Requirement for ubiquinone downstream of cytochrome(s) *b* in the oxygen-terminated respiratory chains of *Escherichia coli* K-12 revealed using a null mutant allele of *ubiCA*

Britta Søballe^{1,2} and Robert K. Poole²

Author for correspondence: Robert K. Poole. Tel: +44 114 222 4447. Fax: +44 114 272 8697.
e-mail: r.poole@sheffield.ac.uk

¹ Division of Life Sciences,
King's College London,
Campden Hill Road,
London W8 7AH, UK

² Department of Molecular
Biology and
Biotechnology, Krebs
Institute for Biomolecular
Research, The University of
Sheffield, Firth Court,
Western Bank,
Sheffield S10 2TN, UK

An *Escherichia coli* knockout *ubiCA* mutant has been constructed using a gene replacement method and verified using both Southern hybridization and PCR. The mutant, which was unable to synthesize ubiquinone (Q), showed severely diminished growth yields aerobically but not anaerobically with either nitrate or fumarate as terminal electron acceptors. Low oxygen uptake rates were demonstrated in membrane preparations using either NADH or lactate as substrates. However, these rates were greatly stimulated by the addition of ubiquinone-1 (Q-1). The rate of electron transfer to those oxidase components observable by photodissociation of their CO complexes was studied at sub-zero temperatures. In the *ubiCA* mutant, the reduced form of haemoproteins – predominantly cytochrome *b*₅₅₅ – was reoxidized significantly faster in the presence of oxygen than in a Ubi⁺ strain, indicating the absence of Q as electron donor. Continuous multiple-wavelength recordings of the oxidoreduction state of cytochrome(s) *b* during steady-state respiration showed greater reduction in membranes from the *ubiCA* mutant than in wild-type membranes. A scheme for the respiratory electron-transfer chain in *E. coli* is proposed, in which Q functions downstream of cytochrome(s) *b*.

Keywords: aerobic respiration, cytochrome *b*, ubiquinone mutants, *Escherichia coli*

INTRODUCTION

Quinones are key constituents of the membrane-bound respiratory electron transfer chains in both prokaryotes and eukaryotes. These lipid components, containing isoprenoid side chains of various lengths, can be divided into two major structural groups, benzoquinones and naphthoquinones. The facultatively anaerobic bacterium *Escherichia coli* is capable of synthesizing octaprenyl quinones of both types, i.e. a benzoquinone, ubiquinone-8 (Q-8), and two types of naphthoquinone, menaquinone-8 (MQ-8) and demethylmenaquinone (DMQ) (Meganathan, 1996). The ratio of the quinones is variable and dependent on the growth conditions. Thus, in aerobically grown *E. coli*, Q is dominant in a 4- to 20-fold excess over MQ (Ingledew & Poole, 1984;

Gennis & Stewart, 1996). Mutants that lack either Q or MQ can grow under appropriate conditions, but the *ispB* gene, which is needed for synthesis of the side chain, appears to be essential for *E. coli* (Okada *et al.*, 1997).

The flexibility of the respiratory system in response to environmental changes is due in part to the mobile quinone pool. All of the low-potential segments (dehydrogenases) are quinone reductases, whereas all the high-potential terminal oxidases become reduced by quinols. The functions of the different quinones have been studied by biochemical and physical characterization of mutants defective in quinone biosynthesis. A major problem in interpretation of some of these studies is that the mutants used have generally been isolated after chemical mutagenesis (see, for example, Gibson, 1973; Nakahigashi *et al.*, 1992) and are unstable or leaky. Nevertheless, a consensus view is that Q-8 is used for oxygen respiration, both Q and MQ are involved in nitrate respiration (Wallace & Young, 1977), while MQ and DMQ are used for anaerobic respiration

Abbreviations: Q, ubiquinone; Q-*n*, ubiquinone-*n* (where *n* is the number of isoprenoid units in the polyprenyl side chain); MQ, menaquinone; MQ-8, menaquinone-8; DMQ, demethylmenaquinone; Km, kanamycin; Cm, chloramphenicol.

with fumarate and other acceptors except nitrate. The dehydrogenases also show a degree of quinone specificity: thus, only Q appears to function in succinate oxidation. This specificity is reflected in part by the midpoint potentials of the respiratory components involved; the value reported for the menaquinone/menaquinol couple ($E_{m,7} -74$ mV) is lower than for the ubiquinone (Q)/ubiquinol (QH_2) couple ($E_{m,7} +113$ mV) (Gennis & Stewart, 1996).

The biosynthetic pathway for Q was determined (Gibson, 1973) by identification of intermediates accumulated by Q-deficient (*ubi*) mutant strains obtained after chemical mutagenesis. The first step of the biosynthetic pathway is the conversion of chorismate to 4-hydroxybenzoate by chorismate lyase. In the following step, 3-octaprenyl-4-hydroxybenzoate is produced by 4-hydroxybenzoate octaprenyltransferase. These enzymes are encoded by *ubiC* and *ubiA* respectively, and catalyse steps in Q biosynthesis that are probably rate-limiting in biosynthesis. The UbiA protein was found to be of special importance, together with the IspB protein, for the overproduction of Q by genetic engineering (Zhu *et al.*, 1995). We have cloned and sequenced three *ubi* genes: the *ubiCA* operon (91.5 to 91.6 min; Wu *et al.*, 1993), and *ubiG* (50.3 min; Wu *et al.*, 1992). The UbiG protein catalyses the *S*-adenosylmethionine-dependent terminal O-methylation step and also the prior O-methylation leading to 2-octaprenyl-6-methoxyphenol (Hsu *et al.*, 1996). The *ubiX* (Nonet *et al.*, 1987; for comments see Meganathan, 1996), *ubiB* (Spyrou *et al.*, 1991; Daniels *et al.*, 1992), *ubiH* (Nakahigashi *et al.*, 1992) and *ubiE* (Lee *et al.*, 1997) genes have also been identified, cloned and sequenced. The Q biosynthetic pathway is strikingly similar in *E. coli* and in eukaryotes (Hsu *et al.*, 1996).

