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AEROBIC RESPIRATION IN MUTANTS OF *ESCHERICHIA COLI* ACCUMULATING QUINONE ANALOGUES OF UBIQUINONE

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SUMMARY

The ability of three naturally occurring analogues of ubiquinone to function in aerobic respiration in *Escherichia coli* has been studied. The compounds, which differ from ubiquinone in terms of the substituents on the quinone ring, accumulate in the cytoplasmic membranes of *ubiE*$^-$, *ubiF*$^-$ and *ubiG*$^-$ mutants. One of the analogues (2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, MMQ), which lacks the 5-methoxyl group of the benzoquinone ring of ubiquinone promoted the oxidation of NADH, D-lactate and α-glycerophosphate but not succinate. Electron transport supported by MMQ was found to be coupled to phosphorylation. In contrast, 2-octaprenyl-6-methoxy-1,4-benzoquinone, which lacks both the 3-methyl and 5-methoxyl groups of ubiquinone, and 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, in which the 5-methoxyl group of ubiquinone is replaced by an hydroxyl group, were virtually inactive in the oxidases tested. The ability of MMQ to function in respiration in isolated membranes is consistent with the findings that the growth rate and yield of a *ubiF*$^-$ strain, unlike other *ubi*$^-$ strains, were only slightly lower than those of a *ubiF*$^+$ strain.

The fact that MMQ is active in some but not all oxidases provides further support for the concept that the quinones link the individual dehydrogenases to the respiratory chain and that each dehydrogenase has specific structural requirements for quinone acceptors.

Abbreviations: Q, ubiquinone; MK, menaquinone; DMK, dimethylmenaquinone; DMK/MK, the naturally occurring mixture of DMK and MK formed by *E. coli*; MQ, 2-octaprenyl-6-methoxy-1,4-benzoquinone; MMQ, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone; MHMQ, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. The number of isoprene units in the sidechains of the different quinone homologues is indicated by the number after the abbreviation (e.g. Q-8).

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INTRODUCTION

Several classes of ubiquinone-deficient mutants have now been used to study the function of ubiquinone in aerobic respiration in *Escherichia coli* K12 [1–3]. The mutants studied to date have been blocked early in the biosynthetic pathway to ubiquinone prior to the generation of quinonoid intermediates. For example a *ubiB*$^-$ strain which accumulates 2-octaprenylphenol was used in the studies of Cox et al. [1] and in the accompanying paper [3] we have made use of a *ubiA*$^-$ strain in which no octaprenyl intermediates accumulate due to a block in the octaprenyltransferase reaction [4].

Three other classes of ubiquinone mutants of *E. coli* (*ubiE*$^-$, *ubiF*$^-$ and *ubiG*$^-$) have recently been described [5, 6]. These mutants are blocked in the final three biosynthetic reactions of the pathway [7] and accumulate octaprenylbenzoquinone derivatives (Fig. 1) in their respiratory membranes. In this paper we report on the ability of these naturally occurring Q-8 analogues to function in aerobic respiration.

MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of *E. coli* K12 and are described in Table I.

Chemicals. Q-3 and MK-1 were kindly donated by Dr. O. Isler of F. Hoffmann La Roche and Co., Basle, Switzerland. Spectinomycin was a generous gift from Dr. G. B. Whitfield, the Upjohn Co., Kalamazoo, U.S.A. 2-Succinylbenzoic acid was synthesized as described previously [8].

Media. Minimal medium [6] was used at double strength. Supplements were sterilized separately and, unless otherwise stated, were added at the following concen-

