Sometimes being flexible is much too rigid.
In Science you have to be fluid.

Summary

Polycyclic aromatic hydrocarbons are found ubiquitously in nature (natural oil seeps, bushfires, volcanoes…) but anthropogenic activities have led to an increased incidence of these recalcitrant pollutants due to, amongst others during the burning, handling or disposal of organic matter including coal tars, crude oil and petroleum products. In our study the catabolic genes from fluorene-degrading *Sphingomonas* sp. strain LB126 and phenanthrene-degrading *Sphingomonas* sp. strain LH128 isolated from the same contaminated environment were investigated. Special attention was given to the genes involved in the first oxidative attack on their respective substrate. These enzymes play an important role in biodegradation since they define the substrate range of each bacterium. The aromatic compounds are transformed to a limited number of central metabolites that are further degraded to Krebs cycle intermediates. Nevertheless, genes for some of these enzymes are evolutionarily related to each other even though they oxidize different pollutants.

The genes involved in the initial attack on fluorene by *Sphingomonas* sp. strain LB126 were investigated. The α and β subunits of a dioxygenase complex (FlnA1A2), showing 63% and 51% sequence identity respectively, with the subunits of an angular dioxygenase from the Gram-positive dibenzofuran degrader *Terrabacter* sp. strain DBF63, were identified. When overexpressed in *E. coli*, FlnA1A2 was responsible for the angular oxidation of fluorene, 9-hydroxyfluorene 9-fluorenone, dibenzofuran and dibenzo-*p*-dioxin. Moreover, FlnA1A2 was able to oxidize polycyclic aromatic hydrocarbons and heteroaromatics, some of which were not oxidized by the dioxygenase from *Terrabacter* sp. strain DBF63. Quantification of resulting oxidation products showed that fluorene and phenanthrene were the preferred substrates of FlnA1A2.

Sphingomonas sp. strain LH128 harbours the terminal component of a ring-hydroxylating dioxygenase (PhnA1fA2f) responsible for the oxidation of PAHs made of up to four rings, monochlorinated biphenyls and dibenzo-*p*-dioxin. PhnA1fA2f shows exceptionally broad substrate specificity towards various pollutants in contrast to other dioxygenases whose activity is limited to two- and three-ring

PAHs. Moreover a conserved catabolic gene cluster could be isolated harbouring 13 genes involved in PAH degradation. Sequence comparison with the initial dioxygenase of *Sphingomonas* sp. strain CHY-1 showed that the amino acids lining the catalytic pocket are conserved. The variation in substrate specificity of the two dioxygenases demonstrates that substitutions outside of the catalytic pocket can have marked effects on the substrate range of dioxygenase enzymes.

Scope of the Thesis

Polycyclic aromatic hydrocarbons are found ubiquitously in nature (natural oil seeps, bushfires, volcanoes…) but anthropogenic activities have led to an increased incidence of these recalcitrant pollutants due to, amongst others, extraction, processing, combustion and disposal of fossil fuel. The constant exposure to these contaminants has led to the evolution of microorganisms that are able to use aromatic hydrocarbons for growth. Up to now many different bacterial strains have been reported to harbour the capability of degrading aromatic compounds. However, many of these substances are recalcitrant or slowly biodegradable, especially when they are halogenated or have increased numbers of aromatic rings. Nonetheless, it is known that indigenous bacteria exposed to these pollutants could often adapt and these chemicals are degraded completely and at considerable rates. Although not many details are known about the molecular events that lead to adaptation of microbial communities, it is generally accepted that recombination, transposition and lateral gene transfer can accelerate the evolution of catabolic pathways by recruiting and combining new catabolic activities. More generally, genomes evolve both by acquiring new sequences and by rearranging existing ones.

Research in our laboratory is directed towards understanding the mechanisms by which different bacterial strains utilize aromatic compounds as carbon and energy source. Projects in the laboratory emphasize on the use of molecular genetic tools in the analysis of gene evolution, the identification of intermediate compounds in catabolic pathways and the functional analysis of the enzymes involved. For instance, different bacterial strains may utilize different biochemical pathways for the degradation of the same aromatic compound. In contrast, different bacterial strains may degrade an aromatic compound by the same catabolic pathway but possesses genes that have diverged widely in their nucleotide sequence. The oxygenases carrying out the first attack on these compounds determine the substrate range and the degradation pattern followed. The aerobic biodegradation of some model PAHs has been observed and investigated to various extends but there is still a persistent lack regarding the ring hydroxylating dioxygenases setting the stage for further oxidation.

Sphingomonas sp. strain LB126 and *Sphingomonas* sp. strain LH128 have been studied in our laboratory for many years, but no information regarding their initial attack on PAHs is available. We therefore focus on these reactions trying to determine what enzyme(s) is/are involved. Characterization of these enzymes will help to understand the sometimes-complicated degradation pathways of polycyclic aromatic hydrocarbon and other heteroaromatics by aerobic bacteria. Chapter 1 illustrates the convergent and divergent points in catabolic pathways involved in the utilization of PAHs by a wide variety of aerobic bacteria. Chapter 2 provides an overview of the enzymes involved in the initial attack on PAHs. This information is necessary to emphasize the importance of the work carried out on *Sphingomonas* sp. strains LB126 and LH128.

Part I – General Introduction

Chapter 1: Genes and pathways involved in the degradation of polycyclic aromatic hydrocarbons by aerobic bacteria

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In preparation for FEMS Microbial Reviews

Summary

Polycyclic aromatic hydrocarbons are a class of organic pollutants that have accumulated in the environment as a result of anthropogenic activities such as the combustion of fossil fuels (338). Their persistence and toxicity is of great concern and urges researchers to investigate the microbial pathways involved in biodegradation. In the last decades several genes and/or gene clusters involved in PAH degradation have been identified in many organisms. The identification of metabolites has allowed establishing degradation patterns that are often well conserved. Some catabolic pathways, especially for low molecular weight polycyclic aromatic hydrocarbons (PAHs) such as naphthalene and phenanthrene have been described in detail. Different enzymes carry out the initial conversion steps but the compounds are transformed to a limited number of central intermediates such as protocatechuic acid and salicylic acid. This generalized scheme of catabolic pathways for aromatic compounds is also known as catalytic funnel. The sequences of these genes, as well as their arrangement give valuable information about evolution of the pathways. This review provides an overview of the variety of aerobic degradation pathways involved in bacterial PAH degradation.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are found ubiquitously in nature (natural oil seeps, bushfires, volcanoes…) but due to anthropogenic activities PAHs have been accumulating in the environment for many decades (246). PAHs are made up of two or more fused benzene rings. Compounds with the substitution of an aromatic ring carbon with nitrogen, oxygen, or sulphur are often included in the category of polycyclic aromatic hydrocarbons and form a heterocyclic subgroup. Based on their toxicity and on their abundance in the environment, the US Environment Protection Agency (USEPA) has identified 16 non-substituted compounds as priority pollutants (153). Structural formulas of selected PAHs are shown in Fig. 1. Many PAHs and their metabolites are toxic and carcinogenic to microorganisms as well as to higher organisms including humans (268, 320, 340).

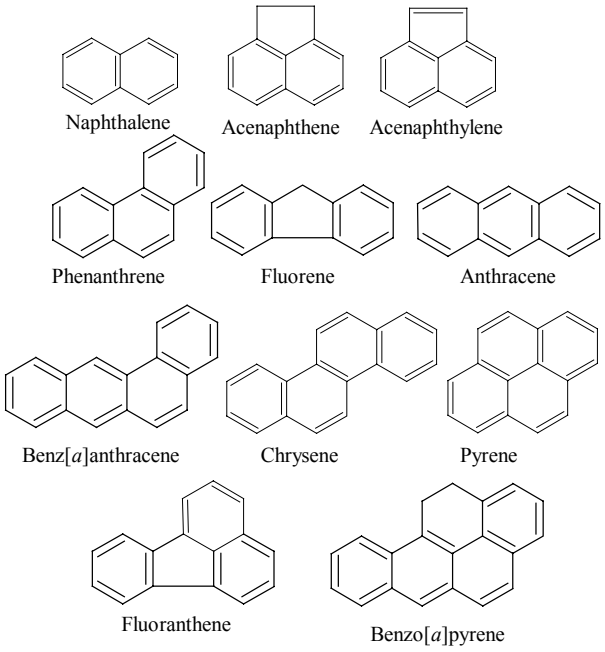

Figure 1. Structures and nomenclatures of selected EPA priority PAHs.

The microbial degradation of PAHs has been investigated over many years. Catabolic pathways have been proposed for various organisms since the 1960's and gene clusters involved in their degradation characterized. In this review we look at the catabolic metabolism of PAHs by a diverse range of PAH-degrading microorganisms by examining the degradation pathways at the genetic level.

Physico-chemical properties of PAHs

The molecular weight of PAHs increases with the number of fused benzene rings. PAHs become less water soluble and more lipophilic, recalcitrant and genotoxic with the increasing number of fused aromatic rings. Bioavailability for instance decreases logarithmically with increasing molecular weight (39, 138). At room temperature, PAHs are solid crystalline substances, whose boiling temperatures rise with increasing molecular weight.

When PAHs are introduced into soil they rapidly escape from the water phase and become associated with sediments and soil particles, to which they bind strongly (39, 138). From the microbial point of view, this dual segregation leads to a highly reduced accessibility – i.e. low bioavailability of the PAHs as potential carbon sources.

Bioremediation

Bioremediation uses living organisms, most often microorganisms, plants or both to degrade toxic chemicals present in natural waters, air and soils. The main principle of this technique is to remove pollutants from the contaminated environment and/or convert the pollutants to a less harmful product using microbial communities. Bioremediation can be adapted for use to treat soil, sediment, sludge, water, or even air. Treatment of contaminated soil or sediment can be either *ex situ* or *in situ*. *Ex situ* procedures involve the removal of the contaminated matrix from a polluted site prior to treatment. *In situ* procedures treat the matrix on the site without moving it to a treatment facility.

Bioremediation, using biological processes to remove hydrocarbons from the environment, is already a successful technology for cleaning up soil and marine sediments (49, 206, 322). Biodegradation leads under optimal conditions to the transformation of pollutants to harmless substances, such a carbon dioxide and water. Hydrocarbon

degrading microorganisms have been found in essentially all environments where they have been diligently searched.

Since the 1960s, research on PAH catabolism has demonstrated the existence of a considerable aerobic degradation potential of various bacteria, fungi, and algae. These organisms possess catabolic abilities that allow them to use these compounds as sources of carbon and energy. At the present time, bioremediation has been shown to be effective in remediating soils contaminated with low molecular weight PAHs (146, 291). However, high-molecular-weight PAHs are generally recalcitrant to microbial attack (20, 90, 111). While the lack of microbial activity towards high-molecular-weight PAHs may be attributed to site-specific environmental factors, such as bioavailability, nutrients, pH etc. (306), an equally important factor is the limited number of microorganisms capable of using high-molecular PAHs as a carbon source (10, 138).

Several basic techniques may be used:

- Stimulation of the activity of indigenous microorganisms by the addition of nutrients, regulation of redox conditions, optimising pH conditions etc. (biostimulation)

- Inoculation of the sites with microorganisms of specific biotransforming abilities (bioaugmentation)

- Application of immobilized enzymes

- Use of plants to remove, contain, or transform pollutants (phytoremediation).

For bioremediation to be more effective for the cleanup of PAH-contaminated soils, a greater understanding of the processes involved, and those that limit the degradation of the high-molecular-weight PAHs, is required. Nevertheless more microorganisms able to degrade PAHs continue to be isolated from different polluted environments (28, 48, 209, 286, 326, 349). These organisms are analyzed for their potential use in bioremediation processes but also to better understand the mechanisms by which these organisms became able to use PAHs as carbon source. Out of all possible approaches, just a handful of patterns have evolved. It is of fundamental scientific interest to

understand their commonalities and differences and to start re-constructing the mechanisms underlying their evolution or recruitment, vertical or lateral.

Microbial degradation pathways

Throughout the course of evolution, microorganisms that had been constantly exposed to a wide range of chemical structures have evolved enzymes required to degrade PAHs. The compounds are degraded by microorganisms either as sole carbon and energy source or by cometabolism. There are a variety of bacteria and fungi, which are capable of cometabolic degradation of polycyclic aromatic hydrocarbons. PAH are cometabolised by microorganisms when these are not able to use them as a sole source of carbon and energy. As cometabolic substrates, they cannot support growth but are modified by enzymes induced by a growth substrate, which is usually a structurally related compound. Not much is known about PAH cometabolic processes and further research is needed to shed light on a mechanism that plays an important role in degrading mixtures of PAHs and in particular high molecular weight PAHs.

Microbial degradation pathways start with the action of multicomponent dioxygenases (205) or through successive monooxygenations of the aromatic ring to produce diols (114) followed by dehydrogenation of the two adjacent hydroxylated carbon atoms (Fig. 2). The next step is ring cleavage of the dihydroxylated ring, either adjacent to the two hydroxylated carbons (*meta*-cleavage) or between them (*ortho*-cleavage) (Fig. 2).

In order to emphasise on the versatility of the catabolic genes involved in the oxidation of the same compound and the different pathways possible, we will discuss each PAH starting from low-molecular weight PAHs to high-molecular weight PAHs. This setup illustrates the presence of conserved catabolic gene clusters involved in PAH degradation by different aerobic bacteria.

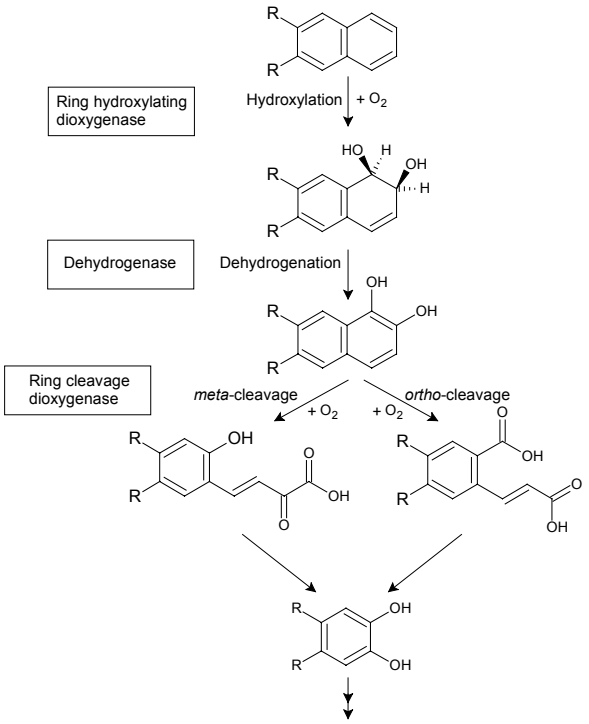

Figure 2. General scheme of the initial steps in a typical aerobic PAH degradation pathway.

Naphthalene

The pathway of naphthalene metabolism by soil pseudomonads was first presented by Davies and Evans in 1964 (50). Since then, genes encoding the degradation pathways of naphthalene have been studied intensively (62, 133, 343) and the pathways completely elucidated.

The oxidation of naphthalene by *Pseudomonas* sp. (23, 57, 59, 62, 133, 135, 191, 309, 337, 343, 344), *Burkholderia* sp. (146, 187, 304) and other bacterial strains (47, 63, 115, 148, 149, 159, 287) (Fig. 3) is initiated by a multienzyme dioxygenase complex and leads to the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene by incorporating dioxygen into the aromatic nucleus (68). The enzyme complex is composed of several subunits: a reductase, a ferredoxin and a hexameric $\alpha_3\beta_3$ oxygenase containing one Rieske [2Fe-2S]

cluster per αβ pair of subunits. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene is dehydrogenated by a dihydrodiol dehydrogenase to 1,2-dihydroxynaphthalene. This intermediate is oxidized by 1,2-dihydroxynaphthalene oxygenase to 2-hydroxychromene-2-carboxylic acid, which is converted by an isomerase to *cis*-o-hydroxybenzalpyruvic acid. This metabolite is then cleaved by a hydratase-aldolase into salicylaldehyde and pyruvate. Salicylaldehyde is further oxidized to salicylic acid by a dehydrogenase. Salicylic acid is oxidized by a monooxygenase to form catechol, which then, depending on the organism, undergoes either *ortho*- or *meta*- cleavage (13). In the *meta*-pathway, catechol is cleaved in the *meta*-position by catechol 2,3-oxygenase and the intermediates formed are degraded to pyruvate and acetaldehyde that are completely mineralised. The *ortho*-pathway described for naphthalene leads to two metabolites of the citric acid cycle: succinate and acetate.

Some organisms such as *Rhodococcus* sp. strain B4, *Polaromonas naphthalenivorans* CJ2 and *Ralstonia* sp. strain U2 (formerly *Pseudomonas*) (105, 134, 351) use a different pathway in which salicylic acid is degraded not via catechol but via gentisate (Fig. 3).

Naphthalene degradation via catechol

The naphthalene-catabolic genes are often encoded on large plasmids. Plasmid NAH7 from *Pseudomonas putida* G7 (62) has been extensively characterized and is often referred to as a model. The upper operon is composed of *nahAa* to *nahD* (10 genes) converting naphthalene to salicylate, the lower operon, *nahG* to *nahY* (12 genes), transforms salicylate to pyruvate and acetaldehyde via *meta*-cleavage (344, 350). A similar genetic organization is found on other plasmids such as pND6-1 from *Pseudomonas* sp. strain ND6 (191), pDTG1 from *P. putida* NCIB9816-4 (57), pKA1 from *P. fluorescens* 5R (270), and on the chromosomes of *P. stutzeri* AN10 (23, 24), *Pseudomonas* sp. strain S47 (AF320981), *P. putida* OUS82 (309) and *P. aeruginosa* PaK1 (D84146) (Fig. 4). Sota et al. (295) pointed out recently that these clusters must have been distributed by lateral gene transfer.

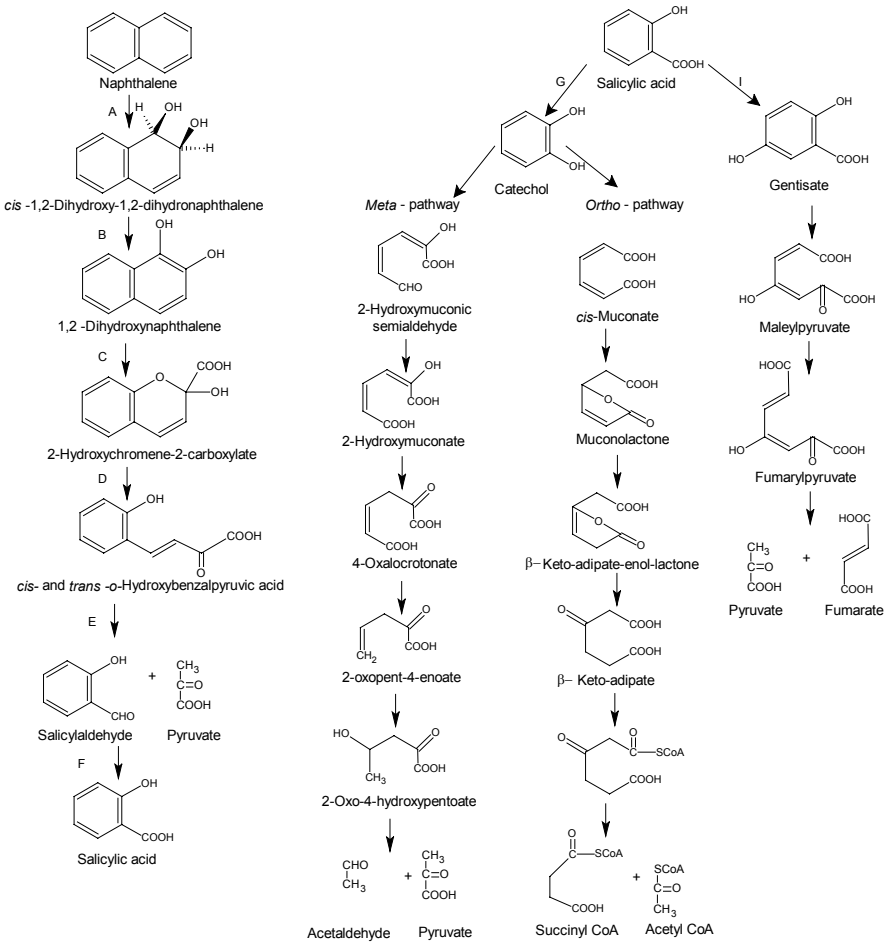

Figure 3. Proposed pathways for naphthalene degradation (101, 344). A, naphthalene dioxygenase; B, *cis*-naphthalene dihydrodiol dehydrogenase; C, 1.2-dihydroxynaphthalene dioxygenase; D, isomerase; E, hydratase and aldolase; F, dehydrogenase; G, salicylate hydroxylase; I, gentisate dioxygenase.

Most of the naphthalene catabolic enzymes encoded on pNAH7 show high amino acid identity to their counterparts encoded on plasmid pDTG1 (57) and by the chromosome in *Pseudomonas stutzeri* AN10 (23-25) except for the absence of *nahQ* (191) in the latter (Fig. 4).

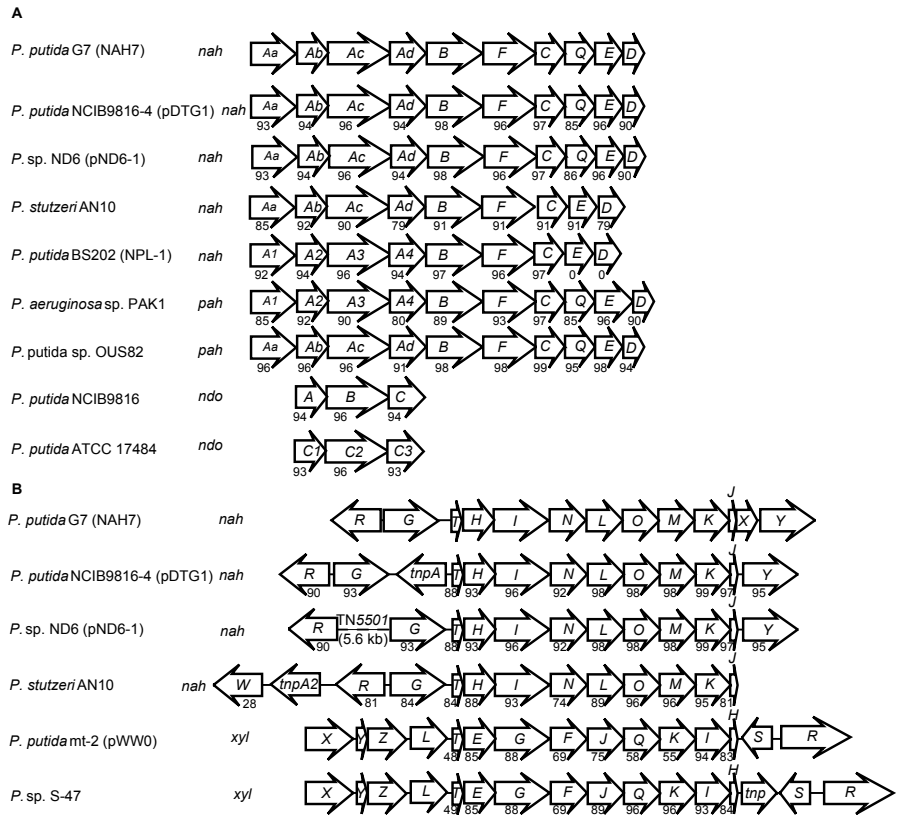

Figure 4. Schematic representation of gene clusters for the upper (A) and lower (B) catabolic pathway of the naphthalene degradation (modified and updated after (95, 295)). The percent amino acid identity of NAH7 proteins to their counterparts is shown. The confusing terms nah, ndo (naphthalene dioxygenase), pah (polycyclic aromatic hydrocarbon degradation), and dox (dibezothiophene oxidation) have been used over the years although the nucleotide sequences are more than 90% identical to each other.

No function has been attributed to *nahQ* to date. NAH7, pDTG1 and pND6 all contain a *nahY* gene, that has not a catabolic function, but is involved in chemotaxis and is cotranscribed with *meta*-cleavage pathway genes (102, 103). The presence of this gene implies that chemotaxis may facilitate biodegradation.

Naphthalene degradation via gentisate

The degradation of naphthalene to gentisate is less well characterized. The genes encoding this pathway have been reported for two species: *Ralstonia* sp. strain U2 and *Polaromonas naphthalenivorans* CJ2 (134). *Ralstonia* sp. strain U2, which was isolated from oil-contaminated soil in Venezuela is able to use naphthalene as the sole carbon source (80). Fuenmayor et al (81) cloned the genes encoding a naphthalene dioxygenase designated *nag* as for naphthalene through gentisate. The genes *nagAaGHAbAcAdBFCQED* (351), were collectively involved in converting naphthalene to gentisate. With the exception of *nagGH*, two genes encoding a salicylate 5-hydroxylase, the *nag* genes share homology with those described for the conversion of naphthalene to salicylate of the NAH7 plasmid. Another set of genes (*nagJIKLMN*) (351) is probably cotranscribed with *nagAaGHAbAcAdBFCQED* as a single large operon. Three of these genes (*nagIKL*) have been shown to encode the enzymes involved in the further catabolism of gentisate to fumarate and pyruvate (351).

In *Polaromonas naphthalenivorans* CJ2 the *nag* genes are grouped into two independently controlled clusters (134). The large gene cluster (*nagRAaGHAbAcAdBFCQEDJI$_1$ORF1tnpA*) is regulated by a LysR-type regulator (*nagR*). The small cluster (*nagR$_2$ORF2I$_2$KL*) is regulated by a MarR-type regulator (*nagR$_2$*) that is not induced by naphthalene, salicylate or gentisate. The catabolic genes of *P. naphthalenivorans* share significant amino acid identity with their counterparts in *Ralstonia* sp. U2. However the genes *nagY*, *nagM* and *nagN*, respectively encoding a chemotaxis protein and two proteins of unknown function were absent in strain CJ2. Moreover, the two clusters in strain CJ2 are separated by an unknown distance. Two copies of a gentisate dioxygenase (*nagI*) are present in strain CJ2 with 77.4 % amino acid sequence identity to each other and 82 % to their counterpart in strain U2 (134).

The naphthalene catabolic genes and their regulation

Understanding the regulatory mechanisms, which govern the expression of catabolic genes, helps elucidating the biodegradation capacities of microorganisms. The molecular partners, the signals triggering pathway expression, and the exact mechanism of activation and repression have become of great interest (315). It soon was

discovered that a large variety of regulatory systems existed for mediating the expression of catabolic pathways (273). Moreover, many related pathways did not carry the same regulatory protein suggesting that regulatory systems and their target operon do not coevolve but seem to become associated independently (37, 53, 273).

LysR transcriptional regulators (LTTRs) comprise the largest family of prokaryotic regulatory proteins identified so far (273, 278, 315). LTTRs are a set of sophisticated proteins with a DNA-binding domain and domains involved in inducer recognition and/or response that inhibit or promote DNA transcription by RNA polymerase (273). Moreover, LTTRs are often oriented in the opposite direction with respect to the transcribed operon (273, 315) and are generally induced/repressed by a pathway intermediate. This type of regulation has been described in detail for naphthalene and serves as a model to understand the regulation of genes involved in the degradation of other PAHs (272-276, 278, 346).

The *nahR* gene product is a master regulator for naphthalene degradation and is responsible for the expression of both, the *nah* and *sal* operons. The system is induced over 20-fold by salicylate and is not catabolite repressed since glucose or succinate did not affect the induction of the two operons (278). NahR is constitutively expressed at low levels. When bound to its inducer salicylate, NahR interacts with DNA binding sequences upstream of the promoter region in the upper and lower *nah* operons (277). Binding of NahR to its target DNA autoregulates its own expression by repressing transcription of *nahR* (276) (Fig. 5).

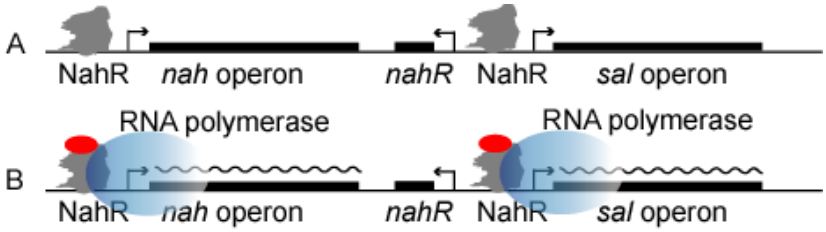

Figure 5. Regulation of the naphthalene catabolic genes. A, without salicylate (red); B, when salicylate interacts with NahR, the latter interacts with the RNA polymerase and triggers transcription of the *nah* and *sal* operon.

The *nag* genes of *Ralstonia* sp. strain U2 are arranged in a single operon regulated by *nagR* showing high similarity to *nahR* (81, 351). NagR regulates the expression of the *nag* genes positively in the presence of salicylate and to a lesser extent in the presence of 2-nitrobenzoate (139).

Besides of naphthalene regulation other families of regulatory proteins have been identified and are summarized in Table 1. A more exhausted list is given in reference (315).

IclR-type regulators have a similar structure as the LysR-type regulators (273), but rather dissimilar amino acid sequences distinguish the two families. IclR-type regulators are generally transcriptional repressors, however those that have been described to regulate catabolic pathways are transcriptional activators. For example, PcaU of *Acinetobacter* sp. strain ADP1 (91) and PcaR of *P. putida* (262) are activators for the *ortho*-cleavage pathways, which they regulate. However, in the absence of inducer they may still act as repressors, as was shown for PcaU (312).

AraC/XylS-type regulators for catabolic operons generally act as transcription activators in the presence of a chemical effector molecule. Most of the genes for XylS-type regulators lie upstream of their target operon, but, in contrast to *lysR*-type genes, they are transcribed in the same direction as the target genes. The best-studied example is XylS. Expression of *xylS* is strongly dependent on another regulatory protein XylR (see further below). XylR stimulates *xylS*-transcription from a σ^{54}-dependent promoter when cells are grown on xylenes (87-89). In the absence of suitable aromatic inducers, the *xylS* gene is expressed at low constitutive levels from a σ^{70}-dependent promoter (87, 200).

MarR-type regulators act as repressor proteins. CbaR is located upstream of and is transcribed in the opposite direction to its target operon.

Table 1. Regulatory proteins involved in expression control of pathways for degradation of aromatic compounds.

Regulatory protein	Bacterial genus	Regulated operon	Pathway substrate	Ref.
LysR family				
CatR	*Pseudomonas*	*catBCA (pheBA)*	Catechol (phenol)	(265)
CatM	*Acinetobacter*	*catBCIJFD, catA, benPK*	Catechol	(224, 261)
PcaQ	*Agrobacterium*	*pcaDCHGB*	Protocatechuate	(236)
BenM	*Acinetobacter*	*benABCDE, benPK*	Benzoate	(45)
NahR	*Pseudomonas*	*nahABCFDE, salGHINL*	Naphthalene, salicylate	(346)
LinR	*Sphingomonas*	*linE-linD*	chlorohydroquinone	(210)
IclR family				
PcaU	*Acinetobacter*	*pcaIJFBDKCHG*	Protocatechuate	(91)
PcaR	*Pseudomonas*	*pcaIJ, pcaBDC, pcaF, pcaK*	Protocatechuate	(262)
AraC/XylS-family				
XylS	*Pseudomonas*	*xylXYZLTE, xylGFKQKIH*	*m*-Toluate	(296)
IpbR	*Pseudomonas*	*ipbABCED*	Isopropylbenzene	(65)
BenR	*Pseudomonas*	*benABC*	Benzoate	(46)
Mar-R family				
CbaR	*Comamonas*	*cbaABC*	Chlorobenzoate	(245)

Acenaphthene

In 1984 Schocken and Gibson were the first to observe the cometabolic conversion of acenaphthene by a biphenyl-degrading

Sphingomonas yanoikuyae B1 (formerly *Beijerinckia* sp. B1) (280). Acenaphthene was hydroxylated to 1-acenaphthenol, subsequently metabolised to 1-acenaphthenone and finally transformed to 1,2-acenaphthenediol and acenaphthenequinone. In 1995 Grifoll et al. (101) observed the co-oxidation of acenaphthene to acenaphthenone, acenaphthoquinone, 1,2-naphthalic anhydride and 1,8-naphthalene dicarboxylic acid by a fluorene utilizing *Burkholderia cepacia* F297.

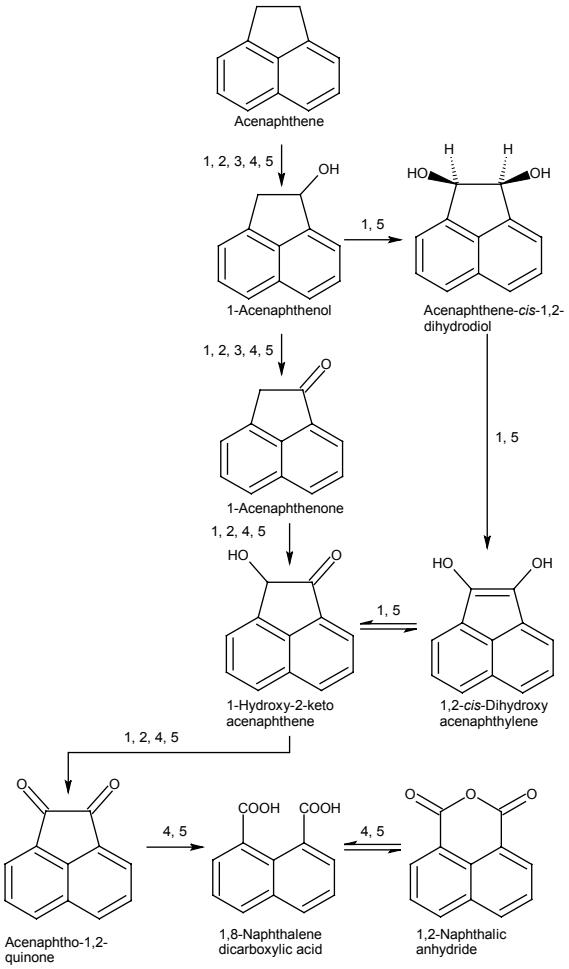

Figure 6. Proposed pathways for acenaphthene degradation. (101, 179, 280, 281) 1, *Sphingomonas yanoikuyae* B1 (280); 2, *Pseudomonas putida* NCIB 9816 (280); 3, *Sphingomonas* sp. strain A4 (179); 4, *Burkholderia cepacia* F297 (101); 5, *Pseudomonas aeruginosa* PAO1 (281).

Pseudomonas aeruginosa PAO1, expressing the *nahA* naphthalene dioxygenase genes from plasmid NAH7, is able to degrade acenaphthene via mono-oxygenation to 1-acenaphthenol with subsequent conversion to 1-acenaphthenone, *cis*- and *trans*-acenaphthene-1,2 diols, 1,2-acenaphthoquinone and 1,8-dicarboxylic

acid (281). The acenaphthene degradation pathways of these bacteria are shown in Fig. 6.

Not much information is available on bacteria that use acenaphthene as their sole source of carbon and energy. Most organisms can only use this substrate cometabolically (76, 101, 115, 286, 325) in the presence of other PAHs. However, *Sphingomonas* sp. strain A4 isolated by Komatsu et al. (179) has no ability to grow on any PAHs except acenaphthene and acenaphthylene. The degradation of acenaphthene by this strain proceeds via acenaphthenol, acenaphthenone and 1,8-naphthalene dicarboxylic acid. In 2004 Pinyakong et al. (239) recovered four complete and two incomplete ORFs on a 5 kb DNA fragment from strain A4. ORFs 3 and 4 encode the two subunits of a ring hydroxylating dioxygenase, ArhA1 and ArhA2 and are most closely related to homologous proteins from *Burkholderia* sp. strain RP007 (56% and 45% identity) (187).

Kouzouma et al. (180) isolated a 16.4 kb DNA fragment from *Sphingomonas* sp. strain A4 harbouring the ferredoxin (*arhA3*) and ferredoxin reductase (*arhA4*) genes. In addition, sixteen other ORFs and a partial ORF were identified, most of which are also involved in acenaphthene degradation. A LysR-type transcriptional regulator, *arhR*, inducible by acenaphthene or its metabolite, is responsible for cotranscribing an operon of 13.5 kb (180). No information is available regarding the oxidation products of 1,8-naphthalene dicarboxylic acid by strain A4.

Acenaphthylene

Similarly to acenaphthene, little is known about the aerobic degradation of acenaphthylene. Sometimes organisms capable of using acenaphthene can also use acenaphthylene either cometabolically (101) or as sole source of carbon and energy (179, 239). Schocken et al. (280) described the cometabolic degradation of acenaphthylene to 1,2-acenaphthenediol by *Sphingomonas yanoikuyae* B1. Grifoll et al. (101) identified from cometabolic acenaphthylene degradation by *Burkholderia cepacia* F297 the same metabolites as from acenaphthene: 1-acenaphthenone, acenaphthoquinone, naphthalic anhydride and 1,8-naphthalenedicarboxylic acid. *Pseudomonas aeruginosa* PAO1 converts acenaphthylene into *cis*-

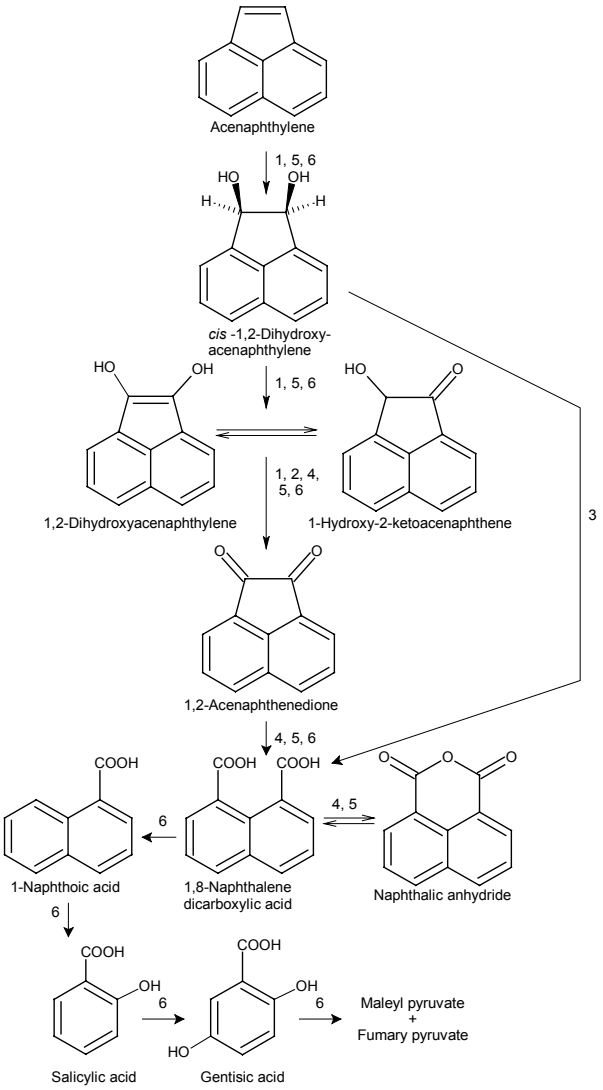

Figure 7. Proposed pathways for acenaphthylene degradation and bacteria involved. 1, *Sphingomonas yanoikuyae* B1 (280); 2, *Pseudomonas putida* NCIB 9816 (280); 3, *Sphingomonas* sp. strain A4 (179); 4, *Burkholderia cepacia* F297 (101); 5, *Pseudomonas aeruginosa* PAO1 (281); 6, *Rhizobium* sp. strain CU-A1 (243)

acenaphthene-1,2-diol, 1,2-acenaphthoquinone, acenaphthylene-1,2-diol and 1,8-naphthalenedicarboxylic (281). Acenaphthylene degradation by *Rhizobium* sp. strain CU-A1 proceeds via gentisate (243). A putative pathway is shown in Fig. 7. *Sphingomonas* sp. strain A4 is capable of utilizing acenaphthene and acenaphthylene as sole carbon and energy sources. Several genes involved in acenaphthene degradation have been described (180).

