The bacterial catabolism of polycyclic aromatic hydrocarbons: Characterization of three hydratase-aldolase-catalyzed reactions

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Summary Polycyclic aromatic hydrocarbons (PAHs) are highly toxic, pervasive environmental pollutants with mutagenic, teratogenic, and carcinogenic properties. There is interest in exploiting the nutritional capabilities of microbes to remove PAHs from various environments including those impacted by improper disposal or spills. Although there is a considerable body of literature on PAH degradation, the substrates and products for many of the enzymes have never been identified and many proposed activities have never been confirmed. This is particularly true for high molecular weight PAHs (e.g., phenanthrene, fluoranthene, and pyrene). As a result, pathways for the degradation of these compounds are proposed to follow one elucidated for naphthalene with limited experimental verification. In this pathway, ring fission produces a species that can undergo a non-enzymatic cyclization reaction. An isomerase opens the ring and catalyzes a cis to trans double bond isomerization. The resulting product is the substrate for a hydratase-aldolase, which catalyzes the addition of water to the double bond of an

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Introduction

Aromatic hydrocarbons are characterized by the presence of the very stable benzene ring (Scheme 1). Polycyclic aromatic hydrocarbons (PAHs) consist of multiple aromatic rings ranging from the simplest one, naphthalene (1, Scheme 1), to more elaborate ones such as phenanthrene (2), fluoranthene (3), and pyrene (4) (Kimes et al., 2014).

Microbial pathways for the degradation of benzene and derivatives such as toluene and xylene have been extensively characterized (Kimes et al., 2014). The meta-fission pathway (Scheme 2) is a major route for the bacterial catabolism of monocyclic compounds (Kimes et al., 2014; Manjasetty et al., 2003). Initially, the aromatic species (e.g., toluene) is converted to catechol (5), or a derivative, which is subjected to ring cleavage adjacent to a hydroxyl group (as indicated). The ring opened compound, 2-hydroxymuconate semialdehyde (6), is then processed by a series of transformations to yield pyruvate and acetyl CoA (Manjasetty et al., 2003). The enzymes comprising this pathway and other degradation pathways for the monocyclic aromatic hydrocarbons have provided many significant insights into enzyme chemistry, specificity, and evolution.

The individual enzymes in the microbial catabolic pathways for PAHs are not as well characterized, if at all. Our interest in these pathways was sparked by the explosion of the Deepwater Horizon drilling rig and subsequent oil spill in the Gulf of Mexico (off the southeastern coast of the United States) in April 2010. An estimated 5 million barrels of crude oil were spilled in the Gulf before the oil well was finally capped about 3 months later. Thus far, the Deepwater Horizon oil spill and the Exxon Valdez oil spill (in 1989) are the two worst spills in the US, and are not expected to be the last major spills. In addition, there have been many smaller spills (Atlas and Hazen, 2011).

The lighter crude oil from the Gulf of Mexico is rich in low molecular weight hydrocarbons and in microorganisms that degrade these compounds. These organisms likely evolved this capability over the years as a result of the natural leakage of oil from the Gulf floor, estimated to be 1.4 million barrels of oil each year (Kimes et al., 2014). It has been suggested that microbial processes removed 43–61% of the oil spilled in the Deepwater Horizon accident (Joye, 2015). Although PAHs are minor components of lighter crude oil, they are the most toxic. Moreover, the fate of PAHs in the Deepwater Horizon oil spill is less clear. For both reasons, it is necessary to have a better understanding of the microbial processes that degrade PAHs.

Characterization of PAH catabolic pathways

There is a considerable body of literature on the microbial degradation of PAHs including the identification of gene clusters, individual enzymes, and the demonstration that many bacterial species can completely catabolize these compounds to cellular metabolites. Examination of the proposed pathways suggests that one major strategy for the degradation of PAHs involves the successive removal of each ring by a round of four transformations. This strategy is illustrated by the naphthalene catabolic pathway, which is the most extensively characterized pathway for the degradation of a PAH.

