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## Ubiquinone Biosynthesis over the Entire O<sub>2</sub> Range: Characterization of a Conserved O<sub>2</sub>-Independent Pathway

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1 **Ubiquinone biosynthesis over the entire O<sub>2</sub> range: characterization of a conserved, O<sub>2</sub>-**  
2 **independent pathway**

3

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23

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25 Ubiquinone, iron-sulfur, anaerobic metabolism, hydroxylation, bioenergetics, proteobacteria, oxygen,  
26 peptidase U32, microaerobic respiration, facultative anaerobe

27

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## 30 **SUMMARY**

31 Most bacteria can generate ATP by respiratory metabolism, in which electrons are shuttled from reduced  
32 substrates to terminal electron acceptors, via quinone molecules like ubiquinone. Dioxygen (O<sub>2</sub>) is the  
33 terminal electron acceptor of aerobic respiration and serves as a co-substrate in the biosynthesis of  
34 ubiquinone. Here, we characterize a novel, O<sub>2</sub>-independent pathway for the biosynthesis of ubiquinone.  
35 This pathway relies on three proteins UbiT (YhbT), UbiU (YhbU) and UbiV (YhbV). UbiT contains an SCP2  
36 lipid-binding domain and is likely an accessory factor of the biosynthetic pathway, while UbiU-UbiV are  
37 involved in hydroxylation reactions and represent a novel class of O<sub>2</sub>-independent hydroxylases. We  
38 demonstrate that UbiU-UbiV form a heterodimer, wherein each protein binds a 4Fe-4S cluster via  
39 conserved cysteines that are essential for activity. The UbiT, -U, -V proteins are found in  $\alpha$ -,  $\beta$ -,  $\gamma$ -  
40 proteobacterial clades including several human pathogens, supporting the widespread distribution of a  
41 previously-unrecognized capacity to synthesize ubiquinone in the absence of O<sub>2</sub>. Together, the O<sub>2</sub>-  
42 dependent and O<sub>2</sub>-independent ubiquinone biosynthesis pathways contribute to optimize bacterial  
43 metabolism over the entire O<sub>2</sub> range.

44

## 45 **IMPORTANCE**

46 In order to colonize environments with large O<sub>2</sub> gradients or fluctuating O<sub>2</sub> levels, bacteria have  
47 developed metabolic responses that remain incompletely understood. Such adaptations have been  
48 recently linked to antibiotic resistance, virulence and the capacity to develop in complex ecosystems like  
49 the microbiota. Here, we identify a novel pathway for the biosynthesis of ubiquinone, a molecule with a  
50 key role in cellular bioenergetics. We link three uncharacterized genes of *Escherichia coli* to this pathway  
51 and show that the pathway functions independently from O<sub>2</sub>. In contrast, the long-described pathway  
52 for ubiquinone biosynthesis requires O<sub>2</sub> as substrate. In fact, we find that many proteobacteria are  
53 equipped with the O<sub>2</sub>-dependent and O<sub>2</sub>-independent pathways, supporting that they are able to  
54 synthesize ubiquinone over the entire O<sub>2</sub> range. Overall, we propose that the novel O<sub>2</sub>-independent  
55 pathway is part of the metabolic plasticity developed by proteobacteria to face varying environmental O<sub>2</sub>  
56 levels.

57

58

## 59 INTRODUCTION

60 Since the oxygenation of the Earth's atmosphere some 2.3 billion years ago, many organisms  
61 adopted dioxygen (O<sub>2</sub>) as a terminal electron acceptor of their energy-producing respiratory chains [1].  
62 Indeed, oxygenic (aerobic) respiration has a superior energetic output compared to anaerobic respiration  
63 or fermentation, which are both O<sub>2</sub>-independent processes [1]. In fact, several microorganisms, including  
64 many important human pathogens, are facultative anaerobes that are able to adopt either an aerobic or  
65 an anaerobic lifestyle depending on the environmental conditions [2,3]. In the laboratory, bacteria are  
66 usually cultured and studied under fully aerobic or completely anaerobic conditions (absence of O<sub>2</sub>) [4],  
67 whereas natural habitats cover the entire range of O<sub>2</sub> concentrations [5]. For instance, large O<sub>2</sub> gradients  
68 are typically encountered in the human large intestine, in biofilms or in transition zones between oxic  
69 and anoxic environments [5]. Moreover, bacteria can experience rapid transitions between  
70 environments with vastly different O<sub>2</sub> contents, as during the infection process of enteric pathogens that  
71 progress along the gastrointestinal tract [3].

72 To maximize their bioenergetic capacities according to the various levels of O<sub>2</sub> encountered in  
73 their environment, bacteria modulate the composition of their respiratory chains, notably the quinone  
74 species and the terminal reductases [3,4,6]. Quinones are lipophilic redox molecules that fuel electrons  
75 to terminal reductases, which reduce O<sub>2</sub> whenever available, or alternative electron acceptors for  
76 instance nitrate, dimethyl sulfoxide (DMSO), trimethylamine N-oxide [7]. Naphtoquinones (menaquinone  
77 (MK) and demethylmenaquinone (DMK)) and ubiquinone (UQ) are the two main groups of bacterial  
78 quinones. (D)MK and UQ differ by the nature of their head group and the value of their redox midpoint  
79 potential [8]. (D)MK are considered "anaerobic quinones" since they function primarily in anaerobic  
80 respiration whereas UQ is considered an "aerobic quinone" because it supplies electrons mostly to the  
81 reductases that reduce O<sub>2</sub> [1,8,9]. Accordingly, UQ is the main quinone of the facultative anaerobe  
82 *Escherichia coli* in aerobic conditions, whereas the naphtoquinones are predominant in the absence of O<sub>2</sub>  
83 [10,11], UQ being nevertheless present.

84 The biosynthesis of UQ requires a total of eight reactions to modify the aromatic ring of the  
85 precursor, 4-hydroxybenzoic acid (4-HB): one prenylation, one decarboxylation, three hydroxylation and  
86 three methylation reactions (Figure 1A) [12]. In addition to the enzymes that catalyze the various steps,  
87 three accessory factors - UbiB, UbiJ and UbiK – are also needed. UbiB has an ATPase activity [13] and we  
88 showed that UbiJ and UbiK [14,15] belong to a multiprotein UQ biosynthesis complex, in which the SCP2  
89 domain (sterol carrier protein 2) of UbiJ binds the hydrophobic UQ biosynthetic intermediates [16]. This

90 UQ biosynthetic pathway is under the dependence of O<sub>2</sub> since all three hydroxylases - UbiI, UbiH and  
91 UbiF – use O<sub>2</sub> as a cosubstrate (Figure 1A) [17,18]. We showed recently that other hydroxylases, UbiL,  
92 UbiM and Coq7, replace UbiI, UbiH and UbiF in some proteobacteria [19]. The six hydroxylases have in  
93 common their dependence to O<sub>2</sub> and thus function in UQ biosynthesis only when sufficient O<sub>2</sub> is  
94 available. Interestingly, Alexander and Young established 40 years ago that *E. coli* was able to synthesize  
95 UQ anaerobically [20], suggesting the existence of an O<sub>2</sub>-independent biosynthesis pathway, which is still  
96 uncharacterized.

97           In this study, we describe the O<sub>2</sub>-independent UQ biosynthetic pathway in *E. coli* and identify  
98 three essential components, the UbiT, UbiU and UbiV proteins, formerly called YhbT, YhbU and YhbV.  
99 We show that the O<sub>2</sub>-independent UQ biosynthetic pathway is widely conserved in proteobacteria. UbiT  
100 likely functions as an accessory factor in the O<sub>2</sub>-independent UQ biosynthetic pathway and we show that  
101 UbiU and UbiV are involved in at least one O<sub>2</sub>-independent hydroxylation reaction. Moreover, we  
102 demonstrate that both UbiU and UbiV bind a [4Fe-4S] cluster essential for activity, which identifies these  
103 proteins as prototypes of a new class of O<sub>2</sub>-independent hydroxylases. Our results highlight that many  
104 proteobacterial species use two different and complementary molecular pathways to produce UQ over  
105 the entire continuum of environmental O<sub>2</sub>.

106

## 107 RESULTS

### 108 4-HB is the precursor of UQ synthesized in anaerobic conditions:

109 4-HB is the precursor of UQ synthesized in aerobic conditions [21]. Accordingly, an *E. coli*  $\Delta ubiC$   
110 mutant impaired in 4-HB biosynthesis [22], is deficient in UQ<sub>8</sub> and is complemented by addition of 4-HB  
111 to the growth medium [23]. In order to evaluate whether 4-HB is also the precursor of the O<sub>2</sub>-  
112 independent UQ biosynthetic pathway, we grew a  $\Delta ubiC$  strain anaerobically. The  $\Delta ubiC$  strain showed a  
113 diminished level of UQ<sub>8</sub>, which was partially recovered by supplementation with 4-HB (Figure 1B).  
114 Furthermore, we grew the  $\Delta ubiC$  strain in a medium supplemented with <sup>13</sup>C<sub>7</sub>-4HB and we analyzed the  
115 labelling of biosynthesized UQ<sub>8</sub> by HPLC-mass spectrometry (MS). In cells grown in aerobic or anaerobic  
116 conditions, the labelled form of UQ (<sup>13</sup>C<sub>6</sub>-UQ<sub>8</sub>, m/z= 750.4 for the NH<sub>4</sub><sup>+</sup> adduct) represented 98.3% and  
117 97.3% (±0.2%) of the total UQ<sub>8</sub> pool, respectively (Figure 1C, 1D). As expected,  $\Delta ubiC$  cells grown  
118 anaerobically with unlabeled 4-HB didn't show any <sup>13</sup>C<sub>6</sub>-UQ<sub>8</sub> (Figure 1E). Altogether, these results  
119 establish that 4-HB is the precursor of the O<sub>2</sub>-independent UQ biosynthetic pathway.

### 120 Ubi enzymes, except hydroxylases, are common to the aerobic and anaerobic UQ biosynthesis 121 pathways:

122 The above result suggests that the UQ biosynthetic pathways decorate 4-HB with the same  
123 chemical groups irrespective of the presence of environmental O<sub>2</sub>. Thus, we evaluated whether the  
124 enzymes of the aerobic pathway are also involved in the O<sub>2</sub>-independent pathway by measuring the UQ<sub>8</sub>  
125 content of knock-out (KO) strains grown in aerobic and anaerobic conditions. Deletion of *ubiA*, *ubiE* or  
126 *ubiG* abrogated UQ<sub>8</sub> biosynthesis in both conditions whereas  $\Delta ubiB$ ,  $\Delta ubiD$  or  $\Delta ubiX$  strains synthesized a  
127 limited amount of UQ<sub>8</sub> but only in aerobic conditions (Figure 1F). In contrast, *ubiJ* and *ubiK* had no effect  
128 on UQ biosynthesis under anaerobic conditions (Figure 1F).

129 In aerobic conditions, the hydroxylation reactions are catalyzed by the flavin monooxygenases  
130 (FMOs) UbiF, UbiH and UbiI that use dioxygen as a co-substrate [17,18,24] (Figure 1A). We previously  
131 reported that cells deleted for a single O<sub>2</sub>-dependent hydroxylase ( $\Delta ubiF$ ,  $\Delta ubiI$  or  $\Delta ubiH$ ) were deficient  
132 in UQ, when cultured in the presence of air, but synthesized UQ in anaerobic conditions [17], consistent  
133 with the existence of an alternative hydroxylation system in the O<sub>2</sub>-independent pathway [20]. Indeed,  
134 we confirmed that all three FMOs are dispensable for the O<sub>2</sub>-independent UQ biosynthetic pathway  
135 since a triple mutant  $\Delta ubiF \Delta ubiI \Delta ubiH$  was deficient for UQ when grown in air, but synthesized wild-  
136 type (WT) level of UQ<sub>8</sub> in anaerobic conditions (Figure 1G). Together, our results demonstrate that the

137 O<sub>2</sub>-dependent and O<sub>2</sub>-independent UQ biosynthetic pathways share the enzymes involved in the  
138 prenylation (UbiA), decarboxylation (UbiX and UbiD) and methylation reactions (UbiE and UbiG), but  
139 differ by their hydroxylases and the accessory factors UbiJ and UbiK (Figure 1H).

#### 140 **Identification of three genes required for UQ biosynthesis in anaerobic conditions:**

141 To identify genes involved in the O<sub>2</sub>-independent UQ biosynthetic pathway, we cultivated  
142 anaerobically a collection of ~ 200 *E. coli* strains that contained deletions covering multiple ORFs [25,26]  
143 and we analyzed their UQ<sub>8</sub> content by HPLC-electrochemical detection (HPLC-ECD). We found a complete  
144 absence of UQ<sub>8</sub> in strains ME4561, ME5034 and ME4746 that carry deletions encompassing, *ubiE-ubiJ-*  
145 *ubiB-ubiD*, *ubiG*, and *ubiX*, respectively (Table S1). Several other strains had a low UQ<sub>8</sub> content and a  
146 poor growth in synthetic medium supplemented with glycerol and nitrate (SMGN). However, those  
147 strains showed better growth and higher UQ<sub>8</sub> content in LB medium (Table S1). Thus we did not  
148 investigate them further, as a genetic defect affecting directly the O<sub>2</sub>-independent UQ pathway was  
149 unlikely. In contrast, ME4641 showed a profound UQ<sub>8</sub> deficiency and robust anaerobic growth in LB and  
150 SMGN media (Table S1). Importantly, ME4641 had a WT UQ<sub>8</sub> level when grown aerobically, suggesting  
151 that only the O<sub>2</sub>-independent pathway was altered (Figure 2A). ME4641 contains a deletion named  
152 OCL30-2 that covers 9 genes, 5 of them lacking an identified function (Figure 2B). To find the candidate  
153 gene involved in the anaerobic biosynthesis of UQ, we obtained 8 single gene KO strains from the Keio  
154 collection [27] and analyzed their quinone content after anaerobic growth (Figure 2C). The  $\Delta yhbT$  and  
155  $\Delta yhbU$  strains were strongly deficient in UQ<sub>8</sub>. We then transduced the  $\Delta yhbT$  and  $\Delta yhbU$  mutations from  
156 the Keio strains into a MG1655 genetic background and also constructed the  $\Delta yhbV$  strain, which was  
157 not available in the Keio collection. We found that all three strains had very low levels of UQ<sub>8</sub> when  
158 grown in anaerobic conditions but showed normal levels after aerobic growth (Figure 2D, Table 1). In  
159 addition, the mutant strains showed a two-fold decrease in MK<sub>8</sub> and a two-fold increase in DMK<sub>8</sub> after  
160 anaerobic growth (Table 1). This effect might indirectly result from the UQ<sub>8</sub> deficiency as it was also  
161 observed in the  $\Delta ubiG$  strain (Figure S1A).

