-P. stutzeri* AN10 (AF039533), *todC1-P. putida* F1 (J04996), *bedC1-P. putida* ML2 (L04642), *bphA Pseudomonas* sp. LB400 (M86348)

Due to the failure to detect the initial PAH dioxygenase from PAH-catabolizing *Comamonas* or *Rhodococcus* (Table 1) using primers specific for the alpha subunits of initial PAH dioxygenases of *Pseudomonas*, it was necessary to develop an alternative strategy to obtain the corresponding genes.

The initial PAH dioxygenases from *Comamonas*

In order to demonstrate the presence of initial PAH dioxygenases, we showed that *Comamonas* strains G, H and M7 (Table 1) catabolizing naphthalene and phenanthrene were able to form a dark blue color from indole when cultivated on MM supplemented with salicylate as inducer (0.05%). As it was not possible to obtain DNA fragments via PCR from *Comamonas* initial PAH dioxygenases using primers for the *Pseudomonas*-type dioxygenases (*nahAc* alleles), a PCR fragment (nahAc1, 0.5 kb) of the initial PAH dioxygenase from *P. putida* OUS82 was used as a heterologous probe to identify the corresponding genes from *Comamonas* strains G, H and M7. Total genomic DNA from these strains was digested with *Pst*I, blotted and hybridized with the nahAc1 probe, showing a single hybridization band for each strain (Fig. 2). As was the case for the positive control *P. putida* NCIB9816, the hybridization signals of strains G and H were about 4.3 kb in size, whereas strain M7 showed a smaller band (2 kb). The negative control strains, *P. putida* F1 and *E. coli* SURE, showed no signal on the blot (Fig. 2).

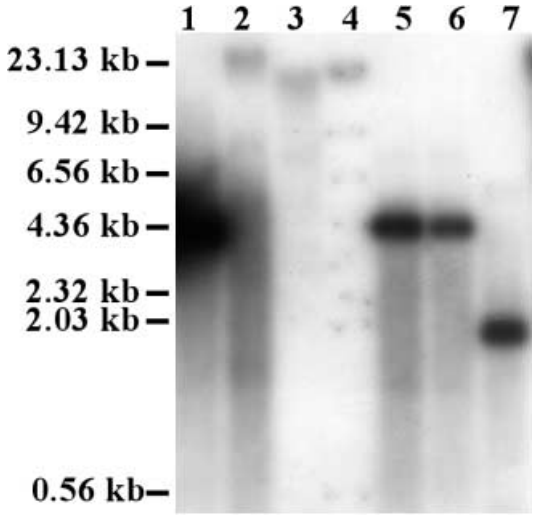

Fig. 2 Southern blot with *Pst*I-digested total genomic DNA of *Comamonas* strains G, H and M7 catabolizing naphthalene and phenanthrene (Table 1). *Lane 1 P. putida* NCIB9816, *lane 2 P. putida* F1, *lane 3 E. coli* SURE; 4, λ-DNA *Hin*dIII marker, *lane 5 Comamonas testosteroni* strain G, *lane 6 C. testosteroni* strain H, *lane 7 C. terrigena* strain M7

Nucleotide sequence analysis of the region encoding parts of the phenanthrene degradation pathway of *Comamonas testosteroni* strain H

A partial gene library was constructed with genomic DNA fragments ranging 2.5–5.5 kb (*Pst*I) in plasmid pUC19. We were able to identify several positive clones by colony hybridization (nahAc1 probe). One of these clones (with a recombinant plasmid named pCORM1) indicated that it contained four complete and two incomplete open reading frames (Fig. 3). A BLASTX search showed that parts of the PAH-degradation gene cluster are located on pCORM1. The similarities of the deduced amino acid sequences of the putative open reading frames to known proteins are shown in Table 3. The Rieske (2Fe-2 S) domain of the alpha subunit of the initial PAH dioxygenase showed the conserved motif Cys-X-His-X-Gly-X_7-Ala-Gly-X_6-Cys-X_2-His (Kauppi et al. 1998).

