Ubiquinone limits oxidative stress in *Escherichia coli*

Britta Søballe and Robert K. Poole

**INTRODUCTION**

Ubiquinone (Coenzyme Q or UQ) is a lipid-soluble component of membrane-bound electron-transport chains, where it is present in large molar excess over other respiratory components (Søballe & Poole, 1999). In animal cells, UQ is found not only in the inner mitochondrial membrane, but also in endoplasmic reticulum, Golgi, lysosomes, peroxisomes, and plasma membrane (Kalén et al., 1989). Recently, eukaryotic UQ has acquired renewed interest due to the increasing body of evidence suggesting that reduced UQ (i.e. ubiquinol or UQH₂) is able to function as a lipid-soluble anti-

Abbreviations: HRP, horseradish peroxidase; SOD, superoxide dismutase; UQ, ubiquinone; UQH₂, ubiquinol (fully reduced form).
oxidant (for a review, see Ernster & Dallner, 1995). UQH₂ scavenges lipid peroxyl radicals and thereby prevents a chain reaction causing oxidative damage to polyunsaturated fatty acids of biological membranes, a process known as lipid peroxidation (Forsmark-Andréee et al., 1995). The amount of UQH₂ and other antioxidants, such as vitamin E, present in low-density lipoprotein, is of vital importance for the prevention of atherosclerosis. The UQH₂ form is maintained by quinone reductases (e.g. hepatocyte DT-diaphorase and lipoamide dehydrogenase) and thus protects against cytotoxic and carcinogenic effects (Beyer et al., 1996; Olsson et al., 1999). Inhibition of quinone reductase activity results in an increase in free radical damage (Beyer et al., 1996). The antioxidant properties of UQH₂ have led to its clinical use in the treatment of various diseases, e.g. heart disease (Ernster & Dallner, 1995).

In the facultatively anaerobic bacterium Escherichia coli, UQ with an 8-unit isoprenoid side-chain is an essential component of the aerobic respiratory chain, whereas an alternative quinone, menaquinone or MQ, is more functional in anaerobic respiration (Gennis & Stewart, 1996). The most commonly shown scheme for the sequence of carriers in the aerobic respiratory chains consists of a single UQ pool located immediately upstream of the oxidases and downstream of the respiratory dehydrogenases (e.g. Gennis & Stewart, 1996). However, this scheme may be oversimplified, since we recently demonstrated that UQ also functions as electron carrier between cytochromes b and the terminal oxidases by using dual-wavelength spectrophotometry to monitor cytochrome reduction levels in a UQ-deficient strain (ubiCA) (Søballe & Poole, 1998). The ubiCA operon encodes the enzymes chorismate lyase and 4-hydroxybenzoate transferase for the first two committed steps of UQ biosynthesis (Søballe & Poole, 1999).

There remains controversy as to whether respiratory-chain quinones and quinone-like compounds such as menadione or anthracycline antibiotics might actually stimulate superoxide production (see Afanas’ev et al., 1990). In E. coli, it was recently demonstrated that NADH dehydrogenase II is a major source of superoxide and hydrogen peroxide production by autoxidation of its reduced FAD cofactor (Messner & Imlay, 1999). Membranes from a UQ- and MQ-deficient mutant produced more superoxide and peroxide than other oxidized quinones in bacterial physiology (e.g. Imlay, 1995) have used UQ-deficient mutants isolated after chemical mutagenesis, which are recognized to be unstable or leaky. Therefore, we have used a stable knockout mutant having a deletion and insertion at the junction of the ubiC and ubiA genes (Søballe & Poole, 1998) and tested the hypothesis that UQ in E. coli acts as an antioxidant in the cell’s defence against oxygen-derived radicals and oxidative stress in the cytoplasmic membrane.

**METHODS**

**Bacterial strains and growth conditions.** The E. coli strains used in this work are described in Table 1. P1 transductions were performed using a modification of the protocol of Miller (see Poole et al., 1996). All cultures were grown at 37 °C with vigorous shaking (200 r.p.m.) in conical flasks containing one-fifth their volume of medium. Culture optical density was measured with a Pye-Unicam SP6–550 spectrophotometer at 600 nm. However, cultures for β-galactosidase assays were grown in 250 ml flasks with matched glass tubes of Klett dimensions as a side arm and the culture densities were measured with a Klett–Summerson photoelectric colorimeter (Manostat Corp.) fitted with a red filter.

