

## Genetic Evidence for an Interaction of the UbiG O-Methyltransferase with UbiX in *Escherichia coli* Coenzyme Q Biosynthesis

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Received 10 May 2006/Accepted 22 June 2006

**IS16 is a thiol-sensitive, Q-deficient mutant strain of *Escherichia coli*. Here, we show that IS16 harbors a mutation in the *ubiG* gene encoding a methyltransferase required for two O-methylation steps of Q biosynthesis. Complementation of IS16 with either *ubiG* or *ubiX*<sub>K-12</sub> reverses this phenotype, suggesting that UbiX may interact with UbiG.**

Ubiquinone (coenzyme Q or Q) is a prenylated, redox-active lipid that functions as an electron carrier in the respiratory electron transport chain in mitochondria of eukaryotes and the plasma membranes of most prokaryotes (5, 8). In addition to the role of Q in the electron transport chain, the redox poise of the quinone/hydroquinone pool (Q/QH<sub>2</sub>) acts as a signal for the global two-component ArcB/ArcA (anoxic redox control) system in *Escherichia coli* (9). Q also functions in the process of disulfide bond formation in *E. coli* periplasm (2).

Previous work has shown that IS16, a Q-deficient mutant strain of *E. coli*, is thiol hypersensitive and unable to grow on succinate. Expression of the *ubiX* gene from *E. coli* K-12 was found to rescue the IS16 Q-deficient phenotypes (30). *E. coli* has two distinct genes, *ubiD* and *ubiX*, thought to be involved in the decarboxylation of 3-octaprenyl-4-hydroxybenzoate (19, 23). The IS16 mutant strain was found to contain an *ubiX* gene sequence identical to that of its parental strain THU (an *E. coli* 15 strain), encoding a single-amino-acid substitution (S98R) relative to the *ubiX* sequence from *E. coli* K-12. It was proposed that strain IS16 harbored a second mutation in *ubiD*, a gene considered to be isofunctional with *ubiX* (30). The physical location of *ubiD* on the *E. coli* chromosome was established (31). The *ubiD* gene sequence was determined for both THU and IS16 strains; however, we found no mutation in the *ubiD* gene of either strain, indicating that the mutation must reside elsewhere.

Here, we show that IS16 harbors a mutation in the *ubiG* gene encoding a methyltransferase required for two O-methylation steps of Q biosynthesis. We show that Q biosynthesis in IS16 is restored by expression of either the *E. coli ubiG* or the *ubiX*<sub>K-12</sub> gene, providing genetic evidence for an interaction of UbiG and UbiX in Q biosynthesis in *E. coli* (Table 1 shows a list of strains).

**Identification of an *ubiG* mutation in the IS16 mutant strain.** To identify the metabolic block of Q synthesis in strain IS16, a plasmid library was constructed from genomic DNA of *E. coli* THU. Chromosomal DNA of strain THU was partially

digested by Sau3AI, and 3- to 12-kb fragments were inserted into the BamHI sites of pUC18 plasmids (Amersham Pharmacia Biotech Inc., Piscataway, NJ). IS16 cells were transformed with this library, and transformants were screened for the ability to grow on media containing succinate. The complementing chromosomal insert contained a 6,023-bp hybrid segment of DNA resulting from the ligation of two chromosomal Sau3AI fragments into the vector; one fragment contained a portion of *gyrA*, complete *ubiG*, and a portion of *xfal*, while the second fragment contained *araB*, complete *araA*, and a small stretch of *araD*. To determine whether IS16 harbors a mutation in the *ubiG* gene, the sequence was amplified from IS16 and THU genomic DNA. Sequence analysis revealed that the *ubiG* gene in IS16 contained a unique nucleotide substitution, T395A, which resulted in an amino acid change, L132Q. The L132Q mutation in IS16 lies adjacent to methyltransferase motif II (Fig. 1). The four motifs shown in Fig. 1 are present in a large family of AdoMet-dependent methyltransferases (18).