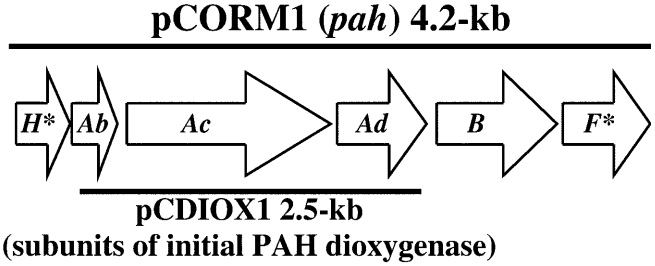

Fig. 3 Genome structure encoding parts of the PAH-degradation gene cluster of *C. testosteroni* strain H. The *pahAb*, *pahAc* and *pahAd* subunits of initial PAH dioxygenase were amplified by PCR and subcloned, resulting in the construction of expression plasmid pCDIOX1. Similarities to known gene products are listed in Table 3. * Indicates incomplete open reading frames

Activity of the initial PAH dioxygenase from *C. testosteroni* strain H

The three open reading frames *pahAb*, *pahAc* and *pahAd* were amplified as one fragment by PCR and cloned in pGEM-T (Fig. 3). Three types of colonies were obtained after transferring the constructs to *E. coli* DH5α and plating the cells on LB/ampicillin/IPTG/X-gal agar: (1) the first type (blue colonies) contained no insert, (2) white clones containing an insert were not able to transform indole and phenanthrene probably due to wrong orientation of the cloned fragment and (3) black clones containing recombinant plasmids exhibited the ability to transform indole and additionally phenanthrene. We therefore conclude that the identified terminal PAH dioxygenase cloned in *E. coli* DH5α is responsible for the initial attack of the aromatic ring structure of PAHs in the wild-type strain *C. testosteroni* H.

Differential detection of initial PAH dioxygenases by PCR and probe-hybridizations

In order to develop a specific PCR and oligonucleotide probe detection system for naphthalene- and phenanthrene-degrading bacteria, various primer pairs were tested. Using the specific primer pair PSE1 (Table 2), we were able to detect the alpha subunits of initial PAH di-

Table 3 Similarities between deduced proteins of PAH-degradation gene cluster *pah* from *C. testosteroni* H and representative orthologs. *aa* Amino acids, *ORF* open reading frame

ORF	Protein size	Functional description of closest relative	% Identity[a]	Source	GenBank accession number
pahH[b] (incomplete)	120 aa	NagH; salicylate-5-hydroxylase (small oxygenase)	99	*Pseudomonas* sp. U2	AF036940
pahAb	104 aa	NagAb; ferredoxin	100	*Pseudomonas* sp. U2	AF036940
pahAc	447 aa	NagAc; naphthalene dioxygenase (alpha subunit)	98	*Pseudomonas* sp. U2	AF036940
pahAd	194 aa	NtdAd; 2-nitrotoluene dioxygenase (beta subunit)	96	*Pseudomonas* sp. JS42	U49504
pahB	259 aa	NagB; *cis*-naphthalene dihydrodiol dehydrogenase	98	*Pseudomonas* sp. U2	AF036940
pahF[b] (incomplete)	186 aa	NagF; salicylaldehyde dehydrogenase	99	*Pseudomonas* sp. U2	AF036940

[a] Percentage of amino acids which are identical when sequences are aligned with BLASTP
[b] These open reading frames are not completely cloned and sequenced

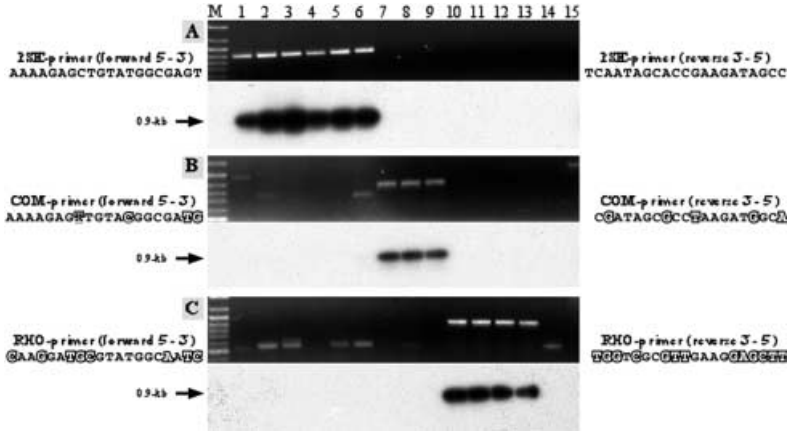

Fig. 4 PCR products obtained using primer pairs PSE1 (**A**), COM1 (**B**) and RHO1 (**C**) and the corresponding southern hybridizations using oligonucleotide probes PSE/COM1α and RHO1α. *Lane M* GeneRuler (MBI Fermentas), *lane 1 P. stutzeri* AN11, *lane 2 P. putida* NCIB9816, *lane 3 P. putida* F, *lane 4 P. fluorescens* A10, *lane 5 P. fluorescens* C4, *lane 6 P. putida* N30, *lane 7 C. testosteroni* G, *lane 8 C. testosteroni* H, *lane 9 C. terrigena* M7, *lane 10 R. rhodochrus* B1, *lane 11 R. erythropolis* G10, *lane 12 Rhodococcus* sp. N22, *lane 13 Rhodococcus* sp. N23. Negative controls: *lane 14 P. putida* F1, *lane 15 Escherichia coli* DSM1576

