Demethoxy-Q, an intermediate of coenzyme Q biosynthesis, fails to support respiration in *Saccharomyces cerevisiae* and lacks antioxidant activity

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Running Title: Demethoxy-Q fails to support respiration in *coq7* mutants
SUMMARY

*Caenorhabditis elegans* *clk-1* mutants cannot produce coenzyme Q<sub>9</sub> and instead accumulate demethoxy-Q<sub>9</sub> (DMQ<sub>9</sub>). DMQ<sub>9</sub> 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<sub>6</sub> in the yeast *Saccharomyces cerevisiae*. Three mutations designed to mirror the *clk-1* mutations of *C. elegans* were introduced into COQ<sub>7</sub>, the yeast homologue of *clk-1*:

- E233K, predicted to disrupt the diiron 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<sub>6</sub> and producing solely DMQ<sub>6</sub> were respiratory deficient and unable to support either NADH-cytochrome *c* reductase or succinate-cytochrome *c* reductase activities. DMQ<sub>6</sub> failed to protect cells against oxidative stress generated by H<sub>2</sub>O<sub>2</sub> 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.*
INTRODUCTION

Coenzyme Q (ubiquinone or Q) 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 Q10, Q9 or Q6, respectively. Q is reversibly reduced (QH$_2$) and oxidized (Q) as it transports electrons from complex I, or complex II, to the cytochrome *bc$_1$* complex in the inner mitochondrial membrane. QH$_2$ is synthesized in the mitochondria, where it acts not only in respiration, but 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 transplasma membrane electron transport system where 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 so are 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). Interestingly, while yeast *coq7* null mutants accumulate
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HHB, yeast bearing the coq7-1 allele (G104D) contain a very small amount of demethoxy-Q₆ (2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone or DMQ₆) (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. E. 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 DMQ₆ (18). Expression of the P. 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 diiron 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).

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 post-embryonic development, slowing of adult behaviors, resistance to UV-induced stress, and a long life-span (21-23). The clk-1 animals fail to synthesize Q₈, but instead produce significant amounts of the Q biosynthetic intermediate demethoxy-Q₉ (DMQ₉) (24), rhodoquinone-9 (RQ₉), and they rely on Q₈, 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 DMQ₉, because it is considered to have fewer prooxidant properties than Q, and is thought to function less efficiently in respiratory chain electron transport (29). However, Q₈ and RQ₉ 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.

Here we use 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 diiron 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 solely DMQ$_6$ are respiratory 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$_2$O$_2$ 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 (MATα, his3Δ1, 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 (34) and included, YPG (1% yeast extract, 2% peptone, 3% glycerol), SDC (0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH2PO4, 0.5% (NH4)2SO4, and complete amino acid supplement) and SD-Ura (SDC, but amino acid supplement lacks uracil). The supplement was modified as described (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, which corresponded to position 697 in the yeast open reading frame. The forward inside primer pQ7XBAR (5’ GGATGACAGCTAGAGCATCTAGACACCGC 3’) was used with the vector primer pQ7HIN (5’ GACGGTATCGATAAGCTTTCTTTTAATTAC 3’), while the inside reverse primer pQ7XBA (5’ GCGGTGTCTAGATGCTCTAGCTTGTATCC 3’) was used with a primer corresponding to positions 218-249 of the open reading frame, pQ7SPH (5’ AACCTGAGCATGCTCCCAAGTGTCAGAATT 3’). This results in an E to K point mutation at aa 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 aa 237. The
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forward inside primer pQ7MUT1 (5' GACGAGCTAGAAGCATTAAGACACCGCTATC 3') was used with pQ7HIN, while the reverse inside primer pQ7MUT2 (5' GATAGCGGTGTTAATGCTCTAGCTGTC 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' GGGAGAAAGTTTTGATTTGCTGTAAGCTGTTACC 3') was used with pQ7HIN, while the reverse inside primer pQ7MUT4-2 (5' TTAACGCAAATCCACAAACTTCTTCCCCCATTTGCAA 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 *Sph*I and *Hin*DIII and ligated into pNMQ71 which had been similarly treated. Each of the mutations were also transferred to multicopy vectors by excising the *Cla*I and *Hin*DIII 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 fragments that overlap which 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 pQ7d30-1F (5'
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AGCTCGGAAATTACCCTCACTAAGGGA 3') and the reverse primer pQ7d30-1R (5'
TCTGGTGATTAGATGCTTAGCTCGTCA 3'). The second fragment was obtained with
the forward primer pQ7d30-2F (5' GAGCATCTATAACCACCAGAAATGGCATA 3') and
the reverse primer pQ7d30-2R (5' ACGTTGTAAAACGACGGCCAGTGA 3'). pQ7d30-1F
and pQ7d30-2R were used as primers for the second PCR reaction.

