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► **To cite this version:**

Yves Jouanneau, Florence Martin, Serge Krivobok, John Christopher Willison. Ring-hydroxylating dioxygenases involved in PAH biodegradation: structure, function, biodiversity. A.-I. Koukkou., Microbial bioremediation of non-metals: current research, Caister Academic Press, Norfolk, UK., pp.149-175, 2011. hal-01070431

HAL Id: hal-01070431

<https://hal.science/hal-01070431v1>

Submitted on 1 Oct 2014

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1 **Ring-hydroxylating dioxygenases involved in PAH biodegradation : structure, function,**
2 **biodiversity**

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17 Runing title : PAH-hydroxylating dioxygenases

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1 **Abstract**

2

3 The first step in the biodegradation of PAHs by aerobic bacteria is catalyzed by
4 metalloenzymes known as ring-hydroxylating dioxygenases (RHDs). Because of the
5 hydrophobic nature and chemical resistance of PAHs, their initial attack by RHDs is a
6 difficult reaction, which is critical to the whole degradation process. This chapter gives an
7 overview of the current knowledge on the genetics, structure, catalytic mechanism and
8 diversity of RHDs involved in PAH degradation. In the past decade, the crystal structures of
9 10 RHDs have been determined, giving insights into the mechanism of substrate recognition
10 and regioselectivity of dioxygenation. The reaction catalyzed by the archetypal naphthalene
11 dioxygenase has been investigated in detail, thus providing a better understanding of the RHD
12 catalytic mechanism. Studies on the catabolic genes responsible for PAH degradation in
13 several bacterial taxa have highlighted the great phylogenetic diversity of RHDs. The
14 implementation of culture-independent methods has afforded means to further explore the
15 environmental diversity of PAH-degrading bacteria and RHDs. Recent advances in this field
16 now allow the *in situ* identification of bacteria responsible for pollutant removal. Further
17 biotechnological developments based on microarrays and functional metagenomics should
18 lead to the conception of molecular tools useful for the bioremediation of PAH-contaminated
19 ecosystems.

20

1 **Introduction**

2

3 In the biodegradation of numerous aromatic hydrocarbons including polycyclic aromatic
4 hydrocarbons (PAHs) by aerobic microorganisms, oxygenases play a central role. Most
5 common oxygenases are either heme-containing cytochromes P450 that catalyze single
6 hydroxylations, or dioxygenases that incorporate both oxygen atoms of the O₂ molecule into
7 the substrate. Dioxygenases are metalloenzymes that generally contain non-heme iron at the
8 active site and fall into two categories: ring-hydroxylating dioxygenases (RHDs), which
9 catalyze a double hydroxylation on two adjacent carbons of the substrate, and ring-cleaving
10 dioxygenases, which catalyze the opening of the ring of catecholic substrates (Harayama et
11 al., 1992). RHDs are multicomponent enzymes (E.C.1.14.12.-) consisting of an oxygenase
12 associated with one or two specific electron carriers. They utilize NAD(P)H as a reductant
13 and transfer electrons to the active site for activation of dioxygen, a prerequisite to substrate
14 hydroxylation. In most cases, the oxygenase component is hexameric ($\alpha_3\beta_3$), with each alpha
15 subunit containing a Rieske-type [2Fe-2S] cluster and a mononuclear iron center where the
16 dioxygenation reaction takes place. RHDs are widely distributed in various taxa among Gram
17 positive and Gram negative bacteria. To date, over 1300 RHDs have been identified on the
18 basis of sequence similarities and have been compiled in the NCBI databases. Current
19 knowledge, based on well-studied enzymes such as naphthalene dioxygenase, indicates that
20 RHDs have relatively broad substrate specificities.

21 This chapter will focus primarily on RHDs involved in PAH degradation with special
22 emphasis on enzyme systems that have been described in the last decade. RHDs from
23 representative PAH-degrading bacteria isolated so far are compared on the basis of their
24 genetic organization, sequence relatedness, and substrate specificity. Aspects related to the
25 structure and catalytic mechanism of RHDs are only briefly discussed, but reviews on these
26 topics have been published recently (Ferraro et al., 2005; Kovaleva and Lipscomb, 2008). A
27 section is devoted to recent studies exploring the biodiversity of PAH-degrading bacteria and
28 associated RHDs in various terrestrial and aquatic environments using culture-independent
29 methods. Finally, we present a brief outlook on the possible strategies to improve PAH
30 bioremediation through bioengineering of RHDs. For general aspects of PAH degradation or
31 RHD biocatalysis that are not covered in this chapter, the reader is referred to recently
32 published reviews (Doyle et al., 2008; Habe and Omori, 2003; Parales and Resnick, 2006;
33 Peng et al., 2008).

34

1 **General properties and classification**

2
3 Ring-hydroxylating dioxygenases, also called Rieske non-heme iron oxygenases are
4 multicomponent enzymes generally consisting of a catalytic component with hydroxylase
5 activity, associated with one or two electron carrier proteins. In most cases, the hydroxylase
6 component is itself composed of two subunits, an alpha subunit of about 50 kDa and a beta
7 subunit of around 20 kDa, which assemble in an $\alpha_3\beta_3$ heterohexamer. However, the
8 hydroxylase component of carbazole dioxygenase contains only alpha subunits (Sato et al.,
9 1997). Each alpha subunit consists of two domains, the N-terminal Rieske domain, which
10 contains a [2Fe-2S] cluster, and the C-terminal catalytic domain, which contains a
11 mononuclear ferrous ion close to the substrate-binding site. The catalytic component needs
12 electrons in order to activate oxygen at each cycle of hydroxylation of the substrate. Two
13 auxiliary proteins, a ferredoxin and a flavin-containing oxidoreductase, usually provide the
14 necessary reductant at the expense of NAD(P)H oxidation. Sometimes, the two proteins are
15 fused in a single polypeptide chain, as is the case for benzoate dioxygenase (Karlsson et al.,
16 2002) and anthranilate dioxygenase (Eby et al., 2001).

17 RHDs share common structural features, including quaternary structure and conserved
18 residues in the Rieske and catalytic domains of the alpha subunit that are involved in binding
19 of the metal centers (see below). However, they differ considerably in terms of amino acid
20 sequence and with respect to the structure of the associated electron carriers. Almost twenty
21 years ago, Batie et al. proposed a classification of dioxygenases based on the number of
22 associated electron carriers and the nature of their redox centers (Batie et al., 1992). Three
23 classes of RHDs were defined, comprising two-component enzyme systems such as the
24 phthalate dioxygenase from *Burkholderia cepacia* (Correll et al., 1992) (class I), three-
25 component enzymes containing an FAD-containing reductase and a [2Fe-2S] ferredoxin, as
26 exemplified by the biphenyl dioxygenase (Furusawa et al., 2004) (class II), and three-
27 component enzymes in which the reductase contains both a FAD and a [2Fe-2S] cluster, as
28 represented by the naphthalene dioxygenase (Kauppi et al., 1998) (class III). Later, new
29 RHDs were identified and characterized, some of which did not fit into this classification
30 system. This was the case for the carbazole dioxygenase from *Pseudomonas* sp. CA10 (Sato
31 et al., 1997) and for three-component enzymes in which the ferredoxin contains a [3Fe-4S]
32 cluster instead of the more common [2Fe-2S] cluster (Martin and Mohn, 1999; Takagi et al.,
33 2005). Nam et al. therefore proposed an alternative classification system based on amino acid
34 sequence similarities between the alpha subunits of hydroxylase components (Nam et al.,

1 2001). RHDs were then organized into four families of proteins according to phylogenetic
2 criteria, each family corresponding to types of enzyme with similar substrate specificities. For
3 example, type II enzymes are represented by benzoate/toluene dioxygenases, type III enzymes
4 by naphthalene and PAH dioxygenases, and type IV enzymes by biphenyl and toluene
5 dioxygenases. Type I enzymes have in common the presence of a hydroxylase composed of
6 alpha subunits only, and show greater disparity than the other families in terms of amino acid
7 sequences and substrate range. An advantage of this classification system lies in the
8 possibility to classify any new oxygenase sequence derived from a nucleotide sequence, even
9 though the associated electron carriers are unknown. Such a situation is encountered in
10 bacterial genomes where catabolic genes are dispersed, and especially when genes encoding
11 hydroxylase component do not lie close to the genes encoding electron carriers. This is
12 frequently the case in PAH-degrading bacteria such as *Mycobacteria* and
13 *Sphingomonadaceae* (see below). Recently, a more sophisticated classification system has
14 been proposed, which takes into account the sequences of associated electron carriers in the
15 phylogenetic analysis of RHDs (Kweon et al., 2008). In addition to the 4 subclasses of RHDs
16 described by Nam et al., a new group was defined comprising enzymes found in Gram-
17 positive PAH-degrading bacteria (Kim et al., 2006; Krivobok et al., 2003; Saito et al., 2000).
18 A distinctive feature of these Type V RHDs is that their associated ferredoxin contains a
19 [3Fe-4S] cluster, in contrast to type III PAH dioxygenases from Gram-negative bacteria,
20 which use [2Fe-2S] ferredoxins. A phylogenetic tree based on alignments of α subunit
21 sequences of representative RHDs is shown in Fig. 1.

22

23 Figure 1

24

25 **Genetics and diversity of RHDs involved in PAH degradation**

26

27 *Genetics and diversity of RHDs in Gram-negative bacteria*

28 Genes responsible for PAH degradation were first studied in *Pseudomonas* strains isolated on
29 naphthalene as carbon source. Naphthalene degradation genes are generally clustered in two
30 closely linked operons carried on a specific plasmid, as initially reported for the NAH7
31 plasmid of *P. putida* G7 (Yen and Gunsalus, 1982; Yen and Serdar, 1988). The upper operon
32 contains genes coding for the naphthalene dioxygenase (*nahAaAbAcAd*) as well as genes
33 specifying the enzymes catalyzing six additional oxidation steps to produce salicylate
34 (*nahABFCQED*). The lower operon also called the *sal* operon, (*nahGTHINLOMKJ*) encodes

1 the enzymes required to convert salicylate to Krebs cycle intermediates. Between the two
2 operons and oriented in the opposite direction lies the *nahR* gene encoding a LysR-type
3 transcriptional regulator, which controls the expression of both operons (Schell, 1985; Tropel
4 and van der Meer, 2004). The NahR regulator activates transcription by binding to specific
5 sequences in the promoter regions of the two *nah* operon and within its own promoter
6 (autoregulation), in a manner dependent on the presence of salicylate. The organization of the
7 naphthalene degradation genes and their nucleotide sequence was found to be very similar in
8 other *Pseudomonas* strains, including NCIB 9816-4 (Simon et al., 1993) and *P. stutzeri*
9 (Bosch et al., 1999; Bosch et al., 2000).

10 In *Comamonas testosteroni* strains isolated on phenanthrene, the genes encoding the initial
11 dioxygenase did not hybridize with the *nahA* probes, indicating that their sequences diverge
12 significantly from that of counterparts found in *Pseudomonas* strains (Goyal and Zylstra,
13 1996). The organization of the phenanthrene catabolic genes (*phd*) was similar to that
14 observed in *Pseudomonas*, except that a gene encoding a dihydrodiol dehydrogenase (*phdB*)
15 was inserted between the genes encoding phenanthrene dioxygenase (Goyal and Zylstra,
16 1997) (Fig. 2). A remarkably similar organization was found in the gene cluster responsible
17 for phenanthrene degradation in *Alcaligenes faecalis* AKF2 (Kiyohara et al., 1982). However,
18 the latter strain degrades phenanthrene via the o-phthalic acid pathway found in Gram
19 positive bacteria, whereas *C. testosteroni* strains utilize the salicylate pathway, like
20 Pseudomonads.

21 Figure 2

22 A recently isolated phenanthrene degrader identified as an *Acidovorax* strain (NA3) was
23 shown to contain a catabolic cluster highly similar to that found in strain AKF2 (Singleton et
24 al., 2009). The alpha subunit of strain NA3 phenanthrene dioxygenase exhibited the highest
25 amino acid sequence identity with counterparts from AFK2 and *Burkholderia* strains. On the
26 basis of transcriptional analysis by real-time qPCR, the phenanthrene degradation genes were
27 found to be highly expressed in cells exposed to phenanthrene or naphthalene, but were not
28 expressed in the presence of other PAHs.