In 2006 a degradation pathway was described for *Rhizobium* sp. strain CU-A1, showing that naphthalene-1,8-dicarboxylic acid was transformed to 1-naphthoic acid which was in turn converted into salicylic acid before mineralisation to gentisic acid (243). This is the first report on the complete acenaphthylene degradation by a bacterial strain. However, no catabolic genes have been identified from strain CU-A1 yet.

Fluorene

Fluorene can be oxidized by diverse bacteria such as *Pseudomonas* sp. strain F101 (formerly *Arthrobacter* sp. strain F101) (36, 98, 100), *Terrabacter* sp. strain DBF63 (formerly *Staphylococcus auriculans* DBF63 (108, 109, 150, 212), *Brevibacterium* sp. strain DPO1361 (314), using at least three distinct degradation pathways. Two of these pathways are initiated by a dioxygenation at the 1,2- (36, 98) or 3,4-position (36, 99, 212) and lead to the formation of central metabolites such as salicylate. The corresponding *cis*-dihydrodiols undergo dehydrogenation, then *meta*-cleavage. The third route (282, 314, 331) is initiated by monooxygenation at the C-9 position to give 9-hydroxyfluorene which is then dehydrogenated to 9-fluorenone. This route is only productive if a subsequent angular dioxygenation forms 1,1a-dihydroxy-1-hydro-9-fluorenone, leading to phthalate, which is degraded in turn via protocathechuate (100, 212, 314). A minor non-productive pathway from 9-fluorenone leads to the accumulation of 4-hydroxy-9-fluorenone. A comprehensive depiction of these pathways is shown in Fig. 8.

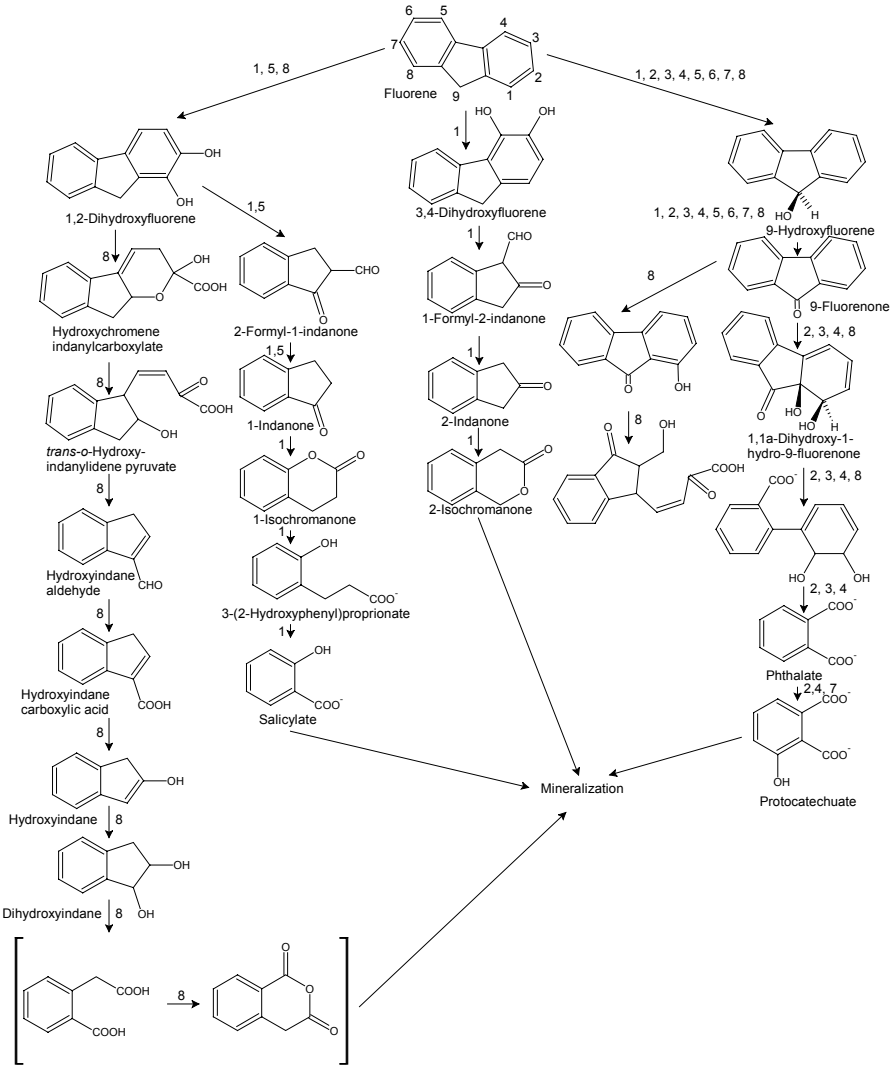

Figure 8. Proposed pathways for fluorene degradation and bacteria involved. 1, *Pseudomonas* sp. strain F101 (36, 98, 100); 2, *Terrabacter* sp. strain DBF63 (212); 3, *Brevibacterium* sp. strain DPO1361 (314); 4, *Pseudomonas* sp. strain F274 (100); 5, *Burkholderia cepacia* F297 (101); 6, *Mycobacterium* sp. strain BB1 (21); 7, *Sphingomonas* sp. strain LB126 (331) 8, *Sphingomonas paucimobilis* EPA505 (300).

Fluorene degradation by Gram-negative bacteria

Little information is available on bacteria using fluorene as a sole source of carbon and energy (36, 98, 212). In contrast, cometabolic degradation of fluorene has been more thoroughly investigated (21, 26, 48, 300, 333) and metabolites identified. However, the specific enzymes or the genes encoding the enzymes involved in the initial attack on fluorene remain unknown.

The fluorene catabolic pathway starting with an oxidation of C9 can be divided into two parts, the upper pathway converting fluorene to protocatechuic acid and the lower pathway, transforming protocatechuic acid into tricarboxylic acid cycle intermediates. The lower fluorene catabolic pathway is well conserved. Genes involved in these steps have been identified in *Sphingomonas* sp. strain LB126 (331), *Sphingomonas* sp. strain SYK6 (202-204, 226) (formerly *Pseudomonas paucimobilis* SYK6), *Comamonas testosteroni* BR6020 (244) and on plasmid pCAR3 of *Sphingomonas* sp. strain KA1 (289).

Pseudomonas sp. strain F101 is able to degrade fluorene as a sole source of carbon and energy by lateral oxygenation at the 1,2- (36, 98) or 3,4-position (98). No information regarding catabolic genes involved in these steps is available.

Fluorene degradation by Gram-positive bacteria

Terrabacter sp. strain DBF63 was isolated for its ability to grow on dibenzofuran, fluorene and dibenzo-*p*-dioxin (212) and is today the best studied fluorene degrader. The genes responsible for both dibenzofuran degradation (150) and fluorene oxidation (108) are encoded on a linear plasmid in strain DBF63 (107). The initial attack on fluorene by strain DBF63 is catalysed by DbfA1A2 to yield 9-hydroxyfluorene. The α-subunit of the dioxygenase (DbfA1) showed low homology to other known proteins and did not cluster with most known dioxygenase α-subunits from Gram-positive bacteria (108). The closest homologues to DbfA1 are NidA from *Rhodococcus* sp. strain I24 (39% protein identity) (313) and PhdA from *Nocardioides* sp. strain KP7 (37% protein identity). Recently many DbfA1 homologues have been identified in various dibenzofuran degrading bacteria (231) and on catabolic plasmid pCAR3 from carbazole degrading *Sphingomonas* sp. strain KA1 (289) (Fig. 9).

Interestingly strain KA1 is not able to grow on fluorene or dibenzofuran even though genes are present that could provide the ability to degrade these compounds (289) (Fig. 9). Moreover, putative genes involved in degradation of anthranilate, catechol (*cat*), 2-hydroxypenta-2,4-dienoate (*carDFE*), dibenzofuran/fluorene (*dbf/fln*), protocatechuate (*lig*), and phthalate (*oph*) were identified (289). Unfortunately no information is available regarding expression analysis and the function of these genes remains unknown.

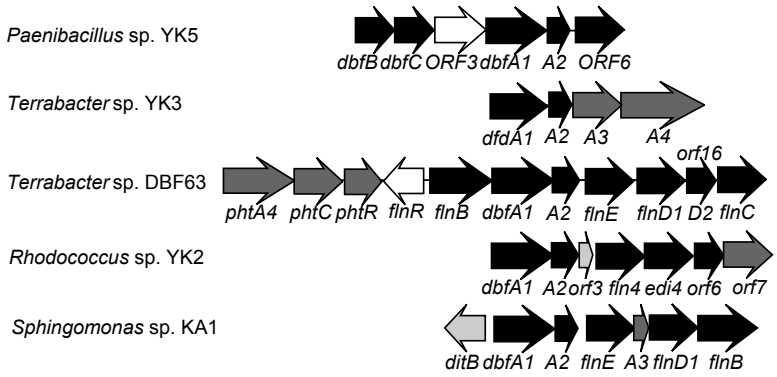

Figure 9. Schematic representation of genes involved in the upper fluorene degradation pathway by *Terrabacter* sp. strain DBF63 aligned with genes from dibenzofuran degrading *Paenibacillus* sp. strain YK5, *Terrabacter* sp. strain YK3, Rhodococcus sp. strain YK2 and carbazole degrading *Sphingomonas* sp. strain KA1.

The initial attack on fluorene by strain DBF63 is catalysed by DbfA1A2 to yield 9-hydroxyfluorene, which is subsequently transformed to 9-fluorenone by a dehydrogenase (FlnB). The next step implies an extradiol ring-cleavage dioxygenase (FlnD1D2). Finally, a hydrolase (FlnE) yields phthalate via *meta*-cleavage which in turn is further degraded to tricarboxylic acid cycle intermediates via protocatechuate (107) (Fig. 10). The *dbf* genes are clustered with genes involved in phthalate- (*pht*) and protocatechuate catabolism (*pca*) (107-109). These results give a new, interesting point of view concerning the upper fluorene degradation pathway genes and open new perspectives for the identification of initial dioxygenases in environmental samples (122, 123, 231).

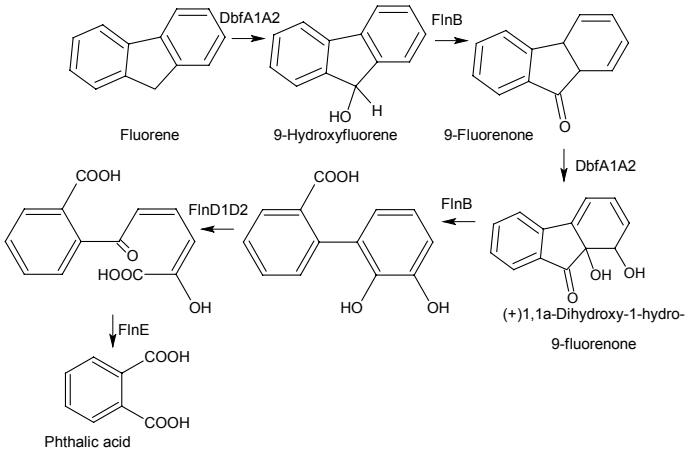

Figure 10. Proposed pathway of fluorene catabolism by Terrabacter sp. strain DBF63 (108). DbfA, 9-fluorenone 1,1a-dioxygenase (fluorene 9-monooxygenase, Dibenzofuran 4,4a-dioxygenase); FlnB, dihydroxyfluorene dehydrogenase; FlnD, 2'-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase; FlnE, 2-hydroxy-6-oxo-6-(2'-carboxyphenyl)-hexa-2,4-dienoate hydrolase.

Concluding remarks on PAHs with two fused aromatic rings

Under aerobic conditions, microorganisms can easily degrade PAHs with two fused aromatic rings such as naphthalene, acenaphthene, acenaphthylene, and fluorene as the sole source of carbon or in cometabolism, while most of the higher PAHs are more recalcitrant. Many organisms have been isolated that are capable of using these PAHs as sole source of carbon and energy. The bacterial metabolism of naphthalene has been fully elucidated both on a metabolomic and genetic level. It starts with the introduction of molecular oxygen by a dioxygenase, which results after further metabolism, in the formation of salicylate. This metabolite is transformed in either catechol or gentisate. Much less is known about the microbial degradation of acenaphthene and acenaphthylene. To date only *Sphingomonas* sp. strain A4 is able to use these substrates as sole source of carbon and energy, for which genetic information is available (239). *Rhizobium* sp. strain CU-A1 is able to degrade 600 mg/l of acenaphthylene completely within three days but the genes encoding the enzymes in these steps remain unknown (243). The bacterial degradation of fluorene has been completely elucidated but the enzymology involved

is poorly documented. Today, the Gram-positive *Terrabacter* sp. strain DBF63 is the best-known fluorene degrader and catabolic genes have been identified (108). No information regarding fluorene catabolic genes from Gram-negative bacteria using fluorene as a sole source of carbon is available.

Phenanthrene

Phenanthrene is initially oxidized at the 1,2 (241) or more commonly, at the 3,4-position to form *cis*-dihydrodiols. The latter is further oxidized by *Pseudomonas*, *Sphingomonas* and *Nocardia* species into 3,4-dihydroxy-phenanthrene, which by *meta*-cleavage is converted to 1-hydroxy-2-naphthoic acid. There are different ways to degrade this metabolite. In one route, utilized by various Gram-negative bacteria such as *Pseudomonas*, *Sphingomonas* and *Burkholderia* sp. RP007, 1-hydroxy-2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene, which is further degraded via the naphthalene pathway to salicylic acid and catechol (71, 136). In contrast *Mycobacterium*, *Nocardioides* and *Micrococcus* use the phthalate pathway where 1-hydroxy-2-naphthoic acid is metabolised through *o*-phthalic acid to protocatechuic acid (21, 106, 175, 176, 298, 323). Interestingly this pathway is also present in Gram-negative *Alcaligenes faecalis* AFK2 (175). The proposed pathways of phenanthrene degradation are shown in Fig. 11.

The phenanthrene catabolic pathway produces 1-hydroxy-2-naphthoic acid as the central metabolite and enters the naphthalene degradation pathway which provides direct biochemical evidence that the naphthalene degradative enzyme system is involved in the degradation of higher-molecular-weight polycyclic aromatic hydrocarbons other than naphthalene. Moreover it has been shown that the enzymes involved in the degradation of naphthalene to salicylate also play an important role in the bioconversion of phenanthrene and anthracene to 1-hydroxy-2-naphthoate and 2-hydroxy-3-naphthoate (178, 207, 270, 341).

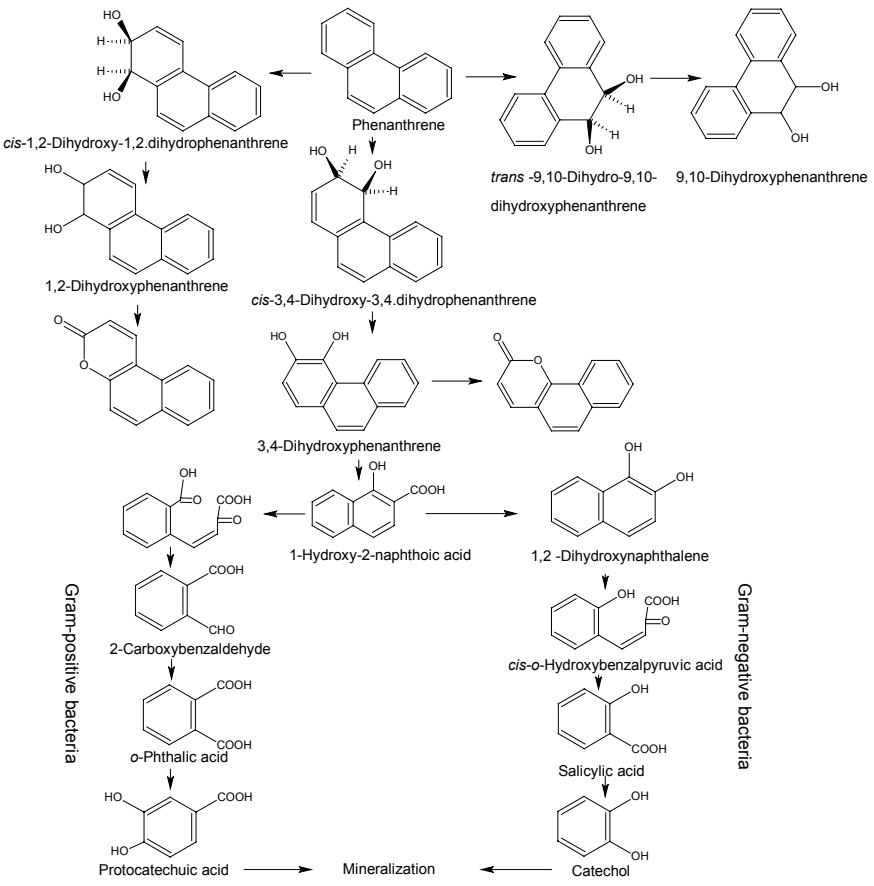

Figure 11. Proposed pathways for phenanthrene degradation (11, 12, 71, 136, 174, 176, 298). Gram-positive bacteria degrade phenanthrene through *o*-phthalic acid whereas Gram-negative bacteria degrade phenanthrene through salicylic acid.

Phenanthrene degradation via salicylate

Sphingomonas species are well known for their ability to degrade a large spectrum of pollutants. The genetic organization of their catabolic genes is unique and the PAH catabolic genes are interspersed with genes involved in the catabolism of monoaromatic compounds (Fig. 12). Sometimes the genes are located on large

plasmids or on the chromosome, but the organization differs compared to other genera like *Pseudomonas*. The genes required for the degradation of one compound are not clustered in the same operon. Many *Sphingomonas* related species like *Sphingobium* sp. strain P2 (241), *Novosphingobium aromaticivorans* F199 (263, 264), *Sphingobium yanoikuyae* B1 (42, 159, 161), *Sphingomonas* sp. strain DJ77 (164, 165, 169, 288), *Sphingomonas* sp. strain CHY-1 (56) and *Sphingobium agrestis* HV3 (347), although belonging to different species, share a conserved gene cluster involved in phenanthrene degradation via salicylic acid (Fig. 12). There are as many as 6 different pairs of dioxygenases ($BphA1_{a-f}A2_{a-f}$) that could be involved in metabolising a variety of aromatic compounds. Since many *Sphingomonas* species present this particular genetic organization, it is possible that these bacteria started as phenanthrene degraders and evolved to degrade other PAHs. In the process of evolving the capability to degrade a wide variety of aromatic hydrocarbons this organism has recruited, modified, and reorganized the appropriate genes and operons needed to construct the required catabolic pathways. The presence of six dioxygenases could result from gene duplication and they became specific to different substrates. *Sphingobium yanoikuyae* strain B1 has been well characterized regarding gene functions (159). Strain B1 is able to grow on monocyclic compounds such as toluene, *m*-xylene, *p*-xylene, biphenyl, naphthalene, phenanthrene and anthracene. This catabolic ability seems to be a common and unique trait of the aromatic hydrocarbon degrading *Sphingomonas* genus. The first operon is composed of five genes (*bphC*: 2,3-dihydroxybyphenyl 1,2-dioxygenase, *bphA3*: ferredoxine, *bphA1cA2c*: α and β subunit of a salicylate 1-hydroxylase, *nahD*: 2-hydroxychromene-2-carboxylate isomerase). This operon thus contains some but not all of the genes needed for an 'upper' pathway for the conversion of polycyclic aromatic hydrocarbons to aromatic acids (159). The recruitment of *nahD* into this operon represents a significant event in the evolution of this catabolic pathway. The second operon resulted from recruitment, modification, and reorganization of genes and operons for aromatic hydrocarbon degradation. This operon is highly similar of the 'lower' TOL plasmid operon (*xylXYZLTEGFJQKIH*) for the catabolism of aromatic acids to pyruvate and acetaldehyde that is found in *P. putida*. However, there are significant differences between these two operons. The 'lower' or *meta*-cleavage TOL plasmid *xyl* operon is organized

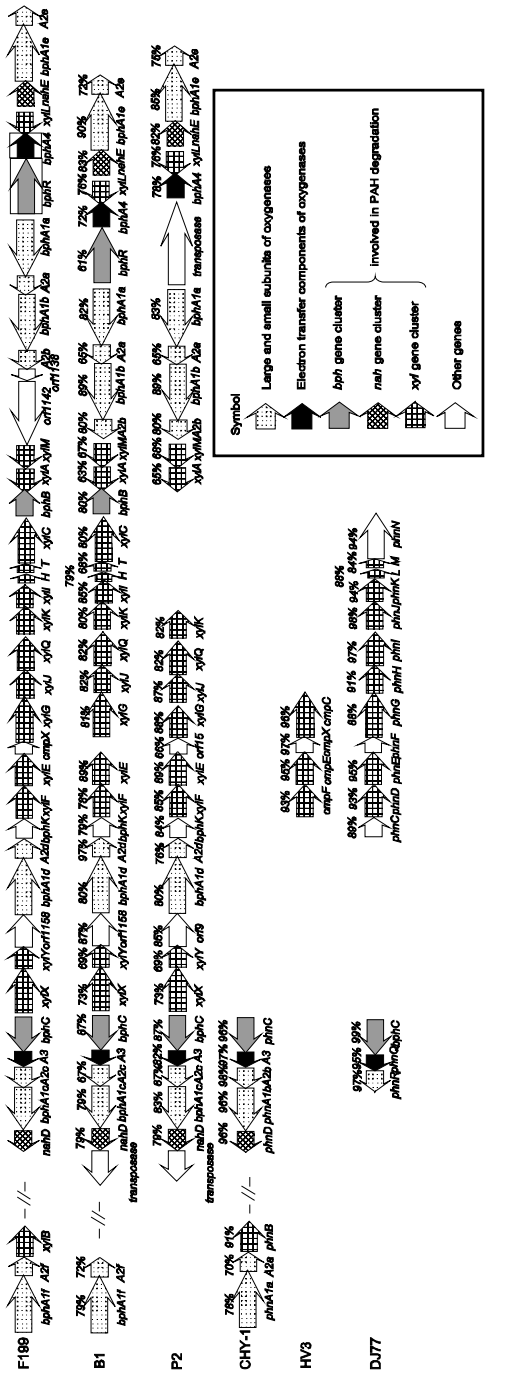

Figure 12. Comparison of the catabolic operons of phenanthrene degrading *Sphingomonas* strains. Many genes of the cluster have been investigated and functional data are available for: *bphC*, a 2,3-dihydroxybiphenyl 1,2-dioxygenase (160); *xylE*, a catechol 2,3-dioxygenase (160); *bphA3*, a ferredoxin component of a dioxygenase (42, 159); *bphA4*, a ferredoxin reductase component (42); *nahD*, 2-hydroxychromene-2-carboxylate isomerase (159); *bphA1c* terminal oxygenase alpha subunit (42, 159); *xylX*, a toluate dioxygenase (159); *bphA1d*, terminal dioxygenase alpha subunit (159).

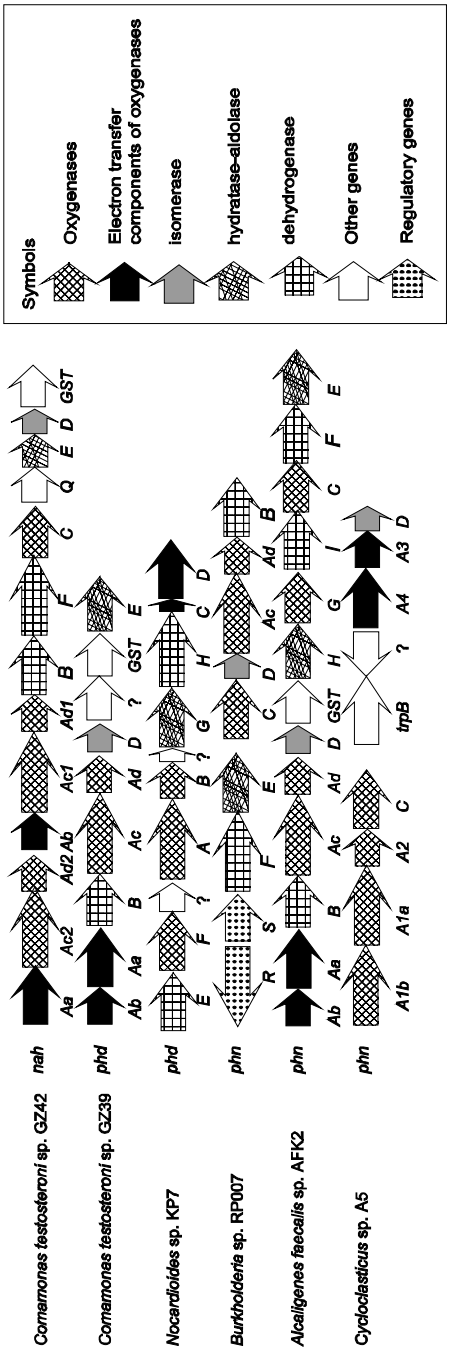

Figure 13. Genetic organization of phenanthrene-degrading genes different from *Sphingomonas* species: *Comamonas testosteroni* GZ42 and GZ39 (95), *Nocardioides* sp. KP7 (267) (AB031319), *Burkholderia* sp. RP007 (187) (AF061751), *Alcaligenes faecalis* AFK2 (AB024945), and *Cycloclasticus* sp. A5 (149) (AB102786).

with the genes in the order *xylXYZLTEGFJQKIH*. *S. yanoikuyae* B1 does not contain the *xylZ* or *xylL* genes in this operon (159). Moreover, another rearrangement occurred through the addition of two genes: *xylC* and *bphB*. The first of these two genes, *xylC*, encodes benzaldehyde dehydrogenase, involved in the conversion of benzaldehyde, *m*-, and *p*-tolualdehyde to the corresponding acids. This gene is normally present in a TOL plasmid 'upper' operon containing the genes for conversion of toluene, *m*-, and *p*-xylene to the corresponding acids (8). The second of these genes, *bphB*, encodes *cis*-2,3-biphenyl dihydrodiol dehydrogenase (Fig. 12).

Many other phenanthrene degraders have been isolated and analyzed. Here we present some examples of bacteria that possess sets of genes that differ from the so-called *Pseudomonas* and *Sphingomonas* gene organizations (Fig. 13).

The phenanthrene degrader *Burkholderia* sp. strain RP007 contains an 11.5 kb *phn* gene cluster (186, 187). This locus encodes the alpha and beta subunits of initial dioxygenase but lacks genes coding for ferredoxin and reductase components (187) indicating that those genes must be located elsewhere. The characterized genes are arranged as follows: *phnRSFECDAcAdB*. *phnR* and *phnS* may play a regulatory role. The sequence similarity of *phnS* suggests that *PhnS* is a LysR type transcriptional activator, while *phnR* is a member of the σ^{54}-dependent family of positive transcriptional regulators (187). Phylogenetic analysis of *phnAc*, *phnAd*, *phnD*, *phnE*, and *phnF* gene products revealed that they fall into the same group as the corresponding *nah*-like genes (24, 292). *phnC* shows homology to extradiol dioxygenases and *phnB* to dihydrodiol dehydrogenase genes (Fig. 13).

Cycloclasticus sp. strain A5 is able to grow on PAHs including unsubstituted and substituted naphthalenes, dibenzothiophene, phenanthrene, and fluorene (149). A DNA fragment of 10.5-kb encoding 10 open reading frames was isolated and sequenced. Seven ORFs showed homology to previously characterized genes for PAH degradation. However the sequence and order of these genes was significantly different from those found in other PAH-degraders. The *phnA1A2A3A4* genes, encoding a ring hydroxylating dioxygenase composed of the α and β subunits of an iron-sulfur protein, a

ferredoxin, and a ferredoxin reductase, respectively was identified (Fig. 13).

Comamonas testosteroni strains GZ39 and GZ42 are able to grow on phenanthrene as sole source of carbon and energy (96). It is interesting to note that the enzymes from strain GZ39 do not show significant amino acid homology to classical *nah*-like enzymes (Fig. 4). In strain GZ39 (95) genes were found coding for ferredoxin (*phdAb*), ferredoxin reductase (*phdAa*), *cis*-dihydrodiol dehydrogenase (*phdB*), the α- (*phdAc*) and β- (*phdAd*) subunit of an iron sulfur protein, isomerase (*phdD*), an unknown ORF, glutathione S-transferase and hydratase-aldolase (*phdE*). Strain GZ42, in contrast, contained all the genes of the classical *nah* operon involved in naphthalene degradation described earlier. The genetic analysis showed that the genes *phdAaAc2Ad2AbAc1Ad1BFCQED* from strain GZ42 were similar to those found in strain *P. putida* NCIB9816. Two exceptions could be observed. The genes *phdAc2* and *phdAd2* were similar to iron sulfur dioxygenases but thought to be non-functional in GZ42 (95) (Fig. 13).

Phenanthrene degradation via o-*phthalic acid*

Alcaligenes faecalis strain AFK2 (175) degrades phenanthrene via *o*-phthalate and protocatechuate. The gene order differs from that found in other bacteria: *phnAbAaBAcAdDHGICFE*. These genes code for ferredoxin (*phnAb*), ferredoxin reductase (*phnAa*), *cis*-dihydrodiol dehydrogenase (*phnB*), the α and β subunit of naphthalene dioxygenases (*phnAc* and *phnAd* respectively), putative 2-hydroxychromene-2-carboxylate isomerase (*phnD*), *trans*-2'-carboxybenzalpyruvate hydratase-aldolase (*phnH*), 1-hydroxy-2-naphthoate dioxygenase (*phnG*), 2-carboxybenzaldehyde dehydrogenase (*phnI*), 3,4-dihydroxyphenanthrene dioxygenase (*phnC*), 1-hydroxy-2-naphthoaldehyde dehydrogenase (*phnF*), *trans*-o-hydroxybenzylidenepyruvate hydratase-aldolase (*phnE*) (177). A glutathione-S-transferase (*gst*) is located between *phnD* and *phnH* (177) (Fig. 13).

Nocardioides sp. KP7 was isolated for its ability to grow on phenanthrene as sole source of carbon (128). It was shown that this strain degrades phenanthrene via the phthalate pathway using the *phdIJK* genes (1, 125-127). These three genes play a role in the conversion of 1-hydroxy-2-naphthoate to phthalate and encode a 1-

hydroxy-2-naphthoate dioxygenases (*phdI*), a trans-2'-carboxybenzalpyruvate hydratase aldolase (*phdJ*) and a 2-carboxybenzaldehyde dehydrogenase (*phdK*) respectively. Saito et al (266, 267) discovered the genes involved in the breakdown of phenanthrene into 1-hydroxy-2-naphthoate. The gene cluster contains 8 ORFs (*phdEFABGHCD*) and is located 6.1 kb downstream from the *phdIJK* cluster. *phdE* encodes a dihydrodiol dehydrogenase, *phdF* an extradiol dioxygenase, *phdAB* the α and β subunit of phenanthrene dioxygenase, *phdG* an aldolase-hydratase, *phdH* an aldehyde dehydrogenase, *phdC* a ferredoxin and *phdD* a ferredoxin reductase (Fig. 13).

Mycobacterium sp. strain PYR-1 has been isolated for its ability to degrade high-molecular-weight polycyclic hydrocarbons (116). To clone the dioxygenase genes involved in PAH degradation, two-dimensional (2D) gel electrophoresis of PAH-induced proteins from cultures of *Mycobacterium* sp. strain PYR-1 was used to detect proteins that increased after phenanthrene and pyrene exposure. Comparison of proteins from induced and uninduced cultures on 2D gels indicated that at least six major proteins were expressed (105, 81, 52, 50, 43, and 13 kDa). The N-terminal sequence of the 50-kDa protein was similar to those of other dioxygenases (157). A 5288 bp DNA fragment encoding three genes (*nidDAB*) could be isolated. *nidAB* genes encode the α and β subunit of a dioxygenase and *nidD* codes for an aldehyde dehydrogenase (157). NidAB was found responsible for the initial attack on pyrene and phenanthrene.

Krivobok et al. isolated two gene clusters from *Mycobacterium* sp. 6PY1 using the same technique as described for strain PYR-1. (181). The first cluster contained the genes *pdoA1B1,* the genes *pdoA2B2* were encoded on a separate cluster. When overexpressed in *E. coli*, PdoA1B1 converted pyrene to pyrene-4,5-dihydrodiol more efficiently than PdoA2B2 (181). On the other hand PdoA2B2 shows better activity against phenanthrene. Both enzymes participate in the oxidation of phenanthrene and pyrene (181).

These data suggest that the enzymes responsible for the oxidative attack on pyrene are also responsible for the attack on phenanthrene which is a pathway intermediate of pyrene. Even though the same enzymes can oxidize pyrene and phenanthrene, one enzyme prefers

phenanthrene to pyrene or vice versa. Since bacteria are able to degrade a large variety of PAHs the existence of such a pool of enzymes is not surprising, but shows that gene duplication and later on adaptation to a specific PAH is the driving force in PAH catabolism.

Anthracene

Anthracene is metabolised by soil pseudomonads and sphingomonads similarly to phenanthrene. One of the first known productive pathways for the bacterial degradation of anthracene was described in 1965 for soil pseudomonads (71). Anthracene is oxidized in the 1,2 position to form 1,2-dihydroxyanthracene, followed by *meta*-cleavage. In cultures of *Burkholderia cepacia* F279 (101), *Alcaligenes denitrificans* WW1 (333), and *Pseudomonas fluorescens* 5R (207) these central metabolites were also detected. *Meta*-cleavage of 1,2-dihydroxyanthracene yields 4-(2-hydroxynaphth-3-yl)- 2-oxobut-3-enoic acid (71). This compound is converted abiotically to 6,7-benzocoumarine or can be further metabolised to 2-hydroxy-3-naphthoic acid. Gram-positive bacteria use a different pathway. Van Herwijnen et al. (323) proposed a pathway for *Mycobacterium* sp. strain LB501T through *o*-phthalic acid and protocatechuic acid. Dean-Ross et al. reported a novel metabolite only identified in *Rhodococcus* strains by *ortho*-cleavage of 1,2-dihydroxyanthracene that produces 3-(2- carboxyvinyl)naphthalene-2-carboxylic acid (55). The degradation pathway is depicted in Fig. 14.

Zylstra et al. (355) reported the cloning of genes involved in naphthalene, phenanthrene and anthracene by *Pseudomonas* sp. strain XPW-2. The genes responsible were probably encoded on a plasmid similar in size to NAH7 (343) and pDTG-1 (284).

In 2006 Hirano et al. (118, 119) characterized a dioxygenase from *Xanthobacter polyaromaticivorans* 127W, capable of degrading compounds such as dibenzothiophene, naphthalene, anthracene and phenanthrene. A terminal oxygenase, DbdCa, and its flanking region was isolated, analyzed and found to be clearly involved in anthracene degradation. Based on amino acid sequence comparisons, the terminal oxygenase does not cluster with other high-molecular-weight PAH dioxygenases but shows high homologies to biphenyl dioxygenases from *Pseudomonas* strains. Gene disruption of *dbdCa* by insertion of a

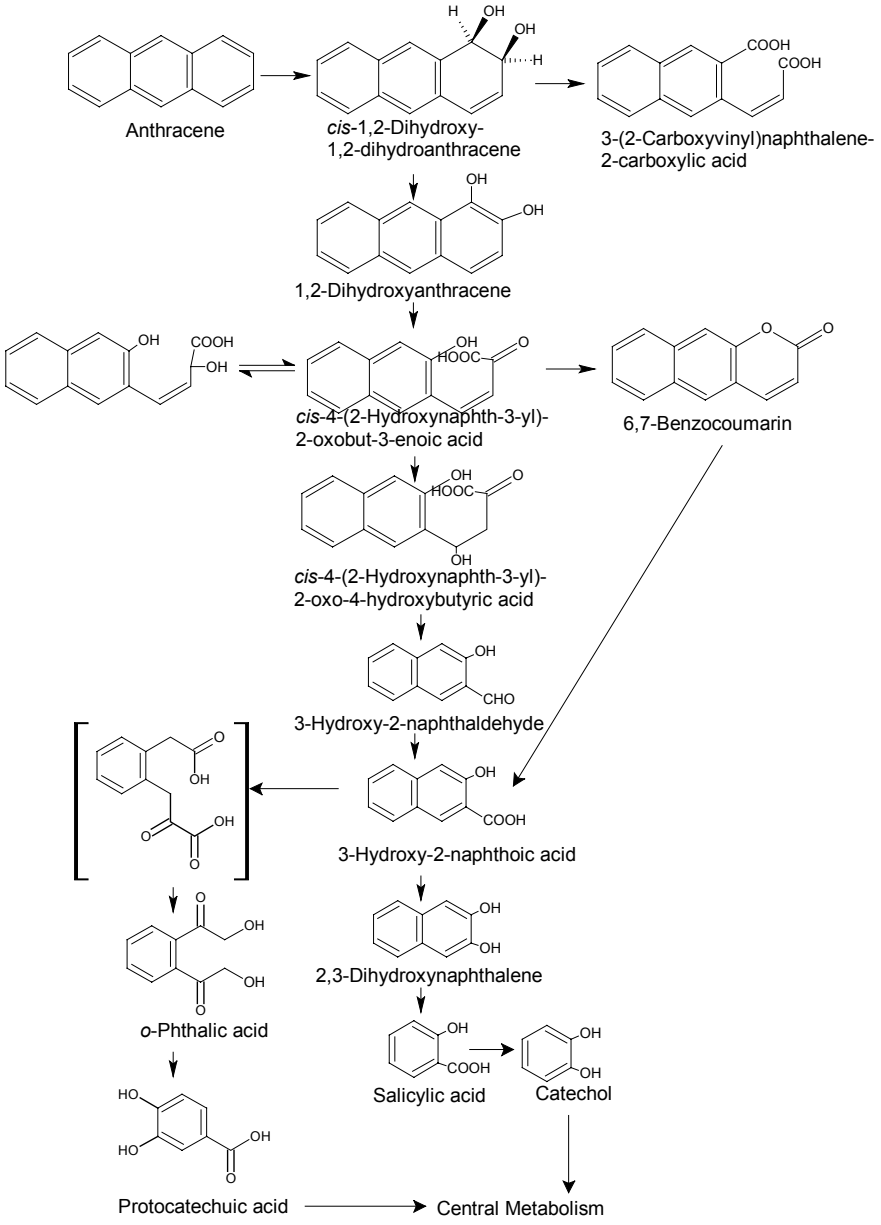

Figure 14. Proposed pathway for anthracene degradation (55, 71, 101, 207, 333).

kanamycin resistance cassette abolished the degradation activity towards biphenyl, dibenzothiophene and anthracene.

Interestingly phenanthrene degradation was hampered but not abolished and low activity could be observed (118). Moreover a putative gentisate 1,2- dioxygenase (DbdB) was identified, suggesting that these PAHs are further degraded via the gentisate pathway.

Fluoranthene

The best known fluoranthene pathway has been partially described for *Mycobacterium* species (154, 155, 333). A more detailed list has been published by Rehman et al. (250).

The bacterial degradation pathways currently proposed indicate that the oxidation of fluoranthene is initiated by dioxygenation at the C-1,2, C-2,3, and C-7,8 positions (155, 196, 250, 283). The C-1,2 and C-2,3 dioxygenation routes degrade fluoranthene via fluorene-type metabolites, whereas the C-7,8 routes oxidize fluoranthene via acenaphthylene-type metabolites. In *Mycobacterium vanbaalenii* PYR-1, dioxygenation was reported to occur at the C-1,2 or C-7,8 position of fluoranthene, producing 9-fluorenone and acenaphthylene-1(2*H*)-one, respectively. Dioxygenation at C-2,3 also occurs in strain PYR-1 since recent molecular cloning and expression studies of NidA3B3, which codes for an initial ring-hydroxylating oxygenase, showed the transformation of fluoranthene to *cis*-2,3-fluoranthenedihydrodiol (167). Nonspecific monooxygenation of fluoranthene with subsequent O methylation of dihydroxyfluoranthene also occurs as a detoxification reaction (184). In *Mycobacterium* sp. strain AP1, the C-7,8 dioxygenation pathway was proposed, followed by further degradation to produce naphthalene-1,8-dicarboxylic acid with subsequent metabolism to form 1,2,3-benzenetricarboxylic acid (154, 155). In strains KR20 (250) and AP1 (196), the 2,3-dihydrodiol is degraded via 9-fluorenone-1-carboxylic acid, which is then decarboxylated to form phthalate. The latter is believed to be funnelled into the central metabolism via the β-ketoadipate pathway. Recently *Mycobacterium* sp. strain JS14 was reported to carry out dioxygenation of fluoranthene at the C-8,9 and C-9,10 positions (184). Both routes yield 1,8-naphthalene dicarboxylic acid, which enters the naphthalene catabolic pathway (Fig. 15).

