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Demethoxy-Q, An Intermediate of Coenzyme Q Biosynthesis, Fails to Support Respiration in *Saccharomyces cerevisiae* and Lacks Antioxidant Activity*

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Caenorhabditis elegans clk-1 **mutants cannot produce coenzyme Q_9 and instead accumulate demethoxy-Q_9 (DMQ_9). DMQ_9 has been proposed to be responsible for the extended lifespan of *clk-1* mutants, theoretically through its enhanced antioxidant properties and its decreased function in respiratory chain electron transport. In the present study, we assess the functional roles of DMQ_6 in the yeast *Saccharomyces cerevisiae*. Three mutations designed to mirror the *clk-1* mutations of *C. elegans* were introduced into *COQ7*, the yeast homologue of *clk-1*: E233K, predicted to disrupt the di-iron carboxylate site considered essential for hydroxylase activity; L237Stop, a deletion of 36 amino acid residues from the carboxyl terminus; and P175Stop, a deletion of the carboxyl-terminal half of Coq7p. Growth on glycerol, quinone content, respiratory function, and response to oxidative stress were analyzed in each of the *coq7* mutant strains. Yeast strains lacking Q_6 and producing solely DMQ_6 were respiratory deficient and unable to support either NADH-cytochrome *c* reductase or succinate-cytochrome *c* reductase activities. DMQ_6 failed to protect cells against oxidative stress generated by H_2O_2 or linolenic acid. Thus, in the yeast model system, DMQ does not support respiratory activity and fails to act as an effective antioxidant. These results suggest that the life span extension observed in the *C. elegans clk-1* mutants cannot be attributed to the presence of DMQ *per se*.**

Coenzyme Q (ubiquinone or Q)[1] is a prenylated benzoquinone involved in respiration (1). The number of isoprene units in the tail of Q varies depending on the species; humans, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* produce Q_{10}, Q_9, and Q_6, respectively. Q is reversibly reduced (Q hydroquinone) and oxidized (Q) as it transports electrons from complex I or II to the cytochrome bc_1 complex in the inner mitochondrial membrane. Q hydroquinone is synthesized in the mitochondria, where it acts not only in respiration but also in fatty acid β-oxidation (2) and uridine synthesis (3). Reports point to a role for Q in the regulation of the permeability transition pore as well (4). Q is transported to other intracellular membranes and to the plasma membrane. It is thought to act in these nonmitochondrial sites as a lipid-soluble antioxidant (5, 6). Plasma membrane Q is known to be involved in a trans-plasma-membrane electron transport system in which external impermeable oxidants are reduced and intracellular NADH serves as an electron donor (7, 8).

Eight complementation groups have been identified as Q-deficient in yeast, *coq1-coq8* (9). As with all of the *coq* mutant strains, yeast *coq7* mutants cannot produce Q and are therefore respiration-defective, unable to grow on nonfermentable carbon sources (9, 10). Yeast *coq7*-null mutants accumulate an early Q biosynthetic intermediate, 3-hexaprenyl-4-hydroxybenzoic acid (HHB), as do mutants in the complementation groups *coq3*, *coq4*, *coq5*, *coq6*, and *abc1/coq8* (Fig. 1) (11). The accumulation of HHB is not diagnostic of the blocked step in the *coq* mutants, and there is accumulating evidence that a multi-subunit complex of Coq polypeptides is required to convert HHB to Q in yeast (12–14). It is interesting that although yeast *coq7*-null mutants accumulate HHB, yeast bearing the *coq7-1* allele ($G_{104}D$) contain a very small amount of demethoxy-Q_6 (2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone or DMQ_6) (10).

The *clk-1* gene of *C. elegans* is an orthologue of yeast *COQ7* (15). Similar to the *coq7/clk-1* orthologues from rat (16) and human (17), *C. elegans clk-1* rescues the Q-biosynthetic defect of a yeast *coq7*-null mutant, indicating functional conservation. *Escherichia coli* lacks a *COQ7* homologue and instead relies on the *ubiF* gene product, a flavin-dependent monooxygenase unrelated to *COQ7* that is essential for the hydroxylation of DMQ_8 (18). Expression of the *Pseudomonas aeruginosa* orthologue of Coq7 restored Q biosynthesis in an *E. coli ubiF* mutant (19). Expression of the *C. elegans clk-1* homologue also rescues Q biosynthesis in an *E. coli ubiF* mutant (20). The Coq7/Clk-1 polypeptide was identified as a member of the di-iron carboxylate family of proteins, including methane monooxygenase, reinforcing the idea that Coq7/Clk-1 polypeptides catalyze the hydroxylation of DMQ, the penultimate step of Q biosynthesis (19).

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[1] The abbreviations used are: Q, ubiquinone (coenzyme Q); Q_n, the coenzyme Q isoform where *n* designates the number of isoprene units; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; DMQ, demethoxy-Q; RQ, rhodoquinone; YPG, yeast extract/peptone/glycerol; HPLC, high performance liquid chromatography; ECD, electrochemical detection/detector; DCIP, dichloroindophenol; MES, 4-morpholineethanesulfonic acid; HE, hydroethidine (dihydroethidium).

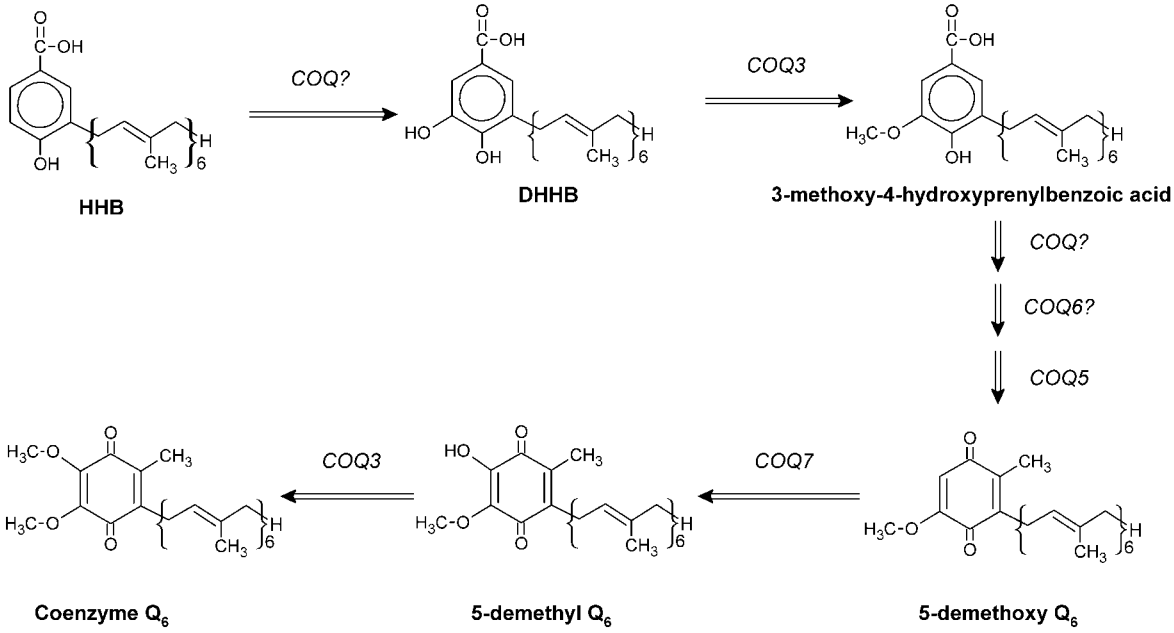

FIG. 1. **Coenzyme Q_6 biosynthesis in yeast.** The first compound in this scheme is 3-hexaprenyl-4-hydroxybenzoic acid (*HHB*), which accumulates in each of the *coq*-null mutants *coq3-coq8*. HHB is converted to 3,4-dihydroxy-5-hexaprenylbenzoic acid (*DHHB*) by an unidentified enzyme. The *O*-methyltransferase encoded by *COQ3* catalyzes the next step to produce 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid. Successive modifications to the head group results in 5-demethoxyubiquinone (*DMQ_6*). Coq7p, a monooxygenase, converts DMQ_6 to 5-demethylubiquinone, which is methylated by the Coq3 *O*-methyltransferase to produce Q_6.

