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1 **A novel quinone biosynthetic pathway illuminates the evolution of aerobic metabolism**

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31 **Abstract**

32 The dominant organisms in modern oxic ecosystems rely on respiratory quinones with high redox
33 potential (HPQs) for electron transport in aerobic respiration and photosynthesis. The diversification of
34 quinones, from low redox potential in anaerobes to HPQs in aerobes, is assumed to have followed
35 Earth's surface oxygenation ~2.3 billion years ago. However, the evolutionary origins of HPQs remain
36 unresolved. Here, we reconstruct the biosynthetic pathway of a novel HPQ, methyl-plastoquinone, that
37 is unique to bacteria of the phylum *Nitrospirota*. We demonstrate that the three extant HPQ biosynthetic
38 pathways, in *Nitrospirota*, *Cyanobacteriota*, and *Pseudomonadota*, share a common origin that predates
39 the emergence of these phyla. We show that aerobic metabolism using HPQs is ancestral to
40 *Cyanobacteriota* and *Pseudomonadota* and significantly predates Earth's surface oxygenation.

41 **Introduction**

42 The oxygenation of Earth's surface environments following the emergence of oxygenic
43 photosynthesis in ancestors of *Cyanobacteriota* enabled the metabolic and genetic diversification of life
44 (1–4). The use of oxygen as a terminal electron acceptor, i.e., aerobic respiration, enabled a higher
45 energy yield compared to anaerobic metabolisms and was a prerequisite for the emergence of
46 eukaryotes (5, 6). However, it remains poorly resolved how and when the electron transport chain (ETC)
47 used for aerobic respiration evolved. While geochemical evidence indicates iron oxidation by
48 acidophilic bacteria must have evolved by the time oxygen accumulated in the atmosphere during the
49 great oxygenation event (GOE; ~2.4–2.3 Ga) (3, 7–9), there is now considerable evidence for an ancient
50 origin of dioxygen-utilizing and detoxifying enzymes as early as 3.1 Ga (10–12). Though these enzymes
51 may not have participated in aerobic respiration (11, 13, 14), their widespread occurrence in bacteria
52 suggests the availability of oxygen in physiologically significant quantities, at least in some niches,
53 before the GOE. Studying the evolution of ETC components, such as oxygen reductases that use
54 electrons derived from the ETC (15–20), can help elucidate the origins of aerobic metabolisms.
55 However, the interpretation of oxygen reductase evolution has remained contentious (15–19, 21), and
56 alternative roles of ancestral oxygen reductases in oxygen detoxification and nitric oxide reduction
57 rather than aerobic respiration have been proposed (16). Exploring the evolution of other ETC
58 components, such as respiratory quinones, may yield new insights into the evolution of ETCs and
59 aerobic respiration.

60 Strict anaerobes use ETCs and quinones with low redox potential (LPQs), while aerobes and
61 facultative aerobes generally use high-potential quinones (HPQs) (22–24). HPQs require all parts of the
62 ETC to operate at high redox potential (24–26) and confer no known benefit over LPQs under anaerobic
63 conditions. However, under aerobic conditions HPQs are advantageous due to their decreased electron
64 leakage to oxygen, thus reducing oxidative stress and minimizing free energy losses (25, 27). The
65 occurrence of HPQs may represent a marker for high-potential ETCs and their evolution may be tied to
66 the history of oxygenic photosynthesis and aerobic respiration. Within bacteria, HPQs have been found
67 only in two phyla, oxygenic *Cyanobacteriota* (here used *sensu stricto*, including only *Cyanophyceae*)

68 and *Pseudomonadota* (formerly Proteobacteria, now comprising the classes Alpha-, Beta-, Gamma-,
69 and Zetaproteobacteria, Acidithiobacillia, and Hydrogenophilalia) (28). The *Cyanobacteriota* and
70 *Pseudomonadota* produce two distinct types of HPQs, plastoquinone (PQ) and ubiquinone (UQ),
71 respectively (22, 23), which became the quinones of plastids (PQ) and mitochondria (UQ) through
72 endosymbiosis during the early evolution of eukaryotes (23, 29). Yet, despite the dominance of HPQ-
73 utilizing organisms in Earth's oxic environments today (30–35), the co-evolution of HPQs and Earth
74 surface oxygenation remains largely unresolved (36).

75 Recent progress in metagenomic coverage of uncultivated bacteria and isolation of novel lineages
76 may help elucidate HPQ evolution through the discovery of new quinone structures and biosynthetic
77 pathways in unstudied lineages of aerobic bacteria. Here, we describe the discovery of a third, novel
78 type of HPQ, methyl-plastoquinone (mPQ). mPQ occurs only in aerobic members of the phylum
79 *Nitrospirota* (formerly Nitrospirae), a metabolically diverse group of bacteria that perform essential
80 transformations in the biogeochemical cycles of iron, nitrogen, and manganese. We characterize the
81 biosynthetic pathway of mPQ using bioinformatic, genetic, and biochemical techniques and use these
82 data to infer the evolutionary history of HPQs. Our study sheds new light on the evolutionary history
83 of ETCs by revealing a single origin of the three HPQ biosynthetic pathways prior to the radiation of
84 crown-group *Cyanobacteriota*, *Nitrospirota*, and *Pseudomonadota*, which evidently preceded the GOE.

