Mutants of *Escherichia coli* affected in respiration: the cloning and nucleotide sequence of *ubiA*, encoding the membrane-bound *p*-hydroxybenzoate:octaprenyltransferase

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A mutant of *Escherichia coli* has been isolated that is unable to grow aerobically on non-fermentable substrates, but able to grow anaerobically on glycerol with alternative electron acceptors such as fumarate. Nitrate as electron acceptor supports anaerobic growth on glycerol, but not on succinate or lactate. Oxygen consumption rates by cell-free extracts with succinate, lactate or glycerol 3-phosphate as substrates were low relative to activities in an isogenic control strain but were restored *in vitro* by adding ubiquinone-1. Transformation of the mutant with a cloned 2.6 kb *ClaI*–*PvuII* fragment of chromosomal DNA restored cellular quinone levels and growth on succinate. The plasmid also complemented a previously isolated *ubiA* mutant for aerobic growth on non-fermentable substrates. The nucleotide sequence of the cloned fragment revealed a fragment of *plsB* (91.7 min on the *E. coli* chromosome map) and three open reading frames (ORFs), one of which (ORF3) encodes a protein with a predicted molecular mass of 32511 Da. The hydrophobicity profile of the ORF3 protein is characteristic of a membrane protein with five hydrophobic regions and is very similar to that of the *Saccharomyces cerevisiae* *COQ2* gene product (*p*-hydroxybenzoate:polyprenyltransferase, required for the second step of ubiquinone biosynthesis) and to the product of the *E. coli* *cyoE* gene. Complementation of *ubi* mutants with various deletion derivatives of the cloned DNA fragment confirms that ORF3 is *ubiA*. ORF3 is closely linked to *ubiC* (ORF2), which encodes chorismate lyase.

Introduction

Quinones play important roles in both aerobic and anaerobic respiratory electron transfer processes. *Escherichia coli* contains isoprenoid quinones of both the benzene and naphthalene series. The major benzoquinone is ubiquinone-8 (the figure referring to the number of prenyl units in the side chain), whereas the naphtho-

quinones are either menaquinone (MQ) or demethylmenaquinone (DMQ). Ubiquinone (UQ) predominates under high aeration conditions in exponential phase and when nitrate is present as alternative electron acceptor (Poole & Ingledew, 1987), whereas MQ predominates under anaerobic conditions and supports respiration with fumarate (Lambden & Guest 1976; Guest, 1977). MQ or DMQ can serve as mediators in the reduction of trimethylamine-*N*-oxide in *E. coli* (Wissenbach *et al.*, 1990).

Genes encoding the enzymes for UQ biosynthesis have been mapped and found to be scattered around the *E. coli* chromosome map (Gibson, 1973). The gene (*ubiG*) encoding the last enzyme of the pathway, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase, has been cloned and sequenced (Wu *et al.*, 1992). Recently, Nichols & Green (1992) described the cloning of *ubiC* and suggested that an adjacent gene is *ubiA*. In yeast, the *COQ3* gene (encoding the first

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Abbreviations: UQ, ubiquinone; MQ, menaquinone; DMQ, demethylmenaquinone; Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; PHB, *p*-hydroxybenzoate; Suc⁺, able to grow on succinate minimal medium; Suc[–], not able to grow on succinate minimal medium.

The nucleotide sequence data reported in this paper from nucleotides 1–1498 in Fig. 3 have been submitted to GenBank and have been assigned the accession number M96268.

Table 1. *E. coli* strains

Strain	Genotype	Origin or reference
AN2342	F ⁻ (referred to as wild type)	Poole <i>et al.</i> (1989)
AN2343	F ⁻ <i>cydD</i>	Poole <i>et al.</i> (1989)
AN2573	F ⁻ <i>ubiA cydD</i> (from AN2343 by NTG mutagenesis)	This work
AN2540	<i>uncC538 argH pyrE entA</i>	This laboratory
AN84	<i>ubiA419 metB thi</i>	I. G. Young*
AN385	F ⁻ <i>ubiA420</i>	I. G. Young*
HW273	F ⁻ <i>ubiA zjb::Tn10</i>	This work
HW274	F ⁻ <i>zjb::Tn10</i>	P ₁ (HW273) × AN2342, this work
XL1-blue	F ⁻ <i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1</i> [F ⁻ <i>proAB lacI^q lacZΔM15 Tn10</i>]	Stratagene

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methyltransferase of UQ biosynthesis) has been isolated and characterized (Clarke *et al.*, 1991) and the molecular cloning and characterization of *COQ2*, identified as the structural gene for *p*-hydroxybenzoate:polyprenyltransferase, has been described recently (Ashby *et al.*, 1992).

Mutants of *E. coli* defective in UQ biosynthesis are able to grow on glucose by fermentation and anaerobically by respiration on fumarate or nitrate, when MQ mediates electron flow between the dehydrogenases and terminal reductases. However, such mutants cannot grow aerobically on non-fermentable substrates such as malate or succinate (Cox & Downie, 1979). In this paper, we report the isolation and characterization of a mutant unable to grow aerobically on non-fermentable substrates except for very slow growth on glycerol. Complementation of the mutant phenotype was achieved by a cloned DNA fragment

bearing two genes, one of which is identified as *ubiA* by its ability to complement authentic *ubiA* mutants and its similarity to the yeast *COQ2* gene. The UbiA protein is predicted to be an integral membrane protein and is shown to resemble proteins of unknown function encoded by genes in cytochrome oxidase operons. A more extensive analysis of sequence data is also presented.

