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Genetic Characterization of the Phenylacetyl-Coenzyme A Oxygenase from the Aerobic Phenylacetic Acid Degradation Pathway of *Escherichia coli*▽

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We show here that the *paaABCDE* genes of the *paa* cluster responsible for phenylacetate degradation in *Escherichia coli* W encode a five-component oxygenase that hydroxylates phenylacetyl-coenzyme A (CoA), the first intermediate of the pathway. The primary structure of the subunits of bacterial phenylacetyl-CoA oxygenases revealed that these enzymes constitute the prototype of a new and distinct group of the large bacterial diiron multicomponent oxygenase family.

The *paa* pathway is the core of the phenylacetyl-coenzyme A (PhAc-CoA) catabolon, a functional unit that integrates several peripheral catabolic pathways and that has been described in many bacteria (1, 2, 5, 12–15, 17). The gene cluster involved in phenylacetyl acid (PhAc) degradation in *Escherichia coli* W contains 14 genes organized in two divergent catabolic operons, *paaABCDEFGHIJK* and *paaZ*, and a regulatory operon, *paaXY*, in which PaaX acts as a transcriptional repressor that becomes inactivated by PhAc-CoA (2, 4, 5). A catabolic scheme where PhAc-CoA undergoes ring hydroxylation, ring opening, and further β-oxidation-type degradation through a proposed pathway that involves CoA thioesters and converges with the classical β-ketoadipate pathway at the β-ketoadipyl-CoA intermediate has been suggested (9). Based on sequence homology, it had been proposed that the *paaABDCE* genes might encode a ring hydroxylation system (5). Moreover, it had been also shown that when these genes were cloned together with *paaK*, which encodes a PhAc-CoA ligase, PhAc could be transformed in vivo into 2-hydroxy-PhAc (2OHPhAc) (5), but a precise demonstration that all these genes are required for ring hydroxylation remained to be presented. It is worth noting that 2OHPhAc is not a true intermediate in the PhAc catabolic pathway, and the 1,2-dihydroxy-1,2-dihydroPhAc-CoA has been proposed as the real product of the hydroxylation reaction (9), with 2OHPhAc being the result of a dehydratation of the dihydrodiol-CoA intermediate (5, 9, 12, 17).

The *paaABCDE* gene products are involved in aromatic ring hydroxylation. To determine if all the *paaABCDE* gene products are involved in the PhAc-CoA ring hydroxylation complex, we used a genetic approach based on two compatible plasmids, pAFK3 (and its derivatives) and pFBP (and its derivatives) (see Fig. 1 for plasmid maps). In brief, plasmid pAFK3 is a pSJ19Not derivative (5) containing the *paaK* gene that encodes the PhAc-CoA ligase and the *paaXY* genes encoding the system that represses the *Pa* promoter, which controls the expression of the *paaABCDEFGHIJK* catabolic operon. On the other hand, plasmid pFBP is a pUC18 derivative that harbors the *paaABCDE* genes encoding the putative PhAc-CoA hydroxylase activity under the control of the *Pa* promoter. When cells of *Escherichia coli* W14 (Δ*paa*), an *E. coli* W derivative strain lacking the *paa* genes (5), harboring plasmids pAFK3 and pFBP were cultured in M63 minimal medium (18) with glycerol as the sole carbon and energy source, plus PhAc as inducer of the *Pa* promoter and substrate of the hydroxylation complex, we observed that PhAc was converted to 2OHPhAc, which was secreted into the culture medium (Table 1). Interestingly, when the same experiment was performed with *E. coli* W14(pFBP), i.e., in the absence of plasmid pAFK3 expressing the PhAc-CoA ligase, we could not observe 2OHPhAc in the culture medium (Table 1), confirming that the synthesis of PhAc-CoA is mandatory to generate the true substrate of the hydroxylation complex encoded by all or some of the *paaABCDE* genes (5).