Despite intensive studies on the structure and function of the respiratory chain components in *E. coli*, there is still considerable uncertainty regarding the number of sites in the respiratory chains where Q acts and the roles of the various *b*-type cytochromes. To date, chemically produced mutants have been used to explore this problem. Cox *et al.* (1970) used a *ubiB* mutant and measured the proportion of respiratory carriers (flavins, cytochromes *b* and *d*) that were reduced in the aerobic steady state accompanying NADH oxidation. The reduction level of cytochrome(s) *b* was higher in the *ubi* mutant than in the wild-type, suggesting a pool of cytochrome *upstream* of Q. Such an organization was supported by the experiments of Downie & Cox (1978) and Kita & Anraku (1981). Wallace & Young (1977) demonstrated quinone specificity in the reactivation of oxygen respiration in a *ubiA menA* mutant using various substrates. This observation led to the proposal of a Q site between the respective dehydrogenases and one or more *b*-type cytochromes, but their findings did not preclude two sites for quinone function. However, the two-quinone pool hypothesis has been contradicted by Au *et al.* (1984). They re-examined the role of quinones in the cytochrome *o*-terminated branch of the *E. coli* respiratory chain (in a *Cyd*⁻ background) and reported

a lower level of cytochrome *b* reduction during steady-state respiration of a *ubiA cyd* mutant.

The objective of the present work was to re-examine the role of Q in *E. coli* using a null mutant constructed by deletion-insertion mutagenesis of the *ubiCA* genes. This mutant was used to explore the role of Q in aerobic and anaerobic growth and respiration. Using continuous multi-wavelength spectrophotometric techniques it was possible to confirm previous claims for enhanced cytochrome *b* reduction during respiration of a *ubi* mutant, supporting a model in which electron transfer from dehydrogenases to oxidases requires two functionally discrete quinone pools, i.e. before and after cytochrome(s) *b*.

METHODS

Bacterial strains and plasmids. Bacterial strains, which were all derivatives of *E. coli* K-12, and plasmids used are described in Table 1.

Growth media and conditions. LB liquid medium and LB plates were prepared as described by Maniatis *et al.* (1982). For membrane preparations, cultures of the *ubi* mutant and wild-type strains were grown with shaking (200 r.p.m.) in LB medium supplemented with 0.5% glucose, in 2 l Erlenmeyer flasks containing 500 ml medium. Anaerobic cultures were grown in Luria/glycerol/nitrate (LGN) medium, containing (g l⁻¹) tryptone (10), yeast extract (5), NaCl (0.5), glycerol (5) and NaNO₃ (10), adjusted to an initial pH of 7.0 with NaOH. For solid medium, 15 g agar was added. Anaerobic cultures were grown overnight in Schott screw-capped bottles, with the caps left loose, in an anaerobic jar having an atmosphere generated using a GasPak (Oxoid). Antibiotics were added to the following final concentrations: chloramphenicol (Cm) 20 µg ml⁻¹ and kanamycin (Km) 50 µg ml⁻¹.

Succinate minimal medium for aerobic growth was as described by Poole *et al.* (1989). Nitrate/fumarate/glycerol minimal medium is a defined medium for anaerobic growth described by Wu *et al.* (1993). Aerobic cultures were grown with vigorous shaking (200 r.p.m.). Anaerobic cultures were grown in sealed test tubes and supplemented with either sodium nitrate (up to 5 mM) or sodium fumarate (up to 5 mM) as electron acceptors.

For growth yield experiments, cells were grown in 10 ml volumes of MOPS medium (Stewart & Parales, 1988), supplemented with limiting concentrations (3–5 mM) of the substrates described in Results. The inocula used (1%) were from overnight cultures grown with 5 mM glucose such that cultures were carbon-depleted when used. Aerobic cultures were shaken (200 r.p.m.) in 250 ml conical flasks with matched glass tubes of Klett dimensions as a side arm. For anaerobic growth, cells were grown in screw-capped glass tubes (Stewart & Parales, 1988), filled to the brim with MOPS medium supplemented with 0.3% Casamino acids (Difco) and containing two glass balls (approx. 1 mm diameter) to aid resuspension of cells that had settled during static culture. Turbidity was measured with a Klett colorimeter (Manostat) fitted with a red filter. Culture turbidities were routinely measured up to 48 h after inoculation or when two successive readings were similar.

DNA manipulations. These were carried out using standard procedures (Maniatis *et al.*, 1982). DNA was recovered from agarose gels using a GeneClean Kit (BioL01). Transformation was performed using CaCl₂-treated competent cells. Chromo-

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> MG1655	F ⁻ , referred to as wild-type	H. D. Williams (Imperial College London, UK)
RKP4151	Cm ^R Km ^R , cointegration of pRKP952 into chromosome of MG1655	This work
RKP4152	Km ^R , $\Delta ubiCA::Km^R$	This work
XL-1 Blue	<i>recA1</i>	Stratagene
Plasmids		
pBRP23	2.6 kb <i>ClaI</i> - <i>PvuII</i> fragment (<i>ubiCA</i> ⁺) cloned in pBluescript II SK(+)	Wu <i>et al.</i> (1993)
pMAK705	Cm ^R , <i>lacZ rep(ts)</i>	Hamilton <i>et al.</i> (1989)
pRKP951	Ap ^R Km ^R cartridge replacing 0.6 kb <i>Bam</i> HI fragment of <i>ubiCA</i> , cloned in pBluescript II SK(+)	This work
pRKP952	Cm ^R , <i>lacZ rep(ts)</i> , 2.8 kb <i>ubiCA::Km^R</i> fragment in pMAK705	This work

somal DNA was purified by ultracentrifugation in CsCl/ethidium bromide gradients. For Southern hybridization, *Eco*RI-digested DNA was transferred from a 0.8% agarose gel to a Hybond-N+ membrane. Three different single-stranded probes were used: the Km^R gene excised with *Sma*I from plasmid pUC4-KIXX (Pharmacia), the wild-type 0.63 kb PCR product shown in Fig. 1, and finally the *Nhe*I-*Bcl*I 517 bp fragment present in *ubiA* in both wild-type and mutant strains. The labelling of the probes and the detection of hybridization were performed using the Enhanced Chemiluminescent Detection System (ECL; Amersham).