29 The phenanthrene degrader *Burkholderia* sp. strain RP007 contains a cluster of genes similar
30 in sequence to those found in strain AKF2 but showing an arrangement different from that
31 described in the two strains above (Laurie and Lloyd-Jones, 1999) (Fig. 2). The catabolic
32 cluster *phnRSFECDAcAdB* contains 9 genes including the *phnAcAd* coding for the
33 hydroxylase component of phenanthrene dioxygenase but lacks the genes specifying

1 associated electron carriers. Sequence analysis suggested that PhnR and PhnS are
2 transcriptional regulators, the latter being of the LysR-type, similar to NahR.

3 In *Ralstonia* sp. strain U2, isolated on naphthalene, the catabolic genes also form a tight
4 cluster *nagAaGHAbAcAdBFCQED* in which two genes, *nagGH*, appear to be inserted
5 between the *nagAa-d* genes encoding the naphthalene dioxygenase (Fuenmayor et al., 1998).
6 In contrast to the previously discussed PAH degraders, strain U2 does not convert salicylate
7 into catechol but into gentisate thanks to a salicylate 5-dioxygenase coded by the *nagGH*
8 genes (Zhou et al., 2001). The *nag* genes are positively regulated via NagR, a regulator very
9 similar to NahR, which is also activated by salicylate (Jones et al., 2003).

10 Another naphthalene degrader, called *Polaromonas naphthalenivorans* CJ2, also exhibited a
11 degradation pathway involving gentisate as an intermediate. The cluster of *nag* genes of CJ2
12 is very similar in organization and sequences to that found in *Ralstonia* U2 (Jeon et al., 2006).
13 These genes are controlled, at the transcriptional level, by the regulator NagR1, which
14 functions in a salicylate-dependent manner as mentioned above. Contrary to strain U2, strain
15 CJ2 lacks the *nagY*, *nagM* and *nagN* genes, one of which encodes a chemotaxis protein.
16 Moreover, strain CJ2 contains another set of genes responsible for the complete degradation
17 of gentisate, which is regulated by NagR2 another type of regulator that is not activated by
18 salicylate nor by naphthalene.

19 Bacteria belonging to the genus *Cycloclasticus* are thought to play a major role in the
20 degradation of PAHs in marine environments (Kasai et al., 2002). In *Cycloclasticus* strain A5,
21 which is able to grow on naphthalene, methylnaphthalenes and phenanthrene, the *phnA1A2*
22 genes encoding the dioxygenase and the *phnA3A4* encoding a ferredoxin and a reductase are
23 part of a 10.5-kb gene cluster (Kasai et al., 2003) (Fig. 2). The dioxygenase of strain A5
24 showed moderate sequence homology with those from other PAH degraders, the closely
25 related enzymes being dioxygenases from Sphingomonads (60% and 52% identities at best
26 for the α and β subunits of the hydroxylase, respectively).

27

28 *Sphingomonads*

29 The genus *Sphingomonas* initially designated a group of bacteria known for their ability to
30 degrade a wide range of organic pollutants, including dibenzo-*p*-dioxin and dibenzofuran
31 (Armengaud et al., 1998), pesticides (Kumari et al., 2002) and PAHs (Leys et al., 2004). This
32 taxonomic group has been split into new genera called *Sphingobium*, *Novosphingobium*,
33 *Sphingopyxis* in 2001 (Takeuchi et al., 2001). Genetic analyses of PAH-degrading strains
34 showed that catabolic genes had a unique organization where genes involved in polyaromatic

1 degradation were interspersed with *xyl* genes responsible for monoaromatic degradation (Fig.
2 3) (Kim and Zylstra, 1999; Pinyakong et al., 2003a; Romine et al., 1999). In
3 *Novosphingobium aromaticivorans* F199, 79 genes thought to associated with the metabolism
4 or transport of aromatic hydrocarbons are carried on a 184-kb plasmid called pNL1 (Romine
5 et al., 1999). As many as six gene pairs possibly encoding the α and β subunits of distinct
6 hydroxylases were identified on pNL1, but their specific function was unknown (Fig. 3). In
7 *Sphingomonas* CHY-1, a strain able to grow on chrysene (Willison, 2004), the pair of genes
8 encoding the initial PAH dioxygenase has been identified and genetic evidence showed that
9 this dioxygenase was the only enzyme required to catalyze the primary oxidation of PAHs
10 (Demaneche et al., 2004). The cluster of catabolic genes in strain F199 and in related strains,
11 contain a single copy each of a gene encoding a ferredoxin and a gene encoding a reductase,
12 located more than 20 kb apart. This observation suggested that the multiple terminal
13 hydroxylases coded by the catabolic cluster shared the same electron carriers. Consistent with
14 this idea, it was found that the initial PAH dioxygenase from strain CHY-1, called PhnI, and
15 another hydroxylase coded by *phnA1_bA2_b* (PhnII), utilized the same electron carriers
16 (Demaneche et al., 2004; Jouanneau et al., 2007).

17 Figure 3

18 The PhnII enzyme, which shares many structural features with classical three-component
19 dioxygenases, has been characterized as a salicylate 1-hydroxylase that converts salicylate
20 and several substituted derivatives of salicylate to catecholic products (Jouanneau et al., 2007).
21 This enzyme substitutes for the monomeric salicylate hydroxylases found in Pseudomonads
22 although the two types of enzymes are phylogenetically unrelated. The three-component
23 enzyme from *Sphingomonas* CHY-1 also differs from the monomeric type in that it is inactive
24 on 1-hydroxy-2-naphthoate, and therefore does not participate in the degradation pathway of
25 3-ring PAHs such as phenanthrene or anthracene. In *Sphingobium* P2, it was found that three
26 of the multiple hydroxylases encoded by the catabolic cluster were salicylate hydroxylases
27 analogous to PhnII (Pinyakong et al., 2003b).

28 Salicylate 5-hydroxylase from *Ralstonia* U2 and anthranilate dioxygenase from *Burkholderia*
29 *cepacia* DBO1 (Chang et al., 2003) are phylogenetically related to three component salicylate
30 hydroxylases with which they form a distinct subgroup among RHDs (Fig. 1).

31 *Sphingomonas macrogolita* TFA has been studied for its ability to utilize tetralin (1,2,3,4-
32 tetrahydronaphthalene) as a carbon source. The *thn* genes responsible for this degradation are
33 clustered in two separate and divergently transcribed operons (Moreno-Ruiz et al., 2003). The
34 *thnA1A2* genes encoding the catalytic component of the initial dioxygenase are located in the

1 main operon, whereas the *thnA3A4* genes coding for the associated electron carriers are part
2 of the other operon. The oxygenase component of this enzyme appeared to be distantly related
3 to PAH dioxygenases from Gram-negative bacteria (Fig. 1).

4 The dioxin dioxygenase from *Sphingomonas* RW1, which catalyzes an angular attack on
5 dibenzo-*p*-dioxin and dibenzofuran, is encoded by genes dispersed in the genome
6 (Armengaud et al., 1998). In the main locus, the *dxnA1A2* genes encoding the hydroxylase
7 component are associated with *dxnB*, which encodes a ring-cleavage dioxygenase that
8 catalyzes the next degradation step. The genes encoding the associated ferredoxin and
9 reductase components are located in two distant and separate loci. The deduced amino acid
10 sequence of the terminal dioxygenase showed weak homology with counterparts from other
11 bacteria, the closest relatives being biphenyl dioxygenases (Armengaud et al., 1998).

12

13 *Genetics and diversity of RHDs in Gram-positive bacteria*

14 Gram-positive bacteria able to degrade PAHs fall into two categories, those which can attack
15 fluorene, dibenzofuran and similar substrates by angular dioxygenation, including
16 *Terrabacter* and *Rhodococcus* strains, and those which can utilize phenanthrene and 4-ring
17 PAHs, including *Nocardioides* and *Mycobacterium* strains.

18 In the first category, *Terrabacter* sp. strain DBF63 is one of the best-studied dibenzofuran and
19 fluorene degraders. The initial attack on these compounds is catalyzed by an angular
20 dioxygenase, the catalytic component of which is encoded by *dbfA1A2* (Habe et al., 2004;
21 Habe et al., 2003). These genes are part of a large cluster including the
22 *flnBdbfA1A2flnEflnD1D2flnC* operon and genes involved in phthalate degradation. A gene
23 (*dbfA3*) was later identified downstream from *dbfA1A2*, which encodes a [3Fe-4S] ferredoxin
24 able to serve as electron donor to the hydroxylase component of the dioxygenase (Takagi et
25 al., 2005). The initial dioxygenase catalyzes two types of reaction on fluorene, first a single
26 hydroxylation on the carbon in C9 position to yield 9-fluoreneol, then an angular
27 dihydroxylation to give 1,1a-dihydroxy-1-hydro-9-fluorenone. This compound is then
28 converted by FlnB to 2'-carboxy-2,3-dihydroxybiphenyl, which is further transformed to
29 phthalate in two steps catalyzed by FlnD1D2 and FlnC. Genes homologous to *dbfA1A2* were
30 identified in other Gram-positive dibenzofuran degraders, including *Terrabacter* sp. strain
31 YK3 (Iida et al., 2002), various *Rhodococcus* strains (Aly et al., 2008), but also in two
32 *Sphingomonas* strains able to metabolize carbazole (Shintani et al., 2007) and fluorene
33 (Schuler et al., 2008). In *Terrabacter* YK3 and *Rhodococcus* YK2, the genes responsible for
34 dibenzofuran degradation, including the *dfdA1A2A3A4* coding for the angular dioxygenase,

1 are carried on a plasmid (Iida et al., 2002). Recently, the *dfd* genes were found to be regulated
2 by a new type of regulator (DfdR) that activates transcription in response to the presence of
3 dibenzofuran or a few other hydrophobic hydrocarbons (Iida et al., 2009b). In *Rhodococcus*
4 HA01, two inducible angular dioxygenases encoded by *DfdA1A2A3A4* and *DbfA1A2*, have
5 been described (Aly et al., 2008). The first set of genes was highly similar to that found in
6 *Terrabacter* YK3, whereas the second set was closely related to that coding for dibenzofuran
7 dioxygenase from strain DBF63. The two enzymes, which appeared to be simultaneously
8 expressed in strain HA01, exhibited complementary activities towards chlorinated substrates,
9 with *DfdA1A2* performing the angular dioxygenation of 3-chlorodibenzofuran and *DbfA1A2*
10 transforming 2-chlorodibenzofuran.

11 Two *Sphingomonas* strains were found to degrade fluorene through a degradation pathway
12 similar to that described in *Terrabacter*, suggesting an initial attack by angular dioxygenation.
13 In *Sphingomonas* sp. LB126, the operon responsible for fluorene degradation exhibited a gene
14 organization similar to that found in strain DBF63, but the gene products were only distantly
15 related, as exemplified by the α and β subunits of the dioxygenase, which showed 63 and 52
16 % sequence identities with *DbfA1* and *DbfA2*, respectively (Schuler et al., 2008). The
17 oxygenase from strain LB126 converted fluorene to 9-fluorenol and subsequently to the
18 angular dihydrodiol product like the DBF63 enzyme. It also transformed dibenzofuran and
19 attacked other 2- and 3-ring PAHs, fluorene and phenanthrene being the preferred substrates
20 (Schuler et al., 2008).