Methods

Bacterial strains and plasmids. The bacterial strains used were derivatives of *E. coli* K12 (Table 1). Plasmids, including those used for complementation analysis, are described in Table 2. Plasmids used for sequencing are shown in Fig. 2 (see below). Generalized transduction was carried out using bacteriophage P1_{vir} as described previously (Wu *et al.*, 1992).

Growth media and conditions. Succinate minimal medium (SMM) was described by Poole *et al.* (1989). SCMM contained, in addition, 0.5% Casamino acids (Difco). In glycerol (GMM) or lactate (LMM) minimal media, the succinate was replaced by 0.5% glycerol or 0.2% Na lactate, respectively. Nitrate-fumarate-glycerol minimal medium (NFGMM) was used for growth under anaerobic conditions. It contained (l⁻¹) Na₂HPO₄ · 12H₂O (10.96 g), KH₂PO₄ (2.7 g), (NH₄)₂SO₄ (1.0 g), trace elements solution (10 ml; Poole *et al.*, 1989), glycerol (5.0 g), fumaric acid (5.0 g), NaNO₃ (10.0 g), 1 µM-ammonium molybdate and 1 µM-potassium selenite. The components were dissolved in 1 litre distilled water and brought to pH 7.0 with NaOH. Sterile 1 M-MgCl₂ · 6H₂O (1 ml) was added before use. For solid media, the components were dissolved in 500 ml water, sterilized and mixed with 500 ml molten 3% (w/v) agar in distilled water. When nitrate was omitted, the medium was called GF; when fumarate was omitted, it was called GN. Where indicated, the glycerol was substituted by succinate (SN) or lactate (LN). Rich meat-glucose medium (MG) was prepared as described by Poole *et al.* (1989); in MGF, the glucose was replaced by 0.5% glycerol and 0.5% fumarate. LB medium is described by Maniatis *et al.* (1982). Anaerobic cultures were grown in a Gaspak anaerobic jar. To study growth rates and yields anaerobically with

Table 2. *Plasmids*

Plasmid	Description	Origin or reference
pBluescript II SK +	Ap ^R	Stratagene
pHC79	Cosmid cloning vector Tc ^R Ap ^R	Hohn & Collins (1980)
pBR328	Plasmid vector Tc ^R Ap ^R Cm ^R	Bolivar <i>et al.</i> (1977)
pBRP23	<i>ClaI</i> - <i>PvuII</i> insert from pRP23 cloned in pBluescript II (SK+) vector.	This work
pCL11	257 bp deletion from the <i>ClaI</i> end of the insert with reference to pBRP23	This work
pCL21	372 bp deletion from the <i>ClaI</i> end of the insert with reference to pBRP23	This work
pRP23Bs	<i>ClaI</i> - <i>BstXI</i> insert of pBRP23 cloned in <i>ClaI</i> and <i>PvuII</i> site of pBR328	This work
pRP23	Ap ^R <i>ubiA</i> ⁺ (2.6 kb <i>ClaI</i> - <i>PvuII</i> fragment cloned in pBR328)	This work
pRP23Sm	<i>ClaI</i> - <i>SmaI</i> insert of pBRP23 cloned in <i>ClaI</i> and <i>PvuII</i> sites of pBR328	This work
pRP23Ss	<i>ClaI</i> - <i>SspI</i> insert of pBRP23 cloned in <i>ClaI</i> and <i>PvuII</i> sites of pBR328	This work

Fig. 1. Anaerobic growth of strains with nitrate as terminal electron acceptor in minimal media with (a) glycerol, (b) succinate or (c) lactate as substrates. The strains used were HW273 (*ubiA*) (△) and HW274 (*ubiA*⁺) (○ and ■). The growth of HW274 in minimal media without nitrate under anaerobic conditions (■) is shown as a control.

nitrate, bacterial cells were mixed with different soft agar media (with 0.5% agar) and incubated anaerobically in test tubes; the growth of cells was followed by measuring the optical density of the tubes with a Corning colorimeter 252 (green filter).

Mutant isolation and construction of *Tn10dTet* insertions close to the *ubi* locus. Strain AN2573 was isolated from AN2343 (*cydD*) by NTG mutagenesis exactly as described by Wu *et al.* (1992). AN2573 was unable to grow on SMM (Suc⁻), but able to grow anaerobically on NFGMM. Strain HW273 was constructed by inserting *Tn10dTet* adjacent to the *ubi* mutation in AN2573, and transducing the mutation with the *Tn10dTet* marker into a wild type genetic background as described by Wu *et al.* (1992). HW274 was selected from the same procedure as having *Tn10dTet* linked to the *ubi*⁺ gene.