Construction of a *ubi* mutant. The method for generating mutations in the *E. coli* chromosome by allele exchange described by Hamilton *et al.* (1989) was used. This relies on the use of a temperature-sensitive recombinant plasmid pMAK705 carrying a mutagenized copy of the gene in question. The recipient strain is transformed with plasmid DNA and cells are selected at 42 °C for integration of the plasmid into the chromosome. Subsequent growth of the cointegrate at 30 °C results in a second crossover, regeneration of the plasmid, and replacement of the chromosomal copy of the mutant allele. The *ubiCA* operon in pBRP23 (Wu *et al.*, 1993) was inactivated by insertion of the 1.2 kb Km^R Tn5 cartridge from pUC4-KIXX (Pharmacia). This cartridge replaced a 587 bp *Bam*HI fragment extending from the 3' end of *ubiC* into the 5' end of *ubiA*. Plasmid pRKP952 was generated by subcloning the *ubiCA::Km^R* fragment into vector pMAK705, which contains a thermosensitive replicon derived from a mutant isolate of pSC101 (Hamilton *et al.*, 1989). Thereafter, the method described by Hamilton *et al.* (1989) was used.

Polymerase chain reaction. PCR reactions were performed in an Omnigene Thermocycler (Hybaid). Two oligonucleotide primers were designed: the forward primer (5' GTAAAGAGATCCCTGCCCT 3') was complementary to a region immediately upstream of the *Bam*HI site in *ubiC*, whereas the reverse primer (5' CCCGCGACAAACACCGCCA 3') was complementary to a region immediately downstream of the *Bam*HI site in *ubiA* (Fig. 1). The PCR mixture contained 50 ng genomic DNA, 7 pmol of each primer, 0.2 mM dNTP, 2 mM DTT, 1 unit *Taq* polymerase and *Taq* buffer with 1 mM MgCl₂. The PCR programme consisted of 30 cycles: denaturation at 94 °C for 1 min, primer annealing at 62 °C and elongation at 72 °C for 3 min. In the first cycle, the template DNA became single-stranded, and in the last step a prolonged elongation time (10 min) permitted the formation

of a large amount of complete double-stranded DNA. The resulting products were visualized on a 1% agarose gel.

Preparation of membranes. One litre of culture was harvested at 10000 *g* and the cells washed in 50 mM Tris/HCl buffer, pH 7.0. The cells were then resuspended in buffer that contained 0.1 M TES, 20 mM magnesium acetate, 0.25 M sucrose and 0.25 mM EGTA. A small quantity of DNaseI and 1 mM PMSF (from a 0.25 M stock in ethanol) were added to the cells, which were frozen overnight at -20 °C. Frozen cells were disrupted by sonication for five 1 min periods, with 1 min chilling between treatments. Cell debris and unbroken cells were removed by centrifugation at 10000 *g* for 20 min. The supernatant was centrifuged at 120000 *g* for 1.5 h. The membrane pellet was resuspended in buffer (50 mM TES, 5 mM EDTA) and homogenized. The membranes were again pelleted by centrifugation and finally resuspended and homogenized in 5 ml of the same buffer. The membranes were stored at -70 °C until use. Protein concentrations were determined by the method of Markwell *et al.* (1978).

Oxygen consumption measurements. Oxygen consumption was measured using an inverted Clark-type oxygen electrode (Rank; Poole, 1977) polarized at 0.65 V. Temperature was maintained with a water jacket around the chamber, which was fitted with a close-fitting deep plug, having a <1 mm diameter hole for injecting reactants with minimum oxygen penetration. Calibration was achieved using 2 ml air-saturated 0.1 M sodium phosphate buffer (pH 7.0), assumed to contain 220 nmol oxygen ml⁻¹ at 37 °C, followed by a few grains of sodium dithionite to remove all dissolved oxygen. For oxygen uptake measurements, cell membranes (about 1.3 mg protein) were added and the oxygen uptake was followed for 5 min or until a linear rate of oxygen consumption was attained. Substrate was added with a Hamilton microsyringe (to give final concentrations of 10 mM), and the rate of oxygen consumption was recorded. Where indicated, Q-1 (80 µM final concentration) was added a few minutes after adding substrate.

Recording of electronic absorbance spectra. Reduced *minus* oxidized difference spectra were obtained at room temperature using a Johnson Foundation SDB-3 dual-wavelength scanning spectrophotometer, similar to that described by Williams & Poole (1987). The pathlength was 1 cm and the reference wavelength was 575 nm. Cells were washed and resuspended in 0.1 M phosphate buffer. A few grains of sodium dithionite were added to reduce a sample, and ammonium persulphate (a

few grains) was used with shaking to oxidize a sample. A baseline was recorded as the difference of two successive scans of an oxidized sample. The data were stored in the instrument memory, and reduced *minus* oxidized spectra were directly plotted, by subtraction of the stored spectrum.

Photodissociation spectra exploit the light sensitivity of carbonmonoxy compounds of haem proteins. Membranes were resuspended in 0.1 M potassium phosphate buffer (pH 7.0). The sample was transferred to a specially constructed metal/Perspex cuvette and ethylene glycol (to a final concentration of 30%, v/v) was added as antifreeze. The sample was reduced with either dithionite or NADH (10 mM final concentration, 1 h at 37 °C). The reduced sample was bubbled with CO and then cooled in an ethanol/solid CO₂ bath at -78 °C for at least 5 min in the dark before further equilibration at -100 °C in the sample compartment of the dual-wavelength spectrophotometer. Electron-transfer events following reaction of oxidases with oxygen were studied by photolysing the CO complex of the reduced oxidase in the presence of oxygen. After reduction with NADH and CO treatment as above, the cuvette was transferred to an ethanol/solid CO₂ bath at -23 °C and, after equilibration, vigorously stirred in the dark with a close-fitting coiled wire (20–30 vertical strokes) to introduce oxygen to about 300 µM (Chance *et al.*, 1975a). The sample was then immediately trapped at -78 °C, then transferred to the sample compartment of the spectrophotometer. The temperature was controlled by a flow of cold N₂ gas, cooled by passing through a coiled copper tube immersed in liquid N₂ and then through a small heater providing a temperature in the cuvette holder of -100 °C ± 1 °C. The sample (reduced + CO, oxygen in frozen solution) was scanned twice generating a reduced + CO *minus* reduced + CO baseline and then photolysed by light from a 200 W slide projector for 1 min. The light source was placed 0.6 m from the afferent limb of a bifurcated light guide that transmitted the actinic light to the temperature-regulated, insulated cuvette holder. Focusing of the source to a spot 6 mm diameter on the light guide was done with a lens of focal length 100 mm. The spectrum of the difference between the photolysed sample and the reduced + CO sample was computed and plotted.