162

culture condition	strain	UQ <sub>8</sub> (pmoles/mg cells)	DMK <sub>8</sub> (247nm peak area/mg cells)	MK <sub>8</sub> (247nm peak area/mg cells)
Air	WT	208 ± 9,4	0,94 ± 0,26	0,15 ± 0,09
Air	<i>ΔyhbT</i>	193,9 ± 12,4	1,34 ± 0,05	0,13 ± 0,03
Air	<i>ΔyhbU</i>	233,8 ± 16,2	0,9 ± 0,26	0,16 ± 0,09
Air	<i>ΔyhbV</i>	227,9 ± 25,1	0,93 ± 0,15	0,13 ± 0,04
anaerobic	WT	114,2 ± 19,1	1,28 ± 0,4	0,41 ± 0,15
anaerobic	<i>ΔyhbT</i>	3,3 ± 0,4	2,88 ± 0,47	0,17 ± 0,01
anaerobic	<i>ΔyhbU</i>	6,1 ± 3,3	2,55 ± 0,55	0,19 ± 0,01
anaerobic	<i>ΔyhbV</i>	7,3 ± 4,7	2,61 ± 0,49	0,21 ± 0,01
anaerobic	<i>ΔyhbT</i> +pK	2 ± 0,4	2,46 ± 0,33	0,16 ± 0,04
anaerobic	<i>ΔyhbT</i> +pK- <i>yhbT</i>	64,6 ± 16,1	1,77 ± 0,29	0,3 ± 0,04
anaerobic	<i>ΔyhbU</i> +pBAD	1 ± 0,6	2,29 ± 0,58	0,18 ± 0,04
anaerobic	<i>ΔyhbU</i> +pBAD- <i>yhbU</i>	82 ± 15,9	1,2 ± 0,18	0,27 ± 0,04
anaerobic	<i>ΔyhbV</i> +pBAD	2,2 ± 3,5	2,14 ± 0,15	0,23 ± 0,01
anaerobic	<i>ΔyhbV</i> +pBAD- <i>yhbV</i>	120,4 ± 18,5	1,87 ± 0,19	0,42 ± 0,06

163

164 **Table 1:** Quinone content of WT and *Δyhb* strains cultured in SMGN either aerobically or anaerobically,  
 165 n=3-5. For strains containing pK or pBAD vectors, SMGN was supplemented with 0.02% arabinose.

166

167 **Deletion of *yhbT*, *yhbU* or *yhbV* causes UQ<sub>8</sub> deficiency specifically in anaerobic conditions:**

168 We then transformed the mutant strains with an empty vector or a vector carrying a WT allele of  
 169 the studied gene. In *yhb* KO strains expressing the corresponding gene from the plasmid, we observed a  
 170 complementation of the UQ<sub>8</sub> deficiency (Table 1) and a normalization of the levels of octaprenylphenol  
 171 (OPP), an early UQ<sub>8</sub> biosynthetic intermediate (Figure 2E). The ~3 fold elevation of OPP in the *yhbT*, -*U*, -  
 172 *V* KO mutants suggested that the O<sub>2</sub>-independent UQ biosynthetic pathway was blocked downstream of  
 173 OPP in these strains. In these experiments, no cross-complementation was observed - for example the  
 174 plasmid with *yhbV* had no effect in *ΔyhbT* or *ΔyhbU* strains - suggesting the absence of redundancy in  
 175 the function of each gene. We also measured a profound UQ<sub>8</sub> deficiency when the *ΔyhbT*, *ΔyhbU* and  
 176 *ΔyhbV* strains were grown anaerobically in various media (glycerol + DMSO, lactate + KNO<sub>3</sub>) (Figure 2F),  
 177 showing that the UQ<sub>8</sub> biosynthetic defect is not linked to a particular carbon source or electron acceptor.  
 178 Altogether, our results demonstrate that the *yhbT*, *yhbU* and *yhbV* genes are part of the O<sub>2</sub>-independent  
 179 UQ biosynthetic pathway, so we propose to rename them *ubiT*, *ubiU* and *ubiV*, respectively.



180 **Complete absence of UQ biosynthesis in  $\Delta ubiT$ ,  $\Delta ubiU$  or  $\Delta ubiV$  mutants grown under strict anaerobic**  
181 **conditions:**

182 As the  $\Delta ubiT$ ,  $\Delta ubiU$  or  $\Delta ubiV$  strains still contained small amounts of UQ<sub>8</sub> after growth in  
183 anaerobic conditions (Table 1, Figure 2F), we wondered how this UQ<sub>8</sub> was synthesized. To verify if the  
184 O<sub>2</sub>-dependent pathway contributed to this synthesis, we inactivated the *ubiH* gene in the  $\Delta ubiU$  strain.  
185 Sensitive HPLC-MS detection established that UQ<sub>8</sub> was completely absent in extracts from the  $\Delta ubiH$   
186  $\Delta ubiU$  cells (Figure 2G). This result supported that the residual UQ<sub>8</sub> synthesized in  $\Delta ubiU$  cells originated  
187 from the O<sub>2</sub>-dependent pathway and suggested that our anaerobic media may contain traces amounts of  
188 O<sub>2</sub> or that O<sub>2</sub>-dependent UQ biosynthesis may occur during the handling of cells, under normal  
189 atmosphere, prior to quinone extraction.

190 To eliminate the traces of O<sub>2</sub>, we took extra precautions in the degassing and inoculation of our  
191 media (see Material and Methods) and also added a reductant (L-Cysteine). In addition, we used the  
192 redox indicator resazurin to verify strict anaerobiosis during the entire culture. We also modified our  
193 sampling procedure to rapidly quench the anaerobic cells in ice-cold methanol, in order to prevent any  
194 O<sub>2</sub>-dependent UQ biosynthesis prior to quinone extraction. HPLC-MS analysis of extracts from cells  
195 cultivated and handled under such strict anaerobic conditions showed the nearly complete absence of  
196 UQ<sub>8</sub> in  $\Delta ubiT$ ,  $\Delta ubiU$  and  $\Delta ubiV$  strains (Figure 2H, 2I). In contrast, the UQ<sub>8</sub> level of the WT strain (Figure  
197 2I) was comparable to those we measured previously in WT cells cultivated in suboptimal anaerobic  
198 conditions (Table 1) [14], establishing that UQ biosynthesis occurred independently from O<sub>2</sub>. Together,  
199 our results show that  $\Delta ubiT$ ,  $\Delta ubiU$  and  $\Delta ubiV$  cells are unable to synthesize UQ under strict anaerobic  
200 conditions unlike WT cells. Furthermore our results support that the low residual UQ content previously  
201 observed in  $\Delta ubiT$ ,  $\Delta ubiU$  and  $\Delta ubiV$  strains (Table 1, Figure 2F) resulted from the function of the O<sub>2</sub>-  
202 dependent pathway.

203 ***ubiT*, -*U*, -*V* strongly co-occur, exclusively in genomes with potential for UQ biosynthesis**

204 We investigated the distribution of *ubiT*, -*U* and -*V* in a large genome dataset gathering 5750  
205 genomes of bacteria and archaea. We found no evidence of genomes harboring matches for more than  
206 one of the three genes of interest outside of Alphaproteobacteria, Betaproteobacteria and  
207 Gammaproteobacteria ( $\alpha$ -,  $\beta$ -,  $\gamma$ -proteobacteria) and three genomes of Acidithiobacillia (Table S2A).  
208 Interestingly, the three former classes are the only known so far to be able to produce UQ [1,8],  
209 consistent with a specific link between UbiT, -U, -V and UQ biosynthesis.

210 This was confirmed by analyzing in more details the distribution of *ubiT*, *-U* and *-V* and of three  
211 marker genes of the UQ biosynthetic pathway (*ubiA*, *ubiE* and *ubiG*) in 611 representative and reference  
212 genomes of  $\alpha$ -,  $\beta$ -,  $\gamma$ -proteobacteria (Table S2B). We chose *ubiA*, *-G*, *-E* because they have a higher  
213 conservation compared to other genes like *ubiC*, *-D*, *-X* [28] and because they are part of the O<sub>2</sub>-  
214 dependent and O<sub>2</sub>-independent pathways (Figure 1H). 575 genomes had positive matches for *ubiA*, *-G*, *-E*  
215 and 589 for at least two of them. Regarding the distribution of *ubiT*, *-U* and *-V*, 221 genomes had  
216 matches for at least one of the three genes, and in 210 cases (95%), the three genes were present.  
217 Importantly, all genomes with *ubiT*, *-U* and *-V* harbored at least two of the three marker genes for the  
218 UQ biosynthetic pathway (Table S2B). In addition, we found that 22 out of the 29 proteobacterial orders  
219 analyzed had up to 50% genomes harboring a complete set of the *ubiT*, *-U* and *-V* genes (Figure 3A),  
220 demonstrating a wide taxonomic distribution. Overall, our analysis indicates a strong pattern of co-  
221 occurrence of *ubiT*, *-U* and *-V*, and demonstrates that they are uniquely found in genomes showing signs  
222 of UQ production.

223 We found *ubiA*, *-G*, *-E* and O<sub>2</sub>-dependent hydroxylase genes in 570 genomes, of which 204 also  
224 contained *ubiT*, *-U* and *-V* (Table S2B and Figure 3B). Only 3 species, *Phaeospirillum fulvum*,  
225 *Magnetococcus marinus* and *Oxalobacter formigenes*, seem to rely exclusively on the O<sub>2</sub>-independent  
226 pathway for UQ production as their genomes contain the *ubiT*, *-U* and *-V* genes but no O<sub>2</sub>-dependent  
227 hydroxylases (Figure 3B). We noticed that the *ubiT*, *-U* and *-V* genes are found next to other *ubi* genes in  
228 *M. marinus* (Figure S1B). Interestingly, *P. fulvum* and *M. marinus* were described to synthesize UQ in  
229 microaerobic conditions [29-31] and *O. formigenes* has been documented as an obligate anaerobe [32].  
230 Together, our results show that the O<sub>2</sub>-independent UQ biosynthesis pathway is widespread in  $\alpha$ -,  $\beta$ -,  $\gamma$ -  
231 proteobacterial orders and co-exists with the O<sub>2</sub>-dependent pathway in 98% of the cases.

232 We then looked into the relative positioning of *ubiT*, *-U* and *-V* in the 210 genomes harboring the  
233 three genes. We found 106 cases, covering  $\alpha$ -,  $\beta$ -,  $\gamma$ -proteobacterial orders, where they were encoded  
234 next to each other ("3-genes loci") and 82 cases of a 2-genes locus with the third gene being encoded  
235 elsewhere in the genome (Figure 3C). Evaluation of the genetic architecture of *ubiT*, *-U* and *-V* revealed  
236 that *ubiU* and *ubiV* were found exactly next to each other in 69% of the loci (all 3-genes loci and in 39/82  
237 of the 2-genes loci) and that the three genes were encoded in three separate parts of the genome only in  
238 21 cases (Figure 3C). Interestingly, as an additional support for their involvement in a same function, we  
239 found an example of a gene fusion between *ubiT* and *ubiU* in two genomes from *Zymomonas mobilis*

240 strains ( $\alpha$ -proteobacteria), which also contain an *ubiV* gene directly downstream of the fused gene  
241 (Figure 3C).

## 242 **UbiT is an SCP2 protein and UbiU-V are required for the O<sub>2</sub>-independent hydroxylation of DMQ<sub>8</sub>**

243 We then analyzed the sequences of the UbiT, UbiU and UbiV proteins. The major part of UbiT  
244 (amino acids 45-133, on a total of 174 aa in the *E. coli* protein) corresponds to a SCP2 domain (PFAM:  
245 PF02036, Figure S2). SCP2 domains typically form a hydrophobic cavity that binds various lipids [33] and  
246 our sequence alignment indeed showed the conservation of hydrophobic amino acids at several  
247 positions in the SCP2 domain of UbiT (Figure S2). Recently, we reported that UbiJ binds UQ biosynthetic  
248 intermediates in its SCP2 domain and organizes a multiprotein complex composed of several Ubi  
249 enzymes [16]. We propose that UbiT and its SCP2 domain may fulfill similar functions in the O<sub>2</sub>-  
250 independent UQ pathway, as UbiJ is required exclusively for the O<sub>2</sub>-dependent biosynthesis of UQ  
251 (Figure 1F).