21
22 The marine strain *Nocardioides* sp. KP7 was one of the first PAH-degrading Gram-positive
23 bacterium to be genetically characterized (Saito et al., 1999; Saito et al., 2000). The genes
24 responsible for the oxidative breakdown of phenanthrene to phthalate were found to be
25 different in terms of organization and sequence from what had been previously reported in
26 Gram-negative bacteria (Fig. 2). The main cluster *phdEFABGHCD* contained the genes
27 coding for the α and β subunits of phenanthrene dioxygenase (*phdAB*), preceded by genes
28 encoding a dihydrodiol dehydrogenase (*phdE*) and an extradiol dioxygenase (*phdF*). The four
29 genes located downstream specified an aldolase-hydratase (*phdG*), an aldehyde
30 dehydrogenase (*phdH*), a ferredoxin (*phdC*) and a reductase (*phdD*), the latter two gene
31 products being the electron carriers specifically associated with phenanthrene dioxygenase.
32 Altogether, the enzymes coded by this cluster catalyzed five oxidation steps to convert
33 phenanthrene to 1-hydroxy 2-naphthoate. The latter intermediate was further metabolized to
34 phthalate by three enzymes coded by the *phdIJK* gene cluster located about 6-kb apart (Saito

1 et al., 2000). The dioxygenase from strain KP7 was the first member of a new subclass of
2 enzymes essentially found in *Mycobacterium* and *Rhodococcus* strains, forming a distinctive
3 phylogenetic group (Fig. 1) and featuring a [3Fe-4S] ferredoxin as typical electron carrier.
4
5 Several members of the genus *Mycobacterium* have been studied for their ability to degrade
6 high molecular weight PAHs, including pyrene and fluoranthene (Heitkamp et al., 1988;
7 Khan et al., 2001; Krivobok et al., 2003; Schneider et al., 1996; Sho et al., 2004; Stingley et
8 al., 2004; Vila et al., 2001). The genes encoding what appeared to be the hydroxylase
9 component of a pyrene dioxygenase were first described in *M. vanbaalenii* PYR-1, and
10 designated *nidBA* (*nid* for *naphthalene induced*) (Khan et al., 2001). Analysis of protein
11 profiles by 2D gel electrophoresis indicated that six proteins were induced by pyrene and
12 phenanthrene, one of which was the alpha subunit of the dioxygenase (NidA). In another
13 pyrene degrader, called *Mycobacterium* 6PY1, a thorough proteomic analysis of pyrene- and
14 phenanthrene-induced polypeptides identified 23 proteins, four of which were subunits of two
15 dioxygenase terminal components named Pdo1 and Pdo2 (Krivobok et al., 2003). The other
16 induced proteins showed significant similarities with enzymes previously implicated in
17 phenanthrene metabolism in *Nocardioides* KP7. The genes encoding Pdo1 and Pdo2 were
18 located in two separate loci, and no genes encoding possibly associated electron carriers were
19 found in either locus. Nevertheless, the two dioxygenases could be successfully expressed in
20 *E. coli* and their activity was enhanced by co-expressing the PhdC and PhdD electron carriers
21 from strain KP7. Amino acid sequence comparison indicated that Pdo1 was highly similar to
22 the pyrene dioxygenase from strain PYR-1, whereas Pdo2 was related to the phenanthrene
23 dioxygenase from strain KP7 (Krivobok et al., 2003). Other *Mycobacterium* strains able to
24 degrade pyrene or fluoranthene were found to express RHDs homologous to Pdo1 or Pdo2 in
25 a regulated manner (Liang et al., 2006; Pagnout et al., 2007; Sho et al., 2004). More recently,
26 Kim et al. assigned a gene product to nearly all steps of the pyrene degradation pathway in
27 strain PYR-1 based on a detailed analysis involving exhaustive genomic and proteomic data,
28 (Kim et al., 2007). However, some of the proposed gene functions remain hypothetical and
29 need to be assessed experimentally. The PAH catabolic genes are part of a large cluster
30 extending over 150 kb and encoding multiple terminal components of oxygenases (Kim et al.,
31 2008). Although many of these oxygenases have still unknown functions, at least four of them
32 have been assigned a catalytic role, including a phenanthrene dioxygenase homologous to
33 Pdo2 from 6PY1, a phthalate dioxygenase encoded by *phtAaAb*, another PAH dioxygenase
34 encoded by *nidA3B3*, and the pyrene dioxygenase discussed above (*nidBA*). The NidA3B3

1 terminal oxygenase showed a preference for fluoranthene (Kim et al., 2006) and was
2 expressed to a relatively high level in *M. vanbaalenii* PYR-1 when grown on this PAH as
3 substrate (Kweon et al., 2007). The entire cluster contains only two electron carrier genes
4 closely linked to *phtAaAb*, *phtAc* encoding a [3Fe-4S] ferredoxin and *phtAd* encoding a
5 reductase, suggesting that the multiple hydroxylases specified by the catabolic cluster share
6 the same electron carriers, a situation similar to that encountered in Sphingomonads.
7 Interestingly, the genomes of four other PAH-degrading *Mycobacterium* strains, including *M.*
8 *gilvum* strain PYR-GCK (NC009338), *Mycobacterium* sp. KMS (NC008705), MCS
9 (NC008146) and JLS (NC009077) appeared to contain a catabolic cluster very similar in gene
10 sequence and organization to that found in strain PYR-1, except that a large portion of the
11 cluster was duplicated on the chromosome (strain PYR-GCK) or on plasmids (strains MCS
12 and KMS).

13 The naphthalene degrader *Rhodococcus* sp. strain NCIMB12038 contains a RHD whose
14 hydroxylase component showed little sequence similarities with naphthalene dioxygenases
15 from Pseudomonads but significant homology with the Gram-positive RHDs described above
16 (Larkin et al., 1999).

17
18

19 **The catalytic mechanism of RHDs : the example of naphthalene dioxygenase from** 20 ***Pseudomonas* sp. NCBI 9816-4**

21

22 Chemically speaking, the initial oxygenation of the aromatic ring is the most difficult
23 catalytic step in the aerobic degradation of PAHs. Bacterial RHDs catalyze the incorporation
24 of the two atoms of molecular oxygen into one of the highly stable aromatic ring, thus
25 allowing further breakdown of the substrate molecule by subsequent dehydrogenation and
26 ring cleavage. The mechanism of the reaction catalyzed by RHDs has been studied in detail
27 with the naphthalene dioxygenase from *P. putida* as model enzyme, taking advantage of the
28 crystal structure of the terminal oxygenase component, which was determined in 1998
29 (Kauppi et al., 1998). The dihydroxylation of naphthalene occurs at the enzyme active site,
30 which is a ferrous ion bound to the alpha subunit through the side chains of two His and one
31 Asp residues. The reaction involves reduction and cleavage of molecular oxygen, and
32 insertion of the oxygen atoms on the C1 and C2 carbon atoms of the substrate to yield *cis*-
33 (*1R,2S*)-dihydroxy-1,2-dihydronaphthalene. Although the reductase and ferredoxin
34 components are required for continuous catalytic turnover of the enzyme, it has been

1 demonstrated that the hydroxylase component alone can catalyze the dioxygenation of
2 naphthalene in single turnover experiments (Wolfe et al., 2001). As isolated, the oxygenase
3 component is unreactive with oxygen. However, upon stoichiometric reduction of the Rieske
4 cluster, naphthalene and oxygen react with the hydroxylase to form the dihydrodiol product.
5 Stopped-flow analyses and spectroscopic measurements indicated that product formation was
6 concomitant with the oxidation of the Rieske and mononuclear iron centers. Moreover, it was
7 found that the oxygenase component could convert naphthalene to dihydrodiol in the presence
8 of hydrogen peroxide, a reaction that stopped after one turnover with the product trapped in
9 the active site (Wolfe and Lipscomb, 2003). This reaction, known as the peroxide shunt,
10 suggested that the normal catalytic cycle of the dioxygenase involves a peroxo intermediate,
11 and that the release of the product requires reduction of the mononuclear iron center.
12 Altogether, the results obtained with naphthalene dioxygenase led Wolfe et al. to propose a
13 mechanism for the reaction cycle of RHDs (Wolfe and Lipscomb, 2003). In this scheme, the
14 oxygenase component is reduced first, then binds sequentially the substrate and O₂ to form an
15 Fe(III)-hydroperoxo intermediate. The exact nature of the reactive species generating the
16 dihydrodiol product is still unknown, but it seems likely that the release of the reaction
17 product requires another reduction step to regenerate the ferrous state of the mononuclear
18 center. Using phthalate dioxygenase as a model, other authors found that this scheme did not
19 fit their data because single turnover reactions yielded one half the expected amount of
20 product and ended with the active site in the ferrous state (Tarasev and Ballou, 2005). It has
21 been proposed that the catalytic cycle of phthalate dioxygenase required the participation of
22 the reductase component to deliver one electron equivalent per cycle.

23 The occurrence of a hydroperoxo intermediate in the reaction cycle has been substantiated by
24 the discovery that oxygen binds side-on at the active site of naphthalene dioxygenase and by
25 the structural description of a ternary complex of the oxygenase component with O₂ and
26 indole (Karlsson et al., 2003). The particular binding mode of oxygen on the active site iron
27 and the rigorous positioning of the substrate would afford a mechanism whereby both atoms
28 of O₂ react in concert with the substrate to form the dihydrodiol. However, it is still uncertain
29 whether it is an Fe(III)-hydroperoxo or an Fe(V)-oxo-hydroxo reactive species that eventually
30 attacks the substrate to yield the dihydrodiol product (Kovaleva and Lipscomb, 2008).

31

32

33 **Comparison of available structures of RHD catalytic components**

34 The catalytic component of the naphthalene dioxygenase from *Pseudomonas* sp. strain

1 NCIB9816-4 (NDO) was the first enzyme to be structurally characterized (Kauppi et al.,
2 1998). Since then, nine crystal structures from other dioxygenase systems have been reported
3 (Ferraro et al., 2005), including three enzymes able to attack PAHs, namely the oxygenases
4 from *Rhodococcus* NCIMB12038 (Gakhar et al., 2005), *Sphingobium yanoikuyae* B1 (Ferraro
5 et al., 2007) and *Sphingomonas* CHY-1 (Jakoncic et al., 2007a; Jakoncic et al., 2007b). All
6 enzymes exhibit an $\alpha_3\beta_3$ quaternary structure, except for the carbazole oxygenase from *P.*
7 *resinovorans* CA10 (Nojiri et al., 2005) and the oxoquinoline 8-monooxygenase from *P.*
8 *putida* strain 86 (Martins et al., 2005), which are homotrimers consisting of alpha subunits
9 only. The following discussion will be focused on enzymes implicated in PAH degradation.

10 All $\alpha_3\beta_3$ oxygenases exhibit a mushroom-like overall structure similar to that reported for
11 NDO, with the trimer of alpha subunits sitting on a base consisting of the beta subunits. The
12 alpha/beta pairs of subunits have almost identical folds and are arranged around a threefold
13 axis of symmetry. The alpha subunits are composed of two domains, the N-terminal Rieske
14 domain that contains the [2Fe-2S] cluster and the catalytic domain containing the substrate
15 binding site and the mononuclear iron center. The fold of the Rieske domain is similar to that
16 found in the cytochrome bc_1 complex of the respiratory chain. The cluster is bound to the
17 protein through four conserved ligand residues, two cysteines and two histidines. In the
18 catalytic domain, the iron atom is coordinated by two histidines and one aspartate (as a
19 bidentate) and one water molecule. The last position of coordination (axial) is occupied by the
20 side chain oxygen atom of a conserved asparagine residue but the O-Fe distance is $> 3.75 \text{ \AA}$.
21 The distance between the Rieske center and the mononuclear iron is about 44 \AA within the
22 same alpha subunit. The Rieske center is however much closer to the iron center of the
23 neighboring subunit (12 \AA), indicating that electron transfer occurs via the metal centers of
24 two adjacent alpha subunits. In addition, these two metal centers are connected through a
25 hydrogen bond network involving a conserved aspartate residue (Asp205 in NDO). This
26 residue binds a histidine ligand of the iron center (His208) and a histidine ligand of the Rieske
27 cluster (His104), thus forming a plausible bridge for electron transfer (Kauppi et al., 1998).
28 The essential role of the Asp205 residue was demonstrated in experiments where its
29 replacement through site-directed mutagenesis resulted in a total loss of enzyme activity
30 (Parales et al., 1999). Its role in electron transfer between the metal centers is, however,
31 controversial. A study using anthranilate dioxygenase and variants carrying substitutions of
32 the conserved Asp residue concluded that this residue rather served to maintain the
33 protonation state and reduction potential of the Rieske cluster (Beharry et al., 2003).