In the proposed naphthalene catabolic pathway in Pseudomonas putida G7, two enzymes catalyze the dihydroxylation of one ring to yield 1,2-dihydroxynaphthalene, 7 (Scheme 3) (Eaton and Chapman, 1992). Subsequently, ring fission produces a chemically reactive dienol (8). Chemical ketonization of 8 generates 9, which reacts non-enzymatically to yield 2-hydroxychromene-2-carboxylate (10). The hemiketal 10 slowly converts to trans-o-hydroxybenzylidenepyruvate (11), but the glutathione-dependent enzyme, 2-hydroxychromene-2-carboxylic acid (HCCA) isomerase (designated NahD), accelerates this transformation. One single enzyme, trans-o-hydroxybenzylidenepyruvate hydratase-aldolase (designated NahE), then catalyzes two reactions: the addition of water to the double bond of 11 to afford 12, and the subsequent retro-aldol cleavage of 12 to produce salicylaldehyde (13) and pyruvate. Oxidation of salicylaldehyde generates salicylate, which is directed to the meta-fission pathway. In this way, naphthalene can be used as a sole source of carbon and energy for the organism.

Each ring of the high molecular weight species (e.g., phenanthrene, fluoranthene, and pyrene, 2, 3, and 4 in Scheme 1) is proposed to undergo a similar set of reactions, but many of these activities have not been experimentally confirmed (Kweon et al., 2011). In addition, the substrates and products are not known for some transformations and might have limited solubility. Nonetheless, the proposed transformations raise many interesting questions about mechanism, specificity, and evolution. The answers to these questions will provide a better understanding of the individual enzymatic transformations and assist in the optimization of the corresponding enzymes for bioremediation.
Characterization of NahE

We began our comprehensive examination of PAH degradation with mechanistic, kinetic, and structural studies of the NahE-catalyzed reaction, which appeared to be the most straightforward one of the three initially targeted hydratase-aldovalues-catalyzed reactions (although this did not turn out to be the case). Sequence analysis identified NahE as a Class I aldolase belonging to the N-acetylneuraminic lyase (NAL) sub-family (Barbosa et al., 2000). In addition to NAL, this sub-family of α/β-barrel enzymes includes dihydronicotinamide synthetase (DHNPS), D-5-keto-4-deoxyglucarate dehydratase (KDGDH), 2-keto-3-deoxysugonate aldolase (KDGA), and trans-2-carboxybenzylidenepeyrurate hydratase-aldolase (designated PhdJ). The common catalytic step in this sub-family is the formation of a Schiff base between the enzyme and the pyruvyl moiety of the substrate.

All members of the NAL sub-family have a conserved lysine to form a Schiff base with the pyruvyl moiety of the substrate (Barbosa et al., 2000). Hence, it was anticipated that the NahE-catalyzed reaction would proceed by a Schiff base mechanism using Lys-183 (the conserved lysine) as shown in Scheme 4. Accordingly, Lys-183 forms a Schiff base with the pyruvyl moiety of 11 to yield 14. The addition of water at C-4 of 14 produces the Schiff base of 12, which can undergo an aldol reaction to yield salicylaldehyde (13) and the pyruvyl enamine, which tautomerizes to the Schiff base of pyruvate. Hydrolysis releases pyruvate and enzyme. The involvement of the Schiff base mechanism is easily determined. However, the two key questions are how the enzyme catalyzes the addition of water to 14 (including whether or not Schiff base formation precedes the addition of water) and what residues are responsible for binding the aromatic portion of the substrate (i.e., 11). These features distinguish NahE (and the related hydratase-aldolases) from the other NAL sub-family members.

Experimental evidence in support of the Schiff base mechanism came from trapping experiments and mutagenesis. In the presence of substrate (i.e., 11) or product (salicylaldehyde or pyruvate) and NaCNBH3, NahE is inactivated by covalent modification at Lys-183 (as indicated by mass spectral analysis). Changing Lys-183 to an alanine resulted in an enzyme that was not covalently modified and had almost no activity. In the forward or reverse direction (following respectively the loss or formation of 11 at 296 nm), the mutated enzyme shows very little activity (at most 1–2% of wild type activity). However, these values are within error limits so there might not be any detectable activity. These observations are all consistent with the anticipated Schiff base mechanism.

