

Ubiquinone limits oxidative stress in *Escherichia coli*

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Ubiquinone is an essential redox component of the aerobic respiratory chains of bacteria and mitochondria. It is well established that mammalian ubiquinone can function in its reduced form (ubiquinol) as a lipid-soluble antioxidant preventing lipid peroxidation. The objective of this study was to test the hypothesis that prokaryotic ubiquinone is involved in the defence against oxidative stress in the cytoplasmic membrane. The rate of superoxide production by rapidly respiring wild-type *Escherichia coli* membranes was twofold higher than in the slowly respiring membranes from a *ubiCA* knockout mutant. However, large amounts of superoxide accumulated in the Ubi⁻ membranes compared to wild-type membranes, which possess superoxide-scavenging ubiquinol. Likewise, the rate of H₂O₂ production was twofold higher in the wild-type, but the overall production of H₂O₂ was again significantly higher in the Ubi⁻ membranes. Inclusion of a water-soluble ubiquinone homologue (UQ-1) effectively decreased the amount of H₂O₂ produced in the Ubi⁻ membranes in a concentration-dependent manner. Addition of UQ-2 to the membranes was even more effective in limiting accumulation of H₂O₂ than was UQ-1, suggesting a role for the side-chain in conferring liposolubility in the antioxidative defence mechanism. Intracellular H₂O₂ concentration was increased 1.8-fold in the *ubiCA* mutant, and expression of the *katG* gene, encoding the catalase hydroperoxidase I, as well as catalase enzyme activity, were increased twofold in this mutant. The *ubiCA* mutant was hypersensitive to oxidative stress mediated by CuSO₄ or H₂O₂; sensitivity to the latter could be abolished by addition of cysteine. This phenotype was also exhibited by a *ubiG* mutant, defective in the last step of UQ biosynthesis and therefore expected to accumulate several UQ biosynthetic intermediates. These observations support the participation of reduced ubiquinone as an antioxidant in *E. coli*. The *ubiCA* mutant exhibited a pleiotropic phenotype, being resistant to heat, linolenic acid and phleomycin. Resistance to the two latter compounds is probably due to reduced uptake. Like mutants unable to synthesize the quinol oxidase, cytochrome *bd*, the *ubiCA* mutant was also sensitive to dithiothreitol, an effect that is attributed to inability of the respiratory chain to maintain an appropriate redox balance in the periplasm.

Keywords: ubiquinone, *Escherichia coli*, superoxide, peroxide, oxidative stress

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

Abbreviations: HRP, horseradish peroxidase; SOD, superoxide dismutase; UQ, ubiquinone; UQH₂, ubiquinol (fully reduced form).

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-

oxidant (for a review, see Ernster & Dallner, 1995). UQH₂ scavenges lipid peroxy radicals and thereby prevents a chain reaction causing oxidative damage to polyunsaturated fatty acids of biological membranes, a process known as lipid peroxidation (Forsmark-Andrée *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; this was attributed to electrons 'backing up' at NADH dehydrogenase II. Sulfite reductase was found to be a second flavin-containing autoxidizable electron-transport enzyme of *E. coli* (Messner & Imlay, 1999). However, in bacteria, it is still not clear whether, in addition to its respiratory roles, UQH₂ acts as an antioxidant or pro-oxidant. *E. coli* does contain a quinone oxidoreductase (Qor) (Thorn *et al.*, 1995), which may be important in maintaining UQ in its fully reduced state by 2-electron reductions, but the role of this enzyme is unknown. In *Saccharomyces cerevisiae*, a UQ-deficient strain showed enhanced sensitivity to

products of autoxidized polyunsaturated fatty acids, indicating a protective role of UQH₂ (Do *et al.*, 1996).