85 **Results & Discussion**

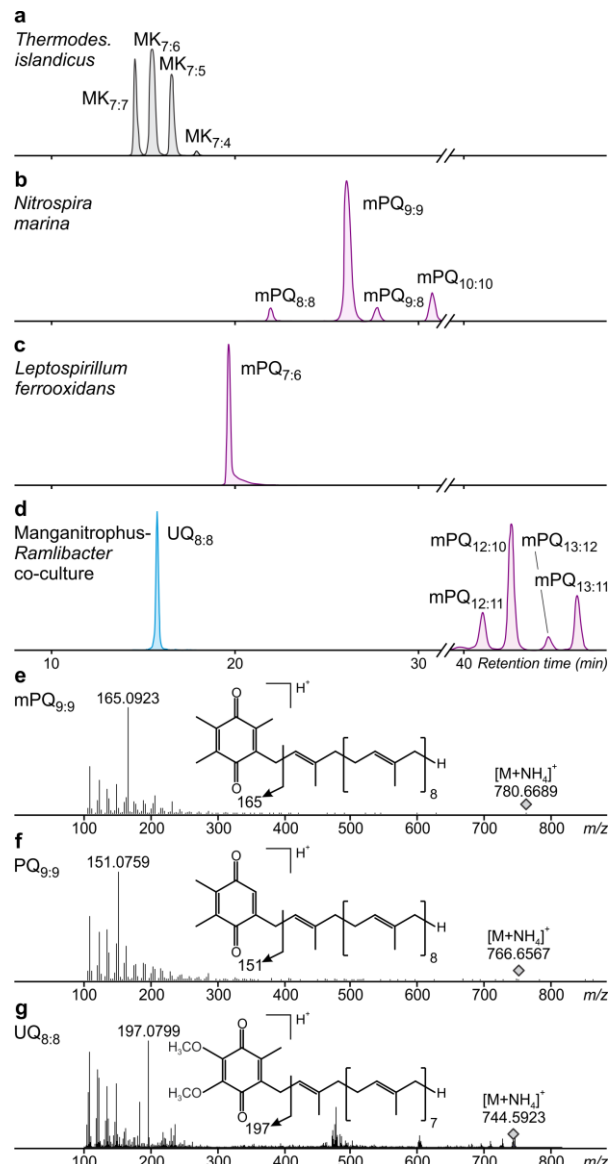
86 *Novel respiratory quinones in Nitrospirota*

87 Despite their widespread distribution and the important roles of *Nitrospirota* in biogeochemical
88 cycles of iron, manganese, and nitrogen (37–41), many aspects of their chemotaxonomy and
89 bioenergetics remain understudied. Genome-based bioenergetic models implicate the presence of ETCs
90 in aerobic and anaerobic *Nitrospirota* (41–45), yet their corresponding respiratory quinones have not
91 been studied. During screening of *Nitrospirota* genomes for lipid biosynthetic pathways (46), we
92 observed that the genomes of aerobic *Nitrospirota* did not contain any of the characterized quinone
93 biosynthesis pathways (23, 36). In contrast, genomes of anaerobic sulfur-reducing *Nitrospirota*, i.e.,
94 *Thermodesulfovibrio* species and some *Nitrospirota* metagenome-assembled genomes from anoxic

95 environments, contained the futasine pathway (MK_{mqn} , composed of *mqn* genes) for biosynthesis of
96 the LPQ, menaquinone (MK; Supplementary Datafile S1).

97 To evaluate the presence of respiratory quinones, we analyzed lipid extracts of one anaerobic and
98 eight aerobic species of *Nitrospirota*, covering all formally described genera (*Thermodesulfovibrio*,
99 *Leptospirillum*, *Nitrospira*, and *Manganitrophus*), using high-performance liquid chromatography
100 coupled to high-resolution tandem mass spectrometry. MKs were detected only in the anaerobic species,
101 *Thermodesulfovibrio islandicus* (Fig. 1a), and we did not find any of the previously known respiratory
102 quinone types in the aerobic *Nitrospirota*. Instead, all eight studied aerobic *Nitrospirota* contained a
103 novel type of quinone, identified as methyl-plastoquinone (mPQ). The polyprenyl chain of mPQ varied
104 in length and saturation depending on species (Fig. 1, Fig. S1, SI results). Mass spectrometric
105 characterization of mPQ revealed fragmentation spectra analogous to PQ but with a dominant ion at
106 m/z 165 instead of 151, indicative of a distinctive trimethyl-benzoquinone headgroup connected to the
107 isoprenoid tail (Fig. 1, Fig. S1, Table S1). Stable isotope labeling experiments and nuclear magnetic
108 resonance spectroscopy of mPQ confirmed the structural assignment of the headgroup (SI Results &
109 Discussion; Fig. S2-4). Specifically, 1H -NMR spectra showed the absence of any proton linked to the
110 C2 of the quinone moiety and 1H -NMR and ^{13}C -NMR confirmed the presence of a third methyl group
111 (Fig. S4; see SI Results & Discussion). mPQ is thus structurally related to both UQ (methylated at C2
112 of the benzoquinone) and PQ (methylated at C5 and C6).

113



114
 115 **Fig. 1. Novel quinones detected in aerobic *Nitrospirota*.** a-d, Chromatograms showing presence of a distinct
 116 quinone type (methyl-plastoquinone, mPQ) in aerobic *Nitrospirota* (*Nitrospira marina*, *Leptospirillum*
 117 *ferrooxidans*, *Ca. Manganitrophus noduliformans*) and canonical menaquinones (MK) in the anaerobic
 118 *Nitrospirota* species *Thermodesulfobacterium islandicus*. Ubiquinone (UQ_{8.8}) in the *Ca. Manganitrophus-Ramlibacter*
 119 co-culture derives from *Ramlibacter* (see Fig. S1). e-g, High resolution mass spectrometric characterization of
 120 mPQ_{9.9} and PQ_{9.9} showing similar fragmentation patterns but suggesting the presence of a trimethyl-benzoquinone
 121 moiety in mPQ_{9.9} (see Fig. S1); structure and fragmentation pattern of UQ_{8.8} from *Ramlibacter* shown in g for
 122 reference.