252 UbiU and UbiV have ~330 and 300 aa respectively, and contain an uncharacterized motif called  
253 peptidase U32 (PF01136) (Figure S3 and S4). Since only the hydroxylation reactions are uncharacterized  
254 in the O<sub>2</sub>-independent pathway (Figure 1H), we hypothesized that UbiU and UbiV might function in these  
255 steps. To test our hypothesis, we developed an *in vivo* assay based on the O<sub>2</sub>-independent conversion of  
256 labeled DMQ<sub>8</sub> into labeled UQ<sub>8</sub>. This assay monitors the C6-hydroxylation and the subsequent O6-  
257 methylation (Figure 4A).  $\Delta ubiC \Delta ubiF$  cells grown aerobically with <sup>13</sup>C<sub>7</sub>-4HB synthesized DMQ<sub>8</sub>, 73% of  
258 which was labelled with <sup>13</sup>C<sub>6</sub>. Upon transfer to anaerobic conditions, the cells gradually converted a  
259 significant part of (<sup>13</sup>C<sub>6</sub>)-DMQ<sub>8</sub> into (<sup>13</sup>C<sub>6</sub>)-UQ<sub>8</sub> (Figure 4B). Inactivation of either *ubiU* or *ubiV* in  $\Delta ubiC$   
260  $\Delta ubiF$  cells did not perturb the accumulation of (<sup>13</sup>C<sub>6</sub>)-DMQ<sub>8</sub> but prevented its conversion into (<sup>13</sup>C<sub>6</sub>)-UQ<sub>8</sub>  
261 (Figure 4C-D). This result demonstrates that UbiU and UbiV are essential for the C6-hydroxylation  
262 reaction of the O<sub>2</sub>-independent UQ biosynthetic pathway.

## 263 **UbiV contains a [4Fe-4S] cluster**

264 To get insights into the potential presence of cofactors in UbiU and UbiV, we attempted to  
265 characterize them biochemically. UbiU was not soluble but we purified UbiV<sub>6His}</sub>, which behaved as a  
266 monomer in solution (Figure S5A-B). UbiV was slightly pink-colored and had a UV-visible absorption  
267 spectrum with features in the 350-550 nm region (Figure 5A, dotted line), suggesting the presence of  
268 iron-sulfur (Fe-S) species [34-36]. We indeed detected substoichiometric amounts of iron and sulfur (0.2  
269 Fe and 0.2 S/monomer), indicating a potential oxidative degradation of the Fe-S cluster during aerobic

270 purification, as already observed with many other Fe-S proteins [37,38]. Consistent with this hypothesis,  
 271 anaerobic reconstitution of the Fe-S cluster yielded a UbiV protein with 3.9 iron and 3.3 sulfur/monomer  
 272 (Table 2) and with a UV-Vis spectrum characteristic of a  $[4\text{Fe-4S}]^{2+}$  cluster [39] (Figure 5A, solid line), that  
 273 was affected by exposure to air (Figure S5C). The electron paramagnetic resonance (EPR) spectrum of  
 274 the cluster reduced anaerobically displayed features characteristic of a  $[4\text{Fe-4S}]^{1+}$  cluster in the  $S = 1/2$   
 275 state (Figure 5B) [38,40]. Overall, we conclude that, under anaerobic conditions, UbiV is able to bind one  
 276 air-sensitive, redox-active  $[4\text{Fe-4S}]$  cluster.

277 Fe-S clusters are typically coordinated by cysteine residues [41,42] and we obtained evidence  
 278 that the  $[4\text{Fe-4S}]$  cluster in UbiV is coordinated by four conserved cysteines arranged in a  $\text{CX}_n\text{CX}_{12}\text{CX}_3\text{C}$   
 279 motif (Figure S4). Indeed, combinatorial elimination of C39, C180, C193, and C197 in double, triple and  
 280 quadruple mutants resulted in proteins incapable of binding  $[\text{Fe-S}]$  clusters *in vivo*, as shown by the  
 281 absence of absorption bands in the 350-550nm region of their UV-vis spectra (Figure S5D). Furthermore,  
 282 after anaerobic reconstitution of the cluster, the Fe and S contents, and the absorbance at 410 nm were  
 283 largely decreased in the double and triple mutants, and were undetectable in the quadruple mutant  
 284 (Table 2 and Figure 5C). At last, we found that the mutation of C180 or C193 altered the function of UbiV  
 285 *in vivo* (Figure 5D), suggesting that the  $[4\text{Fe-4S}]$  cluster is important for activity.

286

proteins	$A_{280}/A_{410}$	Iron content (nmoles/nmole of protein)	Sulfur content (nmoles/nmole of protein)
UbiV WT	5,8	$3.9 \pm 0.13$	$3.3 \pm 0.18$
UbiV C193A C197A	8,4	$2.4 \pm 0.02$	$2.2 \pm 0.13$
UbiV C39A C193A C197A	15,5	$1.9 \pm 0.01$	$1.5 \pm 0.4$
UbiV C180A C193A C197A	26,7	$0.7 \pm 0.05$	$0.6 \pm 0.1$
UbiV C39A C180A C193A C197A	57,4	$0.1 \pm 0.04$	0
UbiU WT - UbiV WT	4,3	$7.9 \pm 0.05$	$7.5 \pm 0.06$
UbiU C169A C176A - UbiV WT	7,3	$4.7 \pm 0.03$	$5.8 \pm 0.1$
UbiU C193A C232A - UbiV WT	8	$3.9 \pm 0.05$	$5.8 \pm 0.1$

287

288 **Table 2:** Characterization of UbiV proteins and UbiU-UbiV heterodimeric complexes (wild-type and  
 289 mutants): metal content, and UV-Vis properties after attempts of anaerobic reconstitution of their Fe-S  
 290 centers.

291

292 **UbiU contains a  $[4\text{Fe-4S}]$  cluster and forms a complex with UbiV**

293 We succeeded purifying UbiU after coexpressing it with UbiV<sub>6His</sub> (Figure S6A). The two proteins  
294 copurified in the form of a heterodimer (Figure S6A-B) that showed traces of Fe-S clusters (Figure 6A,  
295 dotted line), with substoichiometric amounts of iron and sulfide (0.4 Fe and 0.4 S/heterodimer).  
296 Reconstitution with iron and sulfide yielded a heterodimer with about 8 iron and 8 sulfur (Table 2) and a  
297 UV-visible spectrum characteristic of [4Fe-4S]<sup>2+</sup> clusters (Figure 6A, solid line). The EPR spectrum of  
298 reduced UbiU-UbiV was also consistent with the presence of 2 different [4Fe-4S] clusters since it showed  
299 a composite signal, which reflects the presence of two different S=1/2 species (Figure 6B).

300 Four strictly conserved cysteines are also found in UbiU (Figure S3) and we hypothesized that  
301 they might bind the [4Fe-4S]. We eliminated these cysteines in pairs and purified heterodimers  
302 composed of WT UbiV<sub>6His</sub> and mutant UbiU (Figure S6C). After reconstitution, UbiU C169A C176A-UbiV  
303 and UbiU C193A C232A-UbiV had about half the iron as the WT heterodimer (Table 2), and their A<sub>280</sub>/A<sub>410</sub>  
304 ratio were also diminished about two-fold (Figure 6C and Table 2). Altogether, our data clearly  
305 demonstrate that each protein of the heterodimeric UbiU-UbiV complex binds one [4Fe-4S] cluster and  
306 that the iron-chelating cysteines in UbiU are C169, C176, C193 and C232. Finally, an *in vivo*  
307 complementation assay demonstrated that C176 was important for the function of UbiU (Figure 6D).

### 308 **Many U32 proteases display motifs of four conserved cysteines**

309 The presence of Fe-S clusters in UbiU and UbiV, two U32 proteases family members, led us to  
310 evaluate the presence of conserved Cys motifs in other U32 proteins. Kimura *et al.* reported a  
311 phylogenetic tree of 3521 peptidase U32 domains which formed 12 groups, belonging to 10 protein  
312 families [43]. We extracted and aligned the sequences of the 10 protein families and found highly  
313 conserved 4-cysteines clusters (97-100% conservation) in eight of them (Figure 7), suggesting an  
314 important functional role for these residues. Only families PepU32#5 and PepU32#6 had respectively  
315 none, and two mildly (60-80%) plus three poorly conserved cysteines in their sequences (40-65%). The  
316 cysteine motifs for each of the eight families showed a high degree of conservation, and strikingly, most  
317 of them could even be aligned with each other, the CX<sub>6</sub>CX<sub>15</sub>CX<sub>3/4</sub>C patterns appearing recurrently (Figure  
318 7). To be noted, UbiV had a slightly distinct motif, with the first cysteine occurring much upstream of the  
319 three others and outside of the U32 domain. Overall, our data suggest that most members of the U32  
320 peptidase family may contain a 4Fe-4S cluster coordinated by conserved cysteines, similar to what we  
321 demonstrated for UbiU and UbiV.

322

## 323 **DISCUSSION**

### 324 **The O<sub>2</sub>-independent UQ biosynthesis pathway is widespread in proteobacteria:**

325 The evidence for an O<sub>2</sub>-independent synthesis of UQ by *E. coli* was reported more than forty  
326 years ago [20], yet this pathway remained uncharacterized until now. Circumstantial evidence had been  
327 obtained that a few species – limited, to our knowledge, to *E. coli* [20], *Rhodobacter sphaeroides* [44],  
328 *Paracoccus denitrificans* [45], *Halorhodospira halophila* [46] – were able to synthesize UQ in anaerobic  
329 conditions, as demonstrated by biochemical measurements of the quinone content of cells grown  
330 anaerobically. Here, we demonstrate that the O<sub>2</sub>-dependent and O<sub>2</sub>-independent UQ biosynthesis  
331 pathways differ only by three hydroxylation steps (Figure 1), and we identify three genes, *ubiT*, *-U*, *-V* that  
332 are essential for the O<sub>2</sub>-independent biosynthesis of UQ in *E. coli* (Figure 2). The facts that the UbiT, *-U*, *-*  
333 *V* proteins are widespread in  $\alpha$ ,  $\beta$ ,  $\gamma$ -proteobacterial clades (Figure 3) and co-occur with UbiA, *-E*, *-G*  
334 enzymes (Table S2B), reveal UbiT, *-U*, *-V* as key elements of the broadly-distributed, O<sub>2</sub>-independent UQ  
335 pathway. Overall, our data support that many proteobacteria have the previously-unrecognized capacity  
336 to synthesize UQ independently from O<sub>2</sub>.

### 337 **Physiological possibilities offered by UQ biosynthesis over the entire O<sub>2</sub> range:**

338 In our set of reference genomes, only three species (*P. fulvum*, *M. marinus* and *O. formigenes*)  
339 seem to rely exclusively on the O<sub>2</sub>-independent pathway for UQ production (Table S2B). Indeed, the vast  
340 majority of proteobacteria with the O<sub>2</sub>-independent UQ biosynthesis pathway also possess the O<sub>2</sub>-  
341 dependent hydroxylases of the aerobic pathway (207 out of 210) (Figure 3B). This result supports that  
342 both pathways confer physiological advantages, allowing production of UQ over the entire spectrum of  
343 O<sub>2</sub> levels encountered by facultative aerobes.

344 So-called microaerobes, able to respire O<sub>2</sub> in microaerobic conditions, are very abundant in  
345 Nature [5] and *E. coli* is known to respire nanomolar O<sub>2</sub> concentrations [47]. To sustain O<sub>2</sub> respiration in  
346 the microaerobic range, these organisms are equipped with high affinity O<sub>2</sub> reductases [5,47,48]. These  
347 enzymes reduce efficiently the low levels of environmental O<sub>2</sub> present at the cell's membrane, leaving  
348 the cytoplasm devoid of any O<sub>2</sub> [49]. Under such conditions, UQ - which is the main electron donor for  
349 the high affinity O<sub>2</sub> reductases *bdI* and *bdII* of *E. coli* [9] - must therefore be synthesized via the O<sub>2</sub>-  
350 independent pathway. Overall, we believe that the O<sub>2</sub>-independent UQ biosynthesis pathway operates  
351 not only in anaerobic conditions but also in microaerobic conditions, in which UQ is likely crucial for  
352 bacterial physiology.

353           The O<sub>2</sub>-independent UQ biosynthesis pathway may also confer a significant advantage to  
354 facultative bacteria in case of a rapid transition from an anaerobic to an aerobic environment. Indeed,  
355 anaerobic biosynthesis of UQ will result in cellular membranes containing UQ at the time of the  
356 transition, allowing an immediate switch to the energetically favorable metabolism of O<sub>2</sub> respiration. Our  
357 identification of the anaerobic UQ pathway provides the unique opportunity to selectively disrupt UQ  
358 biosynthesis depending on O<sub>2</sub> levels and should foster new research on bacterial physiology in the  
359 microaerobic range. Indeed, apart from *E. coli*, which was thoroughly studied over the microaerobic  
360 range [4,49,50], details on bacterial physiology in microaerobiosis are scarce.

#### 361 ***ubiT,U,V* mutants and pathogenicity:**

362           In addition to bioenergetics *per se*, anaerobic and microaerobic respirations are thought to be  
363 important for pathogenicity [3,51]. Interestingly, homologs of UbiT, -U, -V have been linked to  
364 pathogenicity in several bacterial models. Indeed, the inactivation of *ubiU-V* homologs in *Proteus*  
365 *mirabilis* leads to a decreased infection of the urinary tract of mice [52] and to a diminished virulence of  
366 *Yersinia ruckeri* [53], a pathogen that develops in the gut of fish, an environment with a notoriously low  
367 O<sub>2</sub> content. Furthermore, inactivation of PA3911 [54] (*ubiT*) and PA3912-PA3913 [55] (*ubiU-V*) in  
368 *Pseudomonas aeruginosa*, abolished nitrate respiration, the main anaerobic metabolism used by the  
369 bacterium in the lungs of cystic fibrosis patients [56,57]. Based on our results showing that the deletion  
370 of *ubiT*, *ubiU* or *ubiV* abrogates the O<sub>2</sub>-independent biosynthesis of UQ in *E. coli*, we suggest that the  
371 attenuation of the mutants discussed above results from their UQ deficiency in microaerobic / anaerobic  
372 conditions.

#### 373 **Proposed roles for UbiT, UbiU and UbiV:**

374           UbiT possesses an SCP2 domain, similar to UbiJ that we recently demonstrated to be an  
375 accessory factor that binds the hydrophobic UQ biosynthetic intermediates and structures a multiprotein  
376 Ubi complex [16]. Since UbiJ functions exclusively in the O<sub>2</sub>-dependent pathway, whereas UbiT is  
377 important only for the O<sub>2</sub>-independent pathway, we propose that UbiT may fulfill, in anaerobiosis, the  
378 same functions than UbiJ in aerobiosis. Whether UbiT is part of a complex and is able to bind UQ  
379 biosynthetic intermediates, will be addressed in future studies. Interestingly, PA3911, the homolog of  
380 UbiT in *P. aeruginosa*, was recently shown to bind phosphatidic acid [54], demonstrating an affinity of  
381 UbiT for lipid molecules.