Some studies directed at determining the roles of 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 OD₆₀₀ 0.55. For catalase assays, the strains were also grown to exponential phase (OD₆₀₀ 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 paraquat (50 μ M) or H₂O₂ (50 μ M) 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 μ g ml⁻¹). Heat resistance was analysed by aerobic shock treatment at 52 °C. A portion (0.5 ml) of a mid-exponential

Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference or source
MG1655	F ⁻ , referred to as wild-type	Laboratory collection
RKP4152	<i>ubiCA</i> ::Km	Søballe & Poole (1998)
QC772	Φ (<i>sodA-lacZ</i>) Δ <i>lacU rpsL</i>	Touati (1988)
RKP4228	As QC772 but <i>ubiCA</i> ::Km	This work
BGF931	Tet ^r Φ (<i>katG-lacZ</i>)	Bruce Demple, Harvard School of Public Health, Boston, MA, USA
RKP4241	As BGF931 but <i>ubiCA</i> ::Km	This work
HW271	F ⁻ <i>ubiG zei</i> ::Tn10	Wu <i>et al.</i> (1992)
HW272	F ⁻ <i>zei</i> ::Tn10	Wu <i>et al.</i> (1992)

culture was transferred to 4.5 ml MOPS/glucose medium, preincubated at 52 °C, and shaken at this temperature. When exposed to CuSO₄ (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 (10⁻¹ to 10⁻⁶) 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 H₂O₂ to a final concentration of 2.5 mM, whereas to another was added H₂O₂ (2.5 mM) plus cysteine to a final concentration of 0.83 mM.

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

Superoxide anion detection. The rate of O₂⁻ 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 50 mM potassium phosphate buffer, 20 µM cytochrome *c*, and wild-type or *ubi* membranes (200 µg protein ml⁻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 reduced was calculated using an absorption coefficient, ϵ , of 21.0 mM⁻¹ cm⁻¹.

H₂O₂ production. The amount of H₂O₂ 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⁻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 H₂O₂ generated was determined from a standard curve, in which the H₂O₂ concentration (0–0.15 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 glycerol.

Determination of intracellular H₂O₂ production was based on the assumption that free diffusion of H₂O₂ 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 H₂O₂ 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 ΔA 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 H₂O₂ to a final concentration of 5 mM. The initial rate of decomposition of H₂O₂ was determined using an ϵ_{240} of 43.6 × 10⁻³ mM⁻¹ cm⁻¹. One unit of catalase activity is defined as the change in H₂O₂ concentration (mM) per min.

β -Galactosidase assays. Assays were carried out at room temperature as described before (Søballe & 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 O₂⁻ 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, O₂⁻ 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 O₂⁻ 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%

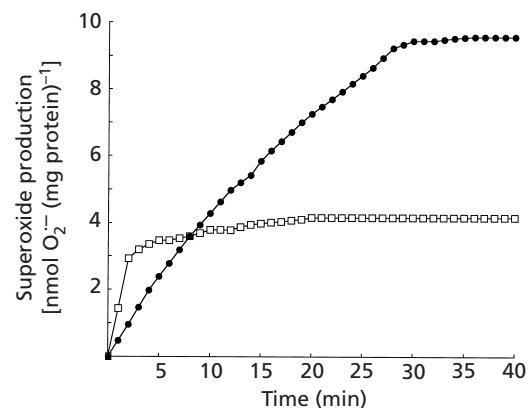


Fig. 1. Effect of UQ deficiency on superoxide production. The SOD-sensitive rate of cytochrome *c* reduction was measured in membranes from wild-type (□) and *ubi* (●) strains. The reaction, containing 20 µM cytochrome *c* and 0.2 mg membrane protein ml⁻¹, was initiated by NADH (100 µM) and monitored at 550 nm. The results shown are typical of three similar determinations.

Table 2. Effects of UQ deficiency on superoxide and H₂O₂ production and metabolism

Production of O₂⁻ and H₂O₂ and catalase activities were measured as described in Methods. The O₂⁻ assay was repeated twice and a typical set of values is given, corresponding to Fig. 1. The values for H₂O₂ and catalase activity are means of three separate experiments.

