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1 **Ubiquinone biosynthesis over the entire O₂ range: characterization of a conserved, O₂-**
2 **independent pathway**

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27
28

29

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 (O₂) 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, O₂-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 O₂-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 α -, β -, γ -
40 proteobacterial clades including several human pathogens, supporting the widespread distribution of a
41 previously-unrecognized capacity to synthesize ubiquinone in the absence of O₂. Together, the O₂-
42 dependent and O₂-independent ubiquinone biosynthesis pathways contribute to optimize bacterial
43 metabolism over the entire O₂ range.

44

45 **IMPORTANCE**

46 In order to colonize environments with large O₂ gradients or fluctuating O₂ 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 O₂. In contrast, the long-described pathway
52 for ubiquinone biosynthesis requires O₂ as substrate. In fact, we find that many proteobacteria are
53 equipped with the O₂-dependent and O₂-independent pathways, supporting that they are able to
54 synthesize ubiquinone over the entire O₂ range. Overall, we propose that the novel O₂-independent
55 pathway is part of the metabolic plasticity developed by proteobacteria to face varying environmental O₂
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 (O₂) 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 O₂-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 O₂) [4],
67 whereas natural habitats cover the entire range of O₂ concentrations [5]. For instance, large O₂ 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 O₂ 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 O₂ 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 O₂ 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 O₂ [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 O₂
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 O₂ since all three hydroxylases - UbiI, UbiH and
91 UbiF – use O₂ 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 O₂ and thus function in UQ biosynthesis only when sufficient O₂ 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 O₂-independent biosynthesis pathway, which is still
96 uncharacterized.

97 In this study, we describe the O₂-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 O₂-independent UQ biosynthetic pathway is widely conserved in proteobacteria. UbiT
100 likely functions as an accessory factor in the O₂-independent UQ biosynthetic pathway and we show that
101 UbiU and UbiV are involved in at least one O₂-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 O₂-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 O₂.

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 UQ₈ 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 O₂-
112 independent UQ biosynthetic pathway, we grew a $\Delta ubiC$ strain anaerobically. The $\Delta ubiC$ strain showed a
113 diminished level of UQ₈, which was partially recovered by supplementation with 4-HB (Figure 1B).
114 Furthermore, we grew the $\Delta ubiC$ strain in a medium supplemented with ¹³C₇-4HB and we analyzed the
115 labelling of biosynthesized UQ₈ by HPLC-mass spectrometry (MS). In cells grown in aerobic or anaerobic
116 conditions, the labelled form of UQ (¹³C₆-UQ₈, m/z= 750.4 for the NH₄⁺ adduct) represented 98.3% and
117 97.3% (±0.2%) of the total UQ₈ pool, respectively (Figure 1C, 1D). As expected, $\Delta ubiC$ cells grown
118 anaerobically with unlabeled 4-HB didn't show any ¹³C₆-UQ₈ (Figure 1E). Altogether, these results
119 establish that 4-HB is the precursor of the O₂-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 O₂. Thus, we evaluated whether the
124 enzymes of the aerobic pathway are also involved in the O₂-independent pathway by measuring the UQ₈
125 content of knock-out (KO) strains grown in aerobic and anaerobic conditions. Deletion of *ubiA*, *ubiE* or
126 *ubiG* abrogated UQ₈ biosynthesis in both conditions whereas $\Delta ubiB$, $\Delta ubiD$ or $\Delta ubiX$ strains synthesized a
127 limited amount of UQ₈ 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 O₂-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 O₂-independent pathway [20]. Indeed,
134 we confirmed that all three FMOs are dispensable for the O₂-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 UQ₈ in anaerobic conditions (Figure 1G). Together, our results demonstrate that the