Multiple-wavelength recordings of cytochrome oxidoreduction kinetics. A spectrophotometer (University of Pennsylvania School of Medicine Research Instrumentation Shop, Room 79E John Morgan, Philadelphia, PA 19104, USA), constructed as described by Chance *et al.* (1975b), was used. In the dual-wavelength mode (for details see D'mello *et al.*, 1994), interference filters (Omega Optical) transmitting maximally at 560 nm and 575 nm provided light appropriate for monitoring absorbance of the α -band of cytochromes *b* and the nearest isosbestic point, respectively. The filters were mounted in an air-driven turbine disc, rotating about an axis parallel to the light beam from a Nikon 50 W halogen source. The speed of the turbine (driven from a filtered, compressed air supply) provided a chopping frequency of 100 Hz. The photo-multiplier was a Thorn-EMI QL-30-RF1 in the 'end-on' configuration. The suspension of cell membranes (2 ml) in buffer (0.05 M PIPES, 8 mM magnesium acetate, pH 7.0) was stirred (see below) continuously for 5 min in a 4 ml open cuvette (1 cm path length) before adding substrate (0.1 M NADH, 100 µl; 1 M sodium D-lactate, 20 µl; 1 M sodium succinate, 20 µl; 1 M glycerol 3-phosphate, 20 µl; 1 M sodium formate, 20 µl). The steady-state reduction level of cytochrome(s) *b* was continuously monitored by plotting $\Delta A_{560-575}$. In Fig. 4(a–e) (see Results), the data are shown as discrete points measured at time intervals from the continuous chart

recordings, of which Fig. 4(f) is an example. Throughout the period of measurement, the sample was continuously aerated by stirring with a magnetic 'flea' driven from below by a rotating button magnet mounted on a DC motor, controlled by a constant speed power supply. A mu-metal magnetic shield was located between the stirrer/cuvette assembly and the photomultiplier to guard it from electromagnetic interference generated by the rotating magnet and motor (N. Graham, University of Pennsylvania, unpublished design).

RESULTS

Construction and verification of a *ubiCA* knockout mutant strain

The *E. coli ubiCA* null mutant was created by homologous recombination. The recombinant plasmid pRKP952 containing the inactivated *ubiCA* genes was used to transform strain MG1655; transformants were selected on Km plates at 30 °C; cointegrates were allowed to form by incubating the transformants overnight at 42 °C on LB plates containing both Km and Cm. Cells from eight colonies were pooled and transferred to rich liquid medium (LGN/Km/Cm) and three growth cycles were performed anaerobically at 30 °C, the permissive temperature for replication of the plasmid. A final growth cycle was performed in LGN at 42 °C to dilute out the resolved plasmids. Following a screen of >1000 Km^R colonies, one Km^R Cm^S isolate was retained as RKP4152.

The genetic construction in the *ubiCA::Km^R* strain was verified by PCR analysis. Primers were designed to hybridize at the 5' side of the *Bam*HI site in *ubiC* and at the 3' side of the *Bam*HI site of *ubiA* (Fig. 1a). The PCR results are shown in Fig. 1(b). As expected, the DNA from the wild-type strain (lane 2) showed one band equivalent to a fragment of 0.64 kb, whereas the DNA from the cointegrate (lane 3) showed two bands (0.64 and 1.3 kb). The presence of the insertion in the mutant is verified in lane 4, as only the 1.3 kb band is seen (Fig. 1b). The construction was also verified by Southern blot analysis of *Eco*RI-restricted genomic DNA from all three strains (results not shown). DNA from the wild-type *Ubi*⁺ parent did not show any hybridization with the Km^R gene probe, while both the cointegrate and the mutant showed a single band of 6.3 kb as expected. When the 'wild-type' PCR product was used as probe, no hybridization was seen with mutant DNA, whereas the wild-type and cointegrate strains gave the expected single bands of 5.7 kb and 2.2 kb, respectively. Finally, genomic DNA from the three strains was probed with a 517 bp *Nhe*I-*Bcl*I fragment from that portion of *ubiA* still anticipated to be present in the mutant construct. A single copy of this fragment was present in both wild-type and mutant strains, whereas two copies were present in the cointegrate (data not shown).

Growth phenotype of the *ubiCA* mutant

In contrast to both wild-type and cointegrate strains, the *ubi* mutant was unable to grow aerobically on succinate minimal medium plates. However, the *ubi* strain was

Fig. 1. Construction and verification of a *ubiCA* mutant. (a) Organization of the *ubiCA* operon in a wild-type strain (top) and the *Bam*HI sites for cloning the *Km^R* cartridge. Convergent arrows indicate the positions of the primers used in PCR analysis and the anticipated product size. Below is shown the mutant construct and the anticipated PCR product size. (b) Gel electrophoresis of the PCR products in wild-type (MG1655, lane 1), cointegrate (RKP4151, lane 2) and the *ubiCA* knockout mutant (RKP4152, lane 3). Lanes marked L show a 1 kb ladder (BRL); the sizes of the observed fragments are marked.

Table 2. Growth yields of strains MG1655 (wild-type) and RKP4152 (*ubi*) grown on similar, limiting concentrations of carbon sources

Growth yields were measured as turbidity, using Klett units. Strains were cultivated in MOPS defined medium supplemented with 0.3% Casamino acids and in the presence of the indicated carbon sources, either aerobically (no further supplements) or anaerobically with nitrate or fumarate as electron acceptor. Turbidities represent maximum growth yields with 3 mM carbon source, which was experimentally verified as growth-limiting.

Carbon source	Electron acceptor	Growth yield (Klett units)	
		MG1655 (<i>ubi</i> ⁺)	RKP4152 (<i>ubiCA</i>)
Glucose	O ₂	194	23
Glycerol	O ₂	168	24
Succinate	O ₂	125	0
Glycerol	NO ₃ ⁻	40	36
Glycerol	Fumarate	26	26

able to grow anaerobically on nitrate/fumarate/glycerol minimal medium plates. We also determined the relative growth yields of the *ubi* mutant and wild-type strains on limiting concentrations of carbon sources under various conditions of growth in MOPS defined media (Table 2). The concentration of each carbon source used was shown to be limiting by the linear dependence of growth yield on substrate concentration in the 1–5 mM range (not shown). The aerobic growth yield of the *ubiCA* mutant was reduced to 12–14% of the yield obtained for the *Ubi*⁺ strain when limiting glucose or glycerol were provided as sole carbon sources. Q is widely reported to participate in electron transfer to nitrate (for a review

see Gennis & Stewart, 1996) but a *ubi* mutation was without effect on anaerobic growth yields on nitrate (Table 2). These data confirm the findings of Cox *et al.* (1970) and also demonstrate that fumarate respiration is unaffected by a *ubi* mutation (Table 2).