1 The substrate-binding site appears as a hydrophobic pocket open to the solvent on one side
2 and ending at the iron center on the other. Seventeen residues lining and shaping the substrate
3 binding site have been identified in NDO (Carredano et al., 2000), three of which are strictly
4 conserved, including the two histidine ligands of the ferrous iron (His208 and His213 in
5 NDO) and the Asp205 residue discussed above. Other semi-conserved residues include
6 Asn201, which is replaced by a glutamine in the biphenyl dioxygenase from *Rhodococcus*
7 RHA1, and Phe202, Gly251 and Phe358, which are replaced by other residues only in
8 trimeric (α_3) oxygenases. Differences observed in the active site residues among
9 dioxygenases might explain in part differences in substrate specificity (Ferraro et al., 2005).
10 Indeed, interactions between substrates and active site residues are believed to control the
11 orientation of the substrate relative to the ferrous center and eventually the regio- and enantio-
12 selectivity of the catalytic reaction.

13 Although the nitrobenzene dioxygenase (NBDO) acts chiefly on monoaromatic substrates, its
14 structure is remarkably similar to that of NDO (Friemann et al., 2005). Only five differences
15 were observed in the residues lining the substrate-binding site. The crystal structure of NBDO
16 in complex with nitrobenzene showed that nitrobenzene is maintained in the correct position
17 for catalysis through hydrogen bonding between the nitro group of the substrate and a specific
18 Asn residue (Asn258). Replacement by this residue by a valine (the analogous residue in
19 NDO) changed the regio-selectivity of the reaction with 2-nitrotoluene, yielding 2-nitro
20 benzyl alcohols rather than catechol (Ferraro et al., 2005).

21 The oxygenase from strain NCIMB12038 is structurally similar to NDO although the two
22 enzymes share only 30 % sequence identity. Moreover, indole was found to bind to the active
23 site of the enzyme in the same position as that described for the NDO-indole complex
24 (Gakhar et al., 2005), although a few differences were observed in the residues lining the
25 active site.

26 The crystal structure of the broad-substrate-range dioxygenase from *Sphingomonas* CHY-1
27 (Jakoncic et al., 2007b) and that of the highly similar biphenyl dioxygenase from
28 *Sphingobium yanoikuyae* B1 (Ferraro et al., 2007) were published almost at the same time.

29 The following description concerns the former enzyme.

30 The RHD from strain CHY-1 is unique in that it can oxidize a very large panel of PAHs
31 ranging from naphthalene to the 5-ring benzo[a]pyrene (Jouanneau et al., 2006). The catalytic
32 component, called PhnI, exhibits a folding pattern very similar to that of NDO, the α carbon
33 chains of the two proteins being superimposable within a 1.2 Å root-mean square deviation.
34 However, PhnI features the largest substrate-binding pocket ever reported for a RHD (Fig. 4).

1 This 12-Å long hydrophobic cavity extends from the iron center on one side to the solvent on
2 the other. The entrance of the cavity is delimited by two flexible loops, which might control
3 the access of the substrates to the active site. Compared to NDO, two leucine residues on loop
4 L1 (Leu223 and Leu226) are moved towards the solvent, thus elongating the catalytic pocket
5 by at least 2 Å (Jakoncic et al., 2007a). Conserved residues line the part of the cavity proximal
6 to the iron center, whereas Phe350, Leu356 and Phe404 shape the central and distal parts.
7 Molecular modeling indicated that the substrate binding cavity was large enough to
8 accommodate the 5-ring substrate benzo[a]pyrene with minor changes in the positions of
9 lining residues (Fig 4). The predicted position of this substrate suggested a hydroxylation of
10 carbons C9 and C10 (Jakoncic et al., 2007a), which is consistent with the position of the
11 hydroxyls on the experimentally obtained dihydrodiol (Jouanneau et al., 2006). Further
12 modeling experiments gave plausible positions for other substrates, such as phenanthrene end
13 benz(a)anthracene, in the substrate binding pocket, which were also in good agreement with
14 the regioselectivity of the hydroxylation reactions. This suggested that the large size and the
15 particular topology of the substrate-binding site of PhnI were the main determinants of its
16 broad substrate specificity.

17 Fig. 4

18 Carbazole dioxygenase is a singular RHD in that it catalyzes angular hydroxylation and
19 because its catalytic component is made up of alpha subunits only. The crystal structure of the
20 trimeric protein exhibits a doughnut-like shape in which interactions between specific loops
21 of adjacent alpha subunits stabilize the trimer (Nojiri et al., 2005). The fold of the alpha
22 subunit is roughly similar to that in the $\alpha_3\beta_3$ NDO structure, although the two proteins share
23 only 17% sequence identity. The substrate-binding site differs from that of other RHDs in that
24 the distal part of the hydrophobic pocket is lined by rather polar residues. Docking simulation
25 of carbazole in the substrate-binding site indicated that, in the predicted position, the carbon
26 C1 and C9a of carbazole were the carbon atoms closest to the iron center, consistent with an
27 angular dioxygenation reaction. Carbazole dioxygenase is the only RHD system for which the
28 structure of a binary complex between the oxygenase and the ferredoxin components has been
29 solved (Ashikawa et al., 2006). In the complex, three ferredoxin molecules were bound at the
30 intersubunit boundary of the trimeric oxygenase, each protein-protein interaction involving a
31 combination of electrostatic, hydrophobic and hydrogen bonds between specific residues of
32 the two partners. Protein interactions and conformational changes brought the Rieske clusters
33 of each partner to a distance of around 12 Å suitable for electron transfer. The ferredoxin
34 binding site has been described as a depression on the surface of the oxygenase trimer at the

1 interface of adjacent alpha subunits. A similar depression has been observed between the
2 alpha and beta subunits of NDO, suggesting that it might be the binding site for the ferredoxin
3 in this hexameric oxygenase (Ashikawa et al., 2006). Analysis of a ternary complex between
4 the oxygenase and ferredoxin components and carbazole showed that upon binding of the
5 substrate, conformational changes resulted in the closure of a lid over it, as if it were trapped
6 in the substrate binding pocket.

7 8 9 **Substrate specificity of RHDs with respect to PAHs**

10
11 RHDs are generally endowed with relaxed substrate specificity, thus allowing bacteria to
12 initiate the degradation of a wide range of aromatic hydrocarbons. In addition, they are
13 remarkable catalysts in that they can perform several types of oxidation reaction, including
14 *cis*-dihydroxylation, monooxygenation, desaturation and sulfoxidation. The impressive range
15 of oxidative reactions catalyzed by naphthalene dioxygenase has been reviewed by Resnick et
16 alnd Gibson (Resnick and Gibson, 1996). Many of the reactions catalyzed by RHDs,
17 especially the generation of various *cis*-dihydroarenediols, might be exploited in the synthesis
18 of products useful in chemistry and medicine (Hudlicky et al., 1999).

19 Despite the fact that RHDs can attack a wide range of substrates, most of them are only active
20 on a limited number of the 16 priority PAHs, essentially those comprising 2 or 3 rings. Some
21 RHDs, like carbazole dioxygenase, specialize in the angular *cis*-dihydroxylation of tricyclic
22 substrates containing one heterocycle. At the other extreme, a few RHDs able to oxidize 4-
23 and 5-ring PAHs have been described, mainly in strains belonging to the Sphingomonads or
24 the *Mycobacterium* genus, and quite different substrate specificities have been observed.
25 Here, the discussion has been focused on the catalytic abilities of the latter category of RHDs
26 as well as on angular dioxygenases.

27 28 *Angular dioxygenases*

29 The carbazole dioxygenase from *Pseudomonas resinovorans* CA10 can perform angular
30 dioxygenation of several carbazole-like compounds, including dibenzofuran, dibenzo-*p*-
31 dioxin, as well as regular *cis*-dihydroxylation on a variety of 2- and 3-ring substrates such as
32 naphthalene and biphenyl. It can also transform dibenzothiophene to the corresponding
33 sulfoxide and fluorene to 9-fluorenol (Nojiri et al., 1999). Studies on variants of the CA10
34 RHD obtained by site-directed mutagenesis indicated that three residues lining the substrate-

1 binding pocket were important for controlling angular *cis*-dihydroxylation (Uchimura et al.,
2 2008). The dibenzofuran dioxygenase from *Terrabacter* DBF63 showed similar substrate
3 specificity except that it could not transform carbazole (Kasuga et al., 2001). The RHD from
4 the fluorene degrader *Sphingomonas* LB126 exhibited a broader substrate range since it
5 proved capable of angular dioxygenation on fluorene and on the three tricyclic substrates
6 mentioned above. In addition, the LB126 RHD could transform phenanthrene and anthracene
7 but generated unusual dihydrodiol products, i.e. *cis*-9,10-dihydro-9,10-dihydroxy-
8 phenanthrene for the former and a compound tentatively identified as 2,3-dihydro-2,3-
9 dihydroxyanthracene for the latter (Schuler et al., 2008). These compounds are considered by-
10 products that cannot be further degraded through the main catabolic pathway of PAH
11 degraders. They might arise from incorrect positioning of the substrates in the enzyme active
12 site.

13

14 *Regular dioxygenases*

15 Naphthalene dioxygenase from *P. putida* sp. strain 9816-4 converts indole to indigo (Ensley
16 et al., 1983) and dihydroxylates biphenyl and PAHs up to three rings with high regio- and
17 enantioselectivity (Parales et al., 2000; Resnick et al., 1996). Studies of variants obtained by
18 site-directed mutagenesis indicated that the enzyme could tolerate single amino acid
19 substitutions near the active site with only minor changes to its specificity (Parales et al.,
20 2000). However, replacement of Phe352 by smaller amino acids resulted in variants with
21 altered regioselectivity. While the wild-type enzyme converted biphenyl to the *cis*-2,3-
22 dihydrodiol with minor amounts of the *cis*-3,4-dihydrodiol, all the variants tested produced a
23 large excess of the 3,4-isomer. Likewise, phenanthrene was mainly hydroxylated in the 3,4
24 positions by the wild-type enzyme, whereas mutant enzymes produced more of the 1,2-
25 dihydrodiol. The enantioselectivity of the reaction was also altered (Parales et al., 2000).
26 Interestingly, replacement of the phenylalanine residue equivalent to NDO Phe352 by smaller
27 residues in other RHDs, including biphenyl dioxygenase (Suenaga et al., 2002) and
28 tetrachlorobenzene dioxygenase (Pollmann et al., 2003), resulted in significant alterations of
29 oxygenase specificity. The important role of the Phe352 residue in NDO has been studied
30 from a structural point of view by elucidating the 3D structure of a F352V variant, either
31 alone or as a complex with substrates (Ferraro et al., 2006). This variant exhibited a broader
32 substrate-binding pocket, allowing phenanthrene to bind in a different position than in the
33 wild-type enzyme in which the C1 and C2 carbons are close to the mononuclear iron center.
34 These results are consistent with previous enzyme assays showing that this variant mainly

1 generated *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene rather than the 3,4-dihydrodiol. They
2 also provide a structural basis for the notion that the orientation of the substrate binding at the
3 active site is the primary determinant of the regio- and stereoselectivity of RHD-catalyzed
4 oxygenation reactions.