We then determined whether all five of the *paaABCDE* genes were required for the PhAc-CoA ring hydroxylation activity by constructing five pFBP derivative plasmids carrying individual deletions in each gene (Fig. 1A). These plasmids were used to transform *E. coli* W14(pAFK3), and the resulting recombinants were tested for the production of 2OHPhAc as described above. The *E. coli* W14(pAFK3) cells transformed with plasmid pAFDA (Δ*paaA paaBCDE*), pAFDB (*paaA* Δ*paaB paaCDE*), pAFDC (*paaAB* Δ*paaC paaDE*), or pAFDD (*paaABC* Δ*paaD paaE*) were unable to convert PhAc into 2OHPhAc (Table 1). However, cells harboring plasmid pAFDE (*paaABCD* Δ*paaE*) showed a low but detectable hydroxylase activity (Table 1). Nevertheless, to eliminate any polar effect of the mutations generated in the five pFBP derivative plasmids and, thus, to confirm that the only gene affected in the mutant plasmids was the corresponding deleted gene, we constructed five pAFK3 derivatives carrying, in addition to the *paaK* and *paaXY* genes,

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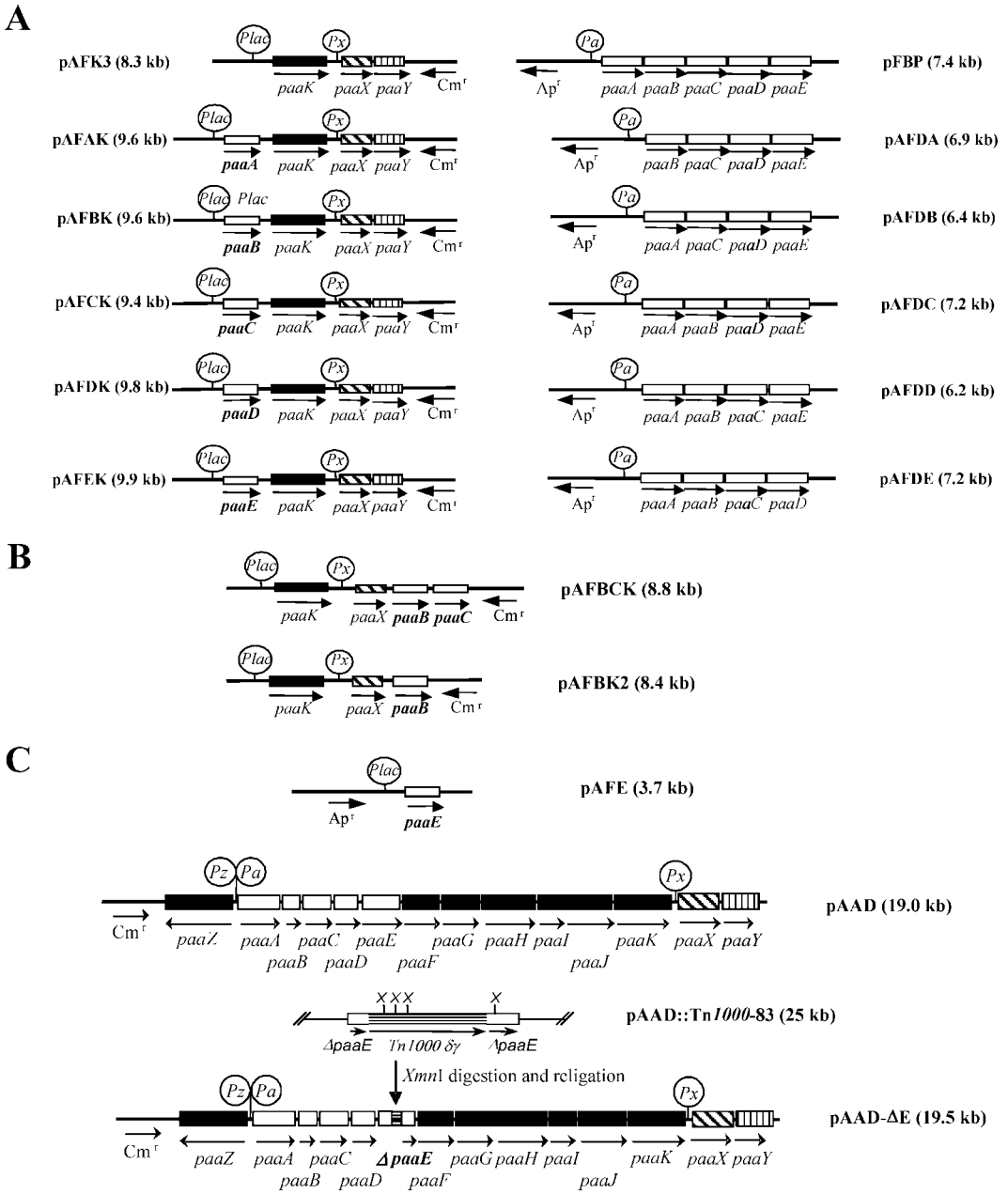