Cloning of *ubi* gene(s). The general methods used have been described by Wu *et al.* (1992). Briefly, partially *Hind*III-digested chromosomal

DNA from strain AN2540 was ligated to *Hind*III-linearized cosmid vector pHC79, packaged into bacteriophage λ particles, and used to transfect strain AN2573. Ap^R transfectants were screened for a Suc⁺ phenotype on SMM and cosmid DNA was prepared from a selected Suc⁺ Ap^R transfectant. The DNA was linearized with *Cla*I and ligated with plasmid vector pBR328, previously linearized with *Cla*I and dephosphorylated with calf intestinal phosphatase. The ligation product was used to transform AN2573, and transformants were selected on SCMM-Cm. One such transformant was again used to prepare plasmid DNA, which was digested with *Pvu*II and recircularized with DNA ligase. The ligation products were used to transform AN2573; transformants were selected on SCMM-Ap and screened for the presence of plasmids that gave the expected fragments on digestion with restriction endonucleases. Plasmid pRP23 (2.6 kb insert) was able to transform AN2573 and HW273 to a Suc⁺ phenotype on SCMM.

Other methods. The determination of nucleotide sequence from double-stranded DNA templates in pBluescript, construction of deletions in such templates, measurement of oxygen consumption, extraction and analysis of quinones and protein assays were all performed as described by Wu *et al.* (1992). Nucleotide and amino acid sequence analyses were performed with the University of Wisconsin sequence analysis package (UWGC), version 7.0 (Devereux *et al.*, 1984) and the Staden package (Seqnet system, SERC Daresbury Laboratory). Hydrophobicity profiles were computed using the GENESYS software of R. Bottomley (CSIRO Division of Plant Industry, Canberra). The program of Engelman *et al.* (1982) was used with a window of 19 amino acids.

Results

Mutant isolation and characterization

NTG mutagenesis of AN2343 (*cydD*) yielded mutants at a frequency of 2.8% that were unable to grow aerobically on succinate but able to grow anaerobically on NFGMM (Wu *et al.*, 1992). One of the mutants selected for study, AN2573, was unable to grow on lactate, malate or succinate, but grew to give small colonies on a rich glucose medium (MG) and grew anaerobically on glycerol with either nitrate or fumarate as sole electron acceptor. To construct a strain in which only the gene of interest was mutated, a *Tn10dTet* marker was inserted adjacent to the mutation and transferred with the mutant allele into a wild type background by P1 transduction. The newly isolated mutant with a single mutated gene linked to the *Tn10* insertion was designated HW273; the isogenic control strain with only the *Tn10* insertion was called HW274. Mutant HW273 grew very slowly on GMM aerobically, even slower than HW271, which carries a *ubiG* mutation (Wu *et al.* 1992), and did not grow either aerobically or anaerobically on SMM or LMM. A DNA fragment that complemented aerobic growth of HW273 on succinate was cloned from a cosmid library and a *Cla*I-*Pvu*II fragment was subcloned into vector pBR328 to give plasmid pRP23.

Both HW273 and HW274 grew well on GN medium (Fig. 1a), although growth of HW273 was almost undetectable for the first 24 h. On SN (Fig. 1b) and LN

Table 3. Oxygen consumption rates of cell-free extracts from mutant HW273 and control strains

Cells were grown in glycerol minimal medium, disrupted, and respiration rates of the unfractionated extracts measured using an oxygen electrode.

Substrate	Strain	Respiration rate [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]		Stimulated rate: initial rate
		Before adding UQ-1	After adding UQ-1	
Glycerol 3-phosphate	AN2342	13.76	24.17	1.75
	HW273	8.52	27.56	3.23
	HW274	11.25	22.72	2.01
Succinate	AN2342	17.42	35.59	2.04
	HW273	7.90	29.70	3.75
	HW274	21.81	31.49	1.44
Lactate	AN2342	32.5	57.3	1.76
	HW273	6.71	52.8	7.86
	HW274	27.40	63.14	2.30

(Fig. 1c) media, HW273 grew very poorly and growth was not significantly different from that of a control culture of HW274 lacking nitrate. The results demonstrate that when cells grow anaerobically on nitrate minimal medium with succinate or lactate as sole carbon source, UQ is an essential component, but cells can grow anaerobically on glycerol-nitrate minimal medium without UQ.

Oxygen consumption rates

Oxygen consumption rates of extracts from mutant HW273 were severely depressed relative to activities of the isogenic control strain HW274 and of AN2342 (Cyd⁺) (Table 3) after growth in GMM. However, respiratory rates with various substrates were differentially affected. For example, the highest rates of respiration, with lactate, were inhibited in HW273 to 24% of the control rate (in HW274), whereas the lowest rate, with glycerol 3-phosphate, was inhibited to only 76% of the control rate. To test whether respiration in HW273 was limited by UQ, reconstitution *in vitro* was attempted using exogenous quinone analogues. A solution of UQ-1 (~130 nM final concn) was added to the reaction chamber during measurements of oxygen consumption. The results (Table 3) show that the respiration rates of HW273 and control strains increased, but the stimulatory effect on the mutant was more marked than the effects on the control strains. Subsequently adding more UQ-1 gave no further enhancement for any strain. The degree of enhancement also differed among the different substrates (Table 3). UQ-10 and UQ-8 extracted from the wild type strain had no effect on respiration (results not shown), presumably due to the solubilities of UQ-8 and UQ-10 in water being much lower than that of

UQ-1. The residual oxidase activity of the quinone mutants may be attributed to the presence of undetectable amounts of UQ, or relatively ineffective electron transfer by MQ.

