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All biological hydroxylation reactions are thought to derive the oxygen atom from one of three inorganic oxygen donors, O_2, H_2O_2, or H_2O. Here, we have identified the organic compound prephenate as the oxygen donor for the three hydroxylation steps of the O_2-independent biosynthetic pathway of ubiquinone, a widely distributed lipid coenzyme. Prephenate is an intermediate in the aromatic amino acid pathway and genetic experiments showed that it is essential for ubiquinone biosynthesis in *Escherichia coli* under anaerobic conditions. Metabolic labeling experiments with ^{18}O-shikimate, a precursor of prephenate, demonstrated the incorporation of ^{18}O atoms into ubiquinone. The role of specific iron–sulfur enzymes belonging to the widespread U32 protein family is discussed. Prephenate-dependent hydroxylation reactions represent a unique biochemical strategy for adaptation to anaerobic environments.

hydroxylation | anaerobiosis | prephenate | ubiquinone | U32 proteins

Significance

Microorganisms colonize anoxic ecological niches and use water as an oxygen donor for hydroxylation reactions in such environments. Here, we identify a unique source of oxygen, namely the metabolite prephenate, which enables the anaerobic biosynthesis of ubiquinone, a lipid important for bacterial bioenergetics. We predict that prephenate is involved as an oxygen donor in several biological pathways, including RNA modification. This study opens prospects for deciphering anaerobic metabolism and designing antimicrobial agents.

Hydroxylation reactions are involved in the biosynthesis, degradation, and modulation of the biological activity of a wide variety of molecules, by introducing a single oxygen (O) atom into a carbon–hydrogen bond. The enzymes that catalyze hydroxylation reactions, collectively referred to as hydroxylases, typically derive the added O atom from O_2 or its reduction product H_2O_2 (1, 2), and therefore require aerobic conditions to function. Enzymes such as flavin monooxygenases or heme/nonheme monooxygenases form large families of O_2-dependent hydroxylases that have been studied for decades (3–7). In anaerobic environments, H_2O is currently the only known O-donor for biological hydroxylation reactions. H_2O-dependent hydroxylases contain a molybdenum cofactor and represent a diverse family with well-known members like xanthine oxidase, resorcinol hydroxylase, or ethylbenzene dehydrogenase (8, 9).

Ubiquinone (UQ) is a redox lipid that contributes to the function of energy-producing respiratory chains in both aerobiosis and anaerobiosis (10–12). The biosynthesis of UQ requires three hydroxylation steps, which are catalyzed by flavin-dependent hydroxylases (UbiI, UbiH, and UbiF in *Escherichia coli*) under aerobic conditions, using O_2 as the O-donor (13) (Fig. 1). Under anaerobiosis, the iron–sulfur (Fe–S) proteins UbiU and UbiV are essential for UQ biosynthesis, but the O-donor used remains unknown (Fig. 1) (14, 15). UbiU-V are widely distributed in Proteobacteria and belong to the U32 peptidase family. Most of the twelve subfamilies of U32 proteins remain uncharacterized, but two have been linked to O_2-independent hydroxylation reactions of RNA molecules (16–18), raising the possibility of a common hydroxylation function with an as yet unidentified O-donor.

Here, we show, using genetics and isotopic labeling, that prephenate, a molecule derived from the shikimate pathway, is the O-donor for the hydroxylation reactions of the UQ biosynthetic pathway operating under anaerobic conditions. We propose a prephenate-dependent hydroxylation chemistry that is likely shared by U32 proteins.

Results

The *aro* Pathway is Required for the Biosynthesis of UQ in Anaerobic Conditions. Preliminary evidence indicated that the aromatic amino acids (*aro*) pathway is involved in O_2-independent UQ biosynthesis in *E. coli* (14). When grown anaerobically in an LB medium containing aromatic amino acids, Δ*aro* mutants showed a strong decrease in the level of UQ (Fig. 2 *A* and *B*), whereas the latter remained unaffected in cells grown aerobically (*SI Appendix*, Fig. S1*A*). Furthermore, the anaerobically grown Δ*aro* mutants accumulated octaprenyl-phenol (OPP) (Fig. 2*C*), a UQ pathway intermediate downstream of 4-hydroxybenzoic acid (4-HB) (Fig. 1), suggesting that the UQ deficiency was independent of 4-HB. Accordingly, the addition of 4-HB to cultures of Δ*aro* mutants did not increase their UQ levels (*SI Appendix*, Fig. S1*B*).

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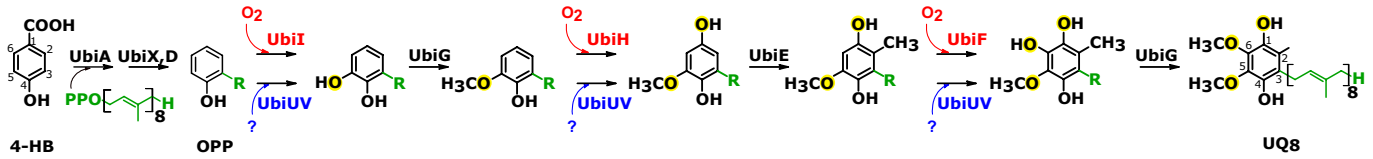

Fig. 1. Aerobic and anaerobic pathways of UQ_8 biosynthesis in *E. coli*. The Ubi-enzymes common to both pathways are in black, those specific to the O_2-dependent pathway are shown in red, and those specific to the O_2-independent pathway are shown in blue (? for the unknown O- donor). The O atoms derived from the three hydroxylation steps are highlighted in yellow. R, octaprenyl chain shown in green in the UQ_8 structure; 4-HB, 4-hydroxybenzoic acid; OPP, 3-octaprenylphenol; UQ_8, ubiquinone-8.

The Δ*aro* mutants were also deficient in demethylmenaquinone and menaquinone, but this was true under both anaerobic and aerobic conditions (*SI Appendix*, Fig. S1 *C* and *D*). This is likely due to a deficiency in the isochorismate precursor, which is derived directly from chorismate in the *aro* pathway (Fig. 2*D*). Overall, this genetic analysis shows that preventing the synthesis of chorismate affects the biosynthesis of UQ under anaerobic conditions at a step downstream of OPP (Fig. 1).

