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The Role of UbiX in *Escherichia coli* Coenzyme Q Biosynthesis

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Abstract

The reversible redox chemistry of coenzyme Q serves a crucial function in respiratory electron transport. Biosynthesis of Q in *Escherichia coli* depends on the *ubi* genes. However, very little is known about UbiX, an enzyme thought to be involved in the decarboxylation step in Q biosynthesis in *E. coli* and *Salmonella enterica*. Here we characterize an *E. coli ubiX* gene deletion strain, LL1, to further elucidate *E. coli ubiX* function in Q biosynthesis. LL1 produces very low levels of Q, grows slowly on succinate as the sole carbon source, accumulates 4-hydroxy-3-octaprenyl-benzoate, and has reduced UbiG *O*-methyltransferase activity. Expression of either *E. coli ubiX* or the *Saccharomyces cerevisiae* ortholog *PAD1*, rescues the deficient phenotypes of LL1, identifying *PAD1* as an ortholog of *ubiX*. Our results suggest that both UbiX and UbiD are required for the decarboxylation of 4-hydroxy-3-octaprenyl-benzoate in *E. coli* coenzyme Q biosynthesis, especially during logarithmic growth.

Introduction

In *Escherichia coli* and *Salmonella enterica*, UbiD and UbiX are generally considered to operate as isofunctional decarboxylases that convert 4-hydroxy-3-octaprenyl benzoic acid (HP₈B) to 2-octaprenylphenol, an early step in the biosynthesis of coenzyme Q (Figure 1) [1]. The *ubiX* gene was first identified in a *S. enterica* mutant TA1851 [2]. Subsequent work by Bar-Tana *et al.*, lent support to the involvement of UbiX in Q biosynthesis [3]. The TA1851 mutant, harboring a deletion including the *ubiX* gene, was deficient in the decarboxylation of HP₈B [4], a reaction already known to be catalyzed by the product of the *E. coli ubiD* gene. Interestingly, 2-octaprenylphenol (and partial rescue of Q₈ biosynthesis) was observed when TA1851 was cultured in the presence of hexanoate or benzoate [4]. The authors speculated that UbiD (or another decarboxylase) might be responsible for the induced decarboxylation activity in the TA1851 mutant.

E. coli ubiD[−] mutants have defects in Q₈ biosynthesis, accumulate HP₈B, and lack decarboxylase activity in vitro [5,6]. *E. coli ubiD*[−] mutants retained the ability to produce about 20-25% of the normal levels of Q [6]. Leppik *et al.* also observed that the amount of HP₈B declined under conditions of prolonged incubation, and the amount of Q₈ increased. Based on

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these observations, Leppik *et al.* concluded that a second enzyme must exist in *E. coli* that functions in vivo to decarboxylate HP₈B [6]. When the homologous *ubiX* sequence was later identified in *E. coli* [7], it was presumed that the residual 20% of Q₈ present in the *E. coli* *ubiD*[−] mutant was due to UbiX [8].

Our interest in the putative UbiX/UbiD protein pair began with the observation that increasing the amount of UbiX in a strain of *E. coli*, hypersensitive to growth inhibition by thioglycerol, restored the cell's ability to cope with this exogenous reducing agent. The actual cause of thiol hypersensitivity was a deficiency of Q [9], a condition that hinders the periplasmic oxidizing system in removing electrons via the electron transport system [10]. The cause for Q deficiency was traced to a mutation in the *ubiG* gene (L132Q) [11] encoding an *O*-methyltransferase required for Q biosynthesis [12]. It was demonstrated that the thiol hypersensitive mutant had normal UbiG protein levels but low *O*-methyltransferase enzyme activity. Expression of wild-type *ubiX* restored *O*-methyltransferase activity and Q₈ synthesis, implicating the interaction of the UbiX and UbiG polypeptides [11]. These observations suggest that UbiX function in Q biosynthesis plays a more central role than previously appreciated.

A homolog of *E. coli* *ubiX* is also present in *Saccharomyces cerevisiae*, the *PAD1* gene (50% amino acid sequence identity). A *S. cerevisiae* *pad1* mutant is defective in decarboxylating phenyl acrylic acids and displays sensitivity to cinnamic acid in plate media [13]. In this previous study, it was assumed that Pad1p functioned as a decarboxylase. However, *E. coli* overexpressing yeast Pad1 lacked decarboxylase activity with a variety of phenyl acrylic acids [14]. Based on its 50% sequence identity with *E. coli* UbiX, the authors postulated that Pad1 might function in Q biosynthesis. Other investigators suspect partner proteins may be required for decarboxylation activity [15]. Thus the functional roles of UbiX and Pad1 in *E. coli* and yeast Q biosynthesis are not known.

