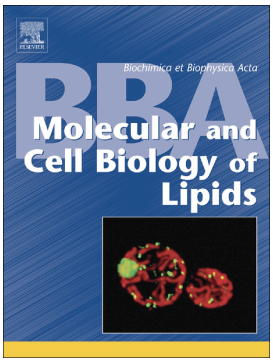

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Recombinant RquA catalyzes the *in vivo* conversion of ubiquinone to rhodoquinone in *Escherichia coli* and *Saccharomyces cerevisiae*

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ABSTRACT

Terpenoid quinones are liposoluble redox-active compounds that serve as essential electron carriers and antioxidants. One such quinone, rhodoquinone (RQ), couples the respiratory electron transfer chain to the reduction of fumarate to facilitate anaerobic respiration. This mechanism allows RQ-synthesizing organisms to operate their respiratory chain using fumarate as a final electron acceptor. RQ biosynthesis is restricted to a handful of prokaryotic and eukaryotic organisms, and details of this biosynthetic pathway remain enigmatic. One gene, *rquA*, was discovered to be required for RQ biosynthesis in *Rhodospirillum rubrum.* However, the function of the gene product, RquA, has remained unclear. Here, using reverse genetics approaches, we demonstrate that RquA converts ubiquinone to RQ directly. We also demonstrate the first *in vivo* synthetic production of RQ in *Escherichia coli* and *Saccharomyces cerevisiae*, two organisms that do not natively produce RQ. These findings help clarify the complete RQ biosynthetic pathway in species which contain RquA homologs.

Keywords: Rhodoquinone, Ubiquinone, Fumarate reduction, Anaerobic respiration, Biosynthesis

1. Introduction

Quinones serve as essential electron carriers in conserved, central metabolic processes such as respiration and photosynthesis. Ubiquinone (Coenzyme Q or Q, Fig. 1) is a core component of the electron transport chain in mitochondria. While Q is near ubiquitous in aerobic organisms, other, more exotic prenylated quinones facilitate energy production in facultative anaerobes (1). Rhodoquinone (RQ, **Fig. 1**) is one of these rarer quinones and is a structural analog of Q. RQ has an amino group at C-2 position of the benzoquinone ring while Q has a methoxy group. This small structural change alters their respective midpoint redox potentials from +100 mV for Q to -63 mV for RQ (2). With this lower midpoint redox potential, RQ can accept electrons from Complex I and the resulting RQH_2 donates electrons directly to fumarate reductase to maintain the chemiosmotic gradient needed for ATP generation in the absence of oxygen (3, 4).