Prephenate Is Essential for the Biosynthesis of UQ under Anaerobic Conditions. Single deletions of genes located downstream of chorismate in the *aro* pathway had no effect on UQ levels (Fig. 3*A*). In contrast, combined inactivation of *pheA* and *tyrA* resulted in a severe UQ deficiency under anaerobic conditions (Fig. 3*A*) associated with increased OPP levels (*SI Appendix*, Fig. S2*A*), while UQ levels were normal under aerobic conditions (*SI Appendix*, Fig. S2*B*). Expression

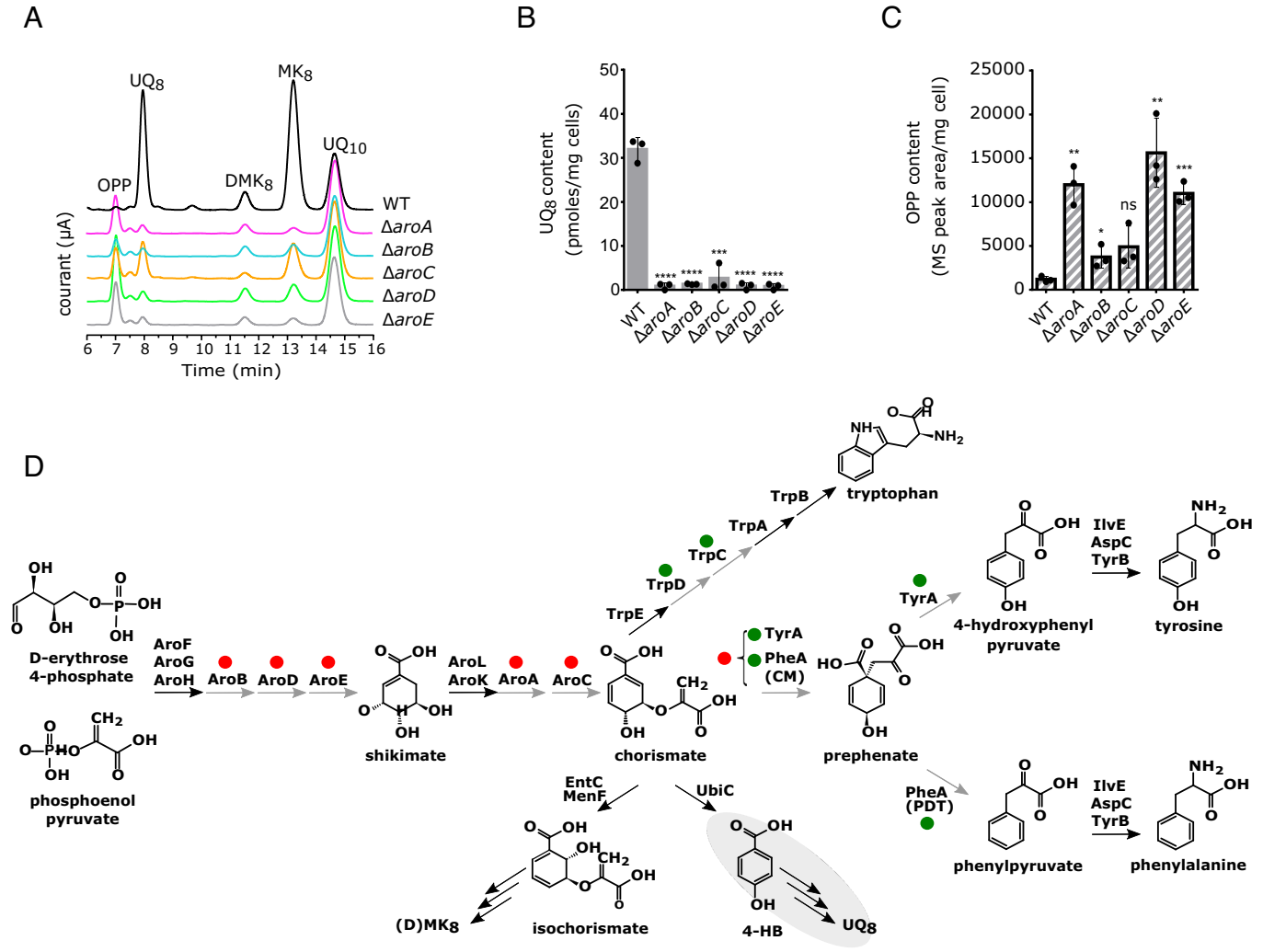

Fig. 2. O_2-independent biosynthesis of ubiquinone depends on the *aro* pathway in *E. coli*. (*A*) High Perfomance Liquid Chromatography-Electrochemical Detection (HPLC-ECD) analysis of lipid extracts from *E. coli* Δ*aro* strains grown anaerobically in LB medium, UQ_{10} added as internal standard. UQ_8 (*B*) and OPP (*C*) content in the strains analyzed in *A*. Mean ± SD (n = 3). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by unpaired Student's *t* test comparing to WT. ns, not significant. (*D*) Metabolic pathway of aromatic amino acids in *E. coli*, modified from ref. 17. The chorismate mutase (CM) and prephenate dehydratase (PDT) domains of PheA convert chorismate to prephenate and prephenate to phenylpyruvate, respectively (19). The black and gray arrows indicate the mutants not tested or tested in this study. The red and green disks indicate the proteins dispensable or essential for UQ_8 biosynthesis under anaerobic conditions, respectively. The Δ*pheA* and Δ*tyrA* strains are not deficient for UQ_8 but the double-mutant Δ*pheA* Δ*tyrA* is. The UQ pathway is highlighted in gray, and (D)MK_8 corresponds to the (demethyl) menaquinone pathway.

of the chorismate mutase (CM) domain of PheA (PheA_CM) complemented the deficiency (Fig. 3*B*), supporting that prephenate, the product of PheA_CM (Fig. 2*D*), is required for the O_2-independent biosynthesis of UQ. Accordingly, the addition of prephenate to cultures of the Δ*pheA* Δ*tyrA* strain increased UQ levels (Fig. 3*C*). We observed a residual level of UQ in the Δ*pheA* Δ*tyrA* strain, which was abolished upon inactivation of the O_2-dependent hydroxylases, UbiI and UbiH (Fig. 3*C*). The residual level of UQ was therefore attributed to the activity of the O_2-dependent hydroxylases using trace amounts of O_2 in the anaerobic culture medium, a hypothesis supported by the complete absence of UQ in the Δ*aroD* Δ*ubiIH* mutant (Fig. 3*D*). Importantly, prephenate increased UQ levels in both strains (Fig. 3*D*). Collectively, the data support that prephenate is essential for UQ biosynthesis under anaerobic conditions.