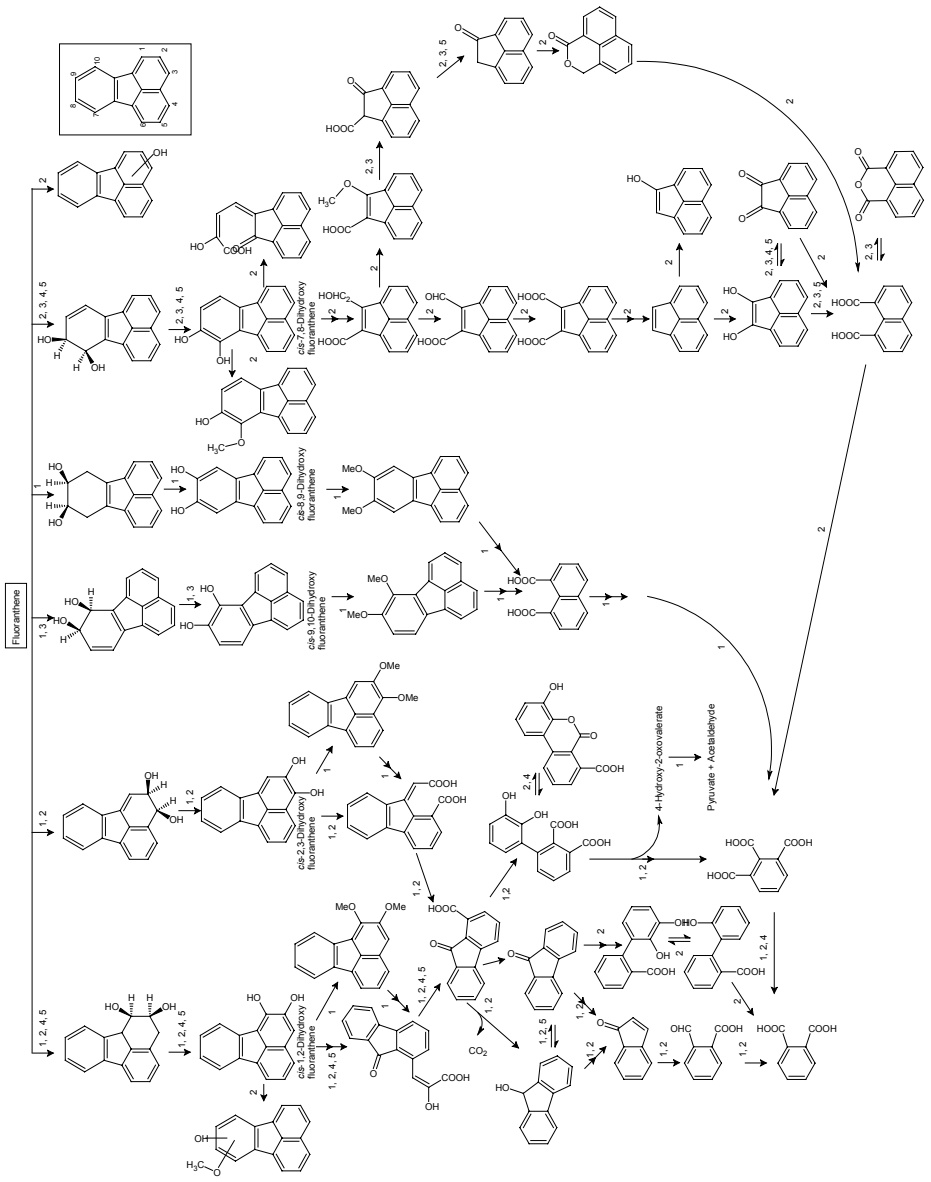

Figure 15: Proposed pathways for fluoranthene degradation. 1, *Mycobacterium* sp. strain JS14 (189); 2, *Mycobacterium vanbaalenii* PYR-1 (154, 155, 184); 3, *Alcaligenes denitrificans* WW1 (332, 333); 4, *Mycobacterium* sp. AP1 (195, 196); 5, *Mycobacterium* sp. strain KR20 (250); 6, *Sphingomonas paucimobilis* EPA505 (219).

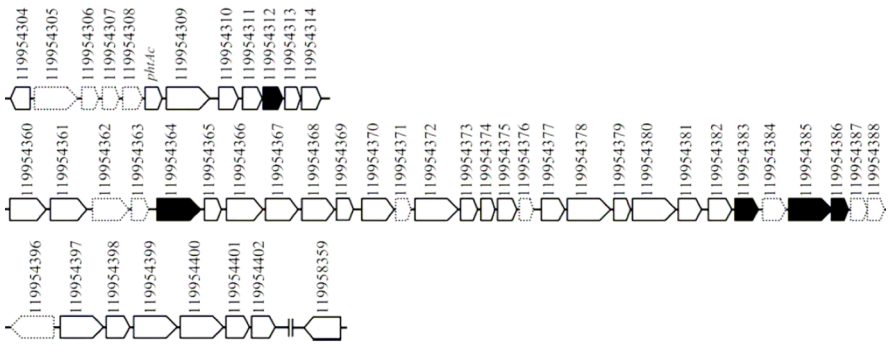

Figure 16. (Taken from Kweon et al. (184)). Organization and expression levels of the genes involved in fluoranthene degradation initiated by dioxygenation of fluoranthene at the C-2,3 positions. Genes/ORFs are represented by arrows: black arrows, up-regulation (more than twofold); arrows with solid lines, no up-regulation; arrows with dotted lines, no expression. The numbers above the arrows indicate the GenBank identifier (gi) numbers from the PYR-1 genome. Vertical lines indicate that genes are not adjacent in the genome.

Dioxygenation at the C-1,2 or C-7,8 position occurs in the gram-negative bacteria *Sphingomonas* sp. strain EPA505 (301), *Pseudomonas alcaligenes* PA-10 (94), and *Alcaligenes denitrificans* WW1 (333). The pathway of 7,8-dihydroxyfluoranthene is continued via *meta*-cleavage and results in the formation of 1-acenaphthenone in *Alcaligenes denitrificans* strain WW1 (333) (Fig. 15).

Many studies have demonstrated the implication of some PAH-degrading enzymes in various bacteria, such as PhnI from *Sphingomonas* sp. strain CHY-1 (56), NidAB from *Mycobacterium vanbaalenii* PYR-1 (298), Pdo1 and Pdo2 from Mycobacterium sp. strain 6YP1 (181) are involved in the pyrene and phenanthrene metabolism.

Mycobacterium vanbaalenii PYR-1 (156, 170) has been investigated for many years and is able to degrade a wide range of high-molecular-weight PAHs, including fluoranthene (116, 154, 214-217). Moreover its genome sequence has recently been released (http://jgi.doe.gov). Kweon et al used a metabolomic, genomic and proteomic approach to study the fluoranthene degradation pathway of *Mycobacterium vanbaalenii* PYR-1 (184). The identification of fluoranthene

metabolites, genome analysis and whole-cell proteome in relation to the degradation of fluoranthene allowed elucidating the fluoranthene degradation pathway in *M. vanbaalenii* PYR-1. 53 gene products were identified and confirmed by expressed proteins in the proteome analysis. Most enzymes found previously to be involved in pyrene degradation in this strain were also identified during fluoranthene degradation. Particularly, NidA3B3 was most highly expressed when total peptides were counted. NidA3B3 has been expressed in *Escherichia coli*, and its fluoranthene oxidation activity has been characterized (167). The 53 genes responsible for fluoranthene degradation by strain PYR-1 are grouped into three different clusters as shown in Fig. 16 (184).

In 1990, Mueller et al. (219) isolated the bacterium *Sphingomonas paucimobilis* EPA505 which was capable of using PAHs with more than three rings as sole carbon source. When pregrown on fluoranthene this strain is able to transform benzo[*a*]fluorene, benz[*a*]anthracene, chrysene and pyrene (219, 342). Mutants carrying Tn*5* insertions in *pbhA* (2,3-dihydroxybiphenyl 1,2-dioxygenase), *pbhB* a Rieske-type ferredoxin subunit, *pbhC* a *trans*-o-hydroxybenzylidene-pyruvate hydratase-aldolase were unable to degrade PAHs. Sequence analysis of the region proximal to *pbhC* revealed a putative promoter that contained a binding site for a LysR transcriptional activator (301, 302). The incubation of these mutants with fluoranthene showed that different metabolites accumulated demonstrating that fluoranthene degradation by this strain goes via acenaphthenol, acenaphtoquinone, naphthalene anhydride and 2,3-dihydroxy-benzoic acid (301) (Fig. 15).

Concluding remarks on PAHs with three fused aromatic rings

Under aerobic conditions some bacteria can degrade PAHs with three fused aromatic rings by using these compounds as a sole source of carbon and energy. The degradation of phenanthrene starts with the formation of *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, which is further converted to 3,4-dihydroxyphenanthrene and 1-hydroxy-2-naphthoic acid. The latter can be mineralised via the naphthalene pathway (Gram-negative bacteria) or via *o*-phthalic acid (Gram-positive bacteria) depending on the bacterial strain. On a genetic level many information are available. *Sphingomonas* species seem to harbour a conserved catabolic cluster encoding six ring-hydroxylating

dioxygenases (56, 241, 264, 354). Many *Mycobacterium* species such as *Mycobacterium vanbaalenii* PYR-1 have been intensively studied and many catabolic genes identified. Gene duplication and enzymes becoming specific to different substrates were probably responsible for the emergence of these conserved patterns. Anthracene is oxidized at the 1,2-position yielding *cis*-1,2dihydroxy-1,2-dihydroanthracene and subsequently 1,2-dihydroxyanthracene. Further metabolism results in the formation of 3-hydroxy-2-napthoic acid, which can be mineralised via *o*-phthalic acid or salicylic acid. A dioxygenase from *Xanthomonas polyaromaticivorans* 127W able to degrade naphthalene, anthracene and phenanthrene has been isolated. Moreover the presence of a gentisate 1,2-dioxygese suggests that these PAHs are further degraded via the gentisate pathway. Fluoranthene can be oxidized at the positions C-1,2, C-2,3, C-8,9, and C-9,10 to form the corresponding dihydrodiols. Recently the fluoranthene pathway of *Mycobacterium vanbaalenii* PYR-1 and *Mycobacterium* sp. strain JS14 were completely elucidated using proteomic, genomic and metabolomic approaches. The major route of dioxygenation, the C-2,3 dioxygenation route via 9-fluorenone-1-carboxylic acid and phthalic acid, involving 18 enzymatic steps. The C-7,8 route oxidizes fluoranthene via acenaphthylene-type metabolites.

Benz[*a*]anthracene

In 1988 Mahaffey et al. (198) presented the first direct documentation of bacterial degradation of benz[*a*]anthracene by *Sphingomonas yanoikuyae* B1 after induction by biphenyl, *m*-xylene or salicylate. The resulting compounds from the oxidative degradation were identified by nuclear magnetic resonance and mass spectral analyses as 1-hydroxy-2-anthranoic acid, 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid. Strain B1 attacks benz[*a*]anthracene at the C-1,2 (73)%, C-8,9 (15%) and C-10,11 (12%) positions. The C-1,2 dioxygenation route degrades benz[*a*]anthracene via anthracene-type metabolites, whereas the C-8,9 and C-10,11 routes oxidize benz[*a*]anthracene via phenanthrene-type metabolites (Fig. 17).

In 1996 Schneider et al. (279) studied the benz[*a*]anthracene degradation by *Mycobacterium* sp. strain RJGII-135. *cis*-5,6-Benz[*a*]anthracene-dihydrodiol, *trans*-10,11-benz[*a*]anthracene-dihydrodiol, *cis*-8,9-benz[*a*]anthracene-dihydrodiol, *trans*-8,9-

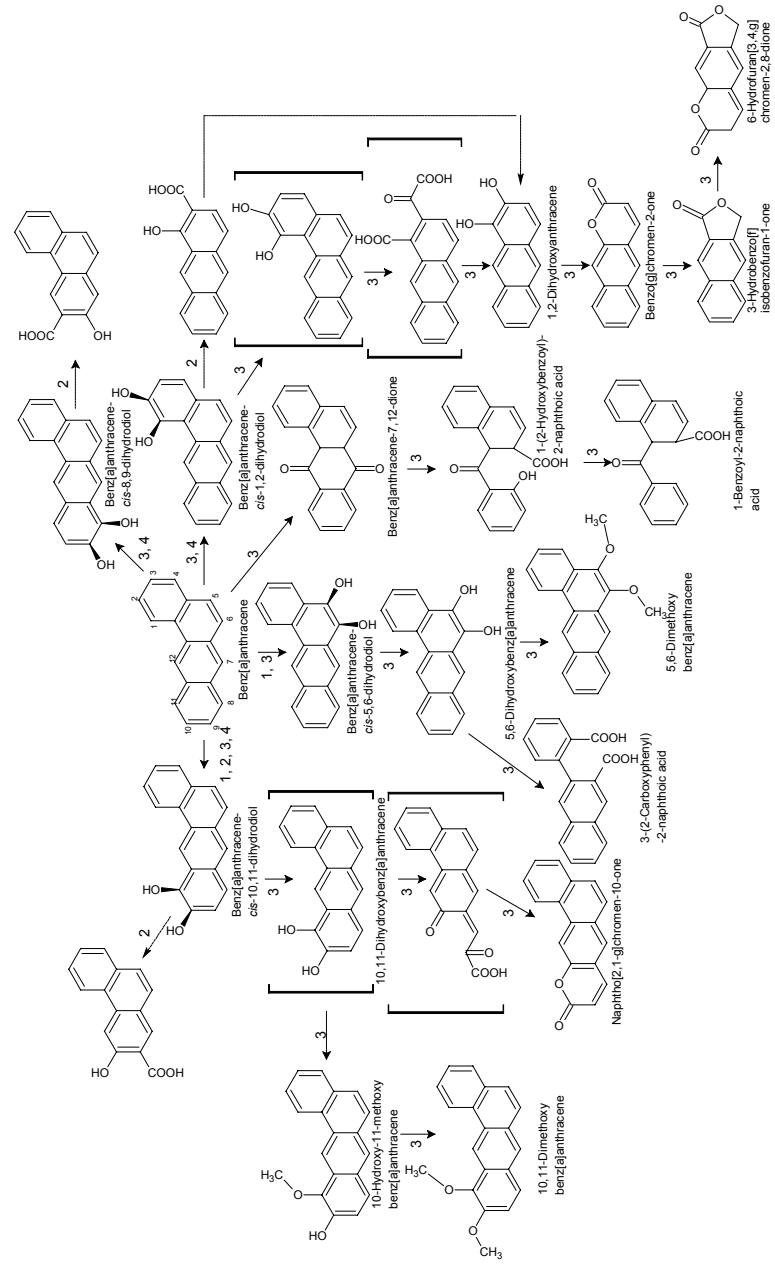

Figure 17. Proposed transformation products in the degradation of benz[*a*]anthracene by 1, *Mycobacterium* sp. strain RJGII-135 (279); 2, *Sphingomonas yanoikuyae* B1 (198); 3 *Mycobacterium vanbaalenii* PYR-1 (214). 4 *Sphingomonas* sp. strain CHY-1 (141).

benz[*a*]anthracene-dihydrodiol were identified. Attack at position 5,6 is only seen in *Mycobacteria*. In 2005 Moody et al (214) reported benz[*a*]anthracene degradation by *Mycobacterium vanbaalenii* strain PYR-1. This strain, although not capable of using benz[*a*]anthracene as sole source of carbon and energy, was able to degrade 15% of the added benz[*a*]anthracene after 12 days in the presence of phenanthrene. It was found that this strain uses at least four points of attack, for benz[*a*]anthracene, the predominant being at position C-10,11 (214) (Fig. 17).

The study of ring hydroxylating dioxygenases such as PhnI from *Sphingomonas* sp. CHY-1 (141) and BphA1fA2f from *Sphingobium yanoikuyae* B1 (198) have brought evidence that the first step of degradation involves the same enzymes that are also responsible for the degradation of smaller PAHs such as naphthalene and phenanthrene.

Pyrene

Pyrene is the most studied four-ring PAH and is mostly degraded by members of the genera *Mycobacterium* (21, 41, 54, 104, 117, 168, 171, 181, 192, 194, 211, 251, 279, 290, 325) and *Rhodococcus* (26, 329). Heitkamp et al. (116) described for the first time a bacterium able to use pyrene as sole source of carbon and energy. This strain, *Mycobacterium vanbaalenii* PYR-1, has become the most studied pyrene degrader (155-157, 166, 168, 172, 173, 213-217, 283, 297, 298, 330). The complete pyrene degradation pathway of this remarkable bacterium was finally elucidated in 2007 (168). Although pyrene is oxidized at the C1 and C2 positions to form pyrene-1,2-diol (173), this pathway is not productive and the predominant pathway is initiated at the C4 and C5 positions. 4,5-Dihydroxypyrene is in turn transformed to 4,5-dicarboxyphenanthrene which is decarboxylated to 4-phenanthroate. A second dioxygenation reaction of *cis*-3,4-dihydroxyphenanthrene-4-carboxylate leads after rearomatization to 3,4-dihydroxyphenanthrene which is subsequently transformed to 1-hydroxy-2-naphthoate. The latter compound is degraded via phthalate and protocatechuate (Fig. 18).

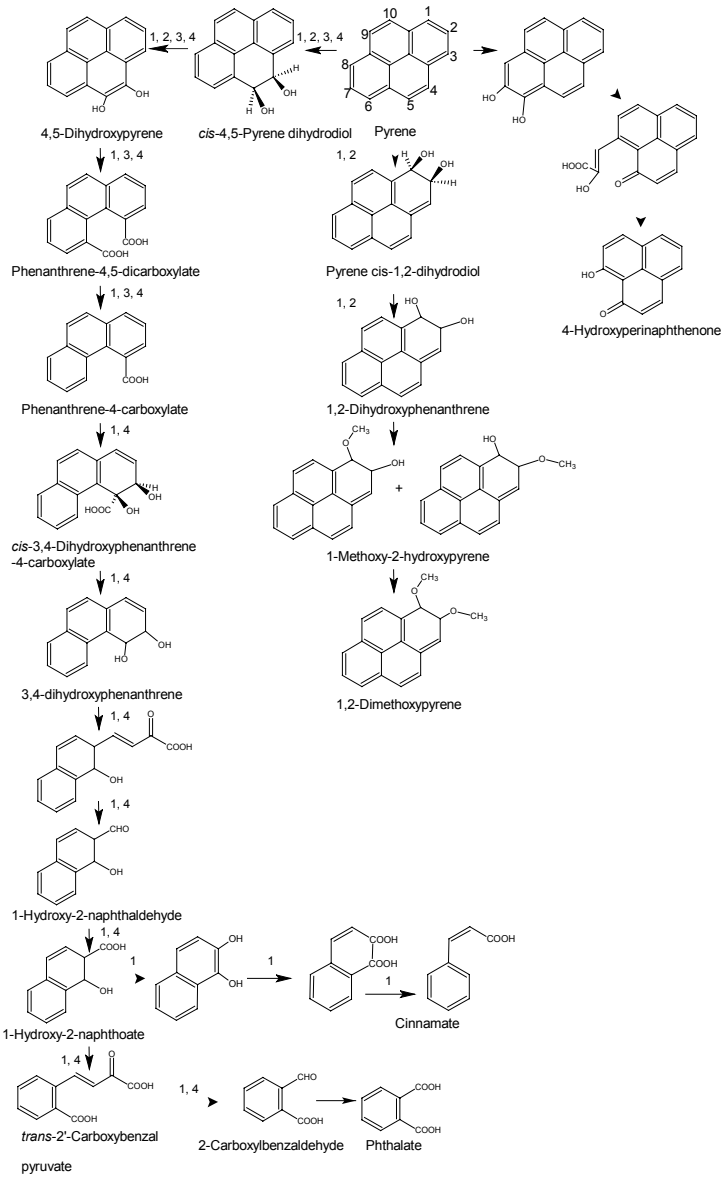

Figure 18. Proposed pathway for pyrene degradation (39, 54, 104, 117, 168, 279, 329). 1, *Mycobacterium vanbaalenii* PYR-1 (39, 117, 166, 168, 171); 2, *Rhodococcus* sp. strain UW1 (329); 3, *Mycobacterium* sp. strain RJGII-135 (104, 279); *Mycobacterium flavescens* ATCC700033 (54).

A proteomic approach was used to detect genes involved in pyrene degradation in *Mycobacterium vanbaalenii* strain PYR-1 (157). An aldehyde dehydrogenase (*nidD*), a dioxygenase β-subunit (*nidB*), a dioxygenase α-subunit (*nidA*) and a partial gene named *nidC* were detected. Phylogenetic analysis showed that the large (α)-subunit did not cluster with most of the known α-subunit sequences but showed some homology with recently described dioxygenases from *Rhodococcus* sp. (313) and *Nocardioides* sp. (267). Functional steps could be assigned to NidAB (pyrene, phenanthrene ring hydroxylating oxygenase), NidA3B3 (fluoranthene, pyrene ring hydroxylating oxygenase), PhtAaAb (phthalate 3,4-dioxygenase), PhtAcAd (ferredoxin, ferredoxin reductase components), PhtB (*cis*-3,4-dihydro3,4-dihydroxyphthalate dehydrogenase, MvanDraft_0992 (catechol O-methyltransferase) (157, 167, 173, 297). For a detailed summary of the gene clusters involved in the catabolism of pyrene by strain PYR-1 see reference (168).

In 2003 Krivobok et al. (181) identified genes involved in pyrene degradation by *Mycobacterium* sp. strain 6PY-1. DNA fragments encoding genes similar to *nidBA* from *Mycobacterium* sp. strain PYR-1 were recovered. Unlike strain PYR-1 the *nidAB* genes in strain 6PY-1 are not inducible by naphthalene. Due to this difference they were called *pdoA1* (α-subunit) and *pdoB1* (β-subunit). PdoA1B1 showed high activity toward pyrene. A second dioxygenase PdoA2B2 was isolated and found to be more active against phenanthrene. Both enzymes are able to oxidise both PAHs (see discussion on phenanthrene).

Pyrene degraders continue to be isolated and functional genes identified (20, 192, 209, 290). The enzymes responsible for the initial attack on pyrene NidAB (157) for strain PYR-1 or Pdo1 and Pdo2 (181) for strain 6YP are also responsible for the oxidation of phenanthrene starting with 3,4-dihydroxyphenanhtrene.

Chrysene

No complete degradation pathway for chrysene has been described to date.

The isolation of bacterial strains able to degrade chrysene as sole source of carbon or by cometabolism has been reported (22, 34, 56,

90, 336, 349). Studies of a mutant of *Sphingomonas yanoikuyae* B1, which has a broad substrate specificity range, showed that chrysene was hydroxylated to *cis*-3,4-dihydrodiol which may correspond to the first intermediate in the chrysene degradation pathway (27). In 2004 Willison et al. (336) isolated and characterized a novel sphingomonad capable of growth with chrysene as its sole carbon source. Although ^{14}C labelled chrysene degradation experiments were carried out, no intermediate metabolite could be identified (56). A proteomic approach allowed the identification of proteins that display similarity with a ring-hydroxylating dioxygenase beta subunit, and an extradiol dioxygenase and the corresponding genes were isolated (see below). They were found in two distinctive loci showing high similarities to corresponding genes found on the catabolic plasmid pNL1 from *Novosphingobium aromaticivorans* F199 (264) (Fig. 7). The genes designated $phnA1_a$, $phnA1_b$ and *phnB* encoded the alpha and beta subunit of a putative ring-hydroxylating oxygenase and an aryl alcohol dehydrogenase respectively. The second locus contained five genes encoding an extradiol dioxygenase (*phnC*), a ferredoxin (*phnA3*), a second oxygenase complex $phnA2_b$, $phnA1_b$ and an isomerase (*phnD*). $phnA1_aA1_b$ gene products were found to be capable of converting several PAHs, including chrysene, to the corresponding dihydrodiols (56). The activity of these genes was greatly enhanced upon coexpression of genes encoding a ferredoxin (*phnA3*) and a reductase (*phnA4*). Disruption of the $phnA1_a$ gene resulted in a mutant strain that had lost the ability to grow on PAHs. This enzyme complex has been recently studied in detail (140) and its crystal structure resolved (129). These insights into the catalytic pocket of the enzyme complex show how the substrate interacts with the protein and gives useful information concerning the degradation spectrum of the enzyme. Moreover a detailed study of the *phnB* gene, a dihydrodiol dehydrogenase has been published recently (140) and the biochemical properties of this enzyme as well as the substrate range described.

Concluding remarks on PAHs with four fused aromatic rings

The polycyclic aromatic hydrocarbons with four fused aromatic rings are more resistant to biodegradation than the lower PAHs. This is partly caused by their low water solubility with results in a poor bioavailability (145). A few bacteria have been isolated that are capable of using pyrene and chrysene under aerobic conditions as a growth substrate. Furthermore many bacterial strains that cannot use

these compounds as a sole source of carbon and energy are able to convert them cometabolically during growth on other PAHs (145). Many oxygenases have been reported to oxidize benz[*a*]anthracene at the positions C-1,2, C-5,6, C-8,9 and C-10,11 (184, 189). Oxidation at position C-5,6 is only seen in *Mycobacteria*. Pyrene is the most studied four-ring PAH and is mostly degraded by members of the *Mycobacterium* genera. *Mycobacterium vanbaalenii* PYR-1 has been isolated on pyrene as growth substrate and the complete degradation pathway was elucidated in 2007 (168). The predominant pathway starts with dioxygenation at the position C-4,5 yielding *cis*-4,5-dihydoxy-4,5-dihydropyrene, which is further metabolised via 3,4-dihydroxyphenanthrene and phthalate using the same enzymes (168, 181). To date no degradation pathway for chrysene was proposed. *Sphingomonas* sp. strain CHY-1 has been isolated on chrysene and the initial ring-hydroxylating dioxygenase identified (56). The dioxygenase of strain CHY-1 was able to oxidize at least eight PAHs made of 2-5 aromatic rings, compared to most other dioxygenases, whose activity is limited to 2 and 3 ring PAHs (141).

Benzo[*a*]pyrene

Degradation of five-ring PAHs has been described but relatively few data are available (143). In 1975 Gibson et al. (92) isolated *Sphingomonas yanoikuyae* B1, capable of oxidizing benzo[*a*]pyrene and benz[*a*]anthracene. This oxidation led to the formation of *cis*-7,8- and 9,10-dihydrodiols, but no ring cleavage metabolites were detected. In 1996 Schneider et al. (279) identified the following metabolites from *Mycobacterium* sp. strain RJGII-135: *cis*-7,8-benzo[a]pyrene-dihydrodiol, 4,5-chrysene-dicarboxylic acid, 7,8-dihydro-pyrene-(7-or-8)-carboxylic acid and *cis*-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid. In 2004 Moody et al. analyzed the degradation of benzo[*a*]pyrene by *Mycobacterium vanbaalenii* PYR-1 (216). This organism initially oxidized benzo[*a*]pyrene with dioxygenases and monooxygenases at C-4,5, C-9,10, and C-11,12. The major intermediates of benzo[*a*]pyrene metabolism that accumulated in the culture media after 96 h of incubation were *cis*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene, *cis*-11,12-dihydro-11,12- dihydroxy benzo[*a*]pyrene, *trans*-11,12-dihydro-11,12- dihydroxybenzo[*a*] pyrene, 10-oxabenzo[*def*]chrysen-9-one, and hydroxymethoxy and dimethoxy derivatives of benzo[*a*]pyrene. The results of this study, in

conjunction with those of previously reported studies, extend the pathways proposed for the bacterial metabolism of benzo[*a*]pyrene (216) (Fig. 19).

No genes directly involved in the degradation of benzo[*a*]pyrene have been isolated to date. It is, however, generally accepted that genes necessary for pyrene degradation are needed for benzo[a]pyrene mineralisation.

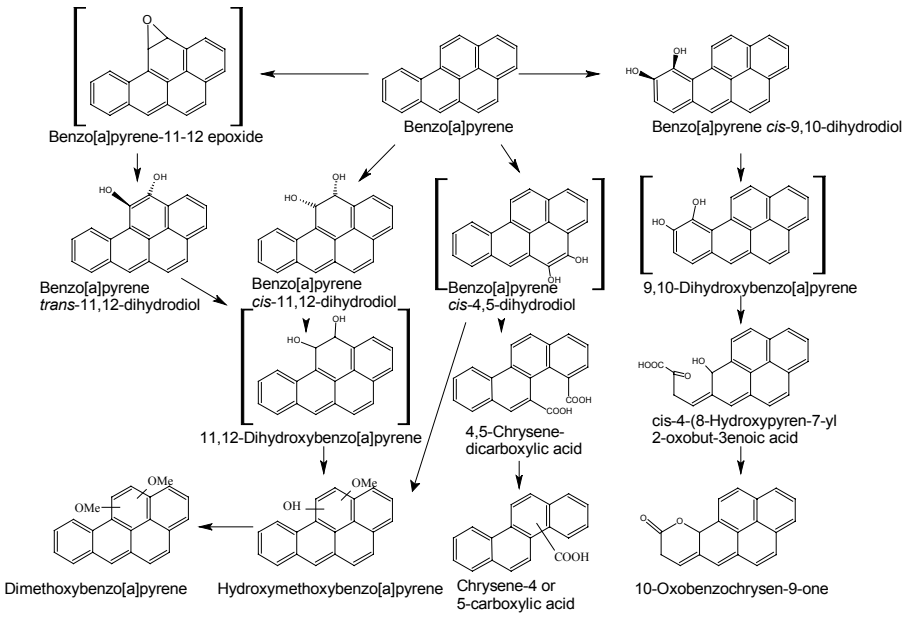

Figure 19, Proposed pathway for benzo[*a*]pyrene degradation (92, 216, 279).

Concluding remarks on PAHs with five fused aromatic rings

Polycyclic aromatic hydrocarbons with five and more fused aromatic rings are considered extremely resistant to microbial degradation (145). Furthermore, microorganisms cannot use five-ring or higher PAHs as a sole source of carbon and energy, although some strains are able to degrade them cometabolically. The pathway for the complete degradation of benzo[*a*]pyrene has not been elucidated, only the initial steps are known. *Sphingomonas* sp. CHY-1 and *Sphingobium*

yanoikuyae B1 possess a dioxygenase with catalytic pockets large enough to accommodate benzo[*a*]pyrene (141, 225).

Final Remarks

To date, several PAH catabolic plasmids like pNL1 from *Sphingomonas aromaticivorans* F199 (264), pADP-1 from *Pseudomonas* sp. strain ADP, pWWO from *Pseudomonas putida* mt-2 (97, 335), pCAR1 from *Pseudomonas resinovorans* CA10 (197), pND6-1 from *Pseudomonas* sp. strain ND6 (191), pCAR3 from *Sphingomonas* sp. strain KA1 (289) have been completely sequenced and their gene organizations have been published. A more extensive list of catabolic plasmids and other mobile genetic elements carrying catabolic genes is given by Nojiri et al. (230). Recently Basta et al. (15, 16) managed to isolate plasmids from xenobiotic degrading *Sphingomonas* strains. In the 16 strains analysed two to five plasmids with sizes of 50 – 450 kb were isolated by using pulsed-field gel electrophoresis. However, with the exception of naphthalene and phenanthrene, we lack information about the dioxygenase genes encoding the initial step in the catabolism of PAHs. In the last few years more information regarding the dioxygenases involved in the initial attack on PAHs has become available, allowing us to get a closer look into the secrets of natural transformation processes that could be profitably adapted into bioremediation practice. This information is an essential prerequisite in order to understand the complex catabolic pathways of potential PAH degraders. The success of any bioremediation strategy is dependent on the characteristics of the microorganisms. It is essential that the initial dioxygenases have relaxed substrate specificity, hence increasing the number of different substrates that can be oxidized. The limiting factor is the metabolic capabilities towards the by-products to reduce the toxicity of the soil.

Many bacteria that have the ability to degrade a wide range of PAHs have been studied, but there is still an urgent need concerning high molecular weight PAH-degraders. An alternative to this problem could be the cometabolic degradation. Expanding the research on the practical application of cometabolic processes is more interesting because soil is often contaminated with a mixture of different PAHs. On the other hand, the composition of bacterial consortia, completing their degradation capabilities, could also be a good tool for bioremediation.

Chapter 2: Ring Hydroxylating Dioxygenases

Introduction

Aerobic bacteria often initiate the oxidation of PAHs by incorporating both atoms of oxygen into an aromatic ring to produce a *cis*-dihydrodiol. Further metabolism of *cis*-dihydrodiols involve NAD^+-dependant dehydrogenation reactions that produce a 1,2-dihydroxylated compound. The latter undergoes ring-cleavage through the action of a ring-cleavage dioxygenase. During the past two decades much interest was given to the metabolic pathways through which microorganisms degrade PAHs. The identification of metabolites has allowed establishing degradation patterns that are often well conserved. Different enzymes carry out the initial conversion steps but the compounds are transformed to a limited number of central intermediates. This generalized scheme of catabolic pathways for aromatic compounds, also known as catalytic funnel (Fig. 1), suggests that the enzymes have extended their substrate range by developing peripheral pathways, which are able to transform initial substrates into central intermediates such as salicylic acid and phthalic acid (319).

Enzymology and Mechanism of Ring-Hydroxylating Dioxygenases

Oxygenases have only been known for less then fifty years. The initial oxidation of the aromatic ring is the most difficult catalytic step in the aerobic degradation of aromatic compounds. Bacterial aromatic ring hydroxylating dioxygenases catalyse the addition of hydroxyl groups to the highly stable aromatic ring, setting the stage for further oxidation, and eventual ring cleavage. This reaction is catalysed by a multicomponent aromatic ring-hydroxylating dioxygenase. These enzyme complexes generally consist of three components, a terminal dioxygenase which has two different subunits α (large) and β (small), a ferredoxin, and a NADH-dependent ferredoxin reductase (Fig. 2). Each α subunit contains two redox centers, a Rieske [2Fe-2S] center and mononuclear Fe^{2+} at the active site. The α subunit of the terminal oxygenase of RHDs represents a useful molecular target to investigate the significance of aromatic hydrocarbon degraders. This protein is

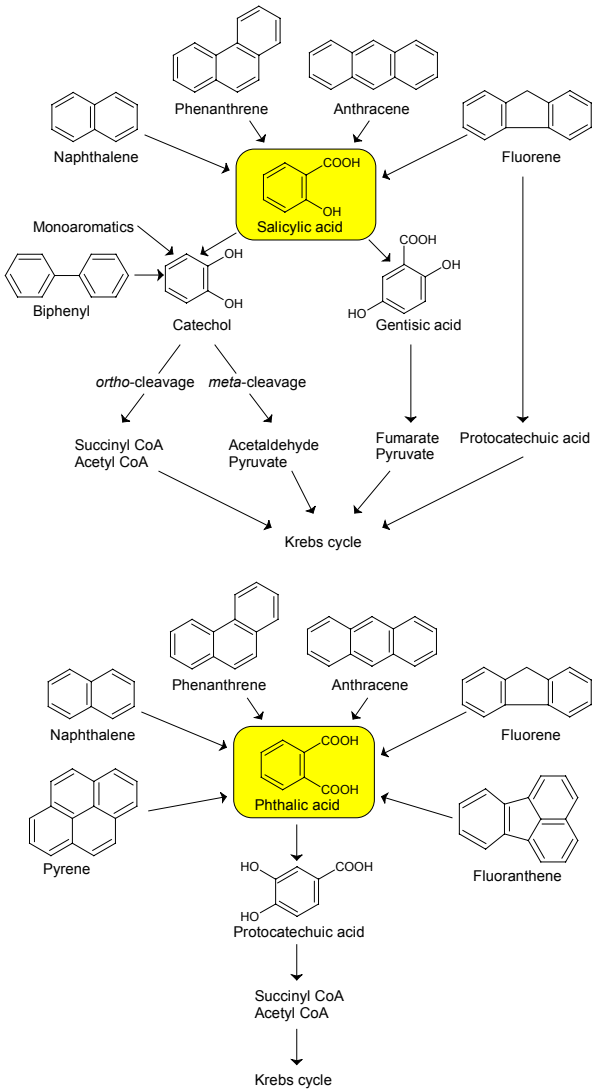

Figure 1. Various polycyclic aromatic hydrocarbons are oxidized by enzymes having relaxed substrate specificity to common intermediates. These compounds are further oxidized to Krebs cycle intermediates by enzymes having a high selectivity. PAH degradation by Gram-negative bacteria generally proceeds via salicylic acid as central metabolite, whereas Gram-positive bacteria produce protocatechuic acid as central metabolite.

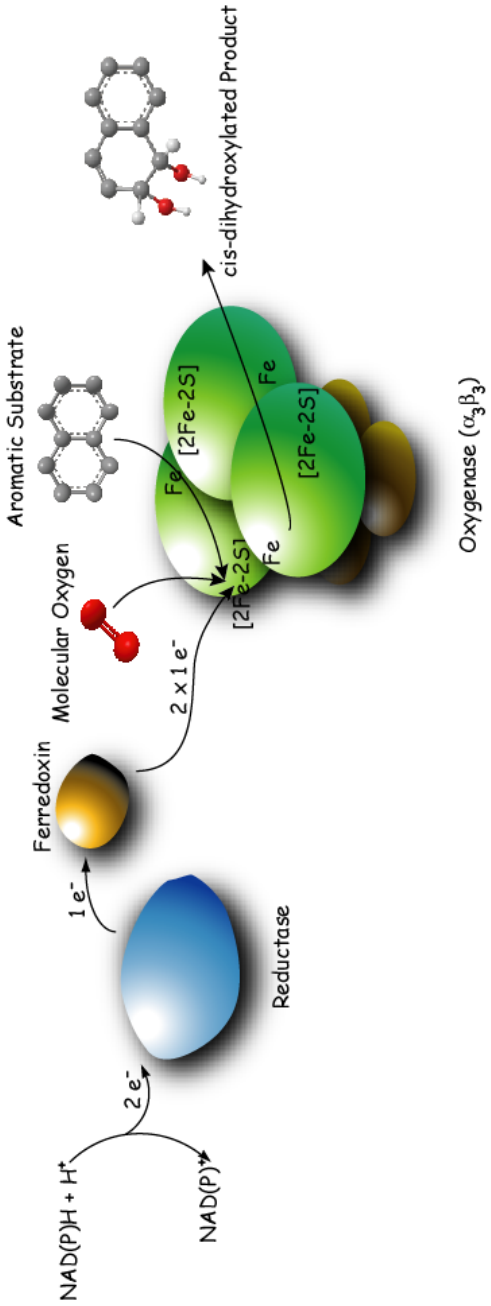

Figure 2. (Adapted from (74)) Reaction catalysed by the three component ring hydroxylating dioxygenase. Electrons from NADH are transferred by the reductase to the Rieske [2Fe-2S] ferredoxin. An electron is then transferred from the ferredoxin to one of the Rieske centers in the dioxygenase. Reduced oxygenase catalyses the addition of both atoms of O2 to naphthalene foming naphthalene *cis*-1,2-dihydrodiol.

thought to primarily determine the substrate range of RHDs (69, 233) by determining the position of the substrate in the active site relative to the iron atom. These enzymes produce *cis*-dihydrodiols from a large variety of substances and molecular oxygen (O_2) (327). Moreover these enzyme complexes are able to perform oxidation with a high degree of stereo-, regio-, and enantiospecificity (254, 255).