382 UbiU and UbiV form a tight heterodimer suggesting that the proteins function together, as  
383 further supported by the fact that the *ubiU* and *ubiV* genes co-occur in genomes in 99% of the cases and  
384 that they are mostly found next to each other (Figure 3). We demonstrated that UbiU and UbiV are both  
385 required for the O<sub>2</sub>-independent C6-hydroxylation of DMQ and the accumulation of OPP in  $\Delta$ *ubiU* or  
386  $\Delta$ *ubiV* mutants suggests that the two proteins may also function in C5-hydroxylation. We want to  
387 emphasize our recent demonstration that a single hydroxylase catalyzes all three hydroxylation steps in  
388 the O<sub>2</sub>-dependent UQ pathway of *Neisseria meningitidis* [19]. This result showed that three different  
389 enzymes are not necessarily required and opens the possibility that UbiU-V may in fact catalyze all three  
390 hydroxylation reactions of the O<sub>2</sub>-independent UQ biosynthesis pathway. Establishing the hydroxylase  
391 activity and the regioselectivity of UbiU-V will require the development of an *in vitro* assay, a challenging  
392 task given that the oxygen donor of the reaction is currently unknown and that the substrates are not  
393 commercial and highly hydrophobic. Of note, one of the O<sub>2</sub>-dependent hydroxylases was shown to  
394 hydroxylate DMQ<sub>0</sub>, a substrate analog with no polyprenyl side chain [58], suggesting that an *in vitro*  
395 assay for UbiU-V might be developed with soluble analogs.

#### 396 **Fe-S clusters in UbiU-V and other members of the U32 peptidase family:**

397 Up to now, members of the peptidase family U32 were not shown to bind Fe-S clusters or to  
398 contain any of the ~30 cysteine motifs found in well-characterized iron-sulfur proteins [59]. The  
399 expression, purification and spectroscopic characterization of UbiV and of the UbiU-UbiV heterodimeric  
400 complex clearly showed that each protein contains one [4Fe-4S] cluster (Figure 5 and Figure 6). Mutation  
401 of the candidate cysteine ligands, arranged in a CX<sub>6</sub>CX<sub>16</sub>CX<sub>38</sub>C motif in UbiU and in a CX<sub>n</sub>CX<sub>12</sub>CX<sub>3</sub>C motif in  
402 UbiV, disrupted Fe-S binding and abolished *in vivo* complementation, suggesting a crucial function of the  
403 [4Fe-4S] clusters in these proteins. The conservation of a CX<sub>6</sub>CX<sub>15</sub>CX<sub>3/4</sub>C motif in other U32 proteases  
404 supports that these proteins likely bind Fe-S clusters. This hypothesis should guide and stimulate  
405 investigations of U32 members, very few of which have currently an established molecular function  
406 ([60], <http://www.ebi.ac.uk/merops/>). Interestingly, RlhA, a member of the U32 proteases family involved  
407 in the C-hydroxylation of a cytidine on *E. coli* 23S rRNA, was recently shown to be connected to iron  
408 metabolism [43], corroborating our suggestion that RlhA is also an Fe-S protein.

409 In biological systems, Fe-S clusters are mainly known to be involved in electron transfer  
410 reactions, but also in substrate binding and activation, in transcription regulation, in iron storage, and as  
411 a sulfur donor [41,61-63]. The role of the Fe-S clusters in UbiU and UbiV is unknown at this stage. Our  
412 current working hypothesis is a role as an electron transfer chain between the substrate (the UQ



413 biosynthetic intermediate to be hydroxylated) and an unidentified electron acceptor required for the  
414 activation of the substrate. Clearly, the Fe-S clusters of UbiU-V are distinct from the molybdenum  
415 cofactor present in molybdenum-containing hydroxylases, the only family currently known to catalyze  
416 O<sub>2</sub>-independent hydroxylation reactions [64]. Together, our results identify UbiU and UbiV as prototypes  
417 of a novel class of O<sub>2</sub>-independent hydroxylases and extend the framework of the chemically-fascinating  
418 O<sub>2</sub>-independent hydroxylation reactions.

419

## 420 **MATERIALS and METHODS**

### 421 **Strain construction:**

422 Strains used in this study are listed in table S3. We obtained the collection of *E. coli* strains containing  
423 large and medium deletions from the National BioResource Project, National Institute of Genetics, Japan  
424 (<http://www.shigen.nig.ac.jp/ecoli/pec/>).

425 The  $\Delta ubiA::cat$ ,  $\Delta ubiD::cat$ ,  $\Delta ubiT::cat$  and  $\Delta ubiV::cat$  mutations were constructed in a one-step  
426 inactivation of *ubi* genes as described [65]. A DNA fragment containing the *cat* gene flanked with a 5'  
427 and 3' region bordering the *E.coli ubi* genes was amplified by PCR using pKD3 as a template and  
428 oligonucleotides 5wanner and 3wanner (Table S3). Strain BW25113, carrying the pKD46 plasmid, was  
429 transformed by electroporation with the amplified fragment and *cat*<sup>R</sup> colonies were selected. The  
430 replacement of chromosomal *ubi* by *cat* gene was verified by PCR amplification in the *cat*<sup>R</sup> clones.  
431 Mutations (including *ubiU::kan* from the keio strain) were introduced into MG1655 strains by P1 *vir*  
432 transduction [66], selecting for the appropriate antibiotic resistance. The antibiotic resistance cassettes  
433 were eliminated when needed using plasmid pCP20 as described [67].

### 434 **Plasmid construction:**

435 All plasmids generated in this study were verified by DNA sequencing. The *yhbU*, *yhbT* and *yhbV* inserts  
436 (UniProtKB: P45527, P64599, P45475) were obtained by PCR amplification using *E. coli* MG1655 as  
437 template and the oligonucleotides pairs *yhbU5/yhbU3*, *yhbT5/yhbT3* and *yhbV5/yhbV3*, respectively  
438 (Table S3). *Yhb* inserts were EcoRI-Sall digested and inserted into EcoRI-Sall-digested pBAD24 plasmids,  
439 yielding the pBAD-*yhbU*, pK-*yhbT* or pBAD-*yhbV* plasmids, respectively.

440 To create a plasmid expressing the *ubiV* (*yhbV*) ORF as C-terminally His-tagged protein, the *ubiV* gene  
441 was amplified using pET-22-UbiV-FW (introducing the NdeI site) and pET-22-UbiV-RV (introducing the  
442 XhoI site) as primers and pBAD-*yhbV* as template. The NdeI and XhoI digested amplicon was ligated to  
443 NdeI and XhoI digested pET-22b(+) plasmid to obtain pET-22-UbiV.

444 The plasmid pETDUET-UbiUV containing UbiU in MCS1 and UbiV in MCS2 was obtained as follows. *ubiU*  
445 was amplified from pBAD-*yhbU* using pETDUET-UbiU-FW (introducing the NcoI site) and pETDUET-UbiU-  
446 RV (introducing the EcoRI site) as primers. The NcoI and EcoRI digested amplicon was ligated to NcoI and  
447 EcoRI digested pETDUET-1- plasmid to obtain pETDUET-UbiU. The *ubiV* gene was then cloned from pET-  
448 22-UbiV into the MSC2 of pETDUET-UbiU by PCR amplification with pET-22-UbiV-FW and pETDUET-UbiV-

449 RV (introducing the C-terminal His<sub>6</sub>-tag) as primers. The NdeI and XhoI digested amplicon was ligated to  
450 NdeI and XhoI digested pETDUET-UbiU to obtain pETDUET-UbiUV.

451 A hexahistidine-tag was fused at the N-terminal extremity of UbiV to create pBAD-UbiV<sub>6His</sub>. The *ubiV*<sub>6His</sub>  
452 gene was obtained by PCR amplification (Phusion High-Fidelity DNA Polymerase) using pBAD-UbiV as a  
453 template and 6HisV5 (introducing the NcoI site) and 6HisV3 (introducing the HindIII site and the DNA  
454 sequence of 6His-tag) as primers. The NcoI/HindIII digested amplicon was cloned into the NcoI/HindIII-  
455 digested pBAD plasmid.

456 Variants of UbiV and UbiU were constructed using the Q5 Site-Directed Mutagenesis Kit (New England  
457 Biolabs) according to the manufacturer's specifications. The plasmids (pET-22b-UbiV, pETDUET-UbiUV,  
458 pBAD-*yhbU* or pBAD-*yhbV*) were used as templates in conjunction with the appropriate primers for each  
459 respective amino acid substitution.

#### 460 **Culture conditions:**

461 *E. coli* strains were grown at 37 °C in lysogeny broth (LB) medium or in synthetic medium (SM) containing  
462 either 0.4% glycerol (w/v), 0.4% lactate (w/v) or 0.2% glucose (w/v) as carbon sources. Autoclaved SM  
463 medium was supplemented with 0.5% casaminoacids (w/v) and with 1/100 volume of a filter-sterile  
464 solution of 1mM CaCl<sub>2</sub>, 200 mM MgCl<sub>2</sub>, 1% thiamine (w/v) [68]. Ampicillin (50 mg/L), kanamycin (25  
465 mg/L), and chloramphenicol (25 mg/L) were added from stocks (1000X solution sterilized through 0.22  
466 µm filters and stored at -20°C), when needed. When needed, 0.02% arabinose was added to induce the  
467 expression of genes carried on pBAD and pK plasmids. External electron acceptors like KNO<sub>3</sub> (100 mM) or  
468 dimethylsulfoxide (DMSO, 50 mM) were added to SM medium for anaerobic cultures. Anaerobic cultures  
469 were performed in Hungate tubes containing 12 mL medium deoxygenated by argon (O<sub>2</sub><0.1 ppm)  
470 bubbling for 25 min before autoclave (in case of LB medium, 0.05% antifoam (Sigma) was added).  
471 Hungate tubes were inoculated through the septum with 100 µL of overnight precultures taken with  
472 disposable syringe and needles from closed Eppendorf tubes filled to the top. Aerobic cultures were  
473 performed in Erlenmeyer flasks filled to 1/10 of the maximal volume and shaken at 180 rpm.

474 For the initial screen, we grew ME strains anaerobically in SMGN (SM medium supplemented with  
475 glycerol and nitrate). Strains that presented a severe growth defect or a low UQ<sub>8</sub> content were  
476 subsequently grown anaerobically in LB medium.

477 Cultures were cooled down on ice before transferring 5-10 mL volumes into 15 mL Falcon tubes for  
478 centrifugation at 3200 g, 4°C, 10 min. Cell pellets were washed in 1 mL ice-cold PBS and transferred to

479 pre-weighted 1.5 mL Eppendorf tubes. After centrifugation at 12000 g, 4°C, 1 min and elimination of  
480 supernatant, the cells wet weight was determined (~10-20 mg) and pellets were stored at -20°C prior to  
481 quinone extraction. We note that these steps were conducted under normal atmosphere and allowed  
482 limited O<sub>2</sub>-dependent UQ biosynthesis in cells grown anaerobically. Thus, modifications (detailed below)  
483 were adopted in additional experiments conducted under strict anaerobic conditions.

#### 484 **Culture under strict anaerobic conditions and cells quenching:**

485 LB medium was supplemented with 100 mg/L L-cysteine (adjusted to pH 6 with NaOH) and 2.5 mg/L  
486 resazurin. The medium was distributed in Hungate tubes and was deoxygenated by argon (O<sub>2</sub><0.1 ppm)  
487 bubbling for 45 min at 60°C. The resazurin was initially purple, then quickly turned to pink, and  
488 eventually became colorless. The Hungate tubes were sealed and autoclaved. Two sequential  
489 precultures were performed in order to dilute the UQ present in the initial aerobic inoculum. The first  
490 preculture was performed overnight and used Eppendorf tubes filled to the top and inoculated with cells  
491 grown aerobically on LB agar. The second preculture was performed for 8 hours in Hungate tubes and  
492 was used to inoculate Hungate tubes that were subsequently incubated overnight at 37°C. Disposable  
493 syringes (1 mL) and needles were flushed 5 times with argon prior to inoculating 50 µL of preculture  
494 through the septum of the Hungate tubes. The resazurin remained colorless at all steps of the culture,  
495 indicating that the medium in the Hungate tubes was strictly anaerobic. At the end of the culture, the  
496 Hungate tubes were cooled down on ice for 45 min and 2 mL medium was sampled through the septum  
497 with argon-flushed syringes (2 mL) fitted with needles. The cells were immediately quenched by transfer  
498 to -20°C precooled glass tubes containing 6 mL methanol, 0.5 mL glass beads (0.5 mm diameter) and 20  
499 mM KCl. The tubes were homogenized by vortex for 30 seconds and kept at -20°C prior to quinone  
500 extraction. In parallel, we also centrifuged 2 mL of culture from the Hungate tubes in order to determine  
501 the weight of the cells and normalize the UQ content of the quenched cells that was subsequently  
502 measured.

503 For the experiments conducted under strict anaerobic conditions (Figure 2H, 2I), we used LB medium  
504 instead of MSGN, since nitrite - produced during the anaerobic respiration of nitrate in MSGN medium -  
505 is able to oxidize resazurin [69].

#### 506 ***Lipid extraction and quinone analysis:***

507 Quinone extraction from cell pellets was performed as previously described [17].

508 Quinone extraction from cells quenched in methanol was slightly adapted from [9]. Briefly, 4  $\mu$ L of a 10  
509  $\mu$ M UQ<sub>10</sub> solution was added as internal standard to the cells-methanol mixture. Then 4 mL petroleum  
510 ether (boiling range 40–60 °C) was added, the tubes were vortexed for 30 sec and the phases were  
511 separated by centrifugation 1 min, 600 rpm. The upper petroleum ether layer was transferred to a fresh  
512 glass tube. Petroleum ether (4 mL) was added to the glass beads and methanol-containing tube, and the  
513 extraction was repeated. The petroleum ether layers were combined and dried under nitrogen.