The Hydroxylation Steps Involving UbiU-V Are Dependent on Prephenate. The expression of UbiU-V is tightly regulated by the transcription factor FNR (Fumarate and Nitrate Reductase); as a result, the two proteins are well expressed under anaerobiosis but very poorly expressed under aerobiosis (15). When expressed under aerobic conditions thanks to a constitutive promoter, UbiU-V can replace UbiI, UbiH, and UbiF in catalyzing the three hydroxylation reactions necessary for UQ biosynthesis, using an unidentified O-donor (15). In line with previous results, UbiU-V allowed robust aerobic growth of the Δ*ubiIH* strain on succinate medium, which is strictly dependent on UQ biosynthesis (Fig. 3*E*). Consistent with the requirement of the 4Fe–4S cluster of UbiU for activity (15), the C176A mutation, which impairs the binding of the Fe–S cluster, abolished growth on succinate (Fig. 3*E*). Deletion of *aroD* (Fig. 3*E*) or *pheA tyrA* (Fig. 3*F*) abolished the growth conferred by UbiU-V on succinate, whereas anaerobic growth on glucose, which is independent of UQ, was maintained in all cases. The addition of shikimate rescued the growth of the Δ*aroD* Δ*ubiIH* strain expressing UbiU-V (Fig. 3*E*), demonstrating that a metabolite downstream of shikimate in the *aro* pathway is required for UbiU-V function. Furthermore, the expression of PheA_CM in the Δ*pheA* Δ*tyrA* Δ*ubiH* strain restored the function of UbiU-V (Fig. 3*F*). Taken together, these results demonstrate that prephenate is required for the UbiU-V-dependent hydroxylation steps and suggest that prephenate could be the O-donor for the O_2-independent hydroxylation reactions of UQ biosynthesis.