TABLE I

STRAINS OF *E. COLI* K12 USED

Strain	Sex	Relevant genetic loci[a]	Other information
AB3311	Hfr	*metB*$^-$	
AN151	Hfr	*metB*$^-$, *ubiG*423	Derived from AB3311 by mutagenesis with NTG[b]
AN329	Hfr	*metB*$^-$, *ubiG*423, *aroB*$^-$, *str*^{R}	*aroB*$^-$ transductant of AN151
AN185	Hfr	*leu*$^-$, *thr*$^-$, *ubiE*$^+$	*metE*$^+$ transductants derived from AB2154
AN130	Hfr	*leu*$^-$, *thr*$^-$, *ubiE*401	
AN570	Hfr	*leu*$^-$, *thr*$^-$, *ubiE*401, *aroE*$^-$, *spc*^{R}	*aroE*$^-$ transductant of AN130
AN147	F$^-$	*ura*$^-$, *ubiF*$^+$	*gltA*$^+$ transductants of W620
AN146	F$^-$	*ura*$^-$, *ubiF*411	
AN572	F$^-$	*ura*$^-$, *ubiF*411, *aroE*$^-$, *spc*^{R}	*aroE*$^-$ transductant of AN146
AN573	F$^-$	*ura*$^-$, *ubiF*$^+$, *aroE*$^-$, *spc*^{R}	*aroE*$^-$ transductant of AN147
W620	F$^-$	*ura*$^-$, *gltA*$^-$	

[a] Genetic nomenclature is that used by Bachmann et al. [11].

[b] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

trations: D-glucose, 30 mM, casamino acids, 0.1 %; succinate, 30 mM; thiamine · HCl, 1 μM; leucine, 0.8 mM; threonine, 0.7 mM; uracil, 0.2 mM; methionine, 0.2 mM; phenylalanine, 0.2 mM; tyrosine, 0.2 mM; tryptophan, 0.1 mM; 4-aminobenzoate, 1 μM; 2,3-dihydroxybenzoate, 0.1 mM; 4-hydroxybenzoate, 1 mM; and 2-succinylbenzoate, 0.1 mM.

Growth tests. Growth tests were carried out using 10-ml volumes in 125-ml sidearm flasks shaken at 37 °C. The turbidity of cultures was measured hourly using a Klett Summerson colorimeter fitted with a blue filter.

Genetic techniques. The *aroB*$^-$ allele was transduced into strains by cotransduction with the *str*^{R} allele as described elsewhere [4]. Using an analogous method, the *aroE*$^-$ allele was introduced into strains as an unselected marker by P_1-mediated transduction from a *spc*^{R}*aroE*$^-$ donor selecting for resistance to spectinomycin (200 μg/ml).

Determination of products of glucose metabolism. The supernatants of cultures grown on limiting glucose (6 mM) were assayed for various fermentation products by chromatography using a Shimadzu GC-IC gas chromatograph with a flame ionization detector. For volatile products, (ethanol and acetic acid) culture supernatants were acidified to pH 1 with 10 M HCl and 4-μl samples injected into the gas chromatograph. The conditions were as follows: glass columns 6 ft × 1/8 inch; packing, porapak Q 100–200 mesh; column temperature, 155–190 °C at 4 °C/min; injection block temperature, 150 °C; detector temperature, 215 °C; flow rates: N_2, 20 ml/min; H_2 and air for flame ionization detector, 25 ml/min and 0.8 kg/cm^2, respectively.

For non-volatile products (succinic and D-lactic acids), 5 ml of culture supernatant was acidified to pH 1 with 10 M HCl and extracted continuously with diethyl ether (20 ml) for 1 h. The ether was evaporated almost to dryness, the flask rinsed with ethanol (1 ml) and the ethanol transferred to a small glass-stoppered tube. The ethanol was evaporated off under reduced pressure and 0.5 ml of silylating agent (0.7 ml "Supelco" hexamethyldisilazane/trimethylchlorosilane +1.8 ml anhydrous pyridine) added. After 5 min at room temperature 2 μl was injected into the gas chromatograph. The conditions for chromatography were as follows: glass columns 6 ft × 1/8 inch; packing, 5 % OV 101 on varaport 30; column temperature, 70–160 °C at 6 °C/min; inlet temperature, 150 °C; detector temperature, 220 °C; flow rates: N_2, 50 ml/min; H_2, 50 ml/min; air, 0.8 kg/cm^2.

In both cases linear standard curves were obtained (based on peak heights) when standards prepared in minimal medium were assayed.

