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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 Q_8) 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 *ubiE* and *ubiB*, all three being likely to constitute an operon. In this work, we showed that the uncharacterized *yigP* gene was involved in Q_8 biosynthesis in both strains, and we have renamed it *ubiJ*. Under aerobic conditions, an *ubiJ* mutant was found to be impaired for Q_8 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 Q_8 biosynthesis and growth of the *ubiJ* mutant. *Salmonella ubiE* and *ubiB* mutants were impaired in Q_8 biosynthesis and in respiration using different electron acceptors. Moreover, *ubiE*, *ubiJ*, and *ubiB* mutants were all impaired for *Salmonella* intracellular proliferation in macrophages. Taken together, our data establish an important role for UbiJ in Q_8 biosynthesis and reveal an unexpected link between Q_8 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 Q_8. 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, Q_8 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 Q_8 biosynthesis were shown to be hypersensitive to H_2O_2, as the production rate of $O_2^{\bullet-}$ and H_2O_2 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, monooxygenases 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 monooxygenase 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 monooxygenases include UbiF and UbiH (Fig. 1) (12, 13). Also, *ubiF*, *ubiH*, and *ubiI* mutants do not synthesize Q_8 under aerobic growth conditions but produce appreciable amounts of Q_8 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 Q_8 and menaquinone (MK_8) biosynthesis, and an *ubiE* mutant synthesizes neither Q_8 nor MK_8 (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 Q_8 biosynthesis under aerobic conditions, and we changed the name of YigP to UbiJ. Evidence that the role of *ubiJ* in Q_8 biosynthesis was carried out by the UbiJ poly-

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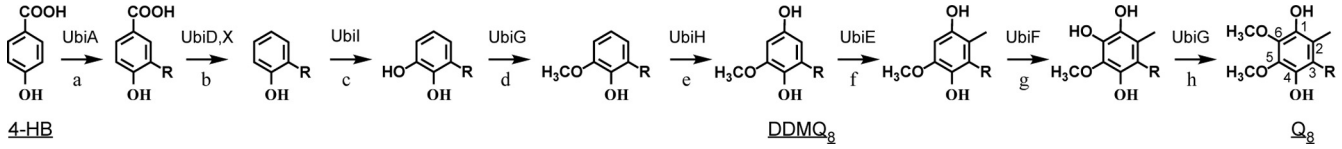

FIG 1 Biosynthetic pathway of coenzyme Q_8 in *Escherichia coli*. The octaprenyl tail is represented by R on carbon 3 of the different biosynthetic intermediates. The names of the enzymes catalyzing the reactions (each labeled with a lowercase letter) are provided. 4-HB, 4-hydroxybenzoic acid; $DDMQ_8$, C-1-demethyl-C-6-demethoxy-Q_8; Q_8, coenzyme Q_8.

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 Q_8 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 (OD_{600}) 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 *ubiJ* 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 p*ubiJ*$_{E.c.}$ and p*ubiJ* 50C$_{E.c.}$. p*ubiJ* 50C$_{E.c.}$ was digested by EcoRI and HindIII, and the insert carrying the *ubiJ* variant was cloned in

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype[a]	Source or reference
Strains		
S. Typhimurium		
12023	Wild type	Laboratory stock
Δ*ubiJ* mutant	12023 Δ*ubiJ*::Kan	This study
Δ*ubiE* mutant	12023 Δ*ubiE*::Kan	This study
Δ*ubiB* mutant	12023 Δ*ubiB*::Kan	This study
E. coli		
MG1655	Wild type	Laboratory stock
Δ*ubiJ* mutant	MG1655 Δ*ubiJ*::Kan	This study
Plasmids		
pBAD24	Cloning vector (Ap^r)	24
pTrc99A	Cloning vector (Ap^r)	Amersham
pBADI*	Cloning vector (Ap^r)	34
pKD4	Template plasmid containing an FRT-flanked kanamycin resistance	23
pKD46	Red recombinase expression plasmid	23
S. Typhimurium		
p*ubiJ*$_{S.t.}$	pBAD24 derivative carrying the *S.* Typhimurium *ubiJ* gene (Ap^r)	This study
p*ubiB*	pBAD24 derivative carrying *ubiB* (Ap^r)	This study
p*ubiJ* N-ter	pBAD24 derivative carrying the 120 amino acids located in the N terminus of *ubiJ* (Ap^r)	This study
p*ubiJ* 63C	pBAD24 derivative carrying the 63 amino acids located in the C terminus of *ubiJ* (Ap^r)	This study
p*ubiJ* 50C	pBAD24 derivative carrying the 50 amino acids located in the C terminus of *ubiJ* (Ap^r)	This study
p*ubiJ* 50C+1	pBAD24 derivative carrying the 50 amino acids located in the C terminus of *ubiJ* and a frameshift of one nucleotide after the start codon (Ap^r)	This study
p*ubiJ* 50C+2	pBAD24 derivative carrying the 50 amino acids located in the C terminus of *ubiJ* and a frameshift of two nucleotides after the start codon (Ap^r)	This study
p*ubiJ* 35C	pBAD24 derivative carrying the 35 amino acids located in the C terminus of *ubiJ* (Ap^r)	This study
E. coli		
p*ubiJ*$_{E.c.}$	pBAD24 derivative carrying the *E. coli ubiJ* gene (Ap^r)	This study
p*ubiJ* 50C$_{E.c.}$	pTrc99A derivative carrying the 50 amino acids located in the C terminus of *ubiJ* (Ap^r)	This study