5 Besides NDO from strain NCIB 9816-4, only a few RHDs have been studied for their ability
6 to utilize PAHs as substrates. In addition, most published data have been obtained with *E. coli*
7 strains producing recombinant RHDs using various expression systems, thus precluding
8 meaningful comparisons of activities towards PAHs. The RHD from *Sphingomonas* CHY-1
9 exhibits one of the broadest substrate ranges ever reported, since it is able to attack 9 of the 16
10 priority PAHs. Also, it is one of the rare cases where the catalytic activity towards each PAH
11 was measured using the purified enzyme, thus allowing the kinetic parameters and the
12 stoichiometry of the dioxygenase reaction to be determined (Jouanneau et al., 2006). The
13 dioxygenase activity was maximal with naphthalene and declined as a function of the
14 substrate ring number, to reach 2% of the maximum with benzo[a]pyrene. With naphthalene
15 as substrate, the CHY-1 dioxygenase exhibited an apparent k_{cat} of $1.82 \pm 0.03 \text{ s}^{-1}$ and a
16 specificity constant of $2.0 \pm 0.3 \mu\text{M}^{-1} \text{ s}^{-1}$. These values are comparable to those reported for
17 biphenyl dioxygenase ($k_{\text{cat}} = 7.0 \pm 0.2 \text{ s}^{-1}$, specificity constant of $1.2 \mu\text{M}^{-1} \text{ s}^{-1}$; (Imbeault et al.,
18 2000)) and 2-nitrotoluene dioxygenase ($k_{\text{cat}} = 2.4 \text{ s}^{-1}$; (Parales et al., 2005)) and indicate that
19 RHDs are intrinsically slow enzymes. Most PAHs were converted to a single dihydrodiol,
20 except for fluorene, fluoranthene and benz[a]anthracene, which yielded up to three diols
21 (Jouanneau et al., 2006). The four-ring benz[a]anthracene was transformed to three
22 dihydrodiol isomers bearing hydroxyls in positions 1,2-, 8,9-, and 10,11-. The 1,2- and
23 10,11-dihydrodiols were found to be substrates of the enzyme and were converted in a
24 subsequent reaction to a *bis-cis*-dihydrodiol hydroxylated on carbon positions 1,2,10 and 11.
25 It was observed that the coupling between substrate oxidation and O₂ (and NADH) utilization
26 varied depending on PAHs and decreased with substrate size. Uncoupling was associated with
27 a release of hydrogen peroxide, which might be toxic for the cells if such a side reaction also
28 occurs in vivo. Hence, the unproductive NADH oxidation that was associated mainly to 4-
29 and 5-ring PAH oxidation might in part explain the slow metabolism and poor yields
30 observed during bacterial growth on such PAHs.

31 The RHD from *Sphingobium yanoikuyae* B1 exhibits 91-93% amino acid sequence identity
32 with its counterpart from strain CHY-1, and yet displays significant differences in substrate
33 specificity (Chadhain et al., 2007). The purified B1 RHD apparently utilizes biphenyl as its
34 preferred substrate and was therefore referred to as a biphenyl dioxygenase (Yu et al., 2007),

1 although it falls into the subgroup of PAH RHDs (from Gram-negative bacteria) based on
2 phylogenetic analysis (Fig. 1) and on its substrate specificity. Like the CHY-1 RHD, the B1
3 enzyme was also shown to dihydroxylate naphthalene, phenanthrene and benzo[a]pyrene, the
4 latter two substrates yielding 2 dihydrodiol isomers instead of one (Yu et al., 2007). Previous
5 studies using a mutant strain derived from strain B1 indicated that the same RHD was capable
6 of converting chrysene (Boyd et al., 1997) and benz[a]anthracene (Gibson et al., 1975), the
7 latter substrate giving three dihydrodiols just like the CHY-1 RHD.

8 The phenanthrene degrader *Sphingomonas* LH128 produces an RHD exhibiting 78%
9 (α subunit) and 63% (β subunit) identities with the CHY-1 enzyme (Schuler et al., 2009). Its
10 substrate range was found to be similar, with a marked preference for naphthalene.
11 Nevertheless, the LH128 RHD was less competent in the dioxygenation of large PAHs than
12 the CHY-1 enzyme, as it failed to transform benzo[a]pyrene.

13 The differences in substrate specificity between the three related enzymes above may be
14 tentatively interpreted in structural terms, based on the crystallographic data known for two of
15 them. The substrate-binding pocket is almost identical in all three enzymes as judged from the
16 conservation of the residues lining this cavity. Two conservative changes are however
17 observed in the B1 enzyme (Ile replaces Val202 and Leu replaces Ile260). Much more
18 variation can be noticed in the sequences of the two loops located at the entrance of the active
19 site, which are thought to control substrate access (Jakoncic et al., 2007b). Differences in
20 substrate specificity might also be due to changes of residues more distant from the active site.

21 This notion is supported by studies regarding biphenyl dioxygenases in which three specific
22 regions of the alpha subunit have been shown to determine the selectivity of enzymes towards
23 PCBs (Furukawa et al., 2004).

24 The RHD from *Cycloclasticus* A5, which is distantly related to the PAH dioxygenases from
25 Sphingomonads, exhibits a markedly different selectivity. Based on biotransformation assays
26 using a recombinant *E. coli* strain overexpressing this RHD, it was found that the enzyme was
27 able to oxidize biphenyl, naphthalene, phenanthrene and methylnaphthalenes, but it failed to
28 transform anthracene and larger PAHs (Kasai et al., 2003).

29 The RHD from the acenaphthene degrader *Sphingomonas* sp. strain A4 was found to oxidize
30 acenaphthylene, naphthalene, phenanthrene, anthracene and fluoranthene, although strain A4
31 could not grow on the latter four hydrocarbons (Pinyakong et al., 2004). Unlike RHDs from
32 other Sphingomonadaceae, the A4 enzyme is related, although distantly, to RHDs from
33 betaproteobacteria, the phenanthrene dioxygenase from *Burkholderia* RP007 being the closest
34 homologue (56% identity).

1
2 Gram-positive bacteria belonging to the *Mycobacterium* genus were found to degrade a range
3 of PAHs up to 5 rings in a process likely involving multiple RHDs since several such
4 enzymes were expressed in PAH-grown cells (see section 2 above) . In *M. vanbaalenii* PYR-
5 1, two RHDs encoded by *nidBA* and *nidA3B3* have been shown to dihydroxylate the 4-ring
6 PAHs pyrene and fluoranthene, respectively (Khan et al., 2001; Kim et al., 2006). Based on
7 biotransformation assays, NidA3B3 was also found to attack several 2- to 4-ring PAHs
8 including naphthalene, phenanthrene, anthracene and pyrene but preferentially transformed
9 fluoranthene, consistent with the finding that this enzyme was maximally induced in cells
10 grown with that PAH as sole substrate. In *Mycobacterium* 6PY1, two RHDs with markedly
11 different PAH specificities have been studied: the Pdo1 enzyme preferentially attacked pyrene
12 but also converted phenanthrene to two dihydrodiols bearing hydroxyls in position 3,4-, and
13 9,10-, whereas the Pdo2 enzyme only dihydroxylated 2- and 3-ring PAHs, phenanthrene
14 being the best substrate (Krivobok et al., 2003). All data reported so far on the PAH
15 selectivity of *Mycobacterium* RHDs were from biotransformation assays using *E. coli*
16 recombinant systems in which dioxygenases were generally poorly expressed. In addition, the
17 associated electron carriers being unknown, oxygenase components were expressed alone or
18 in combination with heterologous electron carriers, thus resulting in non-optimal and
19 probably underestimated enzyme activities. Recently, the Pdo1 and Pdo2 oxygenase
20 components from strain 6PY1 were obtained in highly active soluble forms in *E. coli* using
21 non-commercial expression systems (Krivobok, Meyer and Jouanneau, unpublished work).
22 Moreover, two genes designated *phtAc* and *phtAd* encoding a [3Fe-4S] ferredoxin and a
23 reductase, respectively, were cloned from the *Mycobacterium* 6PY1 genome and successfully
24 co-expressed with the oxygenase components. The co-expression of these electron carriers
25 resulted in a 8-fold (Pdo1) or 2-fold (Pdo2) enhancement of the enzyme activity in
26 biotransformation assays (unpublished results). Pdo1 appeared as a unique RHD in that it
27 could utilize 3- and some 4-ring PAHs as substrates, including phenanthrene, anthracene,
28 fluoranthene and pyrene but was unable to hydroxylate smaller substrates like naphthalene
29 and biphenyl. Confronted with the problem of poor expression of RHDs from Gram-positive
30 actinomycetes in *E. coli*, Pagnout et al. utilized *Mycobacterium smegmatis* as host for
31 overproducing a RHD from the PAH-degrader *Mycobacterium* SNP11 (Pagnout et al., 2007).
32 In this expression system, the SNP11 RHD, which is very similar to Pdo2 (>98% sequence
33 identity), efficiently oxidized phenanthrene and anthracene but also fluorene and
34 fluoranthene. More recently, an expression system involving a *Rhodococcus* strain as host for

1 studying recombinant RHDs has been developed. Interestingly, three RHDs expressed in this
2 system exhibited different regio-specificities and relative preference towards dibenzofurans
3 and chlorinated dioxins compared to the enzymes produced in *E. coli* (Iida et al., 2009).
4 Hopefully, convenient expression systems for overproduction of Actinobacterial RHDs in
5 highly active form will emerge, thus allowing purification and further characterization of
6 these enzymes, which show PAH selectivities quite different from those of their Gram-
7 negative counterparts.

9 **Exploring the natural biodiversity of RHD involved in PAH degradation**

11 Early studies on the biodiversity of PAH-degrading bacteria relied on microbial culture and
12 selection on defined growth media as a means to isolate strains with the desired traits. This
13 approach appeared inappropriate when it was realized that a large majority of the bacterial
14 species present in the environment (>90%) were not culturable under standard laboratory
15 growth conditions. Various culture-independent methods were then developed to study
16 microbial diversity, most of which were based on DNA extraction from environmental
17 samples followed by PCR-amplification of selected molecular markers i.e. genes (or gene
18 regions) encoding the 16S ribosomal RNA subunit (see (Kirk et al., 2004) for review).
19 Bacterial species identification and classification based on sequence analysis of 16S rRNA
20 genes recovered from the environment is now the most commonly used method to probe
21 biodiversity, although some pitfalls and biases due to PCR must be taken into consideration
22 (Wintzingerode et al., 1997). In the last decade, studies targeting a specific population of
23 bacteria in complex environment such as soils have emerged, due to the implementation of
24 new techniques involving *in situ* labeling of a population of interest with a naturally rare
25 stable isotope and subsequent isolation and analysis of the labeled DNA. This approach
26 known as stable isotope probing (SIP) has been used recently to identify PAH degraders in
27 soils or sediments (Jeon et al., 2003; Singleton et al., 2005). Besides, the diversity of RHDs in
28 soils has been assessed directly by PCR amplification and sequence analysis of relevant gene
29 portions recovered from environmental samples (Yeates et al., 2000). Here, the contribution
30 of recent studies to our understanding of the biodiversity of PAH-degraders and associated
31 RHDs is discussed.

33 *Relative abundance and diversity of known PAH degraders in the environment*

1 Since Sphingomonadaceae are very often found on PAH-polluted sites, their diversity has
2 been investigated by PCR-based analysis of 16S rRNA genes using family-specific primers
3 (Leys et al., 2004). Sequence analysis revealed that most 16S rRNA genes were significantly
4 different from those of known isolates, suggesting that species different from those studied so
5 far are present in PAH-contaminated soils. A similar study was conducted to examine the
6 community composition of fast-growing *Mycobacterium* in various contaminated soils (Leys
7 et al., 2005). Species identified included known PAH degraders such as *M. austroafricanum*
8 and *M. frederiksbergense* but also *M. tusciae*, a species not previously reported to degrade
9 PAHs.

10 In marine environment, classical isolation of PAH-degraders from contaminated sediment in
11 the Gulf of Mexico led to 23 strains which all belonged to the *Cycloclasticus* genus
12 (Geiselbrecht et al., 1998). Several culture-independent studies conducted all around the
13 world showed that bacterial communities involved in PAH degradation in coastal and
14 estuarine sediments were actually dominated by *Cycloclasticus* in all marine environments
15 tested (Kasai et al., 2002; McKew et al., 2007; Niepceron et al., 2010; Zhou et al., 2009).
16 Members belonging to this genus were also found to be most abundant in sediment of the
17 deep ocean (Cui et al., 2008). On the other hand, in a coastal environment heavily impacted
18 by oil spill, members of the classes Alphaproteobacteria (Sphingomonadaceae) and
19 Actinobacteria (*Mycobacterium*, *Rhodococcus*) were found to prevail (Alonso-Gutierrez et al.,
20 2009).