Strain	SOD-sensitive O ₂ ⁻ production		H ₂ O ₂ production			Catalase activity (H ₂ O ₂ converted, units mg ⁻¹)
	Initial rate of O ₂ ⁻ (nmol min ⁻¹ mg ⁻¹)	Total amount of O ₂ ⁻ (nmol mg ⁻¹)	Initial rate of H ₂ O ₂ production in membranes (nmol min ⁻¹ mg ⁻¹)	Accumulation of H ₂ O ₂ in membranes (nmol mg ⁻¹)	Intracellular H ₂ O ₂ (µM)	
Wild-type	1.5	4.2	0.46	0.43	14	2.7
<i>ubiCA</i>	0.7	9.6	0.29	0.84	25	5.2

(Søballe & Poole, 1998) and therefore correction of Fig. 1 for the electron transfer rates to O₂ would emphasize the antioxidant role of UQ. Fig. 1 also shows that the rapid production of O₂⁻ in the wild-type strain ceased after about 2–3 min, whereas in the *ubiCA* strain, O₂⁻ production continued until the cytochrome *c* present in the assay was fully reduced (Fig. 1). Thus, the total amount of O₂⁻ produced expressed per mg membrane protein was 2.3-fold higher in the *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 O₂⁻ levels.

Messner & Imlay (1999) used vesicles produced from a chemically produced *ubiA* mutant (AN385) and found a high rate of O₂⁻ production when expressed per electron transferred to O₂. Titration of the substrate for UbiA (4-hydroxybenzoate) to the growing *ubiA* culture resulted in the production of UQ, increasing respiration rate and causing a decrease in the rate of O₂⁻ production.

UQ limits accumulation of H₂O₂ *in vitro*

H₂O₂ production in membranes from wild-type and *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 H₂O₂ (not shown). The initial rate of H₂O₂ production was 37% lower in the *ubiCA* mutant compared to the wild-type (Table 2). However, in the *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 H₂O₂ in the *ubiCA* mutant under these assay conditions (Fig. 2, Table 2).

The effects on H₂O₂ production of adding a water-soluble ubiquinone homologue, UQ-1, to the *ubi* membranes are shown in Fig. 3(a). Over the assay period, the accumulation of H₂O₂ decreased substantially with increasing additions of UQ-1; accumulation of H₂O₂ decreased by 80% in the presence of 2 µM UQ-1. On addition of 0.4 µM UQ-1 [corresponding to 2 nmol (mg protein)⁻¹], H₂O₂ accumulation decreased by 50% to a

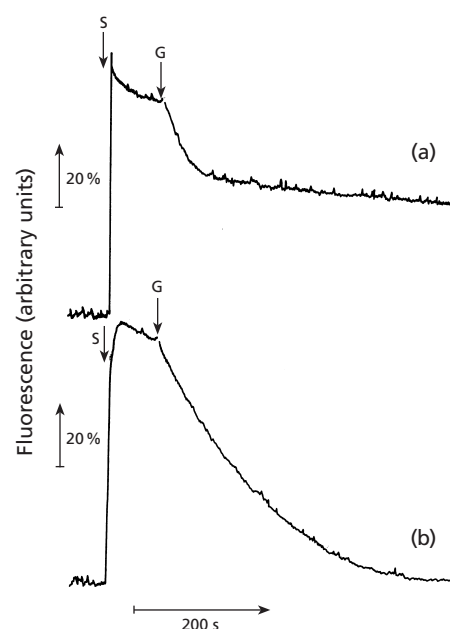


Fig. 2. Effect of UQ deficiency on H₂O₂ production. Quenching of the fluorescent substrate scopoletin by glucose-respiring membranes from wild-type (a) and *ubi* (b) cells was followed in a fluorescence spectrophotometer in time-scanning mode, with excitation at 350 nm and emission at 460 nm. The assay contained 0.2 mg membrane protein ml⁻¹ and 0.76 µM HRP. Addition of 0.2 µM scopoletin (S) and 25 mM glucose (G) initiated the reaction. The results shown are typical of three similar determinations.