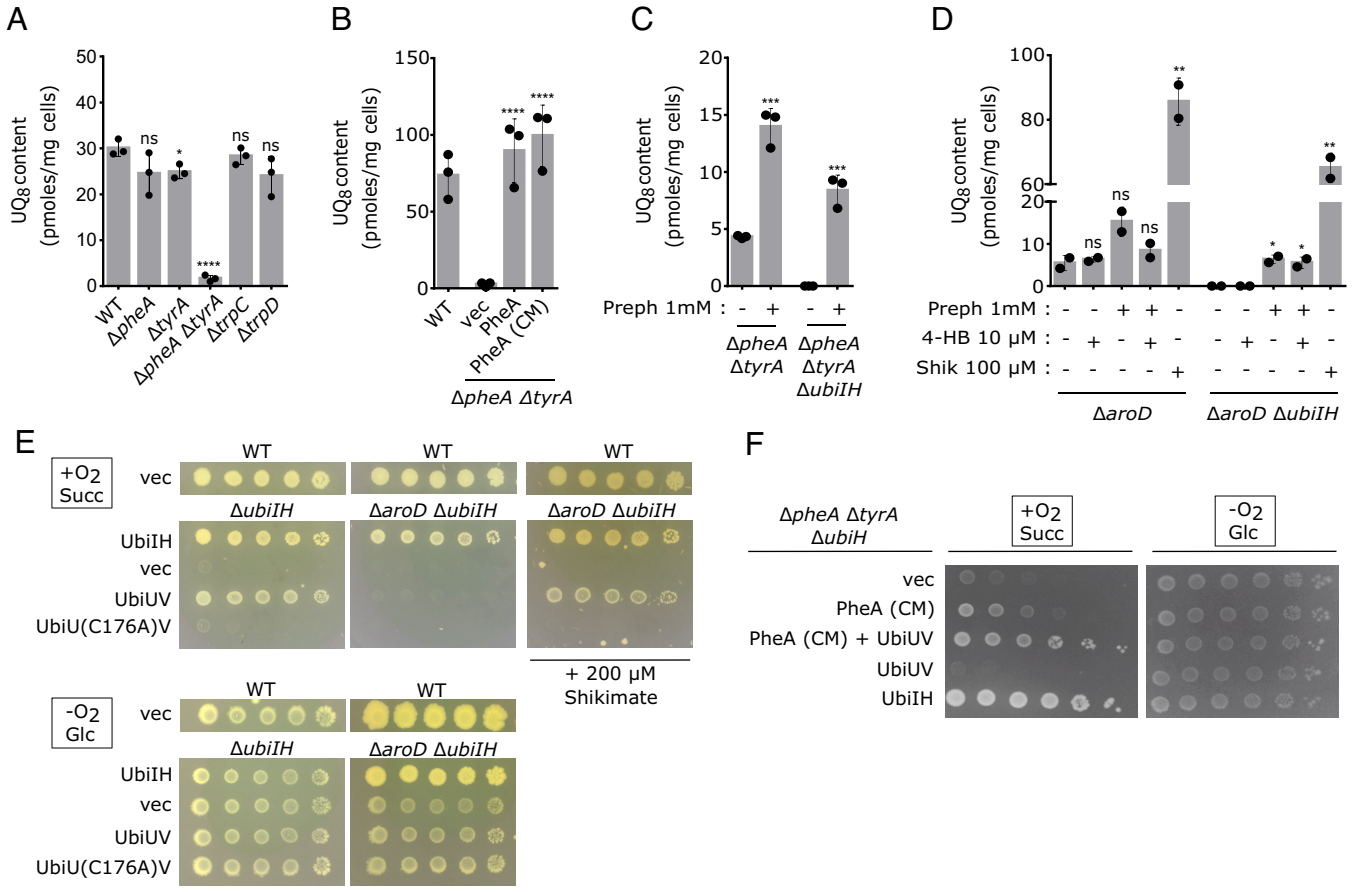

Fig. 3. Prephenate is required for the O_2-independent biosynthesis of ubiquinone. The indicated *E. coli* strains were grown anaerobically in either LB (*A* and *B*) or MOPS (*C* and *D*) media. UQ_8 content in the mutants downstream of chorismate (*A*), in the Δ*pheA* Δ*tyrA* double mutant containing either pTet (vec), pTet-*pheA* (PheA), or pTet-*pheA*-CM [PheA (CM)] (*B*), in the double mutant Δ*pheA* Δ*tyrA* and the quadruple mutant Δ*pheA* Δ*tyrA* Δ*ubiIH* strains supplemented or not with 1mM of prephenate (Preph) (*C*), in the Δ*aroD* and the Δ*aroD* Δ*ubiIH* strains supplemented with 1 mM of prephenate and/or 10 μM 4-HB and/or 100 μM of shikimate (Shik) (*D*). Mean ± SD (n = 3) except for D (n = 2). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by unpaired Student's *t* test comparing to WT for (*A*), to vec for (*B*) and to mutant strains without supplementation for (*C* and *D*). (*E*) Serial dilutions of the mutant strains Δ*ubiH*, or Δ*aroD* Δ*ubiIH* containing either pBAD24 (vec), pES077 (UbiIH), pES154 (UbiUV), or pES185 (UbiU(C176A)V) on M9 succinate plates containing Phe, Tyr, Trp, 0.02% arabinose and supplemented or not with 200 μM shikimate. (*F*) Serial dilutions of the Δ*pheA*Δ*tyrA* Δ*ubiH* strain containing either pTet + pEB067 (vec), pES047 + pEB067 (PheA_CM), pES047 + pES232 (PheA_CM + UbiUV), pTet + pES154 (UbiUV), or pES077 + pEB067 (UbiIH on M9 succinate or glucose plates containing Phe, Tyr and 0.1% arabinose. Incubation at 37 °C was carried out for 48 h under aerobic conditions for succinate plates (+O_2 Succ) or under anaerobic conditions for glucose plates (−O_2 Glc, used as controls) (*E* and *F*). The results are representative of at least two independent experiments (*E* and *F*).

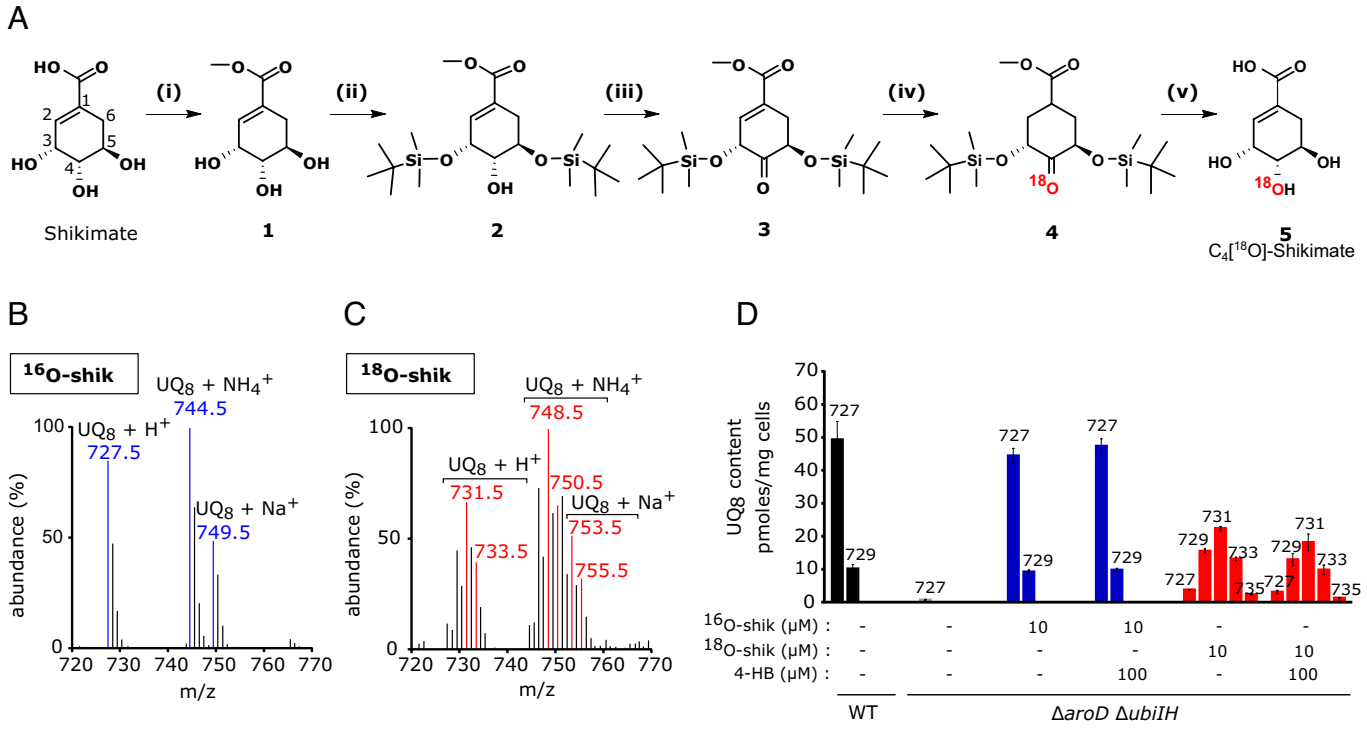

Fig. 4. Addition of $C_4[^{18}O]$-shikimate to the Δ*aroD* strain leads to the biosynthesis of $^{18}O_3$-UQ in anaerobic conditions. (*A*) Synthetic route of ^{18}O-shikimate (3R,4S,5R)-3,4[^{18}O],5-trihydroxy-4-cyclohex-1-ene-1-carboxylic acid. i) APTS, CH_3OH; ii) TBDMSOtf, 2,6-lutidine, CH_2Cl_2; iii) Dess-Martin Periodinane, CH_2Cl_2, rt; iv) $H_2{}^{18}O$, $CH3C[^{18}O]_2H$, THF; v) a) $NaBH_4$, THF/H_2O; b) APTS, THF/H_2O. Mass spectra of UQ_8 from Δ*aroD* Δ*ubiIH* strain grown anaerobically in the presence of 10 µM unlabeled ^{16}O-shikimate (*B*) or $C_4[^{18}O]$-shikimate (*C*). The peaks corresponding to adducts of either unlabeled UQ_8 or UQ_8 labeled with two ^{18}O ($^{18}O_2$-UQ_8) or three ^{18}O ($^{18}O_3$-UQ_8) are represented in blue (*B*) and red (*C*), respectively. Mass spectra representative of three independent experiments. (*D*) Quantification of UQ_8 H^+ adducts (m/z 727 to 735) in WT and Δ*aroD* Δ*ubiIH* cells grown with ^{16}O-shikimate or $C_4[^{18}O]$-shikimate. Mean ± SD (n = 3), see also *SI Appendix*, Fig S3.

