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Genetics of naphthalene and phenanthrene degradation by *Comamonas testosteroni*

AK Goyal and GJ Zylstra

Center for Agricultural Molecular Biology, Cook College, Rutgers University, New Brunswick, NJ 08901-8520, USA

Naphthalene and phenanthrene have long been used as model compounds to investigate the ability of bacteria to degrade polycyclic aromatic hydrocarbons. The catabolic pathways have been determined, several of the enzymes have been purified to homogeneity, and genes have been cloned and sequenced. However, the majority of this work has been performed with fast growing *Pseudomonas* strains related to the archetypal naphthalene-degrading *P. putida* strains G7 and NCIB 9816-4. Recently *Comamonas testosteroni* strains able to degrade naphthalene and phenanthrene have been isolated and shown to possess genes for polycyclic aromatic hydrocarbon degradation that are different from the canonical genes found in *Pseudomonas* species. For instance, *C. testosteroni* GZ39 has genes for naphthalene and phenanthrene degradation which are not only different from those found in *Pseudomonas* species but are also arranged in a different configuration. *C. testosteroni* GZ42, on the other hand, has genes for naphthalene and phenanthrene degradation which are arranged almost the same as those found in *Pseudomonas* species but show significant divergence in their sequences.

Keywords: *Comamonas*; naphthalene; phenanthrene; biodegradation; polycyclic

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in nature. They are formed by a variety of biotic and abiotic reactions in nature. Their major source in the environment is from the combustion of organic matter and, in recent years, from the processing and use of fossil fuels. Due to their aromatic nature and the stabilization afforded by multiple rings, PAHs are very stable and recalcitrant to microbial degradation. Many microbes can, however, rapidly degrade the simple polycyclic compounds naphthalene (two fused rings), phenanthrene (three fused rings, angular arrangement), and anthracene (three fused rings, linear arrangement). Four-ring compounds such as pyrene and chrysene are also metabolizable by bacteria but at a much slower rate. This being the case naphthalene, and to a lesser extent phenanthrene, has often been used as a model compound to study the degradation of PAHs both in the laboratory and the field. *Pseudomonas* strains (especially *P. putida* PpG7 and NCIB 9816-4) have long been used as the organisms of choice for laboratory investigations due to the ease with which they can be isolated from the environment, the rapid rate of growth they exhibit on naphthalene, and their ease of manipulation. These strains have become paradigms for what occurs in nature. However, nature by definition is a diverse environment and many different bacteria, some perhaps unculturable by today's methods, have the ability to utilize PAH compounds as carbon sources for growth. This paper will review what is known about the degradation of naphthalene and phenanthrene by *Pseudomonas* strains and compare that with what has recently been discovered about the ability of *Comamonas* strains to degrade naphthalene and phenanthrene.

Correspondence: Dr GJ Zylstra, Center for Agricultural Molecular Biology, Foran Hall, 59 Dudley Road, Cook College, Rutgers University, New Brunswick, NJ 08901-8520, USA
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Metabolism of naphthalene and phenanthrene by *Pseudomonas* strains

The pathways for the bacterial degradation of naphthalene and phenanthrene have been analyzed for over 30 years. In fact, the degradation of naphthalene (and to a lesser extent the degradation of phenanthrene) is often used as a model system for polycyclic aromatic hydrocarbon degradation. The catabolic pathway was initially described by Davies and Evans [1] and is shown in Figure 1. Clarification of the steps following ring cleavage was supplied in 1992 by Eaton and Chapman [5] who used the cloned *nahCDE* genes to produce and analyze metabolites in the pathway. Utilization of naphthalene begins through dioxygenase attack on the aromatic ring, forming *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) [14]. This reaction is catalyzed by the multicomponent enzyme naphthalene dioxygenase (Figure 2) which has been purified to homogeneity from *P. putida* NCIB 9816-4 by Gibson and coworkers [7]. Two components of naphthalene dioxygenase function to transfer electrons to the third which performs the catalytic reaction. Initially, a reductase functions to accept electrons from NADH or NADPH. This enzyme has been purified [12] and shown to contain both FAD and a plant-type two-iron two-sulfur center. Electrons are then transferred to a small two-iron two-sulfur ferredoxin [11]. The terminal oxygenase component [6] receives the electrons from the ferredoxin and catalyzes the addition of both atoms of molecular oxygen to the aromatic nucleus to form *cis*-naphthalene dihydrodiol. The oxygenase component consists of two nonidentical polypeptide subunits, a Rieske-type two-iron two-sulfur center, and a mononuclear iron [6]. The large subunit of the oxygenase