Quinone levels and their complementation by pRP23

Mutant HW273, HW273 complemented with pRP23, and the isogenic control strain HW274 were grown in defined medium plus glucose (Gibson *et al.*, 1977) for quinone assays. The extracts from HW274 and HW273 complemented with pRP23 each exhibited spots on TLC plates corresponding to UQ-8, the main quinone of aerobically grown *E. coli*. Elution of the material from the plates and spectroscopic analysis confirmed the identification (results not shown). No UQ was detected in HW273. The results suggest that HW273 is deficient in UQ biosynthesis and that plasmid pRP23 restores quinone levels in this mutant. MQ levels appeared to be low in all strains under these growth conditions.

Sequence of the 2.6 kb insert in pBRP23

Plasmid pRP23 contains a 2.6 kb *Cla*I–*Pvu*II fragment of chromosomal DNA subcloned from a cosmid clone into pBR328, and was able to complement the aerobic growth of AN2573 and HW273. This insert was further subcloned into pBluescript II using *E. coli* XL1-blue as host and the plasmid designated pBRP23. Fig. 2 shows the strategy for sequencing the insert. The sequence data from nucleotides 1 to 1498 (Fig. 3) were obtained using both strands as templates and were in agreement. The sequence from nucleotides 367 to 2367 has also been reported by Nichols & Green (1992) and is identical to ours but only a cursory description of its features and

Fig. 2. Map of, and strategy for sequencing, the 2.6 kb *Cla*I-*Pvu*II/*Eco*RV fragment in pRP23 and pBRP23. The ORFs were determined by searching for initiation and stop codons. The deletion clones made by restriction digestions are shown in the upper part of the figure. The nested deletion clones are shown in the lower part. The thicker parts represent the sequence determined with those clones. The dashed arrows show the sequence determined with synthetic oligonucleotides.

interpretation was given. The sequence from nucleotides 1499 to 2390 (Fig. 3) was taken from that of Lightner *et al.* (1983). The sequence at the *Pvu*II end of the fragment is identical to the 3' end of the *plsB* gene, which maps around 91.7 min on the *E. coli* linkage map (Bachmann, 1990).

Three long ORFs were identified on this fragment by searching for stop and initiation codons; ORF2 and ORF3 were closely linked. A perfect ribosome binding site (GAGG) was located 8 bp before the first initiation codon ATG (at nucleotide number 1257) of ORF3; it is likely that this ATG is the translation initiation site. No typical ribosome binding site was found before the first ATG at nucleotide number 636 of ORF2, but a good ribosome binding site (GGAG) was identified 8 bp before the second ATG at nucleotide number 747. This ATG has been confirmed (Nicholls & Green, 1992) as the initiation codon of ORF2, which was shown by these authors to be *ubiC* based on the N-terminal sequence of purified chorismate lyase.

A computer search for promoter-like sequences of the *Cla*I-*Pvu*II fragment from pRP23, by using the Staden package, suggested a medial strength promoter upstream of the second ATG at nucleotide number 747 of ORF2 (results not shown). This prediction is mostly based on the strong -35 region, but the separation between the -10 and -35 regions is not optimal. No strong promoter is predicted upstream of ORF3; the results suggest that *ubiC* and ORF3 are cotranscribed. The adenine nucleotide at nucleotide 723 is possibly the +1 site where RNA synthesis is initiated.

Polypeptides encoded by *ubiC* and ORF3

Resemblances to well-characterized functional domains were sought within the predicted polypeptides from *ubiC* and ORF3. The MOTIF program within the UWGGC package was used. It uses a 'prosite dictionary', which

contains 509 patterns (Release 7.1 August, 1991). An ATP binding site 'motif A' (Walker *et al.*, 1982) was identified in the UbiC polypeptide, chorismate lyase. This motif has been identified in a number of proteins that bind ATP or GTP, which include ATP synthetase α and β subunits, a number of kinases, an ATP-binding protein involved in 'active transport' (Higgins *et al.*, 1990) and GTP-binding elongation factors (EF-Tu, EF-1 α , etc). However, this motif has also been found in a number of other proteins that are not ATP- or GTP-binding (Moller & Amons, 1985; Fry *et al.*, 1986; Higgins *et al.*, 1990). Chorismate lyase activity does not appear to require added ATP *in vitro* (Nichols & Green, 1992).