Oxygen consumption rates

The total electron flux of the respiratory chain is reflected in the oxygen consumption rate. Membranes from the isogenic strains RKP4152 (*ubi*) and MG1655 (wild-type) were prepared; the oxygen utilization rates with NADH and lactate as electron donors are shown in

Table 3. Respiration rates of strains MG1655 (wild-type) and RKP4152 (*ubi*) and the effects of exogenous ubiquinone

Substrate (+ exogenous quinone)	Oxygen uptake [nmol O ₂ min ⁻¹ (mg membrane protein) ⁻¹]	
	MG1655 (<i>ubi</i> ⁺)	RKP4152 (<i>ubiCA</i>)
NADH	34.0	6.5
NADH + Q-1	68.0	35.5
Lactate	15.0	4.3
Lactate + 80 µM Q-1	43.3	23.4

Fig. 2. Photodissociation spectra of dithionite-reduced, CO-ligated membranes from (a) MG1655 (*ubi*⁺) and (b) RKP4152 (*ubiCA*) in the absence of oxygen at -100°C . Each spectrum was recorded immediately after photolysis in the dual-wavelength scanning mode, with 575 nm as reference wavelength at 2.86 nm s^{-1} and a spectral band width of 4 nm. The pre-photolysis reduced + CO *minus* reduced + CO baseline is shown as a dashed line. The protein concentrations were 10 mg ml^{-1} for both (a) and (b).

Table 3. The oxygen uptake rates were clearly much lower in the membranes from the *ubi* strain and were stimulated by the addition of Q-1. Q deficiency affected NADH oxidase dramatically, diminishing the rate of oxygen uptake to only 20% of that in MG1655 membranes. Exogenous Q restored the oxygen uptake of the *ubi* mutant to wild-type levels and increased the rate of oxygen uptake in MG1655 membranes twofold. No

further enhancement was observed by adding Q-1 to higher concentrations. Lactate oxidation was reduced in the *ubi* membranes to 29% of wild-type levels. Addition of Q-1 enhanced respiration rates more than fivefold in the mutant membranes but less than threefold in the wild-type membranes.

Spectral characterization

Reduced *minus* oxidized difference spectra of membranes prepared from wild-type and *ubi* cells grown aerobically and anaerobically were recorded at room temperature (not shown). The most distinctive feature of these spectra was the peak at 556 nm due to cytochrome *b* reduction. The *ubiCA* mutation in strain RKP4152 had no significant effect on the cytochrome *b* content, i.e. both wild-type and *ubi* mutant membranes from anaerobically grown cells contained cytochromes *b* at a concentration of $0.18\text{ nmol (mg protein)}^{-1}$. Cytochrome *d* was also evident at about 630 nm. The ligand binding haem O of cytochrome *bo'* makes a small contribution to the bands near 556 nm and cannot be distinguished spectrally from cytochromes *b* in such samples.

Since static difference spectra obtained after total reduction and oxidation with dithionite and persulfate, respectively, provide no information on the reactivity of the cytochromes with either oxygen or other electron carriers, we exploited the low-temperature photodissociation behaviour of the terminal oxidases of *E. coli* to measure turnover kinetics. Identification of the oxidase components detectable by this approach is illustrated in Fig. 2, which shows that photolysis, of reduced CO-ligated cytochromes in membranes from either wild-type or *ubiCA* strains in the absence of oxygen at -100°C , revealed a distinctive photodissociation spectrum. In such spectra, peaks above the baseline (reduced + CO *minus* reduced + CO) arise from the unligated cytochrome formed by photolysis of the CO adduct, whilst troughs arise from loss of the CO adduct. Photodissociation spectra of both strains reveal a 438 nm peak and a 420 nm trough: these signals are each at wavelengths too high to be attributed to cytochrome *o* (415 nm trough, 430 nm peak; Poole, 1983). Although cytochrome *bd* is present in these membranes, as revealed by the presence of distinctive-

Fig. 3. Absorbance difference changes (438 minus 420 nm) after photodissociation of cytochrome b_{595} in membranes from MG1655 (*ubi*⁺) (□, protein concentration 10 mg ml⁻¹) and RKP4152 (*ubiCA*) (●, protein concentration 20 mg ml⁻¹) in the presence of oxygen at -100 °C. Spectra were recorded after photolysis of the CO-ligated reduced sample and at the time intervals shown. Successive spectra were recorded in the dual-wavelength mode, with 575 nm as reference wavelength at 2.86 nm s⁻¹ and a spectral band width of 4 nm.

α -signals in the 630–650 nm region of reduced *minus* oxidized difference spectra (results not shown), the Soret signals in Fig. 2 cannot be due to cytochrome *d*, because in the absence of oxygen the recombination of cytochrome *d* is too fast to be observed at liquid nitrogen temperature (Poole *et al.*, 1982). The Soret signals in Fig. 2 are indistinguishable from those in photodissociation spectra of a *cyo* mutant of *E. coli* and here, as previously (D'mello *et al.*, 1996), they are attributed to cytochrome b_{595} , the high-spin cytochrome *b* in the cytochrome *bd*-type oxidase complex.

