ubiJ, a New Gene Required for Aerobic Growth and Proliferation in Macrophage, Is Involved in Coenzyme Q Biosynthesis in Escherichia coli and Salmonella enterica Serovar Typhimurium.

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ubij, a New Gene Required for Aerobic Growth and Proliferation in Macrophage, Is Involved in Coenzyme Q Biosynthesis in Escherichia coli and Salmonella enterica Serovar Typhimurium

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Ubiquinone (coenzyme Q or Q₈) is a redox active lipid which functions in the respiratory electron transport chain and plays a crucial role in energy-generating processes. In both Escherichia coli and Salmonella enterica serovar Typhimurium, the yigP gene is located between ubij and ubib, all three being likely to constitute an operon. In this work, we showed that the uncharacterized yigP gene was involved in Q₈ biosynthesis and for growth in rich medium but did not present any defect anaerobically. Surprisingly, the C-terminal 50 amino acids, predicted to interact with lipids, were sufficient to restore Q₈ biosynthesis and growth of the ubij mutant. Salmonella ubie and ubib mutants were impaired in Q₈ biosynthesis and in respiration using different electron acceptors. Moreover, ubij, ubie, and ubib mutants were all impaired for Salmonella intracellular proliferation in macrophages. Taken together, our data establish an important role for Ubij in Q₈ biosynthesis and reveal an unexpected link between Q₈ and virulence. They also emphasize that Salmonella organisms in an intracellular lifestyle rely on aerobic respiration to survive and proliferate within macrophages.

Ubiquinone, also known as coenzyme Q, is a lipid component found in organisms ranging from bacteria to mammals. It consists of a conserved quinone head group and an isoprenoid hydrophobic tail, the length of which differs among species (1). In Escherichia coli and Salmonella enterica serovar Typhimurium, the tail of coenzyme Q has eight isoprene groups and is designated Q₈. This molecule is located in the bacterial plasma membrane and serves as an electron carrier between electron donors, such as NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), or lactate dehydrogenase, and terminal electron acceptors, such as cytochrome oxidases or reductases (2, 3). Thus, Q₈ is an essential element for aerobic respiratory growth and for a series of processes depending upon a functional proton motive force, such as nutrient import, cell motility, and protein secretion (4–7). Moreover, mutants affected in Q₈ biosynthesis were shown to be hypersensitive to H₂O₂, as the production rate of O₂⁻ and H₂O₂ in their membranes was significantly higher than that in a wild-type strain (8).

Biosynthesis of coenzyme Q has been studied for many years in both E. coli and Saccharomyces cerevisiae. Besides the prenyltransferase UbiA and the decarboxylases UbiX and UbiD, two types of enzymes, monoxygenases and methyltransferases, catalyze chemical modifications of the aromatic ring of the 4-hydroxybenzoic acid (4-HB) universal precursor (Fig. 1). Beyond this seemingly well-established pathway, uncertainties remain. For instance, UbiB was described as being involved in the C-5 hydroxylation reaction, but we recently demonstrated that this step was catalyzed by UbiI, a new monoxygenase formally known as VisC (9). The function of UbiB remains unclear, although some circumstantial evidence suggests that it might act as a kinase (10, 11). Other monoxygenases include UbiF and UbiH (Fig. 1) (12, 13). Also, ubij, ubie, and ubib mutants do not synthesize Q₈ under aerobic growth conditions but produce appreciable amounts of Q₈ anaerobically (9, 14, 15). This indicates that an anaerobic biosynthetic pathway exists for which we have no clues as to which genes are involved. Methyltransferases include UbiE and UbiG (Fig. 1) (16–18). UbiE is required for the C methylation reactions in both Q₈ and menaquinone (MK₉) biosynthesis, and an ubie mutant synthesizes neither Q₈ nor MK₉ (6, 17, 19). The ubie gene is predicted to be part of an operon including the ubib gene and the yigP gene, whose function is unknown (20). In Salmonella, a transposon-based mutagenesis screen identified yigP as an essential gene (21). Recently, yigP was also proposed to be essential in E. coli (22). Curiously, in this case, the yigP gene was proposed to encode, within its 3' moiety, a small RNA of 252 nucleotides which exhibits an essential function of unknown nature (22). This predicted small RNA was named esre, for “essential small RNA in E. coli.”

