Isolation and characterization of *Escherichia coli* mutants affected in aerobic respiration: the cloning and nucleotide sequence of *ubiG*

Identification of an *S*-adenosylmethionine-binding motif in protein, RNA, and small-molecule methyltransferases

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We report the isolation and characterization of a mutant of *Escherichia coli* unable to grow aerobically on non-fermentable substrates, except for very slow growth on glycerol. The mutant contains cytochrome oxidases *o* and *d*, and grows anaerobically with alternative electron acceptors. Oxygen consumption rates of cell-free extracts were low relative to activities in an isogenic control strain, but were restored *in vitro* by adding ubiquinone-1 to cell-free extracts. Transformation with a cloned 2.8 kb *Cla*I-*EcoRV* fragment of chromosomal DNA restored the ability of this mutant (AN2571) to grow on succinate and also restored cellular quinone levels in this strain. The plasmid also complemented a previously isolated *ubiG* mutant (AN151) for aerobic growth on succinate. The nucleotide sequence revealed a 0.7 kb portion of *gyrA*. Unidirectional nested deletions from this fragment and complementation analysis identified an open reading frame encoding a protein with a predicted molecular mass of 26.5 kDa. This gene (*ubiG*) encodes the enzyme 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase, which catalyses the terminal step in the biosynthesis of ubiquinone. The open reading frame is preceded by a putative Shine–Dalgarno sequence and followed by three palindromic unit sequences. Comparison of the inferred amino acid sequence of UbiG with the sequence of other *S*-adenosylmethionine (AdoMet)-dependent methyltransferases reveals a highly conserved AdoMet-binding region. The cloned 2.8 kb fragment also contains a sequence encoding the C-terminus of a protein with 42–44% identity to fungal acetyl-CoA synthetases.

Introduction

The diversity of energy-producing pathways in *Escherichia coli* has facilitated genetic studies of respiratory electron transfer and ATP synthesis (Cox & Downie, 1979, Poole & Ingledew, 1987), since mutants totally defective in aerobic respiration may be grown fermentatively or anaerobically with alternative electron acceptors. Similarly, mutations affecting either of the two aerobically-functioning terminal oxidases (cytochromes *bd* and *bo*) can be isolated, since either oxidase alone can support aerobic respiration. Mutants unable to synthesize ubiquinone, an essential intermediate in the transfer of reducing equivalents from low-potential dehydrogenases to the oxidase complexes, were isolated early on (see Gibson, 1973) as being able to grow on glucose by fermentation, but not on malate or succinate. We set out to isolate mutants affected in the aerobic respiratory pathway terminated by the cytochrome *bo* oxidase complex. By mutagenizing a strain carrying a mutation in *cydD* (Poole et al., 1989), one of four genes known to be required for expression and function of the
alternative bd oxidase complex, mutations affecting electron transfer to or within the cytochrome bo complex could be selected. In such a search, retention of the ability to grow anaerobically with fumarate or nitrate can be used (Au et al., 1985) to eliminate mutants affected in haem B biosynthesis or oxidative phosphorylation, since these functions are required for both aerobic and anaerobic respiratory energy conservation. Using a similar approach, Au et al. (1985) obtained mutants defective in cytochrome o and thus identified a new operon (cyo). We report here the isolation and characterization of a mutant unable to grow aerobically on non-fermentable substrates. The mutation responsible has been identified in ubiG, which encodes the enzyme for the terminal step in ubiquinone biosynthesis and which was originally described by Stroobant et al. (1972). The cloning and complete nucleotide sequence of ubiG is reported, together with an analysis of the sequence, which reveals the presence of an S-adenosylmethionine-binding site in UbiG and of three palindromic unit sequences following the ubiG coding region.

Methods

Bacterial strains and plasmids. The bacterial strains used (Table 1) were derivatives of E. coli K12. Generalized transduction by bacteriophage P1vir was as described by Miller (1972) and Sinhayv et al. (1984). Plasmids are described in Table 2.

