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Ubiquinone is essential for denitrification in Pseudomonas aeruginosa

The O₂-independent pathway of ubiquinone biosynthesis is essential for denitrification in Pseudomonas aeruginosa

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Running title: Ubiquinone is essential for denitrification in Pseudomonas aeruginosa

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ABSTRACT

Many proteobacteria, such as Escherichia coli, contain two main types of quinones, benzoquinones represented by ubiquinone (UQ) and naphthoquinones such as menaquinone (MK) and dimethyl-menaquinone (DMK). MK and DMK function predominantly in anaerobic respiratory chains, whereas UQ is the major electron carrier in the reduction of dioxygen. However, this division of labor is probably not very strict. Indeed, a pathway that produces UQ under anaerobic conditions in an UbiU-, UbiV-, and UbiT-dependent manner has been recently discovered in E. coli. However, its physiological relevance is not yet understood because MK and DMK are also present in E. coli. Here, we established that UQ is the major quinone of Pseudomonas aeruginosa and is required for growth under anaerobic respiration (i.e. denitrification). We demonstrate that the ORFs PA3911, PA3912, and PA3913, which are homologs of the E. coli ubiT, ubiV and ubiU genes, respectively, are essential for UQ biosynthesis and thus for denitrification in P. aeruginosa. These three genes hereafter are called ubiTpa, ubiVpa, and ubiUpa. We show that UbiVpa accommodates an iron–sulfur [4Fe-4S] cluster. Moreover, we report that UbiUpa and UbiTpa can bind UQ and that the isoprenoid tail of UQ is the structural determinant required for recognition by these two Ubi proteins. Since the denitrification metabolism of P. aeruginosa is believed to be important for pathogenicity of this bacterium in individuals with cystic fibrosis, our results highlight that the O₂-independent UQ biosynthetic pathway may represent a possible target for antibiotics development to manage P. aeruginosa infections.

INTRODUCTION

The opportunistic pathogen Pseudomonas aeruginosa has a remarkable ability to grow under a variety of environmental conditions such as soil and water as well as animal-, human-, and plant-host-associated environments. P. aeruginosa is responsible for numerous acute and chronic infections and poses a major health risk for patients with severe burns, cystic fibrosis (CF) or in severely immunocompromised states (1,2). The utilization of various carbon sources and energy metabolism (respiration or fermentation) might contributes to the environmental adaptation of P. aeruginosa (3). Its main energy-producing system is respiration, which requires a proton motive force (pmf) used for ATP synthesis. The pmf is produced by the transfer of electrons and protons from reduced donors to oxidized acceptors via the quinone pool. Whereas the
dehydrogenases and reductases involved in respiratory metabolism have been well described and annotated in the genome of *P. aeruginosa* PAO1 (4,5), the composition of its quinone pool has not yet fully established. Studies in the sixties suggested ubiquinone 9 (UQ9) as a major quinone of aerobically-grown *P. aeruginosa* (6) and UQ9 is therefore believed to be essential for aerobic respiration (7).

Proteobacteria contain two main types of quinones, benzoquinones and naphthoquinones, represented respectively by UQ (or coenzyme Q) and menaquinone (MK) / demethylmenaquinone (DMK) (8). Typically, MK and DMK function predominantly in anaerobic respiratory chains, whereas UQ is the major electron carriers used for reduction of dioxygen by various cytochrome oxidases (8). Recent data indicated that the metabolic use of various quinone species according to environmental dioxygen availability might be more complex than initially thought. Indeed, using *E. coli* as a model, we highlighted a pathway conserved across many bacterial species and able to produce UQ under anaerobic conditions (9). The classical UQ biosynthetic pathway requires O2 for three hydroxylation steps (10). Obviously, the flavin-dependent monoxygenases UbiI, UbiF, and UbiH, which catalyze the O2-dependent hydroxylation steps are not involved in the anaerobic pathway, nor are the accessory UbiK and UbiJ proteins implicated in the assembly and/or stability of the aerobic Ubi-complex (11). Seven proteins (UbiA, B, C, D, E, G and X) catalyzing the prenylation, decarboxylation and methylation of the phenyl ring of the 4-hydroxybenzoate precursor are common to both pathways (9). In addition, the anaerobic pathway requires UbiT, UbiU and UbiV proteins. UbiT is homologous to UbiJ and UbiU and UbiV are expected to be involved in O2-independent hydroxylations (9). However, as explained above, the metabolic relevance of the O2-independent UQ pathway is not yet clearly understood to date.

In absence of O2, *P. aeruginosa* is able to carry out anaerobic respiration with nitrate and nitrite as terminal electron acceptors of the respiratory chain. This process called denitrification allows the reduction of soluble nitrate (NO3-) and nitrite (NO2-) to gaseous nitrous oxide (N2O) or molecular nitrogen (N2) (12). Because *P. aeruginosa*-infected mucus in CF airway is depleted of oxygen and enriched in nitrate and nitrite, the anaerobic metabolism of *P. aeruginosa* via the denitrification pathway is believed to be important for its pathogenicity (13). Four sequential reactions involving metalloenzymes are needed to reduce nitrate to N2, i.e. nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. *P. aeruginosa* was considered as a paradigm of the denitrification pathway and all the reductases involved in this metabolism have been widely studied as well as the regulatory network controlling the denitrification genes (3,4,14). However, the anaerobic quinone pool of *P. aeruginosa* has not been characterized so far.

In the present study, we discovered that UQ9 is essential for the growth of *P. aeruginosa* PAO1 strain in denitrification medium. We identified in this bacterium the ORFs PA3911, PA3912 and PA3913 as homologs to *E. coli* ubiT, ubiV and ubiU, respectively. Our results showed that these three genes hereafter called *ubiTpa*, *ubiVpa* and *ubiUpa* are essential components of the O2-independent UQ9 biosynthetic pathway of *P. aeruginosa*. We demonstrated that i) *ubiVpa* binds a [4Fe-4S] cluster and ii) *ubiUpa* and *ubiTpa* copurify with UQ by recognizing the isoprenoid tail. Such a molecular pathway for UQ production was found only in proteobacteria (9) where it can exert an essential role under anaerobic conditions, as demonstrated here. Taken together, our results highlight that this pathway could be an interesting lead for the development of antibiotics targeting the denitrification metabolism.