Kinetic studies were carried out on NahE using trans-benzylidenepeyrurate (15), cis-benzylidenepeyrurate (16), and trans-o-carboxybenzylidenepeyrurate (17) (Table 1) in order to assess the contributions of the o-substituent and stereochemistry (cis vs trans) to activity. It is not yet possible to obtain consistent kinetic parameters for NahE using 11, the biological substrate, for reasons that are not entirely clear. However, the lower limit for the $k_{cat}/K_m$ is estimated to be $8.5 \times 10^4 \text{M}^{-1} \text{s}^{-1}$. The absence of the o-hydroxy substituent (15 and 16) has a detrimental effect on activity as both isomers have comparable kinetic parameters with overall $k_{cat}/K_m$ values of $~600–630 \text{M}^{-1} \text{s}^{-1}$ (down $~14,000$ fold from that measured for 11). The $k_{cat}/K_m$ values also
suggest that the enzyme is indifferent to the stereochemistry (cis vs trans). Replacement of the o-hydroxy group with a carboxylate group decreases the $k_{cat}/K_m$ value (1200 $M^{-1}$s$^{-1}$) from that measured using 11 (~7100-fold), but the effect is not as dramatic as that observed for substrates lacking an o-hydroxy substituent.

The inability to obtain kinetic parameters for NahE and 11 prompted us to look more closely at the previous reaction in the pathway, the NahD-catalyzed conversion of 2-hydroxychromene-2-carboxylate (10) to 11 (Scheme 3). NahD is a glutathione-dependent isomerase that opens the hemiketal by the addition of the glutathione thiolate to C-4 (Scheme 5) (Thompson et al., 2007). The resulting glutathione adduct now has a C3, C4 single bond. Rotational, ketonization, and expulsion of glutathione yield 11, as shown in Scheme 5.

Three separate $^1$H NMR experiments were carried out (data not shown). In the first experiment, NahE was incubated with the hemiketal (i.e., 10). In a second experiment, NahE was incubated with trans-o-hydroxybenzylidene pyruvate (11). In the third experiment, NahD and NahE were incubated with 11. After 30 min, the products (salicylaldehyde, 13, and pyruvate) were observed for all three reactions, but the reaction containing NahD and NahE generated the most product (as assessed by integration of the signal for the methyl group of pyruvate). This reaction showed 26% conversion. In contrast, the reaction containing NahE and 10 showed only 10% conversion and the one containing NahE and 11 showed 16% conversion. In solution, 10 and 11 rapidly equilibrate to form a mixture (55% and 45%, respectively). Hence, over the 30-min time course of the first $^1$H NMR experiment, 11 will be present so that NahE is processing 11 and not 10. The sum of these observations suggests that NahD is required to maintain sufficient quantities of 11 in solution for the NahE reaction to occur (Route A, Scheme 6), or an intermediate between 10 and 11 is the actual substrate (Route B, Scheme 6). This intermediate might be the cis-isomer (i.e., 18) although 18 has not

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Table 1  Kinetic parameters for NahE using various substrates.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ ($M^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>$\ldots$</td>
<td>$\ldots$</td>
<td>$&gt;8.5 \times 10^6$</td>
</tr>
<tr>
<td>15</td>
<td>0.1</td>
<td>160</td>
<td>6 $\times 10^2$</td>
</tr>
<tr>
<td>16</td>
<td>0.07</td>
<td>110</td>
<td>6.3 $\times 10^2$</td>
</tr>
<tr>
<td>17</td>
<td>0.3</td>
<td>240</td>
<td>1.2 $\times 10^3$</td>
</tr>
</tbody>
</table>

\(^a\) The standard errors for the kinetic parameters are estimated to be 10%.

\(^b\) It is not yet possible to obtain kinetic parameters, as discussed in the text.
been observed by UV or \(^1\)H NMR spectroscopy in the course of the reaction. Efforts are underway to resolve this issue.

**Structural analysis of NahE**

Structural analysis of NahE (2.2 Å resolution) shows that it has the signature \(\alpha,\beta\)-barrel motif of a Class I aldolase where Lys-183 is found on \(\beta\)-strand F in the center of the barrel (Fig. 1A) (Barbosa et al., 2000; Choi et al., 2006). A close-up of the active site in this structure shows the presence of Lys-183, Tyr-155, Thr-65, Phe-66, and Trp-224 (Fig. 1B). A second structure of NahE where the \(\varepsilon\)-amino group of Lys-183 forms a Schiff base with pyruvate (1.9 Å resolution) suggests roles for these residues (Fig. 1C). Accordingly, the amide protons of Thr-65 and Phe-66 bind the carboxylate group of pyruvate (and likely bind the carboxylate group of 11). The hydroxyl group of Tyr-155, near Lys-183, interacts with the amide proton of Thr-65 and the hydroxyl group of the side chain. The role of Trp-224 is unknown. Analysis of these structures has not yet shown how the water is added or what determines the specificity for the aromatic portion of substrate.