Growth media. LB liquid medium and LB plates were as described by Miller (1972). MG rich plates (meat infusion agar with glucose) were as described by Poole et al. (1989). For anaerobic growth, MG was modified by omitting glucose and adding glycerol (0.5%). Nitrate (0.5%), and 1 μM-ammonium molybdate and 1 μM-potassium selenite (MGN). Suceinate minimal medium (SMM) was described by Poole et al. (1989). In glycerol minimal medium, succinate was replaced with 0.5% glycerol (GMM). Where indicated, SMM was supplemented with 1 MGN. Succinate minimal medium (SMM) was described by Poole et al. (1989). For anaerobic growth, MG was modified by omitting glucose and adding glycerol (0.5%). A portion of (5) was then made on a pool of ~3000 Tc<sup>R</sup> transductants and used to retransduce AN2571 to Tt<sup>R</sup>. Transductants able to grow aerobically on SMM have Tn<sub>10</sub>Dtet linked to the wild-type allele. A fresh lysate was made on a purified transductant and used to retransduce AN2571 to Tt<sup>R</sup>; transductants were screened for inability to grow on SMM. HW272 was isolated as one such transductant having Tn<sub>10</sub>Dtet linked to the mutant ubiG allele with a cotransduction frequency of 85%. HW272 was isolated from the same procedure as having Tn<sub>10</sub>Dtet linked to the ubiG<sup>+</sup> allele.

Cloning of ubiG. Cloning methods, restriction endonuclease digestion, plasmid isolation, and transformations were essentially as described by Maniatis et al. (1982). Fragments of DNA were isolated from agarose gels using a GeneClean kit from BIO 101 Inc. Small-scale plasmid preparations were performed according to the method of Birnbom (1983). Chromosomal DNA from strain AN2540 was partially digested with HindIII and ligated with 5'-dephosphorylated, HindIII-linearized vector pHCh9. The resulting concatamers were packaged into bacteriophage λ particles and used to transfect strain AN2572. Ap<sup>R</sup> transfectants were screened for a Suc<sup>+</sup> phenotype on SMM. One (of twelve) such transfectants was used to prepare cosmid DNA (pRP7) and the resulting preparation again used to transform strain AN2572 to the Suc<sup>+</sup> phenotype. A clone from a single colony of transformants was selected and used to prepare cosmid DNA which was then linearized with ClaI. Plasmid vector pBR328 was 5'- dephosphorylated with calf intestinal alkaline phosphatase (Maniatis et al., 1982) and ligated with the ClaI-derived fragments using DNA ligase. Strain AN2572 was grown in MGN medium, then transformed with the ligation product; transformants were selected on SCMM-Cm. One such transformant, shown to contain a 9 kb plasmid, was retained as strain RP112 containing plasmid pRP19. Plasmid pRP19 was digested with EcoRV and the fragments recircularized with DNA ligase. The ligation products were used to transform strain 71-18; transformants were selected on MG-Cm and screened for the presence of plasmids that gave the expected fragments on digestion with restriction endonucleases. Plasmid pM226 (2.8 kb insert) was able to transform AN2571 and HW271 to a Suc<sup>+</sup> phenotype on SCMM-Cm.

DNA sequence determination. The ClaI-EcoRV insert in pM226 was cloned into the polylinker region of pBluescript II (Stratagene), and designated pRPB. Deletions in the cloned fragment were obtained using a Nested Deletion kit (Pharmacia). Exonuclease III (5 U μl<sup>-1</sup>) was used without added NaCl at 37 °C, and samples were taken at 1 min intervals. Alkali-denatured double-stranded plasmid DNA (Murphy & Ward, 1989) was used directly as template. DNA sequence was determined by the method of Sanger et al. (1977) using reagents and Sequenase in a kit supplied by USB and [5′<sup>32</sup>P]dATPAs (Amersham) for detection. Annealing of primer and template was achieved by incubation at 37 °C for 15 min or by boiling for 1 min, followed by immediate transfer to ice. The boiling method was preferable for overcoming sequencing problems arising from DNA secondary structure. Regions of the cloned fragment inaccessible from the deletions were sequenced using synthetic oligonucleotide primers.
Oxygen consumption measurement. Cells for oxygen consumption measurement were grown in 400 ml GMM to early stationary phase. Cells were washed and resuspended in 50 mM-phosphate buffer, and then sonicated, while chilled in an ice-salt slurry, five times, each for 15 s interspersed with 1 min intervals of cooling. The cell debris and unbroken cells were removed by centrifugation at 10000 g for 10 min. The supernatant fraction (cell-free extract) was stored at -70 °C. Oxygen consumption was measured according to Poole (1977). Substrates were used at a final concentration of 20 mM, where indicated, ubiquinone-1 (reduced as an ethanolic solution with a few grains of NaBH₄) was added to a final concentration of 0.13 mM. Oxygen consumption was performed as described by Kroppenstedt (1982). Thawed cells were suspended in 50 mM-phosphate buffer, and five, each for 15 s interspersed with 1 min intervals of cooling. Cell debris was removed by filtration through Whatman no. 1 paper; the filtrate was evaporated to dryness, and stored at 70 °C.