The free-living nematode *C. elegans* has provided a powerful model system for the study of development and aging. Mutations in *clk-1* lead to delayed embryonic and postembryonic development, slowing of adult behaviors, resistance to UV-induced stress, and a long life span (21–23). The *clk-1* animals fail to synthesize Q_9 but instead produce significant amounts of the Q biosynthetic intermediate DMQ_9 (24), rhodoquinone-9 (RQ_9), and they rely on Q_8, obtained from their diet of *E. coli*, for growth and fertility (25–27). The long life span and delayed embryogenesis (28) of *clk-1* mutants observed on the standard *E. coli* diet has been attributed to the presence of DMQ_9, because it is believed to have fewer pro-oxidant properties than Q and is thought to function less efficiently in respiratory chain electron transport (29). However, Q_8 and RQ_9 are other components that may influence life span in *C. elegans* (30) and are confounding factors in determining the functional role of DMQ_9 in respiration and as an antioxidant.

In this study, we used the simpler model organism *S. cerevisiae* to study the effects of *clk-1* mutations on growth, respiratory function, and quinone levels. The yeast model enables growth in the absence of dietary or exogenous Q and in the absence of RQ, which is known to function in anaerobic respiration and could possibly function in aerobic respiration as well (31). The mutations introduced into the *COQ7* gene were designed to recapitulate the *C. elegans clk-1* mutations and included: E233K (*e2519*), predicted to disrupt the di-iron carboxylate site thought to be required for hydroxylase activity (19); L237Stop (*qm30*), deleting 36 amino acid residues from the carboxyl terminus; and P175Stop (*qm51*), deleting the carboxyl-terminal half of Coq7p. This study shows that yeast strains producing only DMQ_6 are respiration-deficient and unable to support either NADH-cytochrome *c* reductase or succinate-cytochrome *c* reductase activities. Steady-state levels of cytochromes c_1 and *c* are dramatically decreased in yeast containing DMQ_6 and lacking Q_6. Furthermore, the presence of DMQ_6 does not protect cells against oxidative stress generated by H_2O_2 or linolenic acid. The results presented here indicate that DMQ fails to support respiratory activity and is a less effective antioxidant than Q and suggest that the life span extension effects observed in the *C. elegans clk-1* mutants cannot be attributed solely to the presence of DMQ.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—Four yeast strains were used in this study, CEN.PK2–1C (MAT**a**, *his3-Δ1*, *leu2-3,112*, *trp1-289*, *ura3-52*, *MAL2-8c*, *MAL3*, *SUC3*) (32), CEN.MP3–1A (CEN.PK2–1C, *coq7*::*HIS3*) (32), EG103 (MATα, *his3Δ1*, *leu2-3,112*, *trp1-289*, *ura3-52*) (33), and EG110 (EG103 *sod2*::*HIS3*) (33). Growth media for yeast was prepared as described previously (34) and included YPG (1% yeast extract, 2% peptone, 3% glycerol), SDC (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), and SD-Ura (SDC, but amino acid supplement lacks uracil). The supplement was modified as described previously (35). Yeast were grown at 30 °C with shaking.

Plasmid Construction—The plasmids used in this study are listed in Table I. pNMQ71 contains the wild-type *COQ7* gene in the pRS316 low copy vector (10, 36). PCHQ71 was created by digesting pNMQ7 (10) with ClaI and HindIII and ligating the fragment with pRS316 that had been similarly treated. Primer mediated mutagenesis of PCR fragments was performed with the plasmid pNMQ71. To create pE233K, a G-to-A substitution was made in the primer sequence that corresponded to position 697 in the yeast open reading frame. The forward inside primer pQ7XBAR (5′-GGATGACAAGCTAGAGCATCTAGACACCG-3′) was used with the vector primer pQ7HIN (5′-GACGGTATCGATAAGCTTTCTTTTAATTAC-3′), whereas the inside reverse primer pQ7XBA (5′-GCGGTGTCTAGATGCTCTAGCTTGTCATCC-3′) was used with a primer corresponding to positions 218–249 of the open reading frame, pQ7SPH (5′-AACCTGAGCATGCTCCCAAGTGTCAGAATT-3′). This results in a Glu-to-Lys point mutation at amino acid 233 in the encoded yeast polypeptide. To generate pL237Stop, primers contained an insertion of the TAA stop codon at positions 709–711, thus creating a stop codon at amino acid 237. The forward inside primer pQ7MUT1 (5′-GACGAGCTAGAGCATTAAGACACCGCTATC-3′) was used with pQ7HIN, whereas the reverse inside primer pQ7MUT2 (5′-GATAGCGGTGTCTTAATGCTCTAGCTCGTC-3′) was used with pQ7SPH. In *C. elegans*, the *qm51* allele is a mutation in the splice-site acceptor, resulting in the translation of intron 2, which contains a stop codon after seven amino acids. To create the ochre mutation of pP175Stop, the primer design included an insertion of the first eight amino acids of *C. elegans* intron 2, ending in a TAA stop codon at aa 175 of the yeast sequence. The forward inside primer pQ7MUT3–2 (5′-GGGAAGAAGTTTGTGGATTTGCTGTAAGCTGGTACC-3′) was used with pQ7HIN, whereas

the reverse inside primer pQ7MUT4–2 (5′-TTACAGCAAATCCACAA-ACTTCTTCCCCATTGCAAA-3′) was used with pQ7SPH.

For each construct, the first round of PCR using pNMQ71 as a template resulted in two PCR products that overlap and contain the same introduced mutation. These overlapping products underwent another round of PCR, with each product using the other as template (37). The final PCR product was digested with SphI and HindIII and ligated into pNMQ71 that had been similarly treated. Each of the mutations was also transferred to multicopy vectors by excising the ClaI and HindIII segment and ligating the inserts into pRS426 (36). These were designated pmE233K, pmL237Stop, and pmP175Stop. All constructs were transformed into competent DH5α and amplified.

A truncation representing a true L237Stop C-terminal deletion, ptdL237Stop, was created. The first PCR generated two overlapping fragments that were subsequently used in a second round of PCR to create the final product. This process removed the fragment from position 709 to the penultimate codon, maintaining the original STOP codon. The first fragment was obtained with the forward primer pQ7-d30–1F (5′-AGCTCGGAATTAACCCTCACTAAAGGGA-3′) and the reverse primer pQ7d30–1R (5′-TCTGGTGGTTATAGATGCTCTAGCTC-GTCA-3′). The second fragment was obtained with the forward primer pQ7d30–2F (5′-GAGCATCTATAACCACCAGAAAGTGGCATA-3′) and the reverse primer pQ7d30–2R (5′-ACGTTGTAAAACGACGGCCAGT-GAA-3′). pQ7d30–1F and pQ7d30–2R were used as primers for the second PCR reaction. Each of the constructs described (Table I) was sequenced and found to be identical to wild type except for the site-directed changes specified by the primers.

Rescue Assays for Growth on a Nonfermentable Carbon Source—The constructs pE233K, pL237Stop, pP175Stop, or their multicopy counterparts were each placed into CEN.MP3–1A, a yeast strain harboring a deletion in the *COQ7* gene. Transformants were selected on SD-Ura plates. Samples of each were streaked onto YPG and grown for 4 days at 30 °C. For liquid growth, yeast strains were grown overnight in 5 ml of SD-Ura. The cultures were then diluted into 50 ml of YPG (A_{600} = 0.1). Growth was monitored by A_{600} measurements.

Mitochondrial Isolation and Western Blots—Crude mitochondria were isolated as described previously (38). SDS-PAGE and Western blotting employed standard methods (39). Polyclonal antibodies were used at the following dilutions: yeast porin (1:10,000), yeast cytochrome *c* (1:2,500), yeast cytochrome c_1 (1:10,000), and $F_1\beta$ATPase (1:5,000). Steady-state levels of specific mitochondrial polypeptides were measured by densitometric analysis, carried out with a GS800 densitometer with software analysis by Quantity One (Bio-Rad).