The structure of UbiG is not currently available; hence, it is difficult to ascertain the functional role of this amino acid substitution. However, based on the known crystal structure of the rat catechol O-methyltransferase (COMT), the post-motif II region is known to comprise the active site of the enzyme, with specific residues that contact AdoMet and the catechol

TABLE 1. Genotypes and sources of *E. coli* strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference
<i>E. coli</i> strains		
HW272	Parental strain of GD1	28
GD1	<i>ubiG</i> ::Kan	13
THU	Derivative of strain 15, <i>thy-43 his-67 pyr-37</i>	7
IS16	Mutagenized derivative of THU ( <i>ubiG</i> T395A)	29; this study
Plasmids		
pUbiG <sub>THU</sub>	Harbors <i>ubiG</i> <sub>THU</sub> (Glu8)	This study
pAHG	Harbors <i>ubiG</i> <sub>K12</sub> (Val8)	13
pHZ1	Harbors <i>ubiX</i> <sub>THU</sub> (Arg98)	29
pPZ2	Harbors <i>ubiX</i> <sub>K12</sub> (Ser98)	29

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	Motif I	post I	Motif II	↓(L132Q)	Motif III
UbiG	V L D V G C G G G <sub>68</sub>	V T G L D <sub>85</sub>	G Q Y D V V T C <sub>128</sub>	M E M <span style="border: 1px solid black; padding: 0 2px;">L<sub>132</sub></span>	L V K P G G D V F F <sub>173</sub>
Coq3	V L D V G C G G G <sub>138</sub>	V Q G I D <sub>157</sub>	G Q F D I I T C <sub>199</sub>	M E M <span style="border: 1px solid black; padding: 0 2px;">L<sub>203</sub></span>	L N P E K G I L F L <sub>229</sub>
hCoq3	I L D V G C G G G <sub>99</sub>	V I G I D <sub>116</sub>	E T F D A V V A <sub>162</sub>	S E V <span style="border: 1px solid black; padding: 0 2px;">V<sub>166</sub></span>	V L K P G G S L F I <sub>191</sub>
COMT	<u>β<sub>1</sub></u> V L E L G A Y C G <sub>70</sub>	<u>β<sub>2</sub></u> L L T M E <sub>90</sub>	<u>β<sub>4</sub></u> D T L D M V F L <sub>140</sub>	D H W <sub>143</sub> K <sub>144</sub>	<u>β<sub>5</sub></u> L L R K G T V L L A <sub>168</sub>
	a	a	m a a/s s		

FIG. 1. Alignment of *E. coli* UbiG, *S. cerevisiae* Coq3, human Coq3, and rat COMT amino acid sequences across methyltransferase motifs I, post-I, II, and III. The box designates the L132Q *ubiG* mutation in the IS16 mutant strain. This amino acid substitution is adjacent to methyltransferase motif II. Sequence analysis revealed that *ubiG* genes from THU and IS16 share five nucleotide variances in comparison to the *ubiG* gene from K-12. These variances include T23A, resulting in V8E, and four silent changes, G90T, C109T, A294G, and C321T. Secondary structural elements ( $\beta_1$ ,  $\beta_2$ ,  $\beta_4$ , and  $\beta_5$ ) and important active site residues involved in the binding of ligands are indicated for the rat soluble COMT. a, AdoMet; m, magnesium; s, substrate (27).

substrate (29). The alignment between *E. coli* UbiG, *Saccharomyces cerevisiae* Coq3p, human hCoq3p, and rat COMT amino acid sequences over methyltransferase motifs I, post-I, II, and III is shown in Fig. 1. The UbiG L132Q mutation occurs at the position corresponding to K<sub>144</sub> in COMT, which is shown to be involved in substrate binding. It is reasonable to assume that the L132Q mutation may impact the substrate binding specificity of the UbiG polypeptide. Use of PHYRE, a protein fold recognition server (<http://www.sbg.bio.ic.ac.uk/phyre/>), identified mycolic acid cyclopropane synthases from *Mycobacterium tuberculosis* as protein structures closely related to the UbiG polypeptide sequence. Cyclopropane synthases catalyze the transfer of the methyl group from AdoMet to a

double bond of the acyl substrate. Residues 137 to 144 of mycolic acid cyclopropane synthases are involved in cofactor binding (15), are located in the post-motif II region, and also implicate the UbiG L132Q substitution in UbiG as potentially affecting cofactor or substrate binding.