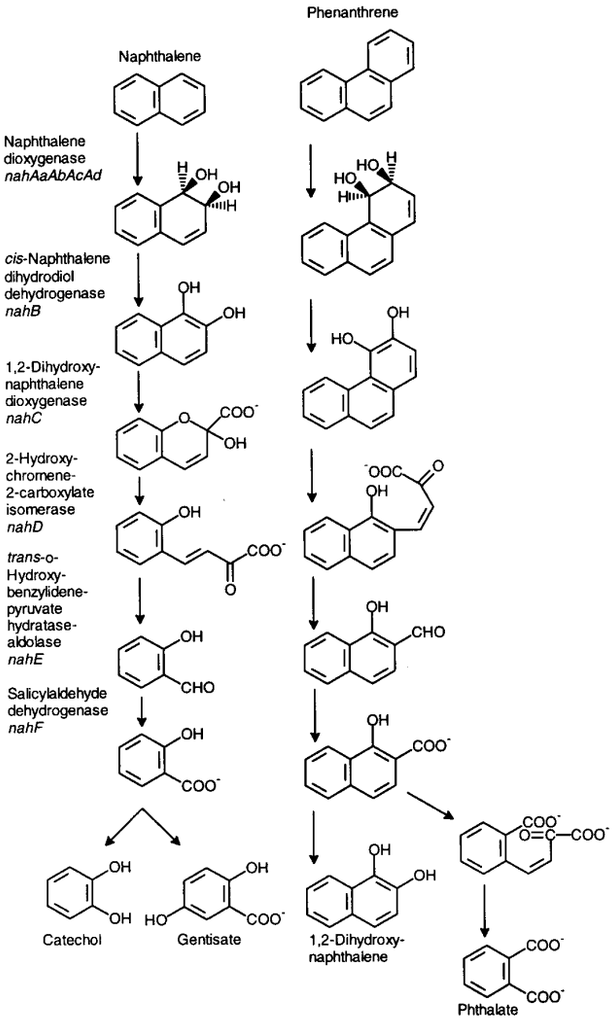

Figure 1 Catabolic pathways for the degradation of naphthalene and phenanthrene by bacteria. The enzymes for naphthalene degradation and the genes that encode them are listed on the left. The same enzymes are able to catalyze the metabolism of phenanthrene by the pathway shown on the right.

component contains both the Rieske-type iron sulfur center [24] which accepts the electrons from the ferredoxin as well as the mononuclear iron [15] involved in the actual catalytic activity. Naphthalene dioxygenase from *P. putida* NCIB 9816-4 has an extremely broad substrate range, having the ability not only to catalyze the addition of oxygen to a wide variety of polycyclic aromatic hydrocarbons to form *cis*-dihydrodiols but also having the ability to carry out monohydroxylation, desaturation (dehydrogenation), *O*- and *N*-dealkylation and sulfoxidation reactions on a variety of substrates (reviewed by Resnick *et al* [20]).

The *cis*-naphthalene dihydrodiol formed by naphthalene dioxygenase is subsequently dehydrogenated to 1,2-dihydroxynaphthalene by a *cis*-dihydrodiol dehydrogenase. This reaction produces NADH, essentially replacing the reducing equivalents utilized in the first catabolic step. The *cis*-naphthalene dihydrodiol dehydrogenase from *P. putida* NCIB 9816-4 has been shown to catalyze the dehydrogenation of *cis*-dihydrodiols of naphthalene, biphenyl, phenanthrene, anthracene, toluene, and, to a limited extent, benzene [19]. The enzyme is not able to catalyze the dehydrogenation of *trans*-naphthalene dihydrodiol. 1,2-Dihydroxynaphthalene is the substrate for *meta*-cleavage of the first aromatic ring by 1,2-dihydroxynaphthalene dioxygenase. Unlike ring cleavage of monocyclic compounds, the product of this reaction is not stable and rapidly forms 2-hydroxychromene-2-carboxylic acid [5]. In order for the metabolism of naphthalene to continue, two specialized enzymes must be present. An isomerase acts to change the *cis* double bond in the cleaved ring to a *trans* double bond, forming *trans*-*o*-hydroxybenzylidenepyruvate. The latter compound is acted upon by a hydratase-aldolase to cleave the side-chain at the position of the *trans* double bond to form salicylaldehyde. A dehydrogenase then catalyzes the conversion of salicylaldehyde to salicylate. Salicyate is subsequently metabolized to tricarboxylic acid cycle intermediates either through the catechol or gentisate pathways depending on the host bacterium (reviewed by Yen and Serdar [29]).