21

22 *Direct analysis of the RHD biodiversity through PCR-based approaches*

23 In recent studies, RHD biodiversity in PAH-contaminated marine environments was
24 investigated by PCR-based analysis of a portion of the dioxygenase alpha subunit gene.
25 *Cycloclasticus*-like RHD genes appeared to be well represented in coastal sediments from
26 Patagonia (Lozada et al., 2008), consistent with the dominance of this genus in such habitats.
27 Seven other RHD gene types were detected, five of which were distantly related to RHD
28 sequences from known bacteria and two of them clustered with *nahAc*-like (*Pseudomonas*)
29 and *phnAc*-like (*A. faecalis* AFK2) genes. On this other hand, RHDs similar to those found in
30 *Pseudomonads* (*nahAc*) and beta proteobacteria (*nagAc*, *pahAc*) were mostly detected in
31 marine microbial mats subjected to an oil contamination (Bordenave et al., 2008). In
32 mangrove sediments, RHD genes similar to those found in Actinobacteria and in
33 Sphingomonads were detected by PCR in bacterial isolates recovered from these habitats
34 (Zhou et al., 2006).

1 Differences in the observed biodiversity of RHDs in PAH-polluted sites might reflect the
2 natural variability of microbial communities in geographically distant sites. On the other
3 hand, differences might also be due to the PCR-based methods used for amplification of the
4 RHD genes. The genes encoding the α subunits of RHDs are highly variable in sequence (see
5 Fig. 1) and various sets of degenerate primers have been used to amplify different gene
6 regions (Bordenave et al., 2008; Lozada et al., 2008; Ni Chadhain et al., 2006). None of the
7 proposed primer sets has the potential to amplify all types of RHD genes and biases in the
8 PCR-based approaches are therefore to be expected. Nevertheless, one can use primer sets
9 specific for a defined group of RHDs, for instance those found in Gram-positive PAH
10 degraders. Analysis of subantarctic marine sediments using a PCR-based approach with
11 primers targeting Gram-positive RHDs led to the identification of 14 groups of genes, most of
12 which were related to dioxygenases genes found in *Mycobacterium*, *Rhodococcus*,
13 *Nocardioides* and *Terrabacter* (Marcos et al., 2009). As a means to detect a wide range of
14 RHD genes without compromising specificity, Ni Chadhain et al. designed generic primers to
15 amplify a small PCR product encoding a relatively conserved region of the α subunit around
16 the Rieske-cluster binding site (Ni Chadhain et al., 2006). Although the primers were
17 successfully tested against DNA from various isolated bacteria, PCR products obtained from
18 environmental samples were essentially related to RHDs from Gram-negative bacteria. No
19 product could be attributed to Actinobacteria RHD while this bacterial group represented a
20 significant part of the microbial diversity as determined from 16S rRNA genotyping. Another
21 drawback of this approach was the limited information gained from the sequences of the short
22 PCR products representing only about 6% of the α subunit gene length. Yet, this study was
23 one of the first to combine the analysis of rRNA profiles and RHD profiles, providing useful
24 information for assessing the bioremediation potential of a contaminated soil. In another
25 study, a similar PCR-based approach was used to examine the RHD diversity of a coal tar
26 contaminated aquifer in relation with the naphthalene degradation potential (Yagi and
27 Madsen, 2009). Results indicated a broad diversity dominated by *nagAc*-type genes, the
28 relative abundance of which fluctuated substantially over a 9-month as determined by
29 quantitative PCR. Good agreement was observed between in field gene expression as
30 monitored by *nagAc* specific mRNA and the naphthalene degradation potential in the aquifer.
31
32 As a means to determine the occurrence or relative abundance of specific RHD genes in a
33 given soil or sediment, methods based on quantitative PCR have been developed using

1 specific primers (Baldwin et al., 2003; Cebren et al., 2008; DeBruyn et al., 2007; Park and
2 Crowley, 2006). Due to the sequence divergence between RHDs from Gram-positive and
3 Gram-negative bacteria, Cebren et al. designed one set of primers for each RHD category.
4 The primer sets were used for quantification of RHD genes in soil by Real Time qPCR. Data
5 could be normalized relative to the 16S rRNA gene copy number, thus allowing an estimation
6 of the PAH degrader population of up to 1% of the whole bacterial community (Cebren et al.,
7 2008). RHD gene enumeration by qPCR was used as a means to monitor biodegradation
8 activity during treatment of a BTEX contaminated site. Fluctuations in the RHD levels
9 detected were correlated with dynamic changes of the microbial population as a function of
10 oxygen availability (Nebe et al., 2009).

11 The presence of RHDs genes as revealed by PCR amplification in a given environmental
12 sample does not prove that the corresponding enzymes are expressed *in situ*. In order to
13 identify RHDs that are expressed *in situ*, one approach consists of analyzing specific
14 transcripts by RT-PCR using bulk RNA extracted from environmental samples. For example,
15 transcripts of *nahAc*-like genes corresponding to 28 gene sequences potentially coding for
16 naphthalene dioxygenases were detected in groundwater from a coal tar contaminated site
17 (Wilson et al., 1999). More recently, real-time PCR of RHD transcripts and microarrays were
18 employed to monitor changes in the diversity and degradation potential of high Arctic soil
19 bacteria upon hydrocarbon pollution (Yergeau et al., 2009).

20

21 *Identification of PAH-degraders in situ by stable isotope probing*

22 Even though DNA or RNA-based molecular methods to investigate the microbial biodiversity
23 of polluted sites have made significant progresses especially during the last decade, it is still a
24 challenge to identify those bacterial species that are most active at removing pollutants *in situ*.
25 In order to meet this challenge, one of the best experimental approach employed so far, called
26 stable isotope probing (SIP), was first described 10 years ago (Radajewski et al., 2000). The
27 method involves incorporation of a rare stable isotope, most often ^{13}C or ^{15}N , into the
28 biological material of a subpopulation of microorganisms that can metabolize the organic
29 compounds of interest, here the PAHs. For this purpose, a fully labeled organic compound is
30 incubated with the microbial population of an environmental sample to allow selective
31 incorporation of the isotope in targeted bacteria. Subsequent isolation of bulk DNA or RNA
32 from the environmental sample, followed by separation and recovery of labeled nucleic acids
33 by density centrifugation on cesium salt gradient, allows analysis of labeled biomarkers (i.e.
34 rRNA gene sequences) and identification of relevant target bacteria (Dumont and Murrell,

1 2005; Kreuzer-Martin and Jarman, 2007; Neufeld et al., 2007). Using a field-based SIP
2 strategy to track naphthalene degraders on a coal tar contaminated site, Jeon et al. found out
3 that bacteria most active on the dicyclic hydrocarbon belonged to the class of beta
4 proteobacteria (Jeon et al., 2003). The dominant naphthalene degrader was isolated and
5 identified as a previously undescribed *Polaromonas* strain, host of a RHD related to that
6 found in *Comamonas* strains. This study brought clear evidence that the bacteria having a
7 major role in pollutant removal *in situ* had nothing to do with the great majority of
8 naphthalene degraders studied so far, mainly Pseudomonads derived from classical
9 microbiological selection and cultivation methods.

10 In a SIP study targeting naphthalene- and phenanthrene-degrading bacteria in a bioreactor
11 treating PAH-contaminated soil, Singleton et al. observed that the bacterial groups detected
12 differed depending on the PAH-substrate used as probe (Singleton et al., 2005). Bacteria
13 selected on naphthalene primarily belonged to the *Pseudomonas* and *Ralstonia* genera,
14 whereas those selected on phenanthrene were dominated by *Acidovorax*. The same authors
15 examined the diversity of pyrene-degrading bacteria from the microbial community of a
16 similar bioreactor (Singleton et al., 2006). The primary species identified based on 16S rRNA
17 genotyping were uncultivated members of the beta and gamma classes of proteobacteria. In a
18 later study also using ^{13}C -pyrene as a probe, relevant degraders were tracked in creosote-
19 polluted soil under static conditions or under agitation (Jones et al., 2008). Although pyrene
20 mineralization was stimulated by agitation, the dominant bacterial degraders identified based
21 on 16S rRNA sequences under either conditions were very similar. The rRNA gene sequences
22 obtained were closely related to those of uncultured gamma proteobacteria, and were also
23 remarkably similar to those previously found as dominant in a slurry bioreactor (Singleton et
24 al., 2006). None of the sequences matched that of Actinobacteria rRNA, although members of
25 this class, especially fast-growing *Mycobacteria*, are the most studied, cultivated group of
26 pyrene-degrading bacteria.

27 SIP techniques eliminate the need for growth outside the natural microenvironment, thereby
28 reducing bias associated with growth in artificial media and permitting the exploration of the
29 vast majority of uncultured bacteria present in natural or contaminated environments. It is
30 also a suitable approach for studying changes in the composition of the subpopulation of
31 degraders as a function of contaminant concentration or availability, although such
32 investigations are still rare. In this respect, we have recently initiated an analysis of the
33 biodiversity of PAH degraders in the soil of a wetland station collecting road runoff. Using
34 ^{13}C -phenanthrene as a probe, we observed that the population of PAH degraders changed

1 dramatically as a function of phenanthrene availability (F. Martin and Y. Jouanneau,
2 unpublished). Possible limitations to the SIP approach are the uncertainties linked to carbon
3 cross-feeding; that is the incorporation of stable isotope into the biomass of trophically related
4 bacteria, through uptake of labeled metabolites or scavenging of labeled biomass. DeRito et
5 al. showed that ^{13}C -labeled biomass from primary phenol-degraders could be used by other
6 soil bacteria (DeRito et al., 2005). However, cross-feeding can be avoided by reducing the
7 incubation of microbial populations with the labeled substrate to a time shorter than that
8 required for its complete mineralization, which can be monitored by $^{13}\text{CO}_2$ respirometry
9 (Padmanabhan et al., 2003).

10 Beyond the advantage of straightforward rRNA-based identification of bacteria responsible
11 for pollutant degradation, the selective labeling of DNA *in situ* offers the possibility of PCR
12 based analysis of any functional gene involved in the degradation. In this respect, the
13 diversity of RHDs involved in PAH degradation might be explored by PCR amplification of
14 relevant portion of the genes.

15

16 **Strategies for improving PAH bioremediation based on current knowledge on RHDs**

17

18 From the current knowledge of their catalytic properties, RHDs appear as rather slow
19 enzymes with limited activity towards large polycyclic hydrocarbons. Most studied enzymes
20 cannot oxidize PAHs composed of more than three aromatic rings, and even RHDs displaying
21 a broader substrate specificity, such as the *Sphingomonas* CHY-1 RHD, are poorly efficient in
22 the oxidation of 4- and 5-ring substrates (Jouanneau et al., 2006). Given that RHDs catalyze a
23 critical step in PAH degradation, which can limit substrate processing through the
24 downstream catabolic pathway, strategies based on genetic engineering to improve enzyme
25 activity or expand substrate specificity might be implemented to enhance bioremediation,
26 especially with respect to recalcitrant 4- and 5-ring PAHs. So far, bioengineering studies were
27 essentially focused on biphenyl dioxygenases in attempts to improve the biodegradation of
28 polychlorobiphenyls (PCb) (Barriault et al., 2002; Furukawa, 2000; Furukawa et al., 2004).
29 Such studies aimed at improving the substrate specificity and catalytic efficiency of biphenyl
30 dioxygenases towards PCB are reviewed in detail in another chapter of this book. Generally,
31 the most successful approach employed to improve the catalytic activity of dioxygenases
32 relied on directed molecular evolution involving DNA shuffling of the gene of interest
33 according to Stemmer (Stemmer, 1994). In the case of RHDs, only the gene encoding the
34 α subunit of the catalytic component has been the target of DNA shuffling, since this subunit

1 contains the active site and the substrate-binding pocket, which determines specificity. In an
2 interesting development of this technique, Vezina et al. amplified partial sequences of the
3 *bphA* gene encoding the dioxygenase alpha subunit from soil DNA and used them in shuffling
4 experiments to improve the activity of biphenyl dioxygenase toward 2,2'-substituted PCB,
5 thus taking advantage of the catalytic biodiversity available in soil for engineering new
6 enzymes (Vezina et al., 2007).