Growth media were prepared as described by Poole et al. (1996). For membrane preparations, strains MG1655 (wild-type) and RKP4152 (ubiCA) were grown aerobically in LB with 0.5% (w/v) xylose and harvested at OD₅₅₀ 0.55. For β-galactosidase assays, the strains were also grown to exponential phase (OD₅₅₀ 0.5) but in LB with 40 mM glucose. For β-galactosidase assays, strains were grown in 10 ml LB with 40 mM xylose added. Strains were treated with parquat (50 μM) or H₂O₂ (50 μM) at 50 Klett units and harvested in the exponential phase of growth at 100 Klett units. Viable counts were performed after treating mid-exponential cultures of wild-type and ubi cells grown in MOPS/glucose (40 mM) medium with 0.03% (w/v) H₂O₂ or phleomycin (10 μg ml⁻¹). Heat resistance was analysed by aerobic shock treatment at 52 °C. A portion (0.5 ml) of a mid-exponential

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>MG1655</td>
<td>F⁻, referred to as wild-type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>RKP4152</td>
<td>ubiCA::Km</td>
<td>Søballe &amp; Poole (1998)</td>
</tr>
<tr>
<td>QC772</td>
<td>Φ(sodA–lacZ)</td>
<td>Touati (1988)</td>
</tr>
<tr>
<td>ΔlacU rpsL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RKP4228</td>
<td>As QC772 but ubiCA::Km</td>
<td>This work</td>
</tr>
<tr>
<td>BGF931 Tet' Φ(katG–lacZ)</td>
<td></td>
<td>Bruce Dempl, Harvard School of Public Health, Boston, MA, USA</td>
</tr>
<tr>
<td>RKP4241</td>
<td>As BGF931 but ubiCA::Km</td>
<td>This work</td>
</tr>
<tr>
<td>HW271 F ubiG zei::Tn10</td>
<td></td>
<td>Wu et al. (1992)</td>
</tr>
<tr>
<td>HW272 F zei::Tn10</td>
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<td>Wu et al. (1992)</td>
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</table>
culture was transferred to 4.5 ml MOPS/glucose medium, preincubated at 52 °C, and shaken at this temperature. When exposed to CuSO\textsubscript{4} (5 mM) or linolenic acid (1 mM) the strains were grown in LB medium with xylose (40 mM). Samples of all the treated cultures were taken at appropriate time intervals and a dilution series (10\textsuperscript{-1} to 10\textsuperscript{-6}) was performed in 0.9% NaCl. A portion (20 µl) of each dilution was spotted on LB agar plates and incubated at 37 °C overnight. DTT sensitivity studies were performed as described by Goldman et al. (1996a). Wild-type and ubi mutant cells were streaked on nutrient agar plates containing various concentrations of DTT (0.8–24 mM) and grown at 30 °C for 2 d. To obtain aerobic growth curves, three flasks were inoculated with 1% of an overnight culture of wild-type or ubiCA cells in LB with glucose (40 mM). To one of the flasks was added H\textsubscript{2}O\textsubscript{2} to a final concentration of 2.5 mM, whereas to another was added H\textsubscript{2}O\textsubscript{2} (2.5 mM) plus cysteine to a final concentration of 0.83 mM.

Preparation of membranes. This was performed as described by Soballe & Poole (1998). Protein concentrations were determined by the method of Markwell et al. (1978).

Superoxide anion detection. The rate of O\textsubscript{2}\textsuperscript{−} production during reactions in vitro was measured as the superoxide dismutase (SOD)-sensitive rate of cytochrome c reduction (Imlay & Fridovich, 1991) in a Beckman DU 650 spectrophotometer. The 1 ml reaction cuvette contained 30 mM potassium phosphate buffer, 20 µM cytochrome c, and wild-type or ubi membranes (200 µg protein ml\textsuperscript{-1}, final concentration). Reduction of cytochrome c was initiated by the addition of 100 µM NADH (final concentration) and monitored spectrophotometrically at 550 nm. Duplicate reactions were performed with the addition of 250 units SOD (Sigma). The extent of cytochrome c reduction was calculated using an absorption coefficient, ε\textsubscript{250/550}, of 21.0 mM\textsuperscript{-1} cm\textsuperscript{-1}.