level equivalent to H₂O₂ 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 H₂O₂ accumulation to an even greater extent than UQ-1; addition of

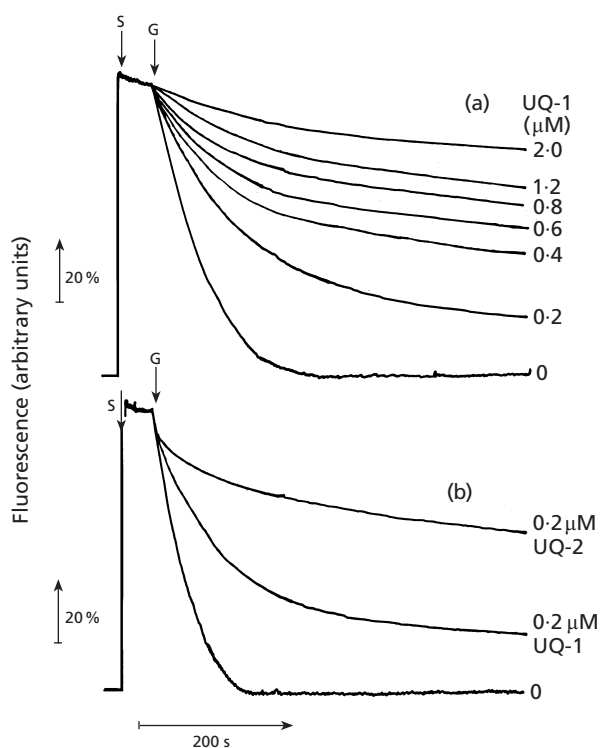


Fig. 3. H_2O_2 production in *ubi* membranes with added UQ homologues, UQ-1 or UQ-2. The scopoletin (S) assay (as described in Fig. 2) was initiated by addition of 5 mM glycerol (G). In (a), various concentrations of UQ-1 (0.2–2.0 μM) were included in the assay. In (b), UQ-1 or UQ-2 was added to a final concentration of 0.2 μM .

0.2 μM UQ-2 decreased the H_2O_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_2O_2 concentration, which was determined after allowing diffusion of H_2O_2 into PBS buffer, was increased 1.8-fold in the *ubiCA* mutant (Table 2). These results mimic the $\text{O}_2^{\cdot-}$ and H_2O_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_2O_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. HPI, 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_2O_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_2O_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 $\Phi(katG-$

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

Mid-exponential cultures of $\Phi(katG-lacZ)$ strains were induced with H_2O_2 (50 μM), whereas induction of $\Phi(sodA-lacZ)$ strains was performed with paraquat (50 μM).

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

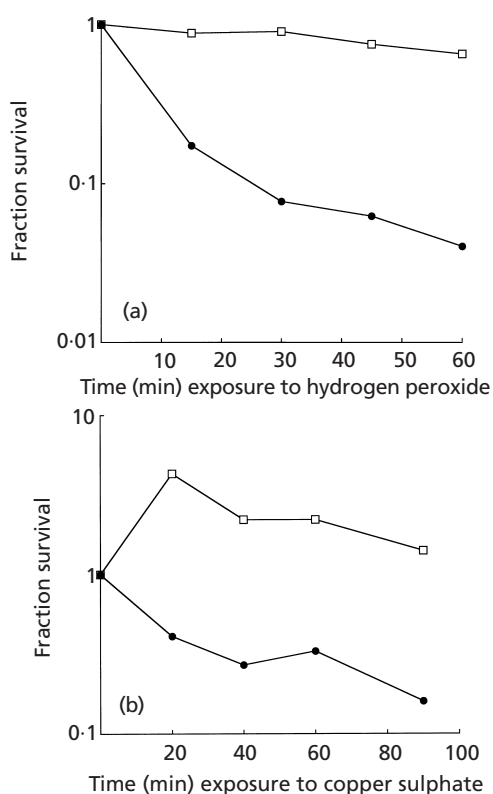


Fig. 4. Sensitivity of the *ubi* mutant to oxidative stress. Exponential cultures of wild-type (□) and *ubi* (●) cells were exposed to 0.03% H₂O₂ (a) and 5 mM CuSO₄ (b) and samples for viable counts were taken at appropriate time intervals. The results shown are typical of three similar determinations.