Biosynthesis of $^{18}O_3$-UQ from $C_4[^{18}O]$-Shikimate under Anaerobic Conditions. To test whether prephenate is the O-donor of the hydroxylation reactions of the anaerobic UQ pathway, we designed an in vivo labeling experiment using shikimate labeled with ^{18}O at position 4 exclusively, which we termed $C_4[^{18}O]$-shikimate. Shikimate was preferred over prephenate, which has a low bioavailability (20) and is unstable in solution, especially under acidic conditions (21–24). Moreover, the C4-hydroxyl group of shikimate remains intact during in vivo conversion to prephenate (25), and shikimate restored higher UQ levels when added to cultures of the Δ*aroD* strains (Fig. 3*D* and *SI Appendix*, Fig. S2*C*). The synthesis of $C_4[^{18}O]$-shikimate was performed in five steps using commercial shikimate as the starting material (Fig. 4*A* and *SI Appendix*, Fig S4-S22). The general strategy was to protect all reactive functions of the molecule, except the hydroxyl at position 4 to selectively label it with ^{18}O and then remove the protecting groups (Fig. 4*A*). LC–MS analysis of the final compound revealed that 64% of shikimate was labeled with ^{18}O, while 36% remained unlabeled and contained ^{16}O (*SI Appendix*, Fig. S3*A*).

$C_4[^{18}O]$-shikimate and unlabeled shikimate, used as control, were added to anaerobic cultures of the Δ*aroD* Δ*ubiIH* strain and yielded similar levels of UQ (*SI Appendix*, Fig. S3*B*). The mass spectra of UQ differed significantly depending on whether the shikimate added to the cultures was labeled or not (Fig. 4 *B* and *C*). With $C_4[^{18}O]$-shikimate, the most prominent ions corresponding to H^+, NH_4^+, and Na^+ adducts of UQ_8 were predominantly shifted by +4 mass units, and lower peaks at +6 were also detected (Fig. 4 *B* and *C*), indicating the incorporation of two and three ^{18}O atoms, respectively. Quantification of these signals by single-ion monitoring of the H^+ adducts (*SI Appendix*, Fig. S3 *C* and *D*) yielded a relative abundance consistent with a quantitative incorporation of three oxygen atoms from a precursor labeled at 64% with ^{18}O (compare Fig. 4*D* and *SI Appendix*, Fig. S3 *E–G*). Importantly, when 4-HB, which provides the hydroxyl group at position C4 of UQ (Fig. 1), was added together with $C_4[^{18}O]$-shikimate, the labeling of UQ_8 was not affected (compare Fig. 4*C* and *SI Appendix*, Fig. S3*H*). This result established that the ^{18}O labels incorporated from $C_4[^{18}O]$-shikimate are present at the three positions C1, C5,

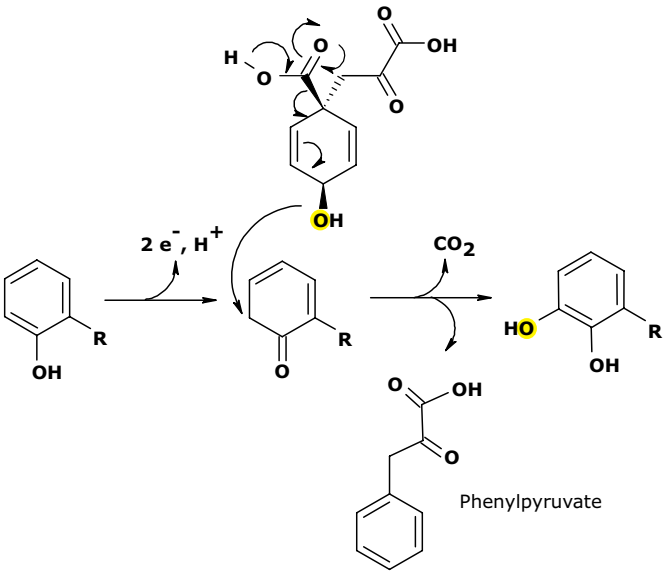

Fig. 5. Proposed molecular mechanism of prephenate-dependent hydroxylation: the reaction exemplifies the first hydroxylation step in the O_2-independent biosynthesis of UQ_8. R, octaprenyl chain.

and C6 of the UQ_8 molecule. Collectively, the data demonstrate that an organic molecule derived from shikimate is the O-donor for the three hydroxylation reactions required to convert OPP into UQ_8 under anaerobic conditions.

Discussion

We have established genetically that prephenate is essential for the biosynthesis of UQ under anaerobic conditions. In addition, isotopic labeling experiments demonstrated that the three O atoms of UQ are derived exclusively from the O atom at position C4 of shikimate, the unique precursor of prephenate. These data show conclusively that the hydroxylation reactions of the O_2-independent pathway leading to the biosynthesis of UQ use prephenate as the O-donor. This is the first example of an organic molecule serving as an O-donor in a biological reaction, with only O_2, H_2O_2, and H_2O previously known as O-donors (1, 2, 8).

We have shown here that prephenate is required for the function of UbiU-V, two Fe–S proteins of the U32 family that are essential for the hydroxylation steps of the O_2-independent UQ biosynthetic pathway (14, 15). Interestingly, two other U32 members, RlhA and TrhP, are involved in O_2-independent hydroxylation reactions of RNA molecules (16–18), but a direct hydroxylation activity has not been demonstrated so far. Overall, an attractive hypothesis is that U32 proteins are bona fide hydroxylases that catalyze prephenate-dependent hydroxylation reactions, consistent with previous proposals (16, 17). While waiting for the development of in vitro activity assays, the availability of C_4[^{18}O]-shikimate, through the chemical synthesis reported here, provides an opportunity to confirm in vivo the importance of prephenate for U32 protein-dependent pathways.