514 The dried lipid extracts were resuspended in 100  $\mu$ L ethanol and a volume corresponding to 1 mg of cells  
515 wet weight was analyzed by HPLC-electrochemical detection-mass spectrometry (ECD-MS) with a  
516 BetaBasic-18 column at a flow rate of 1 mL/min with mobile phases composed of methanol, ethanol,  
517 acetonitrile and a mix of 90% isopropanol, 10% ammonium acetate (1 M), 0.1% TFA: mobile phase 1  
518 (50% methanol, 40% ethanol and 10% mix), mobile phase 2 (40% acetonitrile, 40% ethanol, 20% mix).  
519 Mobile phase 1 was used in MS detection on a MSQ spectrometer (Thermo Scientific) with electrospray  
520 ionization in positive mode (probe temperature 400°C, cone voltage 80V). Single ion monitoring (SIM)  
521 detected the following compounds: OPP (M+NH<sub>4</sub><sup>+</sup>), m/z 656.0-656.8, 5-10 min, scan time 0.2 s; DMQ<sub>8</sub>  
522 (M+Na<sup>+</sup>), m/z 719-720, 6-10 min, scan time 0.2 s; <sup>13</sup>C<sub>6</sub>-DMQ<sub>8</sub> (M+Na<sup>+</sup>), m/z 725-726, 6-10 min, scan time  
523 0.2 s; UQ<sub>8</sub> (M+NH<sub>4</sub><sup>+</sup>), m/z 744-745, 6-10 min, scan time 0.2 s; UQ<sub>8</sub> (M+Na<sup>+</sup>), m/z 749-750, 6-10 min, scan  
524 time 0.2 s; <sup>13</sup>C<sub>6</sub>-UQ<sub>8</sub> (M+Na<sup>+</sup>), m/z 755.0-756, 6-10 min, scan time 0.2 s; UQ<sub>10</sub> (M+NH<sub>4</sub><sup>+</sup>), m/z 880.2-881.2,  
525 10-17 min, scan time 0.2 s. MS spectra were recorded between m/z 600 and 900 with a scan time of 0.3  
526 s. UV detection at 247 nm was used to quantify DMK<sub>8</sub> and MK<sub>8</sub>. ECD, MS and UV peak areas were  
527 corrected for sample loss during extraction on the basis of the recovery of the UQ<sub>10</sub> internal standard  
528 and were then normalized to cell's wet weight. The peak of UQ<sub>8</sub> obtained with electrochemical detection  
529 was quantified with a standard curve of UQ<sub>10</sub> [17]. The absolute quantification of UQ<sub>8</sub> based on the m/z=  
530 744.6 signal at 8 min (Figure 2H, I) was performed with a standard curve of UQ<sub>8</sub> ranging from 0.5 to 150  
531 pmoles UQ<sub>8</sub> (the detection limit was around 0.1 pmole).

#### 532 **Anaerobic <sup>13</sup>C<sub>6</sub>-UQ<sub>8</sub> biosynthesis activity assay:**

533  $\Delta$ ubiC  $\Delta$ ubiF cells containing or not the additional  $\Delta$ ubiU or  $\Delta$ ubiV deletions were grown overnight in MS  
534 medium supplemented with 0.2% glucose. This preculture was used to inoculate at OD<sub>600</sub>=0.1, 100 mL of  
535 fresh medium supplemented with 10  $\mu$ M <sup>13</sup>C<sub>7</sub>-4HB. The culture was grown at 37°C, 180 rpm until  
536 OD<sub>600</sub>=1, at which point 100  $\mu$ M 4HB was added. The cells were pelleted by centrifugation at 3200 g, 4°C,  
537 10 min and suspended in 100 mL MSGN medium. A 10 mL aliquot was taken for quinone extraction

538 (aerobiosis, Figure 4B-D) and the rest of the culture was placed at 37°C in an anaerobic bottle with a two-  
539 port cap fitted with plastic tubing used to inject argon ( $O_2 < 0.1$  ppm) throughout the experiment in order  
540 to create and maintain anaerobiosis. After 5 min bubbling, a 10 mL sample was taken corresponding to 0  
541 min anaerobiosis, then samples were taken every 30 minutes and analyzed for quinone content.

542 ***Overexpression and purification of proteins:***

543 *Overexpression and purification E. coli wild-type UbiV and variants.*

544 The pET-22b(+) plasmid, encoding wild-type UbiV or variants, were co-transformed with pGro7 plasmid  
545 (Takara Bio Inc.) into *E. coli* BL21 (DE3) competent cells. Single colonies obtained from transformation  
546 were grown overnight at 37°C in LB medium supplemented with ampicillin (50 µg/mL) and  
547 chloramphenicol (12.5 µg/mL). 10 mL of preculture was used to inoculate 1 L of LB medium with the  
548 same antibiotics, and the bacteria were cultured further at 37 °C with shaking (200 rpm). At an  $OD_{600}$  of  
549 1.2, D-arabinose was added to the cultures at a final concentration of 2 mg/mL. At an  $OD_{600}$  of 1.8, the  
550 culture was cooled in an ice-water bath, and isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added at  
551 a final concentration of 0.1 mM. Cells were then allowed to grow further at 16 °C overnight. All  
552 subsequent operations were carried out at 4°C. Cells were harvested in an Avanti® J-26XP High-  
553 Performance centrifuge from Beckman Coulter with a JLA-8.1000 rotor at  $5,000 \times g$  for 10 minutes. The  
554 cell pellets were resuspended in 5 volumes of buffer A (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 15% (v/v)  
555 glycerol, 1 mM DTT) containing Complete™ Protease Inhibitor Cocktail (one tablet per 50 mL) (Roche)  
556 and disrupted by sonication (Branson Digital Sonifier, amplitude 40% for 10 min). Cells debris were  
557 removed by ultracentrifugation in an Optima™ XPN-80 ultracentrifuge from Beckman Coulter with a 50.2  
558 Ti rotor at  $35,000 \times g$  for 60 min. The resulting supernatant was loaded onto a HisTrap FF Crude column  
559 (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 10 column volumes of  
560 buffer B (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 15% (v/v) glycerol, 1 mM DTT, 10 mM imidazole) to  
561 remove non-specifically bound *E. coli* proteins then eluted with a linear gradient of 10 column volumes  
562 of buffer B containing 500 mM imidazole . Fractions containing WT UbiV or variants were pooled and  
563 phenylmethylsulfonyl fluoride was added at a final concentration of 1mM. The proteins were then  
564 loaded on a HiLoad 16/600 Superdex 75 pg (GE Healthcare) pre-equilibrated in buffer C (50 mM Tris-HCl  
565 pH 8.5, 25 mM NaCl, 15% (v/v) glycerol, 1 mM DTT). The purified proteins were concentrated to 30-40  
566 mg/mL using Amicon concentrators (30-kDa cutoff; Millipore), aliquoted, frozen in liquid nitrogen and  
567 stored at -80 °C. Overall, a high yield of 150 mg UbiV/L culture was obtained.

568 *Overexpression and purification of UbiU/V complex and variants.*

569 The overexpression of wild-type UbiU/V complex or variants in *E. coli* BL21 (DE3) competent cells were  
570 performed following the same protocol as for UbiV. The expression of these proteins was induced by  
571 addition of IPTG to a final concentration of 0.05 mM. Wild-type UbiU/V complex or variants were  
572 purified with the same procedure as for UbiV, with the exception that the proteins were loaded on the  
573 HiLoad 16/600 Superdex 75 pg with buffer A.

#### 574 **[Fe-S] cluster reconstitution**

575 The [Fe-S] cluster(s) reconstitution of holo-UbiV and holo-UbiU/V were conducted under anaerobic  
576 conditions in an Mbraun LabStar glove box containing less than 0.5 ppm O<sub>2</sub>. Classically, a solution  
577 containing 100 μM of as-purified UbiV or UbiU/V complex, was treated with 5 mM DTT for 15 min at  
578 20°C and then incubated for 1 hour with a 5-fold molar excess of both ferrous ammonium sulfate and L-  
579 cysteine. The reaction was initiated by the addition of a catalytic amount of the *E.coli* cysteine  
580 desulfurase CsdA (1-2% molar equivalent) and monitored by UV-visible absorption spectroscopy. The  
581 holo-UbiV or holo-UbiU/V complexes were then loaded onto a Superdex 75 Increase 10/300 GL column  
582 (GE Healthcare) pre-equilibrated with buffer C or A, respectively, to remove all excess of iron and L-  
583 cysteine. The fractions containing the holo-proteins were pooled and concentrated to 20-30mg/mL on a  
584 Vivaspin concentrator (30-kDa cutoff).

#### 585 **Quantification Methods**

586 Protein concentrations were determined using the method of Bradford (Bio-Rad) with bovine serum  
587 albumin as the standard. The iron and acid-labile sulfide were determined according to the method of  
588 Fish [70] and Beinert [71], respectively.

#### 589 **UV-Vis spectroscopy**

590 UV-Visible spectra were recorded in 1 cm optic path quartz cuvette under aerobic conditions on a Cary  
591 100 UV-Vis spectrophotometer (Agilent) and under anaerobic conditions in a glove box on a XL-100  
592 Uvikon spectrophotometer equipped with optical fibers.

#### 593 **EPR spectroscopy**

594 EPR spectra of frozen solutions were recorded on a Bruker Continuous-Waves (CW) X-Band ELEXSYS E500  
595 spectrometer operating at 9.39 GHz, equipped with an SHQE cavity cooled by an helium flow cryostat  
596 ESR 900 Oxford Instruments under non-saturating conditions and using the following parameters: a  
597 microwave power in the range 2 to 10 mW and a modulation of the magnetic field at 100 kHz with a  
598 modulation amplitude of 0.6 mT. Holo UbiV or holo UbiU-UbiV complex were treated with 10-fold molar  
599 excess of dithionite to reduce the Fe-S cluster. Each solution was introduced into EPR quartz tubes in a  
600 glove box and frozen with liquid nitrogen before the EPR measurements.

#### 601 **Genome datasets**

602 The protein sequences from 5750 complete genomes (“extended dataset”) were downloaded from the  
603 NCBI Refseq database (bacteria and archaea, last accessed in November 2016, Table S2A). A  
604 representative set of complete genomes from a monophyletic group of bacteria that potentially harbor  
605 the ubiquinone biosynthesis pathway was also created: “Reference” and “Representative” genomes  
606 were downloaded from the NCBI Refseq database for 204 Alphaproteobacteria, 103 Betaproteobacteria,  
607 303 Gammaproteobacteria (last accessed in November 2018). In addition to these 610 genomes, the  
608 genome of *Phaeospirillum fulvum* (99.5% estimated completeness according to CheckM,  
609 [http://gtadb.ecogenomic.org/genomes?gid=GCF\\_900108475.1](http://gtadb.ecogenomic.org/genomes?gid=GCF_900108475.1)) was included (Table S2B).

#### 610 **HMM protein profiles creation**

611 An initial set of protein sequences (“curated set”) was retrieved from genomes manually and from a  
612 publication [72], to cover the diversity of UQ-producing organisms. The curated set included 48 pairs of  
613 YhbU and YhbV from 10 alpha, 19 beta and 19 gamma- proteobacteria, 17 sequences for YhbT, 64, 181,  
614 69 and 189 sequences for UbiA, MenA, UbiG and UbiE respectively (Table S4). Then, for each gene family  
615 these sequences were aligned with Mafft (v7.313, “linsi”) [73], and each alignment trimmed at its N-ter  
616 and C-ter extremities based on the filtering results of BMGE (BLOSUM 30) [74]. The core of the  
617 alignments were kept as is, and Hidden Markov Model (HMM) profiles were created directly from the  
618 trimmed alignments using the Hmmbuild program (Hmmer suite, version 3.1b2) [75].

619 To ensure a more sensitive search, and good delineation between homologs, phylogenetic curation was  
620 used, as YhbU and YhbV are known to be part of the larger U32 protease gene family [43]. A search using  
621 the YhbU and YhbV HMM profiles was performed with Hmsearch (Hmmer suite) on the extended 5750  
622 genomes dataset, and sequences with an i-evalue (“independent e-value”) lower than 10E-20 and a  
623 coverage of the profiles higher than 90% were selected. The 4212 sequences obtained were de-



624 replicated using Uclust from the Usearch program suite (80% identity level) [76]. A phylogenetic tree was  
625 built by maximum-likelihood with the IQ-Tree program (best evolutionary model) based on the  
626 alignment (Mafft: "linsi", BMGE with BLOSUM30) of the 480 selected sequences including all curated  
627 YhbU and YhbV sequences [73,74,77]. YhbU and YhbV proteobacterial sequences formed two separate  
628 monophyletic groups, giving credit to our curated set (100% and 80% UF-Boot support respectively). The  
629 other sequences that formed a large monophyletic group of bacterial sequences were categorized as  
630 "U32 proteases" (98% UF-Boot, <https://doi.org/10.6084/m9.figshare.7800614.v1>). The 98 sequences  
631 from this U32 proteases group (Table S4) were used to re-create an HMM profile as described above,  
632 and served as an outgroup for the profile search.

633 For YhbT, the first profile obtained from the curated set of sequences was used altogether with the  
634 YhbU, YhbV and U32 proteases profiles for a search in the 611 proteobacteria genomes dataset. A  
635 second profile was created from YhbT sequences (YhbT2, Table S4) that were co-localizing with YhbU and  
636 YhbV hits (10E-20 i-evalue and 80% profile coverage). The two YhbT profiles matched complementary  
637 sets of sequences and therefore were both used for annotating YhbT in genomes.

