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

GUANGHUI WU,1 HUW D. WILLIAMS,2 FRANK GIBSON3 and ROBERT K. POOLE1*

1Microbial Physiology Research Group, Division of Life Sciences, King's College London, Campden Hill Road, London W8 7AH, UK
2Department of Biology, Imperial College of Science, Technology and Medicine, London SW7 2BB, UK
3Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia

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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 CiaI-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
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 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<sub>gl</sub> 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 (1-')Na<sub>2</sub>HPO<sub>4</sub>, 12H<sub>2</sub>O (10.96 g), KH<sub>2</sub>PO<sub>4</sub> (2.7 g), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (10 g), trace elements solution (10 ml; Poole et al., 1989), glycerol (50 g), fumaric acid (50 g), NaNO<sub>3</sub> (100 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-M<sub>3</sub>C<sub>2</sub>O<sub>4</sub>(5.0 g), NaNO<sub>3</sub>, 1 ml distilled water and brought to pH 7.0 with NaOH. Sterile 1 M-M<sub>3</sub>C<sub>2</sub>O<sub>4</sub> 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 1. E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN2342</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; (referred to as wild type)</td>
<td>Poole et al. (1989)</td>
</tr>
<tr>
<td>AN2343</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;cycD</td>
<td>Poole et al. (1989)</td>
</tr>
<tr>
<td>AN2573</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ubiA cycD</td>
<td>This work</td>
</tr>
<tr>
<td>AN2573</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ubiA cydD (from AN2343 by NTG mutagenesis)</td>
<td>This work</td>
</tr>
<tr>
<td>AN2540</td>
<td>uncC538 argH pyrE entA</td>
<td>This laboratory</td>
</tr>
<tr>
<td>AN84</td>
<td>ubiA419 metB thi</td>
<td>I. G. Young*</td>
</tr>
<tr>
<td>AN385</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ubiA420</td>
<td>I. G. Young*</td>
</tr>
<tr>
<td>HW273</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ubiA zjb::Tn10</td>
<td>This work</td>
</tr>
<tr>
<td>HW274</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;zjb::Tn10</td>
<td>P&lt;sub&gt;1&lt;/sub&gt; (HW273) &lt;br&gt; AN2342, this work</td>
</tr>
<tr>
<td>XL1-blue F&lt;sup&gt;−&lt;/sup&gt; recA1 lac endA1 gyrA96 thi</td>
<td>Stratagene</td>
<td></td>
</tr>
</tbody>
</table>

*Division of Biochemistry and Molecular Biology, Australian National University, Canberra, Australia.

### Table 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Origin or reference</th>
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<td>pBluescript II SK + pHC79</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; Clai–PrulI insert from pRP23 cloned in pBluescript II (SK +) vector.</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBR328</td>
<td>pBR328</td>
<td>Hohn &amp; Collins (1980)</td>
</tr>
<tr>
<td>pRP23</td>
<td>Cosmid cloning vector Tc&lt;sup&gt;R&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bolivar et al. (1977)</td>
</tr>
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<td>pCL11</td>
<td>257 bp deletion from the Clai end of the insert with reference to pBRP23</td>
<td>This work</td>
</tr>
<tr>
<td>pCL21</td>
<td>372 bp deletion from the Clai end of the insert with reference to pBRP23</td>
<td>This work</td>
</tr>
<tr>
<td>pRP23Bs</td>
<td>Clai–BrxiI insert of pBRP23 cloned in Clai and PrulI site of pBR328</td>
<td>This work</td>
</tr>
<tr>
<td>pRP23</td>
<td>Clai–bruA + (2.6 kb Clai–PrulI fragment cloned in pBR328)</td>
<td>This work</td>
</tr>
<tr>
<td>pRP23Sm</td>
<td>Clai–SmaI insert of pBRP23 cloned in Clai and PrulI sites of pBR328</td>
<td>This work</td>
</tr>
<tr>
<td>pRP23Ss</td>
<td>Clai–SpeI insert of pBRP23 cloned in Clai and PrulI sites of pBR328</td>
<td>This work</td>
</tr>
</tbody>
</table>
procedure as having TnI0dTet linked to the nitrate under anaerobic conditions mutagenesis exactly as described by Wu et al. (1992). AN2573 was 05% agar) and incubated anaerobically in test tubes; the growth of nitrate, bacterial cells were mixed with different soft agar media (with 

Mutant isolation and construction of TnI0dTet 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 TnI0dTet adjacent to the ubi mutation in AN2573, and transducing the mutation with the TnI0dTet marker into a wild type genetic background as described by Wu et al. (1992). HW274 was selected from the same interest was mutated, a TnI0dTet marker was inserted adjacent to the mutation and transferred with the mutant allele into a wild type background by