In the work reported here, we generated an *E. coli* *ubiX* null mutant to characterize its functional roles in Q biosynthesis and in supporting the UbiG *O*-methyltransferase. We characterize the growth properties of the *ubiX* deletion mutant, determine its Q₈ content, and identify the accumulating Q-intermediate. We determine whether the absence of UbiX affects *O*-methylation in *E. coli* Q biosynthesis and also test whether expression of yeast Pad1 can rescue the Q-deficiency of the *E. coli* *ubiX* null mutant. The results identify yeast Pad1 as an ortholog of *E. coli* UbiX, and indicate that both UbiD and UbiX are required for the decarboxylation of HP₈B. We present a model implicating 2-octaprenylphenol as a lipid component that is required for later enzymatic steps in Q biosynthesis, including the *O*-methylation steps catalyzed by UbiG.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

Strains and plasmids are listed in Table 1. Bacteria were grown in either Luria-Bertani (LB) or minimal salts [16] media at 30°C or at 37°C with shaking. Glucose 0.3% (w/v), was used as carbon/energy source with minimal salts media. Antibiotics used both in liquid media and in plates were: ampicillin (100 µg/ml), chloramphenicol (20 µg/ml) and kanamycin (50 µg/ml). Growth was measured by optical density at 600 nm with a Kontron DB-3500 UV/VIS spectrophotometer (Research Instruments International). Growth of the LL1/pYDR538W strain was conducted at 30°C in order to optimize expression of the yeast Pad1 polypeptide. Thiol sensitivity was measured as described previously [9].

Construction of the *ubiX* null mutant

The LL1 *ubiX* deletion mutant strain was generated by deletion of the *ubiX* ORF and insertion with a kanamycin cassette as described [17]. Approximately 1-kb regions of DNA, flanking the *ubiX* gene of *E. coli* strain MC4100 were isolated by PCR. Primers used to isolate the upstream flanking segment were 1. flusfwdX: 5'-*cccatccactaaactaaaca*GAGCTCTGTCTGACACTTGCAGGCAGACTTTT-3', 2. flusrevX: 5'-GATCGCGGCCGCATTTACATCACTGAAGAAGGGCAGTTGTTT-3'. Primers used to isolate the downstream flanking segment were 3. fldsrevX: 5'-*tgtttaagttagtgatggg*CTCGACATTTTGTGTTTGTGCCATTC-3', 4. fldsfwd4: 5'-TGACCCGGGCGCTGGCGTGCACCATGATGATCATCC-3'. The 5' end of primer #2 contains a *NotI* site and that of primer #4 a *SmaI* site. The lower case, italicized nucleotides of primers #1 and #3 represent reverse complements. Moreover, primer #1 also contains a *SacI* site. PCR products representing the upstream and downstream flanking regions of *ubiX* were purified by agarose gel electrophoresis and aliquots from each were mixed and subjected to a second PCR, with primers #2 and #4. The resulting 2-kb product was first purified and then digested with the enzymes *SmaI* and *NotI* and inserted into plasmid pKO₃. This construct was digested with *SacI* and ligated with a *SacI* digested-kanamycin cassette [18]. The kanamycin cassette containing *SacI* sites was isolated by PCR from pkaner using primers kansacfwd: 5'-TTGAGAGAGCTCTTAGAAAACTATCGAGCATCAAATGAAA-3' and kansanrev: 5'-TTGAGAGCTCAATATATCATCATGAACAATAAACT-3'. The resulting construct p3A, was then used to generate the *ubiX* null mutant, named LL1. MC4100 cells were transformed with plasmid p3A by electroporation, the culture was allowed to recover 90 min at 30°C in SOC medium, and aliquots were plated onto LB plates with 20 µg/ml chloramphenicol pre-warmed to 43°C. After incubation at 43°C overnight, five colonies were resuspended in 1 ml of LB medium, the cell suspension serially diluted, and immediately plated on LB plates with 50 µg/ml kanamycin. These plates were incubated at 30°C and colonies that tested kanamycin positive and chloramphenicol negative in replica plating were examined for the loss of *ubiX* gene by PCR and by DNA sequencing, performed by Davis Sequencing (Davis, CA).

Plasmid construction

The 570-bp *ubiX* ORF from *E. coli* strain MC4100 was PCR-amplified with a forward primer 5-*Clal-ubiX* 5'-CCATCGATATGAAACGACTCATTGTAG-3' from +1 to +19 of *ubiX* ORF, a reverse primer 3-*KpnI-ubiX* 5'-TTGGTACCTTATGCGCCCTGCCAG-3' from +354 to +370, and *Elongase*® DNA Polymerase (Invitrogen Corp., Carlsbad, CA). The amplified DNA product was digested with *Clal* (underlined) and *KpnI* (double underlined) and was then ligated to the *Clal* and *KpnI* cut high-copy pCH vector [12] to generate the plasmid pUbiX (*ubiX* expression is driven from the *S. cerevisiae* *CYC1* promoter).

RT-PCR assay

For quantitative analysis of *ubiD*, *ubiG*, and *ubiX* gene expression, real-time PCR was performed. The Invitrogen SuperScript™ III first-strand synthesis system was used to generate cDNA from RNA that was prepared with the PureYield™ RNA Midiprep kit (Promega, Madison, WI). To quantify gene-specific cDNAs, the products of the first-strand reactions were added to Absolute™ QPCR SYBR Green Mix (ABgene Inc., Rochester, NY). Real-time PCR was accomplished using a Bio-Rad iCycler iQ™ multi-color real time PCR detector system. The following primers were used:

*ubiD*for: 5'-TTGGTAAAAACAACTGATTATGCGCTGGC-3',

*ubiD*rev: 5'-ACCGGGGAAACGTTTCGCCCCGGATGCGCCGCA-3',

*ubiG*for: 5'-GTGCTCGATGTCTGGTTGTGGCGGCGGCATT-3',

ubiGrev: 5'-CATCTCCATGCAGGTCACCACATCATACTG-3',

ubiXfor: 5'-CTTGTCCCTCGAAACGGATTTTCTCTGCG-3',

ubiXrev: 5'-CATCTGCCGCACGGGTCAGTAAGCCATCAG-3',

recAfor: 5'-GCTTTACCCTGACCGATCTTCTCACTTTTG-3',

recArev: 5'-GGGCGAAAACGTGGTGGGTAGCGAAACCGC-3'.