**RESULTS**

**UQ9 is the major quinone of *P. aeruginosa***.

The quinone content of *P. aeruginosa* PAO1, grown under ambient air or anaerobic conditions (denitrification), was determined using electrochemical detection of lipid extracts separated by HPLC and compared to those obtained from *E. coli*. Whatever the conditions of growth, a major quinone species eluting at 11.5 min was present in the analyses of *P. aeruginosa* lipid extracts, UQ10 being used as standard (Fig. 1A and 1B). Mass spectrometry analysis showed a predominant ammonium adduct (M+NH4+*) with m/z ratio of 812.7 (Fig. 1C), together with minor adducts such as Na+ (817.6) and H+ (795.7) (Fig. S1). These masses identify UQ9 (monoisotopic mass 794.6) as the major quinone produced by *P. aeruginosa*. Membranes of *E. coli* contain UQs and naphthoquinones (DMK8 and MK9). The absence of detectable level of naphthoquinones in *P. aeruginosa* lipid extracts, with or without oxygen (Fig.1A and 1B), is in agreement with the
absence of their biosynthetic pathways (MK or fualosine pathways) in *P. aeruginosa* genomes. It is also interesting to note that the UQ content of *E. coli* was higher under aerobic compared to anaerobic conditions (97±4 versus 42±6 pmol of UQs per mg of cells), whereas we found the opposite for *P. aeruginosa* (95±4 versus 126±4 pmol of UQs per mg of cells). Together, our results establish that UQ₉ is the major quinone of *P. aeruginosa* PAO1 and suggest that UQ₉ is used under denitrification conditions.

**Identification of ubi genes in the genome of *P. aeruginosa* PAO1.**

To identify the Ubi proteins of *P. aeruginosa*, IspB, UbiX, and UbiA from *E. coli* MG1655 were first screened for homologs in the *P. aeruginosa* PAO1 protein sequence data set available at http://www.pseudomonas.com/ using the BLASTP software. As listed in Table S1, the analysis disclosed the presence of 11 homologous proteins (IspB, UbiA to UbiE, UbiG to UbiJ and UbiX). As reported previously, the functional homolog of UbiF is a Coq7-like hydroxylase (15) and the corresponding PA0655 protein was shown to be essential for aerobic UQ₉ biosynthesis (16). Overall, we propose that the O₂-dependent UQ biosynthetic pathways in *P. aeruginosa* and *E. coli* share a similar pattern (Fig. S2).

Under anaerobic conditions, *E. coli* still synthesizes UQ and we recently identified three genes that we called *ubiT*, *ubiV* and *ubiU*, as essential for this process (9). Homologues of *ubiT*, *ubiV* and *ubiU* were also identified in *P. aeruginosa* PAO1 and correspond to ORFs PA3911, PA3912 and PA3913, respectively (Table S1 and Fig. S2). These genes were called hereafter *ubiTₚ₉*, *ubiVₚ₉* and *ubiUₚ₉*. The three genes are predicted to form an operon, *ubiUVT* (www.pseudomonas.com). Interestingly, this operon is located downstream of the genes *moeA₁*, *moeB₁*, *moeE*, *moeD* and *moeC* involved in the biosynthesis of the molybdopterin cofactor (MoCo) (Fig. 2), which is essential for nitrate reductase activity (17). Next, we evaluated the metabolic relevance of the O₂-dependent UQ biosynthetic pathway in *P. aeruginosa* by studying mutants of *ubiTₚ₉*, *ubiUₚ₉* and *ubiVₚ₉* genes.

**Tn mutants of ubiVₚ₉ and ubiUₚ₉ present a growth defect for denitrification and an impaired UQ₉ content.**

The physiological importance of the proteins UbiTₚ₉, UbiUₚ₉ and UbiVₚ₉ was first investigated using transposon (Tn) mutants PW7609 (*ubiTₚ₉*), PW7610 (*ubiVₚ₉*), PW7611 (*ubiVₚ₉*), PW7612 (*ubiUₚ₉*) and PW7613 (*ubiUₚ₉*) and the isogenic parental strain MPA01 (wild-type strain from the Manoil collection) as control (Table S2). Aerobic growth in LB medium was similar between the Tn mutants and the wild-type strain MPA01 (Fig. S3A) and the Tn mutants presented a UQ₉ level comparable to the wild-type (Fig. S3B). Thus, UbiTₚ₉, UbiUₚ₉ and UbiVₚ₉ are not involved in the O₂-dependent UQ biosynthetic pathway of *P. aeruginosa*. In contrast, the growth of the *ubiVₚ₉* and *ubiUₚ₉* mutants was severely impaired in denitrification conditions (Fig. S3C) and their UQ₉ content was strongly lowered (Fig. S3B). These results suggest the overall requirement of *ubiVₚ₉* and *ubiUₚ₉* for denitrification in *P. aeruginosa*, supposedly via their involvement in O₂-independent UQ biosynthesis. Surprisingly, the growth of the *ubiTₚ₉* Tn mutant PW7609 was not affected (Fig. S3C) and it showed around 40% UQ₉ compared to the wild-type in anaerobic cultures (Fig. S3B). In this mutant, the Tn is inserted at the fifth base of the *ubiTₚ₉* gene, potentially leading to only partial inactivation of the gene (Table S2). We note that previous studies with *E. coli* ubi mutants showed that only 20% UQ was sufficient to maintain a wild-type growth phenotype (18,19).

**Denitrification is dependent of ubiTₚ₉, ubiUₚ₉, and ubiVₚ₉ genes via their involvement in O₂-independent UQ biosynthesis.**

As *ubiTₚ₉*, *ubiVₚ₉* and *ubiUₚ₉* are localized next to each other in the genome of PAO1, the transposon inserted in the mutants previously studied might impact the expression of the neighbouring genes. In addition, it is likely that the Tn mutant PW7609 is not disrupting properly the *ubiTₚ₉* gene. Thus, for each of the three genes, we constructed knockouts (KO) as well as complementation mutants in the parental strain PAO1. All deletion mutant strains (called hereafter *ubiTUV-KO*) shared a growth defect under denitrification coupled to a strong decrease of UQ₉ content compared to the wild-type (Fig. 3A and 3B), whereas UQ₉ content and growth were normal in aerobic conditions (Fig. 3B and 3C). In anaerobic conditions, *ubiTUV-KO* strains accumulated an early UQ biosynthetic intermediate corresponding to nonaprenylphenol (NPP) (Fig. S2 and Table S3). This result suggests that the O₂-independent UQ biosynthetic pathway
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As mentioned previously, the *ubiUVT* operon is located downstream of the genes *moeA1*, *moaB1*, *moaE*, *moaD* and *moaC* involved in MoCo biosynthesis. Currently, MoCo-containing hydroxylases constitute the only family known to catalyze O₂-independent hydroxylation reactions (20). Since three O₂-independent hydroxylation reactions are needed to synthesize UQ anaerobically and that UbiU and UbiV are suspected to be involved in these reactions (9), we reasoned that MoCo might be involved in this process, providing a rationale for the co-localization of the *ubi* and *moa/moe* genes. To test this hypothesis, we evaluated the ability of Tn mutants PW7614, PW7615/PW7616, PW7618/PW2470, PW7619/PW1920 and PW7621/PW7622 (Table S2) corresponding respectively to Tn insertions in the ORFs *moeA1*, *moaB1* (two mutants), *moaE* (two mutants), *moaD* (two mutants) and *moaC* (two mutants) to synthesize UQ₉ without O₂. However, MoCo is also essential for nitrate reductase activity and thus for denitrification (17). To overcome this problem, WT and Tn mutants were grown in LB medium using arginine as a fermentable energy source in rich medium. As expected, all the Tn mutants exhibited a growth defect in denitrification. However, anaerobic growth was rescued by addition of arginine as previously described (21), and we were therefore able to measure the UQ content of the cells in these conditions (Fig. S4). The UQ₉ content of the MoCo Tn mutants was comparable to that of the wild-type strain (Fig. S4), suggesting that MoCo is not involved in anaerobic UQ₉ biosynthesis.