638 A similar approach was taken in order to identify the six known aerobic hydroxylases. 51 Coq7, 73 UbiF,  
639 80 UbiH, 58 UbiI, 24 UbiL and 32 UbiM sequences were extracted manually and from publications [72]  
640 [19](Table S4, "version 1") to serve as a reference, annotated set of sequences. Profiles were created as  
641 described above. To ensure their specificity, we ran the HMM profiles against our 5570 genomes dataset  
642 and selected the sequences that had an i-evalue lower than 10E-20 and a coverage of the profiles higher  
643 than 90%. We built two phylogenetic trees as described above: one for Coq7, and another one for  
644 UbiFHILM, which are known to be part of the large FMO protein family [19]. In the latter case, we de-  
645 replicated the 1619 sequences obtained for the FMO protein family before performing the alignment,  
646 alignment filtering, and tree reconstruction steps (using Uclust at the 60% identity level). The Coq7 tree  
647 obtained showed our reference Coq7 sequences covered the whole diversity of retrieved sequences,  
648 suggesting that they all could be *bona fide* Coq7 (<https://doi.org/10.6084/m9.figshare.7800680>). The  
649 FMO tree showed a monophyletic group containing all reference FMO ubiquinone hydroxylases, forming  
650 sub-groups for the different homologs (UbiFHILM) in Proteobacteria  
651 (<https://doi.org/10.6084/m9.figshare.7800620>). Further, a large set of sequences formed an outgroup  
652 consisting of sequences from various clades of bacteria, a lot being found outside of Proteobacteria,  
653 robustly separated from the ubiquinone hydroxylases. We split this large clade into four sub-trees, and  
654 extracted the corresponding sequences to obtain four new HMM profiles (as described above), to be

655 used for precise discrimination between ubiquinone hydroxylases and other members of the FMO family  
656 ("AlloFMO\_1" to "AlloFMO\_4" in Table S4, "version 2"). FMO ubiquinone hydroxylases sub-trees were  
657 also used to re-design improved HMM profiles for UbiFHILM (36, 168, 198, 139, and 65 sequences  
658 respectively, see Table S4 "version 2").

### 659 **Evaluation of genomic distributions with HMMER and MacSyFinder**

660 Two MacSyFinder models were created to i) search sequences of interest in genomes using Hmmer, and  
661 ii) investigate their genetic architecture [78]. A first model was created to focus only on YhbTUV-related  
662 genes. In this model, the YhbTUV components were defined as “mandatory”, and U32 protease as  
663 “accessory”. A second, more comprehensive model “Ubi+YhbTUV” was designed to list the families  
664 corresponding to the 9 profiles obtained (UbiA, MenA, UbiE, UbiG, 2 YhbT, YhbU, YhbV and U32  
665 proteases). For both models, the two YhbT profiles were set as “exchangeable”. The parameter  
666 “inter\_gene\_max\_space” was set to 5 in the “YhbTUV” model, and 10 in the “Ubi+YhbTUV” model.  
667 MacSyFinder was set to run HMMER with the options “--i-value-select 10E-20” and “--coverage-profile  
668 0.8”. Independently of their genetic context, sequences corresponding to selected HMMER hits were  
669 listed for all profiles in all genomes analyzed in order to establish the genomic distribution for each of  
670 the protein families of interest. When several profiles matched a sequence, only the best hit (best i-  
671 evalue) was considered.

### 672 **U32 proteases sequence analysis**

673 We retrieved from the UniProt-KB database 3460 protein sequences of U32 proteases that were  
674 categorized in 12 families by Kimura *et al.* [43], and created a FASTA for each of these families. 50  
675 sequences from different families could not be retrieved as they had been deleted from the Uniprot-KB  
676 database (46 “obsolete”) or were not found based on the published accession numbers. As the “RIhA1”  
677 and “RIhA2” families mostly corresponded to two domains from the same protein sequences that had  
678 been split, we put whole sequences all together into a single fasta file for sequence analysis of the  
679 overall “RIhA” family. For each of the 10 family, sequences were de-replicated at the 80% identity level  
680 with Uclust, in order to limit any potential taxonomic sampling bias, and sequences were aligned (Mafft,  
681 linsi). The alignments were visualized in Jalview [79] and used to create the logo sequences. Images of  
682 alignments were created using the ESPript webserver (<http://esprict.ibcp.fr/ESPript/ESPript/>) [80].

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693

## 694 **COMPETING INTERESTS**

695 The authors declare that they have no competing interests.

696

## 697 **REFERENCES**

698

- 699 1. Schoepp-Cothenet B, van Lis R, Atteia A, Baymann F, Capowiez L, Ducluzeau AL, et al. On the universal  
700 core of bioenergetics. *Biochim Biophys Acta*. 2013; 1827: 79-93.
- 701 2. Marteyn B, Scorza FB, Sansonetti PJ, Tang C. Breathing life into pathogens: the influence of oxygen on  
702 bacterial virulence and host responses in the gastrointestinal tract. *Cell Microbiol*. 2011; 13: 171-  
703 176.
- 704 3. Wallace N, Zani A, Abrams E, Sun Y (2016) The Impact of Oxygen on Bacterial Enteric Pathogens. In:  
705 Sariaslani S, Gadd GM, editors. *Advances in Applied Microbiology*, Vol 95. San Diego: Elsevier  
706 Academic Press Inc. pp. 179-204.
- 707 4. Bettenbrock K, Bai H, Ederer M, Green J, Hellingwerf KJ, Holcombe M, et al. Towards a Systems Level  
708 Understanding of the Oxygen Response of *Escherichia coli*. *Advances in Microbial Systems*  
709 *Biology*. 2014; 64: 65-114.
- 710 5. Morris RL, Schmidt TM. Shallow breathing: bacterial life at low O<sub>2</sub>. *Nature Reviews Microbiology*.  
711 2013; 11: 205-212.
- 712 6. Marreiros BC, Calisto F, Castro PJ, Duarte AM, Sena FV, Silva AF, et al. Exploring membrane respiratory  
713 chains. *Biochimica Et Biophysica Acta-Bioenergetics*. 2016; 1857: 1039-1067.
- 714 7. Lecomte SM, Achouak W, Abrouk D, Heulin T, Nesme X, Haichar FE. Diversifying Anaerobic Respiration  
715 Strategies to Compete in the Rhizosphere. *Frontiers in Environmental Science*. 2018; 6: 16.
- 716 8. Nowicka B, Kruk J. Occurrence, biosynthesis and function of isoprenoid quinones. *Biochim Biophys*  
717 *Acta*. 2010; 1797: 1587-1605.
- 718 9. Sharma P, Teixeira de Mattos MJ, Hellingwerf KJ, Bekker M. On the function of the various quinone  
719 species in *Escherichia coli*. *FEBS J*. 2012; 279: 3364-3373.

- 720 10. Shestopalov AI, Bogachev AV, Murtazina RA, Viryasov MB, Skulachev VP. Aeration-dependent  
721 changes in composition of the quinone pool in *Escherichia coli* - Evidence of post-transcriptional  
722 regulation of the quinone biosynthesis. *Febs Letters*. 1997; 404: 272-274.
- 723 11. Nitzschke A, Bettenbrock K. All three quinone species play distinct roles in ensuring optimal growth  
724 under aerobic and fermentative conditions in *E. coli* K12. *PLoS One*. 2018; 13: e0194699.
- 725 12. Aussel L, Pierrel F, Loiseau L, Lombard M, Fontecave M, Barras F. Biosynthesis and physiology of  
726 coenzyme Q in bacteria. *Biochim Biophys Acta*. 2014; 1837: 1004-1011.
- 727 13. Reidenbach AG, Kemmerer ZA, Aydin D, Jochem A, McDevitt MT, Hutchins PD, et al. Conserved Lipid  
728 and Small-Molecule Modulation of COQ8 Reveals Regulation of the Ancient Kinase-like UbiB  
729 Family. *Cell Chem Biol*. 2018; 25: 154-165 e111.
- 730 14. Loiseau L, Fyfe C, Aussel L, Hajj Chehade M, Hernandez SB, Faivre B, et al. The UbiK protein is an  
731 accessory factor necessary for bacterial ubiquinone (UQ) biosynthesis and forms a complex with  
732 the UQ biogenesis factor UbiI. *J Biol Chem*. 2017; 292: 11937-11950.
- 733 15. Aussel L, Loiseau L, Hajj Chehade M, Pocachard B, Fontecave M, Pierrel F, et al. *ubiJ*, a New Gene  
734 Required for Aerobic Growth and Proliferation in Macrophage, Is Involved in Coenzyme Q  
735 Biosynthesis in *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium. *J Bacteriol*. 2014;  
736 196: 70-79.
- 737 16. Hajj Chehade M, Pelosi L, Fyfe CD, Loiseau L, Rascalou B, Brugiere S, et al. A Soluble Metabolon  
738 Synthesizes the Isoprenoid Lipid Ubiquinone. *Cell Chem Biol*. 2019; 26: 482-492 e487.
- 739 17. Hajj Chehade M, Loiseau L, Lombard M, Pecqueur L, Ismail A, Smadja M, et al. *ubil*, a New Gene in  
740 *Escherichia coli* Coenzyme Q Biosynthesis, Is Involved in Aerobic C5-hydroxylation. *J Biol Chem*.  
741 2013; 288: 20085-20092.
- 742 18. Alexander K, Young IG. Three hydroxylations incorporating molecular oxygen in the aerobic  
743 biosynthesis of ubiquinone in *Escherichia coli*. *Biochemistry*. 1978; 17: 4745-4750.
- 744 19. Pelosi L, Ducluzeau AL, Loiseau L, Barras F, Schneider D, Junier I, et al. Evolution of Ubiquinone  
745 Biosynthesis: Multiple Proteobacterial Enzymes with Various Regioselectivities To Catalyze Three  
746 Contiguous Aromatic Hydroxylation Reactions. *mSystems*. 2016; 1: e00091-00016.
- 747 20. Alexander K, Young IG. Alternative hydroxylases for the aerobic and anaerobic biosynthesis of  
748 ubiquinone in *Escherichia coli*. *Biochemistry*. 1978; 17: 4750-4755.
- 749 21. Cox GB, Gibson F. Biosynthesis of Vitamin K and Ubiquinone. Relation to the Shikimic Acid Pathway in  
750 *Escherichia coli*. *Biochim Biophys Acta*. 1964; 93: 204-206.
- 751 22. Siebert M, Severin K, Heide L. Formation of 4-hydroxybenzoate in *Escherichia coli*: characterization of  
752 the *ubiC* gene and its encoded enzyme chorismate pyruvate-lyase. *Microbiology*. 1994; 140 ( Pt  
753 4): 897-904.
- 754 23. Payet LA, Leroux M, Willison JC, Kihara A, Pelosi L, Pierrel F. Mechanistic Details of Early Steps in  
755 Coenzyme Q Biosynthesis Pathway in Yeast. *Cell Chem Biol*. 2016; 23: 1241-1250.
- 756 24. Kwon O, Kotsakis A, Meganathan R. Ubiquinone (coenzyme Q) biosynthesis in *Escherichia coli*:  
757 identification of the *ubiF* gene. *FEMS Microbiol Lett*. 2000; 186: 157-161.
- 758 25. Hashimoto M, Ichimura T, Mizoguchi H, Tanaka K, Fujimitsu K, Keyamura K, et al. Cell size and  
759 nucleoid organization of engineered *Escherichia coli* cells with a reduced genome. *Mol Microbiol*.  
760 2005; 55: 137-149.
- 761 26. Mizoguchi H, Sawano Y, Kato J, Mori H. Superpositioning of deletions promotes growth of *Escherichia*  
762 *coli* with a reduced genome. *DNA Res*. 2008; 15: 277-284.
- 763 27. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12  
764 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol*. 2006; 2: 2006 0008.
- 765 28. Esposti MD. A journey across genomes uncovers the origin of ubiquinone in cyanobacteria. *Genome*  
766 *Biol Evol*. 2017.