**IS16 can be complemented by either *ubiG* or *ubiX*.** The *ubiG* gene from *E. coli* THU was amplified by PCR and inserted into pNoTA/T7. The resulting plasmid, pUbiG<sub>THU</sub>, restored growth on succinate and partially restored production of Q<sub>8</sub> in the IS16 mutant (Fig. 2). Rescue of Q-deficient phenotypes can be achieved with levels of Q that are significantly lower than the amount normally present in wild-type cells (3, 6, 17). Lower steady-state levels for the UbiG<sub>THU</sub> polypeptide than for

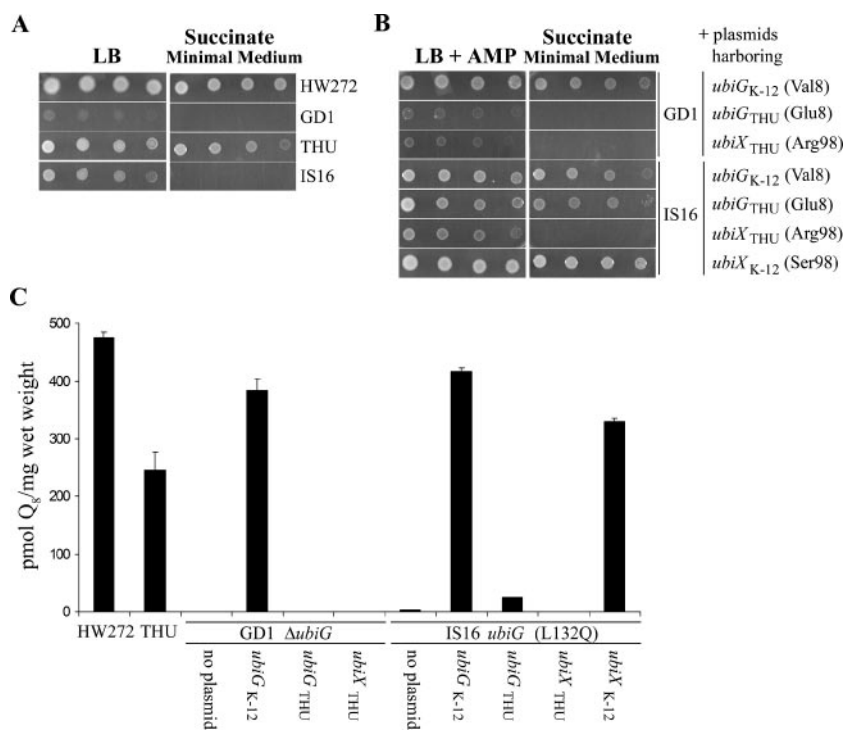


FIG. 2. Succinate growth and Q<sub>8</sub> levels in *E. coli* strains. (A) Serial 10-fold dilutions (starting optical density at 600 nm, 0.2) of the indicated strains were plated on LB and succinate minimal medium plates supplemented with thymine, histidine, and uracil and incubated at 37°C for 1 day. (B) Serial 10-fold dilutions (starting optical density at 600 nm, 0.2) of the indicated strains were plated on LB-AMP and succinate minimal medium plates supplemented with thymine, histidine, and uracil and incubated at 37°C for 1 day. *E. coli* strains harbored plasmids expressing the designated genes as follows: pAHG, UbiG<sub>K-12</sub>; pUbiG<sub>THU</sub>, UbiG<sub>THU</sub>; pHZ1, UbiX<sub>THU</sub>; and pPZ2, UbiX<sub>K-12</sub>. (C) Quinones were extracted and separated by reverse-phase HPLC, and Q<sub>8</sub> content was quantified. A standard for Q<sub>8</sub> was prepared from *E. coli* lipid extracts and verified by mass spectrophotometric methods. External standard curves were created for Q<sub>8</sub> (600, 300, 75, and 30 pmol) and Q<sub>10</sub> (300, 150, and 37.5 pmol). The amounts of Q<sub>8</sub> were corrected by recovery of the Q<sub>10</sub> internal standard.