137 O₂-dependent and O₂-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 O₂-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 UQ₈ content by HPLC-electrochemical detection (HPLC-ECD). We found a complete
144 absence of UQ₈ 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 UQ₈ content and a
146 poor growth in synthetic medium supplemented with glycerol and nitrate (SMGN). However, those
147 strains showed better growth and higher UQ₈ content in LB medium (Table S1). Thus we did not
148 investigate them further, as a genetic defect affecting directly the O₂-independent UQ pathway was
149 unlikely. In contrast, ME4641 showed a profound UQ₈ deficiency and robust anaerobic growth in LB and
150 SMGN media (Table S1). Importantly, ME4641 had a WT UQ₈ level when grown aerobically, suggesting
151 that only the O₂-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 UQ₈. 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 UQ₈ 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 MK₈ and a two-fold increase in DMK₈ after
160 anaerobic growth (Table 1). This effect might indirectly result from the UQ₈ deficiency as it was also
161 observed in the $\Delta ubiG$ strain (Figure S1A).

162

culture condition	strain	UQ ₈ (pmoles/mg cells)	DMK ₈ (247nm peak area/mg cells)	MK ₈ (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 UQ₈ 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 UQ₈ deficiency (Table 1) and a normalization of the levels of octaprenylphenol
171 (OPP), an early UQ₈ biosynthetic intermediate (Figure 2E). The ~3 fold elevation of OPP in the *yhbT*, -*U*, -
172 *V* KO mutants suggested that the O₂-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 UQ₈ deficiency when the *ΔyhbT*, *ΔyhbU* and
176 *ΔyhbV* strains were grown anaerobically in various media (glycerol + DMSO, lactate + KNO₃) (Figure 2F),
177 showing that the UQ₈ 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 O₂-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 UQ₈ after growth in
183 anaerobic conditions (Table 1, Figure 2F), we wondered how this UQ₈ was synthesized. To verify if the
184 O₂-dependent pathway contributed to this synthesis, we inactivated the *ubiH* gene in the $\Delta ubiU$ strain.
185 Sensitive HPLC-MS detection established that UQ₈ was completely absent in extracts from the $\Delta ubiH$
186 $\Delta ubiU$ cells (Figure 2G). This result supported that the residual UQ₈ synthesized in $\Delta ubiU$ cells originated
187 from the O₂-dependent pathway and suggested that our anaerobic media may contain traces amounts of
188 O₂ or that O₂-dependent UQ biosynthesis may occur during the handling of cells, under normal
189 atmosphere, prior to quinone extraction.

190 To eliminate the traces of O₂, 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 O₂-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 UQ₈ in $\Delta ubiT$, $\Delta ubiU$ and $\Delta ubiV$ strains (Figure 2H, 2I). In contrast, the UQ₈ 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 O₂. 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 O₂-
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 (α -, β -, γ -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 α -, β -, γ -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 O₂-
214 dependent and O₂-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 O₂-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 O₂-independent
226 pathway for UQ production as their genomes contain the *ubiT*, *-U* and *-V* genes but no O₂-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 O₂-independent UQ biosynthesis pathway is widespread in α -, β -, γ -
231 proteobacterial orders and co-exists with the O₂-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 α -, β -, γ -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 (α -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 O₂-independent hydroxylation of DMQ₈**

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 O₂-
250 independent UQ pathway, as UbiJ is required exclusively for the O₂-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 O₂-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 O₂-independent conversion of
256 labeled DMQ₈ into labeled UQ₈. This assay monitors the C6-hydroxylation and the subsequent O6-
257 methylation (Figure 4A). $\Delta ubiC \Delta ubiF$ cells grown aerobically with ¹³C₇-4HB synthesized DMQ₈, 73% of
258 which was labelled with ¹³C₆. Upon transfer to anaerobic conditions, the cells gradually converted a
259 significant part of (¹³C₆)-DMQ₈ into (¹³C₆)-UQ₈ (Figure 4B). Inactivation of either *ubiU* or *ubiV* in $\Delta ubiC$
260 $\Delta ubiF$ cells did not perturb the accumulation of (¹³C₆)-DMQ₈ but prevented its conversion into (¹³C₆)-UQ₈
261 (Figure 4C-D). This result demonstrates that UbiU and UbiV are essential for the C6-hydroxylation
262 reaction of the O₂-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_{6His}}, 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 ± 0.13	3.3 ± 0.18
UbiV C193A C197A	8,4	2.4 ± 0.02	2.2 ± 0.13
UbiV C39A C193A C197A	15,5	1.9 ± 0.01	1.5 ± 0.4
UbiV C180A C193A C197A	26,7	0.7 ± 0.05	0.6 ± 0.1
UbiV C39A C180A C193A C197A	57,4	0.1 ± 0.04	0
UbiU WT - UbiV WT	4,3	7.9 ± 0.05	7.5 ± 0.06
UbiU C169A C176A - UbiV WT	7,3	4.7 ± 0.03	5.8 ± 0.1
UbiU C193A C232A - UbiV WT	8	3.9 ± 0.05	5.8 ± 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_{6His} (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]²⁺ 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_{6His} 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 A₂₈₀/A₄₁₀
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 CX₆CX₁₅CX_{3/4}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 O₂-independent UQ biosynthesis pathway is widespread in proteobacteria:**