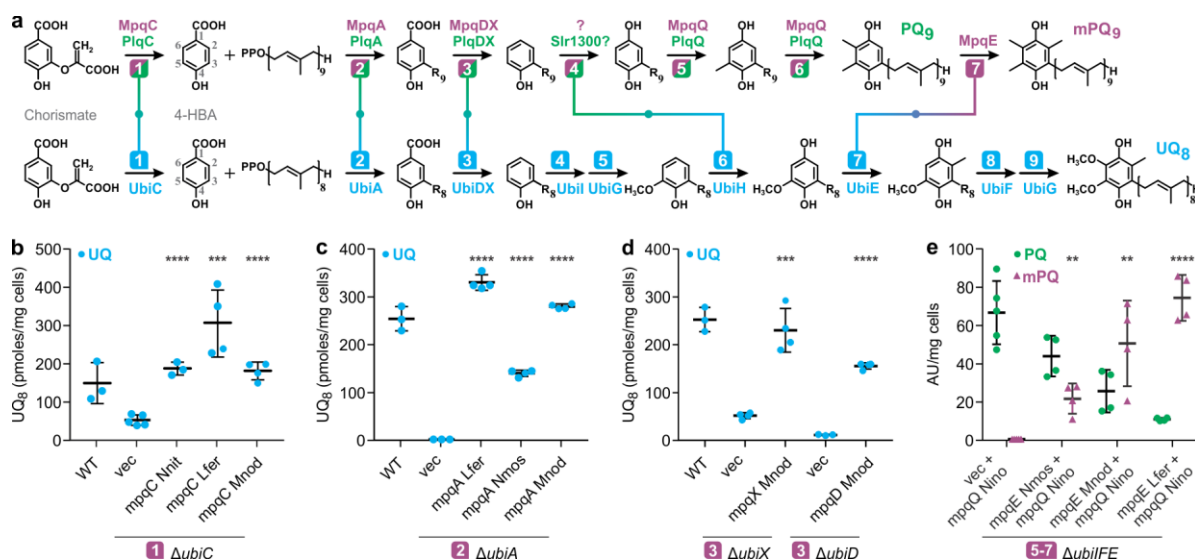
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Characterization of the biosynthetic pathway of mPQ

126 Based on the structure of mPQ, we hypothesized that its biosynthesis pathway might share
 127 characteristics with the UQ and PQ biosynthesis pathways. The PQ biosynthesis pathway of
 128 *Cyanobacteriota* has been partially resolved (47, 48) and contains several enzymes that are homologous
 129 to enzymes involved in the well-characterized bacterial UQ pathway (36, 49). In both pathways, the
 130 conversion of chorismate into 4-hydroxybenzoate (4-HBA) is mediated by a UbiC homolog and the

131 subsequent prenylation, decarboxylation, and hydroxylation of 4-HBA involve the UbiA, UbiD/X, and
132 UbiH homologs, respectively (Fig. 2a) (36). Specific to the UQ pathway, methylation at C2 is mediated
133 by UbiE (50). In the cyanobacterial PQ pathway, methylation at C5 and C6 has been proposed to be
134 mediated by SII0418 (PlqQ) (49, 51).

135 Isotope labeling experiments further point to biochemical similarities between the HPQ
136 biosynthesis pathways. Supplementation of cultures with ring-¹³C₆-labeled substrates demonstrates that
137 4-HBA is the ring precursor in *Nitrospirota*, similar to *Cyanobacteriota* and *Pseudomonadota* (Fig. S5,
138 SI Discussion). Further, experiments with methyl-²H₃ methionine indicate that all three methyl groups
139 of mPQ (at C2, C5, and C6) are derived from methionine via SAM-dependent methyltransferases (Fig.
140 S2). We used this information to find multiple, homologous candidate genes for the biosynthetic
141 pathway of mPQ in genomes of *Nitrospirota*. We suggest a gene nomenclature for the mPQ pathway
142 (*mpq*) analogous to that of the UQ pathway and extend this to the PQ pathway (*plq*; Fig. 2). We
143 identified a four-gene cluster in *Leptospirillum* spp. (Fig. S6), encoding a *ubiA* family prenyltransferase
144 (*mpqA*; LFE_2122), *ubiC*-like chorismate pyruvate lyase (*mpqC*; LFE_2123), a cobalamin-binding
145 radical *S*-adenosyl methionine (SAM) methyltransferase (LFE_2124), and a *ubiE*-like
146 methyltransferase (*mpqE*; LFE_2125). The genes are not co-localized in other *Nitrospirota*, but *mpqA*
147 and *mpqE* homologs are found in all aerobic *Nitrospirota*, in addition to a *ubiB*-like kinase (*mpqB*). By
148 contrast, homologs of *mpqC*, *ubiD/X* (*mpqD/X*) and *plqQ* (*mpqQ*) occur only in a subset of aerobic
149 *Nitrospirota* (Table S2). No clear *ubiH* homologs were identified. Consequently, aerobic *Nitrospirota*
150 contain a mosaic pathway for mPQ biosynthesis composed of well conserved genes (*mpqA*, *mpqB*,
151 *mpqE*) and alternative genes (*mpqC*, *mpqD/X*, *mpqQ*).



152

153 **Fig. 2. Characterization of the mPQ biosynthetic pathway.** **a**, Biosynthetic pathways of quinones showing
 154 homology of pathways for mPQ₉ in *Nitrospirota* (purple), PQ₉ in the cyanobacterium *Synechocystis sp.* PCC6803
 155 (green) and UQ₈ in the gammaproteobacterium *Escherichia coli* (blue). Biosynthetic steps are numbered, and
 156 homologous steps are connected by colored lines. **b-d**, Heterologous complementation experiments using mPQ
 157 biosynthesis gene candidates to restore UQ₈ production in *E. coli* mutants lacking key genes for ubiquinone
 158 biosynthesis ($\Delta ubiC+mpqC$, $\Delta ubiA+mpqA$, $\Delta ubiX+mpqX$, $\Delta ubiD+mpqD$). **e**, PQ production in *E. coli* $\Delta ubiIFE$
 159 mutants complemented with $mpqQ$ from *N. inopinata* as well as PQ and mPQ in *E. coli* $\Delta ubiIFE$ mutants
 160 complemented with $mpqQ$ from *N. inopinata* and $mpqE$ from other *Nitrospirota*. WT=wild type; vec=empty
 161 vector; thick bars represent means and error bars represent standard deviations of the means, $n=3-5$; AU=arbitrary
 162 units. Abbreviations: *Ca. N. nitrificans* (Nnit), *N. moscoviensis* (Nmos), *N. inopinata* (Nino), *L. ferrooxidans*
 163 (Lfer), *Ca. M. noduliformans* (Mnod). The numbering of the carbon atoms on the 4-HBA precursor (panel **a**, light
 164 grey) defines the nomenclature for all intermediates described in the text. The octaprenyl and nonaprenyl chains
 165 are abbreviated with R₈ and R₉, respectively. See Fig. S7-S9 for details on compound identification and
 166 quantification. Stars indicate $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) for unpaired Student's *t* tests
 167 relative to the empty vector.