To quantify the turnover of cytochrome b_{595} identifiable in photodissociation spectra, similar experiments were performed after supplementing the CO-ligated membranes with oxygen immediately before freezing under conditions where the oxygen does not displace CO from the oxidase haem(s). Photolysis of such samples, reduced with 10 mM NADH at 37 °C for 1 h, at -100 °C (not shown) generated photodissociation spectra for membranes from both strains that were similar in shape to those shown in Fig. 2 (recorded in the absence of oxygen). However, in the case of oxygen-supplemented membranes, the changes in absorbance due to ligand recombination were faster than those in the absence of oxygen, as observed previously (Poole & Chance, 1981). The kinetics of combination and reaction with ligands may be determined by measuring loss of the spectral signals assigned to the unligated form (i.e. the 438 nm peak) and/or gain in absorbance due to ligand binding (i.e. the 420 nm trough). Spectra recorded sequentially after photolysis at intervals of several minutes revealed a decrease in the intensity of the 438 nm peak, but no change in the intensity of the 420 nm trough (data not

shown), indicating reoxidation of the reduced cytochrome. Fig. 3 shows time-resolved changes in absorbance at these wavelengths ($\Delta A_{438-420}$). Observation of the reaction progress over 90 min showed the reaction to be markedly faster in the case of *Ubi*⁻ membranes. The simplest explanation of these kinetics is that the faster absorbance changes in *Ubi*⁻ membranes reflects inhibition of electron flux to the oxidase by Q deficiency with consequently faster loss of the intermediate represented by the spectral changes.

Multiple-wavelength recordings of cytochrome oxidoreduction kinetics

Previous attempts have been made to identify those electron-transfer reactions in the respiratory chain mediated by Q. The principle of this method is that, in the aerobic steady state (Chance & Williams, 1956), when both substrate and oxygen are present in excess, the cytochromes become partially reduced, and the amount of this reduction is dependent on the relative activities of dehydrogenases and oxidases. Ubiquinone deficiency equates with 'inhibition' of electron transfer, such that components upstream of Q become more reduced in *Ubi*⁻ membranes, whereas components downstream become more oxidized due to continued electron transfer to oxygen, without replacement of electrons normally donated by Q. Several dual-wavelength and split-beam spectrophotometric experiments involving time-sampling after substrate addition have given conflicting results (see Discussion). In the present work, we exploited the ability to record *continuously* the absorbance changes in cytochrome(s) *b* in turbid suspensions, without the need for sampling or interruption to the stirring and aeration of the sample.

The levels of reduction of cytochrome(s) *b* in aerobic steady states in membranes prepared from aerobically and anaerobically grown cells are shown in Fig. 4. In all cases, levels of cytochrome reduction are presented as a percentage of that attained with sodium dithionite. Membranes were clearly not fully reduced, showing that the sample did not become anaerobic during spectrophotometric observation. When NADH was added to membranes from aerobically grown *Ubi*⁺ cells (Fig. 4a), rapid reduction of cytochrome *b* was observed, reaching a level of about 35% of that attained with dithionite in 1 min. In membranes from the *ubiCA* mutant, reduction was slower, as anticipated for a carrier downstream of Q. However, on prolonged observation, the level of cytochrome *b* reduction attained exceeded that in the *Ubi*⁺ membranes. When lactate was provided as substrate (Fig. 4b), initial rates of cytochrome *b* reduction were similar, but again the level of cytochrome *b* reduction observed in the *Ubi*⁻ membranes after 20 min exceeded that in *Ubi*⁺ membranes. Similar results were obtained with succinate (not shown). In membranes from anaerobically grown cells, which could be grown to higher yields, cytochrome *b* reduction exceeded control levels (*Ubi*⁺ membranes) in experiments in which NADH (Fig. 4c) or lactate (Fig. 4d) were provided, reaching about 50% of the level attained with dithionite

Fig. 4. Kinetics of cytochrome *b* reduction in membranes from MG1655 (*ubi*⁺) (□) and RKP4152 (*ubi*CA) (●) as measured by continuous dual-wavelength spectrophotometry of a constantly stirred suspension oxidizing the following substrates: NADH (a, c, e); lactate (b, d). Traces in (a) and (b) were obtained with membranes from aerobically grown cells; traces in (c), (d) and (e) were obtained with membranes from anaerobically grown cells. In (e), Q-1 was added to membranes from both *ubi* and wild-type strains at the points indicated by the arrows. Trace (f) shows a portion of the continuous ΔA recording from the multi-wavelength spectrophotometer used to obtain the data points in (e) and 100% reduction corresponds to $\Delta A_{560-575}$ of about 0.3. The protein concentrations (mg ml⁻¹) for wild-type and *ubi* strains, respectively, were: (a) 5.7, 3.2; (b) 3.3, 1.5; (c) 5.0, 6.0; (d) 4.0, 13.0; (e) 8.0, 14.0.

in 8 min, compared with 10–20% reduction in *Ubi*⁺ membranes. The same pattern was obtained when formate or glycerol 3-phosphate was used as substrate for anaerobically grown cells. To demonstrate that the enhanced level of reduction in *Ubi*⁻ membranes was a direct consequence of Q deficiency, such membranes were supplemented with exogenous Q-1 during the steady state, when cytochrome *b* reduction had reached about 60% of the dithionite level (Fig. 4e, f). An immediate decrease in reduction level occurred to a value similar to that measured in control membranes. In

contrast, addition of Q-1 to *Ubi*⁺ membranes elicited no significant change in cytochrome *b* reduction.

DISCUSSION

A Q-deficient mutant has been constructed using a gene replacement technique (Hamilton *et al.*, 1989) that obviates the requirement for construction in a strain that is *polA*, *recBC* or *recD*. The method works because the presence of the pSC101 replication origin within the *E. coli* chromosome dramatically reduces the growth rate

of the cell. Therefore, pSC101 cointegrates grow slowly at 30 °C, whereas cells in which the plasmid has been resolved from the co-integrate demonstrate normal growth rates. The mutant genetic construct produced in this study was verified using both Southern blotting and PCR techniques.

The most striking phenotype of the *ubiCA* mutant was an inability to grow aerobically on succinate as carbon and energy source, in agreement with previous studies that exploited the ability of quinone analogues to reconstitute electron transfer in membranes from *ubiA menA* mutants isolated after chemical mutagenesis (Wallace & Young, 1977). Studies with the deletion-insertion mutant used here demonstrate unequivocally that Q cannot be functionally replaced by menaquinone or demethylmenaquinone in growth on succinate. Growth on glucose or glycerol, although diminished in the *ubiCA* mutant, was not abolished, presumably as a result of fermentation (in the case of glucose) or use of alternative quinones between dehydrogenases and oxidases. Anaerobic growth yields on nitrate or fumarate as electron acceptors were unaffected in a *ubiCA* mutant, consistent with earlier literature based on the use of chemically obtained mutants (Gennis & Stewart, 1996). As reported by Wallace & Young (1977), oxidation of lactate or NADH by membranes from the *ubiCA* mutant was restored by addition of a ubiquinone analogue (Q-3 or, in our experiments, Q-1).