- 767 29. Hiraishi A, Hoshino Y, Kitamura H. Isoprenoid quinone composition in the classification of  
768 rhodospirillaceae. *Journal of General and Applied Microbiology*. 1984; 30: 197-210.
- 769 30. Imhoff JF, Petri R, Suling J. Reclassification of species of the spiral-shaped phototrophic purple non-  
770 sulfur bacteria of the alpha-Proteobacteria: description of the new genera *Phaeospirillum* gen.  
771 nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as  
772 transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum*  
773 *molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to  
774 *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis*  
775 comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of  
776 *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *International Journal of*  
777 *Systematic Bacteriology*. 1998; 48: 793-798.
- 778 31. Bazyliński DA, Williams TJ, Lefevre CT, Berg RJ, Zhang CL, Bowser SS, et al. *Magnetococcus marinus*  
779 gen. nov., sp. nov., a marine, magnetotactic bacterium that represents a novel lineage  
780 (*Magnetococcaceae* fam. nov., *Magnetococcales* ord. nov.) at the base of the  
781 Alphaproteobacteria. *Int J Syst Evol Microbiol*. 2013; 63: 801-808.
- 782 32. Stewart CS, Duncan SH, Cave DR. *Oxalobacter formigenes* and its role in oxalate metabolism in the  
783 human gut. *FEMS Microbiol Lett*. 2004; 230: 1-7.
- 784 33. Burgardt NI, Gianotti AR, Ferreyra RG, Ermacora MR. A structural appraisal of sterol carrier protein 2.  
785 *Biochimica Et Biophysica Acta-Proteins and Proteomics*. 2017; 1865: 565-577.
- 786 34. Yano T, Yagi T, Sled VD, Ohnishi T. Expression and characterization of the 66-kilodalton (NQO3) iron-  
787 sulfur subunit of the proton-translocating NADH-quinone oxidoreductase of *Paracoccus*  
788 *denitrificans*. *J Biol Chem*. 1995; 270: 18264-18270.
- 789 35. Ollagnier S, Mulliez E, Gaillard J, Eliasson R, Fontecave M, Reichard P. The anaerobic *Escherichia coli*  
790 ribonucleotide reductase. Subunit structure and iron sulfur center. *J Biol Chem*. 1996; 271: 9410-  
791 9416.
- 792 36. Ollagnier de Choudens S, Barras F. Genetic, Biochemical, and Biophysical Methods for Studying FeS  
793 Proteins and Their Assembly. *Methods Enzymol*. 2017; 595: 1-32.
- 794 37. M NM, Ollagnier-de-Choudens S, Sanakis Y, Abdel-Ghany SE, Rousset C, Ye H, et al. Characterization  
795 of *Arabidopsis thaliana* SufE2 and SufE3: functions in chloroplast iron-sulfur cluster assembly and  
796 Nad synthesis. *J Biol Chem*. 2007; 282: 18254-18264.
- 797 38. Ollagnier-De Choudens S, Sanakis Y, Hewitson KS, Roach P, Baldwin JE, Munck E, et al. Iron-sulfur  
798 center of biotin synthase and lipoate synthase. *Biochemistry*. 2000; 39: 4165-4173.
- 799 39. Ollagnier-de Choudens S, Loiseau L, Sanakis Y, Barras F, Fontecave M. Quinolate synthetase, an  
800 iron-sulfur enzyme in NAD biosynthesis. *FEBS Lett*. 2005; 579: 3737-3743.
- 801 40. Hewitson KS, Ollagnier-de Choudens S, Sanakis Y, Shaw NM, Baldwin JE, Munck E, et al. The iron-  
802 sulfur center of biotin synthase: site-directed mutants. *J Biol Inorg Chem*. 2002; 7: 83-93.
- 803 41. Johnson DC, Dean DR, Smith AD, Johnson MK. Structure, function, and formation of biological iron-  
804 sulfur clusters. *Annu Rev Biochem*. 2005; 74: 247-281.
- 805 42. Fontecave M, Py B, Ollagnier de Choudens S, Barras F. From Iron and Cysteine to Iron-Sulfur Clusters:  
806 the Biogenesis Protein Machineries. *EcoSal Plus*. 2008; 3.
- 807 43. Kimura S, Sakai Y, Ishiguro K, Suzuki T. Biogenesis and iron-dependency of ribosomal RNA  
808 hydroxylation. *Nucleic Acids Res*. 2017; 45: 12974-12986.
- 809 44. Yen HW, Chiu CH. The influences of aerobic-dark and anaerobic-light cultivation on CoQ(10)  
810 production by *Rhodobacter sphaeroides* in the submerged fermenter. *Enzyme and Microbial*  
811 *Technology*. 2007; 41: 600-604.
- 812 45. Matsumura M, Kobayashi T, Aiba S. ANAEROBIC PRODUCTION OF UBIQUINONE-10 BY PARACOCCLUS-  
813 DENITRIFICANS. *European Journal of Applied Microbiology and Biotechnology*. 1983; 17: 85-89.

- 814 46. Schoepp-Cothenet B, Lieutaud C, Baymann F, Vermeglio A, Friedrich T, Kramer DM, et al.  
815 Menaquinone as pool quinone in a purple bacterium. *Proc Natl Acad Sci U S A*. 2009; 106: 8549-  
816 8554.
- 817 47. Stolper DA, Revsbech NP, Canfield DE. Aerobic growth at nanomolar oxygen concentrations.  
818 *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:  
819 18755-18760.
- 820 48. D'Mello R, Hill S, Poole RK. The cytochrome bd quinol oxidase in *Escherichia coli* has an extremely  
821 high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo  
822 by oxygen inhibition. *Microbiology*. 1996; 142 ( Pt 4): 755-763.
- 823 49. Rolfe MD, Ocone A, Stapleton MR, Hall S, Trotter EW, Poole RK, et al. Systems analysis of  
824 transcription factor activities in environments with stable and dynamic oxygen concentrations.  
825 *Open Biol*. 2012; 2: 120091.
- 826 50. Rolfe MD, Ter Beek A, Graham AI, Trotter EW, Asif HM, Sanguinetti G, et al. Transcript profiling and  
827 inference of *Escherichia coli* K-12 ArcA activity across the range of physiologically relevant  
828 oxygen concentrations. *J Biol Chem*. 2011; 286: 10147-10154.
- 829 51. Lee KM, Park Y, Bari W, Yoon MY, Go J, Kim SC, et al. Activation of Cholera Toxin Production by  
830 Anaerobic Respiration of Trimethylamine N-oxide in *Vibrio cholerae*. *Journal of Biological*  
831 *Chemistry*. 2012; 287: 11.
- 832 52. Zhao H, Li X, Johnson DE, Mobley HLT. Identification of protease and rpoN-associated genes of  
833 uropathogenic *Proteus mirabilis* by negative selection in a mouse model of ascending urinary  
834 tract infection. *Microbiology-Uk*. 1999; 145: 185-195.
- 835 53. Navais R, Mendez J, Perez-Pascual D, Cascales D, Guijarro JA. The yrpAB operon of *Yersinia ruckeri*  
836 encoding two putative U32 peptidases is involved in virulence and induced under microaerobic  
837 conditions. *Virulence*. 2014; 5: 619-624.
- 838 54. Groenewold MK, Massmig M, Hebecker S, Danne L, Magnowska Z, Nimtz M, et al. A phosphatidic  
839 acid binding protein is important for lipid homeostasis and adaptation to anaerobic biofilm  
840 conditions in *Pseudomonas aeruginosa*. *Biochem J*. 2018.
- 841 55. Filiatrault MJ, Picardo KF, Ngai H, Passador L, Iglewski BH. Identification of *Pseudomonas aeruginosa*  
842 genes involved in virulence and anaerobic growth. *Infect Immun*. 2006; 74: 4237-4245.
- 843 56. Schobert M, Jahn D. Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int J*  
844 *Med Microbiol*. 2010; 300: 549-556.
- 845 57. Line L, Alhede M, Kolpen M, Kuhl M, Ciofu O, Bjarnsholt T, et al. Physiological levels of nitrate support  
846 anoxic growth by denitrification of *Pseudomonas aeruginosa* at growth rates reported in cystic  
847 fibrosis lungs and sputum. *Frontiers in Microbiology*. 2014; 5: 11.
- 848 58. Behan RK, Lippard SJ. The aging-associated enzyme CLK-1 is a member of the carboxylate-bridged  
849 diiron family of proteins. *Biochemistry*. 2010; 49: 9679-9681.
- 850 59. Estellon J, Ollagnier de Choudens S, Smadja M, Fontecave M, Vandenbrouck Y. An integrative  
851 computational model for large-scale identification of metalloproteins in microbial genomes: a  
852 focus on iron-sulfur cluster proteins. *Metallomics*. 2014; 6: 1913-1930.
- 853 60. Rawlings ND, Alan J, Thomas PD, Huang XD, Bateman A, Finn RD. The MEROPS database of  
854 proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in  
855 the PANTHER database. *Nucleic Acids Research*. 2018; 46: D624-D632.
- 856 61. Rouault TA. Mammalian iron-sulphur proteins: novel insights into biogenesis and function. *Nature*  
857 *Reviews Molecular Cell Biology*. 2015; 16: 45-55.
- 858 62. Lill R. Function and biogenesis of iron-sulphur proteins. *Nature*. 2009; 460: 831-838.
- 859 63. Roche B, Aussel L, Ezraty B, Mandin P, Py B, Barras F. Iron/sulfur proteins biogenesis in prokaryotes:  
860 formation, regulation and diversity. *Biochim Biophys Acta*. 2013; 1827: 455-469.
- 861 64. Hille R. Molybdenum-containing hydroxylases. *Arch Biochem Biophys*. 2005; 433: 107-116.

- 862 65. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using  
863 PCR products. *Proc Natl Acad Sci U S A*. 2000; 97: 6640-6645.
- 864 66. Miller JH (1972) *Experiments in Molecular Genetics*; Laboratory CSH, editor.
- 865 67. Cherepanov PP, Wackernagel W. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the  
866 option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene*. 1995; 158: 9-14.
- 867 68. Alberge F, Espinosa L, Seduk F, Sylvi L, Toci R, Walburger A, et al. Dynamic subcellular localization of a  
868 respiratory complex controls bacterial respiration. *Elife*. 2015; 4.
- 869 69. Jenneman GE, Montgomery AD, McInerney MJ. Method for detection of microorganisms that  
870 produce gaseous nitrogen oxides. *Appl Environ Microbiol*. 1986; 51: 776-780.
- 871 70. Fish WW. Rapid colorimetric micromethod for the quantitation of complexed iron in biological  
872 samples. *Methods in Enzymology*. 1988; 158: 357-364.
- 873 71. Beinert H. Micro methods for the quantitative determination of iron and copper in biological  
874 material. *Methods in enzymology*. 1978; 54: 435-445.
- 875 72. Ravcheev DA, Thiele I. Genomic Analysis of the Human Gut Microbiome Suggests Novel Enzymes  
876 Involved in Quinone Biosynthesis. *Front Microbiol*. 2016; 7: 128.
- 877 73. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in  
878 performance and usability. *Mol Biol Evol*. 2013; 30: 772-780.
- 879 74. Criscuolo A, Gribaldo S. BMGE (Block Mapping and Gathering with Entropy): a new software for  
880 selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol Biol*.  
881 2010; 10: 210.
- 882 75. Eddy SR. Accelerated Profile HMM Searches. *PLoS Comput Biol*. 2011; 7: e1002195.
- 883 76. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010; 26:  
884 2460-2461.
- 885 77. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm  
886 for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015; 32: 268-274.
- 887 78. Abby SS, Neron B, Menager H, Touchon M, Rocha EP. MacSyFinder: a program to mine genomes for  
888 molecular systems with an application to CRISPR-Cas systems. *PLoS One*. 2014; 9: e110726.
- 889 79. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence  
890 alignment editor and analysis workbench. *Bioinformatics*. 2009; 25: 1189-1191.
- 891 80. Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server.  
892 *Nucleic Acids Res*. 2014; 42: W320-324.
- 893 81. Garcia PS, Jauffrit F, Grangeasse C, Brochier-Armanet C. GeneSpy, a user-friendly and flexible  
894 genomic context visualizer. *Bioinformatics (Oxford, England)*. 2019; 35: 329-331.

895

896

897 **FIGURE LEGENDS**

898 **Figure 1: The aerobic and anaerobic UQ biosynthetic pathways differ only in the hydroxylation steps.**

899 A) O<sub>2</sub>-dependent UQ biosynthesis pathway in *E. coli*. The octaprenyl tail is represented by R on the  
900 biosynthetic intermediates and the numbering of the aromatic carbon atoms is shown on OPP.  
901 Abbreviations used are 4-HB for 4-hydroxybenzoic acid, OPP for octaprenylphenol, DMQ<sub>8</sub> for C6-  
902 demethoxy-ubiquinone 8 and UQ<sub>8</sub> for ubiquinone 8. B) UQ<sub>8</sub> quantification of WT and  $\Delta ubiC$  cells grown  
903 anaerobically in glycerol-nitrate medium supplemented or not with the indicated concentrations of 4-HB,  
904 mean  $\pm$ SD (n=3-6), \*\*\*\*: p< 0.0001, unpaired Student's t test. C-E) Mass spectra of UQ<sub>8</sub> obtained by  
905 HPLC-MS analysis of lipid extracts from cells grown with <sup>13</sup>C<sub>7</sub>-4HB either anaerobically (C) or aerobically  
906 (D), or anaerobically with unlabeled 4HB (E). F) UQ<sub>8</sub> quantification from WT and  $\Delta ubi$  cells grown  
907 anaerobically in SMGN medium overnight or aerobically in LB medium until OD 0.8. nd= not detected in  
908 aerobic and anaerobic conditions, nd= not detected in either aerobic or anaerobic conditions, mean  $\pm$ SD  
909 (n=3-4). G) HPLC-ECD analyses (mobile phase 1) of lipid extracts from 1 mg of WT or  $\Delta ubiIHf$  cells grown  
910 in LB medium in air or anaerobic conditions (-O<sub>2</sub>). Chromatograms are representative of n=3  
911 independent experiments (UQ<sub>10</sub> used as standard). H) UQ biosynthesis represented with Ubi enzymes  
912 specific to the O<sub>2</sub>-dependent pathway (red), to the O<sub>2</sub>-independent pathway (green), or common to both  
913 pathways (black). The same color code applies to the accessory factors (circled).

914 **Figure 2: *yhbT*, *yhbU* and *yhbV* are essential to the anaerobic biosynthesis of UQ.**

915 A) HPLC-ECD analysis of lipid extracts from ME4641 strain grown in SMGN either aerobically or anaerobically (-O<sub>2</sub>). B) Genomic  
916 region covered by the OCL30-2 deletion in the ME4641 strain. C) HPLC-ECD analysis of lipid extracts from  
917 knock-out strains of the individual genes covered by the OCL30-2 deletion grown in SMGN anaerobically.  
918 D) HPLC-ECD analysis of lipid extracts from  $\Delta yhbT$ ,  $\Delta yhbU$  and  $\Delta yhbV$  strains constructed in the MG1655  
919 background and grown in SMGN either aerobically or anaerobically. HPLC-ECD analyses with mobile  
920 phase 2 (A, C, D). E) OPP content (as % of WT, mass detection M+NH<sub>4</sub><sup>+</sup>) in cells from table 2. The  $\Delta yhb$   
921 strains contain either an empty plasmid or a plasmid carrying the indicated gene and were cultured  
922 anaerobically in SMGN containing 0.02% arabinose. Mean  $\pm$ SD (n=3-5), \*\*: p< 0.01, unpaired Student's t  
923 test. F) UQ<sub>8</sub> content (as % of WT grown in LB medium) of cells cultured anaerobically in SM containing  
924 the indicated carbon sources and electron acceptors. G) Single ion monitoring for UQ<sub>8</sub> (M+NH<sub>4</sub><sup>+</sup>) in HPLC-  
925 MS analysis (mobile phase 1) of lipid extracts from 1 mg of  $\Delta ubiU$  or  $\Delta ubiU \Delta ubiH$  cells grown in SMGN  
926 under anaerobic conditions. H) Single ion monitoring HPLC-MS analysis (mobile phase 1) of lipid extracts  
927 from 1.6 mg of cells grown in LB medium under strict anaerobic conditions and quenched in methanol.  
928 Chromatograms are representative of n=3 independent experiments (G,H). I) UQ<sub>8</sub> content of cells  
929 described in H (quantification of the signal at 8 min with m/z= 744.6), mean  $\pm$ SD (n=3).