In the present study, we focused on the predicted ubie-yigP-ubiB operon, in both Salmonella and E. coli. Derivatives lacking a functional yigP gene could be constructed in both strains, ruling out the hypothesis of an essential role. Moreover, we found the yigP gene to be required for Q₈ biosynthesis under aerobic conditions, and we changed the name of YigP to Ubij. Evidence that the role of ubij in Q₈ biosynthesis was carried out by the Ubij poly-

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peptide and not by a putative small RNA was collected. Finally, we showed that UbiE, UbiJ, and UbiB were required for *Salmonella* intracellular proliferation in macrophages, establishing a link between Q8 biosynthesis and bacterial virulence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are described in Table 1, and primers are listed in Table S1 in the supplemental material. Strains were routinely grown at 37°C in Luria-Bertani (LB) or M9-based minimal medium. For growth studies, overnight cultures were diluted into medium of the same composition to an optical density at 600 nm (OD600) of 0.05. Growth under anaerobiosis was achieved by using the GENbox Anaer generator (bioMérieux) in a dedicated chamber. Ampicillin (50 μg/ml) and kanamycin (25 μg/ml) were added when necessary. Arabinose, glucose, and glycerol were used at 0.2%. Fumarate (20 mM), nitrate (2 mM), and trimethyl amine oxide (TMAO) (20 mM) were used as electron acceptors. Deletion of various genes and concomitant insertion of an antibiotic resistance cassette was carried out using lambda Red-mediated recombination (23). Mutations were moved to the wild-type *S. Typhimurium* strain 12023 by P22 transductions and to the wild-type *E. coli* strain MG1655 by P1 transductions.

**Plasmid construction.** The cloning vector used was pBAD24 (24). The inserts carrying *ubi* variants, *ubiE*, and *ubiB* were PCR amplified from *S. Typhimurium* 12023 by using the primers described in Table S1 in the supplemental material. PCR products were digested using XbaI and NcoI and cloned into pBAD24. The coding sequences of the *E. coli* *ubiJ* gene were PCR amplified from an MG1655 strain by using the primers described in Table S1, digested using EcoRI and SalI, and cloned into pBADI*, yielding pubiJE.c. and pubiJ50E.c. pubiJ50E.c. was digested by EcoRI and HindIII, and the insert carrying the *ubi* variant was cloned in

![FIG 1 Biosynthetic pathway of coenzyme Q<sub>8</sub> in *Escherichia coli*. The octaprenyl tail is represented by R on carbon 3 of the different biosynthetic intermediates. The names of the enzymes catalyzing the reactions (each labeled with a lowercase letter) are provided. 4-HB, 4-hydroxybenzoic acid; DDMQ<sub>8</sub>, C-1-demethyl-C-6-demethoxy-Q<sub>8</sub>; Q<sub>8</sub>, coenzyme Q<sub>8</sub>.](image)

**TABLE 1 Bacterial strains and plasmids**

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>S. Typhimurium</em></td>
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<tr>
<td>12023</td>
<td>Wild type</td>
<td>Laboratory stock</td>
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<tr>
<td>ΔubiJ mutant</td>
<td>12023 ΔubiJ::Kan</td>
<td>This study</td>
</tr>
<tr>
<td>ΔubiE mutant</td>
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<td>ΔubiB mutant</td>
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<td><em>E. coli</em></td>
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<tr>
<td>MG1655</td>
<td>Wild type</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBAD24</td>
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<td>pTrc99A</td>
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<td>Template plasmid containing an FRT-flanked kanamycin resistance</td>
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<td>Red recombinase expression plasmid</td>
<td>23</td>
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<td>This study</td>
</tr>
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<td>pubiB</td>
<td>pBAD24 derivative carrying <em>ubiB</em> (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>This study</td>
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<td>This study</td>
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<td>pubiJ 50C&lt;sub&gt;E.c&lt;/sub&gt;</td>
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*FRT, Flp recombinase target.*

January 2014 Volume 196 Number 1

*jb.asm.org* 71
the pTrc99A-6his vector at the same restriction sites. The resulting plasmids were verified by DNA sequencing.