Electrons from NAD(P)H are used to activate molecular oxygen, which is then used to oxidize the substrate (Fig. 2). Two more proteins, a ferredoxin and a ferredoxin reductase protein involved in the electron transport chain, assist the dioxygenases. The reductase oxidizes NAD(P)H to $NA(D)P^+$ at the NAD(P)H binding site capturing two electrons. The electrons are stored until the reductase completes a 1-electron reduction of the ferredoxin component. The ferredoxin shuttles the electron received from the reductase to the oxygenase. This step occurs twice for each molecule of product formed at the active site (73). The oxygenase consists of an α subunit, which includes both a Rieske binding domain and the catalytic pocket containing a mononuclear iron centre (75). The β subunit is believed to function as a stabilizer for the α subunit.

Ferredoxins and ferredoxin reductases are small proteins, whose function is generally electron-transfer, which contain one or more Fe-S clusters. They may be divided into classes depending on how many clusters of each type they contain. [Fe-S] clusters are usually ligated by cysteine residues, whose S atoms bind to each Fe atom of the cluster (Fig 3). Iron-sulphur clusters are one of the nature's simplest, functionally versatile and perhaps most ancient prosthetic groups composed of iron and inorganic sulphur. Among the familiar types of Fe-S clusters are [2Fe-2S] and [4Fe-4S], which are usually attached to their protein partner by four cysteine thiol ligands, but other less common forms exist. Proteins containing one or more Fe-S clusters represent a large class of structurally and functionally diverse proteins participating in many metabolic processes. Several critical processes in biology involve dioxygen (O_2) and most of these processes are promoted by transition-metal ions. In 2003 Karlsson et al. showed for the first time molecular oxygen bound to the mononuclear iron and proposed a reactional mechanism activating O_2 in *Pseudomonas* sp. strain NCIB9816-4 (147). In a complex with substrate and dioxygen,

the dioxygen molecule is lined up for attack on the double bond of the aromatic substrate (147) (Fig. 4).

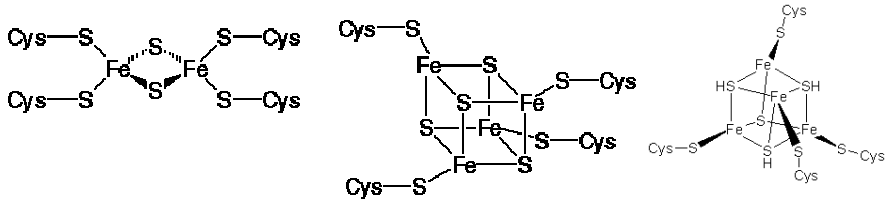

Figure 3. Iron-sulphur clusters (from left to right). [2Fe-2S] cluster: two iron atoms are bridged to one another by two inorganic sulfur atoms and ligated to four cysteines from the peptide backbone. [4Fe-4S]; the four iron atoms are ligated to four cysteines and form a cubic structure. [3Fe-4S]; a single iron of the [4Fe-4S] cluster is absent.

Structure of Ring-Hydroxylating Dioxygenases

To date the structures of several RHD terminal oxygenases have been reported, including that of the naphthalene dioxygenases from *Pseudomonas* sp. strain NCIB9816-4 (NDO_{9816-4}) (35, 152) and *Rhodococcus* sp. strain NCIMB12038 (NDO_{12038}) (86), the nitrobenzene dioxygenase from *Comamonas* sp. strain JS765 ($NBDO_{OJS765}$) (79), the biphenyl dioxygenase from *Rhodococcus* sp. strain RHA1 ($BPDO_{RHA1}$) (85), the cumene dioxygenase from *Pseudomonas fluorescens* strain IP01 (CDO_{IP01}) (61), the 2-oxoquinoline 8-monooxygenase from *Pseudomonas putida* strain 86 (OMO_{86}) (201), biphenyl dioxygenase from *Sphingobium yanoikuyae* B1 (BphA1fA2f) (348), the carbazole-1–9 a-dioxygenase from *Pseudomonas resinovorans* strain CA10 (CARDO) (227) and PhnI from *Sphingomonas* sp. strain CHY-1 (131). Except for OMO_{86} and CARDO, which were found to be homotrimers consisting of α subunits only, all other enzymes exhibited a α3β3 quaternary structure.

Since we investigate terminal components of RHDs from two *Sphingomonas* species we use PhnI from *Sphingomonas* sp. strain CHY-1 as model. In general ring-hydroxylating dioxygenases are

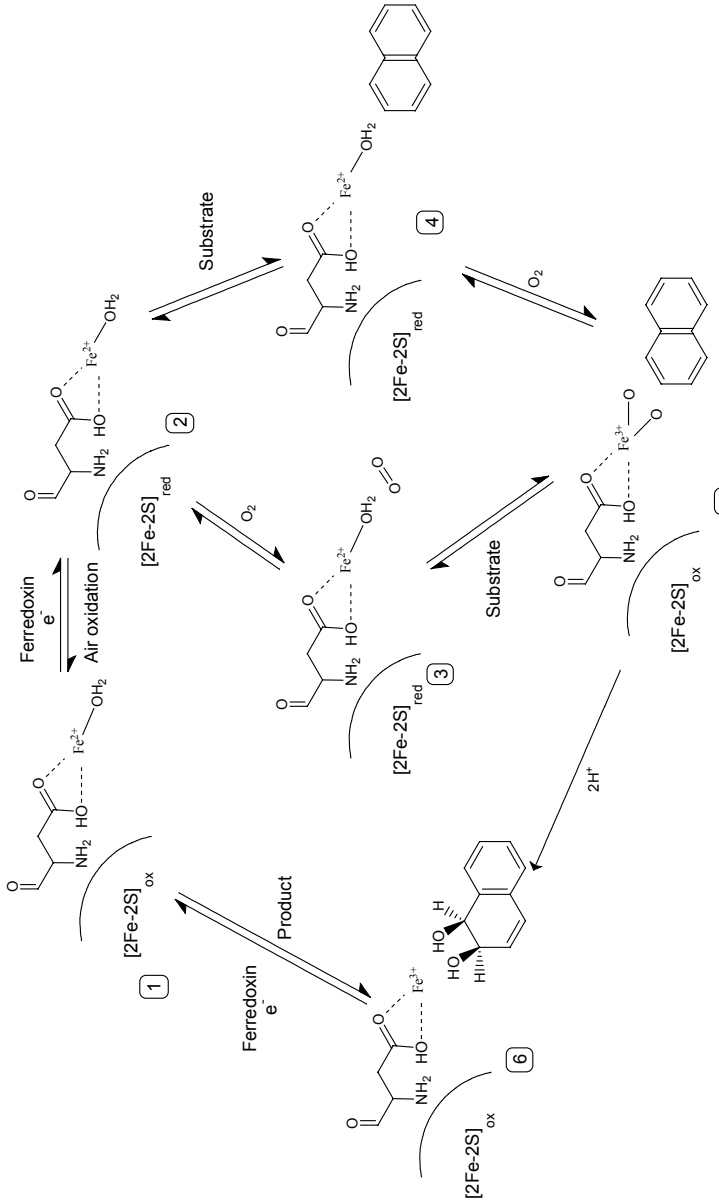

Figure 4. (Taken from (147)) Scheme showing how molecular oxygen interacts with the mononuclear iron at the catalytic site of NDO_{9816-4} before the dihydroxylation reaction is catalysed. The structures are as follows: 1, the resting enzyme with oxidized Rieske center and ferrous active site; 2, the reduced enzyme; 3, binary dioxygen complex; 4, binary substrate complex; 5, ternary substrate dioxygen species; and 6, product naphthalene *cis*-1,2-dihydrodiol. [2Fe-2S] refers to the nearest Rieske iron-sulfur cluster.

α3β3 heterohexamers whose β subunits contain no redox centers and the β subunit residues are distant from the active site in each enzyme. The α3β3 hexamer contains three active sites, which are located at the junctions of adjacent α subunits. Each α subunit contains a Rieske [2Fe-2S] iron sulfur center, which is coordinated by two histidines and two cysteines and mononuclear iron (Fig. 5).

An Asp, totally conserved amongst ring-hydroxylating dioxygenases, buried in a large depression at the junction of the Rieske and the catalytic domains of neighbouring α subunits functions as electron transfer bridge (35, 61, 79, 85, 86, 131, 152, 201, 227, 348). In this key position in PhnI from *Sphingomonas* sp. strain CHY-1, Asp204 provides a bridge between the Rieske cluster and the mononuclear iron center (Fig. 5). Replacement of this Asp by a Ala, Glu, Gln or Asn in $NDO_{9816\text{-}4}$ from *Pseudomonas* sp. strain NCIB9816-4 resulted in a totally inactive enzyme suggesting that this Asp residue is essential for electron transfer or in positioning the two adjacent α subunits (232).

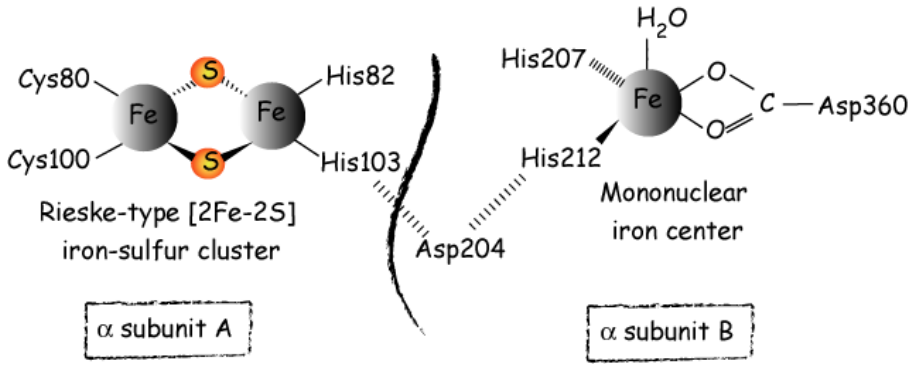

Figure 5. The junction between two adjacent α subunits in PhnI from *Sphingomonas* sp. strain CHY-1. Shown are the Rieske [2Fe-2] center and mononuclear iron at the active site. Amino acids Cys80, His82, Cys100 and His103 coordinate the Rieske center; His212, His207, and Asp360 coordinate mononuclear iron at the active site (131). Asp204 provides a bridge between the Rieske cluster ant the mononuclear iron center of two adjacent α subunits (131).

The PhnI catalytic pocket, formed mostly by hydrophobic amino acids, is surrounded by two loops exposed to the solvent, L1 and L2 (131). The flexible loops L1 and L2 seem to control the substrate access to the catalytic pocket and control pocket length (Fig. 6) (129, 131). The catalytic domain shows a substantially larger hydrophobic substrate-binding pocket, the largest ever reported for this type of enzyme. Compared to other naphthalene dioxygenases, whose selectivities are limited to two and three ring PAHs, these features certainly allow the five-ring benzo[a]pyrene to bind to the catalytic Fe (129).

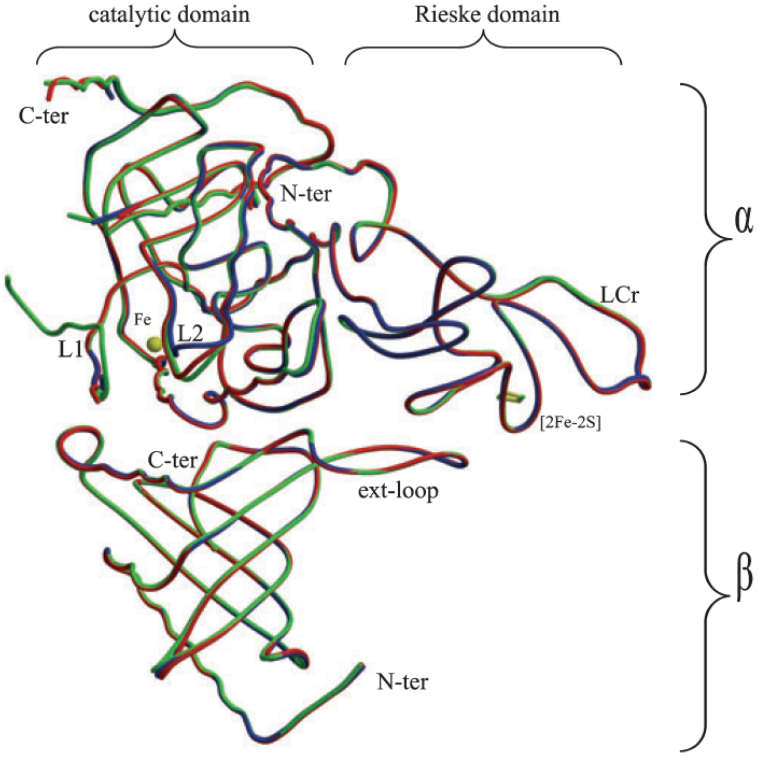

Figure 6. (Taken from (131)). The PhnI αβ heterodimers in red, green and blue are superposed. Each α subunit contains a Rieske domain with the [2Fe-2S] cluster and the catalytic domain with the mononuclear iron. The flexible loops L1 and L2 are believed to control the access to the catalytic pocket (129, 131).

Lateral vs. angular dioxygenation

Dioxygenation is one of the most important reactions involved in the bacterial degradation of various aromatic compounds. Aromatic compounds, such as biphenyl and naphthalene are oxidised at lateral positions of the aromatic ring resulting in the formation of *cis*-dihydrodiols (66). This "normal" type of dioxygenation is termed lateral dioxygenation (Fig. 7A).

On the other hand, the analysis of the bacterial degradation of fluorene analogues, such as dibenzofuran, carbazole, and dibenzothiophene-sulfone and dibenzo-*p*-dioxin revealed the presence of a novel mode of dioxygenation reaction for aromatic nucleus, generally termed angular dioxygenation (Fig. 7B).

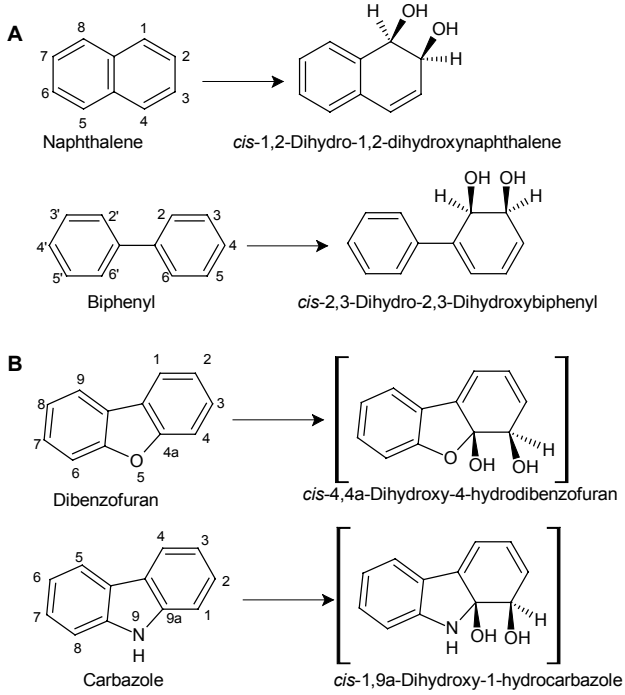

Figure 7. A) Lateral oxygenation for naphthalene and biphenyl. B) Angular dioxygenation for dibenzofuran and carbazole. Products of angular dioxygenation shown in brackets have never been observed directly and are spontaneously transformed to their respective biphenyl intermediate.

In this atypical dioxygenation, the carbon bonded to heteroatoms in the fluorene analogues, and the adjacent carbon in the aromatic ring are both oxidized (228). However, angular dioxygenation of fluorene and dibenzothiophene has not been reported yet. The latter compounds have to be oxidized to 9-fluorenone and dibenzothiophene sulfone. The electronegativity of the atom connecting the two aromatic rings influences the attack of the angular dioxygenase. In dibenzofuran and carbazole, the connecting atoms, O and N respectively, have high electronegativities, and these compounds serve as substrates for angular dioxygenases. In contrast, the connecting atoms in dibenzothiophene and fluorene, S and C respectively, have lower electronegativities, and these atoms must be oxidized before the angular dioxygenases attack these compounds (29) (Fig. 8).

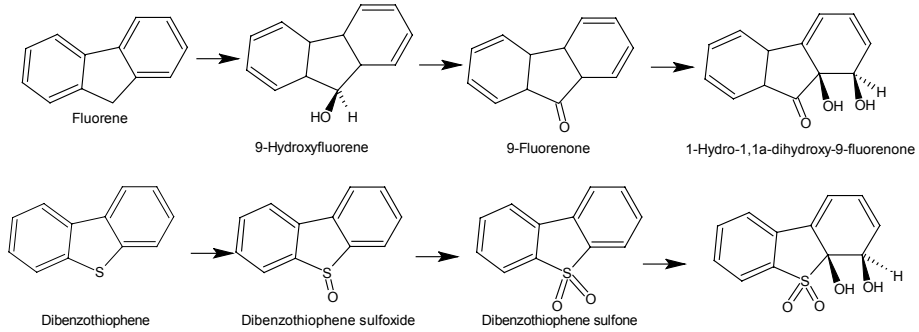

Figure 8. The connecting atom in fluorene and dibenzothiophene, C and S respectively, have lower electronegativities then the connecting atoms, S and N, in dibenzofuran and carbazole. These atoms must be oxidized before angular dioxygenation can occur.

Phylogenetic analysis of ring hydroxylating dioxygenases

Most of the terminal components of RHDs show homology to each other (Fig. 9), but the substrate ranges differ remarkably. Ring-hydroxylating dioxygenases are of central importance to bacterial recycling of aromatic hydrocarbons, and have attracted attention for their potential to degrade anthropogenic pollutants (e.g. PAHs, polychlorinated biphenyls; PCBs).

Different bacterial strains may utilize different biochemical pathways for the degradation of the same aromatic compound. In contrast,

different bacterial strains may degrade an aromatic compound by the same catabolic pathway but possess genes that have diverged widely in their nucleotide sequence. RHDs from aromatic hydrocarbon degraders are defined by a common biochemical function and are therefore not necessarily phylogenetically related. This diversity in nucleotide sequence also plays a role in the specificity and activity of the enzymes produced. Since the substrate range of RHDs has evolved to degrade a remarkable diversity of contaminants (256), one must target genes that are unique to the biochemical pathway in question and are sufficiently conserved to enable their relation to previously described RHDs. The Naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816 is known to catalyse the oxidation of more then 75 different substrates by reactions including *cis*-dihydroxylation, monooxygenation (258, 328), desaturation (93, 311), *O*- and *N*-dealkylation (253) and sulfoxidation (188).

Phylogenetic analysis shows that dioxygenases oxidising monoaromatics (blue) cluster together as well as biphenyl dioxygenases (red) and dibenzofuran dioxygenases (pink) (Fig. 9). This is in accordance with the classification proposed by Nam et al. (223). Oxygenase components were classified into 4 groups (groups I, II, III, and IV). While group I contains broad-range oxygenases sharing low homology, groups II, III, and IV contain some typical oxygenases: benzoate/toluate dioxygenases for group II, naphthalene/polycyclic aromatic hydrocarbon dioxygenases for group III, and benzene/toluene/biphenyl dioxygenases for group IV (223).

PAH oxidizing dioxygenases cluster regarding their respective substrate and are much more dispersed. However it is difficult to group these dioxygenases given the fact that they oxidize a wide range of substances.

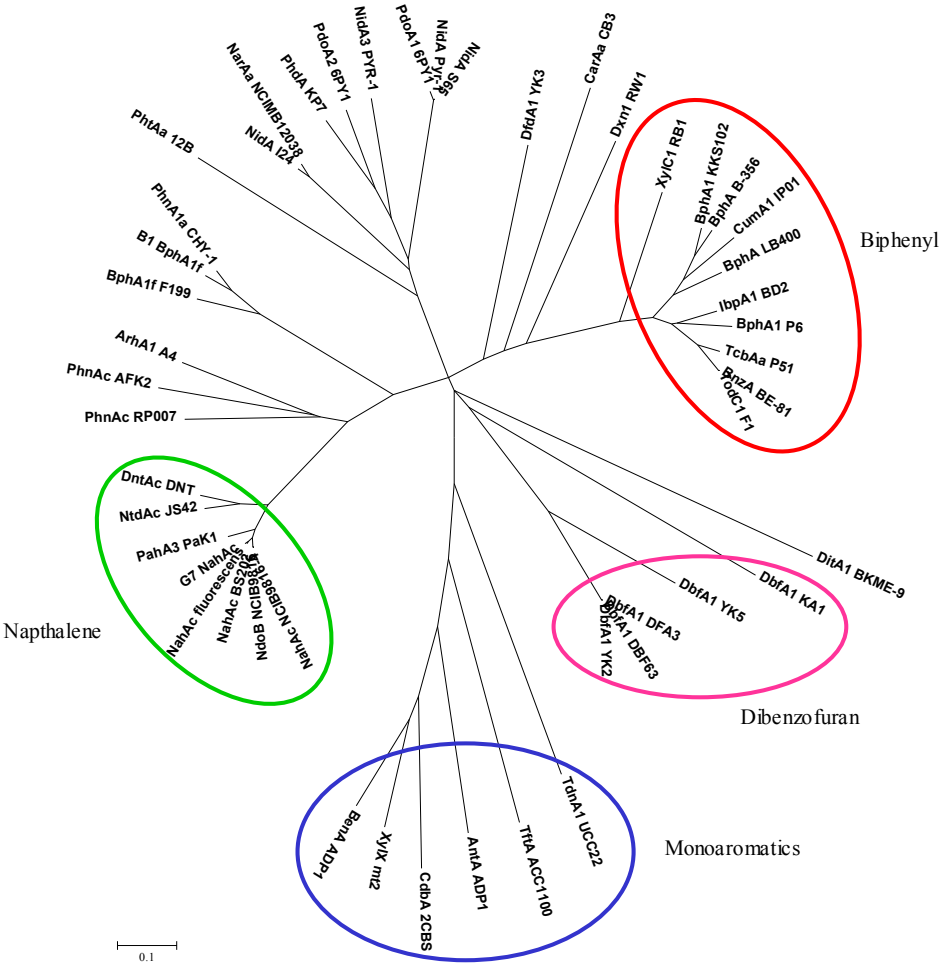

Figure 9. Phylogenetic distribution of initial dioxygenase α subunits. The dendrogram was constructed from a ClustalW alignment by neighbour-joining analysis using MegA3.1 software (183). Sequences were retrieved from GenBank database. The scale bar represents substitutions per amino acid. NidA I24, indene, *Rhodococcus* sp. I24 (AF121905); PhdA KP7, phenanthrene, *Nocardioides* sp. KP7 (AB017794); NarAa NCIMB12038, naphthalene, *Rhodococcus* sp. NCIMB12038 (AF082663); DitA1 BKME-9, diterprenoid, *Pseudomonas abietaniphila* BKME-9 (AF119621); IpbA1 BD2, isopropylbenzene, *Rhodococcus erythropolis* BD2 (U24277); BphA1 P6, biphenyl, *Rhodococcus globerulus* P6 (X80041); TcbAa P51, chlorobenzene, *Pseudomonas* sp. P51 (U15298); BnzA BE-81, benzene, *Pseudomonas putida* BE-81 (M17904); TodC1 F1, toluene, *Pseudomonas putida* F1 (J04996); XylC1 RB1, unknown substrate, *Cycloclasticus oligotrophus* RB1 (U51165); BphA LB400,

biphenyl, *Burkholderia cepacia* LB400 (M86348); BphA1 KKS102, biphenyl, *Pseudomonas* sp. KKS102 (D17319); BphA B-356, biphenyl, *Comamonas testosteroni* B-356 (U47637); CumA1 IP01, cymene, *Pseudomonas fluorescens* IP01 (D37828); TdnA1 UCC22, aniline, *Pseudomonas putida* UCC22 (D85415); TftA ACC1100, 2,4,5-trichlorophenoxyacetic acid, *Burkholderia cepacia* AC1100 (U11420); AntA ADP1, anthranilate, *Acinetobacter calcoaceticus* ADP1 (AF071556); CbdA 2CBS, 2-halobenzoate, *Burkholderia cepacia* 2CBS (X79076); XylX mt2, toluate, *Pseudomonas putida* mt2 (M64747); BenA ADP1, benzoate, *Acinetobacter calcoaceticus* ADP1 (AF009224); DxnA1 RW1, dibenzo-*p*-dioxin, *Sphingomonas* sp. RW1 (X72850); CarAa CB3, carbazole, *Sphingomonas* sp. CB3 (AF060489); NdoB NCIB9816, naphthalene, *Pseudomonas putida* NCIB9816 (M23914); PahA3 PaK1, naphthalene, *Pseudomonas aeruginosa* PaK1 (D84146);NtdAc JS42, 2-nitrotoluène, *Pseudomonas* sp. JS42 (U49504); DntAc DNT, 2,4-dinitrotoluène, *Burkholderia* sp. DNT (U62430); PhnAc RP007, phenanthrene, *Burkholderia* sp. RP007 (AF061751); PhnAc AFK2, phenanthrene, *Alcalingenes faecalis* AFK2 (AB024945); PhtAa, phthalate, *Arthrobacter keyseri* 12B (AF331043); BphA1f F199, biphenyl, *Sphingomonas aromaticivorans* F199 (AF079317); NidA PYR-1, pyrene, *Mycobacterium vanbaalenii* PYR-1 (AF249301); NidA S65, pyrene, *Mycobacterium* sp. S65 (AF546904); DbfA1, carbazole, *Sphingomonas* sp. KA1 (BAF03470); DbfA1, dibenzofuran, *Terrabacter* sp. strain DBF63 (BAC75993); DbfA1, dibenzofuran, *Rhodococcus* sp. strain YK2 (BAC00802); DbfA1, dibenzofuran, *Paenibacillus* sp. strain YK5 (BAE53401); DbfA1, dibenzofuran, *Rhodococcus* sp. strain DFA3 (BAD51811); DbfA1, dibenzofuran, *Terrabacter* sp. strain YK3 (BAC06602); ArhA1, acenaphthene and acenaphthylene, *Sphingomonas* sp. strain A4 (BAD34447); BphA1f, biphenyl, *Sphingomonas yanoikuyae* B1 (ABM91740); PhnA1a, chrysene, *Sphingomonas* sp. strain CHY-1 (AJ633551); PdoA1, pyrene, *Mycobacterium* sp. strain 6PY1 (CAD38647); PdoA2, pyrene, *Mycobacterium* sp. strain 6PY1 (CAD38643); NidA3, pyrene, *Mycobacterium vanbaalenii* PYR-1 (AAY85176); NahAc, naphthalene, *Pseudomonas putida* G7 (YP_534822); NahAc, naphthalene, *Pseudomonas fluorescens* (AAL07262); NahAc, naphthalene, *Pseudomonas putida* BS202 (AAB62707); NahAc, naphthalene, *Pseudomonas putida* NCIB9816-4 (NP_863072). A more exhaustive classification system for oxygenase components is given by Nam et al. (223). Naphthalene dioxygenases cluster together (green) as well as dibenzofuran dioxygenases (pink), monoaromatic dioxygenases (blue) and biphenyl dioxygenases (red).

The *nah*-like group encodes enzymes from various *Pseudomonas* species such as NahAc from strains G7 and NCIB9816-4. *phn*-like genes encoding enzymes such as PhnAc from *Burkholderia* sp. RP007 are not found in the vicinity of *Pseudomonas*-like dioxygenases.

Sphingomonads dioxygenases do not cluster with these enzymes and form a group apart. Even though *Sphingobium yanoikuyae* B1 was isolated on biphenyl, its terminal dioxygenase component α subunit (BphA1f) does not cluster with other biphenyl dioxygenases. This reflects that *Sphingomonas* species harbour broad-range dioxygenases compared to other strains. A more detailed description of dioxygenase classification is given by Nam et al. in ref (223).

Dioxygenases involved in naphthalene, phenanthrene and pyrene degradation have been identified in many Gram-positive strains including *Rhodococcus* (3, 182, 185, 313), *Nocardioides* (266, 267) and *Mycobacterium* (157, 181). These proteins do not cluster with Gram-negative proteins to which they are only distantly related. There are however some exceptions where protein from Gram-positive bacteria were found in sphingomonads. *Sphingomonas* sp. strain KA1 can grow on carbazole as sole source of carbon and energy (289). This strain harbours angular dioxygenase genes that cluster with DBF63 from *Terrabacter* sp. DBF63 (108). However the function of these genes could not be determined yet.

In the past ten years much interest has been turned to the study of these enzymes and novel techniques are adapted to be applied to environmental strains. Metagenomic approaches are starting to complement the standard microbiological methodology (193, 260). Since the different proteins share common domains, the total DNA can be cloned and screened using known genes as probes accelerating the detection of dioxygenases. Furthermore catabolic genes can be induced and expressed by relevant substrates and clones carrying those genes identified.

Proteomic approaches are also starting to play an important role in the isolation of genes involved in PAH degradation (56, 166, 168, 181, 184, 189, 330). The continued detection of PAH induced proteins will allow the identification of the corresponding genes, thus giving further insight in the complex catabolic operons from environmentally useful bacteria.

Part II: Objectives

Since dioxygenases determine the substrate range that can be degraded by a given bacterial strain, the study of this enzyme class is important to gain knowledge of bacterial degradation pathways. In contrast, enzymes involved in the intermediary metabolism catalyse one, and only one, specific reaction. These intermediary-metabolism enzymes need to be highly substrate specific to control pathway fluxes. For example, isomers of simple molecules, such as sugars, are channelled into different pathways. Enzymes must be highly selective to discriminate among similar structures. On the other hand, dioxygenases carrying out the first attack must be able to utilize a variable set of compounds. This relaxed substrate specificity likely arose because similar chemical structures are often found together in nature, for example in petroleum. For instance naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 is known to catalyse the oxidation of more than 75 different substrates (256). We focus on characterizing the enzymes involved in fluorene degradation by *Sphingomonas* sp. strain LB126 and phenanthrene degradation by *Sphingomonas* sp. strain LH128.

The bacterium *Sphingomonas* sp. strain LB126, examined in chapters 1 and 2 of the "Results and Experimental Work" section, is able to metabolise fluorene and cometabolise dibenzothiophene, carbazole, anthracene, phenanthrene and fluoranthene (17). Interestingly strain LB126 cannot use carbazole, dibenzofuran, dibenzothiophene or dibenzo-*p*-dioxin, the heteroaromatic analogues of fluorene, as sole source of carbon and energy. Fluorene degradation by other aerobic bacteria has been investigated and metabolites identified, but no information is available regarding the initial enzymology involved in fluorene degradation in Gram-negative bacteria. For instance. *Pseudomonas* sp strain F101 can grow on fluorene as sole source of carbon and energy (98). Two productive routes are initiated by dioxygenation at positions 1,2 and 3,4, respectively. A third non-productive route accumulates 9-fluorenone (36). However no information regarding catabolic genes from strain F101 is available. PAH catabolic pathways of strain LB126 have already been investigated and metabolites produced during the degradation of fluorene (331), or other PAHs with fluorene as co-substrate have been

identified (324). During a genetic analysis on strain LB126, the genes involved in the breakdown of protocatechuic acid (331), a key metabolite in fluorene degradation, were identified but the genes that govern the initial attacks on fluorene still remain unknown. *Sphingomonas* sp. LB126 initiates fluorene degradation by a monooxygenation at position C9 to yield 9-fluorenone; the second step involves angular dioxygenation of the formed product (331). This pathway, that is non-productive in *Pseudomonas* sp. strain F101, leads to the complete mineralisation of fluorene by strain LB126. It is unclear if the same enzyme is responsible for the mono- and angular oxygenation as shown in *Terrabacter* sp. strain DBF63 (108) or if these reactions need multiple enzymes. Therefore, our objective was to identify and isolate the enzymes involved in the first attack on fluorene. Moreover the selectivities of dioxygenases are important to direct biocatalysis or metabolic engineering towards a specific molecule in bioremediation systems.

The bacterium *Sphingomonas* sp. strain LH128 is able to grow on phenanthrene and, in addition, is able to cometabolise anthracene, dibenzothiophene and fluorene (19). No genetic analysis has ever been undertaken. In order to identify catabolic genes, metabolites produced during phenanthrene degradation were identified and the genes involved in these steps investigated. The fact that all phenanthrene-degrading sphingomonads carry a similar pathway organization as found in *Sphingomonas* sp. strain CHY-1, *Sphingobium yanoikuyae* B1, *Novosphingobium aromaticivorans* F199 and *Sphingobium* sp. strain P2, indicates that this organization is quite stable and well-evolved despite the apparent complex organization compared to the more logical organization of PAH-degradation genes in *Pseudomonas*. The study of *Sphingomonas* sp. strain LH128 initial dioxygenase and catabolic genes can contribute to understand how this organization arose. These data could help to explain that *Sphingomonas* sp. started as phenanthrene degraders and their respective initial dioxygenase became substrate-relaxed in order to oxidize a large variety of PAHs.

Part III – Results and Experimental Work

Chapter 1: Characterization of a novel angular dioxygenase from fluorene-degrading *Sphingomonas* sp. strain LB126

Fluorene, a tricyclic aromatic hydrocarbon containing a five-membered ring, is an environmental contaminant in areas impacted, amongst others, by creosote and oil spills (29). Some oxygenases have been reported to oxidize fluorene but the strains harbouring these enzymes could not use fluorene as a sole source of carbon and energy.

Sphingomonas sp. strain LB126 has been isolated for its ability to use fluorene as sole carbon source and is therefore suitable to study the initial attack on this pollutant. Since almost no data is available regarding fluorene degradation in Gram-negative bacteria, the study of strain LB126 could help to elucidate what enzymes are involved in the upper fluorene catabolic pathway. Fluorene degradation by this strain has already been investigated and metabolites formed during the catabolic pathway were identified suggesting an angular oxidation pathway (331). The methylenic group in fluorene undergoes oxidation before angular dioxygenation occurs (Fig. 1). This mechanism has not been clearly elucidated and the enzymology of the five-membered ring attack on fluorene has not been thoroughly studied. It was impossible to determine if the angular dioxygenase or some other oxygenase was responsible for initial monohydroxylation.

The chapter 1 describes the identification, isolation and functional analysis of an enzyme governing the initial oxidative steps in fluorene degradation by *Sphingomonas* sp. strain LB126. This article was accepted to *Applied and Environmental Microbiology* (under revision).

Authors contributions: LS is the main contributor to designing, performing research and wrote the paper; designed research: LS, SNC, CM, YJ, GZ, PH, SA; performed research: LS, SNC, CM, YJ; analyzed data: LS, SNC, CM, YJ.

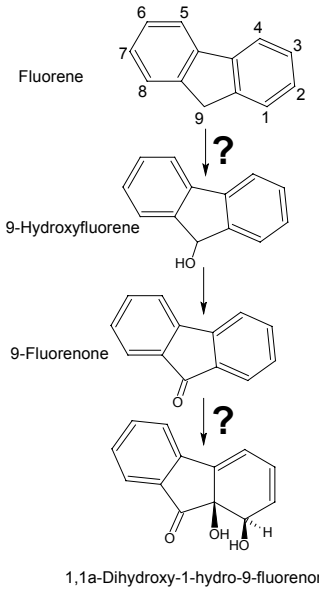

Figure 1. Steps investigated in fluorene oxidation by *Sphingomonas* sp. strain LB126. Metabolites have already been identified but the enzymes responsible for these compounds remain unknown (331). Further metabolism proceeds via protocatechuic acid.

Characterization of a novel angular dioxygenase from fluorene-degrading *Sphingomonas* sp. strain LB126

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Running Title: Angular dioxygenase from *Sphingomonas* sp. LB126

Keywords: Biodegradation, catabolic gene cluster, polycyclic aromatic hydrocarbons, heterologous expression

Abstract

In this study, the genes involved in the initial attack on fluorene by *Sphingomonas* sp. strain LB126 were investigated. The α and β subunits of a dioxygenase complex (FlnA1A2), showing 63% and 51% sequence identity respectively, with the subunits of an angular dioxygenase from the Gram-positive dibenzofuran degrader *Terrabacter* sp. strain DBF63, were identified. When overexpressed in *E. coli*, FlnA1A2 was responsible for the angular oxidation of fluorene, 9-hydroxyfluorene, 9-fluorenone, dibenzofuran and dibenzo-*p*-dioxin. Moreover, FlnA1A2 was able to oxidize polycyclic aromatic hydrocarbons and heteroaromatics, some of which were not oxidized by the dioxygenase from *Terrabacter* sp. strain DBF63. Quantification

of resulting oxidation products showed that fluorene and phenanthrene were the preferred substrates of FlnA1A2.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants and are released during the burning, handling or disposal of organic matter including coal tars, crude oil and petroleum products. There are some natural origins, such as forest fires or natural oil seeps, but PAHs mainly arise from combustion- or oil-related anthropogenic activities. A number of organisms that are able to use PAHs as sole source of carbon and energy have been isolated (39) and bioremediation strategies using these organisms have been proposed (138).

Fluorene, a tricyclic aromatic hydrocarbon containing a five-membered ring, offers a variety of possibilities for biochemical attack. Two of these pathways are initiated by a dioxygenation at the 1,2- (36, 98) or 3,4- positions (36, 99, 212) (Fig. 1). The corresponding *cis*-dihydrodiols undergo dehydrogenation, then *meta*-cleavage. The third route (282, 314) is initiated by monooxygenation at the C-9 position to give 9-hydroxyfluorene, which is then dehydrogenated to 9-fluorenone. This route is only productive if a subsequent angular carbon dioxygenation forms 1-hydro-1,1a-dihydroxy-9-fluorenone, leading to phthalate, which is further degraded via protocatechuate (100, 212, 314) (Fig. 1).

Sphingomonads have been intensively studied for their ability to degrade a wide range of aromatic hydrocarbons (239, 241, 300, 301, 336, 354). The function and organization of PAH catabolic genes in *Sphingomonas* often remains obscure since the genes involved in the degradation of aromatic compounds are not always arranged in discrete operons but are frequently dispersed throughout the genome. *Sphingomonas* sp. strain LB126 was isolated from PAH contaminated

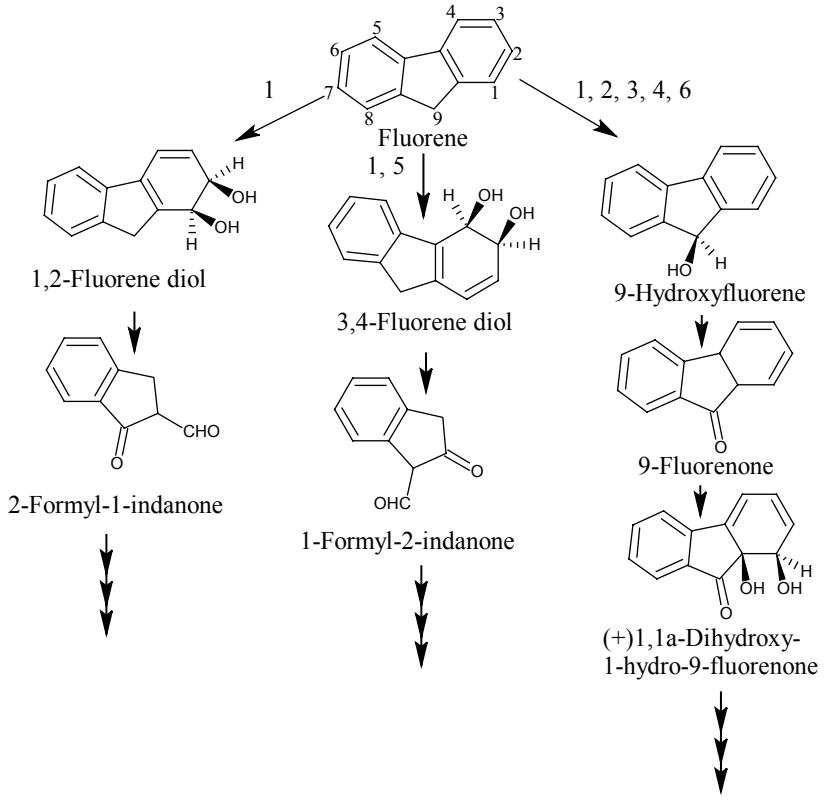

Figure 1. Proposed pathways for fluorene degradation and bacteria involved. 1, *Pseudomonas* sp. strain F101 (36, 98); 2, *Terrabacter* sp. strain DBF63 (212); 3, *Brevibacterium* sp. strain DPO1361 (314); 4, *Pseudomonas* sp. strain F274 (100); 5, *Burkholderia cepacia* F297 (101); 6, *Sphingomonas* sp. strain LB126 (331).

soil and is capable of utilizing fluorene as sole carbon source (19). Fluorene degradation by strain LB126 has been previously investigated (331), but the enzymes that govern the initial attack on fluorene were not identified.