930 **Figure 3: *ubiT*, -U and -V occurrence and genetic architecture in proteobacterial genomes.**

931 A) The proportion of genomes with (green) and without (red) all three genes "*ubiTUV*" (left column) is  
932 indicated for each proteobacterial order known to synthesize UQ. The middle column "3-genes loci"  
933



935 displays the proportion of genomes with the three genes either at a single locus (green) or at different  
936 loci (red). The number of genomes analyzed for each order is given in the right column (“Nb Genomes”).  
937 B) Occurrence in the reference proteobacterial genomes of the marker proteins (UbiA, -E, -G), of the O<sub>2</sub>-  
938 dependent hydroxylases and of the UbiT, -U, -V proteins. The number in bold represents the 3 genomes  
939 (*P. fulvum*, *M. marinus* and *O. formigenes*) containing exclusively the O<sub>2</sub>-independent pathway. C) The  
940 distinct genetic architectures found for *ubiT*, -U and -V in genomes where the three genes were present  
941 are displayed as boxes with different colors. Number of cases corresponding to each depicted  
942 architecture are given on the right. A white box corresponds to a gene found between the genes of  
943 interest, and a white box with dots corresponds to two to five genes between the genes of interest.

944  
945 **Figure 4: UbiU and UbiV are necessary for the anaerobic conversion of DMQ<sub>8</sub> into UQ<sub>8</sub>.** A) Conversion  
946 of DMQ<sub>8</sub> to UQ<sub>8</sub> with enzymes of the O<sub>2</sub>-dependent and the O<sub>2</sub>-independent pathways, indicated  
947 respectively above and below arrows (numbering of carbon atoms shown on DMQ<sub>8</sub> and polyprenyl tail  
948 represented by R). B) Quantification by HPLC-MS (monitoring of Na<sup>+</sup> adducts) of unlabeled (<sup>12</sup>C) and  
949 labeled (<sup>13</sup>C<sub>6</sub>) -DMQ<sub>8</sub> and -UQ<sub>8</sub> in  $\Delta ubiC \Delta ubiF$  cells after aerobic growth and transition to anaerobiosis. C-  
950 D) Same as in B with  $\Delta ubiC \Delta ubiF \Delta ubiU$  cells (C) and  $\Delta ubiC \Delta ubiF \Delta ubiV$  cells (D). nd, not detected,  
951 results representative of two independent experiments (B-D).

952  
953 **Figure 5: UbiV binds a [4Fe-4S] cluster.** A) UV-visible absorption spectra of as-purified UbiV (dotted line,  
954 47  $\mu$ M) and reconstituted holo-UbiV (solid line, 41  $\mu$ M); Inset: enlargement of the 300-700 nm region.  
955 The molar extinction coefficient  $\epsilon_{410nm}$  was determined to be  $10.8 \pm 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$  for holo-UbiV. B) X-  
956 band EPR spectrum of 785  $\mu$ M dithionite-reduced holo-UbiV. Recording conditions: temperature, 10K;  
957 microwave power, 10 mW; modulation amplitude, 0.6 mT. C) Comparative UV-visible absorption spectra  
958 of WT and different Cys-to-Ala mutants of UbiV after Fe-S cluster reconstitution, with the following  
959 concentrations: 41  $\mu$ M WT, 44  $\mu$ M C193A C197A, 46  $\mu$ M C39A C193A C197A, 47  $\mu$ M C180A C193A  
960 C197A, and 54  $\mu$ M C39A C180A C193A C197A. Proteins were in 50 mM Tris-HCl, pH 8.5, 25 mM NaCl,  
961 15% glycerol, 1m M DTT (A-C). D) UQ<sub>8</sub> quantification of  $\Delta ubiV$  cells transformed with pBAD-UbiV<sub>6His</sub>,  
962 pBAD-UbiV<sub>6His</sub> C180A, pBAD-UbiV<sub>6His</sub> C193A or empty pBAD and grown overnight in anaerobic MSGN +  
963 0.02% arabinose. Mean  $\pm$ SD (n=4-5), \*: p< 0.05, \*\*\*\*: p< 0.0001, unpaired Student’s t test.

964  
965 **Figure 6: The UbiU-V complex binds two [4Fe-4S] clusters.** A) UV-visible absorption spectra of as-  
966 purified UbiU-UbiV (dotted line, 17  $\mu$ M) and reconstituted holo-UbiU-UbiV (solid line, 15.5  $\mu$ M); Inset:  
967 enlargement of the 300-700 nm region. B) X-band EPR spectrum of 339  $\mu$ M dithionite-reduced holo-  
968 UbiU-UbiV; Recording conditions: temperature, 10K; microwave power, 2 mW; modulation amplitude,  
969 0.6 mT. C) Comparative UV-visible absorption spectra of Cys-to-Ala mutants of UbiU in the UbiU-UbiV  
970 complex after metal cluster reconstitution with the following concentrations: 15.5  $\mu$ M WT, 16.0  $\mu$ M  
971 UbiU-C169A C176A and 16.0  $\mu$ M UbiU-C193A C232A. Proteins were in 50mM Tris-HCl, pH 8.5, 150mM  
972 NaCl, 15% glycerol and 1mM DTT (A-C). D) UQ<sub>8</sub> quantification of  $\Delta ubiU$  cells transformed with pBAD-

973 UbiU (n=4), pBAD-UbiU C176A (n=2) or pBAD empty vector (n=3) and grown overnight in anaerobic  
974 MSGN+ 0.02% arabinose. Mean  $\pm$ SD, \*\*: p< 0.01, ns: not significant, unpaired Student's t test.

975

976 **Figure 7: Conserved four cysteines motifs in the U32 protease family.**

977 The conserved 4-cysteines motifs and PFAM domains (colored boxes) found in each U32 protease family  
978 are displayed for a set of reference sequences. These motifs were obtained by aligning the sequences  
979 listed by Kimura *et al.* [43]. Conserved cysteines are in red, "x6" indicates that 6 residues were found in  
980 between two conserved cysteines. Positions of the domains are displayed on the outside of the boxes for  
981 the reference sequences. Scrambled extremities show interrupted match for the PFAM domain. No  
982 conserved cysteines were found for U32#5 and U32#6 (see main text). Reference sequences were from  
983 *E. coli* for UbiU, UbiV, YegQ and RhlA (YHBU\_ECOLI, YHBV\_ECOLI, YEGQ\_ECOLI, YDCP\_ECOLI for RhlA).  
984 For the rest of the families, the sequence accession numbers were: R7JPV1\_9FIRM for U32#1,  
985 R6XKQ3\_9CLOT for U32#2, S1NZZ5\_9ENTE for U32#3, H1YXA1\_9EURY for U32#4, H3NJ45\_9LACT for  
986 U32#5, D5MIQ1\_9BACT for U32#6.

987 **SUPPLEMENTARY FIGURES LEGENDS**

988

989 **Figure S1:** A) HPLC-ECD analyses (mobile phase 2) of lipid extracts from 1 mg of WT,  $\Delta ubiT$  and  $\Delta ubiG$   
990 cells grown in LB medium under anaerobic conditions (UQ<sub>10</sub> used as standard). B) Genetic context of the  
991 *ubiT,U,V* genes in *M. marinus* (WP\_011715033.1). The scheme was drawn from a figure obtained using  
992 the GeneSpy program [81].

993

994 **Figure S2:** Multiple sequence alignment of UbiT from representative proteobacteria. Sequences were  
995 aligned using Mafft (linsi), and the output generated using Jalview and Inkscape. Hydrophobic residues  
996 are colored in blue. The position of the SCP2 domain (PF02036) is indicated by a red box. Genbank  
997 accession numbers: *Vibrio cholerae*, NP\_230303.1, *Pseudomonas aeruginosa*, NP\_252600.1, *Escherichia*  
998 *coli*, NP\_312065.1, *Yersinia pestis*, YP\_002348366.1, *Ralstonia solanacearum*, WP\_011004260.1,  
999 *Dechloromonas aromatica*, WP\_011285876.1, *Thiobacillus denitrificans*, WP\_011312695.1,  
1000 *Rhodopseudomonas palustris*, WP\_011666115.1, *Halorhodospira halophila*, WP\_011813831.1,  
1001 *Rhodobacter sphaeroides*, WP\_011909347.1, *Aeromonas salmonicida*, WP\_005310396.1, *Paracoccus*  
1002 *denitrificans*, WP\_011750456.1, *Neisseria sicca*, WP\_080614297.1, *Phaeospirillum fulvum*,  
1003 WP\_074767212.1.

1004

1005 **Figure S3:** Multiple sequence alignment of UbiU from representative proteobacteria. Sequences were  
1006 aligned using Mafft (linsi), and the output generated using ESPript and Inkscape. The four conserved  
1007 cysteines (C169, C176, C193 and C232) involved in iron-sulfur binding are indicated by black columns.  
1008 The position of the domain U32 protease PF01136 is indicated by a green box. GenBank accession  
1009 numbers: *Vibrio cholerae*, NP\_230301.1, *Pseudomonas aeruginosa*, NP\_252602.1, *Escherichia coli*,  
1010 NP\_312066.1, *Yersinia pestis*, YP\_002348368.1, *Ralstonia solanacearum*, WP\_011004262.1,  
1011 *Dechloromonas aromatica*, WP\_011285878.1, *Thiobacillus denitrificans*, WP\_011312698.1,  
1012 *Rhodopseudomonas palustris*, WP\_011666114.1, *Halorhodospira halophila*, WP\_011813833.1,  
1013 *Rhodobacter sphaeroides*, WP\_011909346.1, *Aeromonas salmonicida*, WP\_005310394.1, *Paracoccus*  
1014 *denitrificans*, WP\_041530457.1, *Neisseria sicca*, WP\_049226489.1, *Phaeospirillum fulvum*,  
1015 WP\_074767209.1.

1016

1017 **Figure S4:** Multiple sequence alignment of UbiV from representative proteobacteria. Sequences were  
1018 aligned using Mafft (linsi), and the output generated using ESPript and Inkscape. The four conserved  
1019 cysteines (C39, C180, C193 and C197 - positions in *E. coli*) involved in iron-sulfur binding are indicated by  
1020 black columns. The position of the domain U32 protease PF01136 is indicated by a green box. GenBank  
1021 accession numbers: *Vibrio cholerae*, NP\_230300.2, *Pseudomonas aeruginosa*, NP\_252601.1, *Escherichia*  
1022 *coli*, NP\_312067.2, *Yersinia pestis*, YP\_002348369.1, *Ralstonia solanacearum*, WP\_011004261.1,  
1023 *Dechloromonas aromatica*, WP\_011285877.1, *Thiobacillus denitrificans*, WP\_011312697.1,  
1024 *Rhodopseudomonas palustris*, WP\_011666113.1, *Halorhodospira halophila*, WP\_011813832.1,  
1025 *Rhodobacter sphaeroides*, WP\_011909345.1, *Aeromonas salmonicida*, WP\_005310392.1, *Paracoccus*  
1026 *denitrificans*, WP\_011750458.1, *Neisseria sicca*, WP\_080614296.1, *Phaeospirillum fulvum*,  
1027 WP\_074767477.1.  
1028

1029 **Figure S5:** A) Gel filtration chromatogram of aerobically purified UbiV on a Superdex 75 Increase 10/300  
1030 GL column; Inset: Coomassie staining SDS-PAGE of aerobically purified UbiV. B) Calibration curve,  
1031 standard proteins in rhombi, UbiV in circle. C) UV-visible absorption spectra of 41  $\mu$ M holo-UbiV under  
1032 anaerobiosis (solid line) and after 20 minutes exposure to air (dotted line). D) Comparative UV-visible  
1033 absorption spectra of aerobically purified wild-type (black) and different Cys-to-Ala mutants of UbiV  
1034 (C193A C197A in blue; C39A C193A C197A in green; C180A C193A C197A in pink; C39A C180A C193A  
1035 C197A in brown). Inset: Coomassie staining SDS-PAGE shows the purifications of UbiV and variants. Lanes  
1036 are: M, molecular mass marker; 1, wild-type UbiV; 2, UbiV C193A C197A; 3, UbiV C39A C193A C197A; 4,  
1037 UbiV C180A C193A C197A; 5, UbiV C39A C180A C193A C197. All proteins were in 50 mM Tris-HCl, pH 8.5,  
1038 25 mM NaCl, 15% glycerol, 1 mM DTT (A-D).

1039

1040 **Figure S6:** A) Gel filtration chromatogram of aerobically purified UbiU-UbiV on a Superdex 75 Increase  
1041 10/300 GL column; Inset: Coomassie staining SDS-PAGE of aerobically purified UbiU-UbiV complex. B)  
1042 Calibration curve, standard proteins in rhombi, UbiU-UbiV in circle. C) Coomassie staining SDS-PAGE  
1043 shows the purifications of UbiU-UbiV wild-type and variants proteins: Lanes are: M, molecular mass  
1044 marker; 1, wild-type UbiU-UbiV complex; 2, UbiU C169A C176A - UbiV wild-type; 3, UbiU C193A C232A -  
1045 UbiV wild-type. All proteins were in buffer 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 15% glycerol, 1 mM  
1046 DTT (A-D).

1047

1048 **Table S1:** UQ levels in strains from the medium- and large- deletion collections

1049 **Table S2:** Occurrence of genes from the UQ pathways in bacterial genomes.

1050 **Table S3:** List of oligonucleotides and strains used in this study

1051 **Table S4:** References of the protein sequences used to build the HMM profiles.