In silico genome analysis. The genome analyzed was that of *Salmonella enterica* serovar Typhimurium LT2 (25). BLAST analyses were performed using NPS@ network protein sequence analysis (26). YigP (STM3971) was annotated as a putative uncharacterized protein, and we renamed it UbiJ.

**Quinone extraction and quantification by HPLC-ECD analysis.** Five milliliters of cells in exponential phase was centrifuged, and the pellet mass was determined. Glass beads (100 μl), 50 μl of 0.15 M KCl, and a volume of a Q10 solution (used as an internal standard) proportional to the wet weight were added to cell pellet. Lipid extraction was performed by adding 0.6 ml of methanol, vortexing for 10 min, adding 0.4 ml of petroleum ether (boiling range, 40°C to 60°C), and vortexing for 3 min. The phases were separated by centrifugation for 1 min at 5,000 rpm. The upper petroleum ether layer was transferred to a fresh tube. Petroleum ether (0.4 ml) was added to the glass beads and methanol-containing tube, and the extraction was repeated. The petroleum ether layers were combined and dried under nitrogen. The dried samples were stored at −20°C and were resuspended in 100 μl of 98% ethanol, 20 mM lithium perchlorate. Aliquots were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) with a C18 column (Betabasic-18; 5 mm, 4.6 by 150 mm; Thermo Scientific) at a flow rate of 1 ml/min using 40% ethanol, 40% acetonitrile, and 20% of a mix of 90% isopropanol and 10% lithium perchlorate (1 M) as a mobile phase. Quinones were quantified with an ESA Coulochem III electrochemical detector (ECD) and a 5011A analytical cell (E1, 600 mV; E2, 600 mV). Hydroquinones present in samples were oxidized with a precolumn 5020 guard cell set in oxidizing mode (E, 650 mV). Different volumes of the standard Q10 solution were injected under the same conditions to generate a standard curve which was used for Q8 quantification. The signal of the absorbance at 210 nm was used to estimate the content in DMK8 and MK8 by integrating the respective peaks. Possible sample loss during the organic extraction was corrected on the basis of the recovery of the Q10 internal standard.

**Western blot analysis.** *ubiJ*, *ubiJ*′, *ubiJ*′′, and *ubiB*′′ carrying a His 6 tag at the C terminus were transformed in an MG1655 *E. coli* strain. The resulting strains were grown in LB until an OD600 of 4 was reached and then diluted 1:100 in LB supplemented with the pTrc99A-6his vector at the same restriction sites. The resulting plasmids were verified by DNA sequencing.

**FIG 2** The *ubiJ* mutant exhibited a growth defect under aerobic conditions. (A) Genetic organization of the *ubiE, ubiJ*, and *ubiB* loci in *Salmonella Typhimurium*. (B) Wild-type (filled circles), Δ*ubiE* (filled squares), Δ*ubiJ* (open circles), and Δ*ubiB* (open squares) strains were grown overnight and then diluted at an OD600 of 0.05 in LB medium at 37°C under aerobic conditions. Growth was monitored at 600 nm. (C) The wild type (filled circles) and the Δ*ubiJ* strain transformed with pBAD24 (filled squares), with *ubiJ*′ (open squares), and with pubiB (open circles) were grown overnight and then diluted at an OD600 of 0.05 in LB under aerobic conditions. Growth was monitored at 600 nm. (D) The strains previously described were grown overnight anaerobically and then diluted at an OD600 of 0.05 in LB under anaerobic conditions. Growth was monitored at 600 nm. (E) Wild-type (black bars), Δ*ubiE* (white bars), Δ*ubiJ*′ (light gray bars), and Δ*ubiB*′ (dark gray bars) strains were grown under anaerobiosis in minimal medium supplemented with glucose or glycerol as a carbon source and with nitrate, trimethylamine N-oxide (TMAO), or fumarate as an electron acceptor. OD600 values were recorded after 16 h of incubation at 37°C. The experiments whose results are shown in panels B, C, and D were performed at least in triplicate. Identical patterns were obtained, and results of a representative experiment are shown. Error bars (E) show standard deviations.
0.02% arabinose. The cultures were incubated at 37°C. Cells were harvested, and the pellet was washed twice. The supernatant was removed and the pellet resuspended in SDS buffer (TS-TD). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Bio-Rad Mini-Protein II cells. Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) sheet (Millipore) and probed with HisProbe-HRP (Thermo Scientific). Blots were visualized with an enhanced chemiluminescence (ECL) reagent (Thermo Scientific).