H\textsubscript{2}O\textsubscript{2} production. The amount of H\textsubscript{2}O\textsubscript{2} produced by respiring membranes was measured using the scopoletin assay in a Hitachi F-2500 fluorescence spectrophotometer in time-scanning mode with excitation at 350 nm and emission at 460 nm (Loshen et al., 1971). The reaction mix (2.5 ml) in phosphate-buffered saline (PBS) buffer contained membranes (200 µg protein ml\textsuperscript{-1}, wild-type or ubiCA) and 0.76 µM horseradish peroxidase (HRP). The fluorescent substrate scopoletin was added to a final concentration of 0.2 µM and the reaction was initiated by addition of 25 mM glucose. The amount of H\textsubscript{2}O\textsubscript{2} generated was determined from a standard curve, in which the H\textsubscript{2}O\textsubscript{2} concentration (0–15 M) was directly proportional to the quenching of scopoletin fluorescence. When indicated, water-soluble ubiquinone (UQ-1 or UQ-2, purchased from Sigma) was added to a final concentration of 0.2–2.0 µM (from a 10 mM stock in 1:1 ethanol/water) before the reaction was initiated by the addition of 5 mM glyc erol.

Determination of intracellular H\textsubscript{2}O\textsubscript{2} production was based on the assumption that free diffusion of H\textsubscript{2}O\textsubscript{2} through the cell membrane allows an equilibrium to occur after about 15 min (Gonzalez-Flecha & Demple, 1994). Cells from 2.5 ml of a culture of the wild-type or ubiCA strain, grown in LB to the exponential phase (50 Klett units), were harvested and resuspended in 25 ml phosphate-buffered saline. Samples were taken at 5 min intervals and spun briefly in a microfuge before assaying the H\textsubscript{2}O\textsubscript{2} content of the supernatant using the above fluorometric assay.

Catalase assay. Cell pellets were washed in 100 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication (five periods of 1 min each). Cell debris and unbroken cells were removed by centrifugation in a microfuge for 30 min at 13000 r.p.m. The protein concentration of the supernatant extract was determined by the method of Markwell et al. (1978). The catalase activity was measured by monitoring AA at 240 nm in time-scanning mode in a Beckman DU 650 spectrophotometer (Gonzalez-Flecha & Demple, 1994). The 1 ml UV-cuvette contained 510 µg supernatant protein in potassium phosphate buffer and the reaction was initiated by adding H\textsubscript{2}O\textsubscript{2} to a final concentration of 5 mM. The initial rate of decomposition of H\textsubscript{2}O\textsubscript{2} was determined using an ε\textsubscript{240} of 43.6 x 10\textsuperscript{-3} M\textsuperscript{-1} cm\textsuperscript{-1}. One unit of catalase activity is defined as the change in H\textsubscript{2}O\textsubscript{2} concentration (mM) per min.

β-Galactosidase assays. Assays were carried out at room temperature as described before (Soballe & Poole, 1997). Each culture was assayed in triplicate; results were confirmed in at least two independent experiments.

RESULTS AND DISCUSSION

UQ limits accumulation of superoxide generated in aerobic respiration

A major site of O\textsubscript{2}\textsuperscript{−} generation in E. coli is the respiratory chain of the cell membrane (Gonzalez-Flecha & Demple, 1995; Imlay, 1995). To study the role of UQ in this process, O\textsubscript{2}\textsuperscript{−} was quantified by NADH-induced, SOD-sensitive cytochrome c reduction at 550 nm using membrane preparations of the wild-type (MG1655) and a UQ-deficient strain (ubiCA, RKP4152). Addition of SOD inhibited the initial rate of cytochrome c reduction by 44% in the wild-type and by 36% in the ubiCA mutant. SOD-insensitive reduction of cytochrome c presumably reflects direct interaction with respiratory chain components. Additional quantities of SOD did not further decrease the rate of cytochrome c reduction. The initial rate of O\textsubscript{2}\textsuperscript{−} production expressed per mg membrane protein in the ubiCA mutant was about half of that in wild-type cells (Fig. 1; Table 2). However, the respiration rate of NADH-treated membrane preparations is decreased in ubiCA mutants by about 80%
Table 2. Effects of UQ deficiency on superoxide and \( \text{H}_2\text{O}_2 \) production and metabolism