Quinone Isolation and Identification by HPLC/ECD and Mass Spectrometry—CEN.MP3–1A harboring each of the mutant constructs were grown in SD-Ura and harvested at either log phase or stationary phase. Crude mitochondria were isolated as described previously (38). Lipid extractions of whole cells and quinone quantification were performed as described previously (40). Quinones were quantified in isolated mitochondria after SDS solubilization and lipid extraction as described previously (39). In both cases, Q_9 was used as an internal standard; for whole-cell extractions, 1 μg of Q_9 was added per gram (wet weight) of cell pellets, and 500 ng of Q_9 was added per milligram of mitochondrial protein. Lipid components were separated by a Beckmann 166-126 HPLC system equipped with a 15-cm Kromasil C-18 column in a column oven set to 40 °C, with a flow rate of 1 ml/min and a mobile phase containing 88:24:10 methanol/ethanol/2-propanol and 13.4 mM lithium perchlorate. Quinones were quantified with an ESA Coulochem III electrochemical detector (ECD) and a 5010 analytical cell (E1, −500 mV; E2, 500 mV). Hydroquinones present in samples were oxidized with a pre-column cell set in oxidizing mode (E, +500 mV). Q_6 and Q_9 were quantified with external standards. The Q_6 external standard was used to quantify DMQ_6, assuming a similar redox response in the ECD.

For mass spectrometry analysis, 1 liter of SD-Ura-His cultures were inoculated and harvested after 2 days of growth. A_{600} ranged from 8.3–8.8 A_{600}/ml. Mitochondria were isolated and lipid extracts were resolved over the HPLC/ECD system. The peaks corresponding to Q_6 and DMQ_6 were collected and dried down under nitrogen gas. The samples were analyzed with a PerkinElmerSciex Instruments API III triple quadropole mass spectrometer fitted with a heated nebulizer at 450 °C. The solvent was 0.33% H_2O in acetonitrile at a flow rate of 0.1 ml/min. Instrument conditions for multiple reaction monitoring analysis were 0.3 step size; dwell, 900 ms; scan speed, 4.0 s; and oriface at 50 V; cgt, 220. These samples were compared with Q_6 obtained from Sigma and DMQ_8 obtained from the *E. coli ubiF* mutant strain AN78 (41). Five transition events were monitored by multiple reaction monitoring: *m/z* 591.4 → *m/z* 197.2 and 237.2 (Q_6); *m/z* 561.4 → *m/z* 167.2 and 207.1 (DMQ_6); and *m/z* 697.4 → *m/z* 167.2 (DMQ_8).

Mitochondrial Respiratory Chain Activities—Freshly obtained mitochondria were assayed for NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities. For assays of other respiratory chain activities, mitochondrial samples were subjected to one freeze-thaw cycle. All assays were performed in triplicate.

NADH-DCIP reductase activity was measured as described with minor modifications (42). The reaction was performed at 30 °C with stirring. Mitochondrial protein (10–50 μg) was incubated in reaction buffer (20 mM MES-KOH, pH 6.5, 1 mM EDTA, and 200 mM KCl) for 3 min. The reaction was initiated with 100 μM DCIP, without NADH; after 1 min, 200 μM NADH was added. Specific NADH-DCIP reductase activity was determined by subtracting the initial linear rate before NADH addition from the rate after NADH addition. The specific activity of DCIP reduction was determined at 600 nm with an extinction coefficient of 19.1 $mM^{-1}\cdot cm^{-1}$.

Succinate-decylubiquinone reductase activity sensitive to malonate was used to measure complex II activity (43). Activity was measured in an assay volume of 1 ml with 40 mM sodium phosphate, pH 7.4, 40 mM sodium succinate, 100 μM decylubiquinone, 250 μM potassium cyanide, and 2 μg/ml antimycin A. Samples of mitochondria (20–50 μg of protein) were incubated in the assay buffer for 3 min. The reaction was initiated with the addition of decylubiquinone; after 1 min, succinate was added. As a control, some samples were pre-incubated with 4 mM sodium malonate and this rate was subtracted as background. Reactions were monitored via spectrophotometric measurements of absorbance at 280 nm minus 340 nm. The specific activity was determined with an extinction coefficient of 14.5 $mM^{-1}\cdot cm^{-1}$.

NADH-cytochrome *c* reductase activity was measured in an assay volume of 1 ml in 40 mM sodium phosphate, pH 7.4, 0.2 mM NADH, 50 μM cytochrome *c*, and 250 μM potassium cyanide. Samples of mitochondria (10–50 μg of protein) were incubated in the assay buffer for 3 min. The reaction was initiated by addition of cytochrome *c*; after 1 min, NADH was added. The reaction was monitored via spectrophotometric measurements of absorbance at 550 nm minus 540 nm. The specific activity was determined with an extinction coefficient of 18.5 $mM^{-1}\cdot cm^{-1}$. All assays were performed in triplicate. In some experiments, the activity was measured with the addition of exogenous 10 μM Q_3 and 10 μM DMQ_3 after a 10-min incubation. Given that both compounds were prepared in ethanol, controls with the solvent were performed. To check the nature of cytochrome *c* reduction, 300 units/ml of bovine superoxide dismutase was added as control. Succinate-cytochrome *c* reductase and cytochrome *c* oxidase activities were measured as described previously (44).

Superoxide production was determined in mitochondrial samples by the reduction of acetylated cytochrome *c* according to a method described previously (45). The reaction was measured in final volume of 1 ml in 40 mM sodium phosphate, pH 7.4, 0.2 mM NADH, 40 mM sodium succinate, 10 μM acetylated cytochrome *c*, 250 μM potassium cyanide, and mitochondria samples (50 μg of protein). The reaction was initiated by addition of cytochrome *c* and after 1 min, NADH and succinate were added. The reaction was monitored in a manner similar to that described for NADH-cytochrome *c* reductase. Bovine superoxide dismutase (300 units/ml) was added in control experiments. All assays were performed in triplicate.

Analysis of Oxidative Stress Sensitivity by Colony Counting—Yeast cells were cultured in SDC or SD-Ura media, harvested at exponential growth phase ($A_{600\ nm}$ = 1) or early stationary growth phase ($A_{600\ nm}$ = 4–5), washed and resuspended in sterile 100 mM sodium phosphate buffer, pH 6.2, with 0.2% glucose, at 180 × 10^6 cells/ml. Cell suspensions were incubated with different concentrations of H_2O_2 and linolenic acid for 4 h. Samples from each treatment were diluted and spread onto YPD plates. After 2 days at 30 °C, colonies were counted. For each treatment and strain, three plates were counted.

Measurement of Superoxide Anion Generation by Flow Cytometry—Yeast cells were cultured until stationary phase and treated to produce oxidative stress as described above. Cells treated for 4 h with H_2O_2 (5 and 10 mM) or linolenic acid (750 μM) were harvested, washed, and resuspended at 2 × 10^6 cells/ml in phosphate-buffered saline. To measure cellular superoxide levels, cells were incubated with 4 μM hydroethidine (HE) for 30 min. HE is oxidized by superoxide, resulting in a product that has fluorescent properties similar to those of ethidium and that binds to DNA (46–48). Cellular levels of this oxidized product were measured by flow cytometry after excitation at 488 nm with an argon laser and detection of emitted red fluorescence (>650 nm), with a Coulter Epics XL flow cytometer. The sample fluid pressure was adjusted to acquire ~200 events per second.