Extraction and analysis of quinones. Cells were grown in 1 litre of ‘56 medium (Gibson et al., 1977) supplemented with 1% glucose and 0.1% Casamino acids in a 2 litre flask with shaking until the OD₆₀₀ had reached 1.5, and were then washed with 50 mM-potassium phosphate buffer (pH 7) and stored for up to 2 d at -20 °C. Extraction was performed as described by Kroppenstedt (1982). Thawed cells were suspended in 35 ml acetone (-20 °C) and sonicated, while chilled in an ice-salt slurry, five times, each for 15 s interspersed with 1 min intervals of cooling. Cell debris was removed by filtration through Whatman no. 1 paper; the filtrate was evaporated to -0.5 ml in a rotary evaporator at -35 °C. The sample, which became turbid, was freeze-dried and the residue dissolved in acetonitrile. Samples were applied to a silica gel F₂₅₄ plastic-backed TLC plate (Merck Art. 5735) which was run and developed in hexane/diethyl ether (85:15 v/v; Collins, 1985). The spots, visualized by UV transillumination, were cut out and the quinones eluted with 100% ethanol. The silica powder was removed by centrifugation and spectra of the clear supernatant fractions were recorded with a Varian DMS90 spectrophotometer.

Assay of ubiquinol oxidase activity. The method was based on that of Kita et al. (1984). The reaction mixtures in each of two quartz cuvettes comprised 2.5 ml 60 mM-Tris/HCl (pH 7.5) to which was added 10 μl ubiquinol-1 (final concn 93 μM). This had been previously prepared by adding to 1 ml of an ethanolic solution of the quinone a few grains of NaBH₄ and storing on ice until the solution became colourless and hydrogen bubbles had dissipated. The differential rate of quinol autoxidation was recorded in a split-beam spectrophotometer at 275 nm for a few minutes before adding 10-20 μl of the cell extract to one cuvette only.

Cytochrome determinations. Cells were grown in LB medium plus 0.7% glucose in shaken Erlenmeyer flasks to give OD₆₀₀ values (after 1 in 5 dilution), of 0.20 for ubd mutants and 0.55 for controls. Cells were harvested, then washed with 50 mM-Tris buffer (pH 7.5) and suspended in the same medium for spectroscopy. Spectra were obtained in a Johnson Foundation dual-wavelength scanning spectrophotometer (SDB3) as described by Williams & Poole (1987). The reference wavelength was 575 nm and the scan rate 2.86 nm s⁻¹. For cytochrome d, the wavelength pair 630-650 nm was measured in reduced minus oxidized spectra, using ε = 18.8 mM⁻¹ cm⁻¹. CO difference spectra were used to estimate the levels of cytochrome b₅₉₅ (440-470 nm, ε = 52.8 mM⁻¹ cm⁻¹) and cytochrome o (415-430 nm, ε = 145 mM⁻¹ cm⁻¹). We estimate the coefficient of variation for these measurements to be about 10% (A. Gil & R. K. Poole, unpublished).

Protein measurement. The procedure of Markwell et al. (1978) was used.

Chemicals and enzymes. T4 DNA ligase (from BRL) and all other enzymes (NBL) were used in buffers as specified by the suppliers. Alkaline phosphatase was from Boehringer. Sequencing gel solutions were from National Diagnostics. Ubiquinone-1 was a generous gift from Hoffman LaRoche Co., Basel, Switzerland.