Production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) and catalase activities were measured as described in Methods. The \( \text{O}_2^- \) assay was repeated twice and a typical set of values is given, corresponding to Fig. 1. The values for \( \text{H}_2\text{O}_2 \) and catalase activity are means of three separate experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>SOD-sensitive ( \text{O}_2^- ) production</th>
<th>( \text{H}_2\text{O}_2 ) production</th>
<th>Catalase activity (( \text{H}_2\text{O}_2 ) converted, units mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate of ( \text{O}_2^- ) (nmol min(^{-1}) mg(^{-1}))</td>
<td>Total amount of ( \text{O}_2^- ) (nmol mg(^{-1}))</td>
<td>Initial rate of ( \text{H}_2\text{O}_2 ) production in membranes (nmol min(^{-1}) mg(^{-1}))</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.5</td>
<td>4.2</td>
<td>0.46</td>
</tr>
<tr>
<td>( \text{ubiCA} )</td>
<td>0.7</td>
<td>9.6</td>
<td>0.29</td>
</tr>
</tbody>
</table>

(Søballe & Poole, 1998) and therefore correction of Fig. 1 for the electron transfer rates to \( \text{O}_2^- \) would emphasize the antioxidant role of UQ. Fig. 1 also shows that the rapid production of \( \text{O}_2^- \) in the wild-type strain ceased after about 2–3 min, whereas in the \( \text{ubiCA} \) strain, \( \text{O}_2^- \) production continued until the cytochrome \( c \) present in the assay was fully reduced (Fig. 1). Thus, the total amount of \( \text{O}_2^- \) produced expressed per mg membrane protein was 2.3-fold higher in the \( \text{ubiCA} \) mutant compared to the wild-type strain (Table 2) under these assay conditions. This result clearly indicates the importance of UQ in maintaining low \( \text{O}_2^- \) levels.

Messner & Imlay (1999) used vesicles produced from a chemically produced \( \text{ubiA} \) mutant (AN385) and found a high rate of \( \text{O}_2^- \) production when expressed per electron transferred to \( \text{O}_2^- \). Titration of the substrate for UbiA (4-hydroxybenzoate) to the growing \( \text{ubiA} \) culture resulted in the production of UQ, increasing respiration rate and causing a decrease in the rate of \( \text{O}_2^- \) production.

UQ limits accumulation of \( \text{H}_2\text{O}_2 \) in vitro

\( \text{H}_2\text{O}_2 \) production in membranes from wild-type and \( \text{ubiCA} \) cells was measured using a HRP-dependent assay, in which the quenching of the fluorescent substrate scopoletin is directly proportional to the production of \( \text{H}_2\text{O}_2 \) (not shown). The initial rate of \( \text{H}_2\text{O}_2 \) production was 37% lower in the \( \text{ubiCA} \) mutant compared to the wild-type (Table 2). However, in the \( \text{ubiCA} \) membranes, all the available scopoletin was quenched within 6 min (Fig. 2b), whereas the substrate was only 50% quenched in the wild-type strain in the same time period (Fig. 2a); overall, there was a twofold increase in the accumulation of \( \text{H}_2\text{O}_2 \) in the \( \text{ubiCA} \) mutant under these assay conditions (Fig. 2, Table 2).

The effects on \( \text{H}_2\text{O}_2 \) production of adding a water-soluble ubiquinone homologue, UQ-1, to the \( \text{ubi} \) membranes are shown in Fig. 3(a). Over the assay period, the accumulation of \( \text{H}_2\text{O}_2 \) decreased substantially with increasing additions of UQ-1; accumulation of \( \text{H}_2\text{O}_2 \) decreased by 80% in the presence of 2 \( \mu \text{M} \) UQ-1. On addition of 0.4 \( \mu \text{M} \) UQ-1 [corresponding to 2 nmol (mg protein)\(^{-1}\)], \( \text{H}_2\text{O}_2 \) accumulation decreased by 50% to a level equivalent to \( \text{H}_2\text{O}_2 \) accumulation in wild-type membranes. This is in good agreement with the UQ concentration in membranes from a wild-type strain (AN387) grown aerobically, i.e. about 2.26 nmol (mg protein)\(^{-1}\) (Wallace & Young, 1977). This UQ concentration is anticipated to offer protection against oxidative stress.