TABLE I
Plasmid names and identifications

Plasmid name		Description
Low copy	Multicopy	
pNMQ71, pCHQ71	p7.8	Wild-type Coq7p
pRS316	pRS426	Empty vector
pE233K	pmE233K	$E_{233}K$ of wild-type Coq7p
pL237Stop	pmL237Stop	Stop at amino acid 237 of wild-type Coq7p
ptdL237Stop	pmtd237Stop	True deletion after amino acid 237 of pL237Stop
pP175Stop	pmP175Stop	Stop at amino acid 175 with 7-amino acid insertion

RESULTS

Growth Characteristics of Yeast coq7 Mutant Strains in Glycerol Media—Mutations were introduced into the yeast *COQ7* gene to mirror each of three *C. elegans clk-1* mutant alleles described previously(*e2519*, *qm30*, *qm51*; Table I) (15). Primer-mediated mutagenesis was used to generate the constructs in both low and multicopy plasmids. These plasmids were transformed into the *coq7*Δ strain, CEN.MP3–1A. The transformants were tested for their ability to grow in liquid cultures containing glycerol as the sole carbon source (Fig. 2). The CEN.MP3–1A yeast strain harboring pL237Stop (encoding a truncated Coq7p lacking 36 residues of the carboxyl terminus) was capable of growing on glycerol after a long lag period (Fig. 2). CEN.MP3–1A yeast harboring pE233K (predicted to disrupt the di-iron site) and pP175Stop (lacking the carboxyl-terminal half of Coq7p) failed to grow (Fig. 2*A*). These two transformants were also incapable of growth on media containing ethanol as the sole nonfermentable carbon source (data not shown). Therefore, the lack of growth on glycerol is not a specific consequence of a lack of Q_6 as an electron acceptor for glycerolphosphate dehydrogenase (GUT2) (49) but is caused by a general lack of mitochondrial electron transport mediated by Q_6.

We further investigated the yeast strain CEN.MP3–1A: pL237Stop, which displayed growth after a lag period. When samples of this strain were reinoculated into fresh glycerol media, they did not exhibit a lag and grew like wild type (data not shown). The effect of overexpressing the L237Stop mutation (from the multicopy plasmid pmL237Stop) was dramatic in that this plasmid conferred almost wild-type levels of growth (Fig. 2*B*). It seemed likely that read-through of the introduced stop codon in pL237Stop was responsible for the observed growth adaptation. To investigate this possibility, a C-terminal deletion was prepared by truncating the C-terminal coding region at position 237, and this construct was designated ptdL237Stop. CEN.MP3–1A was transformed with ptdL237-Stop and tested for YPG growth. No rescue of growth was observed on this nonfermentable carbon source (Fig. 2). Therefore, read-through of the introduced stop codon was deemed the most likely explanation for the adaptation phenomenon. Such informational suppression is well documented (50–52) and also accounts for the reproducibility of the lag period observed in response to selective pressure (Fig. 2) (53).

Identification and Quantification of DMQ_6 and Q_6 in coq7 Mutants—To analyze the relationship between growth in YPG and Q synthesis, lipid extracts were prepared from yeast bearing the *coq7* mutant plasmids, and quinones were separated by HPLC and quantified by ECD. Similar trends were observed for quinone content in both whole cells (data not shown) and isolated mitochondria (Table II). Mitochondria isolated from CEN.MP3–1A yeast harboring the wild-type *COQ7* gene and grown in glucose-containing media produced predominantly Q_6, (1950 ng of Q_6/mg of protein), although a significant amount of DMQ_6 (432 ng/mg of protein) was also present. The *coq7* null mutant strain harboring empty vector (CEN.MP3–1A:pRS316), or harboring pP175Stop failed to synthesize either Q_6 or DMQ_6. Q_6 was not detectable in CEN.MP3–1A:pE233K;

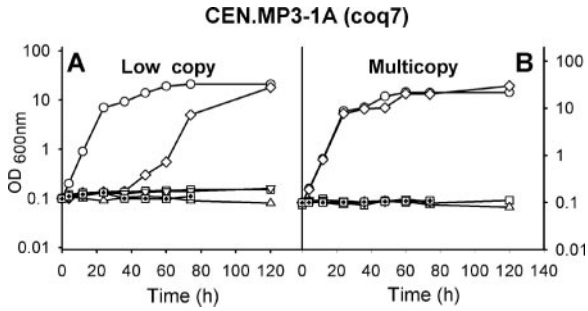

FIG. 2. **Growth and rescue on glycerol of CEN.MP3–1A harboring mutant *coq7* alleles.** Yeast bearing the low copy (*A*) or multicopy (*B*) plasmids were grown overnight in SD-Ura and were subsequently diluted into YPG at A_{600} = 0.1. Growth was monitored by A_{600} measurements. Yeast harbored plasmids bearing either wild-type *COQ7* (○), empty vector (▽), or one of the designated *coq7* mutations, E233K (□), P175Stop (△), L237Stop (◇), or tdL237Stop (☒). *B*, pCHQ71 was used as the wild-type control. Data are representative of at least three experiments.

however this strain accumulated DMQ_6, 861 ng/mg of protein. CEN.MP3–1A yeast bearing pL237Stop produced exclusively DMQ_6 (406 ng/mg of protein) when grown in media containing glucose. The content of Q_6 in some of the mutants was altered in response to growth in media containing glycerol. Mitochondria isolated from CEN.MP3–1A harboring either pL237Stop or pmL237Stop after culture in YPG accumulated significant amounts of Q_6, ranging from 310 to 423 ng of Q_6/mg of protein, although DMQ_6 was still the predominant quinone isolated from these strains. No other quinone containing intermediates were detected.

To verify the identities of the compounds quantified by HPLC/ECD, these fractions were collected, purified, and analyzed by mass spectrometry (*Experimental Procedures*). The expected daughter tropylium ions were present (a 197.2 ion for Q_6 and 167.2 for DMQ_6), verifying their identities.

Analysis of Respiratory Chain Activities in Yeast Mutants Producing Only DMQ_6—To analyze the role of DMQ_6 in respiration, several segments of the mitochondrial respiratory chain were assayed for activity (Table II). The CEN.MP3–1A: pNMQ71 strain was used as a positive control, and these values were set at 100% as a comparison. Yeast that were incapable of synthesizing either Q_6 or DMQ_6, CEN.MP3–1A: pRS316, and CEN.MP3–1A:pP175Stop displayed a decrease in all respiratory chain activities, ranging from 3 to 16% of the wild-type activities. The most dramatic effect was seen in the assays of succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase. Both of these assays rely on endogenous Q_6 present in isolated mitochondria as the electron carrier.

The CEN.MP3–1A strain harboring the plasmids pE233K or ptdL237Stop produces DMQ_6 exclusively and shows a decrease in all mitochondrial respiratory chain activities. This was also the finding for the CEN.MP3–1A:pL237Stop mutant cultured in glucose. However, in each case, NADH-DCIP and succinate-decylubiquinone reductase activities were significantly higher than in the CEN.MP3–1A:pRS316 strain, ranging from 30 to

TABLE II

Mitochondrial respiratory complexes activities in Q_6- and DMQ_6-producing strains

All strains were cultured in SD selective media until stationary phase. Where indicated, strains harboring pL237Stop and pmL237Stop were cultured in YPG. The notation of 2 μM Q_6 refers to the final concentration of Q_6 added to yeast culture media. Lipids were extracted from mitochondria and quantified as described under "Experimental Procedures."