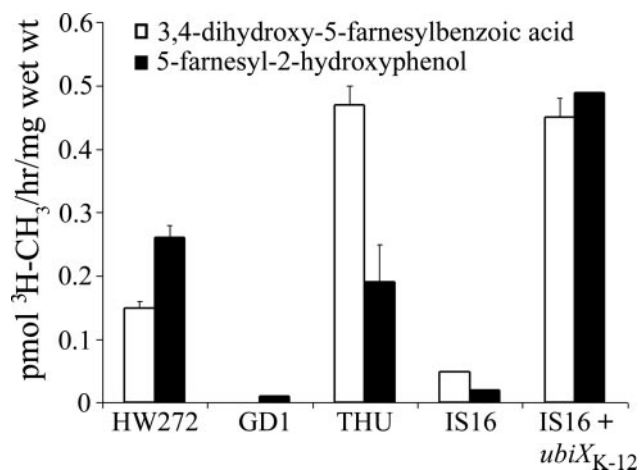


FIG. 3. O methylation of early Q intermediates is defective in IS16 and is restored by expression of *E. coli* UbiX<sub>K-12</sub>. Permeabilized *E. coli* cells were prepared from HW272 (wild-type, parental strain of GD1), GD1 (*ubiG* disruption mutant), THU (parental strain of IS16), IS16 (UbiG-L132Q), and IS16:pPZ2 (IS16 harboring UbiX<sub>K-12</sub> on a plasmid) and incubated with farnesylated analogs of 1 mM 3,4-dihydroxy-5-farnesylbenzoic acid (open bars), 250  $\mu$ M 5-farnesyl-2-hydroxyphenol (closed bars) for 10 min. *S*-Adenosyl-[methyl-<sup>3</sup>H]-methionine (6.9  $\mu$ M, 81.5 Ci/mmol; PerkinElmer Life Sciences) was added to the reaction mixture. Following incubation at 37°C for 45 min, lipids were extracted and separated by reverse-phase HPLC (BetaBasic C18 column, 5  $\mu$ M, 4.6 by 250 mm; Thermo Electron Corporation). Radioactivity present in fractions 6 and 7 (open bars) or fractions 10 to 12 (closed bars) is expressed in pmol of CH<sub>3</sub> groups/hr/mg wet weight and eluted with the <sup>3</sup>H-labeled product standard. Error bars represent standard deviations obtained from two *O*-methyltransferase assays of the same sample, and data shown represent two independent experiments.