Results

Mutant isolation and preliminary characterization

Of 13118 NTG-derived mutants of AN2343 (cydD) screened, 353 (2.8%) failed to grow aerobically on SMM (Suc⁻) but grew anaerobically on a defined medium (NFGMM) that contained fumarate and nitrate as terminal electron acceptors. Of those mutants that on subculturing repeatedly failed to grow on succinate minimal medium (SMM), about one-third also failed to grow, or grew very slowly, on glycerol (GMM). Representatives of this class were tested for cytochrome levels and oxidase activities. The strains that did not grow on succinate but that grew well on glycerol were not studied further; many are likely to have been succinate dehydrogenase mutants. One of the selected mutants, AN2571, which failed to grow on minimal media with succinate, glycerol or lactate as sole carbon source, grew to give small colonies on a rich glucose medium (MG), and grew anaerobically with nitrate as sole terminal electron acceptor. The ubiquinol oxidase specific activity in AN2571 was only 3% of wild-type levels, and NADH oxidase, measured spectrophotometrically, was undetectable. Cytochrome o levels were 16% of wild-type levels. These findings are consistent with a cyo or ubi mutation. The affected gene was cloned by complementation of AN2571 using a cosmid library and subcloned into pBR328 to give pRP19 as described in Methods.

Tn10dTet insertions were made adjacent to the mutation in AN2571 as described in Methods. The mutation was cotransduced with Tn10dTet into the wild-type AN2342. About 85% of TetR transductants were unable to grow aerobically on SMM, indicating that the mutant phenotype is not dependent on the presence of the cydD mutation in AN2343 and, therefore, that the mutation is unlikely to be in cyo. Two TetR transductants, HW272 and HW271, were isolated as being able or unable, respectively, to grow aerobically on SMM. HW271 was also unable to grow on lactate as sole carbon source, but grew very slowly on GMM. Ubiquinol oxidase activities of HW271 were not significantly different from those of HW272 (results not shown). Presumably, the phenotype of AN2571 is a consequence of additional mutation(s) following NTG mutagenesis.

Cytochrome levels and oxygen consumption rates

In contrast to the preliminary measurements of cytochrome levels in AN2571, from which the mutant allele had not been transferred to a genetically ‘clean’ background, the cytochrome o level in HW271 was slightly higher than in the wild-type strain AN2342 (Table 3). However, both cytochrome d and cytochrome
Table 1. Escherichia coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin* or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>71-18</td>
<td>Δ(lac–proAB) thi supE [F' proAB lacF+M15]</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>AN151</td>
<td>ubiG met</td>
<td>Leppick et al. (1976)</td>
</tr>
<tr>
<td>AN1495</td>
<td>unc::mt, argH pyrE entA StrR recA::Tn10</td>
<td>F. Gibson</td>
</tr>
<tr>
<td>AN2342</td>
<td>F– (referred to as wild-type)</td>
<td>Poole et al. (1989)</td>
</tr>
<tr>
<td>AN2343</td>
<td>F– cydD</td>
<td>Poole et al. (1989)</td>
</tr>
<tr>
<td>AN2540</td>
<td>uncC38 argH pyrE entA</td>
<td>Cox et al. (1987)</td>
</tr>
<tr>
<td>AN2571</td>
<td>cydD ubiG from AN2343</td>
<td>This work</td>
</tr>
<tr>
<td>AN2572</td>
<td>F– cydD recA ubiG</td>
<td>P1 (AN1495) × AN2571, this work</td>
</tr>
<tr>
<td>GE1031</td>
<td>supE42/F20 g3-1831::Tn10dTet</td>
<td>G. M. Weinstock</td>
</tr>
<tr>
<td>HW271</td>
<td>ubiG zei::Tn10dTet from AN2571</td>
<td>This work</td>
</tr>
<tr>
<td>HW272</td>
<td>ubiG zei::Tn10dTet</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655</td>
<td>F–</td>
<td>G. M. Weinstock</td>
</tr>
<tr>
<td>RP112</td>
<td>AN2572/pRP19</td>
<td>This work</td>
</tr>
</tbody>
</table>