Plasmid harbored in CEN.MP3–1A	Q_6[a]	DMQ_6[a]	Activity of Respiratory Complexes[b]				
			NADH-DCIP Oxidoreductase	Succinate-decylQ Oxidoreductase	NADH-Cytochrome *c* Oxidoreductase	Succinate-Cytochrome *c* Oxidoreductase	Cytochrome *c* Oxidase
	ng of quinone/mg of mitochondrial protein		*nmol/mg of protein/**min***				
PNMQ71	1950 ± 11	432 ± 12	511 ± 8 (100)	190 ± 27 (100)	1059 ± 67 (100)	301 ± 36 (100)	533 ± 19 (100)
PRS316	ND	ND	74 ± 5 (15)	10 ± 0.3 (5)	33 ± 8 (3)	16 ± 2 (5)	64 ± 2 (12)
PRS316 + 2 μM Q_6	NM	NM	728 ± 15 (142)	97 ± 7 (51)	156 ± 5 (13)	140 ± 12 (47)	223 ± 10 (42)
pL237Stop	ND	406 ± 10	154 ± 16 (30)	87 ± 3 (46)	37 ± 2 (4)	17 ± 2 (5)	63 ± 5 (12)
pL237Stop in YPG	310 ± 22	610 ± 36	365 ± 6 (72)	88 ± 9 (46)	261 ± 6 (25)	195 ± 6 (65)	273 ± 8 (51)
pL237Stop + 2 μM Q_6	NM	NM	478 ± 5 (94)	95 ± 2 (50)	187 ± 11 (16)	163 ± 7 (54)	167 ± 3 (31)
ptdL237Stop	ND	592 ± 20	290 ± 26 (56)	39 ± 1 (20)	45 ± 2 (4)	NM	69 ± 4 (12)
pmL237Stop	56 ± 1	679 ± 12	183 ± 21 (36)	65 ± 7 (34)	120 ± 10 (11)	163 ± 15 (54)	148 ± 7 (28)
pmL237Stop in YPG	423 ± 10	1532 ± 150	739 ± 37 (140)	100 ± 3 (53)	456 ± 8 (43)	225 ± 16 (75)	418 ± 12 (78)
pP175Stop	ND	ND	87 ± 5 (16)	17 ± 1 (9)	66 ± 2 (6)	NM	76 ± 2 (14)
pE233K	ND	861 ± 17	247 ± 32 (48)	62 ± 8 (33)	54 ± 6 (5)	1 ± 0.2 (0.3)	74 ± 3 (14)

[a] Data shown are the average of three injections ± S.D. from two independent extractions. ND, not detected (detection limit is 0.2 ng of Q_6 per injection); NM, not measured.

[b] Results are expressed as the average of three assays ± S.D. from two separate experiments. In parentheses are percentages compared with the wild-type construct. NM, not measured.

56%. Other activities resembled those of the CEN.MP3–1A:pRS316 strain.

The results of Table II suggest that the NADH cytochrome *c* reductase activity is similarly defective in mitochondria isolated from either the *coq7* null or the DMQ_6-producing yeast strains. Because supplementation of the growth media with exogenous Q_6 restores this activity, it was of interest to test whether *in vitro* addition of Q or DMQ analogs to isolated mitochondria would also act to restore activity. As shown in Table III, addition of either Q_3 or DMQ_3 to the assay produced only a very modest increase in NADH cytochrome *c* reductase activity. Most of this increase was inhibited by the addition of superoxide dismutase, suggesting that when either Q_3 or DMQ_3 are added to the assay, most of the cytochrome *c* reduction is mediated by superoxide and not via the bc_1 complex.

Supplementation of growth media with 2 μM exogenous Q_6 restored NADH-DCIP reductase activity, and enhanced the other respiratory chain activities in the CEN.MP3–1A:pRS316 and in the CEN.MP3–1A:pL237Stop strains. Those yeast strains capable of producing significant amounts of Q_6, in response to YPG culture media, namely CEN.MP3–1A:pL237Stop and CEN.MP3–1A:pmL237Stop, showed respiratory enzyme activities much higher than those strains exclusively producing DMQ_6.

The Effect of DMQ_6 on Steady-state Levels of Mitochondrial Polypeptides—Our previous studies with *coq7* null mutant yeast showed that Q_6 is required for stability of the cytochrome c_1 polypeptide (39). To determine whether the exclusive presence of DMQ_6 affected steady-state levels compared with Q_6-producing strains, Western blot analysis of mitochondrial polypeptides, including porin, $F_1\beta$-ATPase, cytochrome c_1, and cytochrome *c*, were performed (Fig. 3*A*). Densitometric analysis showed that steady-state levels of cytochrome *c*, a substrate of complexes III and IV, and cytochrome c_1, a subunit of the bc_1 complex, were dramatically decreased compared with porin and the $F_1\beta$ subunit of complex V (Fig. 3*B*). Levels of cytochromes *c* and c_1 were fully restored when the culture media was supplemented with exogenous Q_6. These results indicate that DMQ_6 is unable to provide for stable steady-state levels of the *c*-type cytochromes.

Sensitivity of coq7 Yeast Mutants to Oxidative Stress—It has been shown that the yeast *coq* null mutant strains are hypersensitive to oxidative stress caused by treatment with linolenic acid (11, 55). These results indicate that Q_6 functions as an important cellular antioxidant, required to protect against the toxicity induced by polyunsaturated lipid autoxidation. To test the efficacy of DMQ_6 as an antioxidant, yeast strains producing Q_6, DMQ_6 or no quinones were subjected to oxidative stress caused by treatment with either H_2O_2 or linolenic acid (Fig. 4*A-D*). Cell viability was analyzed during both log and stationary phase. All strains showed a decrease in cell viability with increasing concentrations of H_2O_2 (Fig. 4, *A* and *B*). Although the wild-type strain was significantly more resistant than the different mutant strains, the CEN.MP3–1A strain harboring pL237Stop was slightly more resistant than the other mutants. Treatment with linolenic acid produced a different effect on the cell viability (Fig. 4, *C* and *D*). Wild-type yeast were not affected by this treatment, whereas all *coq7* mutants were sensitive to linolenic acid at both log and stationary phase, although the cells at log phase showed greatly increased sensitivity. It was interesting that CEN.MP3–1A:pE233K, which produces only DMQ_6, was more sensitive to linolenic acid treatment at stationary phase than was the *coq7* null mutant CEN.MP3–1A, which fails to produce Q_6 or DMQ_6. These data indicate that DMQ_6 fails to act as an effective antioxidant. It was intriguing that the CEN.MP3–1A:pL237Stop mutant was significantly more resistant than the other mutants tested; the effect was most pronounced for cells treated with linolenic acid (Fig. 4, *C* and *D*).

Given that the CEN.MP3–1A:pL237Stop mutant strain adapts to growth under selective conditions (YPG) and synthesizes Q_6 (as a result of the stop codon read-through), we investigated whether linolenic acid treatment also selects for Q_6 production. CEN.MP3–1A cells harboring pL237Stop, ptdL237Stop, or pE233K were cultured in SD-Ura and were treated with 750 μM linolenic acid in phosphate buffer for 4 h. Analysis of lipid extracts prepared from these cells revealed that CEN.MP3–1A harboring pE233K or ptdL237Stop produced only DMQ_6 (data not shown). However, treatment of the CEN.MP3–1A:pL237Stop yeast strain with linolenic acid led to a distinct accumulation of Q6 (Fig. 5), that correlated well with the increased stress resistance (Fig. 4). It is likely that the stress produced by linolenic acid treatment selects for stop codon read-through, similar to the selection imposed by culture in media containing a nonfermentable carbon source.

TABLE III

Enhancement of NADH-cytochrome c reductase activity by Q_3 and DMQ_3

All strains were cultured in S.D. selective media until stationary phase. Results are expressed as the average of three assays ± S.D. from two separate experiments. Samples not incubated with Q_3 or DMQ_3 were incubated with the same volume of ethanol. Q_3 or DMQ_3 were incubated with the samples for 5 min at RT. SOD was present during the incubation.

Strains	NADH-Cytochrome *c* Reductase Activity				
		10 mM Q_3	10 mM Q_3 + 300 units of SOD[a]	10 mM DMQ_3	10 mM DMQ_3 + 300 units of SOD
	nmol/mg of protein/min				
CEN.MP3–1A:pNMQ71	1190 ± 35	NM[b]	NM	NM	NM
CEN.MP3–1A:pL237Stop	28.2 ± 5	71.1 ± 1.2	36.6 ± 1.3	75.3 ± 0.8	40.9 ± 0.8
CEN.MP3–1A:pRS316	9.4 ± 1.2	16.8 ± 1.2	NM	18.6 ± 2.6	NM

[a] SOD, superoxide dismutase.
[b] NM, not measured.