In contrast, more genetic work done in Gram-positive (107, 108) showed that dibenzofuran degrading bacterium *Terrabacter* sp. strain DBF63, can also oxidize fluorene thanks to a cluster of plasmid-borne catabolic genes. The oxygenase component of an angular dioxygenase

complex, encoded by *dbfA1A2*, does not cluster with already known dioxygenases. Few data are available regarding genes involved in fluorene degradation by Gram-negative bacteria. Although many PAH dioxygenases can oxidize fluorene, the respective host strains cannot use fluorene as sole carbon source. Recently, the catabolic plasmid pCAR3 from *Sphingomonas* sp. strain KA1 was described (289). Genes homologous to *dbfA1A2* were found on pCAR3, as well as all genes necessary for the complete degradation of fluorene, but strain KA1 is unable to grow on fluorene as sole source of carbon. We present here the first report, to our knowledge, of genes governing angular attack on fluorene in Gram-negative *Sphingomonas* sp. strain LB126 using fluorene as the sole source of carbon and energy.

Materials and Methods

Bacterial strains, plasmids, and media.

Sphingomonas sp. strain LB126 uses fluorene as the sole source of carbon and energy (19) and was kindly provided by VITO (Vlaamse Instelling voor Technologisch Onderzoek, Belgium). *Escherichia coli* Top10 was used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene expression analysis. PCR amplicons were either cloned into pDrive (Qiagen, Valencia, CA), pGEM-T-easy vector (Promega, Madison, WI), pGEM5Z vector (Promega, Madison, WI) while pET30f (Novagen, San Diego, CA) was used as expression vector. MM284 minimal medium (208) was used for growing *Sphingomonas* sp. strain LB126 and was supplemented with phosphate buffer (50 mM; KH_2PO_4, K_2HPO_4, pH 7.2) instead of Tris buffer. Fluorene was provided as crystals in both solid and liquid media. LB broth (269) was used as complete medium for growing *E. coli* strains. Solid media contained 2% agar. When needed, ampicillin, streptomycin or kanamycin was added to the medium at 100, 200 and 20 µg/ml, respectively. *Sphingomonas* sp. strain LB126 was grown at 30°C, and *E. coli* strains were grown at 37°C. Bacterial growth was determined by optical density readings at 600 nm (OD_{600}).

DNA manipulations and molecular techniques.

Total DNA from pure cultures of *Sphingomonas* sp. strain LB126 was extracted using the UltraClean DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's recommendations or using standard methods (269) when a higher DNA concentration was needed. Plasmid DNA extractions, restriction enzyme digestions, ligations, transformations, sequencing and agarose gel electrophoresis were carried out using standard methods (269).

Polymerase chain reaction (PCR).

Degenerate primers for amplifying conserved sequences of the gene encoding the angular dioxygenase were used as described elsewhere (122). PCR products were purified and cloned into either the pGEM-T or pDrive plasmids. The RT-PCR reactions were performed in 25 µl with 5 ng of total RNA and 20 pmol of each primer with OneStep RT-PCR Kit (Qiagen, Valencia, CA). Total RNA extractions were performed using the RNeasy kit (Qiagen, Valencia, CA) and further purified by spin column and DNase I treatment according to the manufacturer's instructions. The thermocycler program used for the RT-PCR reactions was as follows: 60°C for 30 min, 94°C for 15 min, 30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 45 s), and 72°C for 7 min. The following primer combinations: AD1: 5'-CGTGACGGCGCGCTTC -3' and AD2: 5'-CATGCGAAACTTCATC -3'; flnE-F: 5'- CTGATCGCACTGGCG -3' and flnE-R: 5'- CGTTTCACCGACGTG -3'; cirA-F: 5'-GATCGGCTGGAAGTC -3' and cirA-R: 5'-CCGAGCTTCACGTCG -3' were used to amplify internal fragments of *flnA1* (252 bp), *flnE* (437 bp) and *cirA* (554 bp) respectively. The 16S rDNA specific primers 63F (199) and 518R (220) were used as control.

Southern Blot and cloning of catabolic genes.

Genomic DNA (2 µg) was digested with either *Bam*HI, *Not*I, *Nsi*I or a combination of these enzymes, the fragments were separated by gel electrophoresis, blotted onto a positively charged nylon membrane (Amersham, Buckinghamshire, UK) using standard protocols (269). For Southern Blot detection a PCR-amplified DIG-labelled probe was

prepared according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany) using primers described by Iida et al. (122). Pre-hybridisation and hybridisation were carried out at 68°C. After hybridisation, the membrane was washed twice with 2 x SSC (20 x SSC: NaCl, 3 mol/l; Na-citrate, 0.3 mol/l; pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) (w/v) for 5 min at room temperature and twice with 0.1 x SSC containing 0.1% SDS for 15 min at 68°C. Detection was carried out following standard protocols (269). To isolate catabolic genes, total DNA (10 µg) was digested with *Bam*HI and *Nsi*I separated by gel electrophoresis and DNA fragments of about 7 kb were recovered from the agarose gel using the UltraClean GelSpin DNA Extraction kit (MoBio, Carlsbad, CA). The obtained DNA was cloned into pGEM5Z (Promega, Madison, WI) and transformed into *E. coli* Top10 (Invitrogen, Carlsbad, CA). Resulting clones were screened as pools of 10 clones by PCR using the above-mentioned primers (122). A 6.9-kb plasmid containing the angular dioxygenase gene was identified and sequenced by primer walking.

Construction of plasmids for protein overexpression.

Construction of the plasmids used in this study involved multiple PCR amplifications and cloning steps. The *flnA1A2* fragment (1842 bp) was amplified by PCR with the primer pairs: 5'-*CATATG*GCCACAGCCCTCATGAACCACCC-3' and 5'-*AAGCTT*GGCGCTCACAGGAACACCG-3', introducing *Nde*I and *Hin*dIII sites (italics) at the ends of the amplicon. The PCR product was cloned into pDrive (Qiagen), sequenced, then subcloned into the *Nde*I and *Hin*dIII site of expression vector pET-30f (Novagen). This construct was transformed into *E. coli* BL21(DE3) for expression analysis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Bacterial cells were pelleted by centrifugation and washed with 10 ml ice-cold phosphate buffer (140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM NaH_2PO_4, pH 7.4). 1 ml of ice-cold phosphate buffer was added to the pellet and 550 µl of the suspension was subjected to

sonication on ice for 20 s (5 s pulse interval; 40% of maximum amplitude). After centrifugation the supernatant and the pellet were mixed with an equal volume of loading solution. SDS-PAGE was performed on 13.3 % polyacrylamide mini gels. After electrophoresis, protein staining was performed with Coomassie brilliant blue R-250.

Dioxygenase overexpression and in vivo assays.

Strain BL21(DE3)(pET30f*flnA1A2*) was grown overnight in 3 ml LB medium with the suitable antibiotics. This culture was used to inoculate 25 ml LB medium (0.1% by volume), which was incubated at 42°C until an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM. The cells were further incubated for 7 h at 25°C. For in vivo assays, cells were centrifuged, washed and resuspended to an OD_{600} of approximately 2 in M9 medium (269) containing 0.2% glucose. Cells (25 ml) were incubated for 48 h at 25°C with 5 ml silicone oil containing 0.1 g/l of each tested PAH.

GC-MS analysis of PAH oxidation products.

Water-soluble products resulting from PAH oxidation were extracted from the aqueous phase of bacterial suspension by using columns filled with reverse phase-bonded silica (Upti-clean C18U, 0.5 g, Interchim, Montluçon, France). Columns were washed with 10 ml water then eluted with 1 ml ethyl acetate. The solvent was dried over sodium sulphate and evaporated under nitrogen gas. The dried extracts were then dissolved in 100 or 200 µl acetonitrile, before being derivatized with *N,O-bis*(trimethylsilyl) trifluoroacetamide: trimethylchlorosilane (BSTFA) or *n*-butylboronate (NBB). In order to quantify the dihydrodiols formed upon incubation of BL21(DE3)(pET30f*flnA1A2*) recombinant cells with PAHs, 2.3-dihydrobiphenyl (Sigma-Aldrich, St. Louis, MO) was added to 0.1 µM final concentration in the aqueous phase prior to solid phase extraction, and was used as an internal standard. After derivatization and GC-MS analysis, NBB dihydrodiol derivates were quantified on the basis of peak area using a calibration curve generated by analysing known amounts of anthracene 1,2-dihydrodiol. GC-MS analysis of trimethylsilyl derivatives was carried out as previously described (141). NBB derivatives were separated on MDN-12 capillary column (30 m, 0.25 mm internal diameter; Supelco) using helium as carrier

gas at 1 ml/min. The oven temperature was held at 75°C for 1 min, then increased to 300°C at a rate of 14°C min^{-1}, and held at 300°C for 8 min. The mass spectrometer was operated in the selected ion monitoring mode, selecting *m/z* values corresponding to the expected masses (M^+) of the dihydrodiol derivatives (228 for naphthalene, 278 for anthracene and phenanthrene). The NBB derivative of trihydroxybiphenyl, the oxidation product of dibenzofuran, was monitored at a *m/z* value of 268. The fluorene derivative was detected at a *m/z* of 196 (M^+- OBC_4H_9), because in contrast to other dihydrodiol derivatives, the abundance of the M^+ ion was very low.

DNA and protein sequence analysis.

Sequence analysis was performed using the DNASTAR software package (Lasergene Inc., Madison, WI*)*. The BLAST search tool was used for homology searches (2). Multiple alignments and phylogenetic trees were produced using the DNASTAR and MEGA3.1 softwares (183).

Nucleotide sequence accession number.

The nucleotide sequence described in this report has been deposited in the Genbank database under accession number EU024110.

Results and Discussion

Cloning and sequence analysis of genes encoding a novel angular dioxygenase.

Sphingomonas strain LB126 has been studied for its ability to grow on fluorene and degrade phenanthrene, anthracene and fluoranthene by cometabolism in the presence of pyruvate (324). In order to detect genes potentially involved in the initial attack of PAHs, a PCR strategy was chosen. The genes involved in fluorene oxidation in strain LB126 were expected to display some similarity with counterparts already described in other PAH degrading *Sphingomonas* strains. Several primer pairs corresponding to conserved domains of previously described PAH dioxygenases were tested (56, 144, 185,

218), but no amplification could be obtained (data not shown). Given the dearth of information regarding fluorene degradation genes in Gram-negative bacteria, primers specific to angular dioxygenase genes from Gram-positive bacteria were tested.

Using a set of such primers (122) and total DNA as a template, a 267 bp DNA fragment was amplified, which upon sequencing and translation, revealed 57 % of protein sequence identity with a peptide internal to the dibenzofuran 4,4a-dioxygenase α subunit of *Terrabacter* sp. strain DBF63 (150). The 267 bp fragment was then used as a DIG labelled probe in Southern blot experiments on whole genome extracts of strain LB126. A 6.9-kb fragment encoding four entire open reading frames (ORF) (ORFs 3-6) and three truncated ones (ORFs 1, 2 and 7) was cloned and sequenced as described in Materials and Methods (Table 1). ORF1 gene product did not share amino acid sequence similarities with previously described fluorene catabolic genes, but showed significant homology to the TonB-dependent receptor CirA from *Sphingomonas wittichii* strain RW1 (36%) and *Novosphingobium aromaticivorans* F199 (34%). ORF2 encoded a truncated transposase, suggesting that the adjacent gene cluster was probably acquired by horizontal transfer although no change in GC-content was noticed. ORFs 3-7 showed a genetic organization similar to that of the dibenzofuran catabolic operon from *Terrabacter* sp. strain DBF63 (150) (Fig. 3). Nevertheless, the product of ORF3, a putative dehydrogenase, did not share significant protein sequence similarity with its counterpart (FlnB) from strain DBF63. The highest degree of similarity was found with putative dehydrogenases identified in whole genome sequencing projects of *Mycobacterium* strains MCS and KMS. ORF4 and ORF5 encode the α and β subunits of a putative angular dioxygenase (FlnA1A2) suggested to be involved in fluorene degradation (see below). Their amino acid sequence showed moderate identity (63% and 51%) with DbfA1 and DbfA2 from strain DBF63. Phylogenetic analysis revealed that the ORF4 product did not cluster with dioxygenase α subunits from other sphingomonads, and was only distantly related to the angular dioxygenase from *Sphingomonas witichii* strain RW1 (30) (Fig. 4). The closest homologues within Sphingomonads were the dioxygenase α subunits from the carbazole-degrading strains *Sphingomonas* sp. strain KA1 (36 % amino acid identity) (289) and *Sphingomonas* sp. strain CB3 (35 % amino acid identity) (285).

Table 1. Homology search analyses of the recovered ORFs from *Sphingomonas* sp. LB126.

ORF	Gene	Probable function or product	Homologous protein	Source	Protein Identity (%)	Accession number
ORF1	'*cirA*	TonB-dependent	CirA	*Sphingomonas wittichii* RW1	36	YP_001262040
		receptor	CirA	*Novosphingobium aromaticivorans* DSM 12444	34	YP_001165948
ORF2	'*tnp*'	Transposase	Transposase	*Mesorhizobium loti* MAFF303099	60	NP_085624
			Transposase	*Sinorhizobium medicae* WSM419	57	EAU08642
ORF3	*flnB*	Probable	probable dehydrogenase	*Mycobacterium* sp. MCS	40	ABG07792
		dehydrogenase	probable dehydrogenase	*Mycobacterium* sp. KMS	40	ZP_01286209
			probable dehydrogenase	*Rhodobacterales* sp. HTCC2654	28	ZP_01014534
ORF4	*flnA1*	angular	DbfA1	*Terrabacter* sp. DBF63	63	BAC75993
		dioxygenase α	DbfA1_YK2	*Rhodococcus* sp. YK2	54	BAC00802
		subunit	DbfA1	*Paenibacillus* sp. YK5	52	BAE53401
ORF5	*flnA2*	angular	DbfA2	*Terrabacter* sp. DBF63	52	BAC75994
		dioxygenase β	DbfA2_YK2	*Rhodococcus* sp. YK2	51	BAC00803
		subunit	DbfA2	*Paenibacillus* sp. YK5	48	BAE53402
ORF6	*flnE*	hydrolase	FlnE	*Terrabacter* sp. DBF63	42	BAE45094
			ORF4	*Rhodococcus* sp. YK2	42	BAC00805
			A/b hydrolase_1	*Mycobacterium* sp. MCS	30	YP_642596
ORF7	*flnD1*'	extradiol	FlnD1	*Terrabacter* sp. DBF63	12	BAC75996
		dioxygenase α	BphC6	Rhodococcus rhodochrous	12	BAD10908
		subunit	Edi4	*Rhodococcus* sp. YK2	12	BAC00806

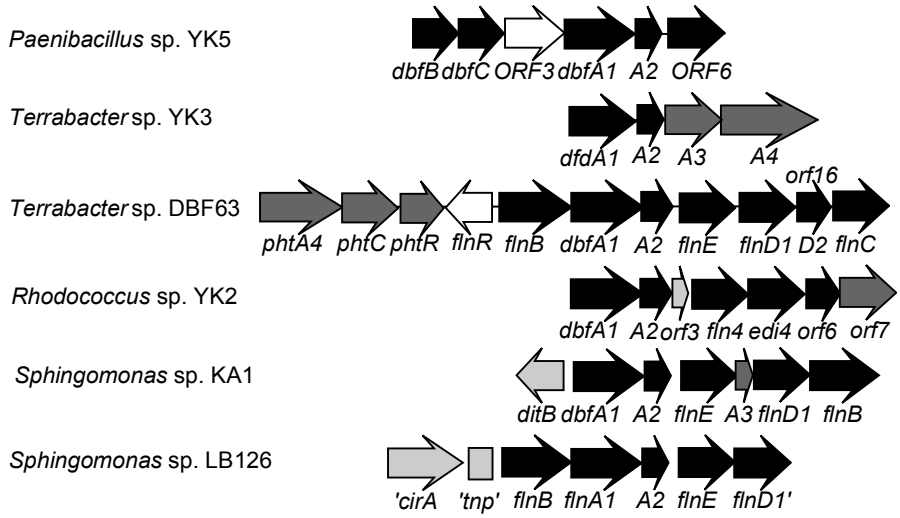

Figure 3. Genetic organization of the 6.9-kb DNA region containing fluorene catabolic genes in *Sphingomonas* sp. strain LB126 compared to *Paenibacillus* sp. strain YK5 (AB201843), *Terrabacter* sp. strain YK3 (AB075242), *Rhodococcus* sp. strain YK2 (AB070456) and *Sphingomonas* sp. strain KA1 (NC_008308) and *Terrabacter* sp. strain DBF63 (AP008980). The arrows indicate location and direction of transcription of the ORFs. Black arrows represent genes involved in the initial attack on fluorene; dark grey arrows indicate genes involved in the electron transport chain or phthalate degradation (*pht*), white arrows indicate regulatory genes and light grey arrows represent genes not directly involved in fluorene oxidation. Tnp; truncated transposase. ', truncated ORF.

Interestingly, in strain LB126, the genes encoding the lower pathway of fluorene via protocatechuate (331), show homologies with the *lig* genes of *Sphingomonas paucimobilis* SYK-6 (204) and the *fld* genes of *Sphingomonas* sp. strain KA1 (289). ORF5 product shows moderate protein identity (48%) to the β subunit DbfA2 from Gram-positive dibenzofuran-degrading *Paenibacillus* sp. strain YK5 (124). DbfA1 and DbfA2 from strain YK5 are the two subunits of a dioxygenase able to oxidize dibenzo-*p*-dioxin, dibenzothiophene, fluorene, and 9-fluorenone, compounds that could however not be utilized as growth substrates by strain YK5 (124). The product of ORF6, located downstream of *flnA1A2*, shares 42% of protein identity with FlnE, a *meta*-cleavage product hydrolase from strain DBF63 (108).

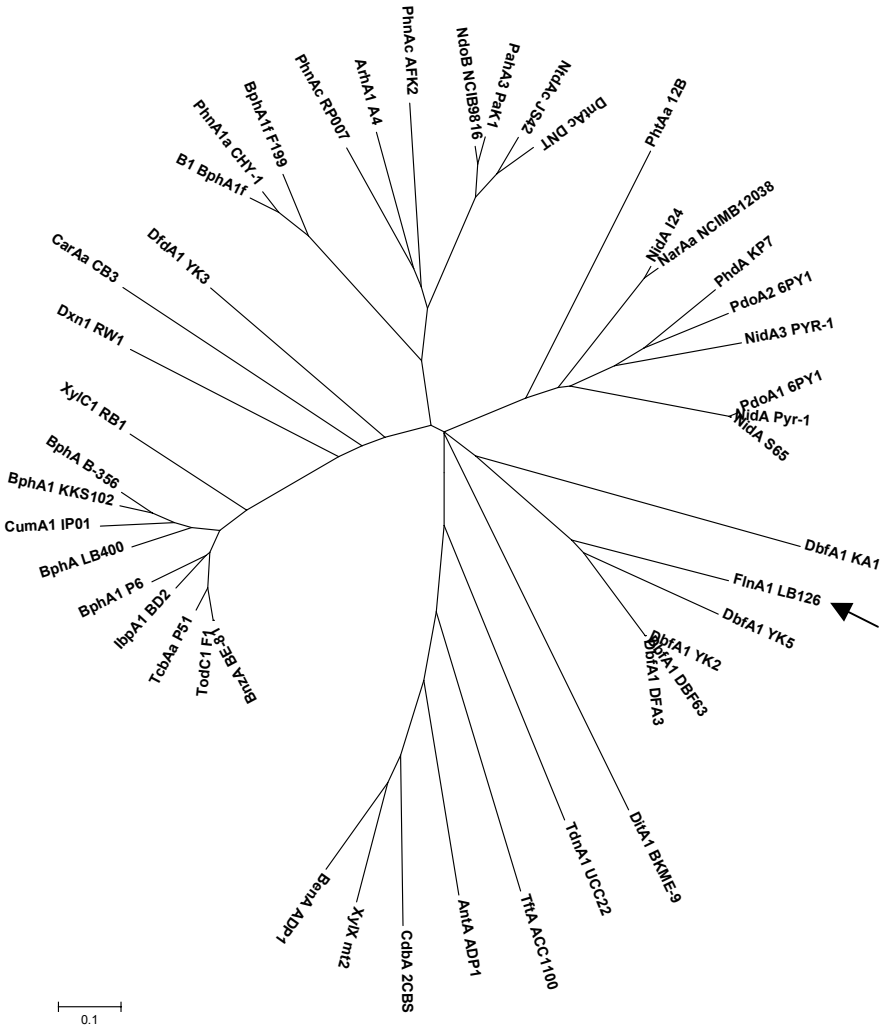

Figure 4. Phylogenetic distribution of initial dioxygenase α subunits. The dendrogram was constructed from a ClustalW alignment by neighbour-joining analysis using MegA3.1 software (183). Sequences were retrieved from GenBank database. The scale bar represents substitutions per amino acid. FlnA1 does not cluster with other *Sphingomonas* dioxygenases and seem more closely related to dioxygenases from Gram-positve strains. NidA I24, indene, *Rhodococcus* sp. I24 (AF121905); PhdA KP7, phenanthrene, *Nocardioides* sp. KP7 (AB017794); NarAa NCIMB12038, naphthalene, *Rhodococcus* sp. NCIMB12038 (AF082663); DitA1 BKME-9, diterprenoid, *Pseudomonas abietaniphila* BKME-9 (AF119621); IpbA1 BD2, isopropylbenzene,

***Rhodococcus erythropolis* BD2 (U24277); BphA1 P6, biphenyl, *Rhodococcus globerulus* P6 (X80041); TcbAa P51, chlorobenzene, *Pseudomonas* sp. P51 (U15298); BnzA BE-81, benzene, *Pseudomonas putida* BE-81 (M17904); TodC1 F1, toluene, *Pseudomonas putida* F1 (J04996); XylC1 RB1, unknown substrate, *Cycloclasticus oligotrophus* RB1 (U51165); BphA LB400, biphenyl, *Burkholderia cepacia* LB400 (M86348); BphA1 KKS102, biphenyl, *Pseudomonas* sp. KKS102 (D17319); BphA B-356, biphenyl, *Comamonas testosteroni* B-356 (U47637); CumA1 IP01, cymene, *Pseudomonas fluorescens* IP01 (D37828); TdnA1 UCC22, aniline, *Pseudomonas putida* UCC22 (D85415); TftA ACC1100, 2,4,5-trichlorophenoxyacetic acid, *Burkholderia cepacia* AC1100 (U11420); AntA ADP1, anthranilate, *Acinetobacter calcoaceticus* ADP1 (AF071556); CbdA 2CBS, 2-halobenzoate, *Burkholderia cepacia* 2CBS (X79076); XylX mt2, toluate, *Pseudomonas putida* mt2 (M64747); BenA ADP1, benzoate, *Acinetobacter calcoaceticus* ADP1 (AF009224); DxnA1 RW1, dibenzo-*p*-dioxin, *Sphingomonas* sp. RW1 (X72850); CarAa CB3, carbazole, *Sphingomonas* sp. CB3 (AF060489); NdoB NCIB9816, naphthalene, *Pseudomonas putida* NCIB9816 (M23914); PahA3 PaK1, naphthalene, *Pseudomonas aeruginosa* PaK1 (D84146);NtdAc JS42, 2-nitrotoluène, *Pseudomonas* sp. JS42 (U49504); DntAc DNT, 2,4-dinitrotoluène, *Burkholderia* sp. DNT (U62430); PhnAc RP007, phenanthrene, *Burkholderia* sp. RP007 (AF061751); PhnAc AFK2, phenanthrene, *Alcalingenes faecalis* AFK2 (AB024945); PhtAa, phthalate, *Arthrobacter keyseri* 12B (AF331043); BphA1f F199, biphenyl, *Sphingomonas aromaticivorans* F199 (AF079317); NidA PYR-1, pyrene, *Mycobacterium vanbaalenii* PYR-1 (AF249301); NidA S65, pyrene, *Mycobacterium* sp. S65 (AF546904); DbfA1, carbazole, *Sphingomonas* sp. KA1 (BAF03470); DbfA1, dibenzofuran, *Terrabacter* sp. strain DBF63 (BAC75993); DbfA1, dibenzofuran, *Rhodococcus* sp. strain YK2 (BAC00802); DbfA1, dibenzofuran, *Paenibacillus* sp. strain YK5 (BAE53401); DbfA1, dibenzofuran, *Rhodococcus* sp. strain DFA3 (BAD51811); DbfA1, dibenzofuran, *Terrabacter* sp. strain YK3 (BAC06602); ArhA1, acenaphthene and acenaphthylene, *Sphingomonas* sp. strain A4 (BAD34447); BphA1f, biphenyl, *Sphingomonas yanoikuyae* B1 (ABM91740); PhnA1a, chrysene, *Sphingomonas* sp. strain CHY-1 (AJ633551); PdoA1, pyrene, *Mycobacterium* sp. strain 6PY1 (CAD38647); PdoA2, pyrene, *Mycobacterium* sp. strain 6PY1; FlnA1, *Sphingomonas* sp. strain LB126 (EU024110).**

The truncated ORF7 gene product showed similarity to an extradiol dioxygenase α subunit (FlnD1) from strain DBF63. Since *flnD1* from strain LB126 lacks a 3' region, no conclusive homology search could be carried out. The genetic organization from the LB126 fragment is similar to the one found in Gram-positive *Terrabacter* sp. strain DBF63 and is unusual to be found in Gram-negative bacteria. The presence of a truncated transposase indicates that this catabolic gene

cluster present in strain LB126 might have been inherited by lateral transfer from other genera of PAH-degrading bacteria (Fig. 3) (240).

Transcriptional analysis by RT-PCR

Transcriptional expression of *cirA, flnA1* and *flnE* was studied by RT-PCR. Total RNA was extracted from cultures in exponential growth (OD_{600} 0.4) of *Sphingomonas* sp. strain LB126 grown on glucose or fluorene. Results indicated that *cirA, flnA1*and *flnE* transcripts are up-regulated in the presence of fluorene and are not or slightly detected when strain LB126 is grown on glucose (Fig. 5). This suggests that the cloned catabolic operon is involved in fluorene degradation by *Sphingomonas* sp. strain LB126.

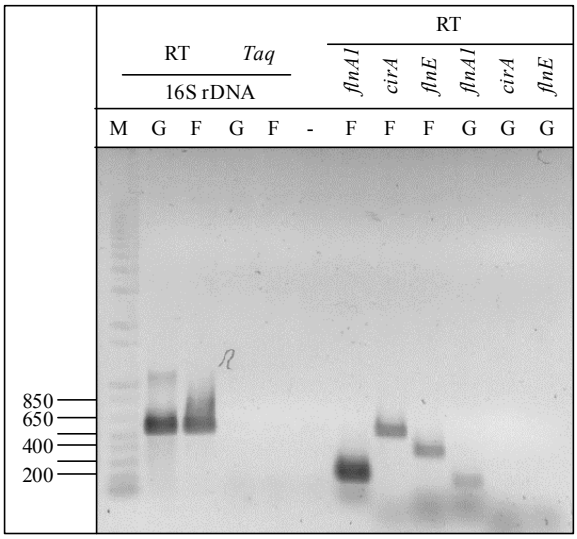

Figure 5. Transcriptional analysis by RT-PCR of the expression of genes *cirA*, *flnA1* and *flnE* induced in *Sphingomonas* sp. strain LB126 during growth on fluorene or glucose. RT-PCR products obtained from template RNA extracted from cells grown on glucose (lanes G) and fluorene (lanes F) were separated by gel electrophoresis. The positions of size markers (in base pairs) (lane M) are indicated on the left (1kb Plus DNA Ladder, Invitrogen, Carlsbad, CA). Differentially amplified RT-PCR fragments indicate differential gene expression in fluorene grown *Sphingomonas* sp. strain LB126 cells. Lane -, negative control without RNA with 16S rDNA primers. RNA quality was checked by amplifying 16S rDNA sequences with reverse transcriptase (RT) and *Taq* polymerase (*Taq*).

Functional expression of FlnA1A2 in *E. coli*.

In order to study the enzymatic activity of FlnA1A2, the corresponding genes were introduced into pET30f and expressed in *E. coli* BL21(DE3). Protein extracts from IPTG-induced cells were separated by SDS-PAGE. The cells overproduced two polypeptides with M_r of 45,000 and 14,000 that did not match exactly the predicted sizes of FlnA1 and FlnA2 as calculated from the deduced polypeptide sequence (49.5 and 19.4 kDa). Differences between the theoretical and apparent molecular masses upon SDS-PAGE gels were also observed for the DbfA1 and DbfA2 dioxygenase components from strain DBF63 (150). Significantly, it was found that the recombinant proteins were inactive and mostly insoluble (Fig. 6). When the recombinant strain was grown at 42°C up to an OD_{600} of 0.5, and subjected to IPTG induction at room temperature, a larger proportion of the FlnA1 and FlnA2 proteins was recovered in the soluble fraction (Fig. 4). In order to assess the enzymatic activity of FlnA1A2 in *E. coli*, biotransformation assays were carried out using induced cells incubated separately with fluorene, carbazole, dibenzofuran, dibenzothiophene and dibenzo-*p*-dioxin, as well as with representative PAHs. Water-soluble oxidation products released into the culture medium were extracted and analyzed using GC-MS. The detection of fluorene oxidation products demonstrated that the recombinant enzyme was active in vivo (Table 2), suggesting that it recruited unspecific electron carriers from the host for function. When strain BL21(DE3)(pET30f), which lacked FlnA1A2, was incubated with the same PAHs under identical conditions, no oxidation product could be detected, demonstrating that FlnA1A2 was indeed responsible for fluorene transformation (Table 2).

Table 2. PAH selectivity of FlnA1A2 from *Sphingomonas* sp. strain LB126 as expressed in *E. coli* and comparison to DFDO (150) and CARDO (229). (Chemical structures are shown in Fig 5)

Substrate	Possible products	Principal fragment ions[a]	Retention Time (min)	Yield (%)[b]	DFDO[c]	CARDO[c]
Naphthalene	*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene[d]	306 (8), 275 (5), 203 (32), 191 (100)	13.455	92.9	+	+
	1-naphthol[d,g]	216 (86), 201 (100), 185 (46), 141 (24)	12.375	7.1	+	+
Biphenyl	*cis*-2,3-Dihydroxy-2,3-dihydrobiphenyl[d]	332 (52), 243 (22), 227 (100), 211(18)	15.134	83.9	+	+
	2-Hydroxybiphenyl[d, g]	242 (48), 227 (76), 211 (100), 165 (7), 152 (20)	12.910	8.5	+	+
	3-Hydroxybiphenyl[d, g]	242 (74), 227 (100), 211 (47), 165 (8), 152 (22)	14.214	7.6	+	+
Phenanthrene	*cis*-9,10-Dihydroxy-9,10-dihydrophenanthrene[e]	356 (16), 253, 191, 147 (100), 73 (99)	16.728	95.1	-	+
	Monohydroxyphenanthrene[g]	266 (100), 251 (65), 235 (27), 176 13)	17.464	4.1	-	+
Anthracene	*cis*-1,2-Dihydroxy-1,2-dihydroanthracene[d, f]	356 (5), 266 (13), 253 (34), 191 (62), 147 (26), 73 (100)	17.348	68.5	-	+
	Anthracenediol	356 (34), 266 (82), 253 (3), 191 (3), 147 (60), 73 (100)	17.874	26.8	-	-
	Monohydroxyanthracene[g]	266 (79), 251 (14), 235 (6), 191 (6), 165 (12), 73 (100)	17.260	4.7	-	+
Fluorene	Dihydroxyfluorene	342 (14), 253 (46), 152 (17), 73 (100)	18.477	28.9	-	+
	1-Hydro-1,1a dihydroxy-9-fluorenone[d]	358 (65), 253 (59), 147 (36), 73 (100)	16.788	63.6	+	+
	Fluorenol-dihydrodiol	360 (39), 270 (95), 242 (100), 181 (55), 165 (13)	16.455	7.4	-	-
9-Fluorenol	1-Hydro-1,1a-dihydroxy-9-fluorenone[d]	358 (40), 253 (41), 147 (39), 73 (100)	16.800	82	+	+
	Fluorenol-dihydrodiol	360 (34), 270 (90), 242 (100), 181 (78), 165 (23)	16.459	18	-	-
9-Fluorenone	1-Hydro-1,1a-dihydroxy-9-fluorenone[d]	358 (56), 253 (51), 147 (43), 73 (100)	16.795	76	+	+
	Fluorenol-dihydrodiol	360 (30), 270 (91), 242 (100), 181 (85), 165 (24)	16.459	24	-	-
Fluoranthene	Monohydroxyfluoranthene[g]	290 (81), 275 (54), 259 (47), 215 (76)	20.038	100	N. T.	+
Carbazole	Monohydroxycarbazole[g]	255 (100), 239 (51), 224 (47), 209 (22), 166 (11)	17.128	56.4	-	-
	Dihydroxycarbazole	343 (100), 327 (34), 252 (7), 164 (2)	18.688	9.7	-	-
	Monohydroxycarbazole[g]	327 (100), 312 (24), 165 (1), 73 (39)	18.997	33.9	-	-

Dibenzofuran	2,2',3-Trihydroxybiphenyl[d]	418 (69), 315 100), 73 (96)	16.357	100	+	+
Dibenzo-*p*-dioxin	2,3,2'-Trihydroxydiphenyl ether[d]	434 (25), 419 (3), 331 (41), 315 (930), 73 (100)	16.988	100	+	+
Dibenzothiophene	Dibenzothiophene-sulfone[d]	200 (7), 184 (100), 171 (7), 139 (18), 73 (4)	17.718	Traces	+	+
	Dibenzothiophene-sulfoxide[d]	216 (5), 200 (5), 184 (100), 147 (72), 73 (9)	17.735	Traces	+	+

[a] Products were identified by GC-MS analysis (TMS derivatisation). Fragment ions are expressed as *m/z* values.
[b] When multiple oxidation products were detected their relative abundance is indicated in %.
[c] The ability and inability to transform each compound are shown by "+" and "-".
[d] Same mass spectrum as that of relevant PAH oxidation products generated by CARDO and DFDO, and previously identified based on ^{1}H and ^{13}C NMR analyses (229).
[e] Same retention time and mass spectrum as *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene produced by Pdo1 (181).
[f] Same retention time and mass spectrum as *cis*-1,2-dihydroxy-12-dihydroanthracene produced by Phn1 (56).
[g] Monohydroxylated products are most probably formed by spontaneous dehydration of the corresponding diols.
N. T.: Not tested

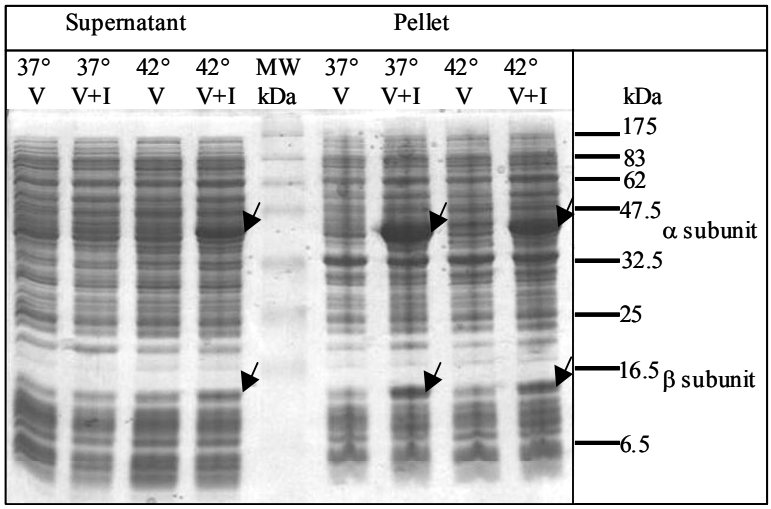

Figure 6. Detection of FlnA1 and FlnA2 overproduced in *E. coli* BL21(DE3). Soluble (supernatant) and insoluble proteins (pellet) were analysed on SDS-PAGE. *E coli* harbouring pET30f lacking the *flnA1A2* insert (V) was used as control. Protein extracts from cells, overexpressing FlnA1A2 (V+I), grown at 37°C and 42°C up to an OD_{600} of 0.5 prior to IPTG induction are shown. Arrows indicate the α and β subunits of the angular dioxygenase. Molecular mass ladder (kDa) (Prestained Protein Marker, Broad Range, New England Biolabs, Ipswich, MA).

Substrate range of FlnA1A2.

The substrate range of FlnA1A2 was investigated and compared with those of the well-studied angular dioxygenases DFDO (dibenzofuran 4,4a-dioxygenase) from *Terrabacter* sp. strain DBF63 (150) and CARDO (carbazole 1,9a-dioxygenase from *Pseudomonas resinovorans* CA10 (229, 271) (Fig. 7). When fluorene was used as substrate, three oxidation products could be detected (Table 2). The major product was identified as 1-hydro-1,1a-dihydroxy-9-fluorenone (63 %) based on the *m/z* fragment pattern of its mass spectrum, which was identical to that of the DFDO-mediated oxidation product of fluorene (150, 212) and the oxidation product of 9-hydroxyfluorene by CARDO (308). Interestingly CARDO does not yield the angular dioxygenation product 1-hydro-1,1a-dihydroxy-9-fluorenone when fluorene is used as substrate (308). Fluorenol-dihydrodiol (7 %) and

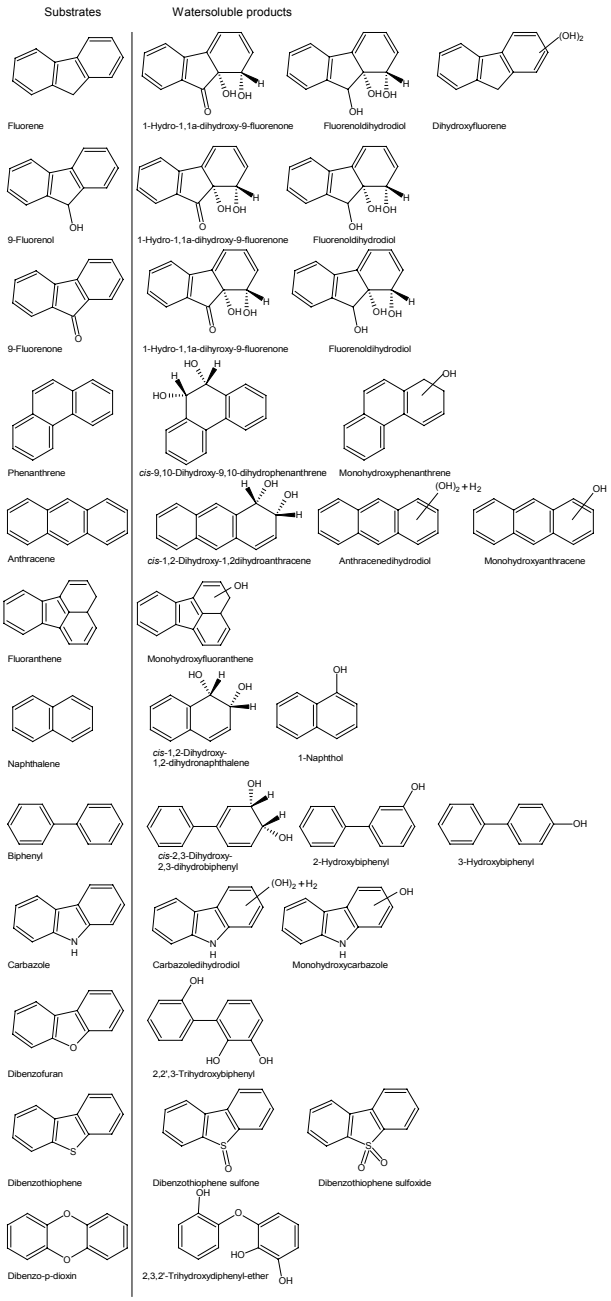

Figure 7. Chemical structures of the compounds formed by FlnA1A2.

dihydroxyfluorene (29 %) were also produced by FlnA1A2 from strain LB126. The latter product was not formed by DFDO. Fluorenol-dihydrodiol probably resulted from spontaneous transformation of 1-hydro-1,1a-dihydroxy-9-fluorenone since this product was not detected after shorter incubations. 9-Hydroxyfluorene is likely oxidized to 9-fluorenone by a non specific dehydrogenase from *E. coli*. Indeed, we also observed such a spontaneous oxidation upon incubation of 9-hydroxyfluorene with the control strain BL21(DE3)(pET30f) lacking the *flnA1A2* construct. This suggests that a dehydrogenase is not essential for the transformation of 9-hydroxyfluorene to 9-fluorenone but that it may be required *in vivo* to catalyse the reaction at a reasonable rate. 1-Hydro-1,1a-dihydroxy-9-fluorenone also accumulated when 9-hydroxyfluorene or 9-fluorenone were used as substrates, showing that FlnA1A2 is involved in at least two steps in fluorene catabolism (Table 2). Given the low activity of FlnA1A2 and the necessity of monooxygenation before angular dioxygenation can occur, 9-hydroxyfluorene and 9-fluorenone are probably instantly consumed and are therefore not present.