Phenanthrene and anthracene are metabolized by the same enzymes that initially act to metabolize naphthalene (for a review see Sutherland *et al* [26]). One aromatic ring is removed in the process with the formation of 1-hydroxy-2-naphthoic acid in the case of phenanthrene and 2-hydroxy-3-naphthoic acid in the case of anthracene. While the continued degradation of anthracene has not been well studied, two possible fates are known for 1-hydroxy-2-naphthoic acid. One pathway, commonly known as the Evans pathway, involves catabolic steps similar to those involved in the catechol pathway for the degradation of naphthalene [1]. That is, the compound is decarboxylated to form 1,2-dihydroxynaphthalene, which subsequently reenters the catabolic pathway at the ring-cleavage step. Thus, the three-ring compound is metabolized twice through the 'upper' pathway to remove the first two aromatic rings and once through the 'lower' pathway to metabolize the last aromatic

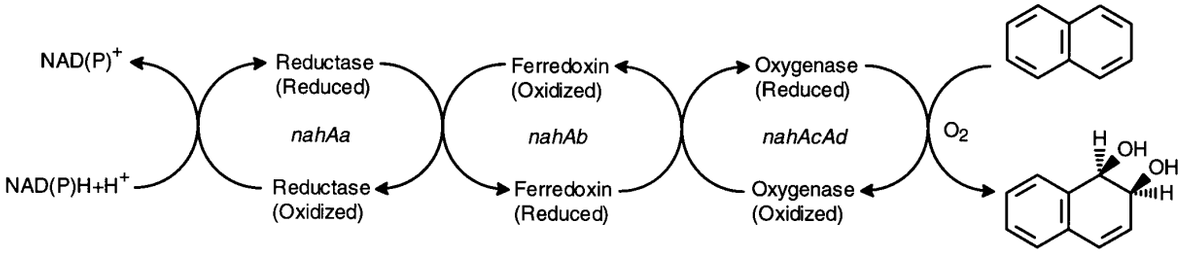

Figure 2 Oxidation of naphthalene to *cis*-naphthalene dihydrodiol by naphthalene dioxygenase.

ring. The second possible pathway, commonly known as the Kiyohara pathway, involves a novel ring cleavage step [16]. In this scenario, 1-hydroxy-2-naphthoic acid itself is the substrate for ring cleavage between the hydroxyl and carboxyl substituents on the aromatic ring. Subsequent metabolic steps involve the removal of the aliphatic side chain and the formation of phthalate which is metabolized through protocatechuate to tricarboxylic acid cycle intermediates.