Both well-characterized terminal oxidases in *E. coli*, cytochromes *bo'* and *bd*, are ubiquinol oxidases. Cytochrome *bd* oxidizes ubiquinol in a lipid bilayer and an inter-helical region on the periplasmic side of the cytoplasmic membrane – the Q-loop – has been proposed as the quinol-binding site. In the cytochrome *bo'* complex, CyoA has been implicated in quinone binding just as subunit II of the mammalian cytochrome *c* oxidase binds and reduces the electron donor (cytochrome *c*). In both *E. coli* oxidases, electrons are assumed to be transferred from ubiquinol to a *b*-type cytochrome of the oxidase complex. This work provides direct confirmation of electron transfer from Q to the haem(s) of cytochrome *bd*. The photodissociation spectra shown in Fig. 2 are attributable to cytochrome *b*₅₉₅ in the cytochrome *bd* complex (see Results). Following photodissociation of CO, the reduced unligated form of the cytochrome is formed. At the temperatures used here, the reduction level of this cytochrome decreased only slowly in membranes from a *Ubi*⁺ strain, when observed over a 60 min period, presumably as a result of electron flux from upstream in the respiratory chain. However, in membranes from a *ubiCA* mutant, the cytochrome is more rapidly re-oxidized as evidenced by the diminishing peak at 438 nm. The observation that the 419 nm trough, due to the loss of the CO-ligated form, does not approach the baseline confirms that the reactions being observed are oxidoreductions and not religation of CO (which would cause symmetrical approach of the trough and peak towards the baseline, representing the fully ligated state).

An important finding from studies of cytochrome *b* reduction levels in respiring membranes is that schemes that implicate only a single pool of Q between respiratory substrates and oxygen are oversimplified. The present data are in agreement with earlier models for the organization of the *E. coli* respiratory chain (Cox *et al.*, 1970; Downie & Cox, 1978) but not the simplified scheme subsequently suggested by Au *et al.* (1984) and widely illustrated. Downie & Cox (1978) used a Q-deficient mutant (*ubiA351 ubiB409*) to determine the sequence of cytochromes and Q in the aerobic electron-transport chain of *E. coli*. The amounts of cytochrome(s) reduced during aerobic steady-state conditions were estimated by freeze-trapping samples at timed intervals and recording difference spectra (i.e. part-reduced *minus* oxidized) on these samples. Two sites for Q involvement were proposed: one between NADH and lactate dehydrogenases and cytochrome *b*, and a second between *b*-type cytochromes and the oxidases. This conclusion was consistent with earlier studies utilizing a *ubi* mutant susceptible to reversion and in which cytochrome reduction levels were assayed by dual-wavelength spectrophotometry (560 nm *minus* 570 nm) after attainment of a steady state during NADH or lactate oxidation (Cox *et al.*, 1970). In both earlier studies and the present work, cytochrome *b* reduction was expressed as a percentage of that attained on depletion of oxygen: Cox *et al.* (1970) reported 23–24% cytochrome reduction in the *ubi* mutant and 8–10% reduction in the *ubi*⁺ strain, whereas Downie & Cox (1978) reported values of 50–60% and 5–10%, respectively. Kita & Anraku (1981) drew similar conclusions based on the effects of the inhibitor piericidin A, a structural analogue of Q. In the aerobic steady state, monitored by freeze-trapping samples and difference spectroscopy, the inhibitor caused cytochrome *b*₅₅₆ to become more reduced than cytochrome *b*₅₆₂, indicating a role for Q upstream of the latter cytochrome.

Subsequent experiments by Au *et al.* (1984) yielded different results. These authors used *ubi* mutants isolated after chemical mutagenesis into which a *cyd* mutant allele was introduced to investigate the electron transfer steps prior to cytochrome *bo'* only. The tendency of these strains to revert to *Ubi*⁺ was noted but 'only those cultures which contained no revertants were used'. In marked contrast to earlier data, cytochrome *b* reduction, measured at 560 *minus* 575 nm at timed intervals on a sample in an oxygen-bubbled cuvette, was lower (0–<20% full reduction) than in membranes from *Ubi*⁺ cells (5–25%). These data suggested that Q exists as a single pool between the dehydrogenases and cytochrome *bo'*. Au *et al.* (1984) attributed the discrepancy to the protocols used earlier for maintaining an aerobic steady state and suggested that the samples trapped in the experiments of Downie & Cox (1978) were oxygen-depleted, leading to artefactually high cytochrome reduction levels.

In the present work, we have attempted to improve the protocols used previously as follows. (1) Most importantly, we have constructed and used a knockout

Fig. 5. Schematic diagram of some components of the aerobic respiratory chains of *E. coli*. NADH is oxidized by one of two dehydrogenases; Nuo comprises 14 subunits (not indicated). LldD and Dld are the dehydrogenases for L- and D-lactate, respectively. A pool of quinone (circled) is shown as the immediate reductant for two terminal oxidases, cytochrome *bo'* (CyoABCD) and cytochrome *bd* (CydAB), each of which is capable of reducing oxygen. Components not enclosed are a putative third oxidase (CbdAB, Sturr *et al.*, 1996) and one or more *b*-type cytochromes, suggested to be upstream of Q in the present work (see Discussion).

mutant having a deletion and insertion at the junction of the *ubiC* and *ubiA* genes, which encode the first two steps of the biosynthetic pathway in *E. coli*. (2) Dual-wavelength spectrophotometry was performed continuously without time sampling or attempting to trap redox states. We regard this approach as preferable to sampling, which would allow recording of difference spectra, since the various *b*-type cytochromes have barely distinguishable spectral properties (see Gennis, 1987). In principle, diode-array spectroscopy should allow direct kinetic analysis of multiple wavelengths (see, for example, Poole *et al.*, 1994), but commercially available instruments do not allow satisfactory spectral sensitivity or resolution with turbid samples. (3) Membrane samples were continuously aerated by a custom-made magnetic stirring device during the experiment, i.e. throughout the period of spectrophotometric recordings. Au *et al.* (1984) reported having to stop the bubbling of oxygen through the cuvette when spectrophotometric recordings were made at about 2.5 min intervals. (4) Reconstitution of oxidase activity *in vitro* by addition of Q to membranes from the *ubi* mutant was used to assign unequivocally the measured redox state to Q deficiency. This was accompanied by a decrease of the reduction level of cytochrome *b* to the level in membranes from *Ubi*⁺ cells.