Three heteroatomic analogues of fluorene, i.e. dibenzofuran, carbazole and dibenzothiophene were tested as substrates for angular oxidation. Dibenzofuran was transformed into 2,2',3-trihydroxybiphenyl by FlnA1A2, as previously found for DFDO (150) and CARDO (229). The initial attack occurred at the 4 and 4a carbon atoms as put forward by Fortnagel et al. in 1989 (77). The dioxygenation of dibenzofuran produces a highly unstable hemiacetal product that could not be observed. Incubation with dibenzothiophene produced traces of dibenzothiophene-sulfoxide and dibenzothiophene-sulfone. These metabolites were previously identified as metabolic intermediates of dibenzothiophene degradation by *Brevibacterium sp*. strain DO (317), DFDO (212) and CARDO (229). Since FlnA1A2 was able to perform angular dioxygenation on fluorene and dibenzofuran, hydroxylation of dibenzothiophene-sulfone at the angular position was expected. The activity of FlnA1A2 towards dibenzothiophene might have been too low to detect an angular dioxygenation product by GC-MS although dibenzothiophene is degraded cometabolically by strain LB126 (324). No angular oxidation product was detected after incubation with carbazole, even though carbazole is a structural analogue of fluorene. Mono- and dihydroxycarbazole were the only oxidation products detected by GC-MS. DFDO from *Terrabacter* sp. strain DBF63 was not able to perform angular dioxygenation on carbazole either.

Detection of monohydroxycarbazole suggests that FlnA1A2 transforms carbazole to the corresponding dihydrodiol by lateral dioxygenation. Resnick et al. reported that carbazole dihydrodiols are unstable and spontaneously form monohydroxycarbazole by dehydration (257). CARDO released 2'-aminobiphenyl-2,3-diol upon angular oxidation of carbazole (229). The crystal structure of CARDO bound with carbazole has been solved and a molecular mechanism of angular dioxygenation for carbazole was proposed (7). Given the low protein identity between CARDO and FlnA1 (16%) no hypothesis could be established why FlnA1A2 does not perform angular dioxygenation on carbazole. Incubation of FlnA1A2 with dibenzo-*p*-dioxin yielded 2,3,2'-trihydroxydiphenylether via angular dioxygenation based on the *m/z* fragments described from DFDO and CARDO.

Since *Sphingomonas* sp. strain LB126 is able to use phenanthrene, fluoranthene and anthracene in cometabolic degradation with pyruvate (324), we tested whether FlnA1A2 would attack these PAHs. *cis*-9,10-Dihydroxy-9,10-dihydrophenanthrene, which was previously identified as a product formed by pyrene dioxygenase from *Mycobacterium* sp. strain 6PY1 (181), was detected as the major oxidation product of phenanthrene. Interestingly, *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene which is produced in the catabolic pathway of known phenanthrene degraders including sphingomonads (56, 241, 354) was not formed. Monohydroxyphenanthrene was detected in low amounts (4 %) and might have resulted from spontaneous dehydration of the corresponding dihydrodiol. In contrast, DFDO did not produce any metabolite when incubated in the presence of phenanthrene (151). When FlnA1A2 was incubated with fluoranthene, trace amounts of monohydroxyfluoranthene could be detected. Anthracene yielded three metabolites. The major compound could be identified as *cis*-1,2-dihydroxy-1,2-dihydroanthracene by comparison to the oxidation product formed by Phn1 from *Sphingomonas* sp. strain CHY-1 (56). Trace amounts of monohydroxyanthracene were also present. CARDO produced the same metabolites but DFDO did not. Moreover, a second putative anthracene-diol was detected. Its mass spectrum was similar to that of *cis*-1,2-dihydroxy-12-dihydroanthracene but the retention time was different. Since no angular attack on anthracene is possible without a preliminary monooxygenation, we suggest that this compound could be *cis*-2,3-dihydroxy-2,3-dihydroanthracene. This metabolite has not been

produced by any other enzyme reported so far. When incubated with biphenyl or naphthalene, FlnA1A2 produced the expected metabolites which were also reported for DFDO and CARDO (150, 229). Our results show that FlnA1A2 from strain LB126 is unique in that it shares characteristics with both DFDO and CARDO.

Quantitative analysis of watersoluble dihydrodiols formed by FlnA1A2

The catalytic activity of FlnA1A2 towards fluorene and other PAHs was compared by estimating the amount of di- or trihydroxylated products formed by strain BL21(DE3)(pET30f*flnA1A2*) after overnight incubation. Products were extracted and quantified as NBB derivatives by GC-MS analysis as described in Materials and Methods. Results showed that 1-hydro-1,1a-dihydroxy-9-fluorenone (97.5 µM) and 9,10-phenanthrene dihydrodiol (96.3 µM) accumulated at highest concentrations indicating that fluorene and phenanthrene were preferred substrates (Table 3).

Table 3: Comparison of the FlnA1A2 dioxygenase activity towards fluorene and other polycyclic substrates

Substrate	Properties of the products formed[a]		
	Retention time (min)	m/z	Concentration (µM)[b]
Fluorene	15.55	280	97.5
Phenanthrene	15.92	278	96.3
Anthracene	14.66	278	9.1
	16.60	278	27.3
Naphthalene	12.11	228	1.92
Dibenzofuran	13.52	418	10.1
Dibenzo-*p*-dioxin	14.12	434	10.0

[a] Characteristics of the NBB derivatives, except for dibenzofuran and dibenzo-*p*-dioxin which were analyzed as the trimehyl silyl derivatives. Anthracene yielded two isomers, one of which was identified as anthracene 1,2-dihydrodiol (retention time: 16.60 min).

[b] Concentrations calculated in the bacterial suspension after 23 h of incubation at 25°C. Values are means of duplicates experiments. The standard error was less than 10%.

GC-MS data from NBB derivatives confirmed that FlnA1A2 attacked fluorene in the angular position. The attack on phenanthrene generated 9,10-phenanthrene dihydrodiol instead of the more common 3,4-isomer. In this respect, the activity of FlnA1A2 is quite different from that of other known phenanthrene dioxygenases. In addition, FlnA1A2 showed a relatively low activity towards naphthalene (Table 3).

Both dibenzofuran and dibenzo-*p*-dioxin yielded low amounts of products, essentially because the trihydroxylated compounds generated from these substrates reacted poorly with NBB (data not shown). The amount of the trihydroxylated products was therefore tentatively determined based on the peak area of the trimethylsilyl derivatives using 2,3-dihydroxybiphenyl as standard (Table 3). These results, together with the fact that neither phenanthrene nor dibenzofuran can support growth of strain LB126, provide additional evidence that FlnA1A2 acts as an angular dioxygenase specifically dedicated to the initial attack of fluorene.

To conclude, the enzymes probably responsible for fluorene oxidation in strain LB126 were most closely related to proteins involved in angular attack on dibenzofuran from Gram-positive bacteria. The genes were likely acquired by lateral gene transfer since a truncated transposase was identified upstream of the catabolic genes. RT-PCR analysis showed that *cirA, flnA1* and *flnE* are expressed and induced when *Sphingomonas* sp. strain LB126 is grown on fluorene compared to glucose. Quantification of FlnA1A2 activity towards several PAHs showed that fluorene and phenanthrene were the preferred substrates although phenanthrene cannot be used as a sole source of carbon and energy by *Sphingomonas* sp. strain LB126. Altogether these data strongly suggest that the genes *flnA1A2* are involved in fluorene degradation by *Sphingomonas* sp. strain LB126. The proteins encoded by *flnA1A2* were quite unique, since no functional counterpart had been described in Gram-negative bacteria to date. FlnA1A2 was able to perform monooxygenations, angular and lateral oxygenations on PAHs and heteroaromatics that were not oxidized by DFDO, the most closely related enzyme from *Terrabacter* sp. strain DBF63.

Acknowledgements

L.S. gratefully acknowledges the Fund for the Promotion of Research in Industry and Agriculture (F.R.I.A.), Belgium, for providing a doctoral fellowship. L.S. also wishes to thank the members of the Unit of Physiological Biochemistry (FYSA), Catholic University of Louvain, for their daily help and constructive remarks for many years. PH is research associate at FNRS.

Chapter 2: Characterization of a new *cis*-dihydrodiol dehydrogenase from fluorene-degrading *Sphingomonas* sp. strain LB126

The preceding chapter demonstrated that FlnA1A2 could catalyse monooxygenation of fluorene to produce 9-fluorenol, which is further oxidized by the same enzyme via angular dioxygenation to 1-hydro-1,1a-dihydroxy-9-fluorenone with non-specific ferredoxin and ferredoxin reductase from *E. coli*. Since a putative short-chain dehydrogenase-reductase protein (FlnB), was found in the vicinity of

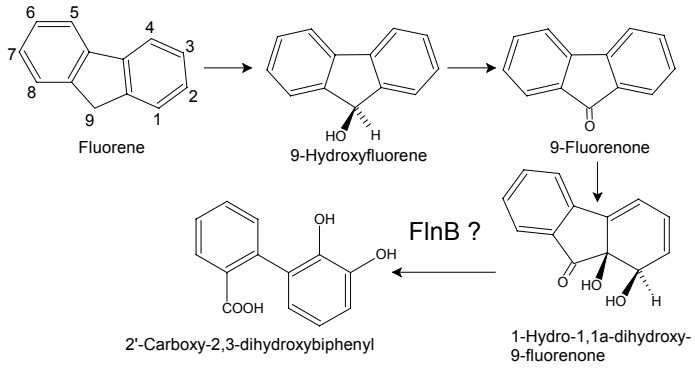

Figure 1. Probable reaction governed by FlnB from *Sphingomonas* sp. strain LB126.

the fluorene degradative gene cluster, we investigated whether this enzyme was responsible for the transformation of 1-hydro-1,1a-dihydroxy-9-fluorenone to 2'-carboxy-2,3-dihydroxybiphenyl (Fig. 1) as shown for *Terrabacter* sp. strain DBF63 (108).

Authors contributions: LS is the main contributor to designing, performing research and wrote the paper; designed research: LS, CM, YJ, PH, SA; performed research: LS, CM, YJ; analyzed data: LS, CM, YJ.

Characterization of a new *cis*-dihydrodiol dehydrogenase from fluorene-degrading *Sphingomonas* sp. strain LB126

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Introduction

The aerobic bacterial degradation of aromatic compounds is usually initiated by dioxygenases that incorporate two hydroxyl groups into the aromatic substrate. The products of such reactions are chiral *cis*-dihydrodiols. Dioxygenases that act on aromatic compounds generally are broad-spectrum enzymes. However, occasional monooxygenation and angular oxygenation may occur, for instance in the bacterial metabolism of fluorene and dibenzofuran (150, 314). During the next step in the degradation of aromatic compounds, the *cis*-dihydrodiols are dehydrogenated to catechols by *cis*-dihydrodiol dehydrogenases. Generally, *cis*-dihydrodiol dehydrogenases are broad-substrate enzymes, and most dehydrogenases are able to transform several *cis*-dihydrodiol isomers with an absolute requirement for NAD^+ as the electron acceptor. The third step in the catabolic pathway is catalysed by a second dioxygenase, which cleaves the aromatic ring (114).

Dihydrodiol dehydrogenases have been described for bacteria degrading monoaromatic compounds (9, 247, 252, 293), biphenyl and polychlorinated biphenyls (158, 305) and naphthalene (14, 237). Recently, an enzyme able to dehydrogenate dihydrodiols composed of more than three rings has been described (140). These enzymes belong to a subgroup of the so-called short-chain alcohol dehydrogenase/reductase family (121).

Sphingomonas sp. strain LB126 is capable of using fluorene as a sole source of carbon and energy (19). FlnA1A2, responsible for the angular oxygenation of fluorene, forming 1,1a-dihydroxy-9-fluorenone (DHF) with a nonspecific ferredoxin and reductase from *E. coli*, has been described in the preceding chapter. Fluorene oxidation by *Terrabacter* sp. strain DBF63 produces DHF that is oxidized by a *cis*-dihydrodiol dehydrogenase to yield 2'-carboxy-2,3-dihydroxybiphenyl (108). In the present study, *flnB* encoding a *cis*-dihydrodiol dehydrogenase from strain LB126 was cloned and overexpressed in *E. coli*, to determine whether FlnB is responsible for this reaction.

Sequence analysis of *flnB* encoding a *cis*-dihydrodiol dehydrogenase from *Sphingomonas* sp. strain LB126.

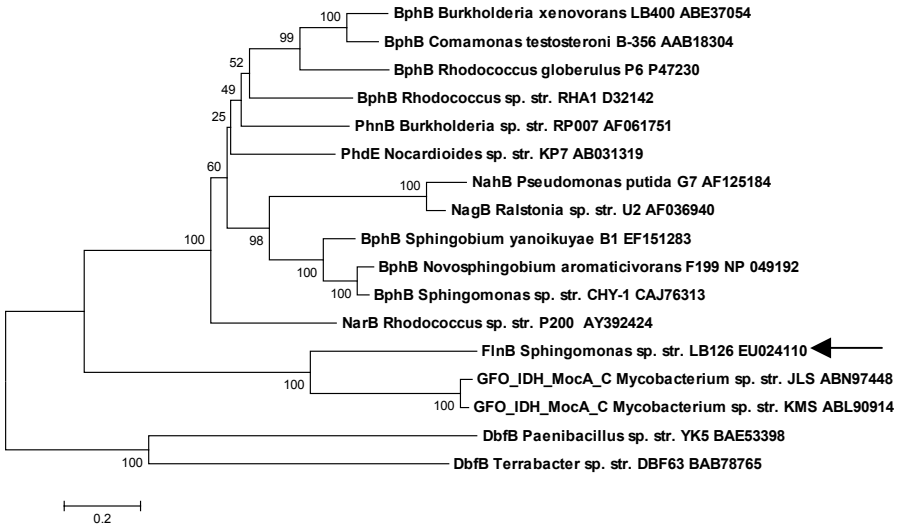

Figure 1. Phylogenetic distribution of FlnB homologous proteins from *Sphingomonas* sp. strain LB126. The dendrogram was constructed from a ClustalW alignment by neighbour-joining analysis using Mega 3.1 software (183). Reference sequences from GenBank include the accession number. The scale bar represents substitutions per amino acid.

In *Sphingomonas* sp. strain LB126, the flnA1A2 genes were arranged in a catabolic cluster and their genetic organization is similar to the catabolic operon from Gram-positive bacteria *Terrabacter* sp. strain DBF63 (150). The *flnB* gene product shows no significant homology to dihydrodiol dehydrogenases from various bacteria including *Novosphingobium aromaticivorans* F199, *Sphingomonas* sp. strain CHY-1, *Sphingobium yanoikuyae* B1 or its counterpart from *Terrabacter* sp. strain DBF63 (Fig. 1). *flnB*, encoding a putative 1,1a-dihydroxy-9-fluorenone dehydrogenase, matches best against proteins from PAH-degrading *Mycobacterium* sp. strain JLS (identity: 41%, ABN97448) (110, 209) identified during whole genome sequencing projects for which no functional data is available.

Cloning, heterologous expression of FlnB and protein extraction.

Since the FlnA1A2 angular oxygenase from *Sphingomonas* sp. strain LB126 was able to convert a wide range of substrates to the corresponding dihydrodiols, it was of interest to examine whether FlnB would further oxidize these dihydrodiols. Construction of the plasmids used in this study involved multiple PCR amplifications and cloning steps. The *flnB* fragment (1299 bp) was amplified by PCR with the primer pairs: 5'- *CATATG*TCTGTATTGACCCAAGCC -3' and 5'- *AAGCTT*CGTGTTAGGTTGGCTCGT -3', introducing *Nde*I and *Hin*dIII sites at the ends of the amplicon. The PCR product was cloned into pDrive vector (Qiagen, Valencia, CA), sequenced, and then subcloned into the *Nde*I and *Hin*dIII sites of expression vector pET30f (Novagen, San Diego, CA). This construct was transformed into *E. coli* BL21(DE3) for expression analysis. In order to assess the overexpression of FlnB under IPTG induction, strain BL21(DE3)(pET30f*flnB*) was grown overnight in 3 ml LB medium with the suitable antibiotics. This culture was used to inoculate 50 ml LB medium (0.1% vol/vol), which was incubated at 37°C until an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM. The cells were further incubated overnight at 25°C. For protein assays, cells were centrifuged, washed with 25 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl, treated with 0.2 mg/ml of lysozyme for 10 min at 37°C. After being cooled on ice, the suspension was subjected to ultrasonication for 3 min at 30% of maximum power using a Vibra cell ultra sonifier (Fisher Bioblock Scientific, Illkirch, France). The

lysate was centrifuged for 10 min at 15 000 x *g* to remove cell debries. Protein extracts from IPTG-induced cells were separated by SDS-PAGE. The cells overproduced at high levels a polypeptide of the expected size (M_r of 48 kDa) (Fig. 2). BL21(DE3)(pET30f) which lacked FlnB served as control. The crude protein extracts were used in protein assays.

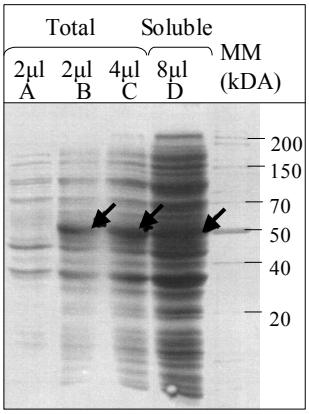

Figure 2. Detection of FlnB overproduced in *E. coli* BL21(DE3). Total induced protein extracts (Lane B 2 µl, Lane C 4 µl) and soluble proteins (Lane D, 8 µl) were analysed on SDS-PAGE. *E coli* harbouring pET30f lacking the *flnB* insert was used as control (Lane A). Molecular mass (MM, kDa): Fermentas, PageRuler prestained Protein Ladder.

Enzyme assays.

Pure DHF, presenting absorption maximum at 314 nm (Fig. 3A), was obtained by incubation of BL21(DE3)pET30f*flnA1A2* in the presence of fluorene (previous chapter). Water-soluble products were passed through solid phase extraction columns (Upti-clean C18U, 0.5 g, Interchim, Montluçon, France). Columns were washed with 10 ml water then eluted with 1 ml ethyl acetate. The solvent was evaporated under nitrogen gas and the extracts were then dissolved in 100 µl acetonitrile and further purified on thin layer chromatography to recover DHF. The recovered metabolite was analysed by GC-MS. The dehydrogenase activity was assayed by spectrophotometric readings of the transformation of DHF at 314 nm.

Reactions were carried out in 1-ml Quartz cuvettes containing 1 ml potassium phosphate (0.1 M, pH 7), 0.1 mM NAD^+ and DHF (4.5 µM final concentration). The reaction was initiated by addition of an appropriate amount of proteins, and the absorption spectrum from 290 to 500 nm was recorded at 5 sec intervals. When cell extracts lacking FlnB (BL21(DE3)pET30f) were used (Fig. 3B), no variation of the UV-spectra could be observed over time. Protein extracts containing

FlnB (Fig. 3C) showed a rapid decline in absorbance at 314 nm indicating that DHF was transformed by FlnB. However no peak appeared at 340 nm, typical for NADH formation. It is possible that NADH, formed during the reduction of NAD^+, is instantly oxidized by other proteins present in the extract and therefore no NADH accumulates in the medium. A decrease of absorbance could also be detected when *cis*-1,2-dihydro-1,2-dihydroxynaphthalene was used as substrate (Fig. 3D). Again no formation of NADH could be detected at 340 nm. Nevertheless the variation in the UV-spectra suggests that there is activity towards the substrate.

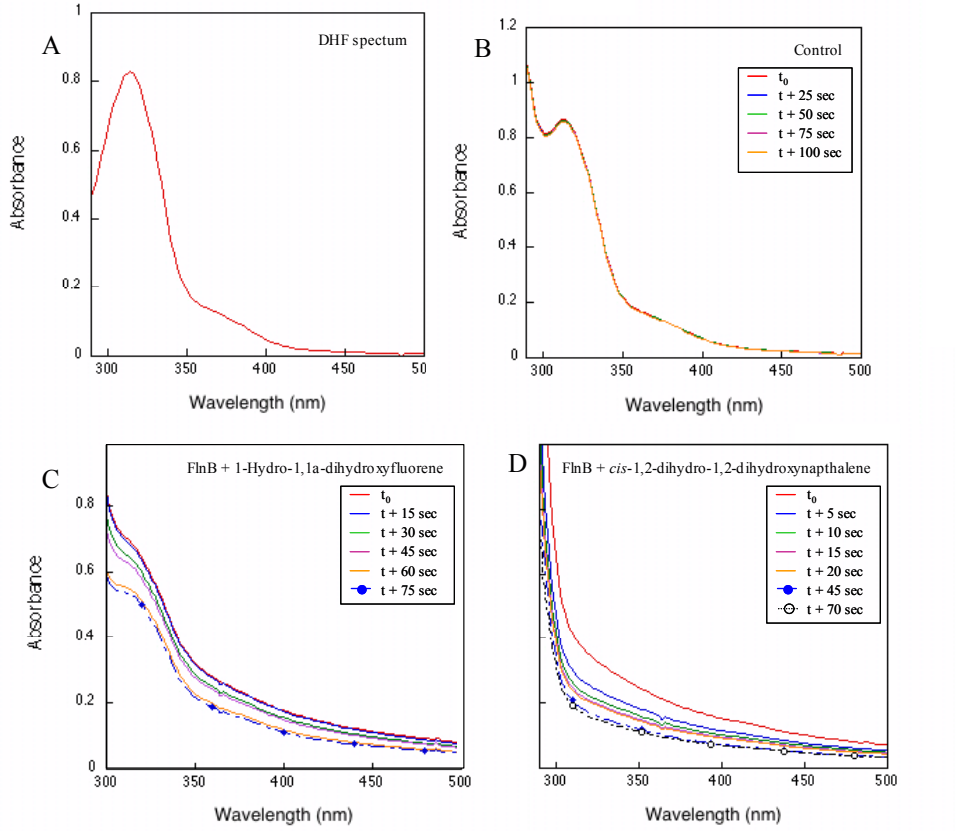

Figure 3. A. UV spectrum of DHF. B. Biotransformation assay with whole-cell extracts lacking FlnB. C. biotransformation assay with whole cell extracts plus FlnB in the presence of DHF. D. biotransformation assay with whole cell extracts plus FlnB in the presence of *cis*-1,2-dihydro-1,2-dihydroxynaphthalene. The time interval for each measurement is indicated in the upper right corner of each sample.

GC-MS analysis of the enzymatic products.

The reaction was initiated by an appropriate amount of enzyme and allowed to reach completion, at which time ethyl acetate extraction was carried out twice with an equal volume of solvent. After being dried with sodium sulphate and solvent evaporation under nitrogen gas, the dry material was dissolved in acetonitrile. Reactions, acidified by adding concentrated HCl, were extracted following the same protocol.

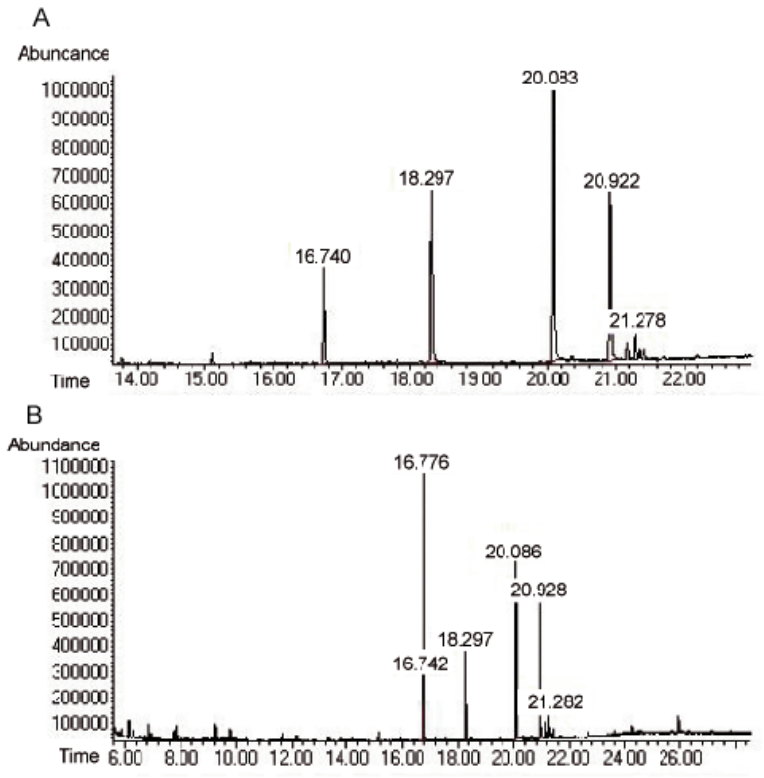

Figure 4. GC-MS chromatograms of A) FlnB + DHF, and B) DHF without FlnB. The peak at Rt: 16.776 is specific to DHF.

Samples were derivatized by *bis*(trimethylsilyl) trifluoroacetamide containing trimethylchlorosilane (BSTFA) and subjected to GC-MS analysis. Non-derivatized neutral and acidic samples were also analyzed on HPLC in neutral and acidic conditions. The control, reaction medium containing DHF but lacking FlnB shows a large peak at a retention time of 16.776 minutes (Fig. 4B) characteristic of DHF. No DHF could be detected in the reaction with FlnB (Fig. 4A), suggesting that DHF was indeed completely transformed. Unfortunately no reaction product could be identified with GC-MS or HPLC in acidic conditions. It is however possible that 2'-carboxy-2,3-dihydroxybiphenyl is spontaneously transformed to 8-hydroxy-3,4-benzocoumarine (Fig. 5) (29, 108) which is difficult to detect in GC-MS. These results do not allow concluding on the function of FlnB, however DHF has been consumed during the reaction.

Concluding Remarks

Dioxygenases catalyse the introduction of two oxygen atoms into the aromatic ring producing *cis*-dihydrodiols. These molecules are further oxidized by an NAD-dependent dehydrogenase before ring cleavage by a second dioxygenase. Little attention has been paid to this second step in PAH degradation and PhnB from *Sphingomonas* sp. strain CHY-1 is the only dehydrogenase reported so far to be able to dehydrogenate dihydrodiols composed of more than three rings (140). The study of FlnA1A2 showed that this enzyme had relaxed substrate specificity and accumulated 1,1a-dihydroxy-9-fluorenone in the presence of fluorene. Fluorene degradation by *Terrabacter* sp. str DBF63 showed that DHF is dehydrogenated by a *cis*-dihydrodiol dehydrogenase to form 2'-carboxy-2,3-dihydroxybiphenyl (108). The aim of this study was to verify whether FlnB was responsible for this transformation step in strain LB126.

Therefore the *flnB* gene was cloned and overexpressed in *E. coli* BL21(DE3). The conversion of DHF by FlnB was monitored by UV absorbance changes upon oxidation by the dehydrogenase reaction media analysed by GC-MS. When protein extracts containing overexpressed FlnB protein were used in enzyme assays, no DHF could be detected after BSTFA derivatisation, suggesting that the reaction was complete. On the other hand, when proteins lacking FlnB were incubated under identical conditions, a large peak of DHF was detected by GC-MS analysis. Nevertheless no transformation product

of DHF could be detected by HPLC and HPLC in acidic conditions. It has been shown that 2'-carboxy-2,3-dihydroxybiphenyl can be spontaneously transformed to 8-hydro-3,4-benzocoumarin (100, 314) which is less volatile and might therefore not be detected by GC-MS analysis (Fig. 5).

A rapid decline could also be observed when FlnB was incubated with *cis*-1,2-dihydro-1,2-dihydroxynaphthalene.

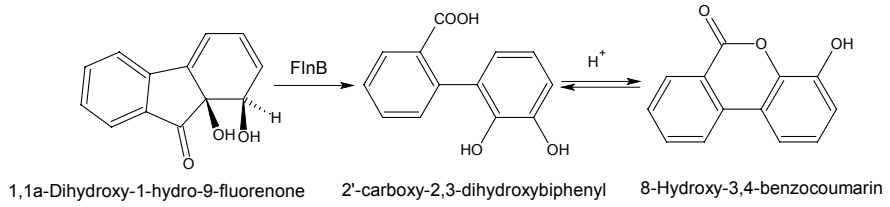

Figure 5. Reaction probably catalysed by FlnB. 2'-carboxy-2,3-dihydroxybiphenyl is spontaneously transformed to 8-hydroxy-3,4-benzocoumarin

However, these data do not allow concluding on the activity of FlnB in crude protein extracts. Overexpression and purification of FlnB is necessary to repeat the enzyme assays. This new series of experiments will provide evidence whether the consumption of DHF is linked to FlnB or to some unspecific protein from *E. coli*. Moreover accumulation of NADH will prove the consumption of NAD^+ by FlnB.

Chapter 3: Identification of genes involved in polycyclic aromatic hydrocarbon degradation by *Sphingomonas* sp. strain LH128 and characterization of a multicomponent ring-hydroxylating dioxygenase

The oxidation of indole to indigo was first shown in *Escherichia coli* strains expressing lateral or naphthalene dioxygenases (NDO) from *Pseudomonas putida* G7 (68). NDO was shown to oxidize indole into an unstable *cis*-indoledihydrodiol, which dehydrates to indoxyl, and undergoes spontaneous oxidation to indigo. Since then indigo formation has been used to discover and isolate strains expressing "naphthalene-like" dioxygenases. *Sphingomonas* sp. strain LH128 is able to transform indole to indigo when grown on phenanthrene, but not on glucose (Fig. 1).

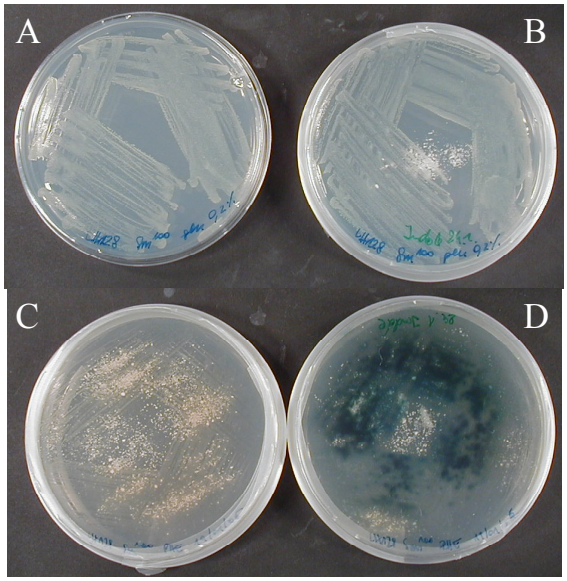

Figure 1. *Sphingomonas* sp. strain LH128 grown on glucose (A), glucose + indole (B) phenanthrene (C) and phenanthrene + indole (D). Indigo formation in the presence of phenanthrene indicates that the dioxygenase is induced by phenanthrene and is not expressed in the presence of glucose.

This shows that strain LH128 harbours a naphthalene-like dioxygenase inducible by phenanthrene. Contrary to angular

dioxygenases, where the methylene group of fluorene or the heteroatoms in its analogues have to be oxidized before angular dioxygenation can occur, lateral dioxygenation or "naphthalene-like attack", do not require this preliminary step. The initial reaction is catalysed by a dioxygenase adding both atoms of molecular oxygen to the aromatic ring of the substrate (Fig. 2) to form the corresponding cis-dihydrodiols.

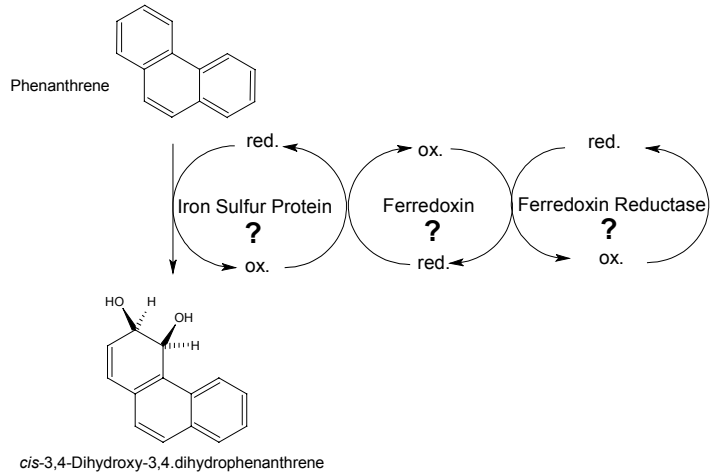

Figure 2. Reaction investigated in phenanthrene oxidation in *Sphingomonas* sp. strain LH128.

Generally these enzymes have relaxed substrate specificity and are able to oxidize a wide range of compounds in addition to their native substrates. Based on already known dioxygenases from other sphingomonads such as *Sphingobium* yanoikuyae B1, *Sphingomonas* sp. strain CHY-1 or *Novosphingobium aromaticivorans* F199, the initial dioxygenase from strain LH128 could be identified and characterized.

This article is in preparation for a submission to *Microbiology*.

Authors contributions: LS is the main contributor to designing, performing research and wrote the paper; designed research: LS, CM, YJ, SNC, GZ, PH, SA; performed research: LS, CM, SNC, YJ, MP; analyzed data: LS, SNC, CM, YJ, MP.

Identification of genes involved in polycyclic aromatic hydrocarbon degradation by *Sphingomonas* sp. strain LH128 and characterization of a multicomponent ring-hydroxylating dioxygenase

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Running Title: Dioxygenase from *Sphingomonas* sp. LH128

Keywords: Biodegradation, *meta*-cleavage operon genes, indigo formation, Rieske non-heme iron oxygenase

Abstract

The catabolic metabolism of phenanthrene as well as the genes involved in PAH degradation by *Sphingomonas* sp. strain LH128 were investigated. This bacterium was isolated from a polycyclic aromatic hydrocarbon (PAH) contaminated soil using phenanthrene as the sole

source of carbon and energy. Degradation experiments showed that strain LH128 was able to completely remove 250 mg/l of phenanthrene within 36 hours. HPLC analysis revealed that the catabolic pathway proceeds via salicylic acid and catechol. The genes encoding a salicylate hydroxylase and a catechol-2,3-dioxygenase were identified and isolated on a continuous 10.5 kb fragment of DNA. Thirteen open reading frames encoding genes involved in the degradation of PAHs were identified which showed high homology and gene organization to genes from *Novosphingobium aromaticivorans* F199, S*phingobium yanoikuyae* B1 and *Sphingomonas* sp. strain CHY-1. Furthermore, *phnA1fA2f* encoding the α and β subunit of a dioxygenase, responsible for the initial attack on PAHs was identified. PhnA1f showed 98%, 78% and 78% amino acid identity to the α subunit of *Novosphingobium aromaticivorans* F199, *Sphingomonas* sp. strain CHY-1 and *Sphingobium yanoikuyae* B1 respectively. When overexpressed in *E. coli*, PhnA1fA2f was able to oxidize low molecular weight PAHs, chlorinated biphenyls, dibenzo-*p*-dioxin and the high molecular weight PAHs benz[*a*]anthracene, chrysene and pyrene. These results demonstrate that PhnA1fA2f was responsible for the oxidation of PAHs of up to four rings.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) have toxic, mutagenic and carcinogenic properties, thus, their persistence in the environment is of great concern (340). They can be found in high concentrations at industrial sites associated with petroleum or gas production and they occur whenever organic matter is burnt. For the purpose of bioremediation, microorganisms able to use these pollutants as the sole source of carbon and energy are extensively studied (39, 138). Amongst these, sphingomonads have received much attention due to their ability to degrade a wide range of aromatic hydrocarbons. *Sphingomonas* species able to degrade mono- and polycyclic aromatic hydrocarbons (241, 301), phenols (33), carbofuran (72, 162), oestradiol (82), dibenzofurans (31, 78), biphenyls (113, 159, 238, 354), dibenzo-*p*-dioxins (5, 120) and herbicides (137, 294) have been isolated. In the last few years, attention has been turned towards identifying and characterizing the genes involved in PAH degradation, allowing a closer look at pathways potentially useful in bioremediation (240).

PAH degradation by aerobic bacteria is generally initiated by the introduction of both atoms of O_2 to the aromatic ring of the substrate (32, 327). This initial reaction, which is catalysed by a multicomponent aromatic ring hydroxylating dioxygenase, involves the dihydroxylation of the carbon-carbon double bond of adjacent carbon atoms. The enzymes responsible for the initial attack on PAHs from *Sphingomonas* sp. strain CHY-1, which was isolated for its ability to degrade chrysene (56, 141) and *Sphingobium yanoikuyae* B1, which was isolated for its ability to degrade biphenyl (225), have been identified and their respective crystal structures were recently determined (129, 130, 348). In *Novosphingobium aromaticivorans* F199, isolated for its ability to degrade PAHs, the genes encoding a putative initial dioxygenase have been found on plasmid pNL1 (264). However, the activity of the encoded enzyme (BphA1fA2f) has not yet been investigated (264).

Sphingomonads harbour multiple copies of genes predicted to encode the terminal component of Rieske-type oxygenases (241, 264, 354). They constitute a large family of two- or three-component metalloenzymes whose catalytic activity component is generally a heteromeric $\alpha 3\beta 3$ hexamer containing one Rieske-type [2Fe-2S] cluster and one nonheme iron atom per α subunit. Moreover the catabolic cluster includes genes involved in the catabolism of monoaromatics (*xyl* genes) interspersed with genes predicted to function in the degradation of polycyclic substances (designated *bph*, *nah*, *ahd*, or *phn*). This unique gene arrangement, which is remarkably conserved among strains of various origins, contrasts with that found in other degraders, such as pseudomonads.

Sphingomonas sp. strain LH128 was isolated from a heavily polluted soil (19) and is capable of growing on phenanthrene as the sole source of carbon and energy. Strain LH128 is also able to transform indole to indigo in the presence of phenanthrene. No indigo formation was observed when the strain was grown in the presence of glucose suggesting that the dioxygenase oxidizing indole must be induced by phenanthrene (data not shown). Moreover strain LH128 is able to degrade anthracene, dibenzothiophene, fluorene (19) and the *N*-heterocyclic PAHs acridine, phenanthridine, benzo[*f*]quinoline and benzo[*h*]quinoline (321). In this study genes involved in phenanthrene degradation by *Sphingomonas* sp. strain LH128 were investigated. To date only a few sphingomonad initial dioxygenases have been well

characterized: BphA1fA2f from strain B1 (225) and PhnI (141) from strain CHY-1 showing 78 % protein sequence identity to the dioxygenase α subunit from strain LH128. Here we present functional data regarding a third multicomponent ring-hydroxylating dioxygenase closely related to BphA1fA2f from *Novosphingobium aromaticivorans* F199 (98 % protein identities) from which no functional data are available.

Materials and Methods

Reagents

PAHs and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). Primers were purchased from Sigma-Genosys. Silicone oil (Rhodorsil 47V20) was purchased from VWR. Restriction enzymes were from New England Biolabs.

Bacterial strains, plasmids, and culture media.