Each of the constructs described above (Table I) were sequenced and were 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 (OD600 = 0.1). Growth was monitored by OD600 measurements.

*Mitochondrial isolation and Western blots*—Crude mitochondria were isolated as
described (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 c1 (1:10,000), and F1βATPase (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 (Biorad, Hercules, CA).
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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 (38). Lipid extractions of whole cells and quinone quantification were performed as described (40). Quinones were quantified in isolated mitochondria following SDS solubilization and lipid extraction as described previously (39). In both cases Q9 was used as an internal standard; for whole cell extractions 1 µg Q9 was added per g wet weight cell pellet, and 500 ng Q9 was added per mg 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, –500mV; E2, 500mV). Hydroquinones present in samples were oxidized with a pre-column cell set in oxidizing mode (E +500mV). Q6 and Q9 were quantified with external standards. The Q6 external standard was used to quantify DMQ6, assuming a similar redox response in the ECD.

For mass spectrometry analysis, 1 L SD-Ura-His cultures were inoculated and harvested after two days of growth. OD600 ranged from 8.3-8.8 OD600/ml. Mitochondria were isolated and lipid extracts were resolved over the HPLC/ECD system. The peaks corresponding to Q6 and DMQ6 were collected and dried down under nitrogen gas. The samples were analyzed with a Perkin-Elmer Sciex API III triple quadropole mass spectrometer fitted with a heated nebulizer at 450°C. The solvent was 0.33% H2O in acetonitrile at a flow rate of 0.1 ml/min. Instrument conditions for multiple reaction monitoring (MRM) analysis were 0.3 step size, dwell = 900
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msec, scan speed = 4.0 sec, and oriface at 50V, cgt=220. These samples were compared to Q$_6$
obtained from Sigma and DMQ$_8$ obtained from the \textit{E. coli ubiF} mutant strain AN78 (41). Five transition events were monitored by MRM: $m/z$ 591.4 $\rightarrow$ $m/z$ 197.2 and 237.2 (Q$_6$); $m/z$ 561.4 $\rightarrow$ $m/z$ 167.2 and 207.1 (DMQ$_6$); and $m/z$ 697.4 $\rightarrow$ $m/z$ 167.2 (DMQ$_8$).