FIG. 1. Genetic constructions used in this work. (A) Maps of plasmids pAFK3 and pFBP and their derivatives. (B) Maps of plasmids pAFBCK and pAFBK2. (C) Maps of plasmids pAFE, pAAD, and pAAD-ΔE. Arrows indicate the direction of gene transcription. Plasmid pAAD-ΔE was constructed by XmnI (X) digestion and religation of plasmid pAAD::Tn*1000*-83 (5), which eliminates most of the Tn*1000* transposon and part of the *paaE* gene. Plasmids pAAD, pCK01, pUC18, and pAFK3 (a pSJ19Not derivative) have been previously described (5). Plasmids of the pAFD(A-E) series, pFBP, and pFBE are pUC18 derivatives. Plasmids of the pAF(A-E)K series are pAFK3 derivatives. Plasmids pAFBCK and pAFBK2 are pCK01 derivatives. Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance. *Plac*, *lac* promoter. *Pa*, *Px*, and *Pz* are the promoters of the *E. coli paa* cluster.

each one of the five *paaABCDE* genes expressed under control of the P_{lac} promoter (Fig. 1A). When *E. coli* W14 cells were transformed with plasmid pAFDA, pAFDB, pAFDC, pAFDD, or pAFDE and the corresponding pAFK3 derivative, the resulting double transformants, with the sole exception of that harboring plasmid pAFBK, which contains the *paaB* gene, acquired the ability to efficiently hydroxylate PhAc-CoA with the concomitant release of 2OHPhAc into the culture medium (Table 1). A possible explanation for the lack of complementation of plasmid pAFDB (*paaA* Δ*paaB paaCDE*) by the *paaB* gene expressed in *trans* from plasmid pAFBK could be that deletion of *paaB* might affect the translation of the downstream *paaC* gene. This assumption was based on the observation that the *paaC* gene has no consensus ribosome binding site (5), which might explain why the PaaC protein requires a cotranslation mechanism to be efficiently synthesized. To test this hypothesis, we constructed two similar

TABLE 1. Production of 2OHPhAc by recombinant *E. coli* W14 cells

Plasmid(s) carried by *E. coli* W14 recombinants	Genes	2OHPhAc (μM)[a] production
pFBP	*paaABCDE*	<1
pFBP, pAFK3	*paaABCDE*, *paaKXY*	700
pAFDA, pAFK3	*paaBCDE*, *paaKXY*	<1
pAFDB, pAFK3	*paaACDE*, *paaKXY*	<1
pAFDC, pAFK3	*paaABDE*, *paaKXY*	<1
pAFDD, pAFK3	*paaABCE*, *paaKXY*	<1
pAFDE, pAFK3	*paaABCD*, *paaKXY*	20
pAFDA, pAFAK	*paaBCDE*, *paaAKXY*	60
pAFDB, pAFBK	*paaACDE*, *paaBKXY*	<1
pAFDC, pAFCK	*paaABDE, paaCKXY*	260
pAFDD, pAFDK	*paaABCE*, *paaDKXY*	210
pAFDE, pAFEK	*paaABCD*, *paaEKXY*	150
pAFDB, pAFBCK	*paaACDE*, *paaKXBC*	240
pAFDB, pAFBK2	*paaACDE*, *paaKXB*	<1