168

169 We verified the mPQ candidate genes by assessing their functions in heterologous
 170 complementation assays using *Escherichia coli* mutants deficient in defined steps of UQ biosynthesis.
 171 When expressed in the *E. coli* $\Delta ubiC$ mutant, the $mpqC$ from *L. ferrooxidans*, *N. nitrificans*, and *M.*
 172 *noduliformans* restored UQ biosynthesis up to wild-type levels (Fig. 2b). Likewise, the $mpqA$ homologs
 173 from *L. ferrooxidans*, *N. moscoviensis*, and *M. noduliformans* restored UQ biosynthesis in an *E. coli*
 174 $\Delta ubiA$ mutant (Fig. 2, S7). Similarly, we observed recovery of UQ levels in *E. coli* $\Delta ubiD$ and $\Delta ubiX$
 175 mutants upon expression of $mpqD/X$ from *M. noduliformans* ($mpqD/X$ do not occur in *Nitrospira* and
 176 *Leptospirillum spp.*; Fig. 2). Expression of the $plqQ$ homolog ($mpqQ$) from *N. inopinata* in an *E. coli*
 177 $\Delta ubiIFE$ mutant yielded PQ₈ and mPQ₈ (Fig. S8b-g). The $\Delta ubiIFE$ strain, in which the *E. coli* $ubiE$ gene
 178 was additionally deleted, showed a strong increase in the amount of PQ₈ and the disappearance of mPQ₈

179 (Fig. 2e, S8b-g). Finally, expression of *mpqE* from *L. ferrooxidans*, *N. moscoviensis*, and *Ca. M.*
180 *noduliformans* in the $\Delta ubiIFE$ strain led to the accumulation of mPQ₈ (Fig. 2e, S8c).

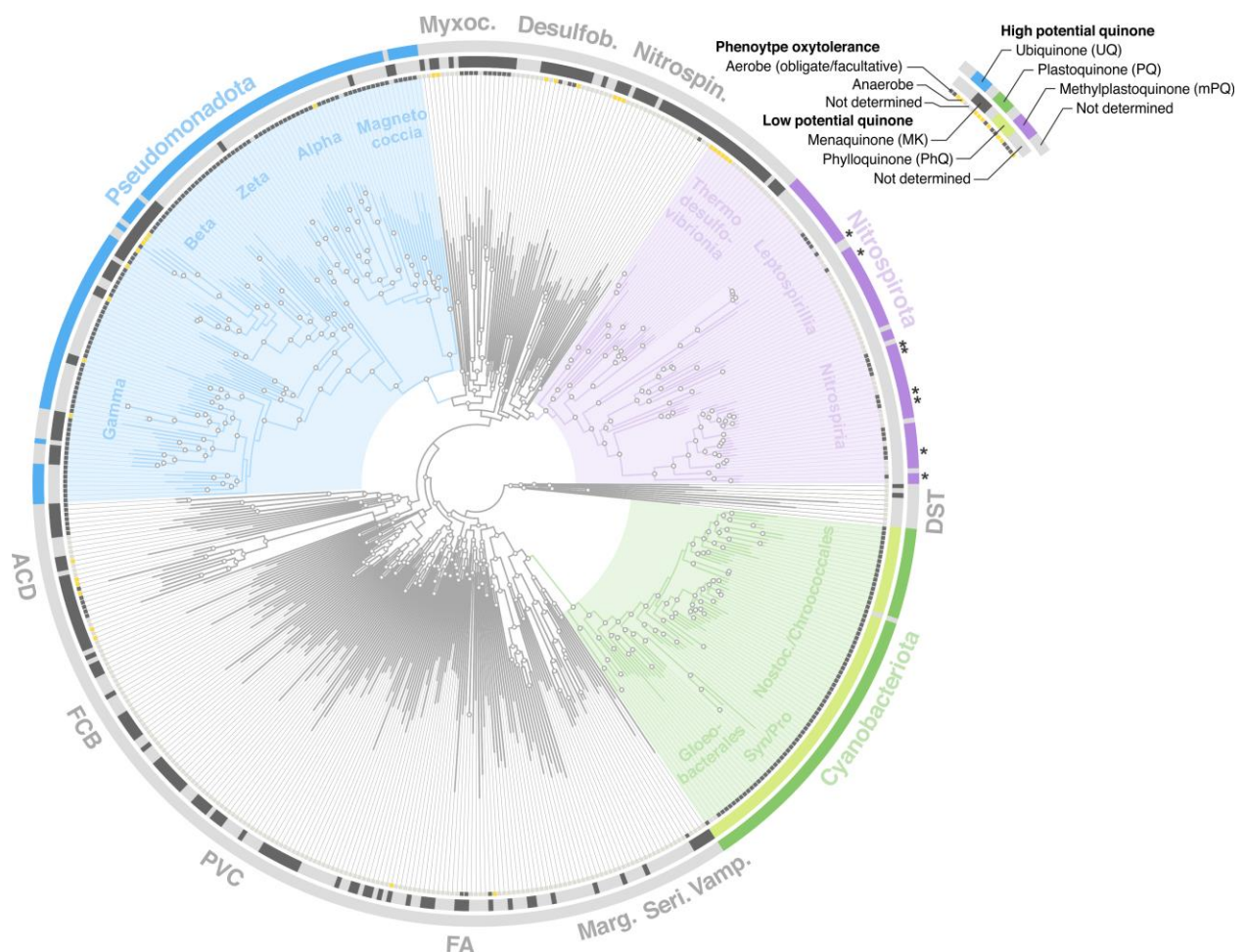
181 Based on these heterologous expression and isotope labeling experiments, we reconstructed a
182 tentative mPQ biosynthetic pathway that shares homology with the UQ and PQ pathways (Fig. 2). The
183 ring precursor 4-HBA is generated from chorismate by MpqC and alternative enzymes, followed by
184 prenylation of 4-HBA by MpqA and decarboxylation by MpqD/X. The following hydroxylation step at
185 C1 is unresolved, but observations from *Pseudomonadota* indicate that a large diversity of
186 benzoquinone C1 hydroxylases exist in nature (36, 52–54). Finally, methylations are introduced at C5
187 and C6 by MpqQ and at C2 by MpqE.