\textit{Mitochondrial respiratory chain activities}—Freshly obtained mitochondria were assayed for NADH-cytochrome \textit{c} reductase and succinate-cytochrome \textit{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\(^\circ\)C with stirring. Mitochondrial protein (10-50 \(\mu\)g) 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 \(\mu\)M DCIP, without NADH, and after 1 min 200 \(\mu\)M 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\(^{-1}\) 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 \(\mu\)M decylubiquinone, 250 \(\mu\)M potassium cyanide and 2 \(\mu\)g/ml antimycin A. Samples of mitochondria (20-50 \(\mu\)g protein) were incubated in the assay buffer for 3 min. The reaction was initiated with the addition of decylubiquinone and 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.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 protein) were incubated in the assay buffer for 3 min. The reaction was initiated by addition of cytochrome c and 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.cm⁻¹. All assays were performed in triplicate. In some experiments the activity was measured with the addition of exogenous 10 µM Q₃ and 10 µM DMQ₃ following 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 U/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 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 similar way described for NADH-cytochrome c reductase. Bovine superoxide dismutase (300 U/ml) was added in control experiments. All assays were performed in triplicate.
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*Analysis of oxidative stress sensitivity by colony counting*—Yeast cells were cultured in SDC or SD-Ura media, harvested at exponential growth phase (OD$_{600nm} = 1$) or early stationary growth phase (OD$_{600nm} = 4$-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$_2$O$_2$ and linolenic acid for 4 hours. Samples from each treatment were diluted and spread onto YPD plates. After 2 days at 30ºC, 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 with H$_2$O$_2$ (5 and 10 mM) or linolenic acid (750 µM) for 4 h were harvested, washed, and resuspended at 2×10$^6$ cells/ml in PBS. 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 with fluorescent properties similar to ethidium, and which 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 approximately 200 events per second.
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 previously described (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 coq7Δ 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 diiron site) and pP175Stop (lacking the carboxyl-terminal half of Coq7p) failed to grow (Fig. 2A). These two transformants were also incapable of growth on media containing ethanol as the sole non fermentable carbon source (data not shown). Therefore, the lack of growth on glycerol is not a specific consequence of a lack of Q6 as an electron acceptor for glycerol-phosphate dehydrogenase (GUT2) (49), but is due to a general lack of mitochondrial electron transport mediated by Q6.

We further investigated the yeast strain CEN.MP3-1A:pL237Stop that displayed growth following a lag period. When samples of this strain were re-inoculated 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 as this plasmid conferred almost wild-type levels of growth (Fig. 2B). 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 ptdL237Stop 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 DMQ₆ and Q₆ 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 electrochemical detection (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 Q₆, (1950 ng Q₆/mg protein), although a significant amount of DMQ₆ (432 ng/mg protein) was also present. The coq7 null mutant strain harboring empty vector (CEN.MP3-1A:pRS316), or harboring pP175Stop failed to synthesize either Q₆ or DMQ₆. Q₆ was not detectable in CEN.MP3-1A:pE233K; however this strain accumulated DMQ₆, 861 ng/mg protein. CEN.MP3-1A yeast bearing pL237Stop produced exclusively DMQ₆, 406 ng/mg protein when grown in media containing glucose. The content of Q₆ 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 following culture in YPG accumulate significant amounts of Q₆, ranging from 310 to 423 ng Q₆ per mg protein, although DMQ₆ was...
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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 (Q₆) and 167.2 for (DMQ₆), verifying their identities.

*Analysis of Respiratory Chain Activities in Yeast Mutants Producing Solely DMQ₆*—To analyze the role of DMQ₆ 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 Q₆ or DMQ₆, 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 Q₆ present in isolated mitochondria as the electron carrier.

The CEN.MP3-1A strain harboring the plasmids pE233K or ptdL237Stop produces DMQ₆ 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 56%. Other activities resembled those of the CEN.MP3-1A:pRS316 strain.
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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 DMQ₆-producing yeast strains. Since supplementation of the growth media with exogenous Q₆ 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 Q₃ or DMQ₃ 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 SOD, suggesting that when either Q₃ or DMQ₃ are added to the assay, most of the cytochrome c reduction is mediated by superoxide, and not via the bc₁ complex.

Supplementation of growth media with 2 µM exogenous Q₆ 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 Q₆, 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 DMQ₆.