Genetics of naphthalene degradation by *Pseudomonas* strains

The genes involved in naphthalene degradation by *Pseudomonas* strains have been extensively studied. Historically, the most information is known for the NAH7 plasmid, originally found in *P. putida* G7. This plasmid contains two operons which contain the structural genes for naphthalene degradation [3,28]. One operon contains the genes for the upper pathway, encoding the enzymes necessary for the conversion of naphthalene to salicylate. The second operon contains the genes for the lower pathway, encoding the enzymes necessary for the metabolism of salicylate through the catechol *meta*-cleavage pathway to pyruvate and acetaldehyde. A gene encoding a regulatory protein responsive to salicylate is located in a third operon. Plasmids, such as pDTG1 found in *P. putida* NCIB 9816-4 [21,22], analogous to the NAH7 plasmid have been found in other organisms (for a review see Yen and Serdar [29]). The genes for naphthalene degradation have been cloned from a number of different *Pseudomonas* strains. The genes are fairly easy to clone, owing to the fact that expression of naphthalene dioxygenase in *E. coli* leads to the synthesis of indigo [8]. This is due to the fact that *E. coli* metabolizes tryptophan in rich medium to indole which is a substrate for naphthalene dioxygenase. Following the enzymatic oxidation of indole to a *cis*-dihydrodiol, spontaneous chemical reactions occur which result in the formation of indoxyl which dimerizes to form indigo. Thus, one need only construct a genomic library of a naphthalene-degrading strain in *E. coli* and screen the resulting colonies for one which turns blue. Currently, genes for naphthalene degradation have been cloned and sequenced from at least nine different *Pseudomonas* strains (summarized in Figure 3). While the majority of these strains were isolated for the ability to degrade naphthalene [17,23], *Pseudomonas* sp strain C18 was isolated for the ability to grow on dibenzothiophene [2] and *P. putida* OUS82 was isolated for the ability to grow on phenanthrene [27]. However, all strains grow on naphthalene and the ability to grow on other substrates may be considered a trait of the broad substrate specificity of the catabolic pathway. Even though all of the *Pseudomonas* strains could be considered to be naphthalene degraders, gene names were assigned based on the characteristics of the strains. The term *nah* has historically been applied to the genes for naphthalene degradation and has been used by researchers working with *P. putida* strains G7 and NCIB 9816-4 [10,23,28]. However, the terms *ndo* for naphthalene dioxygenase [17], *pah* for polycyclic aromatic hydrocarbon degradation (Takizawa *et al*, GenBank accession D84146; [27]), and *dox* for dibenzothiophene oxidation [2] have also been used. These gene designations are confusing as the nucleotide sequences are more than 90% identical to one another.

As shown in Figure 3, a consensus can be formed for the organization of the genes in the upper pathway operon. For many of the strains, only a few of the relevant genes have been sequenced. The operon begins with the four genes necessary for naphthalene dioxygenase: *nahAa, nahAb, nahAc*, and *nahAd* encoding the reductase component, the ferredoxin component, and the large and small subunits of the oxygenase component, respectively. Immediately following these four genes are *nahB* encoding *cis*-naphthalene dihydrodiol dehydrogenase, *nahF* encoding salicylaldehyde dehydrogenase, *nahC* encoding 1,2-dihydroxynaphthalene dioxygenase, *nahQ* encoding a protein of unknown function, *nahE* encoding *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase, and *nahD* encoding 2-hydroxychromene-2-carboxylate isomerase.

Isolation of diverse strains for PAH degradation

As stated above, much of what we know about PAH degradation is based on the study of one catabolic pathway and one family of closely related genes found in *Pseudomonas* species. There are many examples in the literature of bacteria that have been isolated for the ability to degrade naphthalene or phenanthrene that do not fall into this family of closely related organisms and genes. It is possible that these bacteria possess different catabolic pathways for PAH degradation or possess highly divergent genes for the same catabolic pathway as that discussed above. This is consistent with the fact that at least five different catabolic pathways are known for toluene degradation (reviewed by Zylstra [30]). In analyzing diversity one comes to a fuller understanding of the microbial ecology of PAH degradation and gains more tools to study community structure and dynamics at sites where active degradation of PAHs is actually occurring in the environment. We decided to pursue this idea by applying a genetic approach to the isolation of organisms that degrade PAHs. In this approach we would not simply look for the fastest growing organism in the chosen enrichment medium (itself biased for organisms preferring that medium) but would screen for all possible PAH-degrading organisms. This would be accomplished by using a PAH of limited water solubility (phenanthrene) in order to limit the speed of growth and by isolating organisms in the early stages of enrichment before the faster growing strains had a chance to overwhelm them. The organisms obtained would then be screened based on whether their genomic DNA showed hybridization to the *nah* genes. Those not hybridizing would be selected for further analysis for their PAH metabolic pathway, enzymatic traits, and gene structure.