The hydrophobicity of chorismate lyase is characteristic of a soluble protein (not shown), whereas the ORF3 gene product has a hydrophobicity profile characteristic of a membrane protein (Fig. 4), with five hydrophobic regions. Four of these (Fig. 4b) may each be long enough to encompass two transmembrane helices and the other a possible single transmembrane helix at the C-terminal end. A database search showed 49% similarity, allowing for conserved substitutions (Fig. 5) between residues 9 and 289 of ORF3 and residues 79 and 367 of the *COQ2* gene of *S. cerevisiae*, the structural gene for *p*-hydroxybenzoate:polyprenyltransferase (Ashby *et al.*, 1992). The similarity is reflected in the hydropathy plots (Fig 4), the major difference being the number of N-terminal amino acids before the first transmembrane helix of the *COQ2* protein. The number of hydroxylated amino acids and preponderance of positive charges in this region suggest that it is a leader sequence. There is a noticeable similarity in the amino acids at the extremities of the suggested transmembrane regions (Fig. 5). Fig. 4 also shows the striking similarity between the hydrophobicity profiles of the protein encoded by ORF3 and *COQ2* proteins with that of the *E. coli cyoE* gene product. Nichols & Green (1992) also reported 21%

10 30 50 70 90
 ATCGATTGAGGTGAATTTTCGATACCCACAGATGATCGATTTTATTAAACAGATTAAATACGAATAAATGGCTAAATTGATAAAAAATAACAATTTTT
 TAGCTAAGTCACTCCACTTAAAGCTATGGGTGTCTACTAGCTAAAATAATTGCTAAATTATGCTTATTACCGATTAACTATTTTATTGTTAAAAAA
 R F S E V N F D T Q Q M I D F I N R F N T N K W L I D K N N N F F
 ORF1

110 130 150 170 190
 TATCCAATTATCGATCAGGCCTTACGATCAACGGATGATATGATCAAAGCAAATGTTTGGCATCTTTATAAAGAGTGGATTCTAGTGTATGATGTTTCA
 ATAGGTGAATAGCTAGTCCGGAATGCTAGTGCCTACTATAGTTCGTTTACAAACCGTAGAAATATTTCTCACCTAAGCATCACTACTACAAAGT
 I Q L I D Q A L R S T D D M I K A N V W H L Y K E W I R S D D V S

210 230 250 270 290
 CCTATATTTATAGAACTGAAGATAATTAAGAACCTTTAACACGAATGAATTAACACGAAACGATAATATCTTTATCCTGTTCTCCTCAGTCGATGATG
 GGATATAAATATCTTTGACTTCTATAAATCTTGGAAATGTGCTTACTTAATTTGTGCTTGTCTATTATAGAAATAGGACAAAGAGGAGTCAGCTACTAC
 P I F I E T E D N L R T F N T N E L T R N D N I F I L F S S V D D G

310 330 350 370 390
 GGCCAGTTATGGTGGTAAGCTCCAGCGCTTACATGATATGTTGAATCCTACAAAAGATACCAATTGGAATCCACGTATATCTACAAATCCAGACATGA
 CCGGTCAATACCACATTGAGGGTGCAGGATGTACTATACAACTTAGGATGTTTTCTATGGTTAACCTTAAGTGCATATAGATGTTAGGTCTGTACT
 P V M V V S S Q R L H D M L N P T K D T N W N S T Y I Y K S R H E

410 430 450 470 490
 GATGTTGCCTGTTAATCTTACTCAGGAACACTTTTCAGCTCCAAATCTCATGGTAAATATGCGCTTTTCCCATTTTACTGCGAGTGGCGAGCTCAT
 CTACAACGGCAATTAGAATGAGTCTTTGTGAAAAGTCGAGGTTTAGAGTACCATTTATACGCGAAAAGGGGTAAAAATGACGCTCAACCGCTCGAGTA
 M L P V N L T Q E T L F S S K S H G K Y A L F P I F T A S W R A H

510 530 550 570 590
 CGTATAATGAATAAGGGTGTAAAGTAAAGGAAACATCACCGTTCCTGGCATCCTGGACGGTGATGCCCTACGGTTGCCCTCGCCAGCACGGGCATCG
 GCATATTACTTATTTCCCAAAATTCATTTCTTTGTAGTGGCAAGGACCGTAGGACCTGCCACTACGGGGATGCCAACGGGAGCGGTCTGCCCGTAGC
 R I M N K G V *

610 630 650 670 690
 GTAAAGCGTAGGTTCAACATCGTTTACCCTTCATGCGATTGTGCGTTTTTGTGCGTATTAGATCACTTAATTTGCTTTACATCTCCCGTAAACAC
 CATTTCCGATTCCAAGTTGTAGCAAAATGGTAGAGTACGCTAACACCGCAAAACACGCATAATCTAGTGAATTAACGAAATGTAGAGGGCATTGTTG

710 730 750 770 790
 TTTTCTGCGATACAATGCCCTTTACGTTATGTAACGGAGAGTTTCGGCATGTACACCCCGCGTTAACGCAACTGCGTGCCTGCGCTATTGTAAAGAGATC
 AAAAGACGCTATGTTACGGAATGCAATACATTGCGCTCTCAAGCCGTACAGTGTGGGGCGCAATTGCGTTGACGACGCGACGCGATAACATTCTCTAG
 M S H P A L T Q L R A L R Y C K E I
 ORF2

810 830 850 870 890
 CCTGCCCTGGATCCGCAACTGCTCGACTGGCTGTGCTGGAGGATTCCATGACAAAACGTTTGAACAGCAGGGAAGAAACGGTAAGCGTGACGATGATCC
 GGACGGGACCTAGGCGTTGACGAGCTGACCGACACGACCTCCTAAGGTACTGTTTGTGCAAACTTGTGCTCCCTTTTGGCATTCGCACTGCTACTAGG
 P A L D P Q L L D W L L L E D S M T K R F E Q Q G K T V S V T M I R