**The effect of DMQ₆ on Steady State Levels of Mitochondrial Polypeptides**— Our previous studies with coq7 null mutant yeast showed that Q₆ is required for stability of the cytochrome c₁ polypeptide (39). To determine whether the exclusive presence of DMQ₆ affected steady state levels as compared with Q₆-producing strains, western blot analysis of mitochondrial polypeptides including porin, F₁β-ATPase, cytochrome c₁, and cytochrome c were performed (Fig. 3A). Densitometric analysis showed that steady state levels of cytochrome c, a substrate of complexes III and IV, and cytochrome c₁, a subunit of the bc₁ complex, were dramatically
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decreased when compared to porin and the F₁β subunit of complex V (Fig. 3B). Levels of cytochromes *c* and *c₁* were fully restored when the culture media was supplemented with exogenous Q₆. These results indicate that DMQ₆ 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 Q₆ functions as an important cellular antioxidant, required to protect against the toxicity induced by polyunsaturated lipid autoxidation. In order to test the efficacy of DMQ₆ as an antioxidant, yeast strains producing Q₆, DMQ₆ or no quinones were subjected to oxidative stress caused by treatment with either H₂O₂ or linolenic acid (Fig. 4A-D). Cell viability was analyzed during both log and stationary phase. All strains showed a decrease in cell viability with increasing concentrations of H₂O₂ (Fig. 4A 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. 4C and D). Wild-type yeast were not affected by this treatment, while all *coq7* mutants were sensitive to linolenic acid at both log and stationary phase, although the cells at log phase showed greatly increased sensitivity. Interestingly, CEN.MP3-1A:pE233K, that produces solely DMQ₆, was more sensitive to linolenic acid treatment at stationary phase than was the *coq7* null mutant CEN.MP3-1A, which fails to produce Q₆ or DMQ₆. These data indicate that DMQ₆ fails to act as an effective antioxidant. Curiously, the CEN.MP3-1A:pL237Stop mutant was significantly more
Demethoxy-Q fails to support respiration in coq7 mutants resistant than the other mutants tested, with the effect being most pronounced for cells treated with linolenic acid (Fig. 4C and D).

Given that the CEN.MP3-1A:pL237Stop mutant strain adapts to growth under selective conditions (YPG) and synthesizes Q6 (as a result of the stop codon read through) we investigated whether linolenic acid treatment also selects for Q6 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 hours. Analysis of lipid extracts prepared from these cells revealed that CEN.MP3-1A harboring pE233K or ptdL237Stop produced only DMQ6 (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.

Superoxide Anion Generation in Yeast coq7 Mutants Producing DMQ6— In order 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 protein.min). Superoxide production was significantly lower for the coq7 null
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Mutant CEN MP3-1A::pRS316 (5.4 ± 0.5 nmol/mg protein.min) and for the DMQ₆-producing strain CEN MP3-1A::pL237Stop (5 ± 0.2 nmol/mg 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 monitoring the fluorogenic oxidation of HE as described in *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 Mn-SOD. This strain accumulated high levels of superoxide when compared with the isogenic wild type strain EG103 (Fig. 6A). In addition, yeast were also incubated with antimycin A, a compound that produces superoxide by blocking the high potential cytochrome *b* in *bc₁* complex (57). Wild-type yeast showed low levels of superoxide, which increased upon incubation with antimycin A (Fig. 6A). 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 antimycin A did not further increase superoxide levels in these strains. Antimycin A requires functional mitochondria to produce superoxide, providing additional evidence that DMQ₆ fails to support mitochondrial respiratory activity.