**Bacterial infection of macrophages.** RAW 264.7 macrophages were seeded at a density of 4 x 10^5 cells per well in 6-well tissue culture plates containing Dulbecco’s modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) (HyClone). Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) sheet (Millipore) and probed with HisProbe-HRP (Thermo Scientific). Blots were visualized with an enhanced chemiluminescence (ECL) reagent (Thermo Scientific).

**RESULTS**

The ubij gene is required for *Salmonella* aerobic growth. In *E. coli*, the genetic organization of the *ubiE*, *ubiI*, and *ubiB* loci was proposed to form an operon (Fig. 2A) (20). In *S. Typhimurium*, this cluster of genes shares 81.5% sequence identity with that in *E. coli*. Thus, we constructed *ubiE*, *ubiI*, and *ubiB* deletion mutants in *Salmonella*. During aerobic growth in LB at 37°C, the *ubiI* and *ubiB* mutants exhibited a significant growth defect compared to the wild-type strain (Fig. 2B). The *ubiB* mutant was much more severely affected and did not exceed a final OD_{600} of 0.4 (Fig. 2B). To make sure that the *ubiI* mutation did not affect *ubiB* expression, the *ubiI* mutant was transformed with plasmids carrying either *ubiI* or *ubiB*. Whereas the *ubiB* mutant was fully comple-

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**FIG 3** UbiJ is required for Q8 biosynthesis under aerobiosis. (A) Quantification of cellular Q8 content in lipid extracts from WT, ΔubiE, ΔubiJ, and ΔubiB cells grown under aerobiosis (black bars) or anaerobiosis (white bars). Error bars show standard deviations. *, not detected. (B) Demethylmenaquinone (DMK8, black bars) and menaquinone (MK8, white bars) content in lipid extracts from WT, ΔubiE, ΔubiI, and ΔubiB cells grown under aerobiosis (+O_2, left) or anaerobiosis (−O_2, right). Error bars show standard deviations. *, not detected. (C) The wild type (circles) and the *ubiJ* mutant (squares) were grown overnight under either aerobic (open symbols, +O_2) or anaerobic (filled symbols, −O_2) conditions. The cultures were then diluted at an OD_{600} of 0.05 in LB under aerobic conditions. Growth was monitored at 600 nm, and the doubling time was calculated during the exponential phase. (D) The *ubiJ* mutant was grown overnight aerobically and diluted at an OD_{600} of 0.05 in LB. Different amounts of coenzyme Q8 were added at time zero, and growth was monitored at 600 nm. The experiments whose results are presented in panels C and D were performed in triplicate. Similar patterns were obtained, and results of a representative experiment are shown.
mented by its wild-type allele, a plasmid carrying \textit{ubiB} did not restore the \textit{ubi} mutant mutant growth, ruling out any polar effect due to the mutation (Fig. 2C). The wild type and the three mutants were also tested for growth under anaerobic conditions in LB and minimal medium (Fig. 2D and E, respectively). Interestingly, the \textit{ubiJ} mutant was found to grow as well as its wild-type parent in the absence of oxygen in LB, whereas growth of the \textit{ubiB} and \textit{ubiE} mutants was still affected (Fig. 2D). Moreover, the \textit{ubiJ} mutant did not exhibit any significant growth defect in anaerobic minimal medium in the presence of glucose or glycerol supplemented with different carbon sources (Fig. 2E). In contrast, \textit{ubiB} and \textit{ubiE} mutants grew poorly with fumarate, nitrate, or TMAO as the electron acceptor (Fig. 2E), showing that UbiB and UbiE are required for anaerobic respiration, whereas UbiJ is not. Taken together, these data indicate that UbiJ plays an important role in \textit{Salmonella} growth under aerobic conditions but is dispensable under anaerobic conditions.