lacZ) increased 2.2-fold in a wild-type strain when induced with H₂O₂ under our experimental conditions. To seek confirmation that the presence of UQ affects H₂O₂ levels *in vivo*, the effect of introducing the *ubiCA* allele into the $\Phi(katG-lacZ)$ strain was studied. The basal level of *katG* expression was 2.1-fold higher in the *ubi* background, but induction with H₂O₂ still increased the expression a further 1.3-fold (Table 3).

The manganese-containing superoxide dismutase (MnSOD) enzyme is encoded by the *sodA* gene, which can be regarded as a monitor of intracellular oxidative stress as it is induced via *soxRS* regulators by O₂⁻, by increased oxygen pressure and by redox-active compounds (Touati, 1988; Demple, 1991; Compan & Touati, 1993). Paraquat is widely used as a redox-cycling agent; it donates a single electron to oxygen to give superoxide anion and is readily rereduced intracellularly, enabling further rounds of superoxide production. The expression of $\Phi(sodA-lacZ)$ increased 4.2-fold when induced with paraquat, as expected (Table 3). Surprisingly, the introduction of the *ubiCA* mutation did not increase the basal level of the aerobic expression of *sodA* (Table 3). A possible explanation is that, *in vivo*, the slow rate of superoxide generation (seen in membranes; Fig. 1) might be accommodated by spon-

taneous dismutation of superoxide to peroxide. Thus catalase levels are raised but SOD levels are not. Paraquat increased $\Phi(sodA-lacZ)$ expression 2.6-fold in the *ubi* background (Table 3). The fact that paraquat induction was not as marked in the *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 O₂⁻ generation.

A *ubiCA* mutant is hypersensitive to mediators of oxidative stress

In view of the elevated levels of superoxide and peroxide measured in *ubiCA* cells, the ability of cells to tolerate additional exogenous oxidative stress mediated by H₂O₂ or CuSO₄ was examined (Fig. 4). The *ubiCA* mutant appeared to be hypersensitive to treatment with 0.03% H₂O₂ 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*-methyltransferase 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 *ubiG* (e.g. AN86, AN151; Stroobant *et al.*, 1972) were shown to accumulate demethyl-UQ, the last intermediate in UQ biosynthesis. Unlike RKP4152 (*ubiCA* knockout strain), HW271 (*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 H₂O₂. After 1 h of H₂O₂ treatment, the viability of the *ubiG* strain was reduced 28-fold compared to the corresponding wild-type (not shown).

Copper ions (Cu²⁺/Cu¹⁺) participate in a redox-cycle, resulting in the generation of superoxide anion. They have also been reported to catalyse the conversion of H₂O₂ to [•]OH *in vitro* (Kimura & Nishioka, 1997). When treated with CuSO₄, the viability of the *ubiCA* mutant was reduced 10-fold relative to the wild-type strain after 90 min (Fig. 4b). Likewise, the viability of the *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 H₂O₂ or CuSO₄. Sensitivity to oxidative stress in a UQ-deficient mutant of the fission yeast *Schizosaccharomyces 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 *ubiCA* mutant to H₂O₂ (2.5 mM) could be abolished by the addition of this compound to the growing cells (Fig. 5b). In contrast, the presence of H₂O₂ or cysteine did not affect growth of the wild-type