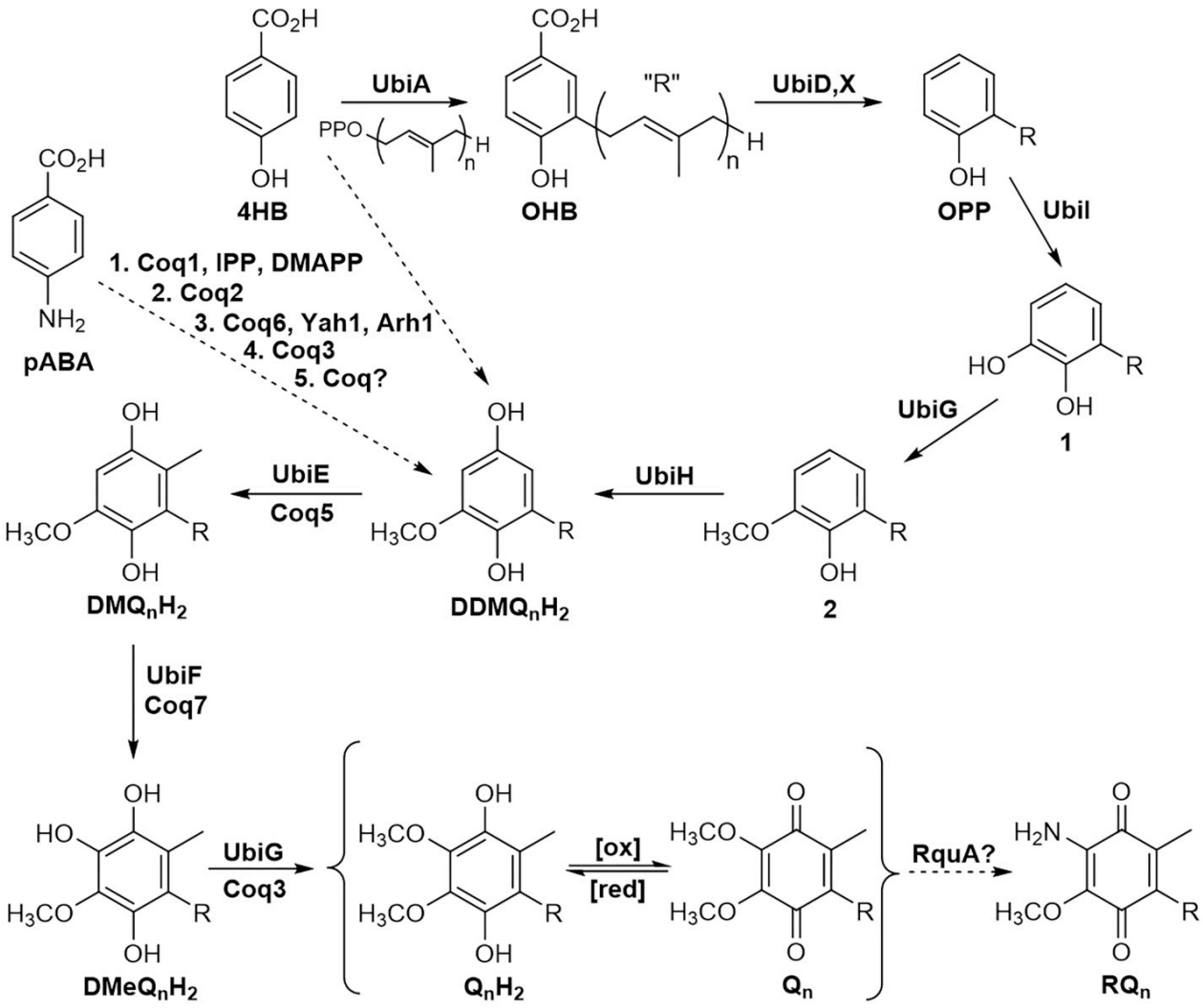

Fig. 1. The biosynthetic pathway of ubiquinone (Q) in *E. coli* (Ubi) and *S. cerevisiae* (Coq), as well as the proposed pathway to RQ biosynthesis in organisms with an RquA homolog. In *E. coli*, 4-hydroxybenzoic acid (4HB) is prenylated by UbiA to form 3-octaprenyl-4-hydroxybenzoate (OHB) which undergoes decarboxylation by UbiD and UbiX. Hydroxylation of the resulting product, 3-octaprenylphenol (OPP), is catalyzed by UbiI to form 3-octaprenyl catechol (compound 1). The first *O*-methylation is catalyzed by UbiG to convert compound 1 to 2-octaprenyl-6-methoxyphenol (compound 2). Hydroxylation of compound 2 with UbiH yields demethyldemethoxyubiquinol ($DDMQH_2$). The Q pathway in yeast can start from 4HB or *para*-aminobenzoic acid (pABA), and after multiple steps, catalyzed by Coq1, Coq2, Yah1, Arh1, Coq3 and other Coq polypeptides, results in the common intermediate, $DDMQH_2$. The *C*-methylation of $DDMQH_2$ with UbiE or Coq5 then produces demethoxyubiquinol ($DMQH_2$). A final hydroxylation of $DMQH_2$ by UbiF or Coq7 yields demethylubiquinol ($DMeQH_2$), which can then be methylated again by UbiG or Coq3 to produce ubiquinol (QH_2), the reduced form of Q. The *R. rubrum* protein, RquA, is proposed to convert Q to RQ. The number of isoprene units in the tail (R) is represented by the letter "n" and varies between species (e.g. in yeast n=6, in *E. coli* n=8, and in *R. rubrum* n=10).

While RQ is an integral compound of core anaerobic bioenergetics, its complete biosynthesis is still not known. Rhodoquinone biosynthesis protein A (RquA) was discovered in a forward genetics screen of *Rhodospirillum rubrum* as a putative methyltransferase-like enzyme that contributes to RQ biosynthesis (5). The *rquA* gene is required for anaerobic growth of *R. rubrum*, and the null mutant, Δ*rquA* is incapable of synthesizing RQ (5). However, the exact function and substrate of the RquA gene product has remained elusive. Q has been hypothesized to be a precursor of RQ from radiolabeling assays (6) and artificial feeding experiments in *R. rubrum* (7), but no genetic evidence has been provided. Furthermore, it is unknown whether this conversion would occur in a single or multi-step process.

A recent phylogenetic analysis of *rquA*'s origin and distribution was reported by Stairs, et al. (4). The authors found that *rquA* is extremely rare and sparingly distributed among the alphaproteobacteria, betaproteobacteria, and gammaproteobacteria classes of bacteria, and four of the five eukaryotic supergroups in which it is found (4). It was proposed that RquA homologs likely evolved from the proteobacterial class I SAM-dependent methyltransferases (4). The closest homologs of RquA are those used in Q biosynthesis: Coq3 and Coq5 in *Saccharomyces cerevisiae*, or UbiE and UbiG in *Escherichia coli* (5). It is possible that RquA evolved from proteins that were capable of binding Q and later gained a new enzymatic function to facilitate RQ biosynthesis (4). Homologs of *rquA* are also found in select eukaryotes that produce RQ such as *Euglena gracilis* (4, 8), and the protist, *Pygsuia biforma* (4). It has been hypothesized that the *rquA* gene was transferred from prokaryotes to eukaryotes by multiple independent lateral gene transfer events after the development of mitochondria (4).

Some higher order eukaryotes such as the metazoans, *Caenorhabditis elegans* and *Ascaris suum*, also produce RQ (9, 10); however, they do not possess a *rquA* homolog in their genome (4). The RQ biosynthetic pathway in species that do not have a gene encoding for RquA appears to differ from the pathway in *R. rubrum;* namely, Q is not a required precursor of RQ. For example, the *C. elegans clk-1* mutant, is deficient in Q_9 and builds up the demethoxyubiquinone-9 (DMQ_9) intermediate; however, the mutant can still produce RQ_9 (11, 12). These data suggest that these metazoans may have convergently evolved the ability to synthesize RQ in adaption to hypoxia and require different RQ biosynthetic intermediates and enzymes (4).

While RQ biosynthesis remains under-studied, Q biosynthesis in prokaryotes and yeast is better understood. Known steps in the Q biosynthetic pathway are outlined in **Fig. 1**. *E. coli ubi* null mutants have been used to elucidate the majority of intermediates and Ubi polypeptides required for Q biosynthesis (13). Polypeptides of interest in this work include UbiG, which performs an *O*-methylation reaction of Compound 1 (**Fig. 1**), and the null mutant, ⅄*ubiG*, accumulates OPP (14). UbiH then facilitates a hydroxylation of Compound 2 (**Fig. 1**) to form $DDMQ_8H_2$, and the corresponding mutant, ⅄*ubiH*, accumulates Compound 2, in addition to OPP (14). The following *C*-methylation step is facilitated by UbiE. The ⅄*ubiE* mutant thus accumulates $DDMQ_8H_2$ (15). UbiF completes the last hydroxylation to produce $DMeQ_8H_2$, and the corresponding mutant, ⅄*ubiF*, accumulates DMQ_8H_2 (16, 17). The final *O*-methylation of $DMeQ_8H_2$ to form Q_8H_2 also requires UbiG (18). *E. coli* UbiE, UbiF, UbiG, UbiH, UbiI, UbiJ and UbiK polypeptides form a high molecular mass soluble metabolon responsible for the ring modification steps in synthesis of Q_8 (19).

The biosynthesis of Q in *S. cerevisiae* (yeast) is known to require a membrane bound complex of at least eight polypeptides, which are products of genes *COQ3-COQ9* and *COQ11* (20). *COQ1* and *COQ2* gene products are required for assembly of the polyprenyldiphosphate

tail and its attachment to the ring precursor (21, 22). Similar to *E. coli*, yeast do not produce or require RQ. The primary metabolic pathways used by yeast are fermentation or respiration, the latter process requiring Q. Yeast can synthesize Q from either 4HB or pABA (**Fig. 1**) (23, 24). Deletion of any of the *COQ* genes results in defects in Q biosynthesis and growth on a non-fermentable carbon source (21, 22). Unlike in *E. coli*, the immediate Q biosynthetic precursors do not accumulate in the yeast null *coq* mutants. For example, the yeast mutant, *coq3*Δ, cannot produce Q_6 and is incapable of respiration; however, the only precursors detected in this mutant are the very early intermediates, 3-hexaprenyl-4-hydroxybenzoic acid (derived from 4HB) and 3-hexaprenyl-4-aminobenzoic acid (derived from pABA). These intermediates accumulate in most of the *coq3* to *coq9* null mutants, which is thought to be due to the required macromolecular protein complex required for Q biosynthesis (20). However, it was demonstrated that the *E. coli* gene homolog, *ubiG,* can rescue respiration in the *coq3*Δ mutant and restore the ability to synthesize Q_6 (25).