The Passaic River in New Jersey was chosen for the initial analysis due to the fact that the river has historically been polluted with a wide variety of chemicals. A number of different strains were isolated for the ability to utilize phenanthrene as the sole carbon source. Six of these strains were chosen for the initial analysis. Each of the six strains was tested for the ability to utilize other aromatic substrates (Table 1). All of the strains were able to utilize naphthalene

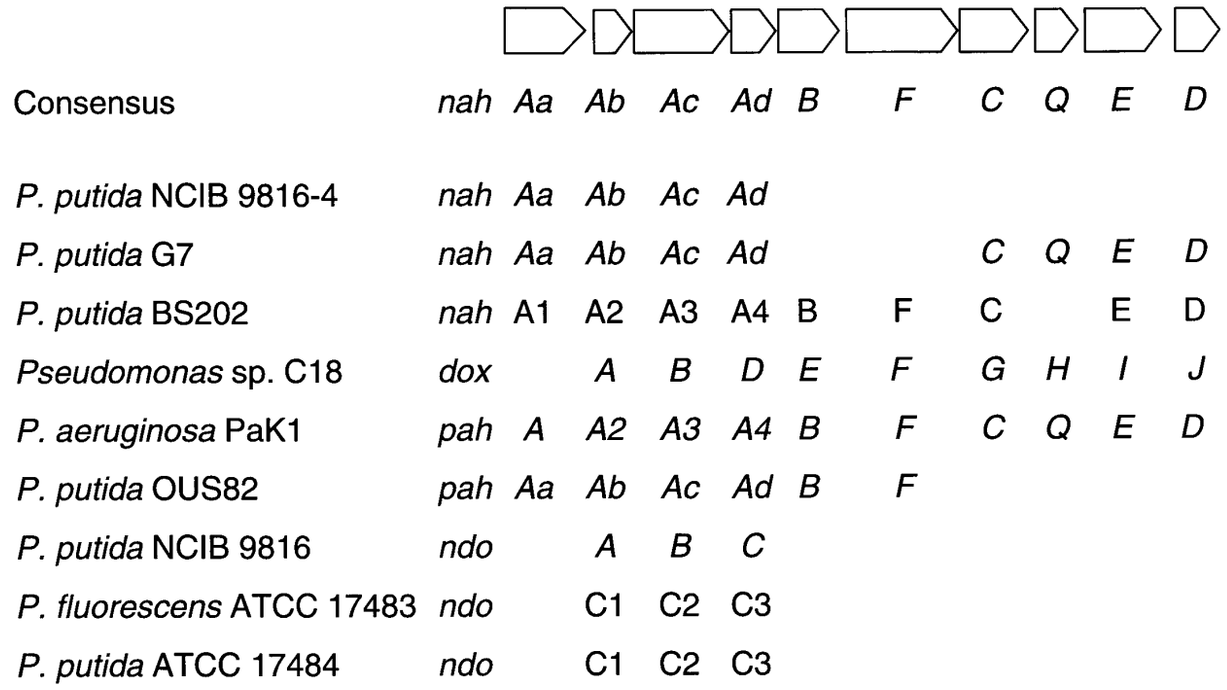

Figure 3 Nucleotide sequences for naphthalene degradation by *Pseudomonas* strains. The sequences from the different organisms are more than 90% identical. References for the sequences are as follows: *P. putida* NCIB 9816-4 ([23] and Parales *et al*, GenBank accession U49496), *P. putida* G7 [4,13,23], *P. putida* BS202 (Bezborodnikov *et al*, GenBank accession AF010471), *Pseudomonas* sp C18 [2], *P. aeruginosa* PaK1 (Takizawa *et al*, GenBank accession D84146), *P. putida* OUS82 [27], *P. putida* NCIB 9816 [17], *P. fluorescens* ATCC 17483 (Hamann, GenBank accession AF004283), and *P. putida* ATCC 17484 (Hamann, GenBank accession AF004284).

Table 1 Growth of Passaic River isolates on various aromatic hydrocarbons

Substrate	*Comamonas testosteroni*			*Pseudomonas putida*		
	GZ38A	GZ39	GZ42	GZ41	GZ44	GZ45-2
Phenanthrene	+	+	+	+	+	+
Naphthalene	−	+	+	+	+	+
Anthracene	+	−	−	−	+	−
Phthalate	+	+	+	−	−	−
Gentisate	−	+	+	−	−	−
o-Hydroxybenzoate	−	+	+	+	+	+
m-Hydroxybenzoate	−	+	+	−	−	−
p-Hydroxybenzoate	−	+	+	+	+	+
Benzoate	−	−	−	+	+	−
Glucose	−	−	−	+	+	+
Succinate	+	+	+	+	+	+