188 ***Distribution and function of mPQ***

189 Analysis of mPQ biosynthesis proteins in a representative selection of high- and medium-quality
190 genomes and metagenome-assembled genomes revealed that mPQ is present in all aerobic lineages of
191 *Nitrospirota* ($n=85$), but not found outside this phylum ($n=482$). A few early-branching lineages of
192 *Nitrospirota*, which are anaerobes using the MK_{mqn} pathway, are devoid of mPQ biosynthesis proteins
193 (Fig. 3). Since mPQ is the only respiratory quinone found in aerobic *Nitrospirota*, it is likely involved
194 in the ETC used for aerobic respiration (41–43), and the structural similarity between mPQ and UQ/PQ
195 suggests that mPQ has a high redox potential. Since *Nitrospirota* grow slowly and to low cell densities,
196 mPQ could not be isolated in quantities required for redox potential measurements. We therefore
197 calculated the redox potential of mPQ, UQ, and PQ using density functional theory (55). For a given
198 biologically relevant prenyl chain length, the calculated redox potential of mPQ ($E^0Q/H2Q = 517 \pm 8$
199 mV) is lower than that of PQ (551 ± 8 mV) but higher than that of UQ (480 ± 8 mV; Table S3).
200 Furthermore, all HPQs are described by significantly higher calculated redox potentials than the LPQ
201 MK (364 ± 8 mV), confirming the validity of our computational approach. Calculations for simple 1,4-
202 benzoquinones indicate that redox potentials decrease by ~ 50 mV per methyl or methoxy group, with
203 methoxy additions having the larger effect, which explains the higher potential of mPQ (trimethyl)
204 relative to UQ (dimethoxy, methyl). These functional group combinations may reflect redox tuning of
205 HPQs to specific components of the ETC in *Cyanobacteriota*, *Nitrospirota*, and *Pseudomonadota*. Due

206 to the tight coupling of redox potentials of quinones to other ETC components (e.g., iron sulfur clusters
 207 of Rieske proteins and hemes) (26), we infer that aerobic *Nitrospirota* have high potential ETCs. Indeed,
 208 we find that Rieske proteins of aerobic *Nitrospirota* contain the ‘SY’ motif (Table S4) characteristic for
 209 Rieske/cytochrome *b* complexes adapted to interact with HPQs in high potential ETCs (56). High
 210 potential ETCs would be advantageous for minimizing ROS generation and maximizing proton motive
 211 force (26) in the low energy-yielding chemoautotrophic metabolisms of aerobic *Nitrospirota*. Since the
 212 use of HPQs requires adaptation of the entire ETC to higher redox potential (26), such a decisive step
 213 may have been linked to a major event, such as Earth’s surface oxygenation (24, 26, 57).

214



215 **Fig. 3. Phylogenetic tree of bacteria showing the occurrence of respiratory quinones.** Quinones with high
 216 redox potential (UQ, PQ, mPQ) occur only in aerobic *Nitrospirota*, *Pseudomonadota*, and *Cyanobacteriota*. Low
 217 potential quinones occur in anaerobic *Nitrospirota* (MK), some *Pseudomonadota* (MK), and all *Cyanobacteriota*
 218 (PhQ). Asterisks indicate strains in which presence of mPQ has been verified experimentally. See Fig. S11-13 for
 219 detailed trees. The maximum-likelihood phylogenetic tree was constructed from 120 concatenated single copy
 220 marker proteins (58) of 547 isolate genomes and metagenome-assembled genomes, covering all bacterial phyla,
 221 and rooted using the DST group to approximate the bacterial root (59). Quinone occurrences were derived from
 222 instrumental analysis of isolates or inferred from the presence of key biosynthesis genes (SI results);
 223

224 Supplementary Datafile S3; including literature data). Phenotype oxytolerance was curated from strain
225 descriptions. Selected classes/orders denoted inside of rings. Selected phyla denoted outside of rings: ACD,
226 *Aquificota-Campylobacterota-Deferribacterota*; Desulfob., *Desulfobacterota*; DST, *Deinococcota-Synergistota*
227 *Thermotogota*; FA, *Firmicutes-Actinobacteriota*; FCB, *Fibrobacterota-Chlorobacteria-Bacteroidota*; Marg.,
228 “*Candidatus Margulisbacteria*”; Myxoc., *Myxococcota*; Nitrospin., *Nitrospinota*; PVC, *Planctomycetota*
229 *Verrucomicrobiota-Chlamydiota*; Seri., “*Candidatus Sericytochromatia*”; Vamp., *Vampirovibrionophyceae*.
230