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Table 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II SK +</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBR328</td>
<td>Plasmid vector Tc&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pEC10</td>
<td>As pRPB, but with 1.262 kb deletion extending from EcoRV site</td>
<td>This work</td>
</tr>
<tr>
<td>PE110</td>
<td>As pRPB, but with 1.009 kb deletion extending from EcoRV site</td>
<td>This work</td>
</tr>
<tr>
<td>pHC79</td>
<td>Cosmid cloning vector Tc&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hohn &amp; Collins (1980)</td>
</tr>
<tr>
<td>pMZ26</td>
<td>ubiG&lt;sup&gt;+&lt;/sup&gt;, pRP19 digested with EcoRV and recircularized</td>
<td>This work</td>
</tr>
<tr>
<td>pND31</td>
<td>As pRPB, but with 1.295 kb deletion extending from ClaI site</td>
<td>This work</td>
</tr>
<tr>
<td>pND42</td>
<td>As pRPB, but with 1.628 kb deletion extending from ClaI site</td>
<td>This work</td>
</tr>
<tr>
<td>pRK972</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, transposase expressed from tac promoter</td>
<td>Elliott &amp; Roth (1988)</td>
</tr>
<tr>
<td>pRP7</td>
<td>Cosmid (pHC79) clone carrying the wild-type region corresponding to the mutation in AN2571</td>
<td>This work</td>
</tr>
<tr>
<td>pRP19</td>
<td>ubiG&lt;sup&gt;+&lt;/sup&gt;, approx. 4 kb ClaI–ClaI fragment from cosmid clone pRP7 in pBR328</td>
<td>This work</td>
</tr>
<tr>
<td>pRPB</td>
<td>2.818 kb ClaI–EcoRV insert from pMZ26 subcloned into pBluescript II</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 3. Cytochrome levels of HW271 and AN2342

<table>
<thead>
<tr>
<th>Cytochrome level [nmol (mg protein)&lt;sup&gt;–1&lt;/sup&gt;]</th>
<th>AN2342</th>
<th>HW271</th>
</tr>
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<tr>
<td>Cytochrome &lt;i&gt;d&lt;/i&gt;</td>
<td>0.258</td>
<td>0.126</td>
</tr>
<tr>
<td>Cytochrome &lt;i&gt;b-595&lt;/i&gt;/haemoprotein &lt;i&gt;b-590&lt;/i&gt;</td>
<td>0.154</td>
<td>0.072</td>
</tr>
<tr>
<td>Cytochrome &lt;i&gt;o&lt;/i&gt;</td>
<td>0.093</td>
<td>0.132</td>
</tr>
</tbody>
</table>

<i>b-595</i> (or haemoprotein <i>b-590</i>, which are not distinguishable in CO difference spectra of intact cells: Lorence et al., 1986; Poole et al., 1986) were reduced approximately twofold in HW271.

Oxygen consumption rates, a measure of total respiratory electron flux, of extracts from mutant HW271 were depressed relative to activities of the isogenic control strain HW272 (Fig. 1a) and of AN2342 (results not shown) after growth in GMM. However, respiratory rates with various substrates were differentially affected. Thus, oxygen consumption rates by HW271 with lactate or succinate as substrate were about 37% of the rates exhibited by HW272 whereas, with α-glycerophosphate, the respiratory activity of HW271 was 60% of that of HW272. Oxygen uptake rates were highest with lactate and lowest with α-glycerophosphate in both mutant and wild-type. Significantly, the respiratory capacity of HW271 varied less with the different substrates than did that of HW272. The basis for continued respiration in the absence of quinone is not understood. The residual oxidase activity of the quinone mutants may be attributable to the presence of undetectable amounts of ubiquinone, or to relatively ineffective electron transfer by menaquinone, or perhaps by a ubiquinone biosynthesis precursor. Wallace & Young (1977a) have demonstrated that the precursor accumulated by a <i>ubiG</i> mutant did not support the oxidation of D-lactate and succinate, but they were unable to assess whether or not this precursor could function in α-glycerophosphate oxidation, because of the low activity of α-glycerophosphate dehydrogenase in these strains when grown on glucose. We do not know whether HW271 carries a leaky mutation that may produce a partially functional polypeptide. We did not attempt to measure respiration rates of intact cells because of the uncertain nature of endogenous substrates. Since cytochromes were still detectable, we tested whether respiration in HW271 was limited by ubiquinone; reconstitution in vitro was attempted using exogenous quinone analogues. A solution of ubiquinone-I (~130 nm final) was added to the
reaction chamber during measurements of oxygen consumption. The results (Fig. 1b) show that the respiration rates of both mutant and control strains were increased, but the stimulative effect on the mutant was more marked than that on the control. Adding more ubiquinone-1 gave no further enhancement for either strain. The degree of enhancement also differed among the different substrates (Fig. 1c). Menaquinone (Vitamin K₃), ubiquinone-10, and ubiquinone-8 extracted from the wild-type strain had no effect on respiration (results not shown), presumably due to the solubilities of ubiquinone-8 and ubiquinone-10 in water being much lower than that of ubiquinone-1. These results are consistent with early reports (Cox et al., 1970; Wallace & Young, 1977b). Long isoprenoid chain ubiquinone homologues have been used to reconstitute the respiration of pentane-extracted, lyophilized mitochondria. In those experiments, quinones were mixed with phospholipid before carrying out the measurement (Lenaz et al., 1975). The partition of water-insoluble long isoprenoid chain ubiquinone homologues into the membrane phase of highly diluted membrane preparations is expected to be slight.