Here, we investigate Q and these earlier Q biosynthetic intermediates as substrates for RquA. Using genetics and analytical biochemistry, we demonstrate that recombinant RquA requires the presence of Q to produce RQ in both a prokaryote and eukaryote model. This work has elucidated a complete pathway for RQ biosynthesis in *rquA*-producing species, a critical compound for anaerobic respiration.

2. Methods

2.1. Yeast and E. coli *strains and plasmids*

A complete list of yeast and *E. coli* strains with their genotypes, specifications, and sources are listed in **Table 1**. A full list of plasmids used in this study and their sources are given in **Table 2.**

2.2. Construction of pET303_RquA

The *rquA* gene [Rru_A3227] was amplified by PCR from chromosomal *R. rubrum* DNA using Pfu Ultra II Hotstart Master Mix (Agilent, La Jolla, CA) with a forward primer containing an XbaI restriction site, p303XbaI_F (5'-CAGTTCTAGAATGACTAAGCACCAAGGTGCGG TCC-3') and a reverse primer with an XhoI cutsite, p303XhoI_R (5'-ACGTCTCGAGAGCGCG TCGCTCCGC-3'). The Champion™ pET303/CT-His vector (Invitrogen, Waltham, MA) and *rquA* amplicon were separately digested with XbaI and XhoI in NEBuffer 4 (NEB, Ipswich, MA) and cleaned with a DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA). Ligation was achieved using T4 DNA ligase and T4 DNA Rapid Reaction Ligase Buffer (NEB, Ipswich, MA) with a 6:1 molar ratio of insert:vector. The ligation mixture was used to transform *E. coli* DH5 and XJb (DE3) autolysis Mix and Go! cells (Zymo Research, Irvine, CA), using ampicillin for selection. The plasmid sequence was verified by Sanger sequencing.

Table 1
Genotype and source of yeast and *E. coli* strains.

Strain designation	Genotype/specifications	Source
S. cerevisiae		
W303-1A	MAT **α** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
CC303	MAT **α** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq3::LEU2*	(26)
E. coli		
K12	Wild-type	
JW2875 Δ*ubiH*	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *ΔubiH758::kan*, *rph-1, Δ(rhaD-rhaB)568*, *hsdR514*	CGSC Keio Collection
JW2226 Δ*ubiG*	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *ΔubiG785::kan, rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	CGSC Keio Collection
JW5581 Δ*ubiE*	F-, *Δ*(*araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-, rph-1, ΔubiE778::kan, Δ(rhaD-rhaB)568, hsdR514*	CGSC Keio Collection
JW0659 Δ*ubiF*	F-, *Δ*(*araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *ΔubiF722::kan, λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514*	CGSC Keio Collection
BL21	One Shot® BL21 Star™ DE3 pLysS cells	Invitrogen
XJb	BL21(DE3) with chromosomally inserted λ lysozyme gene inducible by arabinose	Zymo Research

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University

Table 2
Plasmids used.

Plasmids	Source
pET303_RquA	This work
pBAD24	(28)
pBAD24_RquA	This work
pQM	(25)
pQM_RquA	This work
pQMG	(25)
pRCM_RquA	This work
pRCM	(29)

2.3. Expression of RquA in E. coli *BL21 (DE3) cells*

pET303_RquA was transformed into *E. coli* BL21 (DE3) cells. Three colonies of this transformation were grown overnight in 2 mL culture of M9 minimal media amended with ampicillin. The next day, 500 µL of each of these cultures was added to 30 mL of M9 minimal media amended with ampicillin in 250 mL flasks. Cultures were then incubated at 37 °C with shaking at 250 rpm. Untransformed *E. coli* BL21 (DE3) cells were run in parallel without ampicillin. Once cultures reached an OD_{600} of 0.4 they were cooled to 25 °C and expression of RquA was induced using 100 µM of IPTG. Incubation continued at 25 °C for another 16 h, at which point 15 mL of each culture was harvested by centrifugation. Pellets were then resuspended in 1 mL of milli-Q sterile water and frozen at -80 °C for storage until lipid extraction.

2.4. Q_3 feeding assays in XJb (DE3) E. coli *expressing RquA*

A single colony of XJb::pET303_RquA was used to inoculate 5 mL of Luria-Bertani (LB) broth amended with ampicillin, and the culture was grown overnight at 37 ^{o}C with 250 rpm shaking. Outgrowth cultures (100 mL) were prepared from overnight culture in 500 mL flasks with LB amended with ampicillin and arabinose (3 mM final), at a starting OD_{600} of 0.01, and grown for 2.5 h to an OD_{600} of 0.4-0.6. Cultures were induced with 100 µM of IPTG and then divided into six 15-mL aliquots in 125 mL flasks before adding concentrated Q_3 substrate in ethanol (5 and 10 µM final). The Q_3 substrate was synthesized in two steps from 2,3-dimethoxy-5-methylbenzoquinone using previously published protocols (27). Feeding cultures were grown for 18 h at 25 ^{o}C with shaking, and pellets were harvested from 5 mL of culture and frozen at -80 ^{o}C. Each condition was performed in triplicate, and controls without vectors were prepared without ampicillin, at the same Q_3 concentrations.

2.5. Expression of RquA in E. coli *ubi knockouts*

The *rquA* gene was subcloned from pET303_RquA into pBAD24 (28) using the following primers: forward (5'-CTAGCAGGAGGAATTCATGACTAAGCACCAAGGTGCGG-3') and reverse (5'-GCAGGTCGACTCTAGATTAAGCGCGTCGCTCCGC-3') with *InFusion* cloning technology. An empty pBAD24 vector was used as a control. Expression of RquA was shown to be tightly controlled in the pBAD24 vector and could be expressed at low to high levels using arabinose concentrations ranging from 0.0002% to 0.2% w/v. Wild-type *E. coli* K12 and the Q biosynthetic knockout mutants, Δ*ubiG*, Δ*ubiH*, Δ*ubiE*, and Δ*ubiF* were transformed with pBAD24 and pBAD24_RquA. A 2 mL pre-culture was grown overnight with appropriate selection from individual colonies of each of the transformed mutant strains. Pre-cultures were used to inoculate 30 mL of LB broth amended with appropriate selection in 250 mL flasks. Cultures were incubated at 37 °C with shaking at 250 rpm. Expression was induced with 0.2% w/v (13,000 µM) arabinose when OD_{600} = 0.5. Cultures were then grown until they reached OD_{600} = 1, at which point they were harvested by centrifugation and resuspended in 1 mL of milliQ water. Resuspended pellets were then frozen at -80 °C for storage until lipid extraction and analysis.