[a] Cells were cultured for 24 h at 37°C in M63 minimal medium containing 50 mM glycerol and 1 mM PhAc. The amount of 2OHPhAc was determined by using the colorimetric Gibbs assay (6) in the linear range of the reaction (A_{610} = 1 corresponds to 30 μM 2OHPhAc). The identification of 2OHPhAc as the reactive compound in the Gibbs assay was preformed by high-performance liquid chromatography and gas chromatograph-mass spectrometry as previously described (5). Data are the means of three independent experiments, with standard deviations of <10%.

plasmids expressing either the *paaB* and *paaC* genes from the same promoter (pAFBCK) or just the *paaB* gene (pAFBK2) (Fig. 1B). Whereas *E. coli* W14(pAFDB, pAFBCK) coexpressing the *paaB* and *paaC* genes was able to convert PhAc into 2OHPhAc, *E. coli* W14(pAFDB, pAFBK2) expressing only the *paaB* gene did not transform PhAc (Table 1), strongly suggesting that PaaC must be cotranslated with PaaB. It is worth noting here that the pAFCK plasmid that expresses the *paaC* gene alone is able to complement *E. coli* W14(pAFDC) lacking the *paaC* gene, because in plasmid pAFCK the PaaC protein is cotranslated with the N-terminal region of the LacZ protein.

The observation that 2OHPhAc was formed in *E. coli* W14 containing the cloned *paaABCD* genes in the absence of *paaE* (Table 1) prompted us to study whether PaaE is not strictly required for the hydroxylation of PhAc-CoA in our recombinant (multicopy) system or, on the contrary, whether its function could be replaced by a different *E. coli* gene product. Thus, when we compared the high hydroxylation activity observed in *E. coli* W14(pAFDE, pAFEK), a strain that expresses the cloned *paaE* gene from plasmid pAFEK, with the low activity detected in *E. coli* W14(pAFDE, pAFK3), a strain that does not contain the *paaE* gene (Table 1), we could conclude that PaaE was indeed essential for efficient hydroxylation of PhAc-CoA. To study whether the activity that accounts for the 2OHPhAc formation in our recombinant system could also support the growth in PhAc of an *E. coli* strain lacking *paaE*, we constructed plasmid pAAD-ΔE, which harbors the complete set of *paa* genes with the sole exception of a disrupted *paaE* gene (*paaZ paaABCD* Δ*paaE paaFGHIJK paaXY*) (Fig. 1C). Whereas *E. coli* W14 containing plasmid pAAD, which harbors a wild-type *paa* cluster (5), was able to grow in M63 minimal medium using PhAc (5 mM) as the sole carbon and energy source, the recombinant strain *E. coli* W14(pAAD-ΔE) did not grow. To rule out the possibility that the deletion of the *paaE* gene in plasmid pAAD-ΔE could cause polar effects on the expression of other *paa* genes, the recombinant *E. coli* W14(pAAD-ΔE) strain was transformed with plasmid pAFE, a pUC18 derivative which expresses the *paaE* gene under control of the P_{lac} promoter (Fig. 1C). As expected, *E. coli* W14(pAAD-ΔE, pAFE) grew in M63 minimal medium using PhAc (5 mM) as the sole carbon and energy source. All these results taken together demonstrate that the *paaE* gene is essential for growth of *E. coli* with PhAc as well as for efficient hydroxylase activity when the *paaABCD* genes are expressed from a multicopy plasmid.

Evolutionary considerations on the PhAc-CoA oxygenase. So far, only three ring hydroxylation oxygenases acting on aromatic CoA-acylated compounds have been described in the literature (7, 8, 19), but the PhAc-CoA oxygenase differs from these, since it is the first reported multicomponent enzyme system that hydroxylates the aromatic ring of a CoA-thioester.

Remarkably, the *paaABCD* gene organization is conserved in all bacteria possessing the *paa*-encoded PhAc degradation pathway (1, 2, 12, 14, 15) (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). However, although *paaE* is usually found adjacent to the *paaABCD* genes in the *paa* clusters of many bacteria, a *paaE* homologue is lacking in *paa* clusters from ancestral bacterial groups such as the *Firmicutes* group and the *Thermus*/*Deinococcus* group. This finding suggests that the *paaE* gene has been acquired during the evolution of the *paa* clusters by actinobacteria and gram-negative bacteria to guarantee an efficient hydroxylation of PhAc-CoA (see above).