This analysis is consistent with the previously reported analysis of the NAL sub-family group members (Scheme 7), where the active site residues were divided into two groups (Barbosa et al., 2000). In NAL (left panel, Scheme 7), the carboxylate group of the pyruvyl moiety of substrate interacts with the backbone amide groups of Ser-47 and Thr-48 along with Tyr-136 and Lys-164. These residues are considered the conserved primary group of residues. The pyruvyl moiety of 11 is arranged similarly in NahE (right panel, Scheme 7). Like Ser-47 and Thr-48 in NAL, Thr-65 and Phe-66 are part of a highly conserved GXXGE motif where they are the second and third residues. Tyr-155 is also conserved in the NAL sub-family members and could be involved in Schiff base formation. Allen and co-workers suggested that the conserved tyrosine assists in the dehydration of the carbino-lamine intermediate that forms between the side chain of lysine and substrate (Choi et al., 2006). Other roles have been suggested as well (Daniels et al., 2014). The secondary group of residues (discussed below) is responsible for binding the remaining portion of the substrate and carrying out the specific reaction associated with the sub-family member.

**Characterization of the phenanthrene degradative pathway**

We next turned our attention to the degradation of phenanthrene in *Mycobacterium vanbaalenii* PYR-1. This organism

![Figure 1](image-url)
was cultured from sediments in an oil-contaminated site in Redfish Bay, Texas (near Port Aransas), and is notable for its ability to degrade a broad range of PAHs and derivatives (e.g., naphthalene, anthracene, phenanthrene, fluoranthene, pyrene, and benzopyrene) (Heitkamp and Cerniglia, 1988; Khan et al., 2002). It is the prototype organism to elucidate PAH pathways and has been used to remediate PAH-contaminated soils. In addition, the genome sequence is available, which greatly assists efforts to unravel and understand the PAH catabolic pathways.

Sequence analysis indicates there might be 6 hydratase-aldolases in M. vanbaalenii PYR-1 including PhdG and PhdJ, which are found in a phenanthrene catabolic pathway (Schemes 8 and 9). A third hydratase-aldolase, designated Mvan_0452, is located near the genes for PhdG and PhdJ in a huge cluster of PAH degrading enzymes. The amino acid sequence shows 25% sequence identity with NahE, whereas the amino acid sequences for PhdG and PhdJ show 36% and 45% sequence identity respectively with NahE. The substrates for Mvan_0452 as well as those for the remaining 3 hydratase-aldolases are unknown. In view of the broad range of compounds degraded by the organism, these hydratase-aldolases might segregate themselves into categories for low and high molecular weight PAHs (much like the short chain, medium chain, long chain, and very long chain acyl dehydrogenases in β-oxidation).

PhdG and PhdJ reportedly function as hydratase-aldolases in the proposed phenanthrene catabolic pathway shown in Schemes 8 and 9. In this pathway, phenanthrene is converted to 3,4-dihydroxyphenanthrene, which undergoes ring opening to generate a reactive dienol (Scheme 8) (Stingley et al., 2004).

This product has not been characterized, but the steps leading from the dienol to (E)-4-(1-hydroxynaphthalen-2-yl)-2-oxobut-3-enoate (19) are proposed to parallel those in the degradation of naphthalene (Scheme 3). Accordingly, the dienol undergoes a rearrangement to a hemiketal, which is then isomerized to 19. (Mycobacteria) lack glutathione so the putative isomerase will not be a glutathione-dependent one like NahD). It is further proposed that PhdG converts 19 to 1-hydroxy-2-naphthaldehyde (20) and pyruvate. Oxidation of 20 to 1-hydroxy-2-naphthoic acid followed by ring opening (as indicated in Scheme 9) produces trans-α-carboxybenzyldiene pyruvate (17). The PhdJ-catalyzed reaction then processes 17 to α-carboxybenzaldehyde (21) and pyruvate (Stingley et al., 2004).
Characterization of PhdJ