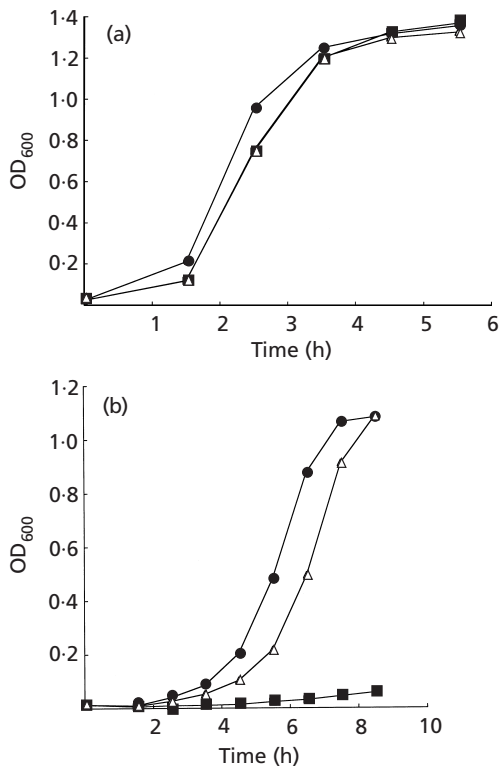


Fig. 5. Rescue of H₂O₂ sensitivity of the *ubi* mutant by cysteine. Growth of (a) the wild-type and (b) the *ubiCA* strain in LB-glucose (●), LB-glucose plus H₂O₂ (2.5 mM) (■), and LB-glucose plus both H₂O₂ (2.5 mM) and cysteine (0.83 mM) (△). The results shown are typical of three similar determinations.

cells (Fig. 5a). Likewise, cysteine or glutathione restores growth of a UQ-deficient (*dps*) mutant of fission yeast in minimal medium (Suzuki *et al.*, 1997).

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 μ g ml⁻¹ (Fig. 6b). The *ubiCA* strain

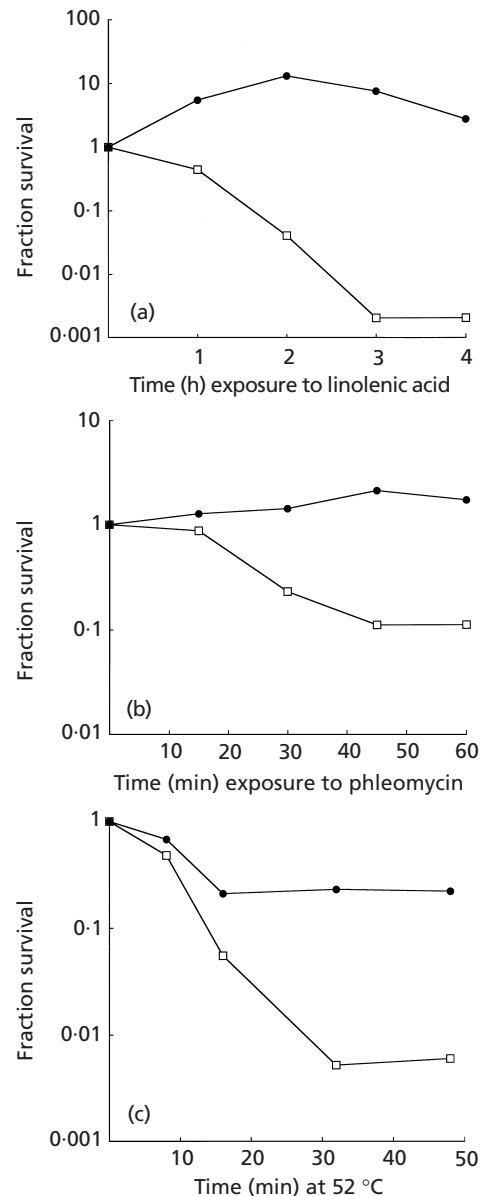


Fig. 6. Pleiotropic phenotype of the *ubi* mutant. Exponential cultures of wild-type (□) and *ubi* (●) strains were exposed to 1 mM linolenic acid (a), 10 μ g phleomycin ml⁻¹ (b) and heat shock (c) at 52 °C. Samples for viable counts were taken at appropriate time intervals. The results shown are typical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

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

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 & Ito, 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 (QH[•]) – 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 (*K_m* 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 perferyls or lipid peroxy 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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