Sequence comparisons of the *paaABCDE* gene products strongly suggest that the PhAc-CoA oxygenase belongs to the bacterial diiron multicomponent oxygenases (BMOs) family, which can be divided into five groups (11, 16). All these enzymes contain a heteromultimeric $(\alpha\beta)_2$ (groups 4 and 5) or $(\alpha\beta\gamma)_2$ (groups 1, 2, and 3) oxygenase component that is responsible for the hydroxylation activity. The evolutionary fingerprint of the different groups of BMOs is also based on the arrangement of the coding sequences within the corresponding operons (Fig. 2A) (11, 16). Sequence comparisons suggest that PaaACD might constitute the α, β, and γ subunits of the heteromultimeric diiron oxygenase component of the PhAc-CoA oxygenase. PaaB and PaaE can be the effector protein and the oxidoreductase, respectively, that mediate electron transfer from NAD(P)H. Although a similar five-subunit composition has been reported for the group 3 BMOs, the order of the genes encoding the five subunits of methane monooxygenases in the *mmo* operon (*mmoXYBZC*) differs from that of the *paaABCDE* genes (Fig. 2A).

A detailed amino acid sequence analysis of each of the five subunits of the PaaABCDE oxygenase from different bacteria (see above) revealed that the equivalent subunits from different organisms showed an extensive conservation of residues (data not shown). This observation is in agreement with the fact that PhAc-CoA oxygenases share a common ancestor, and they constitute a widespread new enzymatic system to hydroxylate PhAc-CoA in bacteria. However, the primary structure of the PaaABCDE subunits clearly differs from that of equivalent subunits of previously reported BMOs. Thus, the α subunits (PaaA proteins) of PhAc-CoA oxygenases show a protein length, around 310 to 320 amino acids, that is significantly shorter than that of the α subunits, around 500 amino acids, from other BMOs (Fig. 2A). Whereas α subunits of most