2.6. Construction of pQM_RquA

The *rquA* gene was amplified from pET303_RquA using Q5® High Fidelity Master Mix (NEB, Ipswich, MA) with the forward primer, pQMClaI_F (5'-CGAAGATCGATACTAAGCAC CAAGGTGCGGT-3') and the reverse primer, pQMKpnI_R (5'-TGATCGGTACCTTAAGC GCGTCGCTCCGCGACGA-3'), containing ClaI and KpnI restriction sites, respectively. The pQM plasmid containing a *COQ3* mitochondrial leader sequence (25) and the *rquA* amplicon were double digested with ClaI and KpnI-HF in CutSmart® buffer (NEB, Ipswich, MA). The linear vector and insert were ligated using the same conditions as for pET303_RquA, and the ligation mixture was transformed into NEB® 5-alpha Competent *E. coli* (NEB, Ipswich, MA) using the Efficiency Transformation Protocol (C2987H/C2987I). The sequence of pQM_RquA was verified with Sanger sequencing.

2.7. Construction of pRCM_RquA

The *rquA* gene was amplified as described in section 2.6 using the pQMClaI_F primer and the reverse primer, pRCMKpnIHis_R (5'-TGATCGGTACCTTAATGATGATGATGATGAT GAGCGCGTCGCTCCGCGACGA-3'). The *rquA* amplicon, containing a C-terminal hexahistidine tag, and the multi-copy pRCM plasmid (29) were both double digested with ClaI and KpnI-HF as described above. The linear pRCM vector was purified by gel extraction using a Zymoclean® Gel DNA Recovery Kit (Zymo Research, Irvine, CA) prior to ligation with the *rquA*-his_6 insert. The sequence of pRCM_RquA was validated using Sanger sequencing.

2.8. Expression of RquA in S. cerevisiae

Growth media for *S. cerevisiae* were prepared as described (30) and included YPD (1% yeast extract, 2%, peptone, 2% dextrose), YPG (1% yeast extract, 2% peptone, 3% glycerol), SD complete and SD-Ura [0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and complete amino acid supplement lacking uracil]. Wild-type W303 yeast was transformed with single copy vectors pQM, pQM_RquA, and pQMG (25), and with multi-copy vectors pRCM and pRCM_RquA (29) using standard protocols (30), and selection was performed on SD-Ura plates. The pQMG vector harboring the *ubiG* gene was previously constructed from pQM, which contains a *COQ3* mitochondrial leader sequence (25). The mutant W303::⅄*coq3* yeast (31) was similarly transformed with the three single copy vectors. Overnight cultures in SD-complete (no vector) or SD-Ura (with vector) of the ten strains were prepared from single colony scrapes and used to inoculate 15-mL cultures in 125 mL flasks. Cultures with no vector or single copy vectors were grown at 30 ^oC for 12 h with 250 rpm shaking, while cultures containing the multi-copy vector required a 24 h growth period to reach similar OD_{600} values (3-4). Aliquots containing 5-mL of culture (15-20 OD_{600} units) were pelleted for lipid extraction and LC-MS analysis. Dilution assays were performed with 2 μL

spots of yeast cells diluted in PBS buffer to OD_{600} 0.2, 0.04, 0.008, 0.0016, and 0.00032 on agar plates containing YPD, YPG, SD-complete or SD-Ura media with 2% bacto agar.

2.9. BL21 E. coli *lipid extraction and HPLC analysis*

Resuspended pellets were thawed at room temperature and transferred to 5 mL Pyrex tubes containing 500 µL of 0.1 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and vortexed at full speed for 120 s. Next, 5 µmol Q_{10} internal standard and 2 mL 95% ethanol was added prior to another 120 s of vortexing. Tubes were then incubated at 70 °C for 15 min with intermitted mixing and then cooled to room temperature. Lipids were extracted twice with 5 mL hexane phase partitions. Hexane fractions were combined and evaporated to dryness under nitrogen gas. The dried lipid extract was then resuspended in 200 µL methanol:dichloromethane (10:1) and transferred to a 1.5 mL Eppendorf tube for centrifugation (21,000 x g; 5 min). From this centrifuged sample, 50 µL was injected on HPLC using a SUPELCO Discovery® C-18 column (25 cm x 4.6 mm x 5 µm) held at 30°C with a flow rate of 1 mL per min of solvent methanol:hexane (90:10). Quinones were detected by diode array spectrophotometry (1260 DAD HS, Agilent Technologies, Germany).

2.10. Lipid extraction of XJb E. coli *and* S. cerevisiae *for LC-MS quantitation*

Cell pellets were thawed and 500 pmol Q_6 internal standard (for *E. coli*) or 1000 pmol Q_3 (for yeast) was added prior to lipid extraction, using methods previously reported for *R. rubrum* (7). Dried lipid extracts were resuspended in 20 µL hexane and 955 µL ethanol, and 30 min prior to LC-MS injection, 25 µL of $FeCl_3$ (100 mM, 2.5 mM final) was added to ensure full oxidation of quinones. Standards were extracted using the same protocol at the following concentrations: For *E. coli*, standards contained Q_6 (5 pmol/10 µL injection), RQ_3 (1.5, 3.0, 4.5, 6.0, or 12 pmol/10 µL injection) and Q_3 (6.0, 12, 24, 36, or 48 pmol/10 µL injection); for yeast, standards contained Q_3 (10 pmol/µL injection) and Q_6 (0.3, 0.6, 1.2, 3.0, or 6.0 pmol/10 µL injection). The standards Q_3 and RQ_3 were synthesized at Gonzaga University using previously published procedures (27, 32). The Q_8 and RQ_8 standards were isolated from BL21::pET303_RquA extracts by preparative HPLC at the University of Florida, Gainesville. The Q_6 and Q_{10} standards were purchased from Sigma-Aldrich (St. Louis, MO). Since an RQ_6 standard was not available, the quantity of RQ_6 was determined using a pmol conversion from the Q_6 standard curve and applying a RQ/Q response correction factor of 2.45 (which was determined from RQ_8/Q_8 and RQ_3/Q_3 standards). The lipid extracts and standards were separated using high performance liquid chromatography (Waters Alliance 2795, Waters Corporation, Milford, MA) and quinones were quantified using a triple quadrupole mass spectrometer in positive electrospray mode (Waters Micromass Quattro Micro, Waters Corporation, Milford, MA). Chromatography was performed at 4 °C using a pentafluorophenyl propyl column (Luna PFP[2], 50 by 200 mm, 3 µm, 100 Å, Phenomenex, Torrance, CA) at a flow rate of 0.5 ml/min and injection volumes of 10 µl. Quinones were eluted between 1.7 and 6.6 min by using a gradient system containing water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). The water and acetonitrile used were liquid chromatography-mass spectrometry (LC-MS)-grade Optima (Fisher Scientific, Pittsburgh, PA),