\textbf{Coenzyme Q synthesis is abolished in an \textit{ubiJ} mutant under aerobic conditions.} UbiE and UbiB were previously shown to be involved in Q\textsubscript{8} biosynthesis in \textit{E. coli}. Therefore, we tested if \textit{Salmonella} paralogs as well as UbiJ were also involved in this biosynthetic pathway. The cellular Q\textsubscript{8} content of the three mutants and of the wild-type strain was measured using electrochemical detection of lipid extracts separated by HPLC (27). None of the three mutants grown aerobically exhibited any detectable Q\textsubscript{8}, whereas wild-type cells reached a level of 107 pmol/mg (wet weight) (Fig. 3A). Surprisingly, under anaerobic conditions, the Q\textsubscript{8} content in the \textit{ubiJ} mutant accounted for 55% of that in the wild-type (35 and 63 pmol, respectively), whereas \textit{ubiB} and \textit{ubiE} mutants did not present any detectable level of Q\textsubscript{8} (Fig. 3A). As previously shown in \textit{E. coli}, an electroactive compound was detected in the \textit{ubiE} mutant grown aerobically with a retention time and UV spectrum similar to those of demethyldehydroxyQ\textsubscript{8} (DDMQ\textsubscript{8}) (see Fig. S1 in the supplemental material) (17). We also measured the cellular content of menaquinone (MK\textsubscript{8}) and demethylmenaquinone (DMK\textsubscript{8}) in the different strains. No MK\textsubscript{8} was detected in the \textit{ubiE} mutant, as UbiE methyltransferase converts DMK\textsubscript{8} into MK\textsubscript{8} (Fig. 3B) (19). In contrast, the levels of MK\textsubscript{8} were not significantly different in the wild type and the \textit{ubiJ} mutant (Fig. 3B), ruling out any role for UbiJ in menaquinone biosynthesis.

As we showed that Q\textsubscript{8} was still produced anaerobically in an \textit{ubiJ} mutant, precultures were performed in LB with or without oxygen, and the strains were grown under aerobic conditions immediately afterwards (Fig. 3C). We did not find any significant difference for the wild-type strain, which presented a doubling time of 19 min in exponential phase when the preculture was performed anaerobically and 20 min when precultured aerobically (Fig. 3C). The doubling time of the \textit{ubiJ} mutant precultured anaerobically was found to be identical to that of the wild type (19 min), whereas it was much longer when the mutant was precultured aerobically (66 min) (Fig. 3C). This result indicates that a preculture performed under anaerobic conditions restores a wild-type growth rate to the \textit{ubiJ} mutant during the first cycles of cell division, suggesting that accumulation of Q\textsubscript{8} sustains aerobic growth for a few generations. To confirm that Q\textsubscript{8} deficiency was the cause of the aerobic growth defect in the \textit{ubiJ} mutant, we added exogenous ubiquinone 1 (Q\textsubscript{1}) in the culture medium, since this component was shown to partially rescue the growth defect of an \textit{ubiCA} mutant (28). Under aerobic conditions, increasing amounts of Q\textsubscript{1} were correlated with an improved growth rate of
the ubiJ mutant in LB (Fig. 3D). Collectively, these data show that UbiJ is required for Q₈ production aerobically and that the absence of coenzyme Q is responsible for the growth limitation observed in the ubiJ mutant.

The 50 C-terminal amino acids of Salmonella UbiJ allow Q₈ synthesis and aerobic growth of the ubiJ mutant. The UbiJ protein is predicted to be composed of two domains: an N-terminal part (amino acids 3 to 120), which was annotated as a sterol-binding domain, and a C-terminal domain, which was found to share 36% identity and 59% similarity with the C terminus of E. coli LpxD, an enzyme involved in lipid A biosynthesis (Fig. 4A). To identify the minimal functional unit of UbiJ, the N-terminal and three different versions of the C-terminal domain of UbiJ were cloned in a vector under the control of a PBAD promoter (Fig. 4A). Neither the N-terminal domain nor the 35 C-terminal amino acids offset the growth defect of the ubiJ mutant (Fig. 4B). In contrast, the plasmids encoding the 50 or the 63 C-terminal amino acids of UbiJ partially restored the growth defect of the ubiJ mutant (Fig. 4B). Accordingly, the constructions carrying the 50 and the 63 C-terminal amino acids in the ubiJ mutant allowed an accumulation of 17% of the wild-type content in Q₈, whereas the full-length UbiJ rescued the wild-type level of Q₈ (Fig. 4C). Altogether, these results show that the minimal functional unit of UbiJ consists of its 50 C-terminal amino acids.