Once the HE assay of superoxide was validated, yeast grown to stationary phase were subjected to oxidative stress by treatment with H₂O₂ (5 or 10 mM) or with linolenic acid (750 µM) (Fig. 6B), and HE oxidation was measured. Treatment with H₂O₂ 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 DMQ₆-producing strains, but not in wild type or null
Demethoxy-Q fails to support respiration in coq7 mutants. The amount of HE oxidation detected in these strains was double the amount detected in the sod2Δ strain. However, HE oxidation dropped to wild-type levels when the CEN.MP3-1A:pL237Stop mutant was cultured in YPG. Thus, under conditions where Q₆ is produced, superoxide is produced at minimal levels, even if DMQ₆ is the predominant quinone present. However, following linolenic acid treatment, and in the absence of Q₆, DMQ₆ appears to have significant prooxidant 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, since this intermediate is considered to have less prooxidant activity than Q, and also is 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 Q8 from the standard E. coli diet, and by endogenously produced rhodoquinone (RQ8), which accumulates in the clk-1 mutants and is required for anaerobic respiratory metabolism (26). Both Q8 and RQ8 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 in order to generate analogs of C. elegans clk-1 mutant alleles: E233K, with a disrupted diiron 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 diiron site is likely to inactivate enzyme activity, as mutation of the corresponding glutamate ligand to alanine (E273A) in the alternative oxidase from Arabidopsis thaliana resulted in loss of the diiron center (58). By expressing these coq7 mutations in a coq7 null mutant, yeast strains were generated that lacked Q6 but produced significant quantities of DMQ6.
Yeast mutants that produced only DMQ₆ failed to grow on media containing glycerol, a nonfermentable carbon source. Mitochondria isolated from these yeast strains lacked Q₆, contained DMQ₆, and failed to support NADH-cytochrome c reductase or succinate cytochrome c reductase activities. The DMQ₆-producing yeast mutant strains also showed decreased steady state levels of cytochrome c₁, a polypeptide component of bc₁ complex, and cytochrome c, a substrate of the bc₁ complex. These data indicate that DMQ₆ fails to support respiratory electron transport in yeast.

To investigate the potential antioxidant properties of DMQ₆, yeast were subjected to treatment with hydrogen peroxide and linolenic acid. When treated with H₂O₂, DMQ₆-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 DMQ₆-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 DMQ₆-producing yeast were treated with linolenic acid. In contrast, yeast strains producing both DMQ₆ and Q₆ showed enhanced resistance to linolenic acid treatment, and decreased levels of superoxide. These results show that the prooxidant attributes of DMQ₆ are negated when Q₆ is also present, presumably because the Q₆/Q₆H₂ redox couple is capable of being regenerated by the electron transport chain. It is also possible that Q₆ may act to reduce and oxidize DMQ₆ via respiratory complex transhydrogenation reactions (59). These data indicate that DMQ₆ on its own fails to act as an effective antioxidant, and, under
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certain stress conditions acts as a prooxidant and is associated with increased levels of superoxide production.

The apparent prooxidant action of DMQ₆ 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 DMQ₆-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 DMQ₆-producing mutants. In yeast, the bc¹ complex is considered 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 DMQ₆. This suggests that the bc¹ complexes in these two strains are similarly defective; an idea supported by the lack of NADH-cytochrome *c* oxidoreductase and succinate cytochrome *c* oxidoreductase activities in both the *coq7* null and DMQ₆-producing mutants (Table II), and by the inability to recover NADH-cytochrome *c* reductase activity in response to addition of either Q₃ or DMQ₃ to isolated mitochondria (Table III). However, it is likely that when these strains are treated with linolenic acid, the DMQ₆-producing strain will uniquely have accumulated DMQ₆H₂, due to significant Segment I and Complex II activities (30% and 46% of wild-type) (Table II). Lipid autoxidation could trigger the oxidation of DMQ₆H₂ 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 multi-subunit Q biosynthetic complex, and mutations in Coq7p are considered to produce the partial or complete disruption of complex
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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 DMQ₆, 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 DMQ₈, 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 DMQ₉, even though 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 DMQ₆ cannot function in yeast mitochondrial electron transport counters previous studies that claimed DMQ₉ 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 DMQ₉, particularly for complex II + III, could very well have been influenced by the presence of either dietary Q₈ or RQ₉. It is noteworthy that in the mouse *clk-1* -/- mutant cells, levels of complex II + III were drastically reduced, while levels of I + III
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activity were only mildly decreased. This is consistent with previous assays of membranes isolated from *E. coli ubiF* mutants; such mutants produce solely DMQ₈ and partial function of complex I was observed, but DMQ₈ was inactive in complex II activity (64). Given this, it is surprising that DMQ₆-producing yeast failed to support activity of NADH-cytochrome *c* oxidoreductase activity. Because NADH-DCIP reductase is only partially decreased in DMQ₆-producing strains, the lack of activity detected for NADH-cytochrome *c* oxidoreductase assays is likely to result from the inability of DMQ₆ to stabilize *bc₁* complex, as judged by the low steady state levels of cytochromes *c* and *c₁*. Addition of exogenous Q₆ to the growth media restores steady state *c₁* polypeptide levels in *coq7* null mutants (39), and as shown here in DMQ₆-producing yeast strains but neither Q₃ nor DMQ₃ could restore any *bc₁* complex activity (Table III) when added in the assay directly. The low levels of complex IV activities detected in DMQ₆-producing yeast strains also supports this idea, as *bc₁* complex and complex IV are coordinately regulated. For example, the near elimination of *bc₁* complex activity is accompanied by a reduction in complex IV activity to about 15% of wild-type (65). Taken together, these data indicate that DMQ₆-producing strains lack a functional and well assembled *bc₁* complex. However it is not yet possible to discriminate whether DMQ₆ is a functional substrate for *bc₁* complex.