Recombinant UbiV₉ is an air-sensitive [4Fe-4S] cluster-containing protein.

To gain insights into their biochemical properties, we produced and purified the three proteins in *E. coli*, UbiV₉ being the most soluble. First, we showed that UbiV₉ purified by size exclusion chromatography (SEC) behaved as a monomer (Fig. S5A and S5B). Moreover, we noticed that the fraction containing the purified protein was slightly pink-colored with a UV-visible absorption spectrum characteristic of the presence of iron-sulfur species (22), with a band at 410 nm and broad and low intensity shoulders between 450 and 600 nm (Fig. 5A, dotted line) (23). However, the amount of iron and sulfur (0.22 iron and 0.22 sulfur/monomer) was largely substoichiometric, suggesting a degradation of the [Fe-S] cluster during the purification of the protein under aerobic conditions, as already

is blocked downstream of NPP in these three mutants. Interestingly, upon complementation, bacterial growth and UQ₉ levels were restored to those of the PAO1 strain used as control (Fig. 4A).

To confirm that UQ was directly involved in the restoration of the anaerobic growth of the *ubiTUV-KO* strains, UQ₉ solubilized in methanol was added to the denitrification medium at 5 and 50 µM final concentrations. After 24 h of anaerobic incubation, colony-forming units per mL (CFU/mL) of each KO strain were estimated and compared to the same strain cultivated without UQ₉. The most significant results were obtained with 50 µM of UQ₉, which increased CFU of *ubiTUV-KO* strains by 8-15 fold (Fig. 4B). We noted a substantial toxicity of methanol on the wild-type strain (Fig. 4B, Ct lane), suggesting that the positive effect of UQ₉ on the *ubiTUV-KO* strains is likely underestimated. Taken together, our results show unequivocally that UbiT₉, UbiU₉ or UbiV₉ are needed for denitrification via their involvement in UQ biosynthesis.

**ubiT₉, UbiU₉ and UbiV₉ are needed for the process of denitrification.**

We used soft-agar experiments to examine dioxygen and nitrate requirements of *ubiTUV-KO* strains with or without the wild-type allele. Soft agar was prepared anaerobically in LB medium containing KNO₃ at 100 mM final concentration and then exposed to ambient air. Oxygen diffuses through the agar to form a gradient, the highest concentration being at the top of the agar (Fig. 4C, lane 1). As shown in Fig. 4C, parental strain PAO1 and complemented *ubiTUV-KO* strains grew throughout the tube because they were able to use aerobic respiration as well as denitrification. In contrast, the growth of the *ubiTUV-KO* strains harboring the empty vector was restricted to the oxygenated part of the medium, whereas the presence of the respective genes on the plasmids allowed growth in the anaerobic medium (Fig. 4C). The bubbles observed in the soft agar correspond to gas evolution of N₂O and/or N₂ (12), suggesting a restoration of the denitrification process in the lower part of the tube. Taken together, these results point to the requirement for *ubiT₉*, *ubiU₉* and *ubiV₉* beyond the nitrate reduction step and support that UQ is probably essential for the entire denitrification process in *P. aeruginosa*.

**Molybdopterin cofactors are not involved in anaerobic UQ₉ biosynthesis.**
observed for many other Fe-S proteins. Consistent with this hypothesis, anaerobic reconstitution of the [Fe-S] cluster allowed to obtain a brown color with a UV-visible spectrum displaying one broad absorption band at 410 nm, which is characteristic of a [4Fe-4S] cluster (Fig. 5A, solid line) (24). The iron and sulfide determination yielded 3.90 ± 0.03 iron and 3.40 ± 0.20 sulfur/monomer of UbiVp, consistent with the presence of one [4Fe-4S] cluster/protein (Table 1). As shown in Fig. S5C, the [Fe-S] cluster of UbiVp was sensitive to air.

Four strictly conserved cysteines (C39, C180, C193, and C197) arranged in a CXXC type protein, suggesting that the four conserved cysteines are good candidates as ligands of the [4Fe-4S] cluster present in UbiVp (9). To test if these four cysteines are important for the chelation of the [4Fe-4S] cluster present in UbiVp, we generated two double mutants (C39AC180A and C193AC197A) and a triple mutant (C39AC193AC197A). All these mutants were colorless after purification under aerobic conditions and did not show any absorption band in the 350- to 550-nm region of their UV-Vis spectra (Fig. S5D), suggesting that they were impaired in their capacity to accommodate a [Fe-S] cluster. After reconstitution under anaerobic conditions, UbiVp C39AC193AC197A precipitated and its UV-vis spectrum could not be recorded. Although they also had a tendency to aggregate, 10% of the double mutants behaved as monomers permitting some assays. Overall, their absorbance at 410 nm (Fig. 5B) and their iron and sulfur contents (Table 1) were largely decreased compared to the wild-type protein, suggesting that the four conserved cysteines are good candidates as ligands of the [4Fe-4S] cluster present in UbiVp.

Recombinant UbiU and UbiT co-purify with UQ in E. coli.

We have recently demonstrated that isoprenoid quinones were able to co-elute with the Ubi-proteins such as UbiJ (11) and that UbiT exhibits a sterol carrier protein 2 (SCP2) domain, which is able to bind lipids (9). To that end, we performed lipid content analysis of the UbiTp, UbiUp, and UbiVp fractions purified from E. coli extracts. No isoprenoid quinones were detected co-eluting with UbiVp (Table S4). In contrast, UQs and DMQs (2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone) were shown to co-purify with UbiUp. This protein was purified only in the presence of detergent, as it was insoluble without it. After a two-step purification protocol, including a Ni-NTA chromatography and a SEC, the solubilized protein had still a tendency to form different oligomeric states covering the fractions 14 to 44 as shown in Fig. 6A and 6B. UQs and DMQs were mainly detected in the elution fractions 33 to 45 (Fig. 6A) corresponding to only a proportion of the purified UbiUp (Fig. 6B). The highest contents, i.e. 488.17 pmoles of UQ per mg of protein and 19 932 AU of DMQs per mg of protein, were assayed in the fraction 39 and 40, respectively (Table S4). This corresponds to a UQs/protein ratio of 1.5%. Taken together, these results show that UQs and DMQs co-purify with UbiUp, depending of its oligomerization state.