as well as phenanthrene except for GZ38A which only grew on phenanthrene. This confirmed the earlier observations (Figure 1) that catabolic pathways for naphthalene and phenanthrene are interchangeable. Three of the six chosen strains were identified as *P. putida* and three were identified as *Comamonas testosteroni* based on substrate profiles (Biolog screening) and by partial 16S rRNA gene sequencing. Southern hybridizations were performed using isolated genomic DNA from each strain cut with *Eco*RI and the *nahAaAbAcAdBFCQED* genes from *P. putida* NCIB 9816-4 as a probe. Genomic Southerns were chosen over simple colony blots for two reasons: to eliminate the background sometimes seen with colony blotting and to obtain a clean hybridization signal with an RFLP pattern that can be compared between the strains. All three of the isolated *P. putida* strains hybridized to the probe with the same size *Eco*RI fragment hybridizing in each case for the unknowns as well as for the control *P. putida* NCIB 9816-4 [9]. This is as expected as *Pseudomonas* strains possessing the *nah* genes are historically the ones which are the easiest to obtain. However, the three newly isolated *C. testosteroni* strains did not show any hybridization to the gene probe containing the *nah* genes from *P. putida* NCIB 9816-4. This was an exciting finding as it suggested that *C. testosteroni* perhaps represented an alternate line of evolution for a phenanthrene/naphthalene catabolic pathway or perhaps had highly divergent genes for phenanthrene/naphthalene degradation.

Molecular analysis of PAH degradation by *Comamonas testosteroni* strains

In order to investigate the differences between the genes for PAH degradation in the *C. testosteroni* strains and the classically studied *P. putida* strains, cloning investigations were initiated. A genomic library was constructed of *C. testosteroni* GZ39 and screened in *E. coli* for the ability to convert indole to indigo [9]. Two clones were obtained that had this ability and thus putatively contained the genes for a dioxygenase. Restriction mapping of the two 40-kbp cosmid clones showed that they contain the same 18-kbp

region of DNA (Figure 4). This region was subcloned and subjected to analysis. Each subclone was tested for the ability to produce indigo from indole, either *cis*-naphthalene dihydrodiol or 1,2-dihydroxynaphthalene from naphthalene, and either *cis*-phenanthrene dihydrodiol or 3,4-dihydroxyphenanthrene from phenanthrene. This allowed the location of the genes for the first two enzymes in the catabolic pathway: naphthalene dioxygenase and *cis*-naphthalene dihydrodiol dehydrogenase. Interestingly enough, the subclone and metabolite assay experiments indicated that the gene for the latter enzyme is located in between the four genes needed for naphthalene dioxygenase [9]. The region of DNA encoding these activities was sequenced (Figure 4). As expected, four genes readily identifiable as encoding naphthalene dioxygenase were seen with a gene for a *cis*-dihydrodiol dehydrogenase located between them. The order of the genes, designated *phd* for phenanthrene degradation, is *phdAb*, encoding the ferredoxin component, *phdAa*, encoding the reductase component, *phdB*, encoding for *cis*-dihydrodiol dehydrogenase, *phdAc*, encoding the large subunit of the oxygenase component, and *phdAd*, encoding the small subunit of the oxygenase component. Although the proteins encoded by the genes were readily identifiable by known characteristics of related enzymes, they show a high degree of divergence from the amino acid sequences of the related proteins from *Pseudomonas* strains. For instance, the large subunit of the PAH dioxygenase (PhdAc) falls into the NahAc family, but is distantly related to the sequences presented in Figure 3. The amino acid sequence of the reductase component (PhdAa), however, shows a high degree of divergence, having more similarity to monooxygenase reductases involved in phenol or toluene oxidation than reductases associated with dioxygenase enzymes. The amino acid sequence of the *cis*-dihydrodiol dehydrogenase (PhdB) shows that it is more similar to *cis*-biphenyl dihydrodiol dehydrogenases than *cis*-naphthalene dihydrodiol dehydrogenases. The amino acid sequences of the small subunit of the oxygenase component (PhdAd) and the ferredoxin (PhdAb) do not show close similarity to any known group of dioxygenase enzymes. Since the genes and encoded proteins showed so much divergence to enzymes known to be involved in aromatic hydrocarbon degradation, further experiments were conducted to prove that the genes cloned actually are involved in phenanthrene degradation. A knock-out mutant was constructed by cassette mutagenesis into the cloned genes followed by homologous recombination with the genome. The resulting mutant strain lost the ability to grow on naphthalene and phenanthrene, indicating that the cloned genes are necessary for growth on the PAHs tested.