Sphingomonas sp. strain LH128, the wild-type strain capable of growing on phenanthrene as the sole source of carbon and energy (19), was kindly provided by VITO (Vlaamse Instelling voor Technologisch Onderzoek, Belgium). *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) was used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene expression analysis. PCR amplicons were either cloned into pDrive (Qiagen, Valencia, CA) or pGEM-T-easy vector (Promega, Madison, WI). pVLT31 (51) and pET30f (Novagen, San Diego, CA) were used as expression vectors. MM284 minimal medium (208) was used for growing *Sphingomonas* sp. strain LH128 and was supplemented with phosphate buffer (50 mM; KH_2PO_4, K_2HPO_4, pH 7.2) instead of Tris buffer. Phenanthrene was provided as crystals in both Petri dishes and liquid media. LB broth (269) was used as complete medium for growing *E. coli* strains. Solid media contained 2% agar. When needed, ampicillin, streptomycin and kanamycin were added to the medium at 200, 100 and 20 μg/ml, respectively. *Sphingomonas* sp. strain LH128 was grown at 30°C, and *E. coli* strains were grown at 37°C. Bacterial growth was determined by optical density readings at 600 nm (OD_{600}).

Degradation experiments.

Phenanthrene degradation experiments were conducted in duplicate in 100 ml Erlenmeyer vials containing 20 ml phosphate buffered minimal medium and 250 mg/l of phenanthrene. Heat killed cell cultures served as negative controls. Phenanthrene-grown cells were used as inoculum (10 % v/v) after filtration with glass wool and resuspension in phosphate buffer. To extract metabolites, the contents of the flasks were acidified by adding concentrated HCl (6M) to obtain a pH around 2 and extracted with two equal volumes of ethyl acetate after magnetic stirring at 300 rpm for one hour. The solvent was volatilised under a gentle stream of N_2 and the residues were dissolved in 100 µl acetonitrile for HPLC analysis. Reversed-phase HPLC was conducted with a Waters 600E system equipped with a photo-diode array UV detector operating at 254 nm. Separation of parent compounds and metabolites was achieved with an Alltech EPS Platinum C18 column (5-µm, 100A, 250 mm*4.6 mm).

DNA manipulations and molecular techniques.

Total DNA from pure cultures of *Sphingomonas* sp. strain LH128 was extracted using the Ultra Clean DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's recommendations or using standard methods (269) when a higher DNA concentration was needed. Plasmid DNA extractions, restriction enzyme digestions, ligations, transformations, sequencing and agarose gel electrophoresis were carried out using standard methods (269).

PCR and primer design.

All PCR reactions were carried out using PCR Master Mix (Abgene, Surrey, UK). All reactions were performed in a programmable T-Gradient Thermocycler (Biometra, Göttingen, Germany). PCR primers were constructed based upon conserved nucleic acid alignments using Clustal X software (310). The primers catF1 and catR1 (Table 1) were specifically designed to match catechol 2,3-dioxygenases (C23DO) in *Sphingomonas* strains. The following sequences retrieved from GenBank were used (the accession number is given in parentheses): *Sphingobium* sp. strain P2 (AB091692), *Sphingomonas* sp. strain A1-8 (AY504990), *Sphingomonas* sp. strain A1-13 (AY504989), *Sphingomonas* sp. (Z84817), *Sphingomonas* sp. strain KMG425 (AF494100), *Sphingomonas paucimobilis*

(AB035539), *Sphingomonas* sp. strain A8AN3 (U73127), *Sphingomonas yanoikuyae* B1 (U23375), *Sphingomonas* sp. strain HV3 (L10655), *Novosphingobium aromaticivorans* F199 (NC_002033), *Sphingomonas chungbukensis* strain DJ77 (formerly *Pseudomonas* sp. strain DJ77 (163)) (U83882), and *Sphingomonas paucimobilis* TZS-7 (AB035678).

Table 1. Primers used for detecting catabolic genes in *Sphingomonas* sp LH128.

Primer[a]	Sequence (5'-3')	Reference
Salicylate hydroxylase		
Block2C	CCAGCAGGAATACTACACCCGC	(15)
Block2Cinv	GCGGGTGTAGTATTCCTGCTGG	(15)
Block2D	CATTGGTGGTAGACGCATTCCAG	(15)
Block2E	TACAATTCCGGAAAATATGTCGAT	(15)
Block2F	CGGGCTGATGAAATCGAAGTAGAA	(15)
cat-R1	GCCGCTGAAIGTYTCGTT	This study
cat-F1	CAYTAYCGYGACCGKAT	This study
bphA1f-F	CACCGCGGCAACCAGAT	This study
bphA1f-R	ACCATGGTATAGGTCCA	This study
bphA1d-F	GCCGAAATAGGTCCAGACCA	This study
bphA1d-R	CAGCAGATCCAGAACACCCT	This study
xylX-R	TCCATCAGGAAGACGTTGGG	This study
xylX-F	CCCAACGTCTTCCTGATGGA	This study

[a]Y = C+T, I = inosine, K = G+T

The PCR temperature program started with an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 10 minutes. All reactions were performed in a total volume of 50 µl. Primers amplifying *Sphingomonas* salicylate hydroxylase gene products were published by Basta *et al* (15). Primers xylX-R, xylX-F, bphA1f-F, bphA2f-R, bphA1d-F and bphA1d-R were constructed based on sequence alignments of the corresponding genes from *Novosphingobium aromaticivorans* F199, *Sphingomonas* sp. strain P2, *Sphingobium yanoikuyae* B1 and *Sphingomonas* sp. strain CHY-1 (Table 1). The same PCR mix as previously described was used with an annealing temperature of 50°C. Long template PCR were carried out using the Extensor Hi-Fidelity PCR Enzyme Mix (Abgene, Surrey, UK) according to the manufacturer's recommendations.

Construction of plasmids for protein overexpression.

The *phnA1fA2f* genes were amplified by PCR using total DNA as a template and the Extensor Hi-Fidelity PCR Enzyme Mix (Abgene, Surrey, UK). The primers 5'- *CATATG*AATGGATCGTCGG -3' and 5'- *AAGCTT*GATCGAATTTGCTTATGCG -3' introduced *Nde*I and *Hin*dIII sites at the ends of the amplicon that was cloned into pDrive (Qiagen). The DNA insert was checked by nucleotide sequencing prior to subcloning into the *Nde*I and *Hin*dIII site of expression vector pET30f. This construct was transformed into *E. coli* BL21(DE3) for expression analysis. The *phnA1fA2f* pair of genes was also transferred into pVLT31 (51) as a *Xba*I - *Hin*dIII fragment from pET30f*phnA1fA2f*.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cells expressing PhnA1fA2f were pelleted by centrifugation and washed with 10 ml ice-cold phosphate buffer (140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM NaH_2PO_4, pH 7.4). One ml of ice-cold phosphate buffer was added to the pellet and 550 µl of the resulting suspension was subjected to sonication for 20 s. After centrifugation the supernatant and the pellet were mixed with an equal volume of loading solution. SDS-PAGE was performed on 13.3 % polyacrylamide mini gels. After electrophoresis, protein staining was performed with Coomassie brilliant blue R-250.

Dioxygenase overexpression and in vivo assays.

Strains BL21(DE3)(pET30f*phnA1fA2f*) or BL21(DE3)(pVLT31*phnA1fA2f*) complemented with pEB431, carrying ferredoxin (*phnA3*) and ferredoxin reductase (*phnA4*) genes from *Sphingomonas* sp. strain CHY-1 (56), were grown up in 5 ml LB medium with the appropriate antibiotics. This culture was used to inoculate 100 ml LB medium (0.1% by volume), which was incubated at 37°C until an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM, the cells were further incubated overnight at 25°C. For in vivo assays, cells were centrifuged, washed and resuspended to an OD_{600} of approximately 2 in M9 (269) medium containing 0.2% glucose. Cells (12 ml) overexpressing PhnA1fA2f, PhnA3 and PhnA4 were

incubated overnight with 2ml silicone oil containing 400 µM of each tested substrate at 25 °C.

GC-MS analysis of PAH oxidation products.

Water-soluble products were analyzed from samples of the aqueous phase, which were centrifuged, then passed through solid phase extraction columns (Upti-clean C18U, 0.5 g, Interchim, Montluçon, France). The columns were washed with 10 ml water then eluted with 1 ml ethyl acetate. The solvent was evaporated under nitrogen gas and the extracts were dissolved in 200 µl acetonitrile. Prior to analysis, extracts were derivatized with *N,O-bis*(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane (BSTFA) or *n*-butylboronate (NBB). In order to quantify the dihydrodiols formed upon incubation of BL21(DE3)(pVLT31*phnA1fA2f)* recombinant cells with PAHs, 2.3-dihydrobiphenyl (Sigma-Aldrich) was added to 0.1 µM final concentration in the aqueous phase prior to solid phase extraction, and was used as an internal standard. After derivatization and GC-MS analysis, NBB dihydrodiol derivates were quantified on the basis of peak area using a calibration curve generated by analysing known amounts of anthracene 1,2-dihydrodiol. GC-MS analysis of trimethylsilyl derivatives was carried out as previously described (141). NBB derivatives were separated on MDN-12 capillary column (30 m, 0.25 mm internal diameter; Supelco) using helium as carrier gas at 1 ml/min. The oven temperature was held at 75°C for 1 min, then increased to 300°C at 14°C min^{-1}, and held at 300°C for 8 min. The mass spectrometer was operated in the selected ion monitoring mode, selecting *m/z* values corresponding to the expected masses (M^+) of the dihydrodiol derivatives.

DNA and protein sequence analysis.

Sequence analysis was performed using the DNASTAR software package (Lasergene Inc., Madison, WI*)*. The BLAST search tool was used for homology searches (2). Multiple alignments and phylogenetic trees were produced using the DNASTAR and MEGA 3.1 software (183).

Nucleotide sequence accession numbers.

The nucleotide sequences described in this report have been deposited in the Genbank database under accession numbers EU024111 and EU024112 for the catabolic operon and the ring-hydroxylating dioxygenase complex, respectively.

Results

Utilization of phenanthrene and metabolite identification.

Phenanthrene is initially oxidised at the 1,2- , 9,10- or more commonly at the 3,4-position to form *cis*-dihydrodiols. (11, 71, 136, 298) (Fig. 1). The latter is degraded through salicylate (Gram-negative bacteria) or phthalate (Gram-positive bacteria) before entering the Krebs cycle. In order to identify the metabolic pathway used by *Sphingomonas* sp. strain LH128 to degrade phenanthrene, time-course degradation experiments were carried out. 250 mg/l of phenanthrene was completely degraded within 36 hours (data not shown). 1-Hydroxy-2-naphthoic acid, salicylate and catechol were identified by HPLC. Based on these results we attempted to identify and isolate the genes encoding the enzymes involved in the formation of the above-mentioned metabolites (Fig. 1).

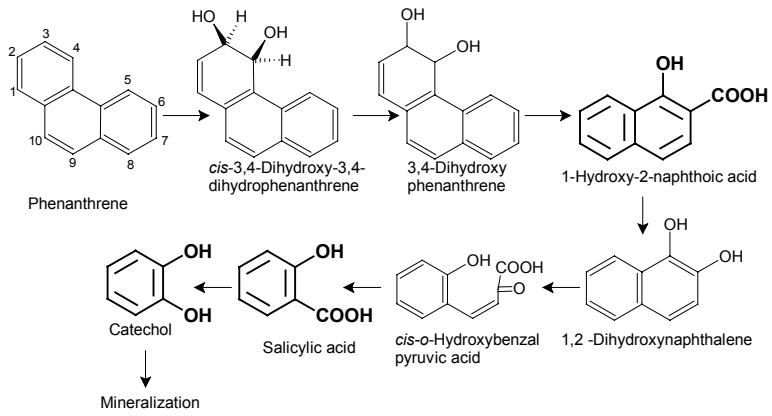

Figure 1. Proposed pathway for phenanthrene degradation by *Sphingomonas* sp. strain LH128. HPLC identified metabolites are shown in bold.

PCR detection of the salicylate hydroxylase and catechol 2,3-dioxygenase encoding genes.

It has been shown that salicylate is a key metabolite in phenanthrene degradation by various *Sphingomonas* species (56, 242, 354). Unlike salicylate 1-hydroxylase from pseudomonads (NahG), which is a well-characterized monomeric flavoprotein (345), the enzyme found in sphingomonads appears to be a three-component Fe-S protein complex (42, 142, 242). The genes encoding the α and β subunit of a salicylate hydroxylase are well conserved and have been identified in *Sphingobium yanoikuyae* B1 (42), *Novosphingobium aromaticivorans* F199 (264), *Sphingobium* sp. strain P2 (242) and *Sphingomonas* sp. strain CHY-1 (142). PCR with primer combinations Block 2C + Block 2D and Block 2E + Block 2F (15) resulted in amplification of overlapping fragments of 1310 and 1444 bp respectively from genomic DNA of strain LH128. DNA sequencing and analysis of the fragments revealed two incomplete (*nahD, phnC*) and three complete open reading frames (ORFs) (*phnA1d, phnA2d, phnA3*) encoded on a 2273 bp fragment. Amino acid sequence analysis revealed that PhnA1cA2c (salicylate 1-hydroxylase) was identical to the protein encoded by the corresponding genes (*phnA1bA2b*) from *Sphingomonas* sp. strain CHY-1 (Table 2). The ferredoxin, PhnA3 had 100 % of protein identity with ferredoxins from strains CHY-1 (CAG17580) and DJ77 (AAC95320). In previously described phenanthrene degradation pathways salicylate is transformed to catechol (42, 56), which is transformed to 2-hydroxymuconic semialdehyde by a catechol 2,3-dioxygenase (XylE). A fragment of the *xylE* gene from *Sphingomonas* sp. strain LH128 (734 bp) was amplified using the primers Cat-F1 and Cat-R1. BLASTx analysis of the obtained fragment showed high identity to known XylE proteins: 86 % identity to *Sphingomonas* sp. strain HV3 (347) and *Sphingomonas chungbukensis* DJ77 (169).

Arrangement of catabolic gene clusters in *Sphingomonas* strains.

In *Sphingobium* sp. strain P2 (240, 242), *Sphingobium yanoikuyae* B1 (225) and *Novosphingobium aromaticivorans* F199 (264) genes encoding salicylate 1-hydroxylase (*bphA1cA2c*) and the *xylE* gene encoding catechol 2,3-dioxygenase are separated by about 10.5 kb (Fig. 2).

Table 2: Summary of the catabolic genes isolated from *Sphingomonas* sp. LH128.

Gene	Probable function or product	Homologous protein	Source	Protein Identity (%)	Accession number
nahD'	2-Hydroxychromene-2-carboxylate isomerase	PhnS	*Novosphingobium aromaticivorans* F199	N.D.	NP_049214
		PhnD	*Sphingomonas* sp. CHY-1	N.D.	CAG17583
		NahD	*Sphingomonas chungbukensis* DJ77	N.D.	AAP84342
phnA1c	Salicylate 1-hydroxylase α subunit	PhnA1b	*Sphingomonas* sp. CHY-1	100	CAG17582
		BphA1c	*Novosphingobium aromaticivorans* F199	96	NP_049213
		AhdA1c	*Sphingomonas* sp. P2	80	BAC65426
phnA2c	Salicylate 1-hydroxylase β subunit	PhnA2b	*Sphingomonas* sp. CHY-1	100	CAG17581
		BphA2c	*Novosphingobium aromaticivorans* F199	98	NP_049212
		AhdA2c	*Sphingomonas* sp. P2	71	BAC65427
phnA3	Rieske-type ferredoxin	PhnA3	*Sphingomonas* sp. CHY-1	100	CAG17580
		PhnR	*Sphingomonas chungbukensis* DJ77	100	AAC95320
		BphA3	*Novosphingobium aromaticivorans* F199	97	NP_049211
phnC	Dihydroxy naphthalene/biphenyl dioxygenase	PhnC	*Sphingomonas* sp. CHY-1	99	CAG17579
		BphC	*Novosphingobium aromaticivorans* F199	96	NP_049210
		DmdC	*Sphingomonas paucimobilis* TZS-7	96	BAB07894
xylX	α subunit toluate/benzoate dioxygenase	XylX	*Novosphingobium aromaticivorans* F199	95	NP_049209
		XylX	*Sphingomonas* sp. P2	69	BAC65430
		ORF6	*Sphingomonas xenophaga* BN6	66	AAD17377

xylY	β subunit toluate/benzoate dioxygenase	XylY	*Novosphingobium aromaticivorans* F199	98	NP_049208
		XylY	*Sphingomonas* sp. P2	69	BAC65431
		BenB	*Pseudomonas fluorescens*	49	ABA06555
orf1158	Hypothetical protein	Orf1158	*Novosphingobium aromaticivorans* F199	100	NP_049207
		Orf9	*Sphingomonas* sp. P2	85	BAC65432
		Orf1	*Cycloclasticus* sp. A5	56	BAC81539
phnA1d	α subunit aromatic oxygenase	BphA1d	*Novosphingobium aromaticivorans* F199	97	NP_049206
		AhdA1d	*Sphingomonas* sp. P2	80	BAC65433
		PhnA1b	*Cycloclasticus* sp. A5	59	BAC81540
phnA2d	β subunit aromatic oxygenase	BphA2d	*Novosphingobium aromaticivorans* F199	78	NP_049205
		AhdA2d	*Sphingomonas* sp. P2	64	BAC65434
		BphA2c	*Novosphingobium aromaticivorans* F199	42	NP_049212
phnK	Glutathione-S-transferase	PhnC	*Sphingomonas chungbukensis* DJ77	97	AAB66314
		BphK	*Novosphingobium aromaticivorans* F199	87	NP_049204
		BphK	*Sphingomonas* sp. P2	81	BAC65435
xylF	2-Hydroxymuconic semialdehyde hydrolase	CmpF	*Sphingomonas* sp. HV3	99	CAB06612
		PhnD	*Sphingomonas chungbukensis* DJ77	99	AAC45091
		XylF	*Novosphingobium aromaticivorans* F199	93	NP_049203
xylE'	Catechol 2,3 dioxygenase	PhnE	*Sphingobium chungbukensis* DJ77	86	AAB41537
		CmpE	*Sphingomonas* sp. HV3	86	CAB06613
		XylE	*Novosphingobium aromaticivorans* F199	83	NP_049202

phnA1f	Ring-hydroxylating dioxygenase α subunit	BphA1f	*Novosphingobium aromaticivorans* F199	99	AAD03858
		BphA1f	*Sphingomonas yanoikuyae* B1	78	ABM91740
		PhnA1a	*Sphingomonas* sp. CHY-1	78	CAG17576
phnA2f	Ring-hydroxylating dioxygenase β subunit	BphA2f	*Novosphingobium aromaticivorans* F199	98	AAD03857
		BphA2	*Sphingomonas yanoikuyae* B1	70	ABM91741
		PhnA2a	*Sphingomonas* sp. CHY-1	63	CAG17577

‘ partial sequence only, N.D. not determined.

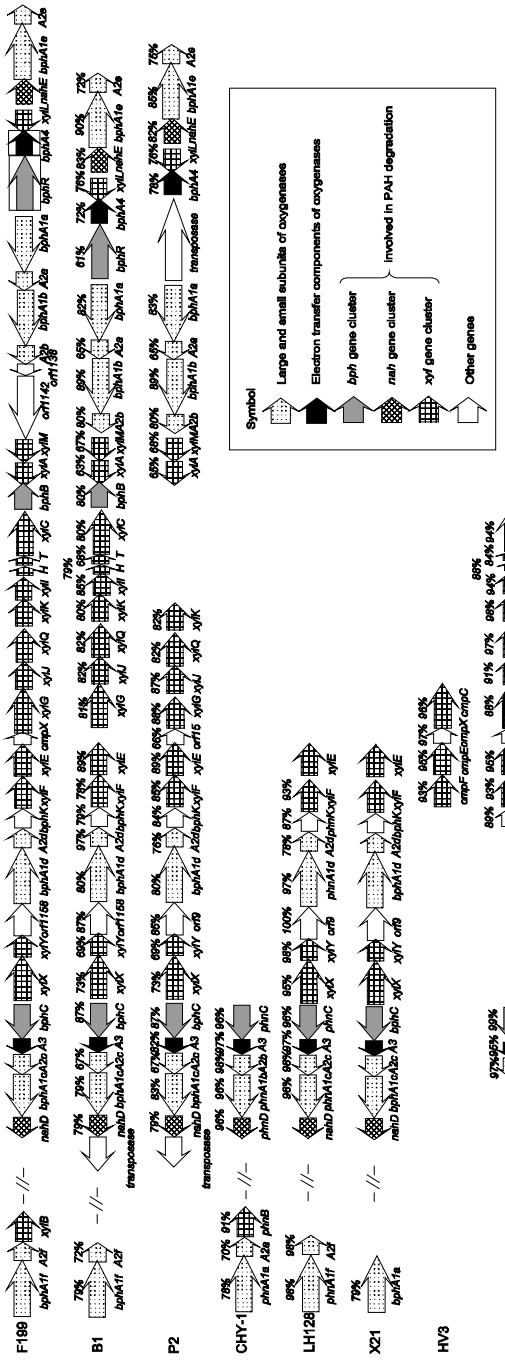

Figure 2. (Modified and updated after (240)). Comparison of the conserved catabolic operon from *Novosphingobium aromaticivorans* F199 (264), *Sphingobium* sp. P2 (242), *Sphingobium yanoikuyae* B1 (225, 354), *Sphingomonas* sp. CHY-1 (56), *Sphingomonas* sp. HV3 (347) and *Sphingomonas chungbukensis* DJ77 (163). The protein sequence identities to the counterparts from strain F199 are indicated.

The high sequence identity of the genes isolated from strain LH128 with counterparts from strains CHY-1, B1, P2 and F199 suggested that the same genetic organization might be found in *Sphingomonas* sp. strain LH128. A long template PCR was therefore performed using primers Block2Cinv and Cat-R1. A fragment of the expected size, about 10 kb, was detected (data not shown). In order to facilitate cloning and handling, intermediate primers were created based on conserved regions of the genes *bphA1d* (aromatic ring hydroxylating dioxygenase) (primers bph1d-F, bphA1d-R) and *xylX* (toluate/benzoate dioxygenase) (primers xylX-F, xylX-R) from *Sphingomonas* sp. strain P2 (239), *Novosphingobium aromaticivorans* F199 (264) and *Sphingobium yanoikuyae* B1 (354). Primer pairs Block2Cinv + xylX-R, xylX-F + bphA1d-F and bphA1d-R + Cat-R1 gave three overlapping fragments that were subsequently cloned and sequenced revealing the presence of 7 more ORFs (Fig. 2). Similarities between representative homologues are detailed in Table 2. Based on protein homology analysis, the identified genes are probably involved in naphthalene (43, 159, 160), biphenyl (159) and xylene (159, 160) degradation. The α and β subunits of a distinct terminal oxygenase were identified (*phnA1dA2d*).

PCR amplification of genes encoding an initial ring hydroxylating dioxygenase.

Based on sequence similarities between the conserved catabolic gene clusters from *Novosphingobium aromaticivorans* F199, *Sphingomonas* sp. strain CHY-1, and *Sphingobium yanoikuyae* B1, we hypothesized that the genes encoding the terminal component of the initial dioxygenase from strain LH128 also showed conserved sequences and could be amplified by PCR using primers bphA1-F and bphA1-R. A fragment of 2048 bp was obtained with genomic DNA from *Sphingomonas* sp. strain LH128 as template. The encoded proteins (PhnA1f and PhnA2f) shared 99%, 78%, 78% identity (α subunit) and 98%, 70% and 63% (β subunit) with counterparts from *Novosphingobium aromaticivorans* F199, *Sphingobium yanoikuyae* B1, and *Sphingomonas* sp. strain CHY-1, respectively (Table 2).

Functional expression of PhnA1fA2f.

In order to investigate the enzymatic activity of PhnA1fA2f, the corresponding genes were PCR-amplified and cloned into the

expression vector pET30f. The resulting construction was introduced into *E. coli* BL21(DE3) for SDS-PAGE analysis of IPTG-induced proteins. Although polypeptides of 50 and 20 kDa were overproduced, the proteins were mainly insoluble (inclusion bodies) and recombinant cells did not show detectable oxygenase activity towards phenanthrene (Fig. 3 A).

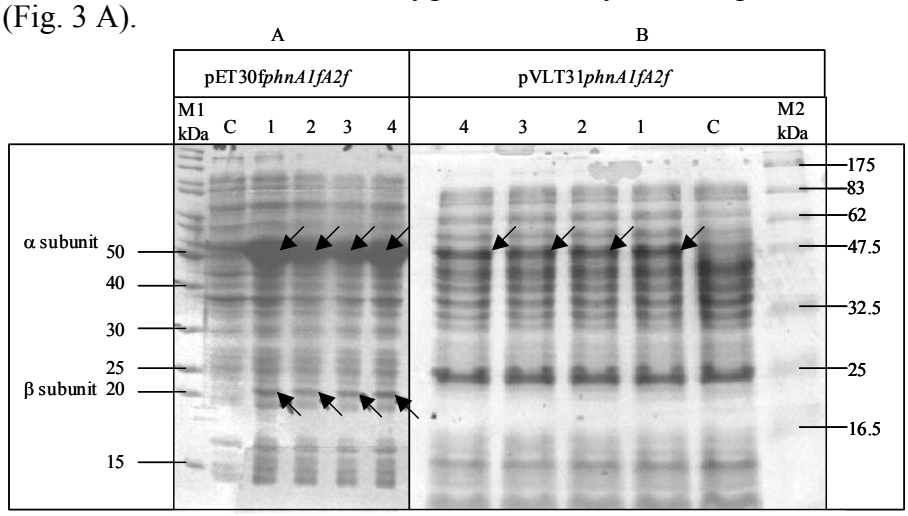

Figure 3. Detection of PhnA1fA2f overproduced in *E. coli* BL21(DE3). A: *E. coli* BL21(DE3)(pET30f*phnA1fA2f*) overproduced high amounts of 50-kDa and 20-kDa that were mainly insoluble. B: *E. coli* BL21(DE3) harbouring pVLT31*phnA1fA2f* produced soluble proteins. However the β subunit could not be detected by SDS-PAGE. *E. coli* BL21(DE3) harbouring pET30f (A) or pVLT31 (B) lacking the *phnA1fA2f* insert were used as controls (C). Protein extracts from 4 clones induced by IPTG are shown (lanes 1-4). Molecular mass (kDa): M1: Prestained PageRuler, Fermentas, St. Leon Rot, Germany , M2: Prestained Protein Marker, Broad Range, New England Biolabs, Ipswich, MA. The arrows show the PhnA1fA2f subunits.

The *phnA1fA2f* sequence was therefore subcloned behind the P*tac* promoter into the broad host-range vector pVLT31 (51) and introduced into *E. coli* BL21 (DE3). When induced with IPTG, the host cells produced appreciable levels of polypeptides, which appeared to form a soluble recombinant protein (Fig. 3B). In order to provide the terminal oxygenase component with an appropriate electron transport chain, plasmid pEB431, allowing the expression of *phnA3* (ferredoxin) and *phnA4* (ferredoxin reductase) from

Sphingomonas sp. strain CHY-1 (56) was co-transformed into *E. coli* BL21(DE3). PhnA3 and PhnA4 formed with PhnA1fA2f a competent enzymatic complex in the *E. coli* host as proved by indigo formation after IPTG induction compared to cells lacking pEB431 (Fig. 4).

Figure 4. To the left: *E. coli* BL21(DE3)(pVLT31*phnA1fA2f*) lacking PhnA3A4 from *Sphingomonas* sp. strain CHY-1 induced by IPTG. To the right: Indigo formation by *E. coli* BL21(DE3) coexpressing PhnA1fA2f and PhnA3A4.

Substrate range of PhnA1fA2f.

The recombinant *E. coli* strain producing PhnA1f, PhnA2f, PhnA3 and PhnA4 was incubated overnight separately with several representative PAHs, dibenzo-*p*-dioxin and PCBs. The water-soluble products released into the culture medium were extracted and analysed by GC-MS (Table 3, Fig. 5) as described elsewhere (181). Since *Sphingomonas* sp. strain LH128 is able to use fluorene, dibenzothiophene, and anthracene in cometabolic degradation (19) we tested whether PhnA1fA2f was responsible for the initial attack on these compounds. The relative activity toward each PAH was calculated from the GC-MS selected ion monitoring peak areas of the NBB (*n*-butylboronate) derivatives compared to an internal standard (2,3-dihydroxybiphenyl). Naphthalene was the preferred substrate (100 %), then, phenanthrene (43.3 %), biphenyl (31.8 %), and anthracene (28.7 %) were converted at significant but lower rates to

the corresponding dihydrodiols. Naphthalene does not support growth of *Sphingomonas* sp. strain LH128 and since the strain was isolated with phenanthrene as the sole source of carbon, the genes were called *phnA1fA2f*. Interestingly, PhnA1fA2f was also able to oxidize the heteroatomic analogues of fluorene i.e. dibenzofuran, dibenzothiophene and carbazole. Strain LH128 is able to degrade fluorene in cometabolism with phenanthrene as the main carbon source (19). However, only traces of fluorenedihydrodiol were detected after NBB derivatization, a result that did not account for the substantial cometabolic activity of strain LH128 towards fluorene. GC-MS analysis of TMS derivates of fluorene oxidation products, identified a large peak of monohydroxyfluorene (RT 16.262 min) with significant fragment ions at m/z 254 (100), 239 (95), 165 (80), 152 (19), 73 (31). Moreover dihydroxyfluorene (RT: 17.577 min; 342 (36), 327 (4), 253 (33), 223 (7), 73 (100) was detected, which most likely resulted from hydroxylation of fluorene on two non-adjacent carbon atoms because it could not be detected by NBB derivatization. Detection of monohydroxycarbazole (RT: 17.092 min; *m/z* 255 (100), 239 (57), 224 (47), 166 (11)) after BSTFA derivatization suggests that PhnA1fA2f transforms carbazole to an unstable dihydrodiol by lateral dioxygenation. Fluoranthene was probably also oxidized to an unstable dihydrodiol, which converted to 8-hydroxyfluoranthene, since the TMS derivate had the same GC-MS characteristics as those reported for the oxidation product of fluoranthene by the PhnI dioxygenase from strain CHY-1 (RT: 20.365 min; *m/z* 290 (100), 275 (55), 215 (15), 201 (19), 200 (18), 189 (30)) (141). Since PhnA1fA2f displayed a relatively high activity towards biphenyl (31.8%), we tested whether PhnA1fA2f could oxidize halogenated biphenyls. Monochlorinated biphenyls such as 2-chlorobiphenyl (6.6 %) and 4-chlorobiphenyl (6.1 %) were oxidized to corresponding dihydrodiols, but 2,3-dichlorobiphenyl was not. Moreover PhnA1fA2f was also able to perform lateral oxygenation of dibenzo-*p*-dioxin. Interestingly, the four-ring PAH benz[*a*]anthracene was transformed into two compounds with masses and retention times consistent with those of two dihydrodiol isomers. These products most likely bear hydroxyls in positions 1,2 and 10,11 since the homologous enzyme from strain CHY-1 preferentially hydroxylated benz[*a*]anthracene on these carbons. Chrysene and pyrene were oxidized to *cis*-3,4-dihydroxy-3,4-dihydrochrysene and *cis*-4,5-dihydroxy-4,5-dihydropyrene based on the retention times of the purified dihydrodiols obtained with Phn1

Table 3. PAH selectivity of PhnA1fA2f from *Sphingomonas* sp. LH128 as expressed in *E. coli*.

Substrate[a]	Products	Molecular mass of NBB derivative	Retention Time (min)	Relative activity (%)[b]	µM Diol/ h mg Prot[c]
Biphenyl	*cis*-2,3-Dihydroxy-2,3-dihydrobiphenyl	254	16.199	31.8	0.097
Naphthalene	*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene	228	14.479	100	0.306
Phenanthrene	*cis*-3,4-Dihydroxy-3,4-dihydrophenanthrene	278	19.239	43.3	0.133
Fluorene[d]	Fluorenedihydrodiol	266	16.043	0.9	0.003
	Monohydroxyfluorene		15.836	N.D.	N.D.
	Monohydroxyfluorene		16.163	N.D.	N.D.
	Dihydroxyfluorene		17.577	N.D.	N.D.
Anthracene	*cis*-1,2-Dihydroxy-1,2-dihydroanthracene	278	19.668	28.7	0.088
Fluoranthene[d]	Fluoranthene-diol	302	21.973	0.1	2.686E-04
	Monohydroxyfluoranthene		20.365	N.D.	N.D.
Benz[*a*]anthracene	*cis*-1,2-Benz[*a*]anthracenedihydrodiol	328	23.563	5.5	0.017
	cis-10,11-Benz[*a*]anthracenedihydrodiol	328	24.513	4.4	0.014
Pyrene	*cis*-4,5-Dihydroxy-4,5-dihydropyrene[e]	302	21.721	Traces	Traces
Chrysene	*cis*-3,4-Dihydroxy-3,4-dihydrochrysene[f]	328	24.801	0.3	9.452E-04
Benzo-*p*-dioxin	Benzo-*p*-dioxindihydrodiol	284	17.936	2.4	0.007
Dibenzothiophene	Dibenzothiophenedihydrodiol	284	18.949	12.6	0.039
Dibenzofuran	*cis*-1,2-Dihydroxy-1,2-dihydrodibenzofuran[g]	268	17.181	17.2	0.053
	Dibenzofurandihydrodiol	268	17.611	5.3	0.016

Carbazole[d]	Carbazoledihydrodiol	267	14.832	0.4	0.001
	Monohydroxycarbazole		17.092	N.D.	N.D.
4-Chlorobiphenyl	4-Chlorobiphenyldihydrodiol	288	19.945	6.1	0.019
2-Chlorobiphenyl	2-Chlorobiphenyldihydrodiol	288	16.806	6.6	0.020

[a] Acenaphthene, benz[*a*]pyrene, benzo[*k*]fluoranthene, benzo[*e*]fluoranthene and 2,3'-dichlorobiphenyl did not give any detectable products.

[b] Calculated from the GC-MS-selected ion monitoring peak areas of the NBB derivatives of the products formed after 24 h of incubation and expressed as percentages of relative activity. The values are averages of two separate determinations.

[c] Calculated from the GC-MS-selected ion monitoring peak areas of the NBB derivatives of the products formed after 24 h of incubation per mg of total proteins. The values are averages of two separate determinations.

[d] Dihydrodiols appear to be unstable and are spontaneously transformed to the corresponding monohydroxylated compounds by dehydration as detected after BSTFA derivatization. Therefore no relative activity is determined for these substrates (N.D.).

[e] Same retention time and mass spectrum as *cis*-4,5-dihydroxy-4,5-dihydropyrene produced by Pdo1 (181)

[f] Same retention time and mass spectrum as *cis*-3,4-dihydroxy-3,4-dihydrochrysene produced by Phn1 (56)

[g] Same retention time and mass spectrum as oxidation products of dibenzofuran from Phn1 (Jouanneau et al. unpublished data)

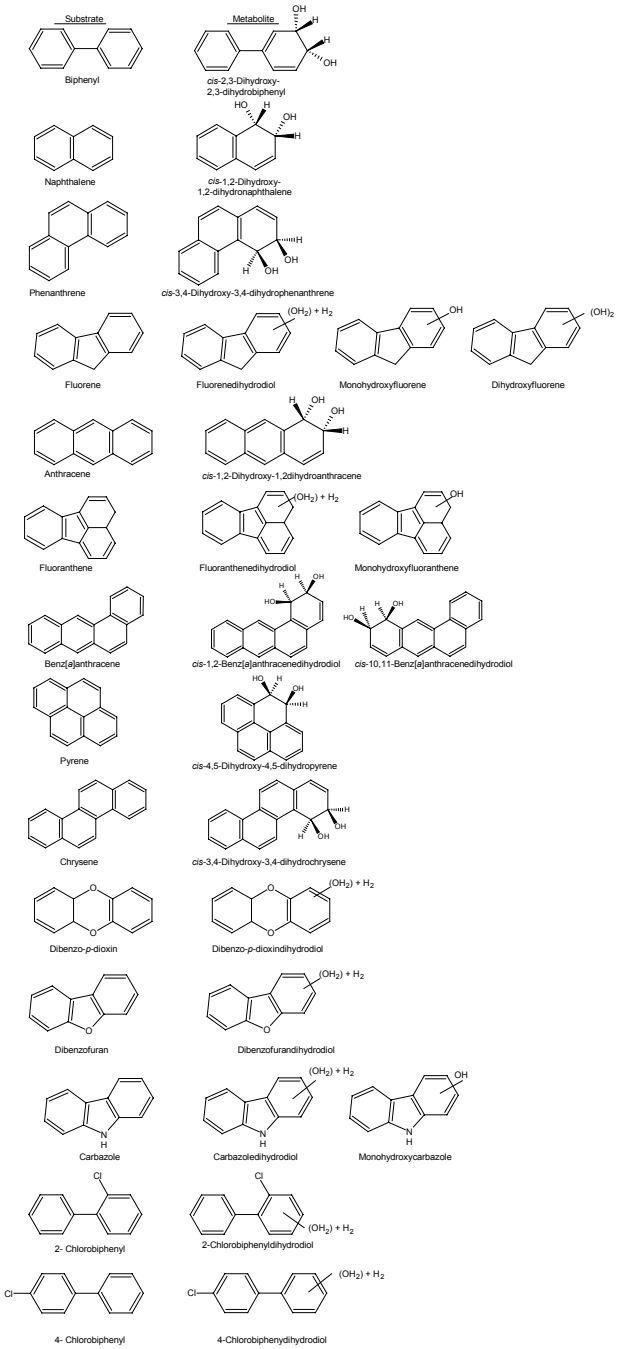

Figure 5. Watersoluble metabolites produced by PhnA1fA2f

(141) and Pdo1 (181), respectively. The 5-ring PAH benz[*a*]pyrene did not produce any detectable dihydrodiol under identical conditions. These data demonstrate that the PhnA1fA2f terminal oxygenase from strain LH128 displays exceptionally broad substrate specificity towards a wide range of aromatic hydrocarbons and other xenobiotics.

Discussion

Sphingomonads are known to degrade a large spectrum of pollutants, ranging from mono- and polycyclic hydrocarbons (241, 301) to naphthalene sulfonate (299), dibenzo-*p*-dioxin (5, 120), and methylated PAHs (60, 354). Most known degradation pathways of homocyclic PAHs start with the formation of a dihydroxy PAH by hydroxylation of two adjacent carbon atoms. This step is catalysed by dioxygenase enzymes that have relaxed substrate specificity, which determines the substrate range of the organism. The compounds are further degraded to a limited number of intermediates such as *o*-phthalic acid or salicylic acid, and then via *ortho* or *meta* cleavage to tricarboxylic acid cycle intermediates. The genes for aromatic hydrocarbon degradation by sphingomonads are quite different from those found in other genera reported both in nucleotide sequence and gene order (240).

By using a PCR-based strategy, a 10.5 kb DNA fragment encoding various hydrocarbon degradation genes was isolated from strain LH128. The gene organization was identical to that found in strains *Sphingomonas* sp. strain CHY-1, *Sphingomonas* sp. strain P2, *Sphingobium yanoikuyae* strain B1 and plasmid pNL1 from *Novosphingobium aromaticivorans* F199 (Fig. 2). These genes were predicted to be required for the conversion of PAHs and biphenyl to aromatic acids (*bph* and *nah* genes) and monoaromatic hydrocarbons to aromatic acids (*xyl* genes) before entering the tricarboxylic acid cycle.

As mentioned before, the ability of a bacterial strain to degrade a given PAH is mainly defined by the initial ring-hydroxylating dioxygenase which determines the strain substrate range. To date only a few sphingomonads initial dioxygenases amongst others have been well characterized: BphA1fA2f from strain B1 (225) and PhnI (141) from strain CHY-1. BphA1fA2f from strain F199 has been identified but further investigation to assess its catalytic abilities is missing.