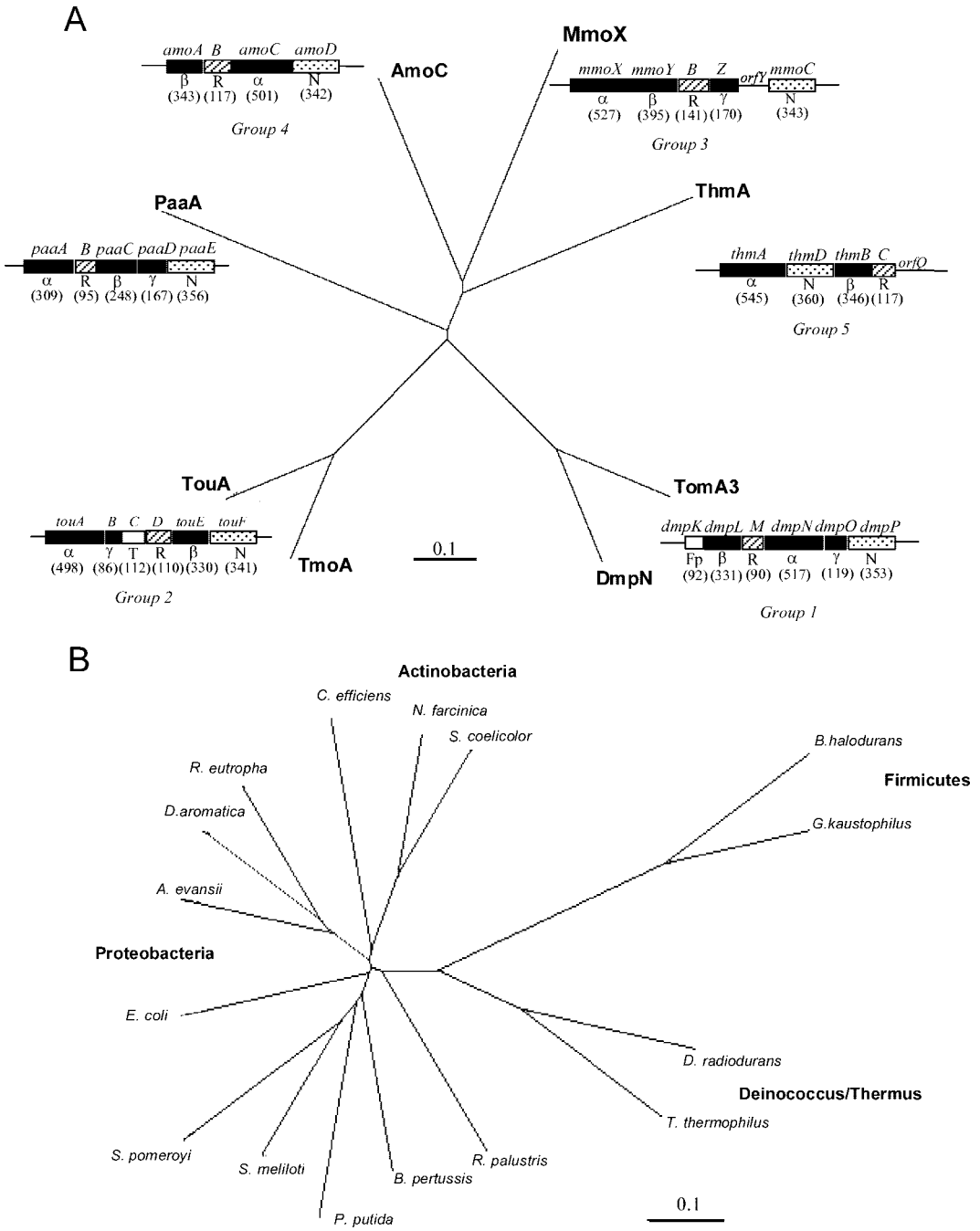

FIG. 2. Phylogenetic trees of the α subunits of BMOs. The bar represents 1 inferred amino acid substitution per 10 amino acids. Phylogenetic analyses were carried out according to the two-parameter method of Kimura (10), and a tree was reconstructed using the neighbor-joining method of the Phylip program (3). (A) Phylogenetic tree of the α subunits of representative members of different groups of BMOs. A schematic representation of the gene arrangement for the different groups of BMOs is also shown. Genes encoding the α, β, and γ subunits of the oxygenase component are indicated with black blocks. The genes encoding the reductase component (N) are indicated with stippled blocks. The genes encoding the effector protein (R) are indicated with hatched blocks. The genes encoding the Fp and T proteins in BMOs of groups 1 and 2, respectively, are shown with empty blocks. The amino acid lengths of the subunits are indicated in brackets below the corresponding proteins. The gene clusters shown are the following (GenBank accession numbers are in parentheses): *dmpKLMNOP* from *Pseudomonas* sp. strain CF600 (M60276), *touABCDEF* from *P. stutzeri* OX1 (AY621080), *mmoXYBZorfYmmoC* from *Methylococcus capsulatus* (M90050), *amoABCD* from *Nocardia coralline* B-276 (D37875), *thmADBCorfQ* from *Pseudonocardia* sp. strain K1 (AJ296087), and *paaABCDE* from *E. coli* W (X97452). The α subunits used for the construction of the phylogenetic tree are the following: DmpN (P19732) and TomA3 (AAL50373) of the phenol monooxygenase and toluene 2-monooxygenase from *Pseudomonas* sp. strain CF600 and *Burkholderia cepacia* G4, respectively; TouA (CAA06654) and TmoA (AAS66660) of the toluene/*o*-xylene monooxygenase and the toluene 4-monooxygenase from *P. stutzeri* OX1 and *P. mendocina* KR1, respectively; MmoX (AAB62392), of the methane monooxygenase from *M. capsulatus*; AmoC (BAA07114) of the alkene monooxygenase from *N. coralline* B-276; ThmA (CAC10506) of the tetrahydrofuran monooxygenase from *Pseudonocardia* sp. strain K1; and PaaA (CAA66090) of the phenylacetyl-CoA oxygenase from *E. coli* W. (B) Phylogenetic tree of the PaaA subunits of phenylacetyl-CoA oxygenases from *E. coli* (CAA66090), *P. putida* (AAC24334), *Bordetella pertussis* (NP_881296), *Azoarcus evansii* (AAG28968), *Dechloromonas aromatica* (YP_283606), *Ralstonia eutropha* (YP_297410), *Rhodopseudomonas palustris* (NP_949105), *Sinorhizobium meliloti* (CAC49959), *Silicibacter pomeroyi* (YP_283606), *Bacillus halodurans* (NP_241062), *Geobacillus kaustophilus* (YP_147897), *Streptomyces coelicolor* (NP_631518), *Corynebacterium efficiens* (NP_737277), *Nocardia farcinica* (YP_118371), *Thermus thermophilus* (YP_004583), and *Deinococcus radiodurans* (AAF11931).