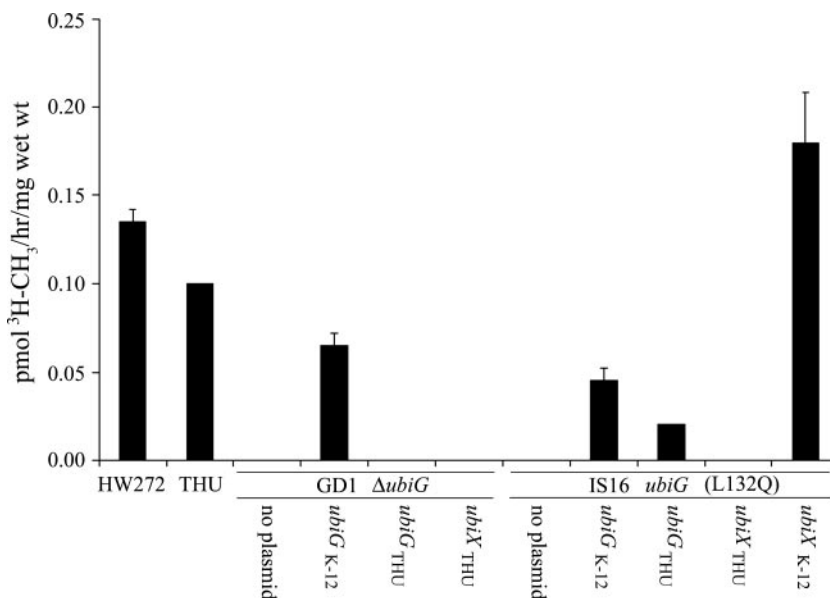


FIG. 4. The final O-methylation reaction is defective in the IS16 mutant and is restored by expression of either UbiG or UbiX<sub>K-12</sub>. In vitro *O*-methyltransferase assays were carried out as described for Fig. 3, except that 50  $\mu$ M 2-farnesyl-5-hydroxy-6-methoxy-3-methyl-1,4 benzoquinone was used as a substrate, and 1 mM NADH was included in all incubations to allow formation of the hydroquinone. At the end of the reaction incubation, 25  $\mu$ l of freshly prepared 1% ammonium cerium (IV) nitrate was added to the reaction mixture prior to lipid extraction (to oxidize hydroquinone products). The elution positions of the methylated products (fractions 11 and 12) were similar to that of the <sup>3</sup>H-labeled product standard and are expressed in pmol of CH<sub>3</sub> groups/hr/mg wet weight. Error bars represent standard deviations obtained from two *O*-methyltransferase assays of the same sample, and data shown represent two independent experiments. *E. coli* strains harbored plasmids expressing the designated genes as follows: pAHG, UbiG<sub>K-12</sub>; pUbiG<sub>THU</sub>, UbiG<sub>THU</sub>; pHZ1, UbiX<sub>THU</sub>; and pPZ2, UbiX<sub>K-12</sub>.

UbiG<sub>K-12</sub> were observed when each construct was expressed in the GD1 mutant strain (data not shown). Both IS16 and *ubiG* null mutant strain GD1 were rescued by *ubiG*<sub>K-12</sub>. Based on this, it seems likely that the differential rescue may be due to efficiency of expression: *ubiG*<sub>K-12</sub> in pAHG is expressed from the yeast *CYC1* promoter, while *ubiG*<sub>THU</sub> is expressed from its native promoter sequence. A plasmid harboring *ubiX* from *E. coli* THU, pHZ1, failed to rescue either IS16 or GD1, while pPZ2, a plasmid harboring *ubiX* from *E. coli* K-12, rescued IS16, as observed previously (30). pPZ2 failed to rescue GD1 (data not shown). For the determination of *E. coli* Q<sub>8</sub> content, cultures were grown in Davis minimal media at 37°C overnight and collected by centrifugation. *E. coli* cells (0.1 to 0.2 g wet weight) were extracted and quinones separated by reverse-phase high-pressure liquid chromatography (HPLC) and quantified with an electrochemical detector as described previously (16). Q<sub>10</sub> (Sigma-Aldrich, St. Louis, MO) was added as an internal standard (final concentration of 20 pmol/ $\mu$ l). The areas of the peaks corresponding to Q<sub>8</sub> and Q<sub>10</sub> samples and standards were determined with Gilson Unipoint version 5.1 software.

***O*-Methyltransferase activity in the IS16 *E. coli* mutant is restored by expression of either *E. coli* *ubiG* or *ubiX*.** The purified *E. coli* UbiG polypeptide has been shown to function as a soluble enzyme and catalyze the *O* methylation of three different farnesylated analogs of intermediates in Q biosynthesis (24). To examine the effect of the L132Q mutation on UbiG *O*-methyltransferase activity in the IS16 mutant, we employed a cell permeabilization assay because this method has been shown to preserve *O*-methyltransferase activity in analyses of