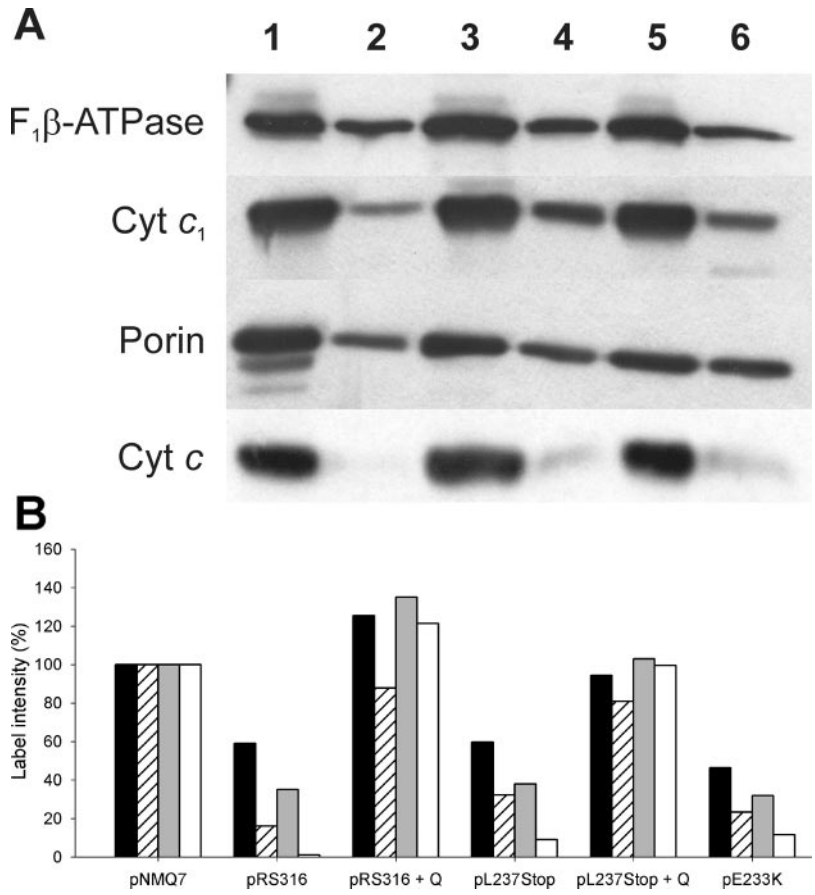

FIG. 3. **DMQ_6-producing strains display lowered steady-state expression of mitochondrial respiratory polypeptides.** *A*, mitochondrial fractions (30 μg of protein/lane) were separated by SDS-PAGE and analyzed by Western blot under standard conditions. Antibodies developed against yeast proteins were used: $F_1\beta$-ATPase (1:5000) for internal mitochondrial membrane (IMM), cytochrome c_1 (1:10,000) for IMM, porin (1:10,000) for outer mitochondrial membrane, and cytochrome *c* (1:2500) for intermembrane space. *Lane 1*, CEN MP3–1A:pNMQ71 (*COQ7*); *lane 2*, CEN.MP3–1A (*coq7*Δ); *lane 3*: CEN.MP3–1A + 2 μM Q_6; *lane 4*, CEN.MP3–1A:pL237Stop; *lane 5*, CEN.MP3–1A:pL237Stop + 2 μM Q_6; and *lane 6*, CEN.MP3–1A:pE233K. *B*, bars designate the steady-state level of polypeptides as quantified by densitometry: *black*, $F_1\beta$-ATPase; *hatched*, cytochrome c_1; *gray*, porin; *white*, cytochrome *c*. The levels of expression in the wild-type strain were set at 100%. The same membrane was imaged after four exposure times (10, 30, 60, and 120 s). All plates were recorded using GPL mode (Good Laboratory Practice) of the Quantity One (Bio-Rad) software, which does not allow image modifications that change the raw image data. The best relationship between time and intensity was selected.

Superoxide Anion Generation in Yeast coq7 Mutants Producing DMQ_6—To analyze the superoxide anion generation by the mitochondrial respiratory chain, the reduction of acetylated cytochrome *c* was measured in mitochondrial samples using NADH and sodium succinate as electron donors. As with cytochrome *c*, acetylated cytochrome *c* can be reduced by superoxide anion; however, the multiple acetyl groups covalently linked to acetylated cytochrome *c* prevent its interaction with respiratory enzyme complexes (54). Mitochondria isolated from CEN.MP3–1A:pNMQ71 produced the highest amounts of superoxide anion (16.3 ± 1.4 nmol/mg of protein/min). Superoxide production was significantly lower for the *coq7* null mutant CEN MP3–1A:pRS316 (5.4 ± 0.5 nmol/mg of protein/min) and for the DMQ_6-producing strain CEN MP3–1A:pL237Stop (5 ± 0.2 nmol/mg of protein/min). These results argue against the idea that partially assembled respiratory complexes in the Q-less yeast contribute to increased levels of superoxide

Cellular superoxide generation during oxidative stress treatment was analyzed with flow cytometry and by monitoring the fluorogenic oxidation of HE, as described under "Experimental Procedures." Conditions that enhance superoxide production in yeast were used to validate this assay. The strain EG110 harbors a *sod2*-null mutation (56) and hence lacks the mitochondrial manganese superoxide dismutase. This strain accumulated high levels of superoxide compared with the isogenic wild-type strain EG103 (Fig. 6*A*). In addition, yeast cells were also incubated with antimycin A, a compound that produces superoxide by blocking the high potential cytochrome *b* in bc_1 complex (57). Wild-type yeast showed low levels of superoxide, which increased upon incubation with antimycin A (Fig. 6*A*). Antimycin A treatment did not affect superoxide production in the *coq7* null strain. The levels of superoxide produced in CEN.MP3–1A mutants harboring pE233K or pL237Stop were significantly higher than wild type, although treatment with

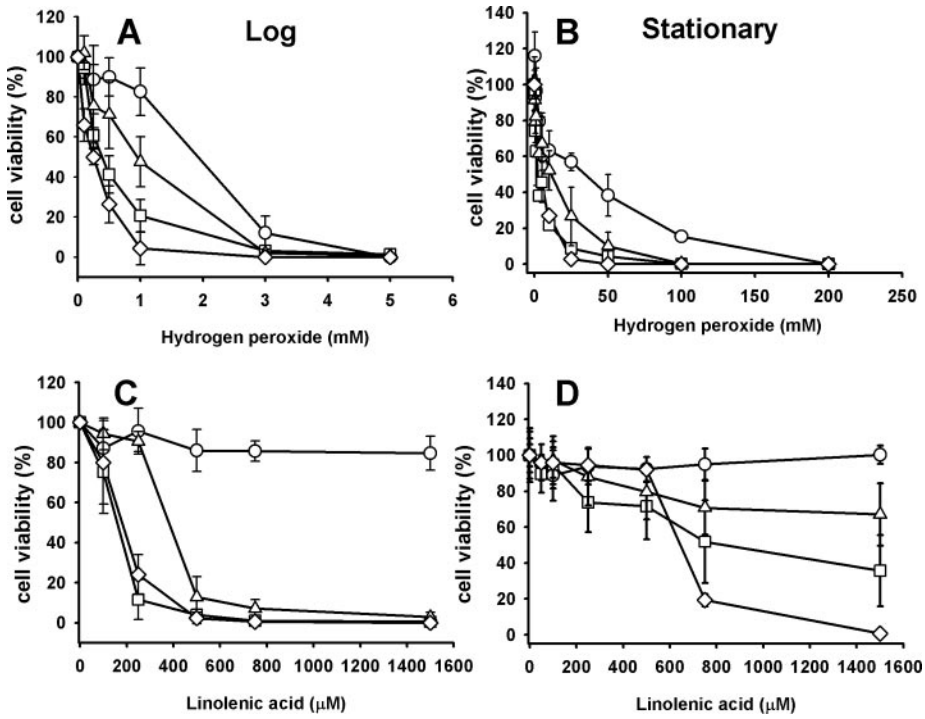

FIG. 4. **Q-producing strains are less sensitive to oxidative stress produced by linolenic acid and H_2O_2.** Cells from wild-type yeast (CEN.PK2–1C, ○) and the null mutant (CEN.MP3–1A) harboring empty vector (□), pE233K (◇), or pL237Stop (△) were cultured, harvested, and subjected to oxidative stress by H_2O_2 or linolenic acid treatment as described under "Experimental Procedures." Shown are H_2O_2 in log phase (*A*), H_2O_2 in stationary phase (*B*), linolenic acid in log phase (*C*), and linolenic acid in stationary phase (*D*).