325 The evidence for an O₂-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 O₂-dependent and O₂-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 O₂-independent biosynthesis of UQ in *E. coli* (Figure 2). The facts that the UbiT, *-U*, *-*
333 *V* proteins are widespread in α , β , γ -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, O₂-independent UQ
335 pathway. Overall, our data support that many proteobacteria have the previously-unrecognized capacity
336 to synthesize UQ independently from O₂.

337 **Physiological possibilities offered by UQ biosynthesis over the entire O₂ 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 O₂-independent pathway for UQ production (Table S2B). Indeed, the vast
340 majority of proteobacteria with the O₂-independent UQ biosynthesis pathway also possess the O₂-
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 O₂ levels encountered by facultative aerobes.

344 So-called microaerobes, able to respire O₂ in microaerobic conditions, are very abundant in
345 Nature [5] and *E. coli* is known to respire nanomolar O₂ concentrations [47]. To sustain O₂ respiration in
346 the microaerobic range, these organisms are equipped with high affinity O₂ reductases [5,47,48]. These
347 enzymes reduce efficiently the low levels of environmental O₂ present at the cell's membrane, leaving
348 the cytoplasm devoid of any O₂ [49]. Under such conditions, UQ - which is the main electron donor for
349 the high affinity O₂ reductases *bdI* and *bdII* of *E. coli* [9] - must therefore be synthesized via the O₂-
350 independent pathway. Overall, we believe that the O₂-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 O₂-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 O₂ respiration. Our
357 identification of the anaerobic UQ pathway provides the unique opportunity to selectively disrupt UQ
358 biosynthesis depending on O₂ 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 O₂ 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 O₂-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 O₂-dependent pathway, whereas UbiT is
377 important only for the O₂-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 O₂-independent C6-hydroxylation of DMQ and the accumulation of OPP in Δ *ubiU* or
386 Δ *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 O₂-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 O₂-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 O₂-dependent hydroxylases was shown to
394 hydroxylate DMQ₀, 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 CX₆CX₁₆CX₃₈C motif in UbiU and in a CX_nCX₁₂CX₃C 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 CX₆CX₁₅CX_{3/4}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 O₂-independent hydroxylation reactions [64]. Together, our results identify UbiU and UbiV as prototypes
417 of a novel class of O₂-independent hydroxylases and extend the framework of the chemically-fascinating
418 O₂-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*^R colonies were selected. The
430 replacement of chromosomal *ubi* by *cat* gene was verified by PCR amplification in the *cat*^R 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 His₆-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_{6His}. The *ubiV*_{6His}
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 CaCl₂, 200 mM MgCl₂, 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 KNO₃ (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 (O₂<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 UQ₈ 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 O₂-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 (O₂<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 μ L of a 10
509 μ M UQ₁₀ 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 μ 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+NH₄⁺), m/z 656.0-656.8, 5-10 min, scan time 0.2 s; DMQ₈
522 (M+Na⁺), m/z 719-720, 6-10 min, scan time 0.2 s; ¹³C₆-DMQ₈ (M+Na⁺), m/z 725-726, 6-10 min, scan time
523 0.2 s; UQ₈ (M+NH₄⁺), m/z 744-745, 6-10 min, scan time 0.2 s; UQ₈ (M+Na⁺), m/z 749-750, 6-10 min, scan
524 time 0.2 s; ¹³C₆-UQ₈ (M+Na⁺), m/z 755.0-756, 6-10 min, scan time 0.2 s; UQ₁₀ (M+NH₄⁺), 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 DMK₈ and MK₈. ECD, MS and UV peak areas were
527 corrected for sample loss during extraction on the basis of the recovery of the UQ₁₀ internal standard
528 and were then normalized to cell's wet weight. The peak of UQ₈ obtained with electrochemical detection
529 was quantified with a standard curve of UQ₁₀ [17]. The absolute quantification of UQ₈ based on the m/z=
530 744.6 signal at 8 min (Figure 2H, I) was performed with a standard curve of UQ₈ ranging from 0.5 to 150
531 pmoles UQ₈ (the detection limit was around 0.1 pmole).