Clearly, DMQ₆ cannot functionally replace Q₆ in the yeast system; in this aspect our data are in agreement with previous studies in both the mouse and nematode models. Homozygous *melk-1* mouse mutants display embryonic lethality (62,63). *C. elegans clk-1* mutants fed a Q-less diet contain significant amounts of DMQ₉, yet are developmentally arrested if deprived of a Q-replete diet as hatchlings or are sterile if deprived of Q during post-dauer development.
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(26,66). Uptake of Q\textsubscript{8} from \textit{E. coli} and transport of dietary Q\textsubscript{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\textsubscript{8} has been shown to shorten life span in adult nematodes. Recently, the developmental and reproductive phenotypes of the \textit{clk-1} mutants have been shown to be unlinked to the longevity phenotype (67). We speculate that the \textit{clk-1} mutant nematodes fed Q\textsubscript{8}-replete diets, or rescued by the maternal mitochondrial contribution of Q\textsubscript{9} and the CLK-1 polypeptide, may have parallels to the yeast strains characterized here that produce both Q\textsubscript{8} and DMQ\textsubscript{9}. In this scenario, the small amount of Q\textsubscript{8} (or in the case of maternal rescue, Q\textsubscript{8}) 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\textsubscript{9}. The increased lifespan of the \textit{clk-1} nematode would then reflect the lowered respiratory chain activity, stemming either from the decreased level of aerobic respiration (due to less Q) and/or from the increase in anaerobic respiration (hypothetically due to increased RQ\textsubscript{9}). This model is consistent with increased reliance on anaerobic metabolism observed in the long lived dauer larvae of \textit{C. elegans}, (68) the life span extensions observed in the nematode model following RNAi-mediated down regulation of mitochondrial respiratory chain components (69,70), and the RNAi silencing of other \textit{COQ} genes (71).

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FOOTNOTES

1The abbreviations used are: DMQ, demethoxy-Q; ECD, electrochemical detection; HE hydroethidine (dihydroethidium); HPLC, high performance liquid chromatography; Q, ubi¬quinone (coenzyme Q); QH2, ubiquinol (Q hydroquinone); Qn, the coenzyme Q isoform where n designates the number of isoprene units; RQ, rhodoquinone.
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**FIGURE LEGENDS**

FIG 1. **Coenzyme Q₆ biosynthesis in yeast.** The first compound in this scheme is 3-hexaprenyl-4-hydroxybenzoic acid (HHB), which accumulates in each if 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 (DMQ₆). Coq7p, a monooxygenase, converts DMQ₆ to 5-demethylubiquinone, which is methylated by the Coq3 O-methyltransferase to produce Q₆.

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 OD₆₀₀ = 0.1. Growth was monitored by OD₆₀₀ measurements. Yeast harbored plasmids bearing either wild type *COQ7* (O), empty vector (V), 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.