We propose the following mechanism for prephenate-dependent hydroxylation: i) a 2-electron oxidation of the substrate aromatic ring would lead to a cation intermediate, followed by ii) a concerted prephenate decarboxylation, releasing a nucleophilic hydroxide molecule that would quench the reactive cation to form the hydroxylated product and phenylpyruvate (Fig. 5). Interestingly, such a decarboxylation coupled to aromatization occurs in prephenate dehydratase (26) and in carboxy-*S*-adenosyl-L-methionine synthase (27). The unknown oxidant involved in step (i) probably has a high redox potential to oxidize the aromatic ring. The [4Fe–4S] clusters bound to UbiU and UbiV are functionally important (14, 15) and may serve as part of an electron transfer chain from the substrate to the yet unidentified electron acceptor. Remarkably, this role may be conserved in other U32 proteins as Fe–S clusters are likely to be a common feature of this family. Indeed, the function of TrhP and RlhA has been shown to depend either directly (18) or indirectly (16) on Fe–S clusters, and the four cysteine residues that coordinate the 4Fe–4S cluster in UbiU-V form a motif that is present in most U32 proteins (14).

The development of chemical analogs of prephenate as inhibitors of U32 proteins may be a promising antimicrobial strategy, considering that the *aro* pathway is found only in microorganisms and plants and that U32 proteins are not present in humans and are almost exclusively distributed in bacteria and to a lesser extent in archaea (17). UbiU-V has recently been implicated in the transition from chronic to acute infection in *Pseudomonas aeruginosa* (28), suggesting that inhibition of the anaerobic metabolism of this potent pathogen with prephenate analogs would affect its pathogenicity.

The existence of the O_2-independent UQ biosynthetic pathway was documented in 1978 (29), and it took 45 y to identify prephenate as the O-donor. Hydroxylation reactions occur in several other anaerobic pathways (30, 31), but the enzymes and O-donors involved remain largely uncharacterized. Of particular interest, one of the pathways for anaerobic microbial degradation of common aromatic pollutants, such as benzene and naphthalene, begins with hydroxylation of the aromatic ring with an unknown O-donor (32–34). Since about one-third of bacterial genomes show no evidence for the use of the molybdenum cofactor required for H_2O-dependent hydroxylation reactions (8, 35), these species must rely on other hydroxylation mechanisms under anaerobic conditions. Given the high conservation of the *aro* pathway among microbes (36), we propose that prephenate acts as an O-donor in some of these hydroxylation reactions.

Materials and Methods

Strains and Plasmids Constructions. All the strains and plasmids used in this study are listed in *SI Appendix*, Tables S1 and S2, respectively. The knockout strains (*SI Appendix*, Table S1) were obtained by generalized Φ P1 transduction using donor strains from the Keio collection (37). For the generation of specific knockouts, PCR recombination with the λRed system was used (38). Alleles were combined in the same strain by generalized Φ P1 transduction. When necessary, the antibiotic resistance marker was removed using flippase (FLP recombinase expression from plasmid pCP20 as described previously (39). Cassette removal and plasmid loss were verified by antibiotic sensitivity and confirmed by PCR amplification.

Expression plasmids for *pheA* and *ubiIH* were constructed using primers listed in *SI Appendix*, Table S3. DNA fragments obtained by PCR on genomic DNA from the MG1655 strain were cloned in EcoRI/XhoI restriction sites of the pTet vector (15). The compatible plasmid pACYC-pBAD-ubiUV was constructed by transferring the EcoRV/HindIII fragment from pES232 into the pACYC184 vector digested by the same enzymes.

Media and Growth Conditions. Strains were grown in lysogeny broth (LB) medium (10 g/L of tryptone, 10 g/L of NaCl and 5 g/L of yeast extract) or MOPS (3-(N-morpholino)propanesulfonic acid, https://www.genome.wisc.edu/resources/protocols/mopsminimal.htm) supplemented with 0.4% glycerol as the carbon source and 19 amino acids (10 mM Ser, 0.8 mM Ala/Gly/Leu, 0.6 mM Gln/Glu/Val, 0.4 mM Arg/Asn/Ile/Lys/Phe/Pro/Thr, 0.2 mM His/Met/Tyr, and 0.1 mM Cys/Trp), 5 vitamins (0.02 mM thiamine hydrochloride, 0.02 mM calcium pantothenate, 0.02 mM 4-aminobenzoic acid, 0.02 mM 4-HB, 0.02 mM 2,3-dihydroxybenzoic acid) according to Sakai et al. (17). Ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (35 μg/mL), 4-HB (10 or 100 μM), prephenate (1 mM) (Sigma-Aldrich) and shikimate (10, 100, or 300 μM) (Sigma-Aldrich) or synthesized labeled ^{18}O-shikimate (10 μM) were added when needed from aqueous stock solutions sterilized by filtration through 0.2-μm filters. PheA and PheA-CM expression from pTet in Δ*pheA*Δ*tyrA* strain was induced by promotor leak.

Aerobic cultures were performed in glass tubes (15 cm long and 2 cm in diameter) at 37 °C, with 180 rpm shaking. 5 mL of fresh medium was inoculated with 100 μL of an overnight culture and the culture was grown overnight.

Anaerobic cultures were performed in Hungate tubes as previously described (14), using LB or MOPS media. LB medium was supplemented with 2.5 mg/L resazurin as an anaerobic indicator, with 100 mg/L L-cysteine (adjusted to pH 6 with NaOH) in order to reduce residual dioxygen, and with anti-foam (Sigma Life Science, 0.5 mL/L). Then, 5 or 13 mL of this medium was distributed in Hungate tubes and deoxygenated by bubbling high-purity argon for 40 min. The Hungate tubes were sealed and autoclaved. The resazurin was initially purple, it turned to pink after deoxygenation and become colorless after autoclaving. The resazurin remained colorless during culture indicating anaerobic conditions. The sterile MOPS medium was supplemented with 100 mM sterile KNO_3 as the final electron acceptor and 5 mL of this medium was distributed to the Hungate tubes and deoxygenated under sterile conditions by bubbling high-purity argon for 40 min. The tubes were sealed and were then used for cultures. The precultures were performed overnight at 37 °C in Eppendorf tubes filled to the top with either LB or MOPS media. The Hungate tubes were then inoculated through the septum with disposable syringes and needles with 100 μL of precultures and incubated at 37 °C without agitation. For Hungate tubes containing LB medium, the shikimate, the prephenate, and the antibiotic were added after autoclave and at the same time as the preculture. In contrast, for the Hungate tubes containing MOPS medium, the supplements were added before deoxygenation.