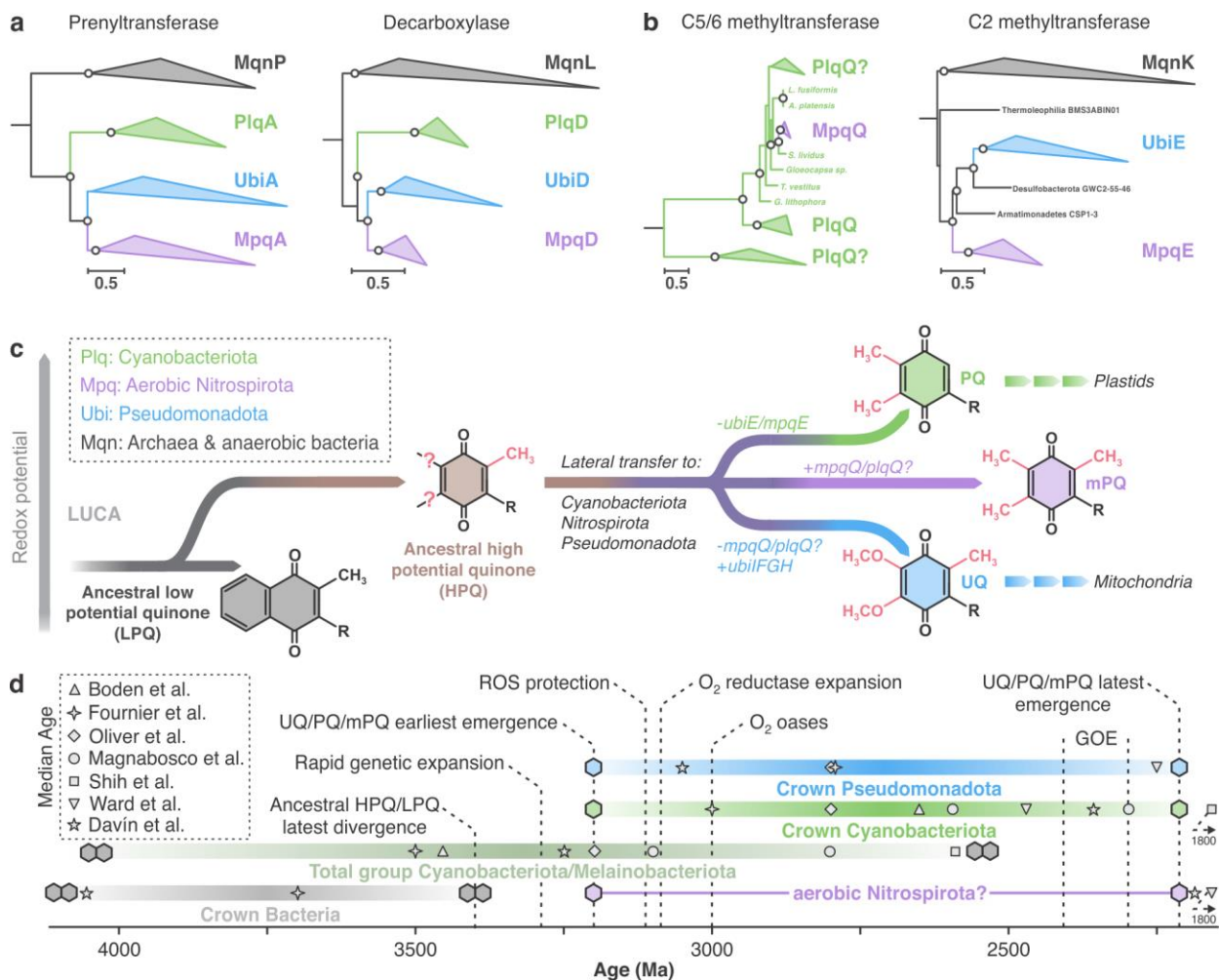
231 ***Ancient origin of high-potential quinones***

232 The biochemical similarity of the HPQ biosynthesis pathways suggests a common ancestry. The
233 mPQ biosynthesis proteins of Nitrospirota are most closely related to homologs from the UQ and PQ
234 pathways of *Cyanobacteriota* and *Pseudomonadota*, which is unexpected given the phylogenetic
235 distance between these phyla (Fig. 3). Specifically, the phylogenies of the key prenyltransferases and
236 decarboxylases exhibit a consistent tree topology, with HPQ proteins being monophyletic relative to
237 homologous proteins of the LPQ biosynthesis pathways (Fig. 4; see SI for expanded discussion). HPQ
238 homologs from *Pseudomonadota* and *Nitrospirota* branch as sister lineages with respect to
239 *Cyanobacteriota*. Other proteins of the HPQ pathways (chorismate-pyruvate lyase, decarboxylase co-
240 factor) generally support this topology, although with lower branch support (Fig. S19-20). These
241 patterns suggest a single, shared origin of the universal core of HPQ biosynthesis in bacteria.

242 The distribution of LPQs, HPQs and their associated biosynthetic genes in Bacteria suggests that
243 HPQ biosynthesis is conserved in all known lineages of *Cyanobacteriota*, *Pseudomonadota*, and
244 aerobic *Nitrospirota* (Fig. 3, S11-13; see SI for expanded discussion). Conversely, HPQs are not found
245 in anaerobic *Nitrospirota*, nor in the sister phyla of *Cyanobacteriota*, *Nitrospirota*, or *Pseudomonadota*,
246 all of which produce LPQs via the MK_{mqn} pathway (Fig. 3; Table S5). Given that *Cyanobacteriota*,
247 *Nitrospirota*, and *Pseudomonadota* are paraphyletic, vertical inheritance of HPQ pathways from a
248 common ancestor is unlikely. Instead, HPQ occurrence and protein phylogenies indicate that an
249 ancestral HPQ pathway was laterally acquired by stem-group *Cyanobacteriota*, *Pseudomonadota*, and
250 aerobic *Nitrospirota* from an unknown or extinct donor lineage.

251 The ancestral HPQ pathway later diversified through changes to the C2, C5, and C6 substituents.
252 Specifically, C2 methyltransferases are present in all LPQ and HPQ biosynthesis pathways except PQ.
253 LPQ and HPQ C2 methyltransferases form sister clades (Fig. 4b) and C2 methyltransferases may thus

254 be as old as the divergence between LPQ and HPQ pathways. Consequently, it is likely that the ancestral
 255 HPQ pathway contained a C2 methyltransferase that was lost prior to the radiation of crown group
 256 *Cyanobacteriota* (Fig. 4c). Lack of C2 methylation increases the redox potential of PQ (Table S3) and
 257 is essential for the functioning of the oxygen-evolving photosystem II (60). Loss of C2-methylation
 258 was likely linked to the evolution of oxygenic photosynthesis and therefore did not occur in
 259 *Pseudomonadota* and *Nitrospirota*. The evolution of the C5/C6 functional groups is less constrained.
 260 The C5/C6 methyltransferases of the PQ/mPQ pathways are poorly conserved in *Nitrospirota* and
 261 *Cyanobacteriota* but at least one subgroup of *Nitrospirota* laterally acquired a C5/C6 methyltransferase
 262 from *Cyanobacteriota* (Fig. 4b; see SI discussion). C5/C6 methylation requires a single enzyme,
 263 whereas methoxylation to yield UQ requires at least two enzymes that are specific to *Pseudomonadota*
 264 (36, 54). Thus, the most parsimonious explanation is that the ancestral HPQ was methylated at C5/C6
 265 in addition to C2, i.e., identical to mPQ, and that methoxylation evolved later (Fig. 4c).