Due to a lack of solubility, UbiTp was overproduced with E. coli thioredoxin (TrxA) as a gene fusion partner, as previously described (25). After the first step of the purification process (Ni-NTA chromatography), the 32 kDa TrxA-UbiTp fusion protein was digested with thrombin. Then in order to remove the TrxA Histagged protein, UbiTp was purified by Ni-NTA chromatography coupled to SEC, as a high oligomeric form (Fig. 6C). The purified UbiTp (pool of fractions 20 to 30) contained 9.75±4.57 pmoles of UQs per mg of protein (Table S4), which corresponds to a UQs/protein ratio of about 0.03%.

Recombinant UbiU and UbiT bind the isoprenoid tail of UQ.

To further confirm the ability of UbiTp and UbiUp to bind UQ, a protein-lipid overlay assay was performed (Fig. 6D). We checked the possibility of these proteins to recognize UQ10, UQ8, solanesol, 3-methylcatechol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and cholesterol. Solanesol is a non-cyclic terpene alcohol that consists of nine isoprene units as found in UQ8. 3-methylcatechol was chosen to mimic the head group of UQ. POPE is the major lipid component of the inner membrane of E. coli (26). Finally, cholesterol was used as a sterol standard. Fig. 6D shows that UbiTp and UbiUp did not interact with 3-methylcatechol, POPE, or cholesterol in our experimental conditions. In contrast, both proteins were able to recognize UQ10, UQ8 and solanesol. We established the ability of UbiTp to bind phosphatidic acid (PA) as previously demonstrated by Groenewold et al. (25) (Fig. S6). Together, we show that UbiTp and UbiUp are able to bind the isoprenoid tail of UQ in agreement with their involvement in the O2-independent biosynthetic pathway of UQ.
DISCUSSION

Ubiquinone is essential for denitrification in Pseudomonas aeruginosa

Ubiquinone (UQ) acts as a membrane-embedded electron and proton shuttle and is a key molecule in the respiratory metabolism of proteobacteria. Biosynthesis of UQ in aerobic conditions has been widely studied and includes a series of enzymatic reactions in which a benzene ring undergoes a series of modifications involving a prenylation, a decarboxylation, three methylations, and three hydroxylations (27). Our chemical analysis identified UQ$_9$ as the major quinone in the membranes of aerobically grown *P. aeruginosa* cells, which is in agreement with the literature (7,16). We found in *P. aeruginosa* homologues of the genes known to be involved in UQ biosynthesis in *E. coli*, except *ubiF* (Table S1). Indeed, as already published, *P. aeruginosa* exhibits a yeast COQ7 protein homolog, which catalyzes the same reaction as UbiF from *E. coli* (15,16). Thereby, both bacteria share a similar UQ biosynthetic pathway involving three hydroxylases - UbiI, UbiH and UbiF or COQ7 - using O$_2$ as a co-substrate (Fig. S2). As no other isoprenoid quinone was detected in lipid extracts of *P. aeruginosa*, we suppose that UQ$_9$ is essential for aerobic growth of this bacterium.

In the absence of oxygen, *P. aeruginosa* can grow by dissimilatory nitrate respiration by using nitrate or nitrite as alternative terminal electron acceptors of the respiratory chain. This metabolic process, known as denitrification, has been widely studied (3). However, the component acting to transfer electrons from primary dehydrogenases to nitrate or nitrite reductases was not clearly identified to date (3). In the present study, we identified UQ$_9$ as the major quinone synthesized in anaerobic conditions. By LC-MS analysis, we also identified two related redox molecules, UQ$_6$ and DMQ$_9$ present in small amounts (see Fig. 1B, the peaks around 8 and 9.5 min) and nonaprenylphenol (NPP, see Fig. S2). NPP and DMQ$_9$ are UQ$_9$ biosynthetic intermediates (see Fig. S2) and UQ$_9$ was already detected in *Pseudomonas* lipid extracts in aerobic conditions (16). Taken together, these results suggest that *P. aeruginosa* possesses an O$_2$-independent UQ biosynthetic pathway, which produces the major quinone species observed under anaerobic conditions. We recently identified such pathway in *E. coli* (9). Here, we characterized three genes, *ubiT$_{Pa}$* (PA3911), *ubiU$_{Pa}$* (PA3913) and *ubiV$_{Pa}$* (PA3912) as essential for the anaerobic UQ$_9$ biosynthesis in *P. aeruginosa* and dispensable for the aerobic one. These genes are homologs to the *ubiT, ubiU* and *ubiV* previously identified in *E. coli*, which grows normally in anaerobic conditions in a UQ-independent manner because of the presence of naphthoquinones (9) (28) (29). In contrast, we demonstrated that these three genes were essential to anaerobic denitrification metabolism of *P. aeruginosa*, which is in agreement with the presence of a single quinone corresponding to UQ. In line with our results, *ubiV$_{Pa}$* (PA3912) and *ubiU$_{Pa}$* (PA3913) are expressed in response to anaerobic conditions (30) and the abundance of UbiU$_{Pa}$ protein was increased during anaerobic growth (31). Moreover, using random transposon mutagenesis, *ubiV$_{Pa}$* (PA3912) and *ubiU$_{Pa}$* (PA3913) were already reported as essential for anaerobic growth of *P. aeruginosa* on nitrate and nitrite as alternative terminal electron acceptors of the respiratory chain (21).

Although its contribution is still poorly understood for within-host growth, anaerobic respiration of *P. aeruginosa* is likely to be significant for promoting virulence mechanisms in chronic lung infections (13). Indeed, the infected endobronchial mucus of CF patients is subject to severe hypoxia or even anoxia (32). A likely hypothesis is that accelerated O$_2$ consumption in the biofilm may result from activated polymorphonuclear leukocytes that produce superoxide (33) and nitric oxide (34). Indeed, high levels of nitrate and nitrite have been measured in sputum from CF patients (35). From all these observations and as UQ is an essential component of the denitrification metabolism in *P. aeruginosa*, we propose that UbiT$_{Pa}$, UbiV$_{Pa}$ and UbiUpa may contribute to the CF lung infection in patients (work in progress in our laboratory). This hypothesis is supported by a recent quantitative proteomics approach revealing increased abundance of the three proteins in anaerobic biofilms grown under conditions of the cystic fibrosis lung (25). Moreover, deduced from a high-throughput sequencing of Tn libraries from *P. aeruginosa* strain PA14, it appears that *ubiT* gene was found to be essential for this bacterium to colonize the murine gastrointestinal tract (36), which suggests that O$_2$-independent UQ biosynthesis could be essential for bacterial virulence. This hypothesis is also supported by the essential contribution of UbiU and UbiV homologs to *Yersinia ruckeri* virulence (37).