Growth of cells and preparation of membranes. All strains, with the exception of AN570, were grown in minimal medium supplemented with glucose and casamino acids in addition to other specific requirements. Membranes were prepared as described elsewhere [3]. The media used for strains AN329 and AN572, which carry *aroB*$^-$ and *aroE*$^-$ alleles, respectively, were supplemented with phenylalanine, tyrosine, tryptophan, 4-aminobenzoate, 4-hydroxybenzoate and 2,3-dihydroxybenzoate. In the case of strain AN570, brain-heart infusion broth (Oxoid) was used in order to minimize the reversion of the *ubi*$^-$ allele which occurred during growth in minimal medium. The inoculum for strain AN570 was prepared using brain-heart infusion plates supplemented with phenylalanine, tyrosine, tryptophan, 4-aminobenzoate, 2,3-dihydroxybenzoate, 4-hydroxybenzoate and 2-succinylbenzoate. The addition of 2-succinylbenzoate, an intermediate of the MK pathway [8] allowed the

synthesis of low levels of MK-8 which further helped to reduce reversion of the *ubi*$^-$ allele. After overnight incubation the cells were harvested and grown in brain-heart infusion broth (10 l), supplemented with 4-hydroxybenzoate (1 mM), for a further three generations during which time MQ-8 continued to accumulate but the MK-8 was diluted out.

Oxidase rates. These were measured as described elsewhere [3].

Protein estimation. Protein concentrations were measured by the method of Lowry et al. [9] using bovine serum albumin fraction V as standard.

Estimation of quinones. The estimation of Q-8, DMK-8, and MK-8 in membrane preparations has been described elsewhere [3]. The same procedure was used for the determination of MQ-8, MMQ-8 and MHMQ-8 with the following variations. After extraction and chromatography, the lemon-coloured bands of MQ-8 and MMQ-8 (R_F 0.4) were eluted with ethanol and rerun on silica gel plates using ethyl-acetate/hexane (25 : 75, v/v) as solvent in order to separate any Q-8 present [5]. In this solvent Q-8 (R_F 0.6) runs slightly ahead of both MQ-8 and MMQ-8. In the case of MHMQ-8 the initial chromatography was carried out on silica gel plates containing 0.7 % (v/v) H_2SO_4 and the orange-brown MHMQ-8 band (R_F 0.6) was eluted with diethyl ether. In this system Q-8 also runs slightly ahead of the MHMQ-8 band. The ether solution containing the MHMQ was evaporated to dryness under reduced pressure and the MHMQ redissolved in ethanol. The concentrations of MQ-8, MMQ-8 and MHMQ-8 were estimated by measuring the reduction in absorbance at 264, 270 and 275 nm, respectively, after the addition of solid $NaBH_4$. The following molar extinction coefficients were used: $\Delta\Sigma_{MQ}$, 9500; $\Delta\Sigma_{MMQ}$, 11 300; $\Delta\Sigma_{MHMQ}$, 4700.

RESULTS

Function of octaprenyl analogues of ubiquinone in aerobic respiration

MQ-8, MMQ-8 and MHMQ-8 (Fig. 1) are intermediates in the biosynthesis of Q-8 and accumulate in the cytoplasmic membranes of *ubiE*$^-$, *ubiF*$^-$ and *ubiG*$^-$ mutants, respectively. These compounds differ from Q-8 in terms of the substituents on the benzoquinone ring but possess the same all-*trans*-octaprenyl sidechain. The ability of these Q-8 analogues to function in aerobic respiration was assessed by in vitro measurements of electron transport rates and coupled phosphorylation as well as by whole cell studies of growth rates, growth yields and the products of glucose metabolism.

To measure the extent to which MQ-8, MMQ-8 and MHMQ-8 can replace Q-8 in respiratory membranes it was necessary to prevent possible interference from

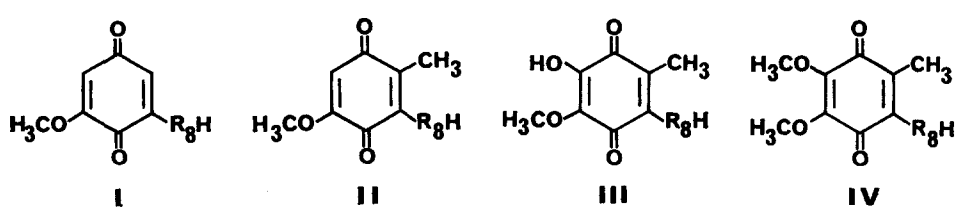

Fig. 1. Structures of the various ubiquinone analogues I, MQ-8; II, MMQ-8; III, MHMQ-8; IV, Q-8. R_8H denotes the all-*trans*-octaprenyl sidechain.