Plate growth assays

E. coli strains MC4100, MC4100/pCH, LL1, LL1/pCH, LL1/pUbiX, LL1/pYDR538W, HW272, and GD1 (*ubiG::Kan*) were grown overnight in 5 ml LB cultures and then diluted to $OD_{600} = 0.1$ in water, followed by three 10-fold serial dilutions in water. Each dilution was spotted (3 μ l) onto Luria Broth and succinate minimal media plates and incubated at 30 °C for one day. Strains harboring plasmids were grown in media supplemented with ampicillin.

Determination of Q₈ content in lipid extracts by Reverse Phase HPLC

For the determination of *E. coli* Q₈ content, ten ml of growth cultures were centrifuged in pre-weighed 15 ml centrifuge tubes. The supernatants were discarded and the wet weights of the pellets were determined. The pellets were re-suspended by vortexing in 980 μ l of water. To each sample 20 μ l of Q₁₀ standard (5 pmol) was added in order to correct for extraction efficiency. Next, 10 ml of methanol/petroleum ether mixture was added (3:2); each mixture was vortexed for 1 minute, and then shaken on a rotary shaker at 4°C overnight in the dark. Following centrifugation, the upper layer from each sample was transferred to a glass tube. The lower phases received 4 ml of petroleum ether; the mixtures were vortexed for 1 minute and were shaken at 4°C in the dark for 1 hour. The upper layers were collected as before and added to the previous organic fractions. The pooled petroleum ether samples were evaporated by a stream of N₂ and the dried residues were re-dissolved in 200 μ l of a methanol/ethanol mixture (9:1). Twenty μ l portions were analyzed by reverse-phase liquid chromatography as described previously [9], with a Beckman Gold chromatographic system (Beckman Coulter, Inc., Fullerton, CA).

Coenzyme Q intermediate labeling and extraction

Aliquots (5 ml) of overnight cultures were used to inoculate 0.5 liter of LB media, containing 3.0 μ Ci of *p*-[¹⁴C]hydroxybenzoic acid (Research Products International Corp., Mt. Prospect, IL) or 1.09 μ Ci of *p*-[¹⁴C]hydroxybenzoic acid (American Radiolabeled Chemicals, Inc., St. Louis, MO). Cultures were harvested ($3500 \times g$, 4 °C, 15 min) in log phase and subject to lipid extraction as described [11] (except no internal standard was added). Briefly, for each gram of wet weight cell pellet, 1 ml water, 9 ml of methanol, and 6 ml of petroleum ether were added. After an overnight incubation, the upper organic phase was removed; two additional extractions with petroleum ether (4 ml) were performed, and added to the previous organic supernatant. This was evaporated under a stream of N₂ and the crude lipids stored at -20 °C.

Normal Phase HPLC

The lipid extracts were resuspended in 0.5 ml hexane. 15 μ l aliquots (1.20×10^3 – 2.87×10^4 dpm) were analyzed by normal-phase HPLC employing a BDS Hypersil Cyano column (Thermo Electron Corporation, Waltham, MA, 250 $\times$ 4 mm, 5 μ particle size) with a cyano guard prior to column fractionation (Thermo Electron Corporation, 10 $\times$ 3 mm, 5 μ particle size). The radioactivity contained in a 15 μ l aliquot of each extract was measured by scintillation counting in 4 ml of ScintiVerse® BD scintillation mixture (Fisher Scientific, Hampton, NH).

with a Beckman scintillation counter (model LS-6500). The efficiency of ^{14}C detection was 87%. The normal phase column was equilibrated for one hour with a mobile phase composed of 99.8% solvent A (heptane) and 0.2% solvent B (isopropanol:heptane:water, 50:45:5) at a flow rate of 1 ml/min. Ten minutes after sample injection, the percentage of solvent B was increased linearly from 0.2 to 20% in 10 min (20 min from the start) and then from 20 to 100% in 20 min (40 min after the start). In the next 2 min, the percentage of solvent B was decreased linearly from 100 to 0.2% and remained at 0.2% for 20 min to equilibrate the column before the next sample injection. The column eluate was monitored (UV absorbance at 274 nm and 250 nm) and the elution of radiolabeled material was detected with an in-line scintillation detector (β RAM Model 2B, IN/US Systems, Tampa, FL). The observed delay between the UV detector signal acquisition and the radioactivity detected was approximately 0.4 minutes. The detector cell volume was 500 μl with In-Flow scintillation fluor (IN/US Systems) to elute ratio of 2:1. The residence time was 10 seconds with a dwell of 2 and the ^{14}C channel settings had lower and upper limits of 50 and 1000, respectively. The efficiency of ^{14}C detection was 89%.