oxygenases of the PAH-degrading *Pseudomonas* strains under investigation (positive signal: 0.9-kb fragment; Fig. 4A and Table 1). As expected, no amplification could be observed for strains belonging to the genera *Comamonas* and *Rhodococcus*, including the negative control strains *P. putida* F1 (catabolizing toluene) and *E. coli* DSM1576 (no reports on initial dioxygenase activity).

Doing PCR with the specific COM1-primer pair (Table 2), the expected PCR fragments (0.9 kb) appeared with DNA from all *Comamonas* strains catabolizing naphthalene and phenanthrene (Fig. 4B and Table 1). In a few cases, *Pseudomonas*, *Rhodococcus* and the negative control strains showed a weak, non-specific signal or no signal at all.

The specific RHO1-primer set (Table 2) was designed according to sequences of alpha subunits of initial dioxygenases from two *Rhodococcus* strains taken from GenBank (Larkin et al. 1999; Treadway et al. 1999). The initial dioxygenases of four strains belonging to the genus *Rhodococcus* were detected with the RHO1-primer pair (expected fragment: 0.9-kb; Fig. 4C, Tables 1 and 2). We were not able to identify the appropriate PCR fragment (0.9 kb) from *Pseudomonas* or *Comamonas* using this primer pair. Only non-specific signals, about 0.5 kb, occurred with the genomic DNA of *Pseudomonas*.

We confirmed these PCR results by using internal oligonucleotides as probes for *Pseudomonas*/*Comamonas* (PSE/COM1α; Table 2) and *Rhodococcus* (RHO1α; Table 2). PSE/COM1α hybridized to all PCR fragments from *Pseudomonas* and *Comamonas* (Fig. 4A, B) and RHO1α hybridized to the corresponding PCR fragments from *Rhodococcus* (Fig. 4C). Comparison of the primer pairs revealed that they can be used for specific detection of *Pseudomonas*, *Comamonas* and *Rhodococcus* harboring PAH dioxygenases.

Discussion

In a previous study (Meyer et al. 1999), we tried to set up a genetic screening strategy for PAH-degradation potential, focusing on key enzymes such as the alpha subunit of multimeric initial PAH dioxygenases. By using specific primers, we were able to detect the corresponding genes in all analyzed *Pseudomonas* strains (Table 1) catabolizing naphthalene or phenanthrene. We have now shown that alpha subunits of initial PAH dioxygenases are highly conserved among *Pseudomonas* strains (*nahAc* alleles). Using the specific *Pseudomonas* dioxygenase primers (Table 2), we were not able to detect initial PAH dioxygenases by a PCR-based approach in other genera, e.g. *Comamonas* and *Rhodococcus*. Hence, one can assume that strains from other genera with the ability to degrade PAH might possess distinct alpha subunit alleles as part of their initial PAH dioxygenases.

Interestingly, we found that *Pst*I-digested genomic DNA from *Comamonas testosteroni* strains G, H and *Comamonas terrigena* M7 showed single bands in Southern hybridization, using a heterologous PCR generated nahAc1 probe (500 bp) derived from *P. putida* OUS82. In addition, these strains were able to form a blue color when cultivated on mineral salt agar containing indole which indicates the formation of indigo (Ensley et al. 1983). Therefore, the presence of an initial PAH dioxygenase seems plausible. Based on the differences in PAH-catabolic gene arrangement of *Pseudomonas* strains (*nahAc* alleles), compared to PAH-degrading *Comamonas* strains (Zylstra et al. 1997), heterologous hybridizations seem to be successful only when using short gene probes (nahAc1, 500 bp) targeted at one gene in a cluster. However, the detection of sequence similarities between differently arranged catabolic gene clusters with long gene probes (15 kb), as demonstrated by Goyal and Zylstra (1996, 1997), was not possible.