In Fig. 3(b) it is shown that ubiquinone with a 2-unit isoprenoid side-chain, UQ-2, decreased \( \text{H}_2\text{O}_2 \) accumulation to an even greater extent than UQ-1; addition of
Ubiquinone limits oxidative stress

0.2 μM UQ-2 decreased the H$_2$O$_2$ accumulation in the $ubi$ membranes by 57%, whereas 0.2 μM UQ-1 decreased it by only 20%. This observation suggests that the length of the side-chain is important in the effectiveness of UQ as an antioxidant. Addition of UQ with longer side-chains, i.e. UQ-6 and UQ-10, was not possible due to their insolubility in water-based assays. In Sacch. cerevisiae, a broad spectrum of UQs (UQ-5 to UQ-10) has been shown to be biologically functional in a UQ-deficient strain, but the original UQ-6 species showed the highest activity (Okada et al., 1998).

**Effects of UQ deficiency on oxidative stress in vivo: peroxide levels, catalase activity and expression of katG and sodA**

The above results demonstrate that isolated membranes from a UQ-deficient strain accumulate substantially more superoxide and peroxide than do membranes containing a normal complement of UQ. To determine if this has physiological consequences for growth, or whether the additional flux of reactive oxygen species can be accommodated by cytoplasmic SOD and catalase activities, we determined the effects of the $ubiCA$ knockout in intact cells.

The steady-state intracellular H$_2$O$_2$ concentration, which was determined after allowing diffusion of H$_2$O$_2$ into PBS buffer, was increased 1.8-fold in the $ubiCA$ mutant (Table 2). These results mimic the O$_2^-$ and H$_2$O$_2$ measurements in membranes and support a protective role of UQ in oxygen radical scavenging.

We also assayed levels of total catalase activity in unfractionated cell extracts. Rates of H$_2$O$_2$ conversion were twofold higher in the $ubiCA$ cells compared to wild-type levels (Table 2).

*E. coli* possesses a bifunctional catalase-peroxidase (HPI, KatG) and a second monofunctional catalase, HPII. HP II, together with an alkyl hydroperoxide reductase, many other proteins with roles in resisting peroxide stress, and a small untranslated RNA are regulated by the OxyR protein in response to peroxide (Demple, 1991; Rosner & Storz, 1997). The intracellular concentration of H$_2$O$_2$ in *E. coli* is normally maintained around 0.1–0.2 μM during aerobic growth and catalase activity is regulated to compensate for changes in H$_2$O$_2$ production rates (Gonzalez-Flecha & Demple, 1995). Thus, *katG* transcription is a useful measure of intracellular peroxide levels (Gonzalez-Flecha & Demple, 1995). Table 3 shows that expression of $\Phi(katG$–sodA–lacZ) strains was performed with paraquat (50 μM).

**Table 3. Effect of UQ deficiency on expression of katG and sodA**

Mid-exponential cultures of $\Phi(katG$–lacZ) strains were induced with H$_2$O$_2$ (50 μM), whereas induction of $\Phi(sodA$–lacZ) strains was performed with paraquat (50 μM).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Inducer (+/-)</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGF931</td>
<td>$\Phi(katG$–lacZ)</td>
<td>-</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>327</td>
</tr>
<tr>
<td>RKP4241</td>
<td>$\Phi(katG$–lacZ) $ubiCA$</td>
<td>-</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>394</td>
</tr>
<tr>
<td>QC772</td>
<td>$\Phi(sodA$–lacZ)</td>
<td>-</td>
<td>1190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>5070</td>
</tr>
<tr>
<td>RKP4228</td>
<td>$\Phi(sodA$–lacZ) $ubiCA$</td>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>2610</td>
</tr>
</tbody>
</table>

![Figure 3. H$_2$O$_2$ production in $ubi$ membranes with added UQ homologues, UQ-1 or UQ-2. The scopoletin (S) assay (as described in Fig. 2) was initiated by addition of 5 mM glycerol (G). In (a), various concentrations of UQ-1 (0.2–2.0 μM) were included in the assay. In (b), UQ-1 or UQ-2 was added to a final concentration of 0.2 μM.](image-url)
membranes; Fig. 1) might be accommodated by spon-

taneous dismutation of superoxide to peroxide. Thus
catalase levels are raised but SOD levels are not.
Paraoquat increased \( \Phi(sodA-lacZ) \) expression 2-6-fold in
the \( \text{ubi} \) background (Table 3). The fact that paraquat
induction was not as marked in the \( \text{ubi} \) background
could be due to the growth defect exhibited by this
strain, especially in the presence of severe oxidative
stress mediators such as paraquat (not shown).
Alternatively, UQ function may be needed for paraquat redox-
cycling and \( \text{O}_2^- \) generation.