As already suggested, homologs of UbiU and UbiV would belong to a new family of O$_2$-independent hydroxylases (9). To date, only the
MoCo-containing hydroxylases using water-derived oxygen are known to catalyze hydroxylations in anaerobic conditions (20). In our study, we have demonstrated that MoCo is not essential to the anaerobic UQ pathway, strengthening the hypothesis that hydroxylation reactions performed in UbiU- and UbiV-dependent manner do not involve MoCo.

In order to better understand their functions, we decided to overproduce in E. coli, purify and biochemically characterize UbiT(Pa), UbiU(Pa) and UbiV(Pa). Our results showed that recombinant UbiV(Pa) is an air-sensitive Fe-S containing protein, as UbiV from E. coli (9), and we demonstrated that cysteines 39, 180, 193 and 197 were ligands to the [4Fe-4S] cluster found in UbiV(Pa). These results confirm the conservation of a four cysteines pattern coordinating a Fe-S cluster across homologs of UbiV. This pattern is also found in RlhA and TrhP (38,39), two proteins that belong to the same proteases U32 family than UbiT. That MoCo, the central hub of phospholipid metabolism (25). Here we showed that UbiT(Pa) binds to UQ₈ and shares with UbiU(Pa) the recognition of the isoprenoid tail of UQ. Taken together, these results support the hypothesis that UbiT may be the counterpart of UbiJ in anaerobic conditions. We propose that UbiT may bind UQ intermediates and may stabilize a putative anaerobic Ubi complex that has yet to be demonstrated.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions.**

*P. aeruginosa* and *E. coli* strains used in this study are listed in Table S2. We obtained the collection of transposon (Tn) mutants in *P. aeruginosa* MPAO1 strain from the Manoil Laboratory, Department of Genome Science, University of Washington, USA (41,42). The Tn insertion site of the mutant strains were verified by sequencing (GATC Biotech, Konstanz, Germany) using PCR primers recommended by the library curators. *P. aeruginosa* strains were aerobically maintained at 37°C on lysogeny broth (LB) agar plates. For quinone assay, aerobic cultures (5 mL) were performed in LB medium at 37°C with rotary shaking at 200 rpm. Anaerobic growth of *P. aeruginosa* were performed in a 12 mL Hungate tubes containing LB medium supplemented with KNO₃ as an electron acceptor (100 mM final concentration) (43), hereafter called denitrification medium, and deoxygenated 30 min by argon bubbling (O₂<0.1 ppm) prior to autoclaving. In some experiments, LB medium was supplemented with arginine at a final concentration of 40 mM instead of KNO₃ (44). Hungate tubes were inoculated through the septum with 100 µL of overnight cultures taken with disposable syringe and needles from closed Eppendorf tubes filled to the top. Cultures in Hungate tubes were used for measuring the quinone contents. For aerobic growth studies, aerobic overnight cultures were used to inoculate a 96-well plate to obtain a starting optical density at 600 nm (OD₆₀₀) of 0.05 and further incubated with shaking at 37°C. Changes in OD₆₀₀ were monitored every 10 min for 12 h using the Infinite 200 PRO microplate reader (Tecan, Lyon, France). For anaerobic growth curve studies, overnight cultures in 50 mL closed tubes of not degassed denitrification medium were used to inoculate 400 mL bottles to obtain a starting OD₆₀₀ of 0.05. Then, bacteria were grown anaerobically by sparging argon (O₂<0.1 ppm) and bacterial cultures were monitored...
spectrophotometrically (OD<sub>600</sub>) at 30 min intervals for 9 h. *E. coli* MG1655 and DH5α were grown on LB agar or in LB liquid. When required, the medium was supplemented with ampicillin at 100 μg/mL for *E. coli*, carbenicillin at 250 μg/mL for *P. aeruginosa*, or tetracycline at 60 μg/mL for *E. coli* and 100 μg/mL for *P. aeruginosa* or gentamicin at 200 μg/mL for *P. aeruginosa* (Table S2). When necessary, UQ<sub>4</sub> or 0.1% (w/v) arabinose final concentration was added to the medium to enhance bacterial anaerobic growth or to induce P<sub>BAD</sub> expression of pSW196-derived plasmids, respectively. *Pseudomonas* Isolation Agar (PIA) medium (from DB) containing irgasan (25 μg/L) was used for triparental mating to counter select *E. coli*. For CFU counting, bacteria were suspended in PBS and cell suspensions were serially diluted in PBS. For each sample, 100 μL of at least four different dilutions were plated on LB plates, incubated for 24 h at 37°C and CFU were counted using a Scan 100 Interscience.

**Plasmids and genetic manipulations.**

The plasmids and the primers used in this study are listed in Tables S2 and S4). All the plasmids produced in this work were checked using DNA sequencing (GATC Biotech, Konstanz, Germany). To generate *P. aeruginosa* deletion mutants, overlapping upstream and downstream flanking regions of *ubiT<sub>Pa</sub>, *ubiU<sub>Pa</sub>* and *ubiV<sub>Pa</sub>* genes were obtained by PCR amplification using PAO1 genome as template and the oligonucleotides described in Table S5. The resulting fragments were then cloned into *SalI*-cut pEXG2 plasmid by ligation-independent cloning (45). To complement the mutants, the *ubiT<sub>Pa</sub>, *ubiU<sub>Pa</sub>* and *ubiV<sub>Pa</sub>* fragments were generated by PCR amplification using the oligonucleotide pairs *ubiT*-PA-F/*ubiT*-PA-R, *ubiU*-PA-F/*ubiU*-PA-R and *ubiV*-PA-F/*ubiV*-PA-R, respectively, and PAO1 genome as template (Table S5). The fragments were *EcoRI*-SacI digested and inserted into P<sub>BAD</sub>-harboring pSW196 plasmid, yielding the *ubiT<sub>Pa</sub>, *ubiU<sub>Pa</sub>* and *ubiV<sub>Pa</sub>* plasmids, respectively (Table S2). The pEXG2- and pSW196-derived vectors were transferred into *P. aeruginosa* PAO1 strain by triparental mating using pRK2013 as a helper plasmid (46). For allelic exchange using the pEXG2 plasmids, co-integration events were selected on PIA (*Pseudomonas* Isolation Agar) plates containing gentamicin. Single colonies were then cultured on NaCl-free LB agar plates containing 10% (w/vol) sucrose to select for the loss of the plasmid, and the resulting sucrose-resistant colonies were checked for mutant genotype by PCR. To overproduce C-terminally His-tagged UbiV<sub>Pa</sub>, the *ubiV<sub>Pa</sub>* gene was cloned into the pET22b(+) vector. The *ubiV<sub>Pa</sub>* insert was obtained by PCR amplification using the oligonucleotide pair pET22-UbiV-F and pET22-UbiV-R and *ubiV<sub>Pa</sub>* ORF as template (Table S5). *NdeI*-Xhol digested amplicon was ligated to *NdeI*-XhoI digested pET22b(+) vector to obtain pET22-UbiV<sub>Pa</sub> (Table S2). Variants of UbiV<sub>Pa</sub> were obtained using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) (UbiV<sub>Pa</sub> C193AC197A and UbiV<sub>Pa</sub> C39AC193AC197A) and the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent) (UbiV<sub>Pa</sub> C39AC180A) according to the manufacturer’s specifications using pET22b-UbiV<sub>Pa</sub> as template (Table S2 and S5). The *ubiU<sub>Pa</sub>* gene was cloned into pET-22b(+) by following the same protocol as that for *ubiV<sub>Pa</sub>* gene. The *ubiT<sub>Pa</sub>* gene was synthesized by Eurofins with *E. coli* codon optimization. The synthetic gene was then cloned into the EcoRI/NotI sites of vector pET32a(+) (Novagen), resulting in plasmid pET32-TrxA-UbiT<sub>Pa</sub> (Table S2).