Quinone levels and complementation

Mutant HW271 complemented with pRP19, and the control strain HW272, were grown in defined medium plus glucose (Gibson et al., 1977) for quinone assays. The extracts from these strains each exhibited spots on TLC plates corresponding to ubiquinone-8, the main quinone of aerobically grown E. coli (results not shown). Extraction of the material and spectroscopic analysis confirmed the identification. No ubiquinone was detected in HW271. The results suggest that HW271 is deficient in ubiquinone biosynthesis and that plasmid pRP19 restores quinone levels in this mutant. Menaquinone levels appeared to be low in all strains.

Identification of the affected gene in mutant HW271 as ubiG

Conventional genetic mapping procedures failed to give satisfactory results. However, preliminary DNA sequence analysis of the insert in pMZ26 (a 2.8 kb ClaI-
Fig. 3. Complete nucleotide sequence and its encoded protein sequence. The sequence from 478 to 696 was taken from Hussain et al. (1987).
EcoRV fragment from pRP19) showed that the Clal end was identical to part of the gyrA gene, which maps at 48 min on the E. coli linkage map adjacent to ubiG (Bachmann, 1990). Confirmation that the gene cloned in pRP19 and pMZ26 is ubiG was obtained by transforming the ubiG mutant AN151 (Leppick et al., 1976) with these plasmids and finding that it was complemented for aerobic growth on SMM. To determine the physical map location of ubiG, HW271 was coinfected with λcl857 and one of seven phage clones, 370–376, from the ordered Kohara miniset library (Kohara, 1990). Only clone 376 (E13A5) gave rise to colonies on SMM medium. This indicates that ubiG is located in the 7 kb region of this clone which does not overlap with clone 375, that is at approximately 2348–2358 kb on the physical map and close to 48 min on the linkage map.

Sequence of the 2·8 kb insert in pMZ26

The insert from pMZ26 was cloned into the DNA sequencing vector pBluescript II, and designated pRPB. Fig. 2(a) shows the strategy for sequencing this 2·818 kb Clal–EcoRV fragment. Nucleotide sequences were obtained using both strands as templates and were in agreement. Fig. 2(b) shows the extent to which the complete nucleotide sequence of the Clal–EcoRV fragment (Fig. 3) includes regions studied by Hussain et al. (1987), Swanberg & Wang (1987) and Yoshida et al. (1988). These workers did not identify the ORF that we assign to ubiG (see below). The Clal end of the sequence (nucleotide numbers 6–478) is identical with the reported gyrA gene (Swanberg & Wang, 1987; Yoshida et al., 1988), but identity starts from a HindIII site (nucleotide 6) adjacent to the Clal site. The Clal site is not part of the reported gyrA sequence and may be a cloning artefact. The sequences reported by Hussain et al. (1987) and Swanberg & Wang (1987) also disagree with each other at a number of sites; for example, at nucleotide number 1029 and 1045, both Gs are reported as A by Swanberg & Wang (1987), and the A at nucleotide 1087 is missing in their sequence. Our sequence (Fig. 3) agrees with that of Hussain et al. (1987).

Several potential long ORFs, shown in Fig. 2(b), were found by searching for initiation and termination codons in the sequence shown in Fig. 3. To identify the complementing region of the insert, the ubiG mutants AN151 and HW271 were transformed with plasmids deleted for several regions (see Fig. 2b); transformants were selected anaerobically on complex medium plus ampicillin and screened aerobically on LB plates and on SMM. Transformation of mutants AN151 and HW271 with pEIl10 restored aerobic growth on LB to levels exhibited by the wild-type strains and by the same mutants transformed with pMZ26. This result suggests that the long ORF extending almost to nucleotide 1679 in Fig. 2(b), and labelled there as ubiG, is the gene that complements these mutants. Plasmid pEC10 is deleted such that the protein product lacks the last four amino acid residues of the presumptive UbiG protein; it gave partial complementation of aerobic growth of these mutants on LB. The other two deletion clones, pND31 and pND42, did not complement either mutant on LB.