Reverse Phase HPLC detection by Mass Spectrometry

For mass spectrometry analysis, the radio-labeled lipid extract was injected (25 μl /injection) onto a reverse phase BetaBasic C18 column (Thermo Hypersil, Keystone Scientific Operations, 20×2 mm, 5 μ particle size) equilibrated in water/acetonitrile (25/75 (v/v)) and eluted (400 $\mu\text{l}/\text{m}$) with an increasing concentration of acetonitrile (min/% acetonitrile: 0/75; 2/87.5; 3/99; 27/99). The eluate was passed directly into an atmospheric pressure chemical ionization source (APCI, nebulizing gas, “zero”-grade air produced by a Zero Air Generator (Peak Scientific, Chicago, IL) at 2-3 L/sec, nebulizer 450°C, orifice 50 volts) attached to a Perkin-Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer operated in the tandem mass spectrometric (MS/MS) mode (99.999% argon collision gas at an instrumental GCT setting of 200). The mass spectrometer had been previously tuned and calibrated by flow injection (100 $\mu\text{l}/\text{min}$) of APCI/APPI calibrant (Agilent technologies, Santa Clara, CA, Cat. No. G2432A). Calibration across the mass range was made using the singly charged phosphazene ions at m/z 121.1, 290.0, 322.1, 622.0, 922.0, 1322.0, 1521.9 and 2121.8 with instrument resolution set so the ^{13}C isotope satellites were resolved with 10-40% valley.

Product ion analyses of HP₈B in *E. coli* extracts

Crude lipid extracts of *E. coli* strains were separated by RP-HPLC as described above before and after treatment with 1/10th volume of 2.0 M trimethylsilane-diazomethane (TMS-DM) (Sigma-Aldrich, St. Louis, MO, Cat. No. 527254). 50 μl of crude lipid extract was incubated at room temperature for 30 min with 5 μl of TMS-DM. Samples (25 μl) with and without TMS-DM were separated by RP-HPLC with location of retention times for the HP₈B compound and its methylated derivative by MS scanning and UV detection at 250 nm. The retention times for HP₈B and its singly methylated derivative were 13.8 and 19.1 minutes respectively. These precursor ions were then selected for MS/MS detection of product ions (daughter fragments) scanning from 75 to 710 m/z (dwell 2.0 msec, 0.05 pause time, step size 0.3 amu).

Preparation of Q₈ standard

Q₈ was isolated from wild-type *E. coli*. Lipids were extracted as described above, except that the sample was vortexed (no overnight incubation) and isolated lipids were concentrated and applied to a TLC silica plate. The band of Q₈ material was scraped from the TLC plate and eluted from the silica with hexane. This material was characterized versus a previously identified Q₈ standard by retention time from a RP-HPLC method, as described [19].

Assays of O-methyltransferase activity were performed as described previously [11].

Results

***E. coli* UbiX is required for production of Q₈ and growth on succinate media**

To determine the function of UbiX in Q biosynthesis, an *E. coli* null mutant strain (LL1) harboring a deletion of the *ubiX* gene was generated as described in Materials and Methods. Deletion of *ubiX* caused a significant reduction of growth rates in LB and in minimal salts plus glucose liquid media under aerobic conditions (Table 2). The *ubiX* null strain also showed slowed growth on LB as compared to the parental strain, and exhibited a significant growth defect on succinate plate media, though not as pronounced as that of the *ubiG* disruptant, GD1 (Figure 2). The diminished growth capacity of LL1 was likely caused by a defect in Q biosynthesis. Table 2 shows the Q₈ content of cells grown under a variety of aerobic growth conditions. During logarithmic growth Q₈ levels were consistently lower in the LL1 *ubiX* mutant than in the MC4100 parental strain. However, Q₈ content in LL1 increased dramatically during overnight incubation and approached near wild-type levels in stationary phase.

The deficiencies in LL1 growth and Q₈ content can be attributed solely to the absence of the *ubiX* gene because expression of *E. coli ubiX* from a high copy plasmid rescued both defects (Figures 2 and 3). In contrast to current schemes of *E. coli* Q biosynthesis, this result suggests that UbiD and UbiX do not function as independent isofunctional decarboxylases. Expression of the *Saccharomyces cerevisiae PADI* gene (50% amino acid identity to UbiX) in LL1 rescued the growth defect on succinate plate media (Figure 2) and Q biosynthesis (Figure 3). The complementation of LL1 by yeast *PADI* identifies a new functional role for this gene product as an ortholog of UbiX, and implicates its involvement in Q biosynthesis in yeast.

We compared the growth rates of strains MC4100 and LL1 in 1-thioglycerol and dithiothreitol. The results show that LL1 was more sensitive to 1-thioglycerol than MC4100 but dithiothreitol inhibited both strains equally (data not shown). Low Q content diminishes the ability of *E. coli* to cope with reductive stress produced by exogenous thiols [9]. Q deficiency stresses the periplasmic disulfide reductase system, which oxidizes all of the essential periplasmic proteins [10]. This disulfide reductase system may be stressed in a similar fashion by exogenously applied mM quantities of neutral thiols, such as 1-thioglycerol or dithiothreitol. Dithiothreitol, a stronger reducing agent than thioglycerol, overwhelmed the disulfide reductase system in both strains at the concentrations tested.