532 **Anaerobic ¹³C₆-UQ₈ biosynthesis activity assay:**

533 Δ ubiC Δ ubiF cells containing or not the additional Δ ubiU or Δ ubiV deletions were grown overnight in MS
534 medium supplemented with 0.2% glucose. This preculture was used to inoculate at OD₆₀₀=0.1, 100 mL of
535 fresh medium supplemented with 10 μ M ¹³C₇-4HB. The culture was grown at 37°C, 180 rpm until
536 OD₆₀₀=1, at which point 100 μ 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 O₂. 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://gtdb.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

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895

896

897 **FIGURE LEGENDS**

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

899 A) O₂-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, DMQ₈ for C6-
902 demethoxy-ubiquinone 8 and UQ₈ for ubiquinone 8. B) UQ₈ 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 UQ₈ obtained by
905 HPLC-MS analysis of lipid extracts from cells grown with ¹³C₇-4HB either anaerobically (C) or aerobically
906 (D), or anaerobically with unlabeled 4HB (E). F) UQ₈ quantification from WT and Δubi 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 (-O₂). Chromatograms are representative of n=3
911 independent experiments (UQ₁₀ used as standard). H) UQ biosynthesis represented with Ubi enzymes
912 specific to the O₂-dependent pathway (red), to the O₂-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 (-O₂). 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+NH₄⁺) in cells from table 2. The Δyhb
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) UQ₈ 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 UQ₈ (M+NH₄⁺) 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) UQ₈ 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
934

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 O₂-
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 O₂-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 DMQ₈ into UQ₈.** A) Conversion
946 of DMQ₈ to UQ₈ with enzymes of the O₂-dependent and the O₂-independent pathways, indicated
947 respectively above and below arrows (numbering of carbon atoms shown on DMQ₈ and polyprenyl tail
948 represented by R). B) Quantification by HPLC-MS (monitoring of Na⁺ adducts) of unlabeled (¹²C) and
949 labeled (¹³C₆) -DMQ₈ and -UQ₈ 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 μ M) and reconstituted holo-UbiV (solid line, 41 μ M); Inset: enlargement of the 300-700 nm region.
955 The molar extinction coefficient ϵ_{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 μ 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 μ M WT, 44 μ M C193A C197A, 46 μ M C39A C193A C197A, 47 μ M C180A C193A
960 C197A, and 54 μ 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) UQ₈ quantification of $\Delta ubiV$ cells transformed with pBAD-UbiV_{6His},
962 pBAD-UbiV_{6His} C180A, pBAD-UbiV_{6His} 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 μ M) and reconstituted holo-UbiU-UbiV (solid line, 15.5 μ M); Inset:
967 enlargement of the 300-700 nm region. B) X-band EPR spectrum of 339 μ 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 μ M WT, 16.0 μ M
971 UbiU-C169A C176A and 16.0 μ M UbiU-C193A C232A. Proteins were in 50mM Tris-HCl, pH 8.5, 150mM
972 NaCl, 15% glycerol and 1mM DTT (A-C). D) UQ₈ 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 (UQ₁₀ 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 μ 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.