By screening a partial *E. coli* DNA library constructed from the *Pst*I-digested genomic DNA of *C. testoste-*

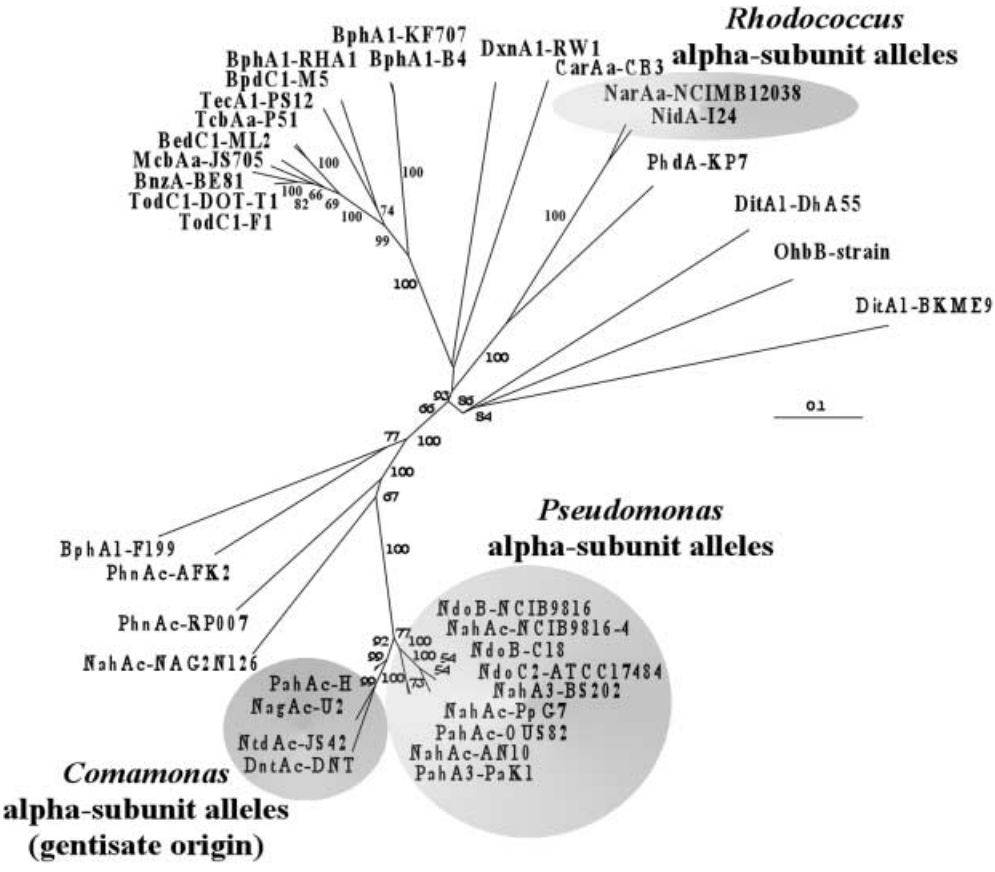

Fig. 5 Relationship of alpha subunits of bacterial initial aromatic dioxygenases based on multiple alignment of the corresponding proteins. The phylogenetic tree was constructed by the CLUSTALX protein distance and neighbor-joining method and the confidence levels of the branching orders were additionally determined by bootstrap analysis. The tree was plotted using TreeView freeware. The *numbers* on the nodes of each branch represent percentage of confidence, from 1,000 replicate analyses. Branch lengths are determined by the *scale bar*. The protein abbreviations, substrate specificity and GenBank accession numbers are as follows: *NdoB-NCIB9816* naphthalene, *P. putida* NCIB9816 (M23914), *NahAc-NCIB9816-4* naphthalene, *P. putida* NCIB9816-4 (M83950), *DoxB-C18* dibenzothiophene, *P. putida* C18 (M60405), *NdoC2-ATCC17484* naphthalene, *P. putida* ATCC17484 (AF004284), *NahA3-BS202* naphthalene, *P. putida* BS202 (AF010471), *NahAc-G7* naphthalene, *P. putida* G7 (M83949), *PahAc-OUS82* polyaromatic hydrocarbon (PAH), *P. putida* OUS82 (AB004059), *NahAc-AN10* naphthalene, *P. stutzeri* AN10 (AF039533), *PahA3-PaK1* naphthalene, *P. aeruginosa* PaK1 (D84146), *PahAc-H* PAH, *Comamonas testosteroni* strain H (AF252550; this work), *NagAc-U2* naphthalene, *Pseudomonas* sp. U2 (AF036940), *NtdAc-JS42* 2-nitrotoluene, *Pseudomonas* sp. JS42 (U49504), *DntAc-RASC* 2,4-dinitrotoluene, *Burkholderia* sp. DNT (U62430), *NahAc-NAG-2N-126* naphthalene, *Neptunomonas naphthovorans* (AF053736), *PhnAc-RP007* PAH, *Burkholderia* RP007 (AF112137), *PhnAc-AFK2* phenanthrene, *Alcaligenes faecalis* AFK2 (AB024945), *BphA1-F199* biphenyl/naphthalene, *Sphingomonas aromaticivorans* F199 (AF079317), *TodC1-F1* toluene, *P. putida* F1 (J04996), *TodC1-DOT-T1* toluene, *P. putida* DOT-T1 (Y18245), *BnzA-BE81* benzene, *P. putida* BE81 (P08084), *McbAa-JS705* chlorobenzene, *Ralstonia* sp. JS705 (CAA06970), *BedC1-ML2* benzene, *P. putida* ML2 (L04642), *TcbAa-P51* chlorobenzene, *Pseudomonas* sp. P51 (U15298), *TecA1-PS12* chlorobenzene, *Burkholderia* sp. PS12 (U78099), *BpdC1-M5* biphenyl, *Rhodococcus* sp. M5 (U27591), *BphA1-RHA1* biphenyl, *Rhodococcus* sp. RHA1 (D32142), *BphA1-KF707* biphenyl, *P. pseudoalcaligenes* KF707 (M83673), *BphA1-B4* biphenyl, *Pseudomonas* sp. B4 (U95054), *DxnA1-RW1* dioxin, *Sphingomonas* sp. RW1 (X72850), *CarAa-CB3* carbazole, *Sphingomonas* sp. CB3 (AF060489), *NarAa-NCIMB12038* naphthalene, *Rhodococcus* sp. NCIMB12038 (AF082663), *NidA-I24* indene, *Rhodococcus* sp. I24 (AF121905), *PhdA-KP7* phenanthrene, *Nocardioides* sp. KP7 (AB017794), *DitA1-DhA55* resin acid, *Mycobacterium* sp. (AF145212), *OhbB-strain 142* halobenzoates, *P. aeruginosa* 142 (AF121970), *DitA1-BKME9* diterpenoid, *P. abietaniphila* BKME9 (AF119621)

roni strain H in pUC19 with the nahAc1-probe, we identified one clone harboring parts of the PAH-degradation gene cluster (the appropriate construct was named pCORM1). The gene arrangement (Fig. 3) and the calculated similarities of the deduced amino acid sequences of the open reading frames of pCORM1 to known proteins are shown in Table 3. Three of the four complete open reading frames of pCORM1 (named *pahAb*, *pahAc* and *pahAd*; Fig. 3) could be identified by sequencing and expression as whole PCR fragment in *E. coli* DH5α as initial PAH dioxygenase able to transform indole and phenanthrene. As the ferredoxin reductase gene (a subunit of multimeric dioxygenases) might be located two open reading frames upstream of the ferredoxin subunit (*pahAb*; Fuenmayor et al. 1998), it is not part of pCORM1. Hence, the expression plasmid pCDIOX1 (harboring *pahAb*, *pahAc* and *pahAd*; Fig. 3) did not contain this reductase gene. However, there are several

examples which indicate that *E. coli* is able to complement missing ferredoxin reductase subunits, leading to active initial dioxygenases (Simon et al. 1993; Eaton et al. 1998). Therefore, we assume a similar complementation mechanism in *E. coli* harboring pCDIOX1, because the recombinant *E. coli* strain expressed an active initial PAH dioxygenase.