The biological activity of Salmonella ubiJ relies on the synthesis of a protein. It was recently proposed that in E. coli, the 3′ region of ubiJ includes a new essential 252-nucleotide RNA, which was designated esre (22). In order to know if the minimal functional unit identified in Salmonella is a small RNA or a polypeptide, we introduced frameshift mutations after the start codon of the pubiJ 50C plasmid, yielding pubiJ 50C/H11001 (frameshift of one nucleotide) and pubiJ 50C/H11001 (frameshift of two nucleotides) (Fig. 5A). Whereas the pubiJ 50C plasmid was found to complement the growth defect of the ubiJ mutant under aerobic conditions, the two plasmids carrying frameshift mutations did not (Fig. 5B). Consistently, when grown on LB plates aerobically, the

FIG 5 Salmonella UbiJ activity relies on protein synthesis. (A) Schematic representation of the S′ ends of the pubiJ 50C derivative plasmids. Frameshift mutations were introduced after the start codon of pubiJ 50C, yielding pubiJ 50C+1 (frameshift of one nucleotide) and pubiJ 50C+2 (frameshift of two nucleotides). (B) The wild type (filled circles), the ΔubiJ mutant (filled squares), and the ΔubiJ mutant transformed with pubiJ 50C (empty squares), pubiJ 50C+1 (empty circles), and pubiJ 50C+2 (empty triangles) were grown overnight and then diluted at an OD₆₀₀ of 0.05 in LB with arabinose under aerobic conditions. Growth was monitored at 600 nm. The experiment was performed at least in triplicate, identical patterns were obtained, and results of a representative experiment are shown. (C) The ΔubiJ strain transformed with pubiJ 50C (left), pubiJ 50C+1 (middle), and pubiJ 50C+2 (right) was grown on LB plates with arabinose for 16 h at 37°C. (D) HPLC separation and electrochemical detection (HPLC-ECD) of lipid extracts from 2 mg of the strains described for panel A and grown in LB with arabinose under aerobic conditions. The peaks corresponding to Q₈, demethylmenaquinone (DMK₈), and menaquinone (MK₈) and to the Q₁₀ standard are marked. (E) Western blot analysis of pubiJ (lane 1), pubiJ 50C (lane 2), pubiJ 50C+1 (lane 3), and pubiJ 50C+2 (lane 4) expressed from a pBAD24-derived plasmid in an MG1655 E. coli strain. UbiJ variants were separated using 15% SDS-PAGE, blotted onto PVDF membranes, and hybridized with a His₆ tag antibody.

Role of ubiJ in Q₈ Biosynthesis and Aerobic Growth

January 2014 Volume 196 Number 1

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75
ubiJ mutant transformed with pubiJ 50C formed individual colonies in 16 h at 37°C, whereas the ubiJ mutant transformed with pubiJ 50C+1 and pubiJ 50C+2 did not (Fig. 5C). The two plasmids carrying the frameshift mutations did not restore Q8 production in an ubiJ mutant grown aerobically, whereas Q8 was still produced in an ubiJ mutant transformed with pubiJ 50C (Fig. 5D). Western blot analysis confirmed that pubiJ 50C encodes a 7-kDa peptide, whereas pubiJ encodes the 23-kDa full-length protein (Fig. 5E). As expected, pubiJ 50C+1 and pubiJ 50C+2 did not present any cross-reaction signal with the antibody (Fig. 5E). Finally, a “scrambled” plasmid was designed by mutation of 30% of the 261 nucleotides located in the 3’ end of the Salmonella ubiJ gene without changing the primary amino acid sequence of the encoded polypeptide (see Fig. S2 in the supplemental material). This plasmid was found to complement the growth defect and to restore Q8 biosynthesis in the ubiJ mutant (see Fig. S2). Altogether, these data show that the aerobic growth-promoting function of the Salmonella ubiJ gene relies on the synthesis of a protein.