910 930 950 970 990
 GCGAAGGGTTTGTGAGCAGAAATGAAATCCCCGAAGAACTGCCGCTGCTGCCGAAAGAGTCTCGTTACTGGTTACGTGAAATTTTGTATGTGCCGATGG
 CGCTTCCCAACAGCTCGCTTACTTTAGGGGCTTCTTACGGCGACGACGGCTTCTCAGAGCAATGACCAATGCACCTTAAACAAATACACGGCTACC
 E G F V E Q N E I P E E L P L L P K E S R Y W L R E I L L C A D G

1010 1030 1050 1070 1090
 TGAACCGTGGCTTGGCGGTGCTACCGTCTGCTGTGTCACGTTAAGCGGGCGGAGCTGGCGTTACAAAAATGGGTAAAACGCGTATAGGACGCTAT
 ACTTGGCACCAGCGCCAGCATGGCAGCAAGGACAGTTGCAATTCGCCCGCTCGACCGCAATGTTTTTAACCCATTTTGGCGCAATCCTGCGATA
 E P W L A G R T V V P V S T L S G P E L A L Q K L G K T P L G R Y

1110 1130 1150 1170 1190
 CTGTTACATCATCGACATTAACCCGGGACTTTATTGAGATAGGCCGTGATGCCGGGCTGTGGGGGCGACGTTCCCGCTTGGGATTAAGCGGTAAACCGC
 GACAAAGTGTAGTGTAAATTTGGGCCCTGAAATAACTCTATCCGGCACTACGGCCGACACCCCGCTGCAAGGGCGGAGCGCTAATTCGCCATTTGGCG
 L F T S S T L T R D F I E I G R D A G L W G R R S R L R L S G K P L

1210 1230 1250 1270 1290
 TGTGCTTAACAGAATGTTTTTACCGCGCTACCGTTGTACTAAGAGGAAAAAATATGGAGTGGAGTCTGACGCAAGTAAGCTGCTGGCGTTTCATCG
 ACAACGATTGCTTGTACAAAAATGGCCGAGTGGCAACATGATTCTCTTTTTTATACCTCACCTCAGACTGCGCTTATTTCGACGACCGCAAAGTAGC
 L L T E L F L P A S P L Y
 M E W S L T Q N K L L A F H R
 ORF3

1310 1330 1350 1370 1390
 CTTAATGCGTACGGATAAGCCAATTTGGCGGTTACTGCTGCTCTGGCCAACATTATGGGCGTTTGGTGGGCGACACCGGGCGTCCCCAGCTCTGGATC
 GAATTACGATGCGCTATTGCGTTAACCGCGCAATGACGACGAGACCGGTTGTAATACCGCAACACCCACCGCTGTGGCCGCAAGGGTTCGAGACCTAG
 L M R T D K P I G A L L L L W P T L W A L W V A T P G V P Q L W I

Fig. 3. The complete nucleotide sequence of the *ClaI*–*PvuII*/*EcoRV* fragment from pBRP23 and its encoded protein sequence. The sequence from nucleotides 1448 to 2390 was taken from Lightner *et al.* (1983). The sequence before nucleotide 110, which contains another two *ClaI* sites, might be a cloning artifact.

overall identity between the gene product of ORF3 and the product of the *cyoE* gene, which lies within an operon encoding the cytochrome *o* ubiquinol oxidase complex of

E. coli (Chepuri *et al.*, 1990). Ashby *et al.* (1992) have deduced the sequence of the putative binding site for the substrate in the prenylation reaction catalysed by the

Table 4. Complementation of *ubiA* mutants for growth by different plasmids

For plasmids used see Table 2. The different extents of growth are indicated by the number of + signs; -, no growth; ND, not determined.

Plasmid	HW273 (<i>ubiA</i>)		AN385 (<i>ubiA420</i>)		AN84 (<i>ubiA419</i>)	
	SCMM	LB	SCMM	LB	SCMM	LB
pRP23	+	++	+(+)	++	-	+(+)
pRP23Ss*	++	+++	++	+++	++	+++
pRP23Bs	-	+	-	+	-	+
pRP23Sm	-	+	-	+	-	+
pBRP23	-	++	-	++	ND	ND
pCL11	-	++	-	++	ND	ND
pCL21	-	++	-	++	ND	ND

* Transformation of mutants by pRP23Ss gave transformants which exhibited irregular 'patchy' growth on SCMM and LB (see Results).

COQ2 protein, namely polyprenyl pyrophosphate. The UbiA protein exhibits 74% similarity, allowing for conservative substitutions, in this region (Fig. 5).

Identification of the complementing ORF

The extensive similarity between the protein encoded by ORF3 and the yeast gene that encodes *p*-hydroxybenzoate:polyprenyltransferase strongly suggests that ORF3 is *ubiA*, that encodes the corresponding biosynthetic enzyme in *E. coli*. Furthermore, although the UbiA protein has not been purified, its enzyme activity is localized in membrane fractions (Young *et al.*, 1972), consistent with the hydropathy data of Fig. 4. Nichols & Green (1992) tentatively attributed ORF3 in Fig. 2 to *ubiA*, based solely on map position. We sought, therefore, to identify ORF2 and ORF3 by complementation studies using both HW273 and two previously described *ubiA* mutants, AN84 (*ubiA419*) and AN385 (*ubiA420*).