and the formic acid was >99% packaged in sealed 1-mL ampoules (Thermo-Scientific, Rockford, IL). The gradient (buffer A-buffer B) method used was as follows: 0 to 3.5 min (30:70), 3.50 to 3.75 min (30:70 to 2:98), 3.75 to 7.25 min (2:98), 7.25 to 7.5 min (2:98 to 30:70), and 7.50 to 9 min (30:70). Quantitation was accomplished using MRM of singly charged ions, and monitored for the mass transition from each quinone precursor ion ($[M + H]^+$) to its respective tropylium product ion ($[M]^+$). Mass Lynx V. 4.1 software was used for data acquisition and processing. Linear slopes were calculated using peak areas with a bunching parameter of 3 and two smoothing functions. The following global conditions were used for MS/MS analysis of all compounds: Capillary voltage, 3.60 kV; Source temp, 120 ^oC; Desolvation temp, 400 ^oC; Desolvation N_2 gas flow, 800 L/h; and Cone N_2 gas flow, 100 L/h. Argon gas was used for the collision gas and was obtained from the boil-off from a bulk liquid argon storage tank. Additional quinone-specific parameters are listed in **Table 3**. Samples were analyzed in duplicate and the pmol quinone was determined from the standard curve and corrected for recovery of internal standard. Samples were then normalized by OD_{600} unit of original culture. Accurate mass determination of RQ was performed using a Waters LCT Premier XE time-of-flight mass spectrometer in positive electrospray mode using a Waters UPLC with the same chromatography conditions.

Table 3
LC-MS parameters for each quinone.

MS parameter	Q_3	RQ_3	Q_6	RQ_6	Q_8	RQ_8
Dwell time (s)	0.1	0.1	0.1	0.1	0.1	0.1
Cone (V)	20	25	31	35	35	39
Collision (V)	20	20	28	28	30	30
Precursor mass $[M+H]^+$ (*m*/*z*)	387.2	372.2	591.4	576.4	727.6	712.6
Ion product mass $[M]^+$ (*m*/*z*)	197.2	182.2	197.2	182.2	197.2	182.2

3. Results

3.1. RquA leads to the production of RQ_8 and a depletion of Q_8 in E. coli

To test if Q was a substrate of RquA, recombinant RquA was expressed in *E. coli*. Wild-type *E. coli* lipid extract profiles contain three major quinone peaks: ubiquinone-8 (Q_8), demethylmenaquinone-8 (DMK_8), and menaquinone-8 (MK_8) (**Fig. 2A.I**). When RquA was expressed in wild-type *E. coli*, a fourth peak accumulated, coinciding with a depletion of the native Q_8. This new peak eluted approximately 2 minutes prior to the native Q_8 peak (**Fig. 2A.II**). This peak had a maximum absorption spectrum identical to that of RQ at 283 nm (**Fig. 2A.II**) (6, 33). Time-of-flight (TOF) MS analysis provided an accurate mass determination of RQ_8, which was within 1 ppm of the calculated exact mass (**Fig. 2B**). In induced cultures of *E. coli* BL21::pET303_RquA, the quantity of RQ_8 averaged 250.6 ± 125.0 pmol RQ_8/OD_{600} unit, compared to only 19.3 ± 10.7 pmol Q_8/OD_{600} unit (Fig. 2C). In the BL21 control cells, there was

an average of 203.2 ± 18.6 pmol Q_8/OD_{600} unit, and no RQ_8 was detected (**Fig. 2C**). The observation that Q_8 quantity is depleted, while RQ_8 is formed, suggests that RquA uses a substrate from the Q pool to form RQ.

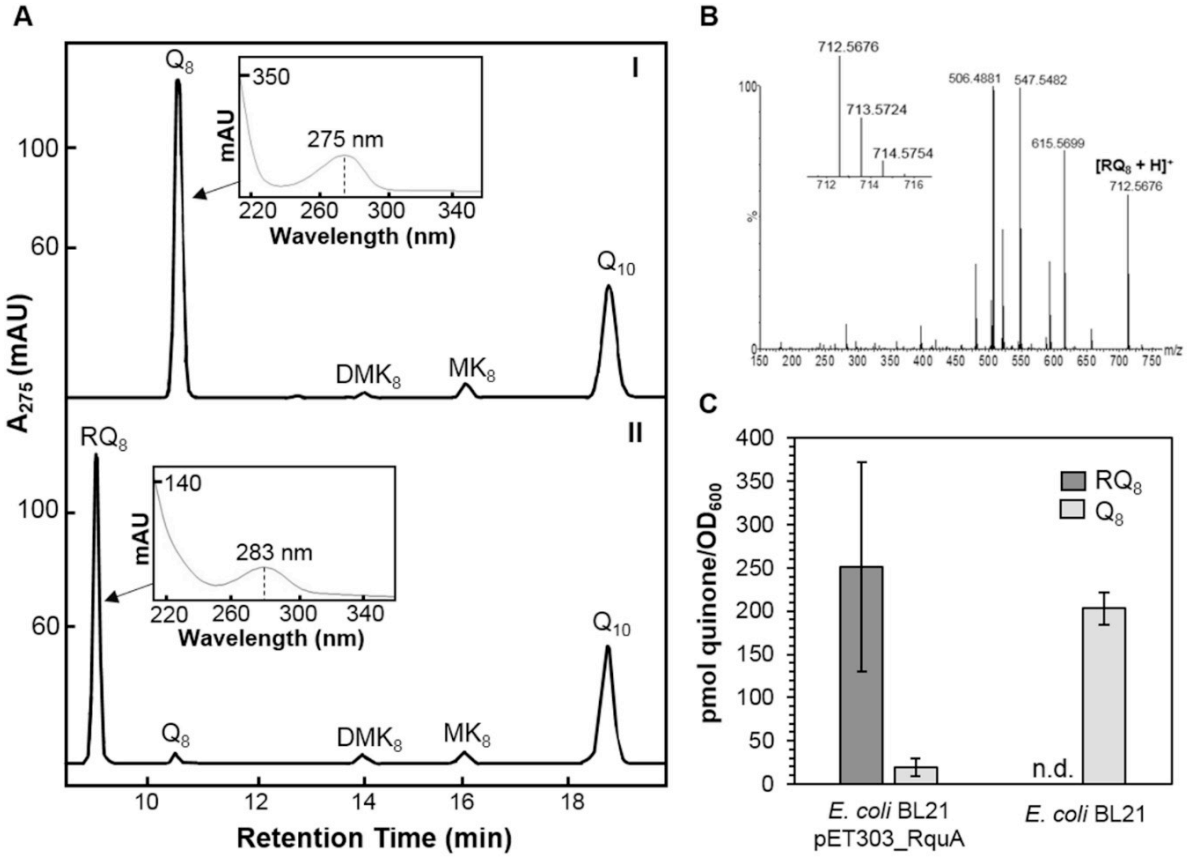

Fig. 2. Expression of RquA in *E. coli*. (A.I) HPLC chromatogram of lipid extracts from *E. coli* BL21 showing the three major lipid peaks: ubiquinone-8 (Q_8), demethylmenaquinone-8 (DMK_8), menaquinone-8 (MK_8) and the internal standard, ubiquinone-10 (Q_{10}); inset plot is the Q_8 absorption spectrum. (A.II) Chromatogram of lipid extracts of *E. coli* BL21::pET303_RquA cells where a new peak is formed with RquA expression; inset, RQ_8 absorption spectrum. (B) The mass spectrum of $[RQ_8 + H]^+$ obtained from an extracted ion chromatogram of *E. coli* BL21::pET303_RquA lipid extracts shows the molecular ion at 712.5676 m/z (exact mass of $C_{48}H_{74}NO_3$, 712.5669 amu). (C) Quantities of RQ and Q produced in *E. coli* BL21 cells with and without induced pET303_RquA.