TABLE II

RATES OF OXYGEN UPTAKE AND QUINONE LEVELS IN MEMBRANES FROM MUTANTS ACCUMULATING ANALOGUES OF UBIQUINONE

Membrane from	Addition[a]	Rate of oxygen uptake[b]				Quinone level[c]	
		NADH	D-Lactate	α-Glycerophosphate	Succinate	Analogue	Q-8
AN570 (*ubiE*$^-$)	Q-3	100 (876)	100 (210)	–	100 (229)	1.7 (MQ-8)	0.04
	Nil	12	16	–	11		
AN572 (*ubiF*$^-$)	Q-3	100 (721)	100 (152)	100 (229)	100 (87)	1.3 (MMQ-8)	< 0.01
	Nil	46	51	63	8		
AN329 (*ubiG*$^-$)	Q-3	100 (837)	100 (424)	–	100 (401)	3.1 (MHMQ-8)	0.16
	Nil	6	17	–	20		

[a] Q-3 was added in 2 μl of ethanol to a final concentration of 32 μM.

[b] Rates of O_2 uptake are expressed as a percentage of the maximally stimulated rates with Q-3 added. The figures in parentheses are the absolute rates in ng atoms O/min per mg protein.

[c] Quinone levels are in nmol/mg protein. No DMK-8 or MK-8 was detected in membranes from any of the three strains. The Q-8 concentration in wild-type membranes is approx. 2 nmol/mg protein [3].

DMK/MK-8. This was achieved by transducing mutations (*aroB*$^-$ or *aroE*$^-$) affecting the common pathway of aromatic biosynthesis into the *ubiE*$^-$, *ubiF*$^-$ and *ubiG*$^-$ strains. The transductants containing the *aro*$^-$ alleles cannot synthesize any of the aromatic end-products including DMK/MK-8 but will accumulate the Q-8 analogues in their respiratory membranes provided the growth medium is supplemented with 4-hydroxybenzoate. With 4-hydroxybenzoate (1 mM) in the growth media the concentrations of MQ-8, MMQ-8 and MHMQ-8 in membranes of *ubiE*$^-$, *ubiF*$^-$ and *ubiG*$^-$ strains, respectively, were found to be similar to the Q-8 concentration in wild-type membranes (Table II). It should be noted that low concentrations of Q-8 were present in membranes of strains accumulating either MQ-8 or MHMQ-8 due to slight reversion of the *ubiE*$^-$ allele in the former case and a slight "leakiness" of the *ubiG*$^-$ allele in the latter. The Q-8 concentrations, however, were too low to influence the oxidase activities except in the case of strain AN329 (*ubiG*$^-$) where Q-8 may have been responsible for some of the low succinate oxidase activity observed. This is because we have observed that the level of Q-3 or Q-8 required for optimal succinate oxidation is less than that for NADH oxidation (unpublished observations).

The rates of oxygen uptake with membranes from both the *ubiE*$^-$ and *ubiG*$^-$ strains and NADH, D-lactate and succinate as substrates were very low. Addition of Q-3, however, stimulated these activities 5–10-fold (Table II) restoring them to wild-type levels. This indicates that neither MQ-8 nor MHMQ-8 can effectively replace Q-8 in the NADH, D-lactate or succinate oxidase systems. It was not possible to assess whether or not MQ-8 and MHMQ-8 could function in α-glycerophosphate oxidation because of the low activity of α-glycerophosphate dehydrogenase in these strains when grown on glucose.