ubiE, ubiJ, and UbiB are essential for Salmonella intracellular proliferation in macrophages. We then investigated the involvement of the ubiE, ubiJ, and ubiB genes in intracellular proliferation of Salmonella. RAW 264.7 mouse macrophages were infected with a wild-type strain, an ubiJ mutant, and the mutant transformed with an empty vector or pubiJ. Bacterial proliferation was assayed by calculating the proliferation index as the ratio of the number of intracellular bacteria at 16 h postinfection to that at 2 h postinfection. The wild-type strain exhibited a proliferation index of 33.6 ± 6.2, whereas the ubiJ mutant presented an index less than 1, indicating that it was unable to proliferate in macrophages (Fig. 6A). Complementation of the ubiJ mutant fully restored its intracellular replication (47.5 ± 3.2), whereas the empty plasmid did not change the proliferation index (Fig. 6A). Since anaerobic growth partially restored the Q8 level in the ubiJ mutant, mouse macrophages were infected with inocula grown aerobically or anaerobically (Fig. 6B). Whereas the ubiJ mutant exhibited a proliferation index of 0.6 in macrophages infected with an aerobic inoculum, it was found to replicate much more efficiently in macrophages infected with an anaerobic inoculum (3.1 ± 0.6) (Fig. 6B). Interestingly, the ratio of the proliferation index of the wild type (WT) to that of the ΔubiJ mutant was found to be about 10 times lower in macrophages infected with an aerobic inoculum (6.6) than in macrophages infected with an anaerobic inoculum (64.6), reflecting the ability of the ubiJ mutant to replicate when precultured under anaerobic conditions (Fig. 6B, inset). Next, we found the ubiB mutant to be totally impaired for intracellular replication and the proliferation index of the ubiJ mutant to range from 2.3 to 4.2 (Fig. 6B). In addition, whereas anaerobic precultures modestly improved the proliferation index of the ubiJ mutant, no change was observed in the ubiB mutant (Fig. 6B). These results are consistent with our previous observations showing that the ubiE and ubiB mutants did not synthesize Q8 under anaerobic conditions (Fig. 3A). Collectively, these data assign a role for UbiE, UbiJ, and UbiB in Salmonella intracellular proliferation and, more generally, highlight the importance of Q8 production for bacterial virulence.

The E. coli ubiJ gene is also required for Q8 biosynthesis. In E. coli, a global approach indicated that the ubiJ (yigP) gene was essential under aerobic conditions (29). In a separate study, no insertion mutant could be obtained using a plasmid shuffling-based strategy, and ubiJ (yigP) was also proposed to be essential for
erologous complementation revealed that the growth defect of the E. coli mutant was rescued by the ubiJ gene of Salmonella (Fig. 7B). Conversely, the ubiJ gene of E. coli complemented the growth defect of the Salmonella ubiJ mutant (Fig. 7B). We also demonstrated that the growth defects of E. coli and Salmonella ubiJ mutants were rescued by a plasmid carrying the 50 C-terminal amino acids of E. coli UbiJ (Fig. 7C). Finally, we tested the involvement of ubiJ in Q8 biosynthesis and did not detect any Q8 in the E. coli ubiJ mutant grown aerobically (Fig. 7D). Taken together, these results show that in E. coli, as in Salmonella, the ubiJ gene participates in Q8 biosynthesis, which is required for aerobic growth.

**DISCUSSION**

Owing to its electron transfer capacity, Q8 is a key molecule for adaptation of bacteria to oxygenic growth conditions. Biosynthesis of Q8 has been studied for some time, but uncertainties remain, and new genes might have to be included in the currently established pathway. In the present study, we identified UbiJ as a new factor involved in Q8 biosynthesis in E. coli and Salmonella under aerobiosis. Under such conditions, the ubiJ mutants of both species were defective for Q8 production and accordingly impaired for growth. Moreover, we showed that Salmonella ubiE, ubij, and ubiB genes were all required for intracellular proliferation. This latest result evidenced the existence of a link between aerobic respiration and Salmonella virulence.