3.2. Feeding of Q_3 to E. coli expressing RquA leads to formation of RQ_3

To test if Q could serve as a direct precursor, synthetic Q_3 was fed to induced cultures of *E. coli* XJb::pET303_RquA at 5 .00M and 10 .00M concentrations, the RQ_3 product was detected using LC-MS with MRM analysis, and validated with a synthetic standard (**Fig. 3**). RQ_3 was not found in the controls without vector (**Fig. 3**). The RQ_3 peak that elutes at 1.68 min corresponds

to a 372.2 > 182.2 m/z mass transition, indicative of fragmentation of the molecular ion, $[RQ_3 + H]^+$, to form the RQ tropylium product ion (see Appendix A, **Fig. A.1** for LC-MS chromatograms). The amount of RQ_3 product that accumulated from increasing concentrations of Q_3 (5 and 10 μM) was similarly proportional at 12.1 ± 0.4 and 25.0 ± 3.5 pmol RQ_3/OD_{600} unit, respectively (**Fig. 3**). RQ_8 was observed under these conditions at 6.40 min with a mass transition of 712.6 > 182.2 m/z at 494.3 ± 38.2 and 433.1 ± 69.2 pmol RQ_8/OD_{600} unit, respectively (**Fig. 3** and **Fig. A.1**). These data help confirm that Q serves as the direct precursor of RQ.

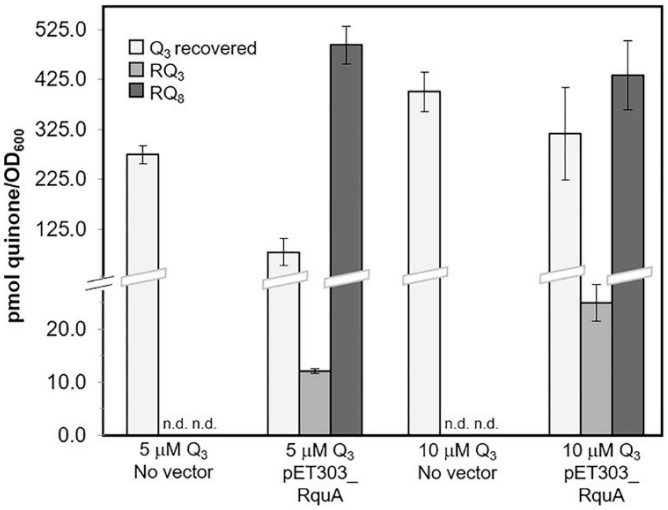

Fig. 3. Levels of RQ_3 (and RQ_8) produced and Q_3 recovered from XJb *E. coli* Q_3 feeding assays with and without the pET303_RquA vector. No RQ_3 or RQ_8 were detected in the absence of vector and the amounts of RQ_3 produced in the presence of the pET303_RquA vector was proportional to the amount of Q_3 added.

3.3. RquA leads to accumulation of RQ_8 only in E. coli strains that produce Q_8

To further confirm if RquA uses Q directly as a substrate, or acts on an earlier Q intermediate, we expressed RquA in a series of Q biosynthetic knockout mutants in *E. coli*. These mutations each halt the production of Q at different enzymatic steps and the corresponding mutants accumulate the respective intermediate as described earlier. The Q biosynthetic enzymes targeted in this study were UbiG, UbiH, UbiE and UbiF. In this experiment, RquA was provided access to four Q biosynthetic intermediates which accumulate in the corresponding null mutants: ⅄*ubiG*, ⅄*ubiH*, ⅄*ubiE*, and ⅄*ubiF*. The respective intermediates tested as potential substrates for RQ biosynthesis were: OPP, 2-octaprenyl-6-methoxyphenol (Compound 2), $DDMQ_8$ and DMQ_8. We found that RquA was not able to produce RQ in any of these mutants (**Fig. 4A**). Again, we observed the formation of RQ with RquA in an *E. coli* strain that contained Q (**Fig. 4A**). It was also observed in this system that higher induction of expression of RquA in *E. coli* K12 pBAD24_RquA correlated with higher RQ_8 and lower Q_8 levels and that the depletion of Q_8 was found to be roughly proportional to the accumulation of RQ_8 in an approximate 1:1 mole ratio (**Fig. 4B**).