The *E. coli ubiX* null mutant accumulates 4-hydroxy-3-octaprenylbenzoic acid (HP₈B), a Q₈ precursor

To identify the Q-intermediate accumulating in LL1, growth media were supplemented with either *p*-[U-¹⁴C]hydroxybenzoic acid (Figure 4) or *p*-[COOH-¹⁴C]hydroxybenzoic acid (data not shown). Lipid extracts of cell pellets were separated by normal-phase HPLC as described in Materials and Methods. Lipid extracts prepared from MC4100 wild-type *E. coli* grown in the presence of *p*-[U-¹⁴C]hydroxybenzoic acid contained a major peak of ¹⁴C-radiolabeled material that eluted at 4.8 min and coincided with a Q₈ standard (Figure 4A and B). *E. coli ubiD* and *S. enterica ubiX* mutants have been hypothesized to have defects in the same biochemical step [4]. Therefore to generate a radiolabeled standard, AN66, an *E. coli ubiD*[−] mutant previously shown to accumulate 4-hydroxy-3-octaprenylbenzoic acid (HP₈B) [5], was grown in the presence of *p*-[U-¹⁴C]hydroxybenzoic acid. A small amount of Q₈ is detected in the lipid extract of the AN66 *ubiD*[−] mutant, and an additional peak (250 nm) eluted at 22 min (Figure 4C), and coincided with the predominant ¹⁴C-radiolabeled material (Figure 4D). A similar HPLC retention time profile was observed in separation of LL1 ¹⁴C-radiolabeled lipid extracts; the predominant radiolabeled material eluted with the same retention time as that observed in the *ubiD*[−] mutant (22 min; Figure 4D and F). The LL1 null *ubiX* mutant reveals very little Q₈ (Figure 4E and F). Importantly, the ¹⁴C-radiolabeled material accumulating in

LL1 at the 22 minute retention time is present when either *p*-[^{14}C]- or *p*-[^{14}C]-hydroxybenzoic acid are added to cells (data not shown). Radiolabeled Q₈ was detected only when the radioactive precursor *p*-[^{14}C]hydroxybenzoic acid was incorporated into cellular lipids, but not when *p*-[^{14}C]hydroxybenzoic acid was incorporated (data not shown), as the Q₈ product does not contain a carboxyl group. Expression of *E. coli ubiX* restores production of ^{14}C -labeled Q₈, and the amount of HP₈B is greatly decreased (Figure 4G and H). Expression of yeast *PAD1* also restores ^{14}C -labeled Q₈, although the rescue is not as robust as that provided by *ubiX* as there is still a large accumulation of HP₈B (Figure 4I and J).

Further analyses with mass spectrometric detection of the abundant peaks detected at 250 nm in the separated extracts from AN66 and LL1 revealed an ion with an *m/z* of 683.6. The fragmentation of this ion was further investigated by MS/MS product ion analyses as shown in Figure 5. Panel A depicts the daughter ion spectra collected from HP₈B isolated from AN66 lipid extracts. An inset in panel A shows the altered *m/z* of the tropylium ion after treatment with the methylating agent TMS-DM. The tropylium-like ion is a characteristic daughter ion of compounds with benzyl or substituted benzoquinone structures [20]. As predicted, the tropylium-like ion shifts from 150/151 *m/z* to 164/165 *m/z* after methylation, showing the addition of *m/z* 14. Panel B shows matching daughter ion spectra obtained from the *m/z* 683.6 ion in the lipid extracts of LL1. Methylation of the LL1 lipid extracts generates daughter spectra essentially identical to the AN66 extracts (compare daughter ions in Figure 5, Panels A and B, and methylated daughter ions in insets to Panels A and B). Importantly, our spectral analyses for the HP₈B intermediate collected and purified from AN66 substantially match those published previously for this same strain [5]. HP₈B is an abundant lipid component in AN66 and can serve as a standard for analysis of the hydroxy-polyprenyl-benzoate compounds found in other species.

UbiG O-methyltransferase activity is decreased in the *ubiX* null mutant

Earlier work showed that *O*-methyltransferase activity in the *E. coli ubiG* mutant IS16 (L132Q) is severely reduced and activity is restored by expression of either *ubiG* or *ubiX*_{K-12} [11]. Thus, it was of interest to determine whether the absence of UbiX protein impacted UbiG activity in the LL1 *ubiX* null mutant. *E. coli* harvested during log phase growth showed an average 3.6-fold decrease in UbiG *O*-methyltransferase activity in LL1 as compared to MC4100. The *O*-methyltransferase activity was restored by expression of either *E. coli ubiX*_{K-12} or yeast *PAD1* (Figure 6A). However, *O*-methyltransferase activity in LL1 stationary-phase cells was nearly normal despite the defect in *ubiX* (Figure 6B).

The decrease in *O*-methyltransferase activity and the accumulation of HP₈B is manifested only during log phase growth. To investigate whether the log phase-dependent deficiencies in LL1 might result from a concomitant decrease in gene expression of *ubiD* and *ubiG*, we performed RT-PCR assays to quantify *ubiD*, *ubiG* and *ubiX* RNAs. However, as shown in Table 3, the expression of the *ubiD* gene was not diminished, and *ubiG* gene expression was in fact elevated in the *ubiX* null mutant LL1 relative to the parental MC4100 strain. However, steady state levels of the UbiG polypeptide, as determined by western blotting of protein extracts prepared from either log- or stationary-phase cells, do not appear to be noticeably affected by deletion of the *ubiX* gene (data not shown).

Discussion

Schemes of *E. coli* Q biosynthesis generally depict UbiD and UbiX as decarboxylases that independently catalyze the decarboxylation of HP₈B [1]. Currently, UbiX is assigned a minor role in the decarboxylation of HP₈B. This is based on the finding that significant amounts of Q₈ (20-25% wild-type levels) were detected in four independently derived *E. coli ubiD*[−] mutants [6]. The residual Q₈ present in the *ubiD*[−] *E. coli* mutants has been ascribed to the

presence of UbiX [1]. However, results presented here argue against UbiX and UbiD functioning as redundant decarboxylases. Both *ubiX*⁻ and *ubiD*⁻ *E. coli* mutants accumulate the same Q₈-intermediate, HP₈B (Figures 4 and 5), and the severely decreased Q₈ content in LL1 is also incompatible with the operation of UbiD and UbiX as decarboxylases acting in parallel. Expression of *ubiX* in the LL1 mutant restores Q₈ synthesis (Figures 3 and 4), even though *ubiD* RNA levels are maintained (Table 3). Similarly, a severe depletion in Q₈ content was observed in a recently generated *E. coli ubiD* deletion mutant [21], and in the previously characterized *S. enterica ubiX* deletion mutant [4].