A \( \text{ubiCA} \) mutant is hypersensitive to mediators of
oxidative stress

In view of the elevated levels of superoxide and peroxide
measured in \( \text{ubiCA} \) cells, the ability of cells to tolerate
additional exogenous oxidative stress mediated by \( \text{H}_2\text{O}_2 \)
or \( \text{CuSO}_4 \) was examined (Fig. 4). The \( \text{ubiCA} \) mutant
appeared to be hypersensitive to treatment with 0.03 \%
\( \text{H}_2\text{O}_2 \) and its viability was reduced about 16-fold relative
to the wild-type strain after 1 h (Fig. 4a).

We considered the possibility that intermediates in the
UQ biosynthetic pathway downstream of the UbiCA-
catalysed steps might act as antioxidants. It has recently
been demonstrated that UbiG catalyses both O-methyl-
transferase steps in UQ biosynthesis, one of which is the
last step (Hsu et al., 1996; Poon et al., 1999). However,
strains harbouring leaky point-mutant alleles of \( \text{ubiG} \)
(e.g. AN86, AN151; Stroobant et al., 1972) were shown
to accumulate demethyl-UQ, the last intermediate in
UQ biosynthesis. Unlike RKP4152 (\( \text{ubiCA} \) knockout
strain), HW271 (\( \text{ubiG} \)) retains considerable respiratory
activity with several oxidizable substrates (Wu et al.,
1992), suggesting that this strain also harbours a leaky
allele. We found that strain HW271 showed increased
sensitivity to \( \text{H}_2\text{O}_2 \). After 1 h of \( \text{H}_2\text{O}_2 \) treatment,
the viability of the \( \text{ubiG} \) strain was reduced 28-fold com-
pared to the corresponding wild-type (not shown).

Copper ions (\( \text{Cu}^{2+}/\text{Cu}^{+} \)) participate in a redox-cycle,
resulting in the generation of superoxide anion. They
have also been reported to catalyse the conversion of
\( \text{H}_2\text{O}_2 \) to \( \text{OH} \) \( \text{in vitro} \) (Kimura & Nishioka, 1997).
When treated with \( \text{CuSO}_4 \), the viability of the \( \text{ubiCA} \)
mutant was reduced 10-fold relative to the wild-type
strain after 90 min (Fig. 4b). Likewise, the viability of the
\( \text{ubiG} \) mutant, HW271, was reduced 4-4-fold after
30 min compared to its isogenic wild-type (not shown).
These results indicate the importance of the presence
of UQ for protection against oxidative stresses generated
by \( \text{H}_2\text{O}_2 \) or \( \text{CuSO}_4 \). Sensitivity to oxidative stress in a
UQ-deficient mutant of the fission yeast \( \text{Schizosaccha}
romyces pombe \) has also been reported recently
(Suzuki et al., 1997).

Cysteine is an amino acid with antioxidant properties
due to the presence of the thiol group (Suzuki et al.,
1997). Sensitivity of the \( \text{ubiCA} \) mutant to \( \text{H}_2\text{O}_2 \) (2.5 mM)
could be abolished by the addition of this compound to
the growing cells (Fig. 5b). In contrast, the presence of
\( \text{H}_2\text{O}_2 \) or cysteine did not affect growth of the wild-type

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**Fig. 4.** Sensitivity of the \( \text{ubi} \) mutant to oxidative stress. Exponential cultures of wild-type (\( \square \)) and \( \text{ubi} \) (\( \bullet \)) cells were exposed to 0.03% \( \text{H}_2\text{O}_2 \) (a) and 5 mM \( \text{CuSO}_4 \) (b) and samples for viable counts were taken at appropriate time intervals. The results shown are typical of three similar determinations.