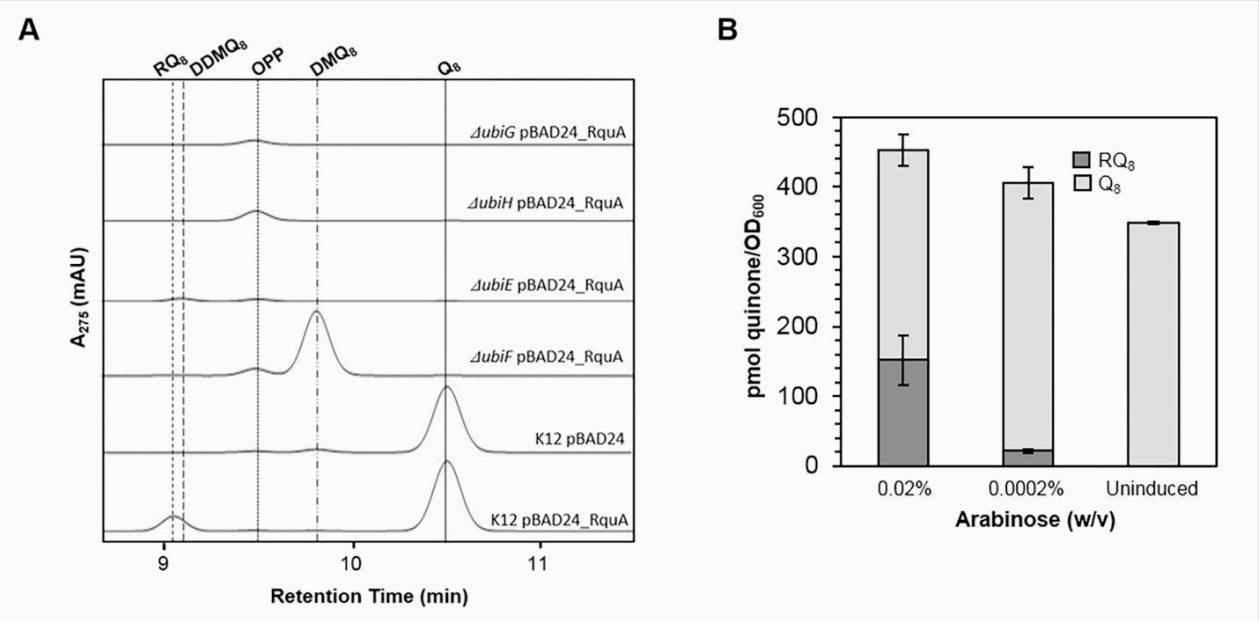

Fig. 4. Expression of RquA in *E. coli* Q biosynthetic knockout strains. (A) HPLC profiles of the lipid extracts of *E. coli* strains tested with and without RquA expressed show that RQ production occurs only in strains which produce Q. Lines mark the retention time of available Q biosynthetic intermediates in the *E. coli* strains tested (OPP, DDMQ and DMQ), as well as the Q and RQ products. (B) Levels of RQ_8 and Q_8 produced in *E. coli* K12 cells with pBAD24_RquA were quantified under high induction (0.02% arabinose), low induction (0.0002% arabinose), and uninduced conditions.

3.4. RquA expression in wild-type yeast leads to formation of RQ_6

W303 yeast transformed with the single copy vectors, pQM, pQM_RquA and pQMG, were capable of growth on YPD, YPG, SD complete and SD-Ura plates (**Fig. 5A**). The rich YPD and YPG media were used to compare growth on a fermentable carbon source (dextrose) versus a non-fermentable carbon source (glycerol). Yeast incapable of performing respiration (i.e. lacking Q) cannot grow on YPG. SD-complete and SD-minus uracil media were used to select for the pQM and pRCM plasmids. Only yeast transformed with these plasmids can grow without uracil. W303 transformed with the multi-copy vectors, pRCM and pRCM_RquA, also showed growth on the four media types (see Appendix A, **Fig. A.2**).. LC-MS with MRM analysis revealed that yeast containing the pQM_RquA and pRCM_RquA vectors produced a new RQ_6 product at 5.85 min with mass transition of 576.4 > 182.2 m/z (see sample chromatograms in Appendix A, **Fig. A.3**). The accurate mass of RQ_6 was confirmed using LC-TOF-MS to within 1 ppm of the calculated exact mass (**Fig. 5C**). The average quantity of RQ_6 in the W303::pQM_RquA cultures was determined to be 12.9 ± 0.3 pmol/OD_{600} (**Fig. 5D**), while the amount of RQ_6 in W303::pRCM_RquA was 15.3 ± 1.5 pmol/OD_{600}. Overall, there was about 10-20 times less RQ_6 produced in yeast than RQ_8 produced in *E. coli* expressing RquA (**Fig. 2C**). These quantities are consistent with the proportions of native Q_6 and Q_8 recovered from yeast and *E. coli*, respectively, in the absence of RquA. This experiment demonstrates that RquA can convert Q to RQ in a eukaryote and that its activity is not restricted to prokaryotic lineages,

consistent with the natural distribution of RquA homologs in the genomes of bacterial and eukaryotic species.