There is a potential-ribosome binding site GGAG (Shine & Dalgarno, 1974) beginning at nucleotide number 835, 11 bp upstream of the ATG codon at nucleotide number 850 (Fig. 3). However, unequivocal determination of the translation initiation site must await determination of the N-terminal sequence of the protein. The potential −35 and −10 regions were also determined, but the actual transcription initiation site must be determined by primer extension experiments or S1 mapping.

After the termination codon, three PU (palindromic unit) sequences are found (Fig. 4). There is no typical rho-independent terminator. However, between the second and third PU, there is a sequence (boxed in Fig. 4) which matches exactly the one found at the 3′ terminus of some rho-dependent terminators (CAATCAA; Lau et al., 1984). This sequence does not suggest a rho-dependent terminator, because the distance between the termination codon and the first PU sequence is only 19 bp (Fig. 3). The rho-dependent termination needs a sequence of at least 70–80 bp without any secondary structure (Bear & Peabody, 1988). Some PU sequences can terminate transcription but most do not (Gilson et al., 1987). It is not yet clear whether they are terminators in this case, but two factor-independent terminators were identified in this region at nucleotide numbers 1641 and 1752 using a computer-based search for terminators (Brendel & Trifonov, 1984).

Sequence comparison of the UbiG protein with other proteins

The nucleotide-derived amino acid sequence (Fig. 3) was used to search the OWL database by using the Prosrch
program through the DAP (Distributed Array Processor; Collins & Coulson, 1988). No protein that showed significant overall similarity was detected, but a number of proteins exhibited some local similarities with the UbiG protein. Thus, the segment of UbiG from amino acid residues 59 to 77 is similar to a sequence found in the UbiG protein, which is reported to map at 40 min (Bachmann, 1990). The 290 amino acids of the protein, sequence (6) from Clarke et al. (1991). The numbers to the left of the sequences indicate amino acid residue numbers in each protein. The three conserved Gs in the AdoMet binding sequence are boxed.

Fig. 5. Alignments of sequences around the putative AdoMet binding site. Predicted secondary structures are also indicated; β, β sheet, α, α helix, T, turn; C, coil. Δ indicates a conserved hydrophobic amino acid; ▲ indicates a conserved charged or polar amino acid. ○, invariant glycine. Proteins were chosen from a multiple sequence alignment (not shown) to represent different groups of methyltransferase. (a) UbiG (this work); (b) 3,4-dihydroxy-5-hexaprenylbenzoate (DHBB) methyltransferase (product of yeast COQ3 gene); (c) protein-β-aspartate methyltransferase, bovine; (d) RNA adenine-N6 methyltransferase, Corynebacterium diphtheriae; (e) rRNA adenine-N4 methyltransferase, Streptococcus sanguis. Sequences (c) and (d) were obtained from Swissprot, sequence (b) is from Clarke et al. (1991). The numbers to the left of the sequences indicate amino acid residue numbers in each protein. The three conserved Gs in the AdoMet binding sequence are boxed.

Another protein encoded by the Clai–EcoRV fragment

The protein encoded by the long ‘orfX’, part of which extends from the EcoRV end of the insert in pMZZ26, was also sought in the OWL database. The 290 amino acids of the C-terminus of this protein are very similar to acetyl-CoA synthetase from Aspergillus nidulans and Neurospora crassa (Connerton et al., 1990) with 44% and 42% identity respectively. Examination of the 48 min region of the E. coli linkage map (Bachmann, 1990) suggests that this protein might belong to the ato operon, which is involved in short-chain fatty acid degradation (Jenkins & Nunn, 1987). The gene for acetyl-CoA synthetase in E. coli is reported to map at 40 min (Bachmann, 1990).

Discussion

E. coli, together with other Gram-negative facultative bacteria, contains isoprenoid quinones of both the benzene and naphthalene series. The major benzoquinone is ubiquinone-8 (with the figure referring to the number of prenyl units present in the side chain), whilst the naphthoquinones are either menaquinone or demethylmenaquinone. The biosynthetic pathways of ubi-
quione and menaquinones 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
AdoMet (except in the case of demethylmenaquinone).
The final step in the biosynthesis of ubiquinone is the
methylation of 2-octaprenyl-3-methyl-5-hydroxy-6-meth-
oxoy-1,4-benzoquinone, a reaction catalysed by the
product of the ubiG gene (Leppick et al., 1976). The gene
described in this paper is identied as ubiG by
complementation experiments and by the map position,
adjacent to gyrA, provided by the sequence data.