By comparing the amino acid sequence of the *C. testosteroni* strain H alpha subunit of the initial PAH dioxygenase with known alpha subunits of aromatic dioxygenases, a separate alpha subunit allele cluster adjacent to the highly conserved *nahAc*-alleles of *Pseudomonas* occurred (Fig. 5). The sequence comparison (Table 3 and Fig. 5) showed that the *Comamonas* alpha subunit is very similar to alpha subunits originating from *Burkholderia* sp. DNT, *Pseudomonas* sp. JS42 and *Pseudomonas* sp. U2 (Parales et al. 1996; Suen et al. 1996; Fuenmayor et al. 1998). Even though the strains harboring this alpha subunit allele belong to different genera, they share a common feature in the arrangement of their catabolic genes; two open reading frames in *Burkholderia* sp. DNT and *Pseudomonas* sp. U2 and one truncated variant of the first of the two open reading frames in *Pseudomonas* sp. JS42 are integrated between the ferredoxin reductase and the ferredoxin subunit of their initial dioxygenases (Parales et al. 1996; Suen et al. 1996; Fuenmayor et al. 1998). This is an exception to the arrangement *nahAa*, *nahAb*, *nahAc* and *nahAd* of naphthalene dioxygenases in *Pseudomonas* strains (Kurkela et al. 1988; Simon et al. 1993; Takizawa et al. 1994). Fuenmayor et al. (1998) showed that the proteins encoded by these reading frames are required for the enzyme salicylate 5-hydroxylase, catalyzing the hydroxylation of salicylate to gentisate. Based on the findings that: (1) pCORM1 contains the C-terminal part of the salicylate-5-hydroxylase (named *pahH*; Table 3) and (2) catechol 2,3-dioxygenase (*meta*-cleavage) activity in *C. testosteroni* strain H could not be measured (Meyer et al. 1999), we assume that this strain might also use the gentisate pathway (work in progress).

We were able to show by PCR and probe hybridization that the *C. testosteroni* H alpha subunit allele is also present in *C. testosteroni* strain G and *C. terrigena* strain M7, but not in the tested *Pseudomonas* strains (Table 1). Hence it is assumed that these distinctive alpha subunits of initial dioxygenases are related to the gentisate pathway; and this is supported by an additional report on a PAH-degrading *Comamonas* strain harboring this unusual degradation gene cluster (Zylstra et al. 1997). Therefore, we presume that the alpha subunits associated to the gentisate pathway might be specific for PAH-degrading strains of the genus *Comamonas*. Strains like *Pseudomonas* sp. U2 and *Burkholderia* sp. DNT have possibly obtained these catabolic genes via horizontal gene transfer (HGT) from *Comamonas*, because the respective degradation clusters in these strains are located on plasmids (Suen and Spain 1993; Fuenmayor et al. 1998). HGT seems also to have played a significant role in the distribution of *Pseudomonas nahAc* alleles to a single Gram-positive and several Gram-negative naphthalene-degrading bacteria (not further characterized; Herrick et al. 1997; Stuart-Keil et al. 1998) and to *Sphingomonas* sp. strains 107 and PL1 (Dagher et al. 1997). This HGT mechanism was also reported to be responsible for the transfer of the highly conserved haloalkane dehalogenase gene (*dhaA*) to strains of the genera *Rhodococcus*, *Pseudomonas* and *Mycobacterium* (Poelarends et al. 2000).

In the light of the presented sequence divergences of alpha subunits of initial PAH dioxygenases from *Comamonas* and *Pseudomonas* (*nahAc* allele), it is not surprising that it was impossible to detect this allele in our *Comamonas* strains using specific primers for the *Pseudomonas nahAc* allele. Therefore, we designed primer pair COM1 (Table 2) to detect the alpha subunit alleles of *Comamonas* strains (Fig. 4B). Additionally we were able to detect the corresponding genes in strains of the genus *Rhodococcus* under investigation, using modified *nahAc* allele primers (RHO1; Table 2 and Fig. 4). As it was possible to amplify DNA fragments of the corresponding alpha subunit alleles in a DNA mixture of *P. putida* NCIB9816, *C. testosteroni* H and *R. rhodochrus* B1, using the respective PCR primers and probes (Table 2; results not shown), we presume that they might be useful for the molecular characterization of an activated sludge community exhibiting PAH-catabolizing potential.

According to the heterogeneity of alpha subunits of initial PAH dioxygenases, such as from *Rhodococcus* sp. I24 and NCIMB12038, *Nocardioides* sp. KP7, *Burkholderia* RP007 and *Alcaligenes faecalis* AFK2 (Fig. 5; Larkin et al. 1999; Laurie and Lloyd-Jones 1999; Saito et al. 1999; Takizawa et al. 1999; Treadway et al. 1999), it seems necessary to design a unique alpha subunit primer pair for each genus in order to detect the alpha subunit allele of interest. If one assumes that initial PAH dioxygenases appeared very early in the evolution of aromatic oxygenases, one can imagine that divergent evolution led to variations of the alpha subunits of terminal PAH dioxygenases. However, the characteristic of catabolic genes spreading and appearing in different genera could make it difficult, working from the detection of a target sequence, to infer the original host strain harboring the corresponding gene.

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