**Soft-agar study to evaluate the O<sub>2</sub>-dependency of growth.**

Soft-agar studies were performed in denitrification medium supplemented by agar 0.7% (w/v) final concentration. After argon bubbling (O<sub>2</sub>&lt;0.1 ppm) for 30 min, the suspension (13 mL) was autoclaved in Hungate tubes. They were then placed in a 40°C incubator and they were inoculated through the septum with 100 μL of overnight cultures taken with disposable syringe and needles from Eppendorf tubes filled to the top, mixed by inverting, and incubated at room temperature 30 min to allow the agar to solidify. Then, the tubes were incubated under aerobic conditions with caps loosened at 37°C for 24 h. A control experiment was performed with resazurin (0.25 μg/mL final concentration) used as an indicator of medium oxygenation. When required, the medium was supplemented with antibiotics.

**Lipid extractions and quinone analysis.**

Cultures (5 mL under ambient air and 13 mL under anaerobic conditions) were cooled down on ice before centrifugation at 3200 g, 4°C, 10 min. Cell pellets were washed in 1 mL ice-cold PBS and transferred to pre-weighted 1.5 mL Eppendorf tubes. After centrifugation at 12 000 g,
4°C, 1 min and elimination of supernatant, the cell wet weight was determined (~5-30 mg) and pellets were stored at -20°C. Quinone extraction from cell pellets was performed as previously described (18). Lipid extracts corresponding to 1 mg of cell wet weight were analyzed by HPLC electrophoeresis detection-mass spectrometry (ECD-MS) with a BetaBasic-18 column at a flow rate of 1 mL/min with mobile phases composed of methanol, ethanol and a mix of 90% isopropanol, 10% ammonium acetate (1 M), 0.1% TFA: mobile phase 1 (50% methanol, 40% ethanol and 10% mix). When necessary, MS detection was performed on a MSQ spectrometer (Thermo Scientific) with electrospray ionization in positive mode (probe temperature 400°C, cone voltage 80V). Single ion monitoring (SIM) detected the following compounds: UQ$_1^-$ (M+NH$_4$)$^+$, m/z 744-745, 6-10 min, scan time 0.2 s; UQ$_9^-$ (M+NH$_4$)$^+$, m/z 812-813, 9-14 min, scan time 0.2 s; UQ$_{10}^-$(M+NH$_4$)$^+$, m/z 880.2-881.2, 10-17 min, scan time 0.2 s., DMQ$_6^-$ (M+NH$_4$)$^+$, m/z 714-715, 5-10 min, scan time 0.2 s and NPP (M+NH$_4$)$^+$, m/z 724-725, 8-13 min, scan time 0.2 s. 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 and were then normalized to cell wet weight. The peaks of UQ$_8^-$ and UQ$_9^-$ obtained with electrochemical detection were quantified with a standard curve of UQ$_{10}^-$ as previously described (18).

**Overproduction and purification of UbiV, UbiU and UbiT from P. aeruginosa in E. coli.**

Wild-type and variants UbiV$_{pa}$ were expressed and purified as previously described for *E. coli* proteins (9). Briefly, the pET-22b(+) plasmid, encoding wild-type or variants UbiV$_{pa}$, were co-transformed with pGro7 plasmid (Takara Bio Inc.) into *E. coli* BL21 (DE3) ΔubiUV competent cells grown at 37 °C in LB medium, which was supplemented with ampicillin (50 µg/mL), kanamycin (50 µg/mL) and chloramphenicol (12.5 µg/mL). At an OD$_{600}$ of 1.2, D-arabinose was added to the cultures at a final concentration of 2 mg/mL. At an OD$_{600}$ of 1.8, cultures were cooled down on ice for 20 min, and IPTG was added at a final concentration of 0.1 mM. Cells were then allowed to grow further at 16°C overnight. Wild-type UbiV$_{pa}$ and the different variants were purified by Ni-NTA chromatography followed by SEC in buffer A (50 mM Tris-HCl, 25 mM NaCl, 15% (v/v) glycerol, pH 8.5) containing 1 mM DTT. The purified proteins were concentrated to 30-40 mg/mL using Amicon concentrators (30-kDa cutoff; Millipore).

The overproduction of wild-type UbiU$_{pa}$ was performed in *E. coli* BL21 (DE3) ΔubiUV cells by following the same protocol as that for UbiV$_{pa}$, except that UbiU$_{pa}$ over-expression was induced with 0.05 mM of IPTG and the cell pellets were resuspended in buffer B (50 mM Tris-HCl, 500 mM NaCl, 15% (v/v) glycerol, pH 8.5) containing 0.2% (w/v) N-lauroylsarcosine sodium salt. After cell disruption by sonication, the clarified cell-free extracts were loaded onto a His-Trap FF crude column (GE Healthcare) pre-equilibrated with buffer B containing 0.1% (w/v) N-lauroylsarcosine sodium salt. The column was washed with 10 column volumes of buffer C (50 mM Tris-HCl, 500 mM NaCl, 15% (v/v) glycerol, 10 mM imidazole, pH 8.5) containing 6 mM N,N-dimethyldecylamine N-oxide (LDAO) to remove non-specifically bound *E. coli* proteins and then eluted with a linear gradient of 10 column volumes of buffer C containing 500 mM imidazole and 6 mM LDAO. Fractions containing UbiU$_{pa}$ were pooled and then loaded on a HiLoad 16/600 Superdex 200 pg (GE Healthcare) pre-equilibrated in buffer D (50 mM Tris-HCl, 150 mM NaCl, 15% (v/v) glycerol, pH 8.5) containing 3 mM LDAO. The purified proteins were concentrated using Amicon concentrators (100-kDa cutoff; Millipore), aliquoted, frozen in liquid nitrogen, and stored at -80°C. For protein-lipid overlay, fractions 34-43 were pooled.