FIG. 3. **DMQ₆-producing strains display lowered steady-state expression of mitochondrial respiratory polypeptides.** (A) Mitochondrial fractions (30 µg protein per lane) were separated by SDS-PAGE and analyzed by Western blot with using standard conditions. Antibodies developed against yeast proteins were used: F₁β-ATPase (1:5000) for IMM, cytochrome c₁ (1:10,000) for IMM, porin (1:10,000) for OMM, and cytochrome c (1:2500) for IMS. Lane 1:
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CEN MP3-1A:pNMQ71 (\textit{COQ7}), 2: CEN.MP3-1A (\textit{coq7Δ}), 3: CEN.MP3-1A + 2 µM Q₆, 4: CEN.MP3-1A:pL237Stop, 5: CEN.MP3-1A:pL237Stop + 2 µM Q₆ and 6: CEN.MP3-1A:pE233K. (B) Bars designate the steady state level of polypeptides as quantified by densitometry; \textit{black}, F₁β-ATPase; \textit{hatched}, cyt c₁; \textit{grey}, porin; \textit{white}, cyt 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 seconds). All plates were recorded using GPL mode (Good Laboratory Practice) of the Quantity One (Biorad) software that does not allow image modifications that changes the raw image data. The best relationship between time and intensity was selected.

FIG. 4. \textbf{Q-producing strains are less sensitive to oxidative stress produced by linolenic acid and H₂O₂.} Cells from wild type yeast (CEN.PK2-1C, ○) and the null mutant (CEN.MP3-1A) harboring empty vector (□), pE233K (◊), or pL237Stop (Δ), were cultured, harvested, and subjected to oxidative stress by H₂O₂ or linolenic acid treatment as described in \textit{Experimental Procedures}. (A) H₂O₂ in log phase, (B) H₂O₂ in stationary phase, (C) linolenic acid in log phase, and (D) linolenic acid in stationary phase.

FIG. 5. \textbf{Linolenic acid treatment stimulates Q₆ production in mutants bearing pL237Stop.} The CEN.MP3-1A:pL237Stop mutant strain was treated with H₂O₂ or linolenic acid, 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 correspond to 100 ng of commercial Q₆ injection. Two controls are defined, 0 h control that indicates cells at start of incubation and 4
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h control indicating the cells after 4 hours incubation without linolenic acid that measured possible quinone changes during the incubation.

FIG. 6. \textbf{DMQo-producing strains have increased levels of superoxide anion generation.}

Cells from the indicated strains were cultured and treated as described in \textit{Experimental Procedures} in order to quantify the amount of superoxide anion produced after oxidative stress. (\textit{A}) Superoxide generation in untreated cells (\textit{black bars}) or treated with antimycin A (2 \(\mu\)g/ml) (\textit{white bars}). (\textit{B}) Superoxide generation in different strains subjected to oxidative stress produced by the following treatments: \textit{White}, Control; \textit{hatched}, 5 mM H\textsubscript{2}O\textsubscript{2}; \textit{grey}, 10 mM H\textsubscript{2}O\textsubscript{2}; and \textit{black}, 750 \(\mu\)M Linolenic. Data showed correspond to the average of four measures from the same sample. The data are representative of three experiments.
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Table I

**Plasmid names and identifications**

| Low copy     | Multicopy  | Description                                      |
|--------------|------------|--------------------------------------------------|
| pNMQ71, pCHQ71 | p7.8       | wild type Coq7p                                  |
| pRS316       | pRS426     | empty vector                                     |
| pE233K       | pmE233K    | E_{233}K of wild type Coq7p                      |
| pL237Stop    | pmL237Stop | Stop at aa 237 of wild type Coq7p                 |
| ptdL237Stop  | pmtd237Stop| True deletion after aa 237 of pL237Stop           |
| pP175Stop    | pmP175Stop | Stop at aa 175 with 7 aa insertion                |
Table II. Mitochondrial respiratory complexes activities in Q₆ and DMQ₆ producing strains.¹