7 The naphthalene dioxygenase from *Ralstonia* strain U2 was engineered to oxidize
8 nitroaromatic compounds (Keenan et al., 2005). Saturation mutagenesis of the residue F350 in
9 the alpha subunit of the enzyme led to the isolation of a variant with acquired dioxygenase
10 activity towards dinitrotoluenes. Additional mutagenesis through DNA shuffling identified
11 two other residues that influenced the regiospecificity of the enzyme and allowed the
12 generation of variant with three substitutions, which exhibited enhanced activity towards
13 dinitrotoluenes. As another option to expand the range of substrates oxidized by a given
14 bacterium, it has been proposed to co-express in a single cell catalytic components of
15 dioxygenases sharing the same electron carriers. An *E. coli* strain carrying two such
16 recombinant oxygenases could simultaneously oxidize naphthalene and dinitrotoluene
17 (Keenan and Wood, 2006).

18 In techniques such as DNA shuffling, large mutant libraries are generated, and the selection of
19 enzymes with the desired activity requires the implementation of efficient screening strategies
20 (Tee and Schwaneberg, 2007). For biphenyl dioxygenases, screening is based on the detection
21 of a yellow product generated from biphenyl or PCBs by two enzymes that are co-expressed
22 with the RHD in recombinant cells, which convert the biphenyl dihydrodiol to a colored
23 cleavage product. A similar strategy might not be applicable for PAH dioxygenases, because
24 the cleavage product obtained from the first ring cleavage of PAHs is not easy to detect.
25 Hence, the selection of RHDs with higher activity towards PAHs, especially 4- and 5-ring
26 hydrocarbons, through DNA shuffling would require another high throughput assay for
27 measuring the activity of recombinant RHDs. In this respect, Joern et al. have developed a
28 solid-phase screen for the detection of dioxygenase activity in recombinant clones (Joern et
29 al., 2001). In the assay, the dihydrodiol produced by the dioxygenase reaction is indirectly
30 measured after chemical or enzymatic conversion to an oxidation product that reacts with
31 Gibbs reagent to form a blue complex. After screening 9000 clones using this procedure, a
32 variant of toluene dioxygenase was selected with an activity twice as high as that of the wild-
33 type enzyme (Joern et al., 2001). In practice, we found that the method is laborious, not very
34 sensitive and inadequate for the detection of highly unstable dihydroxylated PAH such as 1,2-

1 dihydroxyanthracene. As an alternative, we recently developed an assay based on the direct
2 fluorescence detection of the dihydrodiols formed, which has been adapted to the microplate
3 format. Although the assay is limited to PAH yielding dihydrodiols with high enough intrinsic
4 fluorescence (anthracene, chrysene, pyrene, benzo[a]pyrene), preliminary results suggest that
5 it is suitable for the screening of RHDs with activity towards 4- and 5-ring PAHs (Jouanneau,
6 unpublished).

7 8 **Conclusions and perspectives** 9

10 Over the last decade, considerable progress in our understanding of RHD catalytic function
11 has been achieved thanks to detailed structural analyses of representative oxygenase
12 components through X-ray crystallography. The regio- and stereo-selectivity of RHDs can be
13 easily explained from studies on enzyme-substrate complexes showing the critical role of the
14 substrate-binding pocket in the positioning of the substrate relative to the active-site iron
15 atom. The size and topology of the substrate-binding site are certainly important factors in
16 controlling enzyme specificity, but the structural determinants responsible for substrate
17 recognition are still largely unknown. This is an important issue in the context of PAH
18 biodegradation, because the most genotoxic 4- and 5-ring hydrocarbons are poor substrates
19 for most RHDs. Taking advantage of the accumulated structural information on RHDs, one
20 can try to improve RHD catalytic efficiency or enlarge substrate range toward recalcitrant
21 PAHs by employing bioengineering strategies based on rational design or directed molecular
22 evolution. Such strategies, especially those involving DNA shuffling, have been successfully
23 implemented to improve the substrate range of biphenyl dioxygenases towards PCBs
24 (Furukawa, 2000; Furukawa et al., 2004). Hence, provided that efficient screening systems
25 are developed to select for enzymes with desired characters, significant improvements in the
26 performances of PAH oxidizing RHDs may be expected from future bioengineering studies,
27 especially those based on molecular directed evolution.

28 In recent years, new tools have emerged to explore the biodiversity of highly complex
29 microbial populations in their environmental habitats. In particular, it becomes possible to
30 identify bacteria that are most active in the degradation of pollutants *in situ* without the need
31 to cultivate and isolate them. In the case of PAH polluted sites, the use of approaches such as
32 stable isotope probing led to the discovery that the dominant PAH degraders were different
33 from the bacterial species that had previously been isolated and studied under standard
34 laboratory conditions. This observation is consistent with the general assumption that >90%

1 of bacterial species present in the environment are unknown, uncultured microorganisms.
2 Consequently, knowledge derived from PAH degraders studied so far need to be revisited by
3 focusing on species that play a major role in pollutant removal *in situ*. In this respect, it will
4 be necessary to develop novel cultivation conditions and isolation procedures in order to
5 study these bacterial species and eventually to identify their PAH catabolic enzymes. Further
6 developments of culture-independent methods might combine stable isotope labeling with *in*
7 *situ* hybridization for the analysis of single cells (Huang et al., 2009), or with metagenomics
8 to couple sequence information from a specific subpopulation with metabolic function
9 (Kalyuzhnaya et al., 2008; Leigh et al., 2007).

10 PCR-based methods were also developed to analyze directly the biodiversity of RHDs in
11 environmental samples. Since the genes encoding RHDs display great sequence variability,
12 and no region is sufficiently conserved to design universal primers, many studies relying on a
13 single set of degenerate primers introduce biases resulting in a limited sequence sampling of
14 the biodiversity. This problem can be overcome by using multiple sets of primers to amplify
15 different families of RHDs. For instance, two sets of primers were designed to amplify
16 separately RHDs from Gram-positive and Gram-negative PAH degraders (Cebren et al.,
17 2008). Yet, these PCR-based methods do not provide unambiguous evidence that the detected
18 RHD genes are all related to the metabolic function of interest and expressed under the
19 studied conditions. Again, labeling methods such DNA-based or RNA-based stable isotope-
20 probing have the potential to specifically target pollutant degraders and their associated
21 RHDs. For example, bacteria and functional genes associated with biphenyl degradation were
22 identified in a PCB-polluted site using DNA-SIP coupled to analyses including gene detection
23 by DNA microarray and RHD-specific PCR amplification (Leigh et al., 2007). Similar
24 approaches might be used to target RHDs and other functional genes involved in PAH
25 degradation in polluted sites. The resulting genetic information might be useful for the
26 development of quantitative analysis of specific catabolic genes such as real time qPCR, and
27 for the conception of specific microarrays designed to detect relevant genes and their
28 transcripts *in situ*. The development of such molecular tools to detect, quantify and monitor
29 bacterial populations involved in PAH degradation, as well as their specific catabolic
30 enzymes, is likely to be of benefit to bioremediation technologies in the near future.

31

32 **Acknowledgements**

1 We thank L. Schuler for providing figures 2 and 3. This work was supported by a PhD
2 fellowship from the Région Rhône-Alpes to F. Martin, and by grants from the CNRS and the
3 University of Grenoble Alpes.

4

1 **Legends to figures**

2
3 Figure 1 : Phylogenetic tree of alpha subunits of selected RHDs. Sequence alignment and the
4 generation of a tree by neighbour-joining analysis were performed using Clustal X software.
5 The scale bar represents substitutions per amino acid. Sequences were grouped by subfamilies
6 according to substrate specificity. Alpha subunits were from the following
7 dioxygenase/organism systems : PhnAc_AFK2, *Alcaligenes faecalis* AFK2 (AB024945);
8 ArhA1_A4, *Sphingomonas* sp. strain A4 (BAD34447); PhnAc_RP007, *Burkholderia* sp.
9 RP007 (AF061751); PahA3_PaK1, *Pseudomonas aeruginosa* PaK1 (D84146); NagAc_U2,
10 *Ralstonia* sp. U2 (AF036940); NahAc_G7, *Pseudomonas putida* G7 (YP_534822);
11 NdoB_NCIB9816, *Pseudomonas putida* NCIB9816 (M23914); NahAc_NCIB9816-4,
12 *Pseudomonas putida* NCIB9816-4 (NP_863072); NtdAc_JS42, *Pseudomonas* sp. JS42
13 (U49504); DntAc_DNT, *Burkholderia* sp. DNT (U62430); PhnA1_A5, *Cycloclasticus* sp.
14 strain A5 (AB102786); BphA1f_F199, *Sphingomonas aromaticivorans* F119 (AF079317);
15 PhnA1f_LH128, *Sphingomonas* sp. strain LH128 (EU024112); BphA1f_B1, *Sphingomonas*
16 *zanoikuyae* B1 (ABM91740); PhnA1a_CHY1, *Sphingomonas* sp. strain CHY-1 (AJ633551);
17 PhtAa_12B, *Arthrobacter keyseri* 12B (AF331043); NidA_I24, *Rhodococcus* sp. I24
18 (AF121905); NarAa_NCIMB12038, *Rhodococcus* sp. NCIMB12038 (AF082663);
19 NidA3_PYR-1, *Mycobacterium vanbaalenii* PYR-1 (AAY85176); PhdA_KP7, *Nocardioides*
20 sp. KP7 (AB017794); PdoA2_6PY1, *Mycobacterium* sp. strain 6PY1 (CAD38643);
21 NidA_PYR-1, *Mycobacterium vanbaalenii* PYR-1 (AF249301); NidA_S65, *Mycobacterium*
22 sp. S65 (AF_546904); PdoA1_6PY1, *Mycobacterium* sp. strain 6PY1 (CAD38647);
23 DitA1_BKME-9, *Pseudomonas abietaniphila* BKME-9 (AF119621); AntA_ADP1,
24 *Acinetobacter calcoaceticus* ADP1 (AF071556); CbdA_2CBS, *Burkholderia cepacia* 2CBS
25 (X79076); XylX_mt2, *Pseudomonas putida* mt2 (M64747); BenA_ADP1, *Acinetobacter*
26 *calcoaceticus* ADP1 (AF009224); OphA2_DBO1, *Burkholderia cepacia* DBO1 (AF095748);
27 IphA2_YZW-D, *Comamonas testosteroni* YZW-D (AY923836); CarAa_CA10,
28 *Pseudomonas resinovorans* CA10 (AB088420); OxoO_86, *Pseudomonas putida* 86
29 (Y12655); DbfA1_KA1, *Sphingomonas* sp. KA1 (BAF03470); DbfA1_YK5, *Paenibacillus*
30 sp. strain YK5 (BAF_53401); FlnA1_LB126, *Sphingomonas* sp. strain LB126 (EU024110);
31 DbfA1_DBF63, *Terrabacter* sp. strain DBF63 (BAC75993); DbfA1_YK2, *Rhodococcus* sp.
32 strain YK2 (BAC00802); DbfA1_YK3, *Terrabacter* sp. strain YK3 (BAC06602);
33 CarAa_CB3, *Sphingomonas* CB3 (AF_060489); DxnA1_RW1, *Sphingomonas* sp. RW1
34 (X72850); XylC1_RB1, *Cycloclasticus oliotropus* RB1 (U51165); BphA1_P6, *Rhodococcus*

1 *globerulus* P6 (X80041); TcbAa_P51, *Pseudomonas* sp. P51 (U15298); TodC1_F1,
2 *Pseudomonas putida* F1 (J04996); BphA_B-356, -356, *Comamonas testosteroni* B-356
3 (U47637); BphA_LB400, *Burkholderia xenovorans* LB400 (M86348); ThnA1_TFA,
4 *Sphingopyxis macrogoltabida* TFA (AF157565);

5
6

7 Figure 2 : Genetic organization of PAH catabolic genes in representative Proteobacteria.

8 The relevant gene cluster from the phenanthrene-degrading Gram-positive bacterium
9 *Nocardioides* sp. KP7 is also shown for comparison. The figure was adapted from Schuler
10 (Schuler, 2008) with permission.