In contrast to membranes containing MQ-8 and MHMQ-8, those containing MMQ-8 gave rates of oxygen uptake with NADH, D-lactate, α-glycerophosphate and succinate that were 46, 51, 63 and 8 %, respectively, of the maximally stimulated rates with Q-3 added (Table II). Thus MMQ-8 functions fairly efficiently in the NADH, D-lactate and α-glycerophosphate oxidase systems but poorly in the oxidation of succinate. P/O ratios were measured to determine whether or not electron transport supported by MMQ-8 was coupled to phosphorylation. As Table III shows, membranes from a *ubiF*$^-$ strain containing MMQ-8, gave P/O ratios which were similar to those of membranes containing Q-8 (*ubiF*$^+$ strain). This suggests that electron transport promoted by MMQ-8 is via the normal phosphorylating pathway. It should be

TABLE III

P/O RATIOS FOR MEMBRANES CONTAINING Q-8 AND MMQ-8

P/O ratios were measured as described elsewhere [3].

Membranes from	Quinone present	Substrate	P/O
AN573 (*ubi*$^+$)	Q-8	NADH	0.06
		D-lactate	0.06
		α-glycerophosphate	0.06
AN572 (*ubiF*$^-$)	MMQ-8	NADH	0.06
		D-lactate	0.04
		α-glycerophosphate	0.05

TABLE IV

AEROBIC GROWTH RATES AND GROWTH YIELDS OF QUINONE MUTANTS IN GLUCOSE-MINIMAL MEDIUM

Strain	Generation time[a] (min)	Growth yield (Klett units)	
		2 mM glucose	4 mM glucose
AN185 (*ubiE*$^+$)	105[b]	115	192
AN130 (*ubiE*$^-$)	210	44	99
AN147 (*ubiF*$^+$)	72	97	183
AN146 (*ubiF*$^-$)	96	82	143

[a] Strains were grown on 30 mM glucose.

[b] The different genetic backgrounds of strain AN185 and strain AN147 are presumably responsible for the different growth rates of the two *ubi*$^+$ strains.

noted that the P/O ratios for both the *ubiF*$^+$ and *ubiF*$^-$ membranes were low (generally 0.06) as expected for this type of membrane preparation. Moreover since the P/O ratios obtained with NADH were not significantly higher than those for D-lactate or α-glycerophosphate it is likely that the phosphorylation being measured was predominantly at site 2.

Physiology of strains accumulating MQ-8 or MMQ-8

The conclusion that MMQ-8 can promote electron transport and oxidative phosphorylation in respiratory membranes of a *ubiF*$^-$ strain is supported by the results of studies of growth rates, growth yields and products of metabolism of glucose of *ubiF*$^-$ and *ubiF*$^+$ strains. For comparison strains AN130 (*ubiE*$^-$) and AN185 (*ubiE*$^+$) were also included in these tests.

The mean generation time of the *ubiE*$^-$ strain on glucose-minimal medium was double that of the *ubiE*$^+$ strain and its growth yield was reduced to about 40 % of the *ubiE*$^+$ strain (Table IV). Moreover, the culture supernatant of the *ubiE*$^-$ strain contained two-thirds of the original glucose carbon as lactate and acetate (Table V). These results indicate that the *ubiE*$^-$ strain cannot oxidize the products of glycolysis as efficiently as the *ubiE*$^+$ strain. This is consistent with the observation (see above) that MQ-8 has little activity in electron transport from NADH, D-lactate and suc-

TABLE V

PRODUCTS OF GLUCOSE METABOLISM FROM UBIQUINONE-DEFICIENT STRAINS

Cultures were grown on limiting glucose (6 mM) under the same conditions as for the growth yield experiments.

Strain	Concentration (mM) in culture supernatant	
	Acetate	Lactate
AN185 (*ubiE*$^+$)	0.5	< 0.1
AN130 (*ubiE*$^-$)	3.2	5.9
AN147 (*ubiF*$^+$)	3.0	< 0.1
AN146 (*ubiF*$^-$)	5.7	1.8

cinate. The growth characteristics of the *ubiE*$^-$ strain are similar to those of the *ubiA*$^-$ strain described in the accompanying paper [3]. A detailed comparison of the two strains suggests that residual electron transport in the *ubiA*$^-$ strain may be slightly lower than that in the *ubiE*$^-$ strain resulting in a slightly lower growth yield, an increased accumulation of lactate, and a mean generation time for the *ubiA*$^-$ strain which is three times that of the *ubiA*$^+$ strain.