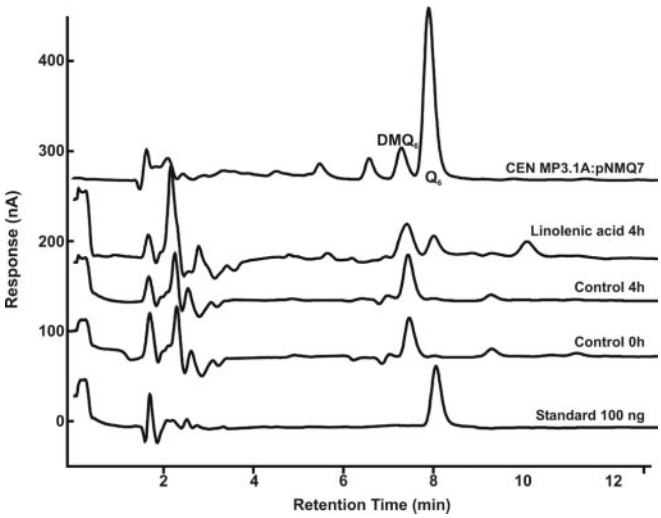

FIG. 5. **Linolenic acid treatment stimulates Q_6 production in mutants bearing pL237Stop.** The CEN.MP3–1A:pL237Stop mutant strain was treated with H_2O_2 or linolenic acid and harvested, and the lipids were extracted and analyzed by HPLC/ECD. Chromatograms from cells treated with 750 μM linolenic acid are displayed. A chromatogram from the strain CEN.MP3–1A:pNMQ71 is shown as control. Standard chromatogram corresponds to 100 ng of commercial Q_6 injection. Two controls are defined: 0 h control that indicates cells at start of incubation and 4-h control indicating the cells after 4-h incubation without linolenic acid that measured possible quinone changes during the incubation.

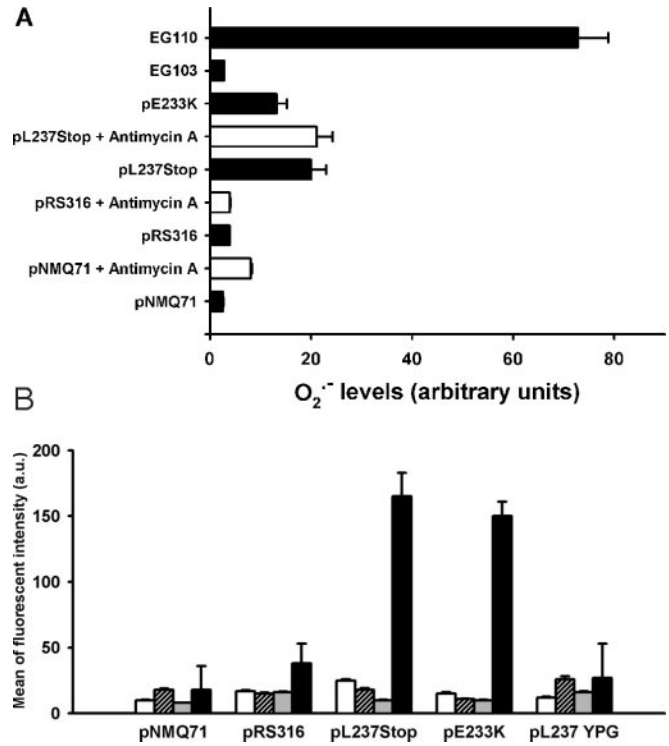

FIG. 6. **DMQ_6-producing strains have increased levels of superoxide anion generation.** Cells from the indicated strains were cultured and treated as described under "Experimental Procedures" to quantify the amount of superoxide anion produced after oxidative stress. *A*, superoxide generation in untreated cells (*black bars*) or cells treated with antimycin A (2 μg/ml) (*white bars*). *B*, superoxide generation in different strains subjected to oxidative stress produced by the following treatments: *white*, control; *hatched*, 5 mM H_2O_2; *gray*, 10 mM H_2O_2; *black*, 750 μM linolenic acid. Data shown correspond to the average of four measures from the same sample. The data are representative of three experiments.

antimycin A did not further increase superoxide levels in these strains. Antimycin A requires functional mitochondria to produce superoxide, providing additional evidence that DMQ_6 fails to support mitochondrial respiratory activity.

Once the HE assay of superoxide was validated, yeast grown to stationary phase was subjected to oxidative stress by treatment with H_2O_2 (5 or 10 mM) or with linolenic acid (750 μM) (Fig. 6*B*), and HE oxidation was measured. Treatment with H_2O_2 did not produce a significant increase in superoxide levels in any strain tested. In contrast, linolenic acid treatment produced high levels of HE oxidation in the DMQ_6-producing strains but not in wild-type or null mutants. The amount of HE oxidation detected in these strains was double the amount detected in the *sod2*Δ strain. However, HE oxidation dropped to wild-type levels when the CEN.MP3–1A:pL237Stop mutant

was cultured in YPG. Thus, under conditions in which Q_6 is produced, superoxide is produced at minimal levels, even if DMQ_6 is the predominant quinone present. However, after linolenic acid treatment, and in the absence of Q_6, DMQ_6 seems to have significant pro-oxidant activity.

DISCUSSION

Analysis of *clk-1* nematodes has introduced some intriguing questions regarding the functional roles of Q and DMQ in development and aging. In particular, the extended lifespan of the *C. elegans clk-1* mutants has been hypothesized to result from the presence of DMQ, because this intermediate is considered to have less pro-oxidant activity than Q and is also thought to be less efficient in mediating electron transport through the respiratory chain (29). However, hypotheses regarding these attributes of DMQ have been difficult to test, because the nematode system is complicated by the contributions of dietary Q_8 from the standard *E. coli* diet, and by endogenously produced rhodoquinone (RQ_9), which accumulates in the *clk-1* mutants and is required for anaerobic respiratory metabolism (26). Both Q_8 and RQ_9 constitute other parameters that have the potential to influence lifespan in *C. elegans* (30). To dissect the functionality of DMQ we turned to a simpler model and employed *S. cerevisiae* and its *clk-1* homologue, *COQ7*.

Three mutations were introduced into the yeast *COQ7* gene to generate analogs of *C. elegans clk-1* mutant alleles: E233K, with a disrupted di-iron binding site; L237Stop, missing 36 carboxyl-terminal amino acids; and P175Stop, missing the carboxyl-terminal half of the polypeptide. The E233K substitution for the putative bridging carboxylate ligand of the yeast Coq7 di-iron site is likely to inactivate enzyme activity, in that mutation of the corresponding glutamate ligand to alanine (E273A) in the alternative oxidase from *Arabidopsis thaliana* resulted in loss of the di-iron center (58). By expressing these *coq7* mutations in a *coq7* null mutant, yeast strains were generated that lacked Q_6 but produced significant quantities of DMQ_6. Yeast mutants that produced only DMQ_6 failed to grow on media containing glycerol, a nonfermentable carbon source. Mitochondria isolated from these yeast strains lacked Q_6, contained DMQ_6, and failed to support NADH-cytochrome *c* reductase or succinate cytochrome *c* reductase activities. The DMQ_6-producing yeast mutant strains also showed decreased steady-state levels of cytochrome c_1, a polypeptide component of bc_1 complex, and cytochrome *c*, a substrate of the bc_1 complex. These data indicate that DMQ_6 fails to support respiratory electron transport in yeast.