DNA sequencing was continued downstream of the *phdAbAaBAcAd* genes to determine if any other genes involved in PAH degradation could be detected. Open reading frames in this case were identified solely on the similarity of the deduced amino acid sequences with other enzymes in the database. Genes identified include *phdD*, encoding 2-hydroxychromene-2-carboxylate isomerase, an orf encoding a polypeptide of unknown function, an orf encoding a glutathione S-transferase, and *phdE*, encoding 2-hydroxybenzalpyruvate hydratase-aldolase (Figure 4). Thus, the *D* and *E* genes are no longer adjacent to one another and two new genes are placed between them. In addition, genes for 1,2-dihydroxynaphthalene dioxygenase and salicylaldehyde dehydrogenase have not yet been located.

One question that remains is whether the genes for phenanthrene and naphthalene degradation are the same in each of the three *C. testosteroni* strains isolated from the Passaic River sediment. A Southern hybridization experiment was carried out using the cloned *phd* genes and genomic DNA isolated from the six strains listed in Table 1 [9]. None of the three *P. putida* strains hybridized to this probe, as expected. However, the two other *C. testosteroni* strains showed unexpected results. *C. testosteroni* GZ38A showed hybridization but with a different RLFP pattern. Whether this is the result of the genes being organized in a different configuration, or the result of loss of certain genes, or simply a change in one base in an *Eco*RI site, remains to be

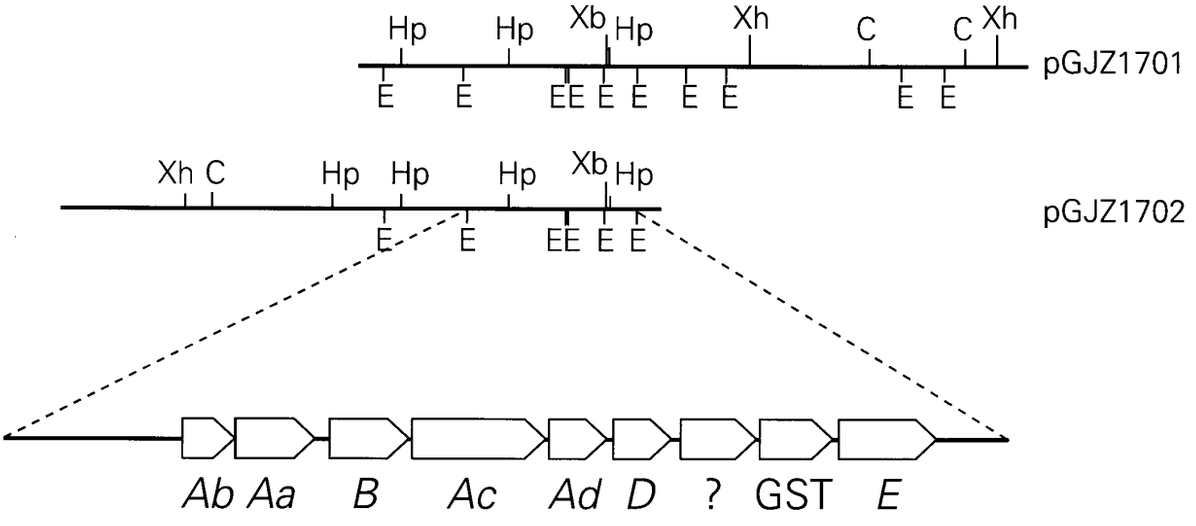

Figure 4 Genes for naphthalene and phenanthrene degradation (designated *phd*) from *C. testosteroni* GZ39. Abbreviations: C, *Cla*I; E, *Eco*RI; Hp, *Hpa*I; Xb, *Xba*I; Xh, *Xho*I.