266

267 **Fig. 4. High-potential quinones (HPQ) share a single origin predating the great oxygenation event. a,**
268 Phylogenetic trees of HPQ biosynthesis proteins demonstrating that prenyltransferases and decarboxylases of the
269 ubiquinone (UQ, UbiAD), plastoquinone (PQ, PlqAD), and methylplastoquinone (mPQ, MpqAD) pathways form
270 sister clades of the archaeal and bacterial fufalosine pathway for biosynthesis of menaquinone (MK, MqnPL). **b,**
271 Phylogenetic trees of quinone C5/C6 (PlqQ, MpqQ) and C2 methyltransferases (UbiE, MpqE), showing a nested
272 topology of C5/C6 methyltransferases and that C2 methyltransferases form a sister lineage of menaquinone-
273 associated methyltransferases (MqnK). Outgroups used for rooting the trees are discussed in the Supplementary
274 Information. Scale bars indicate 0.5 substitutions per site. Open circles indicate ultra-fast bootstrap support ≥ 0.9 .
275 **c,** Conceptual sketch of HPQ evolution and resulting redox potentials. **d,** Timescale of LPQ and HPQ evolution
276 (colors as in panel c) in relation to geochemical changes (evidence for localized O₂ oases (61–63), the great
277 oxygenation event, GOE(7)) and biological innovations (Archean rapid genetic expansion (10), evolution of
278 enzymes protecting against reactive oxygen species (ROS) (12), expansion of O₂ reductase diversity (11)). Shaded
279 hexagons indicate minimum and maximum estimates of HPQ evolution timescale. Open symbols indicate median
280 ages (colored bars: uncertainty range; quinone symbols: upper/lower estimate) of relevant clades estimated by
281 previous molecular clock analyses (Boden et al. (12); Davín et al. (64); Fournier et al. (65); Oliver et al. (66);
282 Magnabosco et al. (67); Shih et al. (68); Ward et al. (69)).

283 It has been proposed that LPQs were present in the last universal common ancestor or evolved
284 shortly thereafter, given their nearly universal presence in archaea and bacteria (70). Of the two LPQ
285 biosynthetic pathways, the MK_{mqn} pathway is considered ancestral to basal archaea and bacteria,
286 whereas the MK_{men} pathway was laterally transferred from bacteria to a subset of archaea (70).
287 Homologous proteins suggest that the LPQ and HPQ biosynthetic pathways are evolutionarily related.
288 The HPQ pathways share five homologs with the MK_{mqn} pathway (prenyltransferase, two-component
289 decarboxylase, C2 methyltransferase, kinase) and two with the alternative MK_{men} pathway
290 (prenyltransferase, C2 methyltransferase) (36). Our analysis shows that the MK_{mqn} homologs from
291 archaea and bacteria form sister groups to the HPQ proteins, whereas the two homologs of the MK_{men}
292 pathway are more distantly related to both HPQ and MK_{mqn} proteins (Fig. 4, S14-16, and SI discussion).
293 This topology suggests that contrary to previous conclusions (71), the HPQ pathways did not descend
294 directly from extant MK pathways.

295 Instead, the HPQ and MK pathways likely evolved from an ancestral quinone biosynthesis pathway
296 that, like all extant pathways, used a chorismate derivative as precursor. In the case of HPQ, this
297 precursor is prenylated in the second step, whereas prenylation is a late step in MK biosynthesis. The
298 specificity of prenyltransferase for its quinone substrate (72) combined with early prenylation in the
299 HPQ pathways may have facilitated evolutionary divergence of the HPQ and MK pathways. Existing
300 machinery from the ancestral quinone biosynthesis pathway such as decarboxylase, C2
301 methyltransferase, and kinase were then co-opted by these new pathways. The deep phylogenetic
302 divergence between HPQ and LPQ proteins (Fig. S14-16) suggests that the ancestral HPQ pathway

303 could have emerged before the radiation of bacteria and archaea (70) 4.1-3.4 Ga ago in an extinct lineage
304 coeval to the evolution of the extant LPQ pathways (Fig. 4c-d). Such an early origin of HPQs is not
305 necessarily linked to aerobic respiration or oxygenic photosynthesis using high-potential ETCs. Instead,
306 ancestral HPQs could have been involved in different functions, such as oxygen detoxification or a
307 primordial form of high-potential photosynthesis (73, 74), and only later adopted into high-potential
308 ETCs used for oxygenic photosynthesis and respiration using oxygen or other high-potential electron
309 acceptors, such as nitric oxide (16, 75).