Previous attempts to isolate ubiquinone-deficient
mutants have exploited the fact that such mutants are
unable to grow on succinate, have low oxidase activities,
and grow slowly on glucose aerobically, yet normally on
anaerobic growth at glucose. However, after nucleotide number 696, the two se-
quences are no longer identical. No known restriction site has been located in this region, so it is unlikely to be
an artificial rearrangement. Mapping the genes around
gyrA has always been problematic. Bachmann (1987)
suggested that perhaps one (or more) of the strains which
had been used in several laboratories in the mapping of
this region carry an inversion of the chromosome between
udk and gyrA. It is interesting to note that the
sequence of Hussain et al. (1987) has only two PU
sequences but ours has three. The PU sequences are
highly conserved; this suggests that they may be sites
where recombination of the chromosome between
homologous region has occurred (see Gilson et al.,
1987).

A database search has revealed a short stretch of the
UbiG polypeptide which is highly similar to a sequence
that occurs in some, but not all, methyltransferases.
Within this family of methyltransferases, the sequence
motif $\Delta A D / E A G X G X G X A X X X X A A \wedge$ is highly con-
served, while other parts of the proteins show very little
resemblance to each other. This common motif may
reflect a common tertiary structural element which
Corresponds to the common nature of the reactions
catalysed, and of the methyl group donor. This region
corresponds to Region I identified by Ingrosso et al.
(1989), but we are unable to locate in UbiG sequences
that correspond to regions II and III in that paper. Since
regions II and III are present in only a few methyltrans-
ferases, and are not conserved in recently sequenced
AdoMet-dependent methyltransferases (see Haydock et

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**Fig. 6.** The 48 min region of the E. coli physical map (Kohara et al., 1987). The PstI fragment is probably that which Gibert et al. (1988)
used to construct a ubiG–lacZ fusion. The dotted arrow shows the direction of transcription proposed by Gibert et al. (1988), and the
shaded region is the sequence that differs from that reported by Hussain et al. (1987).
genes, the product of both being required for synthesis of ubiquinone by Escherichia coli. Biochemical Journal 236, 134-144.

Recently, Clarke et al. (1991) sequenced the COQ3 gene coding for the 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase involved in ubiquinone biosynthesis in Saccharomyces cerevisiae. The predicted amino acid sequence of the gene product showed 40% identity with the predicted protein product of the open reading frame on the E. coli genome identified as ubiG in the present work. The COQ3 gene product codes for one of the early reactions in ubiquinone biosynthesis in the yeast whereas ubiG codes for the terminal methylation in E. coli. It seems likely, therefore, that the other transmethylation enzymes in the ubiquinone biosynthetic pathways in E. coli and S. cerevisiae will also show sequence homology.

Gibert et al. (1988) used a BamHI site on a cloned PsI–PstI fragment to make a ubiG–lacZ fusion. This fragment is known to contain the rrdAB gene and part of gyrA: ubiG is between them (Platz & Sjoberg, 1980). These genes are known to be located at the 48 min region of the E. coli chromosome, and the restriction sites of this fragment match the E. coli physical map of Kohara et al. (1987, Fig. 6). Presumably, the BamHI site in the middle of this fragment is that which Gibert et al. (1988) used to construct the ubiG–lacZ fusion. They concluded that ubiG is transcribed towards the gyrA gene, and subject to catabolite repression. Our sequence analysis results show an opposite transcriptional direction, as indicated in Fig. 6. No Cap binding site (Ebright et al., 1987), as reported by Gibert et al. (1988), has been identified and no BamHI site exists in the fragment on which we are working. The BglII site on the insert of pMZ26 is close to the EcoRV end (Fig. 3). The results suggest a chromosomal rearrangement of our fragment on the left-hand side of the BglII site which is close to ubiG (Fig. 6). One possible explanation is that the ubiG mutant (AN86) which Gibert et al. (1988) used, and AN151 used in the present study, each carry a mutation in one of two closely linked genes, the product of both being required for synthesis of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase. In this case the UbiG protein might consist of two different subunits. However, recent complementation experiments with AN86 (G. Wu, unpublished results) using plasmid pMZ26 described in this paper gave results identical with AN151 described here.

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