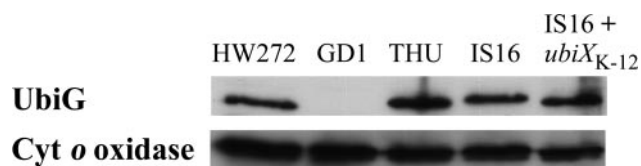


FIG. 5. Steady-state levels of UbiG and cytochrome *o* oxidase in *E. coli* strains. Purified *E. coli* protein His<sub>6</sub>-UbiG (24) was used to generate antisera in rabbits (Cocalico Biologicals, Inc.). Steady-state levels of UbiG were analyzed for *E. coli* HW272 (wild-type, parental strain of GD1), GD1 (*ubiG* disruption mutant), THU (parental strain of IS16), IS16 (UbiG-L132Q), and IS16:pPZ2 (IS16 complemented with UbiX<sub>K-12</sub>) cells. Cytochrome *o* oxidase was used as a loading control.

another *E. coli ubiG* mutant (14). In vitro assays of *O*-methyltransferase activity employed the farnesylated analogs of the *E. coli* intermediate (5-farnesyl-2-hydroxyphenol), demethyl-Q<sub>3</sub> (2-farnesyl-5-hydroxy-6-methoxy-3-methyl-1,4-benzoquinone), or the yeast intermediate (3,4-dihydroxy-5-farnesylbenzoic acid) and *S*-adenosyl-[methyl-<sup>3</sup>H]L-methionine as previously described (22, 24). *O*-methyltransferase activity assays were linearly dependent on time of incubation, substrate concentration, and amount of permeabilized cells. *O*-methyltransferase activity with 3,4-dihydroxy-5-farnesylbenzoic acid (Fig. 3, open bars) or 5-farnesyl-2-hydroxyphenol (Fig. 3, closed bars) was readily detected in the HW272 and THU *E. coli* parental strains, while activity was either not detected or present at significantly lower levels in the GD1 and IS16 *ubiG* mutant strains. Complementation of IS16 with either *ubiX* or *ubiG* from K-12 or *ubiG* from THU rescued *O*-methyltransferase activity with the demethyl-Q<sub>3</sub> substrate (Fig. 4). However, *O*-methyltransferase activity in the *ubiG* disruption mutant GD1 was restored only by *ubiG* from K-12.

**Steady-state levels of UbiG polypeptide in the IS16 mutant strain are similar to those in the wild type.** The steady-state levels of UbiG were examined to determine whether the deficiency in *O*-methyltransferase activity in the IS16 strain was attributed to UbiG polypeptide levels. UbiG steady-state levels for *E. coli* HW272, GD1, THU, IS16, and IS16:pPZ2 (plasmid har-

boring *ubiX*<sub>K-12</sub>) cells were assessed by Western blot analysis as previously described (12). Levels of UbiG polypeptide in IS16 were similar to those in the parental strain THU (Fig. 5).

Complementation studies revealed that multiple copies of *ubiX*<sub>K-12</sub> restore growth on succinate, Q<sub>8</sub> levels (30), and *O*-methyltransferase activity in IS16 (Fig. 2, 3, and 4). This is a surprising finding because the *O*-methyltransferase activity of the purified UbiG polypeptide does not require other Ubi polypeptides (24). It is possible that overexpression of UbiX may stimulate another *O*-methyltransferase with overlapping substrate specificity to UbiG. We consider this unlikely; the rescue is unique to the L132Q UbiG point mutant. Interactions between Ubi polypeptides were not identified by use of tagged constructs to identify multisubunit complexes (7). However, an interaction was detected between UbiX and FldA. FldA plays an essential role in the synthesis of isoprenoid precursors in *E. coli* (26) and suggests that synthesis of the isoprenoid tail occurs in complex with ring modifications. Based on the genetic evidence presented here, UbiX, a protein thought to be involved in a decarboxylation step in Q biosynthesis, may be required to stabilize the catalytic activity of UbiG L132Q by direct interaction or by channeling substrates (Fig. 6). These results provide support for a polypeptide complex involved in *E. coli* Q biosynthesis, first described by Knoell (20, 21). Knoell demonstrated that a complex of membrane-associated polypeptides in *E. coli* converts 2-octaprenylphenol to Q<sub>8</sub> in vitro.