The data presented here support the proposal by Howlett and Bar-Tana that the products of both the *ubiD* and *ubiX* genes are necessary for the decarboxylation of HP₈B [4], especially during logarithmic growth. As shown in the scheme depicted in Figure 7, mutations in either *ubiD* or *ubiX* result in log phase cells that accumulate HP₈B and produce only very low levels of Q₈. In contrast, stationary phase *ubiD*⁻ or *ubiX*⁻ mutants synthesize near normal levels of Q₈. (Table 2 and [6]), presumably via expression of a distinct stationary phase decarboxylase. The absence of UbiD and UbiX homologues in gamma proteobacteria known to produce Q (e.g. *Xanthomonas campestris* and *Acinetobacter* sp.) [22] suggest that these species must possess one or more decarboxylases distinct from either UbiD or UbiX.

Expression of the yeast *PAD1* gene complemented the *ubiX* null mutant LL1; the succinate growth defect was rescued and Q₈ content increased to 67% of wild-type levels (Figures 2 and 3). These findings indicate that yeast Pad1 can replace UbiX and support decarboxylation by UbiD (Figure 7). Hence, our results identify *S. cerevisiae PAD1* as an ortholog of *ubiX*, and implicate its involvement in yeast Q₆ biosynthesis. Intriguingly, the *S. cerevisiae PAD1* gene is located on chromosome 4 and is adjacent to *YDR539W*, a homolog of *E. coli ubiD*. Thus it is tempting to speculate that these genes are required for the related but distinct decarboxylation step in yeast Q₆ biosynthesis (Figure 1). There is evidence that decarboxylases acting in yeast Q biosynthesis may be promiscuous with regard to substrates [31]. However, yeast null mutants lacking either *pad1* or *ydr539w*, or null mutants lacking both *pad1* and *ydr539w*, have Q₆ levels comparable to wild-type yeast [23]. These results indicate that one or more gene products in addition to *PAD1* and *YDR539W* function redundantly to catalyze the decarboxylation reaction of yeast Q biosynthesis.

How do UbiX and UbiD act in concert to decarboxylate HP₈B in *E. coli*? Recent studies indicate that *ubiX*-like genes in many prokaryotes are located within operons that encode partner proteins (including homologs of *ubiD*) and both are required to decarboxylate a variety of hydroxyarylic acids [15]. For example, an operon in *Streptomyces* sp D7 containing the *vdCB* and *vdCC* genes show 50% and 29% amino acid sequence identity with *E. coli* UbiX and UbiD respectively, and both must be present in order to reconstitute the aerobic decarboxylation of vanillate when expressed in *Streptomyces lividans* [15]. However, when expressed in *E. coli*, the *vdCB* gene is no longer needed. It is presumed that an *E. coli* protein (e.g. UbiX) is supplying the necessary partner protein function for hydroxyaryl decarboxylase activity.

Do UbiX and UbiD form a decarboxylase protein complex? The UbiD HP₈B decarboxylase activity eluted from gel filtration chromatography with a mass of 360 kDa [6]. Certainly the high molecular mass of *E. coli* UbiD decarboxylase activity is consistent with the presence of partner proteins. However, UbiD and UbiX polypeptides were not identified as binding partners in a global protein interaction screen [30]. Yeast and *E. coli* Q biosynthetic pathways are proposed to branch at the decarboxylation step (Figure 1), suggesting that the decarboxylases in the two species recognize different substrates [31]. Thus, it seems unlikely that yeast Pad1 would be able to substitute for *E. coli* UbiX and form a Pad1p/UbiD protein complex. Indeed, because yeast Pad1 also rescues *E. coli* UbiG O-methyltransferase activity, one would need to

invoke its interaction with *E. coli* UbiG as well, yet there is only 38% sequence identity of yeast Coq3 and *E. coli* UbiG *O*-methyltransferases [32].

We speculate that the interspecific complementation shown here is not due to the interaction of yeast Pad1 with *E. coli* UbiG, but is rather due to the formation of the 2-octaprenylphenol Q-intermediate. As shown in Figure 7, the 2-octaprenylphenol Q-intermediate produced in response to expression of yeast *PAD1*, or by stationary phase bypass, functions as a crucial lipid component of the Ubi polypeptide biosynthetic complex described by Knoell [26,27]. This 2×10^6 dalton soluble complex was shown to contain 2-octaprenylphenol, multiple polypeptides, and in the presence of oxygen converted 2-octaprenylphenol to Q₈. Moreover, 2-octaprenylphenol is a normal lipid component of wild-type *E. coli* and is present at approximately 15-fold lower levels as compared to Q₈ [28]. Based on these studies and the data presented here, we suggest it is the presence of 2-octaprenylphenol that accounts for the restoration of UbiG *O*-methyltransferase activity in LL1 in response to either expression of the yeast Pad1 polypeptide, or stationary phase growth. It is possible that 2-octaprenylphenol may function as an essential lipid component required for UbiG activity, similar to the role of cardiolipin in stabilizing the respiratory chain complexes [29], or it may function as an allosteric modulator.