For testing complementation of the Δ*pheA* Δ*tyrA* Δ*ubiH* mutant by *ubiUV*, in the presence or absence of the PheA CM activity and for testing complementation of the Δ*aroD* or Δ*aroD* Δ*ubiIH* or Δ*ubiIH* mutants by *ubiUV*, in the presence or absence of shikimate, strains were transformed and selection was performed in $-O_2$. Precultures were done in $-O_2$ in LB at 37 °C. The cultures were diluted in series (10-fold dilution at each step) in an M9 medium and 5 μL drops were plated on a minimal medium with 50 mM succinate and incubated at 37 °C in $+O_2$ or with 0.1% glucose and incubated in the absence of O_2. Arabinose was added at the concentrations indicated in figure legends for inducing expression of *ubiUV* genes.

Lipid Extraction and Quinone Analysis. First, 5 mL of each culture was cooled on ice for at least 30 min before centrifugation at 3,200 g at 4 °C for 10 min. Cell pellets were washed in 1 mL ice-cold phosphate-buffer saline and transferred to preweighted 1.5-mL Eppendorf tubes. After centrifugation at 12,000 g at 4 °C for 1 min, the supernatant was discarded, the cell wet weight was determined and pellets were stored at −20 °C until lipid extraction, if necessary. Quinone extraction from cell pellets was performed as previously described (40). The dried lipid extracts were resuspended in 100 μL ethanol, and a volume corresponding to 1 mg of cell wet weight was analyzed by HPLC electrochemical detection–mass spectrometry with a BetaBasic-18 column (Thermo Scientific) at a flow rate of 1 mL/min with a mobile phase composed of 50% methanol, 40% ethanol, and 10% of a mix [90% isopropanol, 10% ammonium acetate (1 M), and 0.1% formic acid]. When necessary, MS detection was performed on an MSQ spectrometer (Thermo Scientific) with electrospray ionization in positive mode (probe temperature, 400 °C; cone voltage, 80 V). Single-ion monitoring detected the following compounds: UQ_8 ($M+H^+$), m/z 727 to 728, 6 to 10 min, scan time of 0.2 s; ^{18}O-UQ_8 ($M+H^+$), m/z 729 to 730, 6 to 10 min, scan time of 0.2 s; 2(^{18}O)-UQ_8 ($M+H^+$), m/z 731 to 732, 6 to 10 min, scan time of 0.2 s; 3(^{18}O)-UQ_8 ($M+H^+$), m/z 733 to 734, 6 to 10 min, scan time of 0.2 s; 4(^{18}O)-UQ_8 ($M+H^+$), m/z 735 to 736, 6 to 10 min, scan time of 0.2 s; UQ_8 ($M+NH_4^+$), m/z 744 to 745, 6 to 10 min, scan time of 0.2 s; UQ_{10} ($M+NH_4^+$), m/z 880 to 881, 10 to 17 min. MS spectra were recorded between m/z 600 and 900 with a scan time of 0.3 s. ECD and MS peak areas were corrected for sample loss during extraction on the basis of the recovery of the UQ_{10} internal standard (40). The absolute quantification of UQ_8 based on the m/z 744 to 745 signal at 7.8 min was performed with a standard curve of UQ_8 ranging from 6.25 to 50 pmol UQ_8.

Synthesis of C_4[^{18}O]-Shikimate. The synthesis of C_4[^{18}O]-Shikimate is described in *SI Appendix, Supplementary text*. To determine the percentage of labeled shikimate, 3 or 9 μL of an aqueous solution of the synthetized product (0.34 mg/mL) was analyzed by HPLC–MS with a BetaBasic-8 column (Agilent C8 (4.6 × 150 mm; 5 μm) at a flow rate of 0.7 mL/min with a mobile phase composed of 0.1% formic acid in water. MS detection was performed on an MSQ spectrometer (Thermo Scientific) with electrospray ionization in negative mode (probe temperature, 450 °C; cone voltage, 60 V). Single-ion monitoring detected the following compounds: ^{16}O-shikimate (M-H), m/z 172.5 to 173.5, 0 to 6 min, scan time of 0.2 s; ^{18}O-shikimate (M-H), m/z 174.5 to 175.5, 0 to 6 min, scan time of 0.2 s. MS spectra were recorded between m/z 150 and 300 with a scan time of 0.3 s. The percentage of C_4[^{18}O]-Shikimate was calculated as a ratio (A175/(A175+A173))*100 in which A175 and A173 correspond to the peak area of the SIM signals at m/z 175 and m/z 173, respectively. The value obtained (64 ± 1.2%) corresponds to the average of the values measured for the two injections.

Data, Materials, and Software Availability. All data are available in the main text or the *SI Appendix*.