Sequence analysis identified both PhdG and PhdJ as Class I aldolases in the NAL sub-family of enzymes (Barbosa et al., 2000). For both enzymes, Lys-180 is the conserved lysine that forms the Schiff base with the pyruvyl moiety of substrate (19 or 17). Experimental evidence for the presence of a Schiff base mechanism in both enzymes was obtained from NaCNBH₃ trapping experiments. Incubation of PhdJ with substrate (17) or either product (21 or pyruvate) and NaCNBH₃ results in the enzyme’s inactivation and covalent modification of Lys-180 (as assessed by mass spectral analysis). PhdG is modified (with covalent modification of Lys-180) by incubation with pyruvate and NaCNBH₃. Similar experiments have not been carried with the proposed biological substrate, 19, or with 20, the other proposed product. Incubation of PhdG with 17 (and NaCNBH3) did not result in modification of the enzyme.

PhdJ was characterized first due to the availability of substrate and enzyme. Accordingly, a kinetic analysis of PhdJ was performed using the biological substrate, 17, and two derivatives, trans-benzylidene-pyruvate, 15 (which is missing the o-carboxy group) and 11, the substrate for NahE (Table 2). The biological substrate shows a kₐₐₜ/Kₘ value of 8.1 × 10⁴ M⁻¹ s⁻¹. The absence of the o-carboxy group results in a ∼1000-fold decrease in the kₐₐₜ/Kₘ value (∼83 M⁻¹ s⁻¹) and an inability to saturate the enzyme. Using the NahE substrate (i.e., 11) results in a kₐₐₜ/Kₘ value of 3.1 × 10³ M⁻¹ s⁻¹, only a ∼26-fold decrease from that observed for the biological substrate. It is interesting that it is possible to obtain parameters for PhdJ with 11, but not for NahE with 11. This suggests that a non-enzymatic process is not responsible for our inability to obtain kinetic parameters for NahE and 11.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kₐₐₜ (s⁻¹)</th>
<th>Kₘ (µM)</th>
<th>kₐₐₜ/Kₘ (M⁻¹ s⁻¹)</th>
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<tbody>
<tr>
<td>![CO₂⁻][CO₂⁻]</td>
<td>6.4</td>
<td>79</td>
<td>8.1 × 10⁴</td>
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<tr>
<td>![CO₂⁻][O][O]</td>
<td>_b</td>
<td>_</td>
<td>83</td>
</tr>
<tr>
<td>![CO₂⁻][O][O]</td>
<td>0.28</td>
<td>90</td>
<td>3.1 × 10³</td>
</tr>
</tbody>
</table>

a The standard errors for the kinetic parameters are estimated to be 10%.

b It was not possible to achieve saturation with 15.

Structural analysis of PhdJ

Crystal structures of PhdJ (2.0 Å resolution) and PhdJ with pyruvate bound at Lys-180 (2.7 Å resolution) were obtained (Fig. 2A and B). As expected, the enzyme shows the characteristic Class I aldolase TIM barrel fold where the active site is located in the middle of the barrel and the conserved Lys-180 is on β-strand F (Fig. 2A) (Barbosa et al., 2000; Choi et al., 2006). A close-up of the active site with the bound pyruvate shows Lys-180, Tyr-152, Thr-62, Phe-63, and Trp-225 (Fig. 2B). The structure shows that the amide protons of Thr-62 and Phe-63 bind the carboxylate group and Tyr-152 interacts with the side chain hydroxyl group of Thr-62. The role of Trp-225 is unknown.

The addition of the PhdJ active site features to the schematic diagram for the hydratase-aldolases in the NAL sub-family highlights the conservation of the primary group of active site residues interacting with the pyruvyl portion of the substrates (left panel, Scheme 10). The residues are lysine, tyrosine, (forming the Schiff base) and the backbone amides of the two “X” residues in the conserved GXXGE motif (interacting with the carboxylate group). These same residues for NAL are also shown (right panel, Scheme 10). The secondary group of residues in the NAL sub-family consists of the active site residues associated with binding the remaining portion of substrate and the catalytic steps associated with each individual enzyme of the sub-family. For NahE and PhdJ, the residues in this secondary group (binding of the aromatic portion and hydration reaction) are not known. However, it has been suggested that two inserts in the NahE sequence (Thr-267 to Arg-271 and Ala-287 to Gly-293) and one equivalent to the latter in the PhdJ sequence (Ala-283 to Gly-289) might be involved in the mechanism (Barbosa et al., 2000). The corresponding structural elements for these inserts are shown in yellow in Figs. 1A and 2A. Roles for these elements and other active site residues are under investigation.