11
12

13 Figure 3 : Genetic organization of PAH, biphenyl and xylene catabolic genes in
14 Sphingomonads. Gene clusters are from *Novosphingobium aromaticivorans* F199,
15 *Sphingobium yanoikuyae* B1, *Sphingobium* sp. P2, *Sphingomonas* sp. LH128, *Sphingomonas*
16 sp. CHY-1, *Sphingobium agrestis* HV3, and *Sphingomonas chungbukensis* DJ77. The figure
17 was adapted from Schuler (Schuler, 2008) with permission.

18
19

20 Figure 4 : Representation of the substrate-binding pocket of the RHD from *Sphingomonas*
21 CHY-1 and modeling of a benzo[a]pyrene molecule bound at the active site. The entrance
22 into the pocket is indicated by the broad arrow. Residues that are thought to be important for
23 regioselectivity (Phe 350) or substrate specificity (Leu 223 and Ile 260) are highlighted. The
24 figure was reproduced from Jakoncic et al. (Jakoncic et al., 2007a) with permission.

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Fig. 1

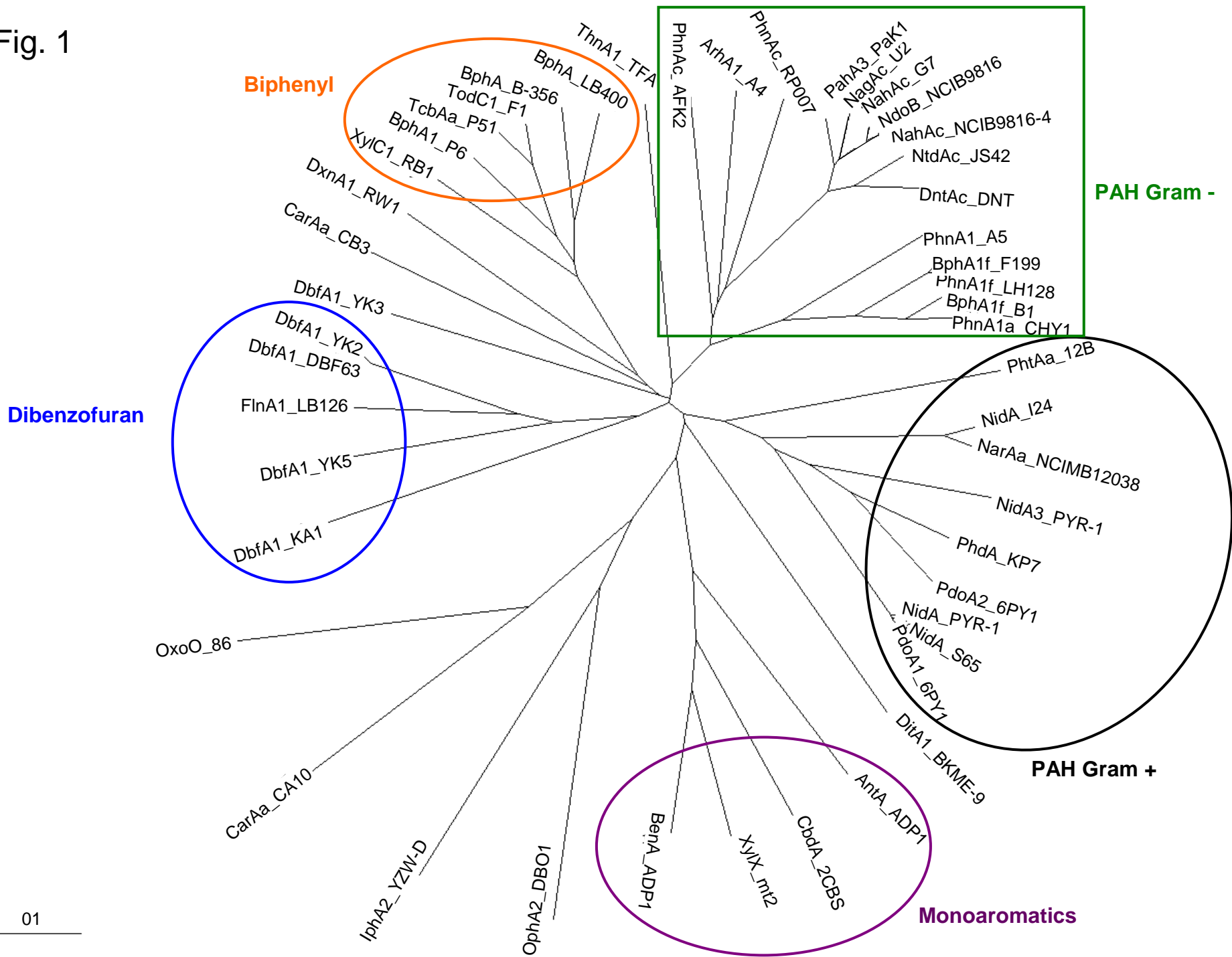


Fig. 2

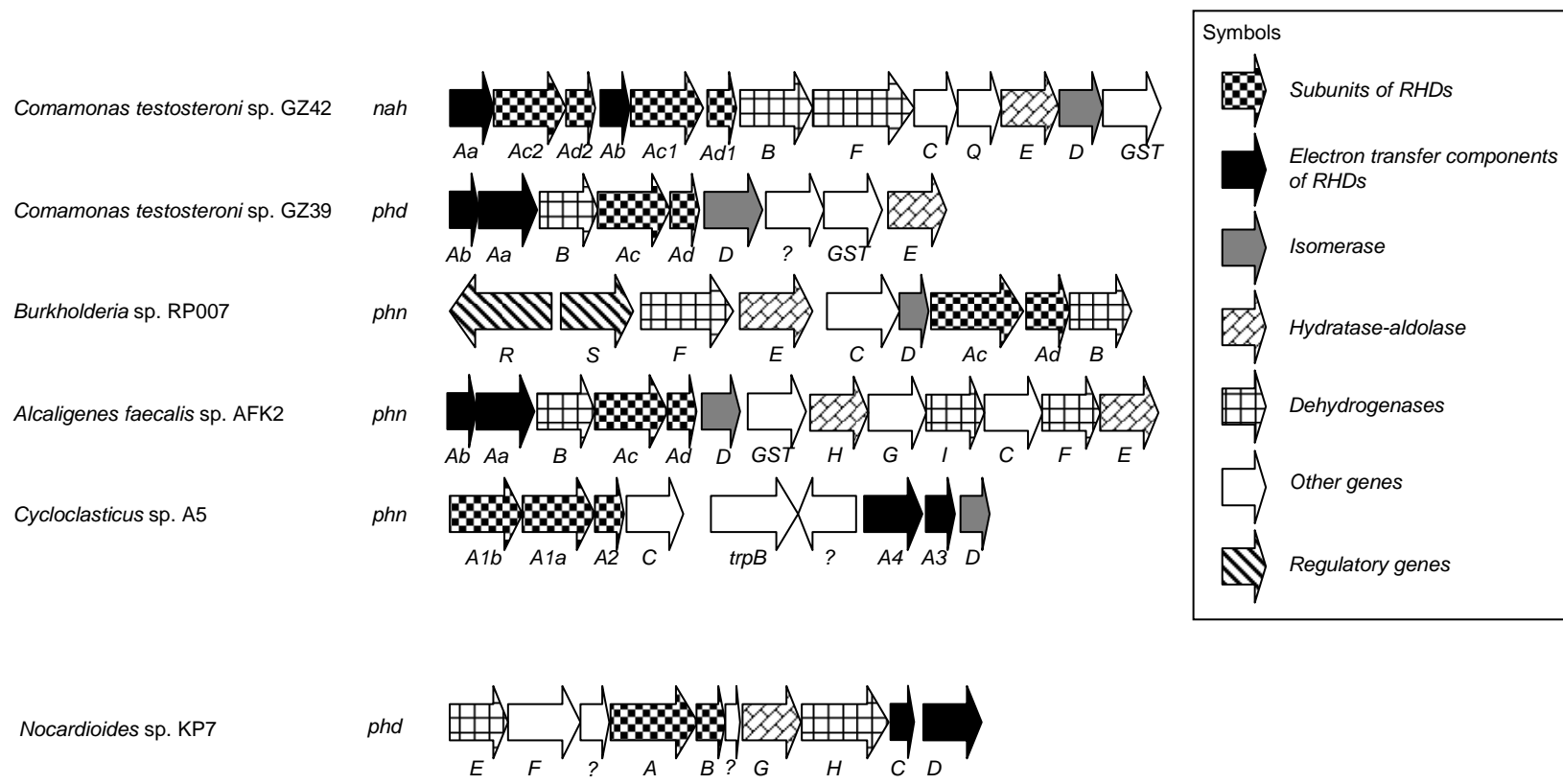


Fig. 3

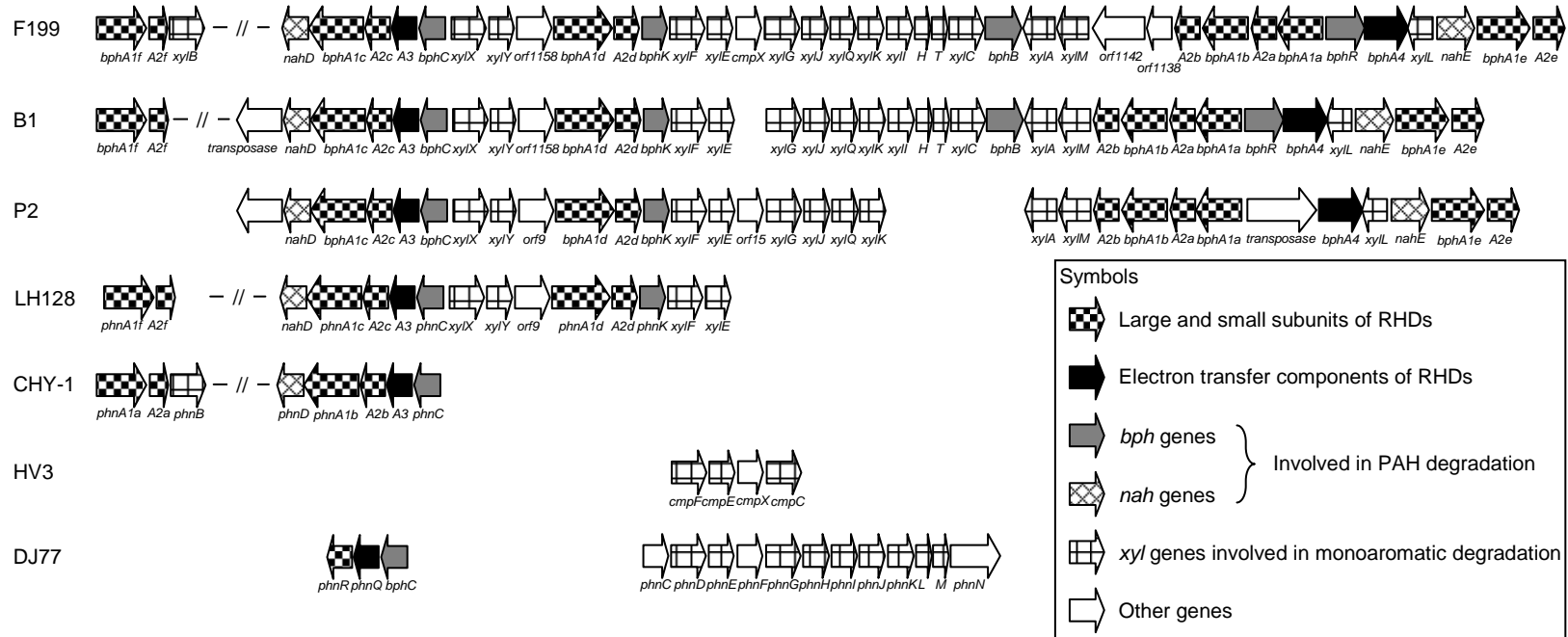


Fig. 4