310 ***Early evolution of aerobic metabolism***

311 The association of HPQ biosynthesis with oxygenic photosynthesis and aerobic respiration in
312 extant bacteria suggests that these traits became inseparably linked during evolution. The phylogeny of
313 HPQ biosynthesis proteins therefore allows dating the origin of aerobic metabolisms using HPQs
314 relative to Earth's oxygenation. Oxygen first accumulated permanently in the atmosphere during the
315 GOE(7) but geochemical tracers suggest oxygen was locally present during the late Archean (61, 62,
316 76). The likely presence of oxygen during the Archean aligns with the diversification of electron
317 transport pathways, oxygenases, oxidoreductases, and antioxidant enzymes around 3.3-2.9 Ga (10-12,
318 77), i.e., long before the GOE. Alternative proposals place the emergence of crown group
319 *Cyanobacteriota*, oxygenic photosynthesis, and aerobic respiration coeval to, or after, the GOE (4, 68,
320 78). Regardless of whether oxygenic photosynthesis emerged during the Archean or was coeval to the
321 GOE, the phylogenetic split between HPQ and LPQ proteins and the presence of the MK_{mqn} pathway
322 in the non-photosynthetic sister lineages (*Vampirovibrionophyceae*, “*Candidatus* Margulisbacteria”,
323 “*Candidatus* Sericytochromatia”; Fig. 3, 4; Table S5; SI discussion) together indicate that the HPQ
324 pathway in *Cyanobacteriota* originated from lateral transfer after their divergence from these sister
325 lineages. Because PQ is central to the functioning of photosystem II in all extant oxygenic
326 photosynthesizers (23, 79), emergence of PQ biosynthesis was likely tied to the evolution of oxygenic
327 photosynthesis and thus may have existed before the radiation of crown group *Cyanobacteriota*. This
328 supports earlier proposals that the extant oxygenic photosynthetic machinery originated in a lineage that
329 diverged from the non-photosynthetic sister lineages (78, 80), but pre-dated the radiation of crown

330 group *Cyanobacteriota* (74). Collectively, these constraints indicate that HPQs are at least as old as
331 oxygenic photosynthesis by *Cyanobacteriota* and therefore predate the GOE.

332 Aerobic metabolism preceding the GOE is supported by the near-universal occurrence of aerobic
333 respiration in crown group *Cyanobacteriota* and *Pseudomonadota*. All basal clades of *Cyanobacteriota*
334 and *Pseudomonadota* possess HPQs and are capable of aerobic respiration, with only few late-
335 branching *Pseudomonadota* being obligate anaerobes (Fig. 3, S10, S12-13). Molecular clocks calibrated
336 using cyanobacterial fossils place the last common ancestor of crown group *Cyanobacteriota* and the
337 emergence of basal, aerobic *Pseudomonadota* (Magnetococci) around 2.5-3.2 Ga (10, 12, 64–66, 81),
338 whereas aerobic *Nitrospirota* may have emerged shortly after the GOE (64, 69). HPQs were thus likely
339 used for aerobic respiration by the time of the radiation of extant *Cyanobacteriota* and
340 *Pseudomonadota*. Given the constraint that HPQs must have been present in stem-group
341 *Cyanobacteriota* and *Pseudomonadota*, the minimum age of extant HPQs is between 2.5-3.2 Ga,
342 whereas the ancestral HPQ pathway may be as old as crown group bacteria (3.4-4.1 Ga; Fig. 4d). We
343 therefore suggest that aerobic respiration with high potential ETCs may have originated up to 800 Ma
344 before oxygen permanently accumulated in the atmosphere during the GOE. Microbial mats could have
345 provided a niche for chemoautotrophs and heterotrophs consuming oxygen provided by
346 *Cyanobacteriota* directly (31), preventing escape to the atmosphere.

347 In modern ecosystems, some aerobic bacteria continue to use LPQ- rather than HPQ-dependent
348 ETCs for aerobic respiration (22). In the presence of O₂, reduced HPQs are relatively stable but reduced
349 LPQs are rapidly autoxidized, resulting in the loss of reducing equivalents to O₂ (25, 82). Further,
350 aerobic respiration with LPQs leads to increased formation of deleterious reactive oxygen species (27,
351 83), requiring energy to be expended on the mitigation of cellular damage, thereby decreasing growth
352 rates (27, 84). Finally, the use of LPQs instead of HPQs for proton pumping by complex I is less
353 efficient (85, 86). Given these drawbacks, one might question why all aerobes have not switched from
354 LPQs to HPQs to use oxygen without these disadvantages. The evolution of HPQs was a complex
355 process closely tied to the evolution of the ETC itself, demanding not only the acquisition of a dedicated
356 pathway for quinone biosynthesis, but also an upshift in redox potential of all other ETC components,

357 including hemes and iron-sulfur clusters (26). These complex requirements may explain why the
358 evolution of HPQ was successful only once and lateral transfers were rare. Distinct redox-tuning then
359 led to the extant diversity of HPQ structures and biosynthetic pathways in *Cyanobacteriota*,
360 *Pseudomonadota*, and *Nitrospirota*. Two of the three HPQ pathways were later obtained by eukaryotes,
361 UQ via incorporation of an alphaproteobacterium as the mitochondrion and PQ via incorporation of a
362 cyanobacterium as the chloroplast (57, 87), while mPQ remained exclusively bacterial. Through their
363 high potential ETCs, these lineages were able to rise to dominance in modern oxic ecosystems (31–35),
364 as evidenced by the prevalence of HPQs in modern oxic environments (30, 88).

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387

388 **Author Contributions**

389 F.J.E., F.P., S.S.A., and A.P. designed the research. F.P., L.P., and J.S. performed heterologous
390 expression experiments. F.J.E., P.B., S.L., E.S., and J.R.L. grew cultures and provided biomass samples.
391 F.J.E. and V.N. performed isotope labeling experiments. A.P., F.P., S.S.A., K.-U.H., and R.E.S
392 provided oversight and access to instruments and facilities. F.J.E., T.W.E, J.C., K.W.B., and F.P.
393 performed chromatographic and mass spectrometric analyses. F.P., A.R., and A.B. performed mPQ
394 purification and NMR characterization. J.D.H. performed redox potential calculations. F.J.E., S-C.C,
395 and S.S.A. performed phylogenetic analyses. F.B. performed sequence analyses of Rieske/cytb
396 complexes. F.J.E. wrote the manuscript with contributions from all authors.

397 **Data Availability**

398 All data are available in the supplementary materials and in the Dryad data repository:
399 [10.5061/dryad.2ngf1vhz1](https://doi.org/10.5061/dryad.2ngf1vhz1)

400 **Code Availability**

401 All code for the calculation of redox potentials is available in the Dryad data repository:
402 [10.5061/dryad.2ngf1vhz1](https://doi.org/10.5061/dryad.2ngf1vhz1)

403 **Competing Interests**

404 The authors declare no competing interests.

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