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1. D. E. Torres Pazmiño, M. Winkler, A. Glieder, M. W. Fraaije, Monooxygenases as biocatalysts: Classification, mechanistic aspects and biotechnological applications. *J. Biotechnol.* **146**, 9–24 (2010).
2. M. Hobisch *et al.*, Recent developments in the use of peroxygenases–Exploring their high potential in selective oxyfunctionalisations. *Biotechnol. Adv.* **51**, 107615 (2021).
3. C. C. Hansen, D. R. Nelson, B. L. Møller, D. Werck-Reichhart, Plant cytochrome P450 plasticity and evolution. *Mol. Plant* **14**, 1244–1265 (2021).
4. C. E. Paul, D. Eggerichs, A. H. Westphal, D. Tischler, W. J. H. van Berkel, Flavoprotein monooxygenases: Versatile biocatalysts. *Biotechnol. Adv.* **51**, 107712 (2021).
5. A. J. Jasniewski, L. Que, Dioxygen activation by nonheme diiron enzymes: Diverse dioxygen adducts, high-valent intermediates, and related model complexes. *Chem. Rev.* **118**, 2554–2592 (2018).
6. X. Huang, J. T. Groves, Oxygen activation and radical transformations in heme proteins and metalloporphyrins. *Chem. Rev.* **118**, 2491–2553 (2018).
7. E. Romero, J. R. G. Castellanos, G. Gadda, M. W. Fraaije, A. Mattevi, Same substrate, many reactions: Oxygen activation in flavoenzymes. *Chem. Rev.* **118**, 1742–1769 (2018).
8. R. Hille, J. Hall, P. Basu, The mononuclear molybdenum enzymes. *Chem. Rev.* **114**, 3963–4038 (2014).
9. P. I. Darley, J. Hellstern, B. Schink, B. Philipp, Resorcinol hydroxylase of Azoarcus anaerobius: Molybdenum dependence, activity, and heterologous expression. *Curr. Microbiol.* **77**, 3385–3396 (2020).
10. T. Franza, P. Gaudu, Quinones: More than electron shuttles. *Res. Microbiol.* **173**, 103953 (2022).
11. S. S. Abby, K. Kazemzadeh, C. Vragniau, L. Pelosi, F. Pierrel, Advances in bacterial pathways for the biosynthesis of ubiquinone. *Biochim. Biophys. Acta Bioenerg.* **1861**, 148259 (2020).
12. C.-D.-T. Vo *et al.*, The O2-independent pathway of ubiquinone biosynthesis is essential for denitrification in Pseudomonas aeruginosa. *J. Biol. Chem.* **295**, 9021–9032 (2020).
13. K. Kazemzadeh *et al.*, Diversification of ubiquinone biosynthesis via gene duplications, transfers, losses, and parallel evolution. *Mol. Biol. Evol.* **40**, msad219 (2023).
14. L. Pelosi *et al.*, Ubiquinone biosynthesis over the entire O2 range: Characterization of a conserved O2-independent pathway. *mBio* **10**, e01319-19 (2019).
15. R. Arias-Cartin *et al.*, Role of the Escherichia coli ubiquinone-synthesizing UbiUVT pathway in adaptation to changing respiratory conditions. *mBio* **14**, e0329822 (2023).
16. S. Kimura, Y. Sakai, K. Ishiguro, T. Suzuki, Biogenesis and iron-dependency of ribosomal RNA hydroxylation. *Nucleic Acids Res.* **45**, 12974–12986 (2017).
17. Y. Sakai, S. Kimura, T. Suzuki, Dual pathways of tRNA hydroxylation ensure efficient translation by expanding decoding capability. *Nat. Commun.* **10**, 2858 (2019).
18. C. T. Lauhon, Identification and characterization of genes required for 5-hydroxyuridine synthesis in Bacillus subtilis and Escherichia coli tRNA. *J. Bacteriol.* **201**, e00433-19 (2019), 10.1128/JB.00433-19.
19. S. Zhang, D. B. Wilson, B. Ganem, Probing the catalytic mechanism of prephenate dehydratase by site-directed mutagenesis of the Escherichia coli P-protein dehydratase domain. *Biochemistry* **39**, 4722–4728 (2000).
20. G. B. Cox, F. Gibson, The role of shikimic acid in the biosynthesis of vitamin K2. *Biochem. J.* **100**, 1–6 (1966).
21. H. Maeda *et al.*, RNAi suppression of arogenate dehydratase1 reveals that phenylalanine is synthesized predominantly via the arogenate pathway in petunia petals. *Plant Cell* **22**, 832–849 (2010).
22. J. Dayan, D. B. Sprinson, "[70a] Preparation of prephenic acid" in *Methods in Enzymology* (Elsevier, 1970), vol. 17, pp. 559–561, https://linkinghub.elsevier.com/retrieve/pii/0076687971172400.
23. H. Plieninger, Prephenic acid: Properties and the present status of its synthesis. *Angew. Chem Int. Ed. Engl.* **1**, 367–372 (1962).
24. L. O. Zamir, R. Tiberio, R. A. Jensen, Differential acid-catalyzed aromatization of prephenate, arogenate, and spiro-arogenate. *Tetrahedron Lett.* **24**, 2815–2818 (1983).
25. M. Kanehisa *et al.*, Data, information, knowledge and principle: Back to metabolism in KEGG. *Nucleic Acids Res.* **42**, D199–D205 (2014).
26. J. Van Vleet, A. Kleeb, P. Kast, D. Hilvert, W. W. Cleland, 13C isotope effect on the reaction catalyzed by prephenate dehydratase. *Biochim. Biophys. Acta* **1804**, 752–754 (2010).
27. J. Kim *et al.*, Structure-guided discovery of the metabolite carboxy-SAM that modulates tRNA function. *Nature* **498**, 123–126 (2013).
28. P. Cao *et al.*, A Pseudomonas aeruginosa small RNA regulates chronic and acute infection. *Nature* **618**, 358–364 (2023).
29. K. Alexander, I. G. Young, Alternative hydroxylases for the aerobic and anaerobic biosynthesis of ubiquinone in Escherichia coli. *Biochemistry* **17**, 4750–4755 (1978).
30. R. Rabus *et al.*, Anaerobic microbial degradation of hydrocarbons: From enzymatic reactions to the environment. *J. Mol. Microbiol. Biotechnol.* **26**, 5–28 (2016).
31. L.-B. Shou *et al.*, New evidence for a hydroxylation pathway for anaerobic alkane degradation supported by analyses of functional genes and signature metabolites in oil reservoirs. *AMB Express* **11**, 18 (2021).
32. T. Zhang *et al.*, Anaerobic benzene oxidation via phenol in geobacter metallireducens. *Appl. Environ. Microbiol.* **79**, 7800–7806 (2013).
33. M. E. Bedessem, N. G. Swoboda-Colberg, P. J. S. Colberg, Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol. Lett.* **152**, 213–218 (1997).
34. C. Chen, Z. Zhang, P. Xu, H. Hu, H. Tang, Anaerobic biodegradation of polycyclic aromatic hydrocarbons. *Environ. Res.* **223**, 115472 (2023).
35. T. Peng, Y. Xu, Y. Zhang, Comparative genomics of molybdenum utilization in prokaryotes and eukaryotes. *BMC Genom.* **19**, 691 (2018).
36. X.-Y. Zhi, J.-C. Yao, H.-W. Li, Y. Huang, W.-J. Li, Genome-wide identification, domain architectures and phylogenetic analysis provide new insights into the early evolution of shikimate pathway in prokaryotes. *Mol. Phylogenet. Evol.* **75**, 154–164 (2014).
37. T. Baba *et al.*, Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 (2006).
38. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640–6645 (2000).
39. P. P. Cherepanov, W. Wackernagel, Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14 (1995).
40. M. Hajj Chehade *et al.*, *ubiI*, a new gene in *Escherichia coli* coenzyme Q biosynthesis, is involved in aerobic C5-hydroxylation. *J. Biol. Chem.* **288**, 20085–20092 (2013).