To investigate the potential antioxidant properties of DMQ_6, yeast were subjected to treatment with hydrogen peroxide and linolenic acid. When treated with H_2O_2, DMQ_6-producing yeast mutant strains were found to be as sensitive as the *coq7* null mutant. In previous work, yeast *coq* null mutants have been shown to be exquisitely sensitive to treatment with linolenic acid (11, 55). In data presented here, we show that DMQ_6-producing yeast mutant strains were even more sensitive to linolenic acid treatment than the *coq7* null mutant. The fluorogenic oxidation of HE was used as an indicator of superoxide. This assay showed that superoxide levels were profoundly increased when DMQ_6-producing yeast were treated with linolenic acid. In contrast, yeast strains producing both DMQ_6 and Q_6 showed enhanced resistance to linolenic acid treatment and decreased levels of superoxide. These results show that the pro-oxidant attributes of DMQ_6 are negated when Q_6 is also present, presumably because the Q_6/Q_6H_2 redox couple is capable of being regenerated by the electron transport chain. It is also possible that Q_6 acts to reduce and oxidize DMQ_6 via respiratory complex transhydrogenation reactions (59). These data indicate that DMQ_6 on its own fails to act as an effective antioxidant and, under certain stress conditions, acts as a pro-oxidant and is associated with increased levels of superoxide production.

The apparent pro-oxidant action of DMQ_6 in response to linolenic acid treatment is curious. It is possible that unstable or improperly assembled respiratory complexes are responsible for generating high levels of superoxide. However, mitochondria isolated from either the null *coq7* mutant or the DMQ_6-producing strains produced similar and very low levels of superoxide, as measured by the reduction of acetylated cytochrome *c*. Similar results have been described for null coq7 mutants with regard to hydrogen peroxide production in mitochondria (60). These results indicate that sources of oxidative stress in mitochondria are similar for the *coq7* null and the DMQ_6-producing mutants. In yeast, the *bc1* complex is believed to generate the highest levels of superoxide, yet the steady-state levels of cytochromes c_1 and *c* are profoundly decreased in both strains, regardless of the presence or absence of DMQ_6. This suggests that the bc_1 complexes in these two strains are similarly defective, an idea that is supported by the lack of NADH-cytochrome *c* oxidoreductase and succinate cytochrome *c* oxidoreductase activities in both the *coq7*-null and DMQ_6-producing mutants (Table II) and by the inability to recover NADH-cytochrome *c* reductase activity in response to addition of either Q_3 or DMQ_3 to isolated mitochondria (Table III). However, it is likely that when these strains are treated with linolenic acid, the DMQ_6–producing strain will uniquely have accumulated DMQ_6H_2, because of significant segment I and complex II activities (30 and 46% of wild-type) (Table II). Lipid autoxidation could trigger the oxidation of DMQ_6H_2 producing the semiquinone radical, which in turn would contribute to superoxide formation and enhance the oxidation of hydroethidium.

In yeast, data suggest that Coq7p is required for a multisubunit Q biosynthetic complex, and mutations in Coq7p are considered to produce the partial or complete disruption of complex integrity and activity. CEN.MP3–1A mutants expressing the Coq7 polypeptide bearing the E233K mutation were able to produce the full-length form of Coq7p (data not shown). Because the polypeptide is stable, it is likely to maintain contacts with other Coq polypeptides or respiratory complexes. This allows for production of DMQ_6, whereas *coq7* null yeast mutants produce only the early intermediate HHB (10).

Is the Coq7/Clk-1 polypeptide part of a multisubunit Q biosynthetic complex in other eukaryotes? Although the answer to this question will depend on its physical characterization, there is evidence that *C. elegans* CLK-1 can function independently. An *E. coli ubiF* mutant known to have a defect in the hydroxylation of DMQ_8 was shown to be rescued by expression of the *C. elegans clk-1* gene, indicating that the CLK-1 polypeptide is active when expressed on its own in *E. coli* (20). Mice and *C. elegans* mutants with deletions in the *clk-1* gene continue to produce DMQ_9 although no CLK-1 polypeptide or RNA can be detected (61–63). This is distinct from the phenotype of *coq7*-null yeast mutants that produce only HHB. These findings indicate that in the mouse and nematode, progression of Q biosynthesis from HHB to DMQ proceeds independently of the CLK-1 polypeptide.

Our finding that DMQ_6 cannot function in yeast mitochondrial electron transport counters previous studies that claimed DMQ_9 supported mitochondrial respiratory chain activities in *C. elegans clk-1* mutants (24) and in mouse *clk-1*−/− embryonic stem cells (62). In the nematode model, the activity attributed to DMQ_9, particularly for complexes II and III, could very well have been influenced by the presence of either dietary Q_8 or

RQ_9. It is noteworthy that in the mouse *clk-1*−/− mutant cells, levels of complexes II and III were drastically reduced, whereas levels of complex I and III activity were only mildly decreased. This is consistent with previous assays of membranes isolated from *E. coli ubiF* mutants; such mutants produce only DMQ_8, and partial function of complex I was observed, but DMQ_8 was inactive in complex II activity (64). Given this, it is surprising that DMQ_6-producing yeast failed to support activity of NADH-cytochrome *c* oxidoreductase activity. Because NADH-DCIP reductase is only partially decreased in DMQ_6-producing strains, the lack of activity detected for NADH-cytochrome *c* oxidoreductase assays is likely to result from the inability of DMQ_6 to stabilize bc_1 complex, as judged by the low steady-state levels of cytochromes *c* and c_1. Addition of exogenous Q_6 to the growth media restores steady-state c_1 polypeptide levels in *coq7* null mutants (39) and, as shown here, in DMQ_6-producing yeast strains, but neither Q_3 nor DMQ_3 could restore any bc_1 complex activity (Table III) when added in the assay directly. The low levels of complex IV activities detected in DMQ_6-producing yeast strains also supports this idea, in that bc_1 complex and complex IV are coordinately regulated. For example, the near elimination of bc_1 complex activity is accompanied by a reduction in complex IV activity to about 15% of wild type (65). Taken together, these data indicate that DMQ_6-producing strains lack a functional and well-assembled bc_1 complex. However, it is not yet possible to discriminate whether DMQ_6 is a functional substrate for bc_1 complex.

It is clear that DMQ_6 cannot functionally replace Q_6 in the yeast system; in this aspect, our data are in agreement with previous studies in both the mouse and nematode models. Homozygous *mclk-1* mouse mutants display embryonic lethality (62, 63). *C. elegans clk-1* mutants fed a Q-less diet contain significant amounts of DMQ_9 yet are developmentally arrested if deprived of a Q-replete diet as hatchlings or are sterile if deprived of Q during post-dauer development (26, 66). Uptake of Q_8 from *E. coli* and transport of dietary Q_8 to the mitochondria are needed to prevent the arrest and sterility phenotypes (27). These data reveal an interesting dual nature of Q; it is required to sustain larval growth and germline development, yet dietary Q_8 has been shown to shorten life span in adult nematodes. The developmental and reproductive phenotypes of the *clk-1* mutants have recently been shown to be unlinked to the longevity phenotype (67). We speculate that the *clk-1* mutant nematodes fed Q_8-replete diets, or rescued by the maternal mitochondrial contribution of Q_9 and the CLK-1 polypeptide, may have parallels to the yeast strains characterized here that produce both Q_6 and DMQ_6. In this scenario, the small amount of Q_8 (or in the case of maternal rescue, Q_9) in the nematode, may act to stabilize respiratory electron transport complexes, support a low level of redox activity (or involve DMQ directly via transhydrogenation reactions (59)), and act to mask the pro-oxidant activity of DMQ_9. The increased lifespan of the *clk-1* nematode would then reflect the lowered respiratory chain activity, stemming either from the decreased level of aerobic respiration (because of less Q) and/or from the increase in anaerobic respiration (hypothetically caused by increased RQ_9). This model is consistent with increased reliance on anaerobic metabolism observed in the long lived dauer larvae of *C. elegans* (68) , the life span extensions observed in the nematode model after RNA interference-mediated down-regulation of mitochondrial respiratory chain components (69, 70), and the RNA interference silencing of other *COQ* genes (71).

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