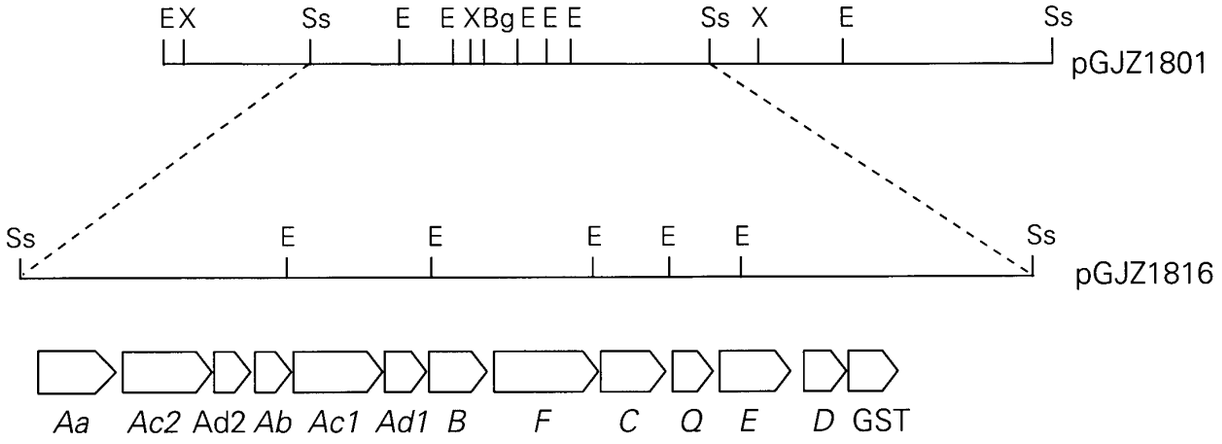

Figure 5 Genes for naphthalene and phenanthrene degradation (designated *nah*) from *C. testosteroni* GZ42. Abbreviations: Bg, *Bgl*II; E, *Eco*RI; Ss, *Sst*I; X, *Xho*I.

determined. However, this change in RFLP pattern may help explain the fact that while strain GZ39 is able to grow on both phenanthrene and naphthalene, strain GZ38A is only able to grow on phenanthrene. In contrast to GZ38A, *C. testosteroni* GZ42 showed no detectable homology to the *phd* genes used as a probe. This suggests that the genes for PAH degradation in strain GZ42 represent a third class of genes for naphthalene/phenanthrene degradation.

The genes for PAH degradation were cloned from *C. testosteroni* GZ42 in order to investigate the genetic basis for the hybridization difference seen. A cosmid library was constructed and screened for the ability to produce indigo from indole in *E. coli*. Subclones of the obtained cosmid clone were constructed and tested for the ability to oxidize naphthalene and phenanthrene. An *Sst*I fragment identified as containing the genes for PAH degradation was sequenced (Figure 5). The nucleotide sequence reveals that although the genes found in *C. testosteroni* GZ39 discussed above are novel, the genes found in *C. testosteroni* GZ42 are divergent relatives of the *nah* genes. The level of divergence is such that the genes would not hybridize to each other except under very low stringency conditions and with a highly labeled probe. The amino acid sequences are highly similar, more so than the nucleotide sequence. The major difference between the genes for PAH degradation found in *C. testosteroni* GZ42 and those in *Pseudomonas* is the presence of new genes. Between the gene for the reductase component (*nahAa*) and the ferredoxin component (*nahAb*) of the initial dioxygenase are two genes encoding proteins of unknown function. The one, designated *nahAc2*, encodes a protein showing similarity to large subunits of oxygenase components of dioxygenases. The other, designated *nahAd2*, encodes a protein showing similarity to small subunits of oxygenase components of dioxygenases. The rest of the operon shows an identical gene organization as that found for the *nah* genes in *Pseudomonas* species. Following the *nahD* gene is a gene encoding a protein with similarity to glutathione S-transferases. Interestingly, the *nahAaAc2Ad2AbAc1Ad1* genes are highly similar to those for 2-nitrotoluene and 2,4-dinitrotoluene degradation [18,25]. In fact, a clone in *E. coli* containing these genes is capable of oxidizing 2,4-dinitrotoluene to the corresponding dihydroxylated product. This then provides direct evidence that the 2-nitrotoluene and 2,4-dinitrotoluene pathways may have evolved directly from an existing PAH degradation pathway.

Acknowledgements

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