All mutants failed to grow on succinate minimal media (SCMM), grew poorly on LB, but grew satisfactorily on glucose-enriched media, or anaerobically on NFGMM. HW273 was complemented for growth on LB by plasmids pCL11 and pCL21, the insertions in which were deleted 257 and 372 bp, respectively, from the *Cla*I site in pBRP23 (Table 4). Similarly, AN385 was complemented by pCL21, demonstrating that ORF1 is not required for complementation of HW273 or a *ubiA* mutant. Plasmid pRP23 complemented mutants HW273 and AN385 for growth on SCMM (Table 4) but, surprisingly, was ineffective at complementing AN84. Plasmid pRP23 enhanced the growth of all three mutants aerobically on MGF (results not shown). Plasmid pRP23 was digested with *Ssp*I and *Pvu*II and the resulting blunt ends religated. This left ORF1, ORF2 and the first 0.52 kb of ORF3 (see Fig. 2). The plasmid (pRP23Ss), when

introduced into HW273, AN385 or AN84, gave patchy growth aerobically on either SCMM or LB, which is probably attributable to recombination of plasmid-borne genes with the chromosome. The contribution of ORF3 was examined unambiguously by digesting pRP23 with *Pvu*II and *Bst*XI (see Fig. 2) to remove ORF3, and treating with T4 DNA polymerase in the presence of dNTPs at 12 °C for 30 min. The resulting blunt ends were ligated with T4 DNA ligase. Neither this plasmid (pRP23Bs) nor pRP23Sm, which also lacks ORF3 plus the last 0.115 kb of ORF2, were able to complement any of the mutants tested for growth on SCMM or LB. These results demonstrate that ORF3 is *ubiA* and are not affected by the use of a *recA*⁺ host, since ORF3 was deleted from the plasmid. It would have been preferable to use *recA* mutants for all complementation tests; however, *ubi* mutants grow very slowly, and *recA ubi* mutants grow more slowly still.

Some *ubiA* mutants have been found to response to *p*-hydroxybenzoate (PHB) as a growth factor, but at a concentration of about 0.1 mM rather than 10 nM as would be expected for a mutant requiring this compound (Cox *et al.*, 1968). The *ubiA* mutant AN385 was found to respond to 0.1 mM-PHB as a growth factor, but not at 10 nM, whereas HW273 did not respond to PHB at all. It seems that AN385 might have a mutated octaprenyltransferase with a diminished affinity for its substrate, whereas HW273 may lack the enzyme completely.

Discussion

Mutants defective in UQ and/or MQ biosynthesis have proved valuable in determining quinone function in the multiply branched respiratory chains of *E. coli*. Some routes of electron transfer can be supported by either quinone but others show marked preference for a particular quinone type (Poole & Ingledew, 1987).

Substrates such as D-lactate, glycerol 3-phosphate and NADH are relatively poor donors of reducing equivalents to nitrate by comparison with formate. However, Wallace & Young (1977) showed that membranes from anaerobically growth cells could oxidize glycerol 3-phosphate and reduce nitrate with either UQ or MQ, but that lactate-driven reduction of nitrate required UQ, and that MQ was ineffective. Consistent with these results, but apparently in disagreement with Scheme 2 of Wallace & Young (1977), and with Wissenbach *et al.* (1990), we show here that lactate utilization, as well as that of succinate, has an absolute requirement for UQ, at least under anaerobic growth conditions.

The biosynthetic pathways of UQ and MQ have been studied in detail (for reviews see Gibson, 1973; Bentley & Meganathan, 1987). Both quinones comprise a nucleus derived from chorismate, a prenyl side chain derived from prenyl pyrophosphate, and a nuclear methyl group derived from *S*-adenyosylmethionine (except in the case of DMQ). The first step in the biosynthesis of UQ is the conversion of chorismate to PHB by chorismate lyase, product of the *ubiC* gene. The second step of the pathway is the transfer of the octaprenyl chain to the PHB ring to generate *p*-hydroxy-3-octaprenyl benzoate. The UbiA enzyme catalyses this reaction.

The nucleotide sequence of a cloned 2.6 kb DNA fragment able to complement HW273 for aerobic growth indicated two complete ORFs, adjacent to a fragment of *plsB*, suggesting that the cloned genes map at about 92 min on the *E. coli* chromosome as do *ubiA* and *ubiC*. Nichols & Green (1992) have also obtained the sequence of these two ORFs and concluded that ORF2 is *ubiC*, based on analysis of the N-terminal sequence of purified chorisate lysate (product of *ubiC*). They also suggested that ORF3 is *ubiA* but presented no evidence for this except for map position. While this paper was in preparation, Nishimura *et al.* (1992) have reported in a note the same conclusion, based on complementation of AN385 by a fragment from the Kohara library, subcloned into a pUC vector. However, the deletion analysis used to assign *ubiA* to the ORF between *ubiC* and *plsB* was not presented. Siebert *et al.* (1992) have also cloned and sequenced *ubiA* and *ubiC*. It could be noted that although several nucleotide databank entries now include the *ubiA* gene, the sequence has not been as extensively analysed in the previous accompanying publications. In the present paper, we additionally report similarity between the predicted sequence and hydrophobicity of the protein encoded by ORF3 with the enzyme involved in prenylation of PHB, the first committed step of UQ synthesis in yeast. This protein is the product of the *COQ2* gene (Ashby *et al.*, 1992). This homology has also been noted by Siebert *et al.* (1992). Identification of

Fig. 4. Predicted hydrophobicity plots of the polypeptides encoded by (a) *cyoE*, (b) *ubiA* (ORF3) and (c) *COQ2*.