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\( \text{lacZ} \) increased 2-2-fold in a wild-type strain when
induced with \( \text{H}_2\text{O}_2 \) under our experimental conditions.
To seek confirmation that the presence of UQ affects
\( \text{H}_2\text{O}_2 \) levels \( \text{in vivo} \), the effect of introducing the \( \text{ubiCA} \)
allele into the \( \Phi(katG-lacZ) \) strain was studied. The
basal level of \( \text{katG} \) expression was 2-1-fold higher in the
\( \text{ubi} \) background, but induction with \( \text{H}_2\text{O}_2 \) still increased
the expression a further 1-3-fold (Table 3).

The manganese-containing superoxide dismutase
(MnSOD) enzyme is encoded by the \( \text{sodA} \) gene, which
can be regarded as a monitor of intracellular oxidative
stress as it is induced via \( \text{soxRS} \) regulators by \( \text{O}_2^- \), by
increased oxygen pressure and by redox-active com-
ounds (Touati, 1988; Demple, 1991; Compan &
Touati, 1993). Paraoquat is widely used as a redox-
cycling agent; it donates a single electron to oxygen to
give superoxide anion and is readily rereduced intra-
cellarily, enabling further rounds of superoxide pro-
duction. The expression of \( \Phi(sodA-lacZ) \) increased 4-2-
fold when induced with paraquat, as expected (Table 3).
Surprisingly, the introduction of the \( \text{ubiCA} \) mutation
did not increase the basal level of the aerobic expression
of \( \text{sodA} \) (Table 3). A possible explanation is that, \( \text{in vivo} \),
the slow rate of superoxide generation (seen in
membranes; Fig. 1) might be accommodated by spon-

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\( \text{ubiCA} \)
Ubiquinone limits oxidative stress

Fig. 5. Rescue of H₂O₂ sensitivity of the ubi mutant by cysteine. Growth of (a) the wild-type and (b) the ubiCA strain in LB-glucose (○), LB-glucose plus H₂O₂ (2.5 mM) (●), and LB-glucose plus both H₂O₂ (2.5 mM) and cysteine (0.83 mM) (▲). The results shown are typical of three similar determinations.

Pleiotropic phenotype of the ubiCA mutant

Linolenic acid is a polyunsaturated fatty acid prone to autoxidation and breakdown into toxic products (Do et al., 1996); UQ-deficient Sacch. cerevisiae (coq3) and Schiz. pombe have been reported to be hypersensitive to this compound (Do et al., 1996). The hypersensitivity could be abolished by addition of the COQ3 gene on a single-copy plasmid, butylated hydroxytoluene, α-tocopherol or trolox, a vitamin E analogue (Do et al., 1996). Surprisingly, when we analysed the sensitivity of E. coli wild-type and ubiCA strains to linolenic acid (1 mM) we found the ubiCA mutant to be highly resistant to this compound, whereas the viability of the wild-type was reduced 500-fold after 3 h (Fig. 6a).

A similar result was obtained when the cells were treated with phleomycin, which is an antibiotic and antitumour agent produced by Streptomyces verticillus. The drug induces DNA breaks and cell death in prokaryotes and eukaryotes (Collis & Grigg, 1989). The wild-type and ubiCA strains were treated with phleomycin at a final concentration of 10 μg ml⁻¹ (Fig. 6b). The ubiCA strain appeared resistant to this concentration, whereas the viability of the wild-type was decreased 10-fold after 1 h of treatment (Fig. 6b). It is likely that the resistance to both linolenic acid and phleomycin is due to reduced uptake of these compounds in the ubiCA strain.

The effects of heat shock at 52 °C on the viability of wild-type and ubiCA strains are shown in Fig. 6(c). The ubiCA mutant proved to be extremely resistant to the lethal effects of heat shock. After 48 min at 52 °C the surviving fraction of the ubiCA mutant was reduced

Fig. 6. Pleiotropic phenotype of the ubi mutant. Exponential cultures of wild-type (□) and ubi (●) strains were exposed to 1 mM linolenic acid (a), 10 μg phleomycin ml⁻¹ (b) and heat shock (c) at 52 °C. Samples for viable counts were taken at appropriate time intervals. The results shown are typical of three similar determinations.
4-5-fold, whereas that of the wild-type was reduced about 170-fold (Fig. 6c).