The present data showing enhanced cytochrome *b* reduction in membranes from a *ubi* mutant are not consistent with the simplest schemes that propose a single Q pool upstream of a single location for cytochrome *b*. A number of possible explanations exist. Since both oxidase complexes interact with ubiquinol, yet cytochrome *b* reduction is increased in *Ubi*[−] membranes, a second pool of cytochrome(s) *b* might occur upstream of a single Q site, as proposed by Downie & Cox (1978), Kita & Anraku (1981) and Cox *et al.* (1970). The identity of this cytochrome is unknown, but Downie & Cox (1978), on the basis of liquid nitrogen temperature spectroscopy of trapped samples, proposed that both cytochrome *b*₅₆₂ and *b*₅₅₆ are upstream of a Q pool. We have made no attempt to distinguish between the *b*-type cytochromes, favouring continuous monitoring of a single wavelength pair. In the case of reduction by NADH in membranes from aerobically

grown cells, we noted that cytochrome *b* reduction was slower after adding substrate to *Ubi*[−] membranes than in the case of *Ubi*⁺ membranes (Fig. 4a). A similar observation was made by Downie & Cox (1978) and attributed to a site for Q function upstream of cytochrome *b*. Interestingly, the slower reduction of cytochromes *b* by added NADH (Fig. 4a) was not observed in membranes from anaerobically grown cells (Fig. 4c); indeed, the reduction rates for *Ubi*⁺ and *Ubi*[−] membranes were reversed. This may reflect the differential regulation of two NADH dehydrogenases (NDH) by oxygen. The level of NDH-II is repressed under anaerobic or low-aeration conditions (Green & Guest, 1994), suggesting that under such conditions (Fig. 4c) NADH oxidation is via NDH-I. One subunit of this 14-subunit intrinsic membrane protein, NuoH, is presumed to be a Q-binding subunit (Weidner *et al.*, 1993), although conflicting views exist (Friedrich *et al.*, 1994). The possibility remains that NDH-I donates electrons to a bound Q, thence to cytochrome(s) *b* of unknown identity (see below), and then to a mobile Q pool which is the reductant for the terminal oxidases.

These data predict that cytochrome(s) *b* should be kinetically heterogeneous following reaction of the reduced respiratory chain with oxygen. Indeed, Haddock *et al.* (1976) resolved two components in stopped-flow experiments: a fast-reacting component (probably comprising haems in the oxidase complexes) and a much more slowly oxidized species (half-time for oxidation about 30 ms) which may be the *b*-type cytochrome upstream of Q shown in Fig. 5.

There are several possible candidates for the upstream cytochrome *b*. (1) Cytochrome *b*₅₅₆ (SdhC, CybA) which is a highly hydrophobic oligomer with a redox potential of *E*_m −45 mV has been purified (Kita *et al.*, 1978) and is an anchor polypeptide of the succinate dehydrogenase complex (Murakami *et al.*, 1985). However, this is unlikely to be the sole explanation since we have observed enhanced cytochrome *b* reduction also with anaerobically grown cells (where succinate dehydrogenase synthesis is repressed) and with a variety of substrates other than succinate. (2) Another candidate is cytochrome *b*₅₆₁, encoded by *cybB* (Murakami *et al.*, 1984) and purified and characterized by Murakami *et al.*

(1986). It is a low-potential dihaem cytochrome *b* ($E_m + 20$ mV) of unknown function but might function upstream of *Q* ($E_m + 113$ mV). This cytochrome is reducible by NADH and D-lactate. However, a knock-out *cyb* mutant decreased NADH oxidase activity by 24% and L-lactate by 18% only, compared to a wild-type (Yamato *et al.*, 1988). (3) A further, less likely possibility is that a soluble cytochrome such as b_{562} (CybC), which is found in the periplasm (Trower, 1993), might be membrane-associated. Neither of the NADH dehydrogenases or lactate dehydrogenases has been reported to contain haem B (Gennis & Stewart, 1996).

Another explanation of these results could be the presence of an intramolecular *Q* tightly bound within a respiratory component receiving electrons from a low-spin haem *b*. Indeed, a novel high-affinity binding site (Q_H) distinct from the low-affinity quinol oxidation site (Q_L) has been identified in the *E. coli* cytochrome *bo'*-type ubiquinol oxidase (Sato-Watanabe *et al.*, 1994). The Q_H site is located close to the Q_L site in subunit II and low-spin haem b_{562} in subunit I. It is proposed that Q_H mediates intramolecular electron transfer from UQH_2 in the Q_L site to low-spin haem *b* as a one-electron transfer gate, producing a highly stabilized ubisemiquinone radical (Sato-Watanabe *et al.*, 1995). Tight binding of the semiquinone radical confers kinetic and thermodynamic stabilization and prevents destructive side reactions. Using flow-flash and stopped-flow techniques, the reaction of cytochrome *bo'* with oxygen showed either multiphasic kinetics as in cytochrome *c* oxidase or monophasic kinetics, depending on the isolation procedure – i.e. with or without the presence of a bound ubiquinone molecule (Puustinen *et al.*, 1996). The reduction of dioxygen to water requires four electrons obtained from oxidation of two quinols to quinone. Both cytochrome *bo'*- and *bd*-type terminal oxidases possess only three metal centres each. This suggests that the bound semiquinone, which remains after reduction of all three metal centres, provides the fourth electron (Ingledew *et al.*, 1995). However, the *bd*-type oxidase is the major oxidase identified in our membrane preparations and in this oxidase the presence of a tightly bound ubiquinone has not yet been reported.

Similarly, the UV-sensitive component of purified *E. coli* nitrate reductase has been identified as MQ-9. Stoichiometric amounts of MQ-9 were found specifically bound to the $\alpha\beta$ -dimer and are suggested to participate in intraprotein electron transfer to nitrate (Brito *et al.*, 1995). Likewise, participation of a specifically bound quinone has also been suggested in *E. coli* fumarate reductase (Westenberg *et al.*, 1993). Clearly, further work is required to unravel the complexities of respiratory electron transport in the multiply branched respiratory chains of this bacterium.

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