Characterization of PhdG

We finally turned to the characterization of PhdG, which is also a Class I aldolase in the NAL sub-family of enzymes. It has the conserved lysine (Lys-180) and tyrosine (Tyr-152),
along with the GXXGE motif where Ser-62 and Ala-63 are the two "X" residues. Because the substrate (i.e., 19) is not available, the reverse reaction was followed using pyruvate and 1-hydroxy-2-naphthaldehyde (20) (Scheme 8). There was no change in the absorbance (at \(\lambda_{\text{max}} \sim 400\) nm) suggesting that the enzyme is not catalyzing the formation of 19 from the two products. This observation prompted us to examine the proposed phenanthrene catabolic pathway more closely. The substrate for PhdG (i.e., 19) is allegedly generated in a two-step process from the 3,4-dihydroxyphenanthrene (22, Scheme 11). Ring opening (catalyzed by PhdF) is proposed to generate a chemically reactive dienol, 23, which undergoes a rearrangement to produce 24. Isomerization then yields 19. These compounds have not been synthesized or characterized and their reactivity in solution is unknown. One possibility is that 23 rearranges to 19 or exists in equilibrium with 19. To address these questions, we have synthesized 22 and are currently using PhdF to generate sufficient quantities of ring-opened product, 23 (or 24), for \(^1\)H NMR analysis. With sufficient quantities of the ring-opened product in hand, its solution chemistry will be examined.

Along with these experiments, we looked more closely at the evidence for the functional assignment of PhdF. The assignment was based on the 65% sequence identity observed with 2,3-dihydroxybiphenyl dioxygenases from \textit{Rhodococcus rhodochrous} and \textit{R. sp} HA99 (Stingley et al., 2004). In addition, all the residues required for 2,3-dihydroxybiphenyl dioxygenase activity are present. Consistent with these observations, we determined that PhdF functions as a 2,3-dihydroxybiphenyl dioxygenase based on spectral changes (increases in absorbance at 346 nm and 434 nm) and the isolation and tentative characterization of the ring-opened product (25 in Scheme 12) (Seah et al., 2000). This compound will likely tautomerize to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (26), which is a substrate for a so-called meta-cleavage product (MCP) hydrolase (Ruzzini et al., 2013). (The tautomerization of 25 to 26 might also be catalyzed by the MCP hydrolase.) Hydrolysis of the C–C bond is initiated by attack of water
Bacterial catabolism of polycyclic aromatic hydrocarbons

or a nucleophile at C-6. The products are benzoate and 2-hydroxy-2,4-pentadienoate.

However, PhdF does process 3,4-dihydroxyphenanthrene (22) as well, but the product(s) have not been identified. The supporting evidence consists of spectral changes (decreases in absorbance at 246 nm and 279 nm and an increase in absorbance at 305 nm). The changes in absorbance presumably correspond to ring-opened compounds, but this has not been confirmed. Interestingly, PhdG processes something in the incubation mixture containing 22 and PhdF, based on an increase in absorbance at 387 nm. The identification of this compound is under investigation.

Conclusion

Threehydratase-aldolases involved in PAH degradation have been examined. All are Class I aldolases in the NAL sub-family. Hence, all will proceed by a Schiff base mechanism, which is supported by inactivation experiments (NahE, PhdJ, and PhdG), structural analysis (NahE and PhdJ), and mutagenesis (NahE). The active site residues involved in Schiff base formation (lysine and tyrosine) and the binding of the pyruvolyl moiety (the backbone amides of the two "X" residues in a conserved GXXGE motif) have been tentatively identified. These residues are considered the primary group of residues in the NAL sub-family. The secondary group of residues will distinguish the hydratase-aldolases from the other NAL sub-family members. These residues are involved in the binding of the remaining portion of substrate and the addition of water. Efforts to identify these residues are ongoing.

Conflict of interest

The authors declare that there is no conflict of interest.

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