Does yeast Pad1 expressed in LL1 act on its own to decarboxylate HP₈B? Several studies suggest this is unlikely. Although yeast Pad1 is required for decarboxylation of phenylacrylic acids in vivo, attempts to detect Pad1 decarboxylase activity in vitro have not been successful [13,24,25]. In the enterohaemorrhagic *E. coli* strain O157:H7 a homolog of yeast Pad1p is present in addition to UbiX. The crystal structure of *E. coli* O157:H7 Pad1p revealed a noncovalently bound FMN present in each monomer of a homo-dodecameric complex [14]. The structure revealed residues contacting the FMN and also implicated strictly conserved residues likely to be involved in substrate binding. Is the FMN involved in the decarboxylation reaction? Neither purified Pad1p from *E. coli* O157:H7 nor extracts from *E. coli* overexpressing yeast Pad1p showed significant decarboxylase activity with a variety of soluble phenyl acrylic acids [14]. Thus, it is tempting to speculate that *E. coli* UbiX, *E. coli* Pad1 and yeast Pad1 homologues function to deliver the substrate to their partner decarboxylase (UbiD-like) proteins, which perform the decarboxylation step. Characterization of the interaction of UbiD/UbiX and the identification of the decarboxylase protein(s) required in yeast Q biosynthesis will help elucidate these questions.

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Figure 1. Coenzyme Q biosynthetic pathway of *Escherichia coli* and *Saccharomyces cerevisiae*
 Polyisoprenyl diphosphate synthases, Coq1p (*S. cerevisiae*) and IspB (*E. coli*), catalyze the formation of the isoprenoid tail. The tail lengths among different organisms vary and are specified by the specific polyisoprenyl diphosphate synthase enzyme present. For example, *S. cerevisiae* synthesize Q₆, bearing six isoprene units, and *E. coli* produce Q₈. In *S. cerevisiae*, the isoprene tail is attached to 4-hydroxybenzoic acid (4-HB) by Coq2p to form 3-hexaprenyl-4-hydroxybenzoate (HP₆B); in *E. coli*, UbiA catalyzes this reaction to form 4-hydroxy-3-octaprenylbenzoate (HP₈B). At this point the pathways are proposed to diverge between prokaryotes and eukaryotes. *E. coli* gene products are identified as Ubi (and include IspB) and *S. cerevisiae* gene products are identified as COQ.

Figure 2. Expression of yeast *PAD1* or *E. coli ubiX*_{K-12} rescues LL1 *ubiX* null growth on succinate minimal media

Serial 10-fold dilutions (starting $A_{600} = 0.1$) of the designated bacterial strains were plated on Luria Broth (LB) and succinate minimal media at 30 °C incubated for one day. HW272 (wild-type, parental strain of GD1), and GD1 (*ubiG::Kan*) were included to provide comparison for growth proficiency and deficiency, respectively. Doubling times were determined in succinate minimal salts liquid media and are presented as (average minutes $\pm$ SD) for each of the following strains: MC4100 (wild-type, parental strain of LL1; 70.9 ± 0.2), MC4100/pCH (empty vector; 76.0 ± 9.2), LL1 (*ubiX::Kan*; 206 ± 12), LL1/pCH (empty vector; 141 ± 22), LL1/pUbiX (plasmid expressing *ubiX*; 85.3 ± 1.1), and LL1/pYDR538W (plasmid expressing *S. cerevisiae PAD1*; 91.8 ± 2.7).

Figure 3. Q₈ content in LL1 *ubiX* null mutant is restored by expression of either *E. coli ubiX* or *S. cerevisiae PAD1*

Strains were grown to logarithmic growth ($OD_{600} \approx 0.5$) in Luria-Bertani media. Growth media for LL1 were supplemented with kanamycin (50 $\mu\text{g/ml}$) or ampicillin (100 $\mu\text{g/ml}$). LL1 strain harboring plasmids expressing the designated genes: pUbiX, *ubiX*; and pYDR538W, *PAD1*. The content of Q₈ for each strain was determined by high performance liquid chromatography as described in [11]. *Error bars* represent standard deviation from three separate injections of the identical sample.

Figure 4. Disruption of the *ubiX* gene in *E. coli* leads to an accumulation of radioactive lipid co-eluting with 4-hydroxy-3-octaprenylbenzoate (HP₈B)

Lipid extracts from *E. coli* strains labeled with *p*-[U-¹⁴C]hydroxybenzoic acid were separated by normal-phase HPLC. Absorbance was monitored at 274 nm (to detect Q₈) and 250 nm (to detect HP₈B); only one wavelength is depicted and is designated for each of the panels on the left side. The ¹⁴C radioactivity (dpm) present in each fraction is depicted in (B, D, F, H, and J). Unless noted otherwise, the amount of lipid extract injected was 15 μl of 500 μl total; chromatograms shown in panels (I and J) represent 15 μl of 50 μl total lipid extract. The paired chromatograms of absorbance and radioactivity (amount of dpm injected is indicated for each panel) correspond to separated lipid extracts from *E. coli* strains, MC4100 (A and B [6.67 ×

10^3 dpm]; AN66, *ubiD* mutant (C and D [2.87×10^4 dpm]); LL1, *ubiX* mutant with pCH empty vector (E and F [1.87×10^3 dpm]); LL1 harboring *ubiX* on a high copy plasmid (G and H [5.02×10^3 dpm]); and LL1 harboring yeast *PADI* (I and J [4.42×10^3 dpm]). There was a 0.4 min time delay between the UV and in-line radiochromatography detector.