Overproduction of UbiT$_{pa}$ fused with the thioredoxin (TrxA-UbiT$_{pa}$) in *E. coli* BL21 (DE3) ΔubiUV cells was performed by following the same protocol as that for UbiU$_{pa}$, except that over-expression of chimeric gene was induced at an OD$_{600}$ of 0.5 and the cell pellet was resuspended in buffer B containing 5% (w/v) sodium cholate. TrxA-UbiT$_{pa}$ was first purified, following the same protocol as that for UbiU$_{pa}$, by Ni-NTA chromatography, except that the HisTrap FF crude column was pre-equilibrated with buffer B containing 0.5% (w/v) sodium cholate and then eluted with buffer C containing 500 mM imidazole and 0.5% (w/v) sodium cholate. Fractions containing TrxA-UbiT$_{pa}$ were pooled and detergent was removed using a HiPrep 26/10 Desalting column (GE Healthcare) pre-equilibrated with buffer D. The fusion protein was digested with thrombin (10 units/mg of TrxA-UbiT$_{pa}$) at room temperature and then loaded on a HiLoad 16/600 Superdex 200 pg (GE Healthcare) coupled with a HisTrap FF crude
column (GE Healthcare) pre-equilibrated with buffer D. The purified proteins were concentrated using Amicon concentrators (100-kDa cutoff; Millipore), aliquoted, frozen in liquid nitrogen, and stored at -80°C.

[Fe-S] cluster reconstitution.

The [Fe-S] cluster(s) of holo-UbiV$_p$, and holo-variants was reconstituted as previously described (9). Briefly, a solution containing 100 μM of as-purified proteins was treated with 5 mM DTT for 15 min at 20°C and then incubated for 1 h with a 5-fold molar excess of both ferrous ammonium sulfate and L-cysteine. The reaction was initiated by the addition of a catalytic amount of the E. coli cysteine desulfurase CsdA (1-2% molar equivalent) and monitored by UV-visible absorption spectroscopy. After 1 h of incubation, the holo-proteins were then loaded onto a Superdex 75 Increase 10/300 GL column (GE Healthcare) pre-equilibrated with buffer A. The fractions containing the holo-proteins were pooled and concentrated to 20-30 mg/mL on a Vivaspin concentrator (30-kDa cutoff).

Protein-lipid overlay.

To assess the lipid-binding properties of UbiT$_p$, and UbiU$_p$, a protein–lipid overlay was performed as previously described (47). Briefly, 2 μL of 20 mM lipids in dichloromethane were spotted onto PVDF membrane and allowed to dry at room temperature for 1 h. The membranes were blocked in 3 % (w/v) fatty acid-free BSA in TBST (50 mM Tris-HCl, 150 mM NaCl and 0.1% (v/v) Tween-20, pH 7.5) for 1 h. The membranes were then incubated overnight at 4 °C with gentle stirring in the same solution containing 0.2 μg/mL of the indicated proteins. After washing six times during 30 min in TBST buffer, the membranes were incubated for 1 h with a 1/1000 dilution of anti-polyHis monoclonal antibody (Sigma) and then for 1 h with a 1/10 000 dilution of anti-mouse–horseradish peroxidase conjugate (Thermo Fisher Scientific). His-tagged proteins bound to the membrane by virtue of its interaction with lipid, were detected by enhanced chemiluminescence using Clarity Max Western ECL Substrate (Bio-rad).

Quantification methods.

Protein concentrations were determined using the method of Bradford (Bio-Rad) with bovine serum albumin as the standard. The iron and acid-labile sulfide were determined according to the method of Fish (48) and Beinert (49), respectively, before and after [4Fe-4S] cluster reconstitution.

UV-vis spectroscopy.

UV-visible spectra were recorded in 1-cm optic path quartz cuvettes under aerobic conditions on a Cary 100 Uv-vis spectrophotometer (Agilent) and under anaerobic conditions in a glove box on a XL-100 Uvikon spectrophotometer equipped with optical fibers.

The abbreviations used are: MK, menaquinone; UQ, ubiquinone; DMQ, dimethyl-menaquinone; C6, C6-dimethoxy-ubiquinone; NPP, nonaprenylphenol; SEC, size exclusion chromatography; SCP2, sterol carrier protein 2; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ECD, electrochemical detection; PA: phosphatidic acid; POPE: 3-methylcatechol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.

Author contributions: L. P., F. B., M. F., M. L. conceived and designed the experiments. C. -D. -T., J. M., S. E., E. B., and L. P. performed the experiments. E. B. and B. F. contributed reagents/materials/analysis tools. L. P., M. L., and F. P. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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Competing interests: The authors declare that they have no competing interests.

Data availability: All data is contained within the manuscript and supplemental materials.
REFERENCES


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Ubiquinone is essential for denitrification in Pseudomonas aeruginosa


**Ubiquinone is essential for denitrification in Pseudomonas aeruginosa**

Table 1: Spectral characterization of UbiV<sub>Pa</sub> and its variants.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>A&lt;sub&gt;280&lt;/sub&gt;/A&lt;sub&gt;410&lt;/sub&gt;</th>
<th>Iron</th>
<th>Sulfur</th>
</tr>
</thead>
<tbody>
<tr>
<td>UbiV&lt;sub&gt;Pa&lt;/sub&gt; WT</td>
<td>5.8</td>
<td>3.90 ± 0.03</td>
<td>3.40 ± 0.20</td>
</tr>
<tr>
<td>UbiV&lt;sub&gt;Pa&lt;/sub&gt; C39AC180A</td>
<td>9.0</td>
<td>1.10 ± 0.16</td>
<td>1.60 ± 0.19</td>
</tr>
<tr>
<td>UbiV&lt;sub&gt;Pa&lt;/sub&gt; C193AC197A</td>
<td>7.8</td>
<td>2.90 ± 0.05</td>
<td>3.00 ± 0.12</td>
</tr>
<tr>
<td>UbiV&lt;sub&gt;Pa&lt;/sub&gt; C39AC193AC197A</td>
<td>nd</td>
<td>nd</td>
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</table>