| Plasmid harbored in CEN.MP3-1A | Q₆     | DMQ₆    | Activity of Respiratory complexes (nmol / mg protein • min)³ |
|---------------------------------|--------|---------|-----------------------------------------------------------|
|                                 | ng quinone per mg mitochondrial protein² | NADH-DCIP oxidoreductase | succinate-decylQ oxidoreductase | NADH-cytochrome c oxidoreductase | succinate-cytochrome c oxidoreductase | cytochrome c oxidase |
| 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 + 2μM Q₆                 | 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 + 2μM Q₆              | 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) |
| pmlL237Stop                     | 56 ± 1  | 679 ± 12 | 183 ± 21 (36) | 65 ± 7 (34) | 120 ± 10 (11) | 163 ± 15 (54) | 148 ± 7 (28) |
| pmlL237Stop 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) |

¹ All strains were cultured in SD selective media until stationary phase. Where indicated, strains harboring pL237Stop and pmlL237Stop were cultured in YPG. The notation of 2 μM Q₆ refers to the final concentration of Q₆ added to yeast culture media.

² Lipids were extracted from mitochondria and quantified as described in Experimental Procedures. Data shown are the average of three injections ± SD from two independent extractions. ND: not detected (detection limit is 0.2 ng Q₆ per injection); NM: not measured.

³ Results are expressed as the average of three assays ± SD from two separate experiments. In parentheses are percentages compared to the wild type construct. NM: not measured
Table III. Enhancement of NADH-cytochrome c reductase activity by Q₃ and DMQ₃

| Strains¹ | 10 mM Q₃ | 10 mM Q₃+ 300 U SOD | 10 mM DMQ₃ | 10 mM DMQ₃+ 300 U SOD |
|----------|----------|----------------------|------------|----------------------|
| CEN.MP3-1A:pNMQ71 | 1190 ± 35 | NM | NM | NM |
| CEN.MP3-1A:pL237Stop | 28.2 ± 5 | 71.1 ± 1.2 | 36.6 ± 1.3 | 75.3 ± 0.8 |
| CEN.MP3-1A:pRS316 | 9.4 ± 1.2 | 16.8 ± 1.2 | NM | 18.6 ± 2.6 |

¹ All strains were cultured in SD selective media until stationary phase.  
² Results are expressed as the average of three assays ± SD from two separate experiments.  NM: not measured.  
Samples not incubated with Q₃ or DMQ₃ were incubated with the same volume of ethanol.  Q₃ or DMQ₃ were incubated with the samples for 5 min at RT.  SOD was present during the incubation.
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A

![Image of protein bands](https://example.com/image1.png)

| Lane | Description |
|------|-------------|
| 1    | pNMQ7       |
| 2    | pRS316      |
| 3    | pRS316 + Q  |
| 4    | pL237Stop   |
| 5    | pL237Stop + Q |
| 6    | pE233K      |

B

![Graph of label intensity](https://example.com/graph.png)

Label intensity (%)

- pNMQ7
- pRS316
- pRS316 + Q
- pL237Stop
- pL237Stop + Q
- pE233K
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**Figure: Cellular Viability**

- **A** (Log phase): Hydrogen peroxide (mM) vs. cell viability (%)
- **B** (Stationary phase): Hydrogen peroxide (mM) vs. cell viability (%)
- **C**: Linolenic acid (µM) vs. cell viability (%)
- **D**: Linolenic acid (µM) vs. cell viability (%)

**Legend:**
- Black line: Control
- Red line: 50 µM Demethoxy-Q
- Blue line: 100 µM Demethoxy-Q
- Green line: 200 µM Demethoxy-Q
- Orange line: 500 µM Demethoxy-Q

**X-axis:**
- Hydrogen peroxide (mM) for A and B, Linolenic acid (µM) for C and D

**Y-axis:**
- Cell viability (%) for A, B, C, and D

**Note:**
- The diagrams illustrate the effect of hydrogen peroxide and linolenic acid on cell viability in both log and stationary phases, with and without Demethoxy-Q treatment.
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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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