Figure 5. LC-APCI-MS/MS mass spectra of radiolabeled HPgB intermediate

The products of the compound designated as 4-hydroxy-3-octaprenyl-benzoate (HPgB) (see Figure 6) were produced by MS/MS analyses of m/z 683.6. Radiolabeled extracts of AN66 (A) and LL1 (B) were separated by RP-HPLC and MS/MS analysis revealed a transition $683.6 \rightarrow 150/151$ characteristic of the tropylium product ion for HPgB. For details of the analysis, see "Experimental Procedures." Treatment with the methylating agent trimethylsilane-diazomethane shifted the tropylium-like ion from $150/151$ m/z to $164/165$ m/z (Panel insets).

Figure 6. *O*-methyltransferase activity is decreased in *ubiX* null in log phase

A) Permeabilized *E. coli* cells grown to log phase in LB ($OD_{600} \approx 0.5$) were prepared from MC4100 (wild-type, parental strain of LL1), LL1 (*ubiX::Kan*), LL1/pUbiX (plasmid expressing *ubiX*), and LL1/pYDR538W (plasmid expressing *S. cerevisiae PAD1*), and incubated with 50 μ M of farnesylated DMeQ₃ and 1 mM NADH for 10 min. *S*-adenosyl-[methyl-³H]-L-methionine (6.9 μ M) was added to the reaction mixture. Following incubation for 45 min, lipids were extracted and separated by reverse-phase HPLC. Radioactivity present in fractions 9-11 is expressed as pmol of ³H-CH₃/hr/mg wet weight and eluted with the ³H-product standard. Error bars represent standard deviation from four independent experiments for MC4100 and LL1 and at least 2 independent experiments for LL1/pUbiX and LL1/pYDR538W. An asterisk (*) indicates statistical significance ($p = 0.012$ by Student's two-tailed *t* test) when comparing the *O*-methyltransferase activity of MC4100 and LL1. B) In vitro *O*-methyltransferase assays were carried out as described in panel A, except MC4100 and LL1 were grown to stationary phase (overnight growth in LB). Error bars represent standard deviation from two separate injections of the identical sample.

Figure 7. Functional roles of UbiD, UbiX, UbiG and 2-octaprenylphenol in Q₈ biosynthesis

During logarithmic growth, both UbiD and UbiX are required to catalyze the decarboxylation of 4-hydroxy-3-octaprenyl-benzoate (HP₈B) and produce 2-octaprenylphenol. Mutations in either UbiD (D) or UbiX (X) result in log phase cells that accumulate HP₈B, and produce only low levels of Q₈. Therefore, the data suggest that both UbiD and UbiX polypeptides are necessary to produce Q₈ in *E. coli* during log phase growth. For a detailed explanation of the model refer to discussion text. D: UbiD; X: UbiX.

Table 1

Strains and plasmids used in this study

Strain or plasmid	Relevant description or genotype	Source
<i>Strains</i>		
MC4100	K-12, <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL150 relA1 flb-5301 deoC1 ptsF25</i>	[33]
LL1	MC4100, <i>ubiX::Kan</i>	This work
HW272	K-12, <i>ubiG</i> ⁺ , <i>zei::TN10dTet</i>	[34]
GD1	HW272, <i>ubiG::Kan</i>	[12]
AN66	Hfr, <i>thr-1</i> ⁻ <i>leu-6</i> ⁻ , <i>ubiD</i> ⁻	[5]
<i>Plasmids</i>		
pKO ₃	gene replacement vector with a temperature sensitive replication origin	[17]
pkanaer	plasmid harboring pUC4K cassette	G. Harding [18]
p3A	pKO ₃ harboring the <i>ubiX</i> downstream flank-pUC4K- <i>ubiX</i> upstream construct	This work
pCH	High-copy <i>E. coli</i> plasmid (empty vector control)	[12]
pUbiX	pCH harboring <i>ubiX</i> _{K-12}	This work
pYDR538W	harboring <i>Saccharomyces cerevisiae PAD1</i> gene	Open Biosystems

Table 2
Growth rates & Q₈ content (expressed as nmol Q₈ mg wet wt⁻¹) of strains MC4100 and LL1 under various growth conditions.

Growth Medium	Doubling rate (min)		Growth phase	Q ₈ content (nmol Q ₈ mg wet wt ⁻¹)	
	MC4100	LL1		MC4100	LL1
Luria-Bertani	35	55	Logarithmic	0.67	0.04
			Early stationary	0.40	0.06
	70	130	Stationary (O/N)	0.48	0.23
			Logarithmic	0.72	0.02
Minimal Salts plus glucose			Stationary (O/N)	0.29	0.23

Table 3**RT-PCR of the *ubiD*, *ubiG*, and *ubiX* Q biosynthetic genes**

Expression levels were normalized to the housekeeping gene *recA*. Results are presented as average $\pm$ standard deviation from three independent experiments.

<i>Gene</i>	<i>Strains</i>	MC4100	LL1
<i>ubiD</i>		13.6 $\pm$ 0.22	15.31 $\pm$ 2.25
<i>ubiG</i>		2.89 $\pm$ 0.36	8.40 $\pm$ 1.43
<i>ubiX</i>		1.00 $\pm$ 0.44	0.00 $\pm$ 0.48