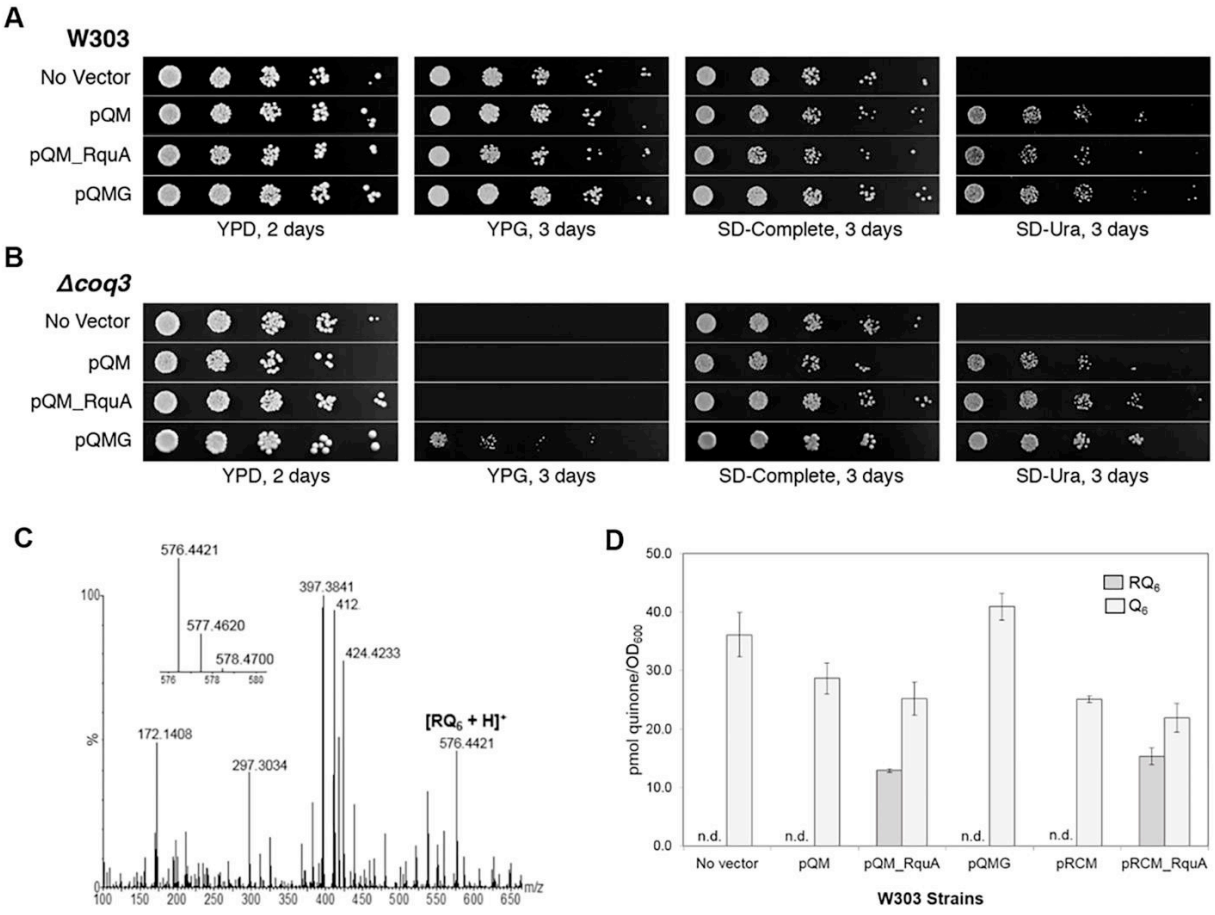

Fig. 5. Expression of RquA in yeast. Yeast dilution assays on YPD, YPG, SD-complete, or minus uracil (SD-Ura) plates: (A) Wild-type W303 yeast containing pQM, pQM_RquA, and pQMG vectors grow on all four media types. The W303 yeast control without a vector cannot grow on SD-Ura. Additional dilution assay plates for W303::pRCM and W303::pRCM_RquA are shown in **Fig. A.2**. (B) Mutant W303 Δ*coq3* yeast strains grown on the same media show survival on non-fermentable carbon (YPG) only in the presence of the pQMG vector containing the *ubiG* gene. (C) The mass spectrum of $[RQ_6 + H]^+$ from yeast W303::pQM_RquA lipid extracts shows the molecular ion at 576.4421 m/z (exact mass of $C_{38}H_{58}NO_3$, 576.4417 amu). (D) RQ_6 is produced only in W303 yeast transformed with the pQM_RquA and pRCM_RquA vectors.

3.5. Expression of RquA in Δ**coq3** *yeast does not produce RQ or rescue respiration*

The yeast *coq3*Δ null mutant cannot grow on glycerol (YPG) (**Fig 5B**); however, transformation with pQMG, containing the *E. coli ubiG* gene, recovered the synthesis of Q_6 and

restored respiration and the mutant's ability to grow on YPG (25) (**Fig. 5B**). Since RquA is annotated on NCBI as a methyltransferase, and shares sequence similarity to UbiG, we tested the *rquA* gene under the same experimental conditions. We found that the pQM_RquA vector did not rescue respiration in the Δ*coq3* yeast (**Fig. 5B**), nor was RQ_6 detected in the corresponding lipid extracts by LC-MS (**Fig. A.3**). LC-MS analysis confirmed that the only Δ*coq3* strain to produce Q_6 contained pQMG (**Fig. A.3**). This experiment demonstrates that RquA cannot functionally replace Coq3.

Discussion

Due to its central function in anaerobic bioenergetics, RQ is a molecule of high interest; yet, information on its biosynthetic pathway is limited. Understanding RQ biosynthesis is worthwhile as it has been cited as a possible target for control of helminth parasites (7). While several recent discoveries have been made in *R. rubrum* (5, 7, 34), the complete pathway for RQ biosynthesis has not been reported. Prior to the work presented here, the enigma for RQ-producing species containing the *rquA* gene included whether Q was a substrate of RquA and whether RQ was a product of the RquA reaction.

In this work, we demonstrate that expression of RquA from *R. rubrum* yields RQ in two species that do not naturally produce RQ, *E. coli* and yeast. The RQ generated varied in tail length (RQ_3, RQ_6, and RQ_8) depending on the Q substrate available to RquA. RquA was unable to utilize any of the Q biosynthetic intermediates tested as substrates in *E. coli*. It was shown that in the absence of Q, no RQ was made in either *E. coli* or yeast. These results provide direct evidence that RquA is necessary and sufficient to convert Q to RQ.

The presence of RQ in yeast did not appear to decrease cell viability in plate dilution assays on the four different types of media. However, yeast transformed with the pRCM_RquA multi-copy vector required double the growth time to achieve the same cell density as cultures containing single-copy vectors. This could be due to Q-cycle bypass reactions that have been previously reported with addition of exogenous RQ to yeast (32), or to reduced levels of Q. Despite lower levels of Q_8 in *E. coli* expressing RquA, there was no effect on growth on plates or in liquid media. It is possible that RQ can act as a substitute for menaquinone, the low potential quinone found naturally in *E. coli* (35).

RquA shows sequence homology to class I *S*-adenosylmethionine (SAM)-dependent methyltransferase enzymes, such as Coq3 in yeast and UbiG in *E. coli* (7, 18, 25). However, certain residues within the RquA SAM-binding motif differ from those observed in close homologs for which methyltransferase activity has been demonstrated (5). There are several examples in the literature where methyltransferase-like proteins have alternate functions, and SAM is implicated as an electrostatic catalyst rather than as a methyl donor (36-38). The data presented here do not support the role of RquA as a methyltransferase, and we propose that RquA may instead be catalyzing a transamination for the direct conversion Q to RQ.

A candidate gene approach was recently used in *R. rubrum* to screen for other genes that may be involved in RQ biosynthesis (34). Gene targets were selected using transcriptome data obtained from RNA sequencing of aerobically and anaerobically grown *R. rubrum,* using *rquA* as a standard for comparison. Targets were further screened using comparative genomic data between *Rhodoferax ferrireducens* and *Rhodobacter sphaeroides*, a RQ-producing and non-RQ-producing species, respectively. Candidates were chosen that were differentially expressed

under anaerobic conditions, and had homologs in the RQ-producing species, *R. ferrireducens*, but not in *R. sphaeroides*. Knockout mutants were generated for each new candidate, and RQ and Q levels were measured. No candidate was found to be as essential as *rquA* for RQ biosynthesis, though two genes were found to modulate Q biosynthesis in anoxic conditions, which had a direct effect on RQ levels (e.g. increased Q production yielded higher RQ levels). This work provides further evidence that Q is a required precursor to RQ, and RquA may be acting alone in this conversion.

Conclusion

Our findings have shed new light on the RQ biosynthetic pathway in species containing the *rquA* gene. Discovering that RquA uses Q as a substrate, and catalyzes the conversion of Q to RQ, was unexpected. The conversion of Q to RQ involves the addition of ammonia and elimination of methanol. This reaction has been observed non-enzymatically *in vitro* (39) supporting that it could also occur *in vivo*. The need to convert Q to RQ directly, despite the addition-elimination reaction required to do so, provides hints at the demand of RQ-synthesizing organisms to quickly convert the midpoint redox potential of their electron carrying quinones in changing environmental conditions. Future work in our laboratories will explore the mechanism of RquA and its regulation in changing oxygen environments.

Acknowledgements

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Recombinant RquA catalyzes the *in vivo* conversion of ubiquinone to rhodoquinone in *Escherichia coli* and *Saccharomyces cerevisiae*

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Highlights

- This work has elucidated a complete pathway for rhodoquinone biosynthesis in *rquA*-producing species, a critical compound for anaerobic respiration.
- Recombinant RquA expressed from the *Rhodospirillum rubrum rquA* gene is active in two non rhodoquinone-producing species, *Escherichia coli* and *Saccharomyces cerevisiae*.
- Expression of RquA in *E. coli* and *S. cerevisiae* facilitates the *in vivo* synthesis of rhodoquinone.
- Ubiquinone is a required substrate of RquA, and the product of the RquA reaction is rhodoquinone.