Similarly, a growing body of evidence suggests that a complex of Coq polypeptides is involved in Q biosynthesis in *S. cerevisiae*. Gel filtration chromatography shows that Coq3p, Coq4p, Coq6p, and Coq7p coelute as a high-molecular-weight complex (22, 28). Coq3p, Coq4p, and Coq7 comigrate as high-molecular-mass complexes as assessed by two-dimensional blue native analysis (22, 28). *O*-Methyltransferase activity is decreased in *coq* null mutants relative to that in *atp2* and *cor1* respiratory deficient mutants (13). Deletions in any of the *COQ* genes affect the steady-state levels of Coq3p, Coq4p, and Coq6p (11). Unlike yeast *coq3-coq9* mutants that accumulate

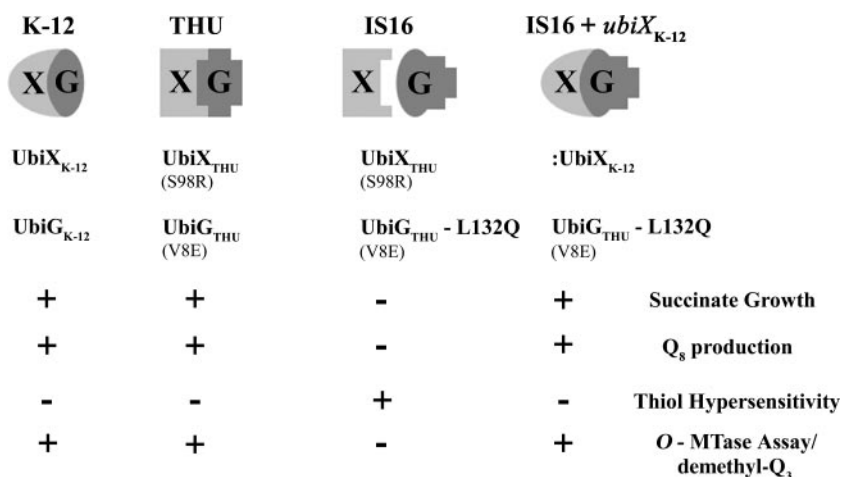


FIG. 6. Genetic evidence for the interaction of UbiX and UbiG. IS16 harbors a mutation in UbiG (L132Q) that catalyzes the *O*-methylation step in *E. coli* coenzyme Q biosynthesis. Lack of growth on succinate, Q deficiency, thiol hypersensitivity (29), and inactive *O*-methyltransferase activity in IS16 are restored with wild-type *ubiX*<sub>K-12</sub>, suggesting that UbiX may be interacting with UbiG (L132Q). X, UbiX; G, UbiG.

an early predominant intermediate, 3-hexaprenyl-4-hydroxybenzoic acid (HHB) (1, 4, 12, 25), *E. coli ubi* mutants tend to accumulate immediate precursor substrates at the blocked steps (10). However, no *ubiG* mutant has been reported to accumulate 3,4-dihydroxy-5-octaprenyl-benzoic acid (14, 27), possibly due to the instability of the catechol moiety. We are currently investigating the lipid quinone intermediate formed in the IS16 strain to further characterize the polypeptide Q-biosynthetic complex in the THU genetic background strain of *E. coli*.

We thank J. N. Shepherd for the farnesylated Q intermediate analogs and W. W. Poon for the generation of the UbiG antibody. We thank N. Lee for the purified *E. coli* protein His<sub>6</sub>-UbiG and R. H. Kaback for the generous gift of the *E. coli* cytochrome oxidase antibody. We also thank members of the Clarke laboratory for helpful suggestions in this study.

This work was supported in part by National Institutes of Health grant GM45952.

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