The **ubiCA** mutation causes sensitivity to DTT

Like *ubi* mutants, mutants (*cydAB* or *cydDC*) unable to synthesize the quinol oxidase, cytochrome *bd*, have a pleiotropic phenotype, including sensitivity to *H₂O₂* (Wall *et al*., 1992; Goldman *et al*., 1996b). In addition, certain reducing compounds can suppress the temperature-sensitive phenotype of both *cydAB* and *cydDC* mutants. DTT was found to inhibit Cyd*−* mutants at 3 and 10 mM, concentrations at which the isogenic wild-type strains were resistant (Goldman *et al*., 1996a, b). A firm explanation for these effects is not available, but it has been suggested (Goldman *et al*., 1996a) that the thiol periplasmic environment of a *cydC* mutant is oxidized in comparison to the wild-type strain. Using the same assay as Goldman *et al*. (1996a), we found that the *ubiCA* mutant was sensitive to 8 mM DTT, whereas the wild-type was resistant to DTT at this concentration (not shown). Thiol hypersensitivity has also been reported for *ubi* mutants isolated after chemical mutagenesis (Zeng *et al*., 1998). Thus the DTT sensitivity is not due to the ability to assemble cytochrome *bd per se*, but to respiratory chain function. We hypothesize that *ubi* mutants are sensitive to DTT because of the requirement for the respiratory chain to oxidize the essential redox-active CXXC motif of DsbB. This membrane protein in turn oxidizes the active-site cysteines in DsbA, the disulfide bond formation factor in the periplasm (Kobayashi & Iro, 1999). Indeed, *ubiA menA* mutants and *hemA* mutants do accumulate a reduced form of DsbA (Kobayashi *et al*., 1997) and, intriguingly, *dsbA/dsbB* mutants are sensitive to DTT (Missiakas & Raina, 1997). These findings and the work of Bader *et al*. (1999) clearly link the function of the respiratory chain with maintenance of an appropriate redox environment in the periplasm.

**Conclusions**

The importance of UQ as a component of *E. coli* respiratory chains terminated by oxygen and nitrate is well established. The present studies using a defined knockout allele of *ubiCA* clearly demonstrate additional roles for UQ in limiting the accumulation of superoxide and peroxide. This contradicts the view that quinones might constitute an important source of superoxide by virtue of the spontaneous autoxidation of the radical form (for references, see Afanas’ev *et al*., 1990). Skulachev (1997) has suggested that respiration that does not involve the Q-cycle – a mechanism that can produce long-lived semiquinone (QH²) – might serve as a defence against reactive oxygen species. Further protection from superoxide production in functioning respiratory chains might be afforded by high rates of electron transfer to the terminal oxidase, thereby avoiding ‘excessive electronegativity of respiratory carriers’ (Papa *et al*., 1997) and the potential for undesirable single-electron donation to oxygen. Both these conditions appear to be met in *E. coli*, in which a Q cycle need not be invoked (Poole & Ingledew, 1987) and in which operation of cytochrome *bd*, which is not a proton pump, allows very rapid rates of respiration with a phenomenally high apparent affinity for oxygen (*Kₘ* about 5 nM; D’mello *et al*., 1996). Both cyanide (Imlay, 1995) and quinone deficiency (Messner & Imlay, 1999) increase superoxide production in membrane vesicles, possibly by electron ‘leakage’ from an upstream component such as NADH dehydrogenase II.

The complex phenotype of *ubi* mutants is not surprising and arises from at least two important aspects of UQ function. First, UQ is able to limit accumulation of superoxide and peroxide due to its ability to rapidly abstract electrons from upstream dehydrogenases and transfer them to the oxidases. Second, reduced UQ is able to react with superoxide in vitro (Nakayama *et al*., 1997) and functions as an antioxidant in scavenging oxygen radicals such as perferryls or lipid peroxyl radicals (Ernster & Dallner, 1995). These studies do not really distinguish between these two mechanisms. Consequent damage in *ubi* mutants by lipid peroxidation may therefore explain the oxidative-stress-sensitive phenotype and poor growth, as well as the changes in membrane permeability and heat tolerance. Finally, other phenotypes such as sensitivity to DTT are more likely a consequence of impaired respiratory chain function.

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**REFERENCES**


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