Cloning of ubi gene(s). The general methods used have been described by Wu et al. (1992). Briefly, partially HindIII-digested chromosomal DNA from strain AN2540 was ligated to HindIII-linearized cosmid vector pHC79, packaged into bacteriophage \( \lambda \) particles, and used to transfect strain AN2573. Ap\(^8\) transfectants were screened for a Suc\(^+\) phenotype on SMM and cosmid DNA was prepared from a selected Suc\(^+\) Ap\(^8\) transfectant. The DNA was linearized with Clai and ligated with plasmid vector pBR328, previously linearized with Clai 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 PvuII 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 (UWGCG), version 7.0 (Devereux et al., 1984) and the Staden package (Seqnet system, SERC Daresbury Laboratory). Hydrophobicity profiles were computed using the GENETYX 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 TnI0dTet 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 TnI0 insertion was designated HW273; the isogenic control strain with only the TnI0 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 Clai-PvuII 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

Corning colorimeter 252 (green filter).

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.

ubz4
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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Strain</th>
<th>Before adding UQ-1</th>
<th>After adding UQ-1</th>
<th>Stimulated rate: initial rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol 3-phosphate</td>
<td>AN2342</td>
<td>13.76</td>
<td>24.17</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>HW273</td>
<td>8.52</td>
<td>27.56</td>
<td>3.23</td>
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<tr>
<td></td>
<td>HW274</td>
<td>11.25</td>
<td>22.72</td>
<td>2.01</td>
</tr>
<tr>
<td>Succinate</td>
<td>AN2342</td>
<td>17.42</td>
<td>35.59</td>
<td>2.04</td>
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<tr>
<td></td>
<td>HW273</td>
<td>7.90</td>
<td>29.70</td>
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<td></td>
<td>HW274</td>
<td>21.81</td>
<td>31.49</td>
<td>1.44</td>
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<tr>
<td>Lactate</td>
<td>AN2342</td>
<td>32.5</td>
<td>57.3</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>HW273</td>
<td>6.71</td>
<td>52.8</td>
<td>7.86</td>
</tr>
<tr>
<td></td>
<td>HW274</td>
<td>27.40</td>
<td>63.14</td>
<td>2.30</td>
</tr>
</tbody>
</table>

(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 Clal–PvuII 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
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 PvuII 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 *ClaI–PvuII* 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 UWGCG 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-1α, 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. 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%
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 \textit{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.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>HW273 (\textit{ubiA})</th>
<th>AN385 (\textit{ubiA420})</th>
<th>AN84 (\textit{ubiA419})</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SCMM LB</td>
<td>SCMM LB</td>
<td>SCMM LB</td>
</tr>
<tr>
<td>pRP23</td>
<td>+ + +</td>
<td>+ (+) ++</td>
<td>- (+)</td>
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</tr>
<tr>
<td>pCL21</td>
<td>- + +</td>
<td>- +</td>
<td>ND ND</td>
</tr>
</tbody>
</table>

* 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 \textit{ubiA}, that encodes the corresponding biosynthetic enzyme in \textit{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 \textit{ubiA}, based solely on map position. We sought, therefore, to identify ORF2 and ORF3 by complementation studies using both HW273 and two previously described \textit{ubiA} mutants, AN84 (\textit{ubiA419}) and AN385 (\textit{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 NFGGM. 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 \textit{ClaI} site in pBRP23 (Table 4). Similarly, AN385 was complemented by pCL21, demonstrating that ORF1 is not required for complementation of HW273 or a \textit{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 \textit{SspI} and \textit{PvuII} 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 \textit{PvuII} and \textit{BstXI} (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 \textit{ubiA} and are not affected by the use of a \textit{recA}+ host, since ORF3 was deleted from the plasmid. It would have been preferable to use \textit{recA} mutants for all complementation tests; however, \textit{ubi} mutants grow very slowly, and \textit{recA} \textit{ubi} mutants grow more slowly still.

Some \textit{ubiA} mutants have been found to respond 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 \textit{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 octaprenyl-transferase 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 \textit{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 chorismate lyase (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 hydropathy 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

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’.
similar gene, COXI10 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 polyisoprenyl pyrophosphate binding site suggested by Ashby et al. (1990) 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 the ability of the 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 pBR23 (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 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 pRP23B, 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 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 BglII 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 BglII site approximately 0.4 kb away from the identified BglII 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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References

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