BMOs show the consensus patterns **E**X_2AX_{25}D**E**XR**H** and **E**X_{18}GDX_8SX_3D**E**XR**H** (iron cluster ligands are in boldface; an X means any residue) for Fe1 and Fe2 binding (11), the PaaA subunits show different consensus patterns, **E**X_2GX_{25} D**E**XG**H** and **D**X_{13}SYX_8RX_3E**E**XF**H**, respectively, although they still maintain the conserved **E**X_2**H** motif. Interestingly, the C-terminal end of the α subunits of most BMOs is lacking in the PaaA proteins. A phylogenetic analysis based on the amino acid sequences of the α subunits from different BMOs reveals that PaaA proteins constitute a different branch of the phylogenetic tree (Fig. 2A).

The suggested translational coupling of PaaC with the PaaB protein in *E. coli* (see above) is in agreement with the predicted essential role of PaaB on the activity of the PaaACD oxygenase component. Unlike the oxygenase component, the oxidoreductase subunit of BMOs is closely related to isofunctional subunits of Rieske-type oxygenases (11). Thus, the oxidoreductase subunit comprises an N-terminal plant-type ferredoxin domain and a C-terminal reductase domain with flavin adenine dinucleotide- and NAD(P)-ribose binding regions, which corresponds to the molecular architecture of the class IB reductases according to the Batie classification system (11, 16). Interestingly, the predicted oxidoreductase subunit of the PhAc-CoA oxygenases (PaaE proteins) belongs to class IA reductases, since it has an N-terminal reductase domain and a C-terminal plant-type ferredoxin domain (5). Therefore, whereas BMOs reported so far have recruited class IB reductases, the PhAc-CoA multicomponent oxygenase has recruited a class IA oxidoreductase, suggesting a distinct evolutionary history for the latter.

Conclusions. The data presented here suggest that the PhAc-CoA oxygenase constitutes the first member of a new and distinct group of BMOs. The phylogenetic tree of the α subunits of PhAc-CoA oxygenases from different bacteria reveals the existence of three major branches, i.e., *Deinococcus*/*Thermus*, *Firmicutes*, and *Proteobacteria*/*Actinobacteria* (Fig. 2B). The same phylogenetic distribution can be extended to the other subunits of the PhAc-CoA oxygenases (data not shown). This observation, together with the facts that the *paaABCDE* genes are chromosomally located and have an average G+C content that corresponds with that of the host bacterium, suggest that the genes encoding the multicomponent PhAc-CoA oxygenase have been the subject of vertical rather than horizontal transmission. The hydroxylation of PhAc-CoA by a five-subunit oxygenase appears to be a general principle in bacteria that degrade PhAc and/or some other aromatics of the PhAc-CoA catabolon, since alternative PhAc degradation pathways have not been reported so far. The PhAc-CoA oxygenase constitutes, therefore, the first reported bacterial multicomponent oxygenase acting on a CoA-activated aromatic compound. Interestingly, although all BMOs described so far are monooxygenases, the proposed product of the reaction catalyzed by the PhAc-CoA oxygenase is a dihydrodiol, and therefore this enzyme could be a hydroxylating dioxygenase (9). Nevertheless, the characterization of the reaction product and whether the PhAc-CoA oxygenase is a monooxygenase or a dioxygenase will require further research.

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