A previous study of the ubiJ gene of E. coli concluded that it is essential and that it encodes a small RNA rather than a polypeptide (22). Our present characterization of ubiJ does not support either of these two notions. First, we could not confirm that ubiJ was an essential gene in either Salmonella or E. coli. Mutants were obtained using standard protocols. Growth analysis of the mutants revealed an alteration in growth rate and maximal OD value reached in liquid cultures, as well as a small-size colony phenotype. However, in both species, the ubiJ mutants were fully viable. Regarding the small-RNA issue, in Salmonella, Northern analysis and RACE (random amplification of cDNA ends) experiments did not allow us to identify any small RNA in the ubiJ gene (data not shown). Moreover, we demonstrated that whereas a DNA region encoding the 50 C-terminal amino acids allowed synthesis of Q8, two types of frameshift mutations within this DNA piece abolished it. Then, a “scrambled” ubiJ allele, including the mutation of 30% of the nucleotides without changing the amino acid sequence, complemented the growth defect and restored Q8 biosynthesis in the Salmonella ubiJ mutant. Finally, no transcript was found to be initiated within ubiJ in the transcriptional landscape and in a small-RNA study which combined three RNA-
sequencing and two sequencing platforms in Salmonella (30). We therefore believe that in Salmonella at least, the biological function of ubiJ is mediated by a protein. That the same situation might prevail in E. coli is strongly supported by the observation that the 50 C-terminal amino acids of UbiJ were sufficient to restore aerobic growth in the E. coli ubiJ mutant. Additional studies dedicated to in-depth analysis of the coding capacity of the ubiJ gene of E. coli are in progress.

UbiE was previously characterized as a methyltransferase (17). UbiB was long thought to intervene at an early step during biosynthesis of Q8, but our recent study revealed this step to be catalyzed by a newly discovered monoxygenase called UbiI (9). ubiE and ubiB mutants did not accumulate Q8 in Salmonella grown either aerobically or anaerobically. Conversely, Q8 was still produced in the ubiJ mutant grown anaerobically. To date, the only mutants known to recover Q8 biosynthesis under anaerobic conditions are E. coli strains in which the aerobic monoxygenase UbiI, UbiH, or UbiF is inactivated (9, 15). UbiJ is unlikely to be a monoxygenase, but it may assist the function of the aerobic monooxygenases. A clue of the role of UbiJ in Q8 biosynthesis may come from the observation that the C-terminal region of UbiJ shares 36% identity and 59% similarity with the C-terminal domain of E. coli LpxD, an enzyme catalyzing the acylation of the lipid A. In LpxD, the C-terminal helical domain caps the hydrophobic cleft that binds the acyl chain (31). This domain seems to be of primary importance, because C-terminal truncations of LpxD yielded inactive proteins (31). Interestingly, our deletion analysis of ubiJ revealed that the 50 C-terminal amino acids were sufficient to sustain Q8 biosynthesis and growth. Thus, an attractive possibility is that UbiJ exhibits, like LpxD, the ability to interact with lipid components. As a working hypothesis, we propose that UbiJ could serve as a carrier of the isoprenoid hydrophobic tail prior to the action of monooxygenases and methyltransferases. Alternatively, it could chaperone prenylated intermediates during the biosynthetic process and, more specifically, during aerobic hydroxylation steps.

The ubiJ mutant retained the ability to grow aerobically in LB to an OD of 1 and was found to be killed within macrophages (proliferation index < 1). Macrophages infected with an anaerobic inoculum of the ubiJ mutant, in which Q8 was still produced, yielded a proliferation index of >5. This clearly established the requirement for Q8 for efficient intracellular proliferation. Several possibilities for connecting Q8 defects and virulence attenuation can be envisioned. A first obvious possibility is that Salmonella uses aerobic respiration to grow intracellularly. Q8 being necessary for aerobic respiration, any defect in its biosynthesis would be predicted to lead to defects in intracellular growth. A second possibility relates to the role of Q8 as an antioxidant in reducing the production rate of O2·− and H2O2 in the plasma membrane (8). However, we previously reported that a multiplicity of catalases and peroxidases endow Salmonella with a high capacity to cope with oxidative stress (32), and it would be surprising if a lack of Q8 unbalanced the enzymatic defenses. A third possibility is that a defect in Q8 prevents proton motive force-dependent processes needed for virulence. For instance, flagellar motility, a key process employed by pathogenic bacteria during the course of infection, is dependent upon proton motive force (33). In summary, our results highlight the importance of Q8 in bacterial virulence and strongly support the idea that Salmonella intracellular lifestyle relies on aerobic respiration and oxygen availability to survive and proliferate within macrophages.

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REFERENCES

16. Hsu AY, Poon WW, Shepherd JA, Myles DC, Clarke CF. 1996. Complementation of coq3 mutant yeast by mitochondrial targeting of the