In contrast to the *ubiE*$^-$ strain, the mean generation time of the *ubiF*$^-$ strain was only 1.3 times that of the *ubiF*$^+$ strain and its growth yield was about 80 % that of the *ubiF*$^+$ strain (Table IV). In addition, the concentration of lactate in the supernatant of the *ubiF*$^-$ strain was reduced compared to the *ubiE*$^-$ strain (Table V). These findings are consistent with the ability of MMQ-8 to promote electron transport from NADH, D-lactate and α-glycerophosphate although not as efficiently as Q-8 (Table II). While the in vitro studies (Table III) indicate that MMQ-mediated electron transport is coupled to phosphorylation it is not possible from the P/O ratios or the data on growth yields to be certain about the efficiency of energy conservation in the *ubiF*$^-$ strain. However, since 47 % of the original glucose carbon remains in the supernatant of the *ubiF*$^-$ strain compared to 17 % in the *ubiF*$^+$ strain it would appear that the 20 % decrease in growth yield of the *ubiF*$^-$ strain is due to reduced rates of electron transport rather than inefficient energy conservation.

DISCUSSION

Studies with quinone-deficient strains of *E. coli* [1, 3] have shown that quinones are obligatory carriers in electron transport from a variety of substrates to oxygen or nitrate. As with ubiquinone in mitochondrial membranes [10], the quinones in *E. coli* appear to behave as a common pool which links the dehydrogenases to the terminal electron transport pathways [3]. The fact that both Q-8 and MK-8 promote the oxidation of α-glycerophosphate and D-lactate whereas Q-8 (but not MK-8) promotes succinate and NADH oxidation [3] indicates that the various dehydrogenases possess a degree of selectivity with respect to the quinone acceptors. This selectivity is further illustrated by the results of the present paper. Thus the Q analogue MMQ-8, which lacks the 5-methoxyl group of Q-8, promotes the oxidation of NADH, D-lactate and α-glycerophosphate but not succinate, suggesting that the 5-methoxyl group of Q-8 is a structural feature of the quinone molecule which is essential for its interaction with succinate dehydrogenase but not with the other dehydrogenases. Loss of the 3-methyl group in addition to the 5-methoxyl group results in a Q-8 analogue (MQ-8) which is inactive in the oxidation of NADH and D-lactate, as well as succinate. Replacement of the 5-methoxyl group of Q-8 by an hydroxyl group (MHMQ-8) also yields a quinone which functions poorly in electron transport.

The inability of particular quinones to function as acceptors for the various dehydrogenases could be due to unsuitable redox potentials or the absence of particular structural features required for reaction. For example, the lower redox potential of MK relative to Q [10] could explain why the former cannot function in succinate oxidation [3].

In contrast to the ring substituents, the length of the isoprenoid side chain is a less critical feature of the Q-8 molecule for activity in the four oxidases tested. As shown in the accompanying paper [3] when Q-3 was added to *ubiA*$^-$ *menA*$^-$ mem-

branes the rates of oxidation of NADH, D-lactate, α-glycerophosphate and succinate were approximately equal to those found in *ubi*$^+$ *men*$^-$ membranes. Moreover electron transport promoted by Q-3 was found to be coupled to phosphorylation.

In summary, these findings indicate that certain substituents of the benzoquinone ring of Q-8 are important in determining whether or not the quinone can interact with each of the various dehydrogenases involved in electron transport in *E. coli*. The structural requirements vary with the dehydrogenase and may in some cases reflect a necessity for a particular redox potential. Succinate dehydrogenase appears to be the most demanding since of all the quinones tested only Q-8 promoted the oxidation of succinate. In contrast, electron transfer from quinones to cytochrome *b* of the terminal chain appears to be less sensitive to structural variations of the quinone moiety.

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