1Iron and sulfur quantification of UbiV<sub>Pa</sub> and its variants. Shown are metal content and UV-vis properties after anaerobic reconstitution of their [Fe-S] clusters for wild-type and variants. nd: not determined.
Ubiquinone is essential for denitrification in Pseudomonas aeruginosa

![Graph](image)

Figure 1: UQ₉ is the major quinone used by *P. aeruginosa* in aerobic and anaerobic conditions. HPLC-ECD analysis of lipid extracts from 1 mg of cells after growth of *E. coli* MG1655 (E. c.) and *P. aeruginosa* PAO1 (P. a.) aerobically (+O₂) in LB medium (A) or anaerobically (-O₂) in denitrification medium (B). The chromatograms are representative of three independent experiments. The peaks corresponding to UQ₈, UQ₉, DMK₈, MK₈ and the UQ₁₀ as a standard are indicated. C, Mass spectrum of the quinone eluting at 11.5 min from extracts of *P. aeruginosa* grown in aerobic and anaerobic cultures.
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**Figure 2:** Genomic localization of the *ubiUVT* operon in *P. aeruginosa* PAO1. ORFs of the genes *ubiT*<sub>Pa</sub>, *ubiU*<sub>Pa</sub> and *ubiV*<sub>Pa</sub> are represented by grey arrows.
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Figure 3: *ubiU*<sub>Pa</sub>, *ubiV*<sub>Pa</sub> and *ubiT*<sub>Pa</sub> are essential genes for anaerobic UQ<sub>9</sub> biosynthesis and for denitrification. Representative growth curves of wild-type PAO1 and *ubiTUV*-KO strains grown (A) in denitrification medium or (C) aerobically in LB medium. B, Quantification of cellular UQ<sub>9</sub> content (*n*=3) in lipid extracts from wild-type PAO1 and KO cells grown aerobically in LB medium (white bars, *P* > 0.05 by unpaired Student’s *t* test) or in denitrification medium (grey bars, **** *P* < 0.0001 by unpaired Student’s *t* test). Error bars represent S.D.
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**Figure 4:** Complementation of *ubiTUV*-KO strains restores bacterial growth over the entire O₂ range in a UQ-dependent manner. A, Photographs of culture tubes after overnight growth under anaerobic conditions in denitrification medium of *ubiTUV*-KO strains transformed with the empty vector pSW196 or the same vector carrying the corresponding wild-type allele (*ubiT*<sub>Pa</sub>, *ubiU*<sub>Pa</sub> and *ubiV*<sub>Pa</sub>). The parental strain PAO1 was used as a control and UQ<sub>9</sub> content of wild-type and *ubiTUV*-KO strains cultured anaerobically was assayed (n=3). B, *ubiTUV*-KO strains were cultured in denitrification medium supplemented with methanol-solubilized UQ<sub>4</sub> at 5 or 50 µM final concentration. After 24h of incubation, colony-forming units per mL (CFU/mL) of each KO strain were estimated and compared to the same strain grown without UQ<sub>4</sub>. As a control (*P > 0.05 by unpaired Student’s t test), toxicity of methanol was tested on the parental strain grown in the same medium but supplemented with 4.5% (v/v) methanol (Ct), which is the final concentration of methanol corresponding to the adding of 50 µM of UQ<sub>4</sub>. Data are representative of three independent experiments (**P < 0.01; ***P < 0.001; ****P < 0.0001; by...
Ubiquinone is essential for denitrification in *Pseudomonas aeruginosa*

unpaired Student’s t test compared to condition without addition of UQ; ns: no significant). C, As in A, but in soft-agar tubes after overnight culture. All the strains studied were inoculated into anaerobic tubes and then exposed to ambient air to create an oxygen gradient. The controls (Ct) correspond to soft agar tubes supplemented with 2.5 μg/mL resazurin and then incubated with (1) or without air (2). Oxic and anoxic parts of the agar are indicated. For all strains containing pSW196 vectors, denitrification medium was also supplemented with 0.1% (w/v) final concentration of arabinose to induce *P*<sub>BAD</sub> promoter.
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Figure 5: Recombinant UbiV<sub>Pa</sub> is a [4Fe-4S] cluster-containing protein. A, UV-visible absorption of as-purified UbiV<sub>Pa</sub> (dotted line, 32.6 µM) and reconstituted holo-UbiV<sub>Pa</sub> (solid line, 22.7 µM). The inset is an enlargement of the 300 to 800 nm region. The molar extinction coefficient, ε<sub>410nm</sub> was determined to be 12.95 ± 0.5 mM<sup>-1</sup> cm<sup>-1</sup> for holo-UbiV<sub>Pa</sub>. B, Comparative UV-visible absorption spectra of wild-type and different Cys-to-Ala mutants of UbiV<sub>Pa</sub> after metal cluster reconstitution. Proteins were analyzed at the following concentrations: 22.7 µM WT, 34.8 µM C39AC180A and 15.9 µM C193AC197A. Proteins were suspended in buffer 50 mM Tris-HCl, 25 mM NaCl, 15% (v/v) glycerol, 1 mM DTT, pH 8.5.
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Figure 6: Recombinant UbiU_{Pa} and UbiT_{Pa} bind UQ_{8}. A, Elution profile of UbiU_{Pa}. 70 mg of protein were loaded on a Superdex 200 16/60 chromatography column. A quantification of UQ_{8} and DMQ_{8} in each fraction was performed by HPLC-ECD MS. A recovery of 73 and 76%, respectively for UQ_{8} and DMQ_{8}, was calculated from the total content of all fractions compared with content of the UbiU_{Pa} purified fraction deposited on the Superdex 200 column. B, Fractions 10 to 44 analyzed by SDS-PAGE for purity. C, Elution profile of UbiT_{Pa} on a Superdex 200 16/60 column. Inset, SDS-PAGE, lane 1, 32 kDa TrxA-UbiT_{Pa} fusion protein, lane 2, after digestion with thrombin (UbiT_{Pa}, 19.6kDa and TrxA, 12.1 kDa), lane 3, pooled fractions 20-30 of UbiT_{Pa}. Quantification of UQ_{8} (pool of fractions 20 to 30) was performed by HPLC-ECD MS. D, Protein-lipid overlay assay between UbiU_{Pa} and UbiT_{Pa} and different lipid ligands. 2 µL of six different lipid/compound potential candidates (1, UQ_{8}; 2, UQ_{10}; 3, solanesol; 4, 3-methylcatechol; 5, cholesterol; 6, POPE) at 20 mM final concentration were spotted on a PVDF membrane and then incubated with UbiT_{Pa} or UbiU_{Pa} (both proteins at 0.2 µg/mL final concentration). Detection of bound proteins was performed by chemiluminescence, as described in Experimental Procedures section. Mw: molecular weights.