ORF3 as *ubiA* is also consistent with the known association of the UbiA protein with the membrane (Young *et al.*, 1972). Fig. 4 shows that the UbiA protein is predicted to be highly hydrophobic. We predict up to nine transmembrane helices whereas Ashby *et al.* (1992) predict six for the yeast *COQ2* protein. The hydrophobicity profile of UbiA is also similar to that of *CyoE* (Fig. 4). *cyoE* has been shown previously to be closely related to ORF1 (whose gene product is uncharacterized) of *Paracoccus denitrificans*, situated between the genes encoding subunits II and III of the cytochrome *c* oxidase in this organism (Chepuri *et al.*, 1990). ORF1 of *P. denitrificans* and *cyoE* of *E. coli* are also homologous to *ctaB* in *Bacillus subtilis* (Saraste *et al.*, 1991), a gene upstream of the structural gene for cytochrome *c* oxidase, and which may encode an 'assembly factor'. A

Fig. 5. Sequence alignment of the polypeptide encoded by *ubiA* and the polyprenyltransferase (PPT) encoded by the yeast *COQ2* gene. Identical residues and conservative replacements are indicated by the vertical bars. Bold sequences indicate the predicted transmembrane helices, whilst underlined residues indicate homologies at the beginning of possible transmembrane helices. The starred residues indicate the putative substrate binding site identified in the yeast protein by Ashby *et al.* (1992).

similar gene, *COX10* in the yeast chromosome is required for an oxidase-positive phenotype (Nobrega *et al.*, 1990).

Since mutant HW273 and the authentic *ubiA* mutant AN385 are complemented by pRP23, and a deletion derivative (pRP23Ss) in which about 50% of *ubiA* is absent, this assignment requires that complementation can be achieved in *trans* by a truncated UbiA protein or, more likely, that recombination between plasmid and chromosome complements the mutation. It is noteworthy, however, that the putative polyprenyl pyrophosphate binding site suggested by Ashby *et al.* (1992) for the protein encoded by *COQ2* is located at a position corresponding to a region (see Fig. 5) that would be intact in the predicted truncated UbiA protein synthesized from pRP23Ss. An alternative explanation of the ability of the *ClaI*-*SspI* fragment to complement *ubiA* mutants is that the *Suc*⁺ phenotype results from over-production of the substrate for the UbiA protein, namely PHB and that the mutant prenyltransferase has a lowered affinity for this substrate. Thus, in multicopy, ORF2 (*ubiC*), whose gene product, chorismate lyase, synthesizes PHB, might complement *ubiA* mutants. However, the inability of pRP23Bs, containing an intact *ubiC* gene, but lacking ORF3, to complement AN385 does not support this hypothesis.

Plasmid pRP23 (having the 2.6 kb fragment cloned in pBR328) did not complement AN84 for aerobic growth on SCMM (and indeed inhibited the very faint growth observed for the untransformed mutant); the plasmid enhanced growth on LB (Table 4), but stimulated markedly growth on a richer medium (MGF), on which alternative routes of energy conservation can be used and biosynthetic demands are minimized. The complementation observed for pRP23Ss was greater than for pRP23 with all *ubiA* strains tested, and we were unable (Table 4) to demonstrate complementation of two *ubiA* mutants for growth on SCMM by the 2.6 kb

insert cloned in pBluescript (pBRP23), which has an even higher copy number than pBR328. One explanation might be that the striking similarity between the UbiA and CyoE proteins (Fig. 4) allows UbiA to compete specifically with CyoE for oxidase assembly.

The present data are consistent with the findings of Nichols & Green (1992), Siebert *et al.* (1992) and Nishimura *et al.* (1992) in showing that the transcriptional direction of *plsB* shown on the *E. coli* linkage map (Bachmann, 1990) is incorrect. Only one *BglI* site is predicted by computer analysis of the nucleotide sequence of the *ClaI*-*PvuII* insert in pRP23, using the compiled data of our own sequencing and that of Lightner *et al.* (1983). However, the restriction digestion pattern of this fragment (results not shown) reveals another *BglI* site approximately 0.4 kb away from the identified *BglI* site at nucleotide number 2381. Thus, the second site should be located approximately between nucleotide numbers 1800 and 2000 (Figs 2 and 3). The result indicates a possible error in the published sequence (Lightner *et al.*, 1983) or the presence of a mutation in that clone or ours. The error (or mutation) probably only results in a substitution, not a frame-shift, since the coding probability remains high in the same reading frame (results not shown).

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