While the initial dioxygenases from strains LH128 and CHY-1 are closely related (78 % identity), strain CHY-1 is able to grow on chrysene as the sole source of carbon (336) while strain LH128 cannot use chrysene as a substrate. Likewise, the dioxygenases from strains CHY-1 and B1 show apparent differences of substrate specificity despite sharing an almost identical structure (56, 141, 225). These observations suggest that there exists a pool of highly conserved multicomponent dioxygenases in sphingomonads, with subtle structural variations that would be responsible for differences in selectivity toward PAHs. Phylogenetic analysis showed that dioxygenases from *Sphingomonas* species cluster together and are only distantly related to dioxygenases from other genera (Fig. 6). Six homologues to both large and small substrate binding components of ring hydroxylating dioxygenases were identified within the same strain. The genes (*bphA1*$_{[a\text{-}f]}$-*bphA2*$_{[a\text{-}f]}$) can be found in *Sphingomonas yanoikuyae* B1 (354), *Sphingomonas* sp. strain P2 (242) and *Novosphingobium aromaticivorans* F199 (264). Since the genes isolated from strain LH128 display high homologies to catabolic genes from these species, one can expect to find the missing dioxygenase encoding genes (*bphA1*$_{[a,b,e]}$-*bphA2*$_{[a,b,e]}$) in strain LH128. Moreover, it has been shown that *Sphingomonas* population structures of several PAH-contaminated soils by PCR-DGGE revealed that soils containing the highest phenanthrene concentrations showed the lowest *Sphingomonas* diversity (190). This indicates that *Sphingomonas* species share a set of dioxygenases that probably originated as phenanthrene catabolic genes, then, by duplication, evolved to degrade different substrates. For instance, the enzymes involved in the initial step of PAH degradation exhibit a greater variety than those involved in the catabolism of central metabolites such as salicylate. (Table 4). The overall identities between salicylate 1-hydroxylase are higher then the identities between the respective ring-hydroxylating dioxygenases of the different strains. This clearly indicates that the enzymes involved in the upper PAH catabolic pathways have a more relaxed substrate specificity then the enzymes involved in the lower pathway.

When overexpressed in *E. coli* BL21(DE3), PhnA1fA2f was found to be responsible for the oxidation of low and high molecular weight PAHs, dibenzo-*p*-dioxin and monochlorinated biphenyls but not 2,3-dichlorobiphenyl. Traces of carbazole dihydrodiol were detected after NBB derivatization, but monohydroxycarbazole was abundant.

Table 4. Salicylate 1-hydroxylase and the initial ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain P2, *Novosphingobium aromaticivorans* F199, *Sphingomonas* sp. strain LH128, *Sphingobium yanoikuyae* B1 and *Sphingomonas* sp. strain CHY1 were compared to each other.

Ring-hydroxylating dioxygenase	BphA1 P2[a]	BphA1f F199[a]	PhnA1f LH128[a]	BphA1f B1[a]	PhnA1a CHY-1[a]
BphA1 P2 (BAC65448)	100	42	42	38	39
BphA1f F199 (YP_001165670)		100	92	75	74
PhnA1f LH128 (EU024112)			100	79	78
BphA1f B1 (2GBW_A)				100	91
PhnA1a CHY-1 (2CKF_A)					100
Salicylate 1-hydroxylase	**BphA1c P2**	**BphA1c F199**	**PhnA1c LH128**	**BphA2c B1**	**PhnA1b CHY-1**
BphA1c P2 (BAC65426)	100	79	79	96	79
BphA1c F199 (NP_049213)		100	97	76	97
PhnA1c LH128 (EU024111)			100	76	76
BphA2c B1 (ABM79781)				100	76
PhnA1b CHY-1 (CAG17582)					100

[a] Amino acid identity to their respective counterparts is shown.

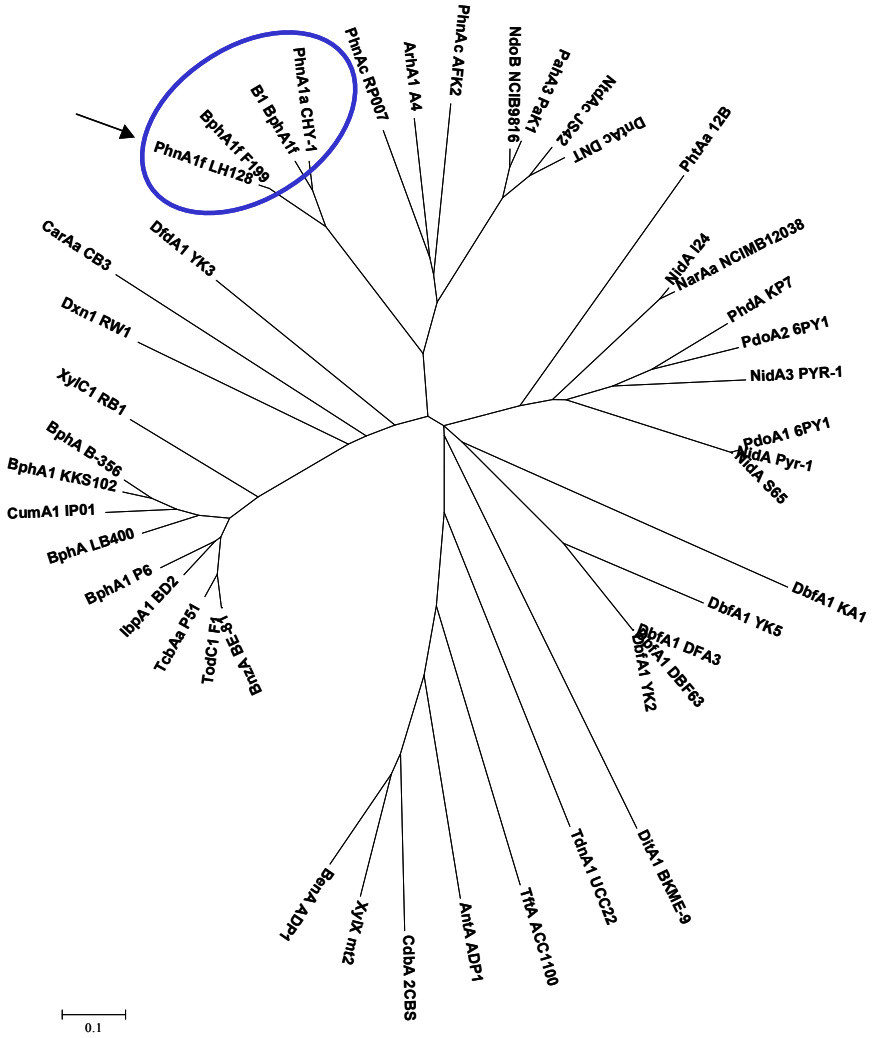

Figure 6. Phylogenetic distribution of initial dioxygenase α subunits. The dendrogram was constructed from a ClustalW alignment by neighbour-joining analysis using MegA3.1 software (183). Sequences were retrieved from GenBank database. Dioxygenase α subunits from *Sphingomonas* strains sharing a conserved catabolic cluster group together (circle). The scale bar represents substitutions per amino acid. NidA I24, indene, *Rhodococcus* sp. I24 (AF121905); PhdA KP7, phenanthrene, *Nocardioides* sp. KP7 (AB017794); NarAa NCIMB12038, naphthalene, *Rhodococcus* sp. NCIMB12038 (AF082663); DitA1 BKME-9, diterprenoid, *Pseudomonas abietaniphila* BKME-

9 (AF119621); IpbA1 BD2, isopropylbenzene, *Rhodococcus erythropolis* BD2 (U24277); BphA1 P6, biphenyl, *Rhodococcus globerulus* P6 (X80041); TcbAa P51, chlorobenzene, *Pseudomonas* sp. P51 (U15298); BnzA BE-81, benzene, *Pseudomonas putida* BE-81 (M17904); TodC1 F1, toluene, *Pseudomonas putida* F1 (J04996); XylC1 RB1, unknown substrate, *Cycloclasticus oligotrophus* RB1 (U51165); BphA LB400, biphenyl, *Burkholderia cepacia* LB400 (M86348); BphA1 KKS102, biphenyl, *Pseudomonas* sp. KKS102 (D17319); BphA B-356, biphenyl, *Comamonas testosteroni* B-356 (U47637); CumA1 IP01, cymene, *Pseudomonas fluorescens* IP01 (D37828); TdnA1 UCC22, aniline, *Pseudomonas putida* UCC22 (D85415); TftA ACC1100, 2,4,5-trichlorophenoxyacetic acid, *Burkholderia cepacia* AC1100 (U11420); AntA ADP1, anthranilate, *Acinetobacter calcoaceticus* ADP1 (AF071556); CbdA 2CBS, 2-halobenzoate, *Burkholderia cepacia* 2CBS (X79076); XylX mt2, toluate, *Pseudomonas putida* mt2 (M64747); BenA ADP1, benzoate, *Acinetobacter calcoaceticus* ADP1 (AF009224); DxnA1 RW1, dibenzo-*p*-dioxin, *Sphingomonas* sp. RW1 (X72850); CarAa CB3, carbazole, *Sphingomonas* sp. CB3 (AF060489); NdoB NCIB9816, naphthalene, *Pseudomonas putida* NCIB9816 (M23914); PahA3 PaK1, naphthalene, *Pseudomonas aeruginosa* PaK1 (D84146);NtdAc JS42, 2-nitrotoluène, *Pseudomonas* sp. JS42 (U49504); DntAc DNT, 2,4-dinitrotoluène, *Burkholderia* sp. DNT (U62430); PhnAc RP007, phenanthrene, *Burkholderia* sp. RP007 (AF061751); PhnAc AFK2, phenanthrene, *Alcalingenes faecalis* AFK2 (AB024945); PhtAa, phthalate, *Arthrobacter keyseri* 12B (AF331043); BphA1f F199, biphenyl, *Sphingomonas aromaticivorans* F199 (AF079317); NidA PYR-1, pyrene, *Mycobacterium vanbaalenii* PYR-1 (AF249301); NidA S65, pyrene, *Mycobacterium* sp. S65 (AF546904); DbfA1, carbazole, *Sphingomonas* sp. KA1 (BAF03470); DbfA1, dibenzofuran, *Terrabacter* sp. strain DBF63 (BAC75993); DbfA1, dibenzofuran, *Rhodococcus* sp. strain YK2 (BAC00802); DbfA1, dibenzofuran, *Paenibacillus* sp. strain YK5 (BAE53401); DbfA1, dibenzofuran, *Rhodococcus* sp. strain DFA3 (BAD51811); DbfA1, dibenzofuran, *Terrabacter* sp. strain YK3 (BAC06602); ArhA1, acenaphthene and acenaphthylene, *Sphingomonas* sp. strain A4 (BAD34447); BphA1f, biphenyl, *Sphingomonas yanoikuyae* B1 (ABM91740); PhnA1a, chrysene, *Sphingomonas* sp. strain CHY-1 (AJ633551); PdoA1, pyrene, *Mycobacterium* sp. strain 6PY1 (CAD38647); PdoA2, pyrene, *Mycobacterium* sp. strain 6PY1 (CAD38643); NidA3, pyrene, *Mycobacterium vanbaalenii* PYR-1 (AAY85176); PhnA1f, phenanthrene, *Sphingomonas* sp. LH128 (EU024112).

Resnick et al. (257) reported the formation of monohydroxycarbazole, possibly as a result of dehydration of unstable dihydrodiols. Phenanthrene (43.3 %), biphenyl (31.8 %) and anthracene (28.7 %) were transformed to high levels of the corresponding *cis*-diols. Interestingly oxidation products of benz[*a*]anthracene, chrysene and pyrene (Table 3) were also identified in contrast with naphthalene

dioxygenases whose selectivity is limited to only two and three ring PAHs (73, 86, 152). The five ring PAH benz[*a*]pyrene did not give any detectable products. These results suggest that benz[*a*]pyrene probably does not fit into the catalytic pocket of PhnA1fA2f.

The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain CHY-1 has been recently described on the basis of its crystal structure, and the amino acids lining the catalytic pocket were identified (129, 130). These residues are conserved in the enzymes from *Sphingomonas* sp. strain LH128, *Novosphingobium aromaticivorans* F199 and, with only two substitutions, in *Sphingobium yanoikuyae* B1 (129) (data no shown), suggesting that the topology of the substrate binding pocket is almost identical. However, these structural resemblances do not explain the differences in substrate specificity of the dioxygenases. The crystal structure of the ring hydroxylating dioxygenase from strain CHY-1 showed that the entrance of the catalytic pocket is covered by two flexible loops L1 and L2, exposed to the solvent. These loops are predicted to control the substrate's access to the catalytic pocket (130). Since the sequence of these loops is only partly conserved in the LH128 enzyme (83 % and 63 % identities for L1 and L2, respectively), it seems plausible that these structural differences are responsible for the lower activity of the LH128 dioxygenase towards high molecular weight PAHs and its inability to oxidize benz[*a*]pyrene. The effects on the catalytic activity of residue substitutions in the active site have been well investigated in the case of naphthalene dioxygenase and biphenyl dioxygenases (232-235), but the effect of substitutions outside the catalytic pocket is less well documented (84, 352, 353). Our results indicate that residues in the loops at the entrance of the catalytic pocket are potentially interesting targets for mutagenesis as a means to better understand the structural determinants of selectivity.

To conclude, we identified the genes encoding the dioxygenase responsible for the initial attack on various PAHs by *Sphingomonas* sp. strain LH128 and expressed them in *E. coli.* The dioxygenase PhnA1fA2f was closely related to BphA1fA2f from *Novosphingobium aromaticivorans* F199 and, to a lower extent, to PhnI from *Sphingomonas* sp. strain CHY-1 and BphA1fA2f *Sphingobium yanoikuyae* B1. Characterization of the activity of the dioxygenase in *E. coli* showed significant differences in catalytic activity compared to the proteins PhnI from strain CHY-1 and BphA1fA2f from strain B1,

indicating that small differences in amino acid sequence outside the catalytic pocket can have substantial effects on dioxygenase selectivity. PhnA1fA2f was able to oxidise the four ring PAH benz[*a*] anthracene and yielded to dihydrodiols.

Acknowledgements

L.S. gratefully acknowledges the Fund for the Promotion of Research in Industry and Agriculture (F.R.I.A.), Belgium, for providing a doctoral fellowship. L.S. also wishes to thank the members of the Unit of Physiological Biochemistry (FYSA), Catholic University of Louvain, for their daily help and constructive remarks for many years. PH is research associate at FNRS.

Part IV - General Conclusions & Perspectives

This work focused on the mechanisms by which environmental bacteria utilize aromatic compounds as carbon and energy sources. Therefore we investigated the enzymes involved in the initial attack on fluorene by *Sphingomonas* sp. strain LB126 and on phenanthrene by *Sphingomonas* sp. strain LH128. Both strains were isolated from the same contaminated environment (17).

Fluorene degradation by *Sphingomonas* sp. strain LB126

Fluorene degradation by strain LB126 has already been investigated and the genes required in protocatechuic acid degradation were identified (331). Based on metabolites identified during growth on fluorene, strain LB126 performs an angular dioxygenation of 9-fluorenone to produce 1,1a-dihydroxy-1-hydro-9-flurenone. Even though some strains (29, 100, 314) have been reported to be able to form this metabolite, the enzymology involved in these steps, especially in Gram-negative bacteria, remained unknown. Therefore the focus of this work was to shed light on these particular steps in *Sphingomonas* sp. strain LB126 and the angular dioxygenase involved in the initial attack on fluorene was characterised. This report was the first providing evidence for fluorene oxidation in Gram-negative bacteria. Several techniques such as transposon mutagenesis and PCR-based methods amplifying conserved sequences from known genes were evaluated for their use in gene identification experiments in this bacterium.

Transposon mutagenesis and *Sphingomonas* spp.

Transposon mutagenesis is a powerful tool for genetic analyses. The random insertion of transposons may be of great value for organisms with poorly described genetic systems. A transposon mutagenesis approach was used on *Sphingomonas* sp. strain LB126 in order to knockout genes involved in fluorene degradation. Due to the lack of positive results, this strategy was abandoned for a PCR-based approach.

Today transposons are widely used at a genomic level as well as on individual genes. To overcome problems with the size of transposons, mini-transposons arrange the cognate transposase outside of the transposons' inverted repeats (52). By doing this, the mini-transposons can stably integrate in the target genome without their transposase preventing further transposition. Mini-transposons have been constructed for the mapping of random insertional mutations, promoter probing and to create translational or transcriptional fusions with a variety of promoterless reporter genes (18, 132, 316). Among these the most popular reporter genes are *gfp* (303, 339) and *luxCDABE* (38, 249, 334). They have been used for the detection of toxic pollutants (18, 70, 112), for genetic studies in thermoregulated genes (248, 249) or in monitoring of bacteria (70, 303).

A variant of the plasposon pTn*Mod*-OTc (58), a self cloning mini-transposon derivative of Tn*5* that carries the transposase in *cis* to the transposable element just outside the inverted repeats to prevent multiple insertions was used. Into this plasposon we cloned the promoterless *gfp* gene to create transcriptional fusions with the target DNA to allow the positive selection of promoters being induced by several substrates (fluorene vs. glucose) (Fig. 2). To facilitate cloning and manipulations, the plasposon harbouring the *ori* pMB1 origin of replication that is not functional in bacteria other than *Escherichia coli* was used. This origin requires the π replication protein to function properly. The plasposon will not replicate unless the bacterial host can supply the π gene product in *trans*. As this protein is usually carried on the plasmid, this suicide-plasmid behaviour allows the selection of cells whose plasposon has been inserted in the bacterial genome (58).

Because Tn*5* has a broad host range, the Tn*Mod*-OTc-*gfp* should work efficiently in gram-negative bacteria. An advantage of this method is certainly the rapid recovery of the mutated region and can hence be used to identify novel genes in environmental bacteria. Because of the promoterless *gfp* gene, this plasposon could be used to create GFP transcriptional fusions and show that a metabolic pathway is broken down.

During this work it has become clear that transposon mutagenesis is not a suitable technique for studying *Sphingomonas* sp. strain LB126. During the bacterial mating step, a passage on LB medium is necessary for *E. coli* S17-1λpyr to pass the genetic information on to

Sphingomonas strains. Unfortunately this step, as well as the screening of recombinant *Sphingomonas* mutants, has to be performed in the presence of an alternative carbon source such as glucose (4 g/l) or protocatechuic acid (0.5 g/l) (331). Contrary to our initial expectation, these experiments showed that the wild type strain quickly lost the ability to degrade PAHs. Therefore it was unclear whether the observed Flu$^-$ phenotype was due to the knockout of fluorene catabolic genes, some rearrangements triggered by the conjugation on rich medium or the loss of essential genes carried on a conjugative catabolic plasmid (264). Indeed such plasmids are common in *Sphingomonas* spp. (16) but sometimes the catabolic genes are chromosome encoded as for *Sphingobium yanoikuyae* B1 (354) and mutant generation can be achieved. This technique is apparently not suitable for the study of *Sphingomonas* sp. strain LB126 and was abandoned for a PCR based approach amplifying small conserved regions of catabolic genes.

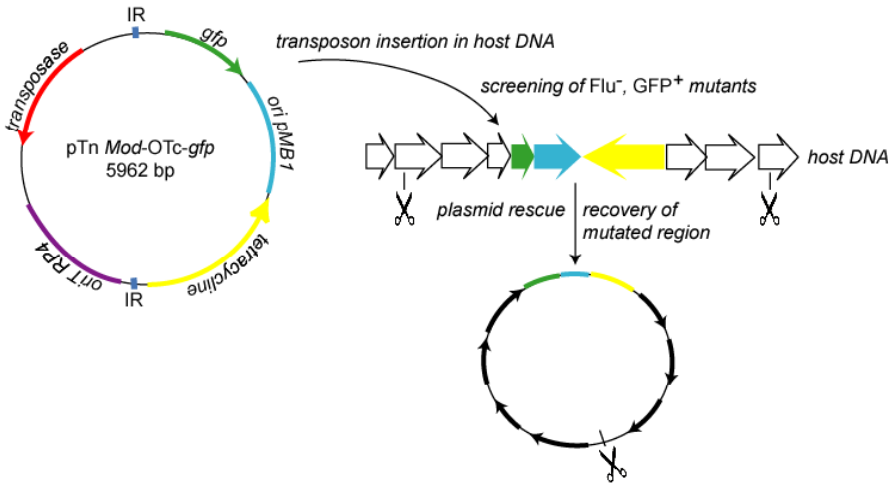

Figure 2. Transposon mutagenesis: the mini Tn*5* derivative inserts randomly into the *Sphingomonas* sp. strain LB126 genome. GFP$^+$ mutants in the presence of fluorene will be selected and the mutated region analyzed. A plasmid rescue strategy allows recovering of the mutated region.

Fluorene is oxidized by an angular dioxygenase probably acquired by lateral gene transfer

Gram-positive *Terrabacter* sp. strain DBF63 was the first strain from which the genes involved in the initial attack on fluorene were identified and isolated (150). Interestingly, a similar set of genes is apparently also responsible for the conversion of fluorene in strain LB126. The fact that a catabolic operon showing a similar organization was found in *Sphingomonas* sp. strain LB126 underlines that horizontal gene transfer is responsible for the distribution of catabolic genes amongst genera.

The angular dioxygenase from *Sphingomonas wittichii* strain RW1, responsible for the oxidation of dibenzofuran and dioxins is only distantly related to those of strains DBF63 and LB126 (31, 150). One could expect that angular dioxygenases from strains RW1 and LB126 share some homologies. Moreover the dioxygenase complex FlnA1A2 isolated from strain LB126 showed relaxed substrate specificity towards PAHs and was able to perform monooxygenations, lateral and angular dioxygenations on PAHs such as phenanthrene, anthracene and carbazole that were not oxidized by its closest homologue DbfA1A2 from strain DBF63 (150).

The most interesting feature of FlnA1A2 is the production of two different dihydroanthracenediols formed by lateral oxygenation that showed the same principal fragment ions as determined by GC-MS. One was identified as *cis*-1,2-dihydroxy-1,2-dihydroanthracene, the second is believed to be *cis*-2,3-dihydroxy-2,3-dihydroanthracene. This metabolite has not been described for any other strain so far. Since the ferredoxin and ferredoxin reductase genes have not yet been identified, it is difficult to produce sufficient metabolite for NMR studies.

With FlnA1A2 expressed together with adequate ferredoxin and ferredoxin reductase genes, the activity of the angular dioxygenase can be enhanced. Since the genes FlnA1A2 show high identities to Gram-positive genes it is possible that the ferredoxin and reductase were also acquired by lateral transfer and do not share homologies with sphingomonad's electron transport proteins. In *Terrabacter* sp. strain DBF63 the ferredoxin *dbfA3* gene was recently identified within

2.5 kb from *dbfA1A2* (307), but the reductase remains unknown. Ferredoxins in the CARDO system from *Pseudomonas resinovorans* CA10 (222, 271), *Terrabacter* sp. strain YK2 (122) and *Sphingomonas wittichii* RW1 (4, 6) possess a [2Fe-2S] cluster. In strain *Terrabacter* sp. strain DBF63 however; the candidate ferredoxin-encoding gene is part of a protein fusion of an extradiol dioxygenase small subunit and a putative ferredoxin (108). The deduced C-terminal 100-amino acid sequence showed homology to the [3Fe-4S] and [4Fe-4S] ferredoxins, the N-terminal 90-amino acid peptide functions with FlnD1 to catalyse ring cleavage (108). Hence, it is of great interest to elucidate the downstream region of *flnA1A2* in strain LB126 since a partial gene encoding FlnD1 (ring cleavage dioxygenase) has been identified.

Biotransformation assays have been carried out using the electron transport proteins from Gram-positive *Nocardioides* sp. strain KP7 (267). The ferredoxin (PhdC) and ferredoxin reductase (PhdD), have been cloned to give plasmid pBRCD (181). The PhdC sequence showed features of a [3Fe-4S] or [4Fe-4S] type of ferredoxin, not of the [2Fe-2S] type of ferredoxin that has been found in most of the reported ring-hydroxylating dioxygenases (267). Expression of *phdC* and *pdhD* from strain KP7 in strain BL2(FlnA1A2)(pBRCD) resulted in an eightfold increase in the rate of anthracene dihydrodiol formation compared to the strain BL21 expressing FlnA1A2 lacking PhdCD.

Quantifying the mRNA levels of the *flnA1A2* genes in the presence of different substrates by real-time PCR could provide information regarding gene regulation and expression. This approach can be extended to other genes whose function is unclear. This approach could also be applied to analyse the cometabolism of several PAHs. Since soils are generally contaminated by a mixture of PAHs, the analysis of gene expression levels could provide information regarding the overall degradation and investigate the effect of a co-substrate on the degradation rate.

Even though Tn*5* insertions have been carried out on strain LB126 without success, the knockout of the *flnA1A2* genes could be obtained by using an alternative strategy. Since the ability to degrade fluorene by strain LB126 was probably lost during the growth phase on glucose after conjugation, one could bypass this problem by inserting a

functional copy of the *dbfA1A2* genes from *Terrabacter* sp. strain DBF63 on a thermosensible plasmid. The selection of a mutant strain could then be carried out in the presence of fluorene. A temperature shift leading to the loss of that plasmid would show the importance of the target gene. A *flnA1A2* linear DNA fragment harbouring an antibiotic resistance cassette could directly be introduced into *Sphingomonas* sp. strain LB126 by electroporation as described for *Sphingomonas* sp. strain CHY-1 (56). Keeping a selective pressure, the inserted fragment will integrate the host DNA by homologous recombination.

In order to identify and isolate genes potentially involved in PAH degradation, one could investigate the sequences flanking the angular dioxygenase cluster. Genome walking based on the already known sequences allows a quick isolation of adjacent DNA fragments and makes the construction of a clone library obsolete. This strategy was also applied to *Terrabacter* sp. strain DBF63 to identify the phthalate catabolic gene cluster that is linked to the angular dioxygenase genes. Phthalate is a metabolic intermediate of fluorene, phenanthrene and anthracene degradation; it has been studied in detail for aerobic bacteria and two pathways have been identified. While phthalate is metabolised to protocatechuate via 4,5-dihydroxyphthalate in Gram-negative bacteria (40, 221, 259), Gram-positive bacteria degrade phthalate via 3,4-dihydroxyphthalate and protocatechuate (44, 64). In *Terrabacter* sp. strain DBF63 the genes encoding seven catabolic enzymes: oxygenase large subunit of phthalate 3,4-dioxygenase (*phtA1*), oxygenase small subunit of phthalate 3,4-dioxygenase (*phtA2*), cis-3,4-dihydroxy- 3,4-dihydrophthalate dehydrogenase (*phtB*), *phtA3, phtA4*, ferredoxin and ferredoxin reductase of phthalate dioxygenase, 3,4-dihydroxyphthalate decarboxylase (*phtC*) and putative regulatory protein (*phtR*), were found in the upstream region of the angular dioxygenase gene (*dbfA1A2*) (109). Since a truncated transposase was found upstream from FlnA1A2 in strain LB126 it would be interesting to check what genes are located downstream of the already known sequence and give information about the size of the horizontally acquired gene cluster and the possible presence of a ferredoxin fused to FlnD2 (307). The identification of the genes responsible for the conversion of phthalate to protocatechuate will complete the fluorene catabolic pathway in strain LB126 and clarify if these genes share homologies to Gram-positive genes or not.

Moreover the study of already identified genes might give a deeper insight into fluorene degradation by this strain and one could attribute a function to these genes. This was attempted with FlnB, a novel *cis*-dihydrodiol dehydrogenase. Even though the genetic organization of the catabolic genes isolated from strain LB126 was similar to *Terrabacter* sp. strain DBF63, the *flnB* gene product did not show any homology to its counterpart from strain DBF63 (150). FlnB was overexpressed in *E. coli* BL21(DE3) to investigate whether this protein was responsible for the further oxidation of 1,1a-dihydroxy-1-hydro-9-flurenone (DHF) to 2'-carboxy-2,3-dihydroxybiphenyl. DHF showed a major absorption peak at 314 nm, which was used to monitor changes in UV absorption. Even though DHF was probably transformed by FlnB, as measured by spectrophotometric readings, and confirmed by GC-MS analysis, the transformation product could not be identified. Therefore a new recombinant expression clone, allowing the purification of FlnB as a His-tagged protein is necessary to clarify if the observed changes in UV spectra are due to FlnB or not.

FlnA1A2 carried out the initial step of the degradation of dibenzo-*p*-dioxin, the non-halogenated backbone of the highly toxic 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin. This reaction consists in a stereospecific angular dioxygenation of one of the aromatic rings leading to the formation of cyclic hemiacetals, which spontaneously cleave to yield 2,2',3-trihydroxydiphenyl ether. Polyhalogenated monocyclic and polycyclic aromatics are important and widely dispersed pollutants that are difficult to treat due to their occurrence in the environment at low but toxicologically relevant concentrations. Most of these xenobiotics are chemically stable and thus biochemically recalcitrant and are of considerable concern due to their accumulation in the food chain (318). Therefore it is important to include these contaminants in future analysis in order to extend the substrate range of FlnA1A2. Moreover it would be worth assessing the degradation rate of these compounds by the wild type strain for possible bioremediation techniques.

The fluorene catabolic genes are encoded on linear plasmid pDBF1 in *Terrabacter* sp. strain DBF63 (107). Therefore it would be interesting to analyse the plasmidic content of strain LB126 and check whether the fluorene degradation genes are plasmid or chromosome encoded. This knowledge could shed light on the distribution of these genes in

environmental bacteria. Especially the *dbf/fln* genes seem to be good candidates to study their distribution in the environment since they can be found in various strains (231).

Although many ring hydroxylating dioxygenases have now been characterized, the properties of this particular enzyme (FlnA1A2) are interesting. Phylogenetic analysis showed that this protein, isolated from Gram-negative *Sphingomonas* sp. strain LB126 clusters with proteins isolated from Gram-positive bacteria and is only distantly related to other sphingomonad dioxygenases. This raises the question why this enzymes is able to use substrates that are not used by *Paenibacillus* sp. strain YK5 (dibenzo-*p*-dioxin, dibenzothiophene, fluorene and fluoranthene) (124), *Sphingomonas* sp. KA1 (fluorene) (289) and *Terrabacter* sp. DBF63 (phenanthrene, anthracene, carbazole) (150).

The resolution of its crystal structure could give valuable information regarding the substrate specificity of FlnA1A2 that might be explained by the shape and size of its substrate-binding pocket. Further studies involving replacements of specific residues of the substrate-binding pocket by site-directed mutagenesis should bring new insights into the role of the residues lining the catalytic pocket of the enzyme.

Sphingomonas sp. strain LH128 harbours a conserved catabolic cluster and a naphthalene dioxygenase

Sphingomonas sp. strain LH128 is capable of using phenanthrene as the sole carbon source and can also degrade three-ring azareenes (321), but no information about catabolic genes is available. Strain LH128 is able to transform indole to indigo in the presence of phenanthrene, a property of "naphthalene-like" dioxygenases (67). The metabolites formed by *Sphingomonas* sp. strain LH128 during growth on phenanthrene were analyzed by HPLC and the genes involved in these steps were identified. Salicylate hydroxylase (*bphA1cA2c*) and catechol 2,3-dioxygenase (*xylE*) genes were identified by using a PCR-based method. Sequence analysis showed high identities to already known genes isolated from *Sphingomonas* sp. strain CHY-1 (56), *Sphingobium yanoikuyae* B1 (354), *Sphingomonas* sp. strain P2 (242) and *Novosphingobium aromaticivorans* F199 (264). These findings allowed isolating a continuous fragment of ~10.5 kb harbouring 13 open reading frames encoding putative catabolic genes. These genes were predicted to be required for the conversion of PAHs and biphenyl to aromatic acids (*bph* and *nah* genes) and monoaromatic hydrocarbons to aromatic acids (*xyl* genes) before entering the tricarboxylic acid cycle (354). Based on what is known about the genetics of aromatic hydrocarbon degradation in related organisms (i.e. *Pseudomonas* species (8, 344)), one might expect these genes to be organized into operons consisting of catabolic segments. For instance, one operon would contain those genes required for conversion of polycyclic aromatic hydrocarbons to simple aromatic acids (similar to the *bph* operon (83) or the upper *nah* operon (59, 344), a second operon might contain those genes required for conversion of monocyclic aromatic compounds to aromatic acids (similar to the upper *xyl* operon (8)), and a third operon would contain those genes required for conversion of aromatic acids to tricarboxylic acid cycle intermediates (similar to the lower *xyl* operon (8)). However, this does not seem to be the case for *Sphingomonas* sp. strain LH128, *Sphingobium* sp. strain P2 (242), *Sphingobium yanoikuyae* B1 (354) and *Novosphingobium aromaticivorans* F199 (264). In the process of evolving the capability to degrade a wide variety of aromatic hydrocarbons these organisms have recruited, modified, and reorganized the appropriate genes and operons needed to construct the required catabolic pathways. This genetic organization

suggests that the members of this genus have the ability to adapt more quickly or more efficiently to the degradation of new compounds in the environment than members of other bacterial genera (16). This could indicate that in the evolution of degradative pathways, some kind of barriers must have existed between sphingomonads and other *Proteobacteria* explaining that this particular genetic organization is specific to the genus *Sphingomonas*.

In future work, a genome walking approach could further identify the missing genes of the catabolic cluster in strain LH128. In particular the dioxygenases *bphA1aA2a*, *bphA1bA2b, bphA1dA2d* and *bphA1eA2e* could be characterized. The study of these genes could clarify how these sets of genes have evolved. It is unclear if these dioxygenases are duplicates or if they are specific to different substrates. Since duplication is a major force of evolution the study of these enzymes could give valuable information as to how the catabolic cluster has evolved in *Sphingomonas* species and provide knowledge about new biological processes or new insights into known processes.

Due to similarities encountered in this catabolic cluster to other sphingomonads genetic organization, we investigated whether the initial dioxygenase carrying out the first attack on phenanthrene also shares homologies with enzymes from the above-mentioned sphingomonads. A terminal dioxygenase (PhnA1fA2f) was isolated showing high identities (98 %) to BphA1fA2f the terminal component of an initial dioxygenase from *Novosphingobium aromaticivorans* F199. When overexpressed in *E. coli* BL21(DE3) together with PhnA3A4, the ferredoxin and reductase from *Sphingomonas* sp. strain CHY-1 (56), PhnA1fA2f showed high activity towards naphthalene, biphenyl, phenanthrene and anthracene. Since phenanthrene was the preferred substrate and not biphenyl, the genes were named *phnA1fA2f* instead of *bphA1fA2f* as in *Sphingobium yanoikuyae* B1 and *Novosphingobium aromaticivorans* F199. Interestingly high molecular weight PAHs such as fluoranthene, chrysene, pyrene and benz[*a*]anthracene were also transformed to the corresponding *cis*-dihydrodiols even though these compounds do not sustain growth of strain LH128. Moreover monochlorinated biphenyls, dibenzo-*p*-dioxin and the fluorene analogues carbazole, diebenzothiophene and dibenzofuran were oxidized. It would be interesting to check whether these compounds can be used as sole source of carbon and energy by strain LH128.

The ability to generate mutants of *Sphingomonas* spp. will facilitate a deeper understanding of this important genus. The absence of efficient transposon systems in these organisms can be overcome by using the same strategy described earlier for *Sphingomonas* sp. strain LB126. To achieve this, electroporation efficiencies and antibiotic selection should be optimised.

The crystal structure of PhnI, the initial dioxygenase from *Sphingomonas* sp. strain CHY-1 was published recently (130). Based on this study, the amino acids lining the catabolic pocket (129) were identified and it turned out that these amino acids are conserved within strains LH128, F199, and with only two substitutions, in strain B1. Given the fact that the dioxygenase complexes from strains CHY-1 and B1 are able to oxidize the 5 ring PAH benz[*a*]pyrene but not PhnA1fA2f, even though the catalytic pocket is conserved, other factors must be responsible for the absence of activity towards this compound. Indeed, PhnA1fA2f shows greater variation in the loops L1 and L2 (83 % and 63 % respectively) surrounding the catalytic pocket predicted to regulate the access of the substrate to the active site. These loops are probably less mobile in strain LH128 and therefore do not allow a bulky substrate to enter the pocket. However it is not proven yet that the loops are involved in substrate specificity. The effects of residue substitutions in the active site of various dioxygenases have been well investigated (232-235). Our results indicate that substitutions in the structures surrounding the catalytic pocket can have marked effects on the substrate range of dioxygenase enzymes. Therefore point mutations of several amino acids especially in the loops L1 and L2 could allow bulky substrates to enter the catabolic pocket and probably enhance microbial degradation of aromatics in the environment.

The catabolic cluster is plasmid encoded in *Novosphingobium aromaticivorans* F199 (264) and chromosomally encoded in *Sphingobium yanoikuyae* B1 (354). No information is available concerning *Sphingobium* sp. strain P2 and *Sphingomonas* sp. strain CHY-1. The degradative plasmids in sphingomonads generally appear to be larger (160 to 240 kb) (15, 16, 264) than the previously studied degradative plasmids from pseudomonads, such as the TOL (117 kb) (97) or NAH (usually 80 to 120 kb) plasmids (344). The analysis of the plasmidic content might give information as to how this conserved cluster has been passed on amongst sphingomonads. More general

information about the presence, importance, and host range of plasmids in xenobiotic-degrading sphingomonads might help understanding the uniqueness of the sphingomonads genetic organization compared to other *Proteobacteria*.

During this work it has become clear that the information gathered by molecular techniques have a significant impact on all aspects of biodegradation. The detailed studies of the enzymes FlnA1A2 and PhnA1fA2f, together with the convergence of different degradation patterns are a prerequisite in the construction of hybrid catabolic pathways for the degradation of recalcitrant compounds that eliminate non-productive side reactions and reduce the formation of toxic intermediates. The combination of the two catabolic pathways in one strain could expand the substrate range and produce a good candidate for bioremediation strategies.

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Remerciements

Au terme de cette thèse je tiens à remercier les personnes qui m'ont aidé à la réaliser.

Tout d'abord mes co-promoteurs. Je remercie Mr Spiros N. Agathos de m'avoir permis de découvrir le monde au travers de deux voyages au VietNam, trois mois à la Rutgers New Jersey State University et deux stages au CEA à Grenoble c'est pas mauvais. Pascal Hols, qui a aussi pris le temps de s'investir dans mon travail. Ses conseils, suggestions et corrections ont été d'une très grande valeur pour moi.

Gerben Zylstra, qui m'a accueilli dans son laboratoire au sein du Biotech Center à Rutgers. Sinéad Ni Chadhain, qui m'a suivi tous les jours. Danielle Rhine pour les excursions et visites à gauche et à droite. On s'est même perdu sur les vastes highways, mais bon, là-bas ça arrive. Les barbecues et surtout la visite au Comedy Club à New York resteront dans ma mémoire.

Yves Jouanneau, qui m'a reçu à deux reprises au CEA à Grenoble. Je n'oublierai pas non plus de remercier sa famille de m'avoir hébergé pendant mon deuxième séjour et de l'avoir rendu très agréable. J'espère vous accueillir une fois chez moi. Christine Meyer pour son suivi au quotidien, Serge et Elodie pour l'ambiance dans le bureau.

Les membre de l'unité FYSA, en particulier Pierre Morsomme et Françoise Foury, qui m'ont laissé utiliser leur matériel dans leurs laboratoires respectifs. Mathieu Trincal et Sébastien Leidgens de m'avoir écouté et encouragé.

Les gens de mon année, Kevin, Gaetan, Vincent, Bernard, Sébastien, Marie-Christine, Laurence, et tout les autres pour les moments inoubliables lors des petites soirées ou le week end dans l'écurie (chhht ça reste entre nous).

Les membres de l'unité GEBI, Estelle et Michaël pour l'ambiance qu'il y avait au bureau. C'est vrai qu'on rigolait pas mal.

Tous mes copains du tir à l'arc, Manu, (un jour je te battrai au tennis….) Toni, Steve, Eric et les incontournables Marina a.k.a. Coach et Thierry a.k.a. Tchet. Bien sûr je n'oublierai pas tout les autres trop nombreux à citer qui ont contribué à ces moments de détente.

Ma famille qui m'a supporté jusque maintenant. Tom qui a toujours eu une oreille ouverte. Voilà, et bien sur tous ceux que j'ai oubliés.