[a] FRT, Flp recombinase target.

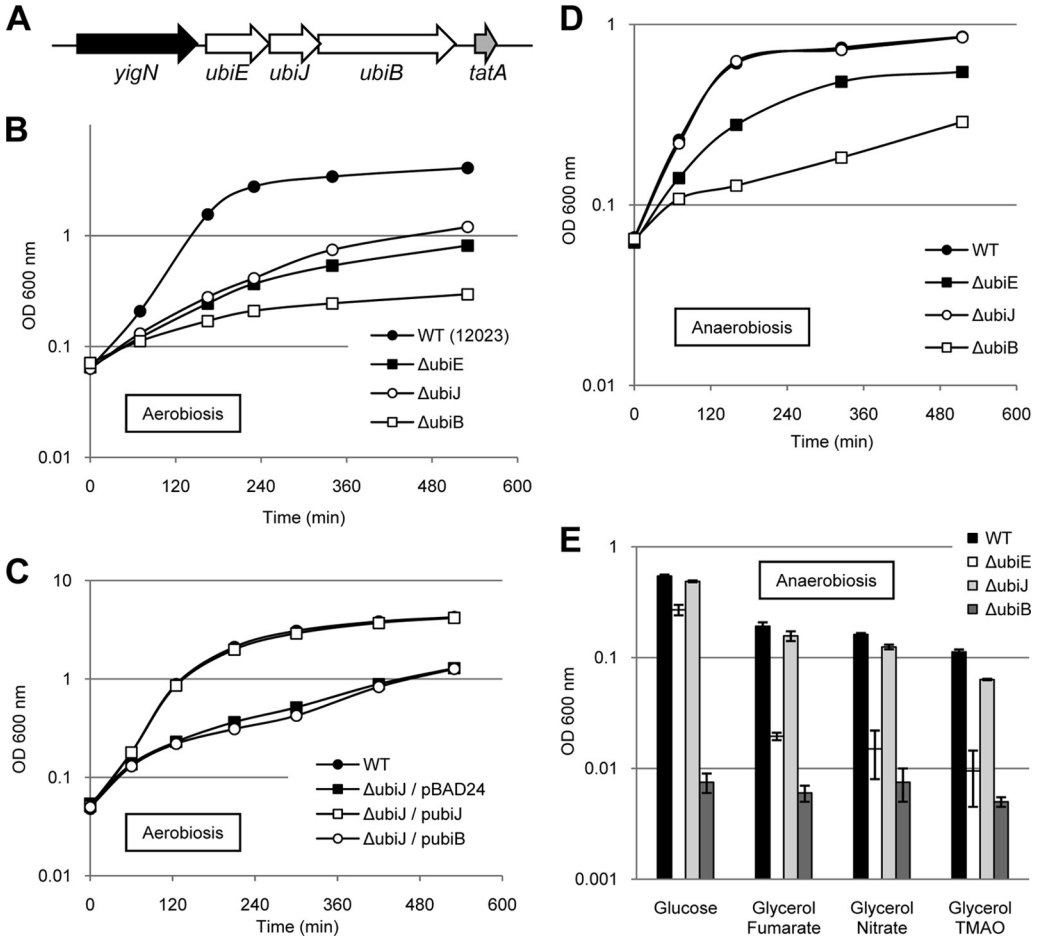

FIG 2 The *ubiJ* mutant exhibited a growth defect under aerobic conditions. (A) Genetic organization of the *ubiE*, *ubiJ*, and *ubiB* loci in *Salmonella* Typhimurium. (B) Wild-type (filled circles), Δ*ubiE* (filled squares), Δ*ubiJ* (open circles), and Δ*ubiB* (open squares) strains were grown overnight and then diluted at an OD_{600} 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 p*ubiJ* (open squares), and with p*ubiB* (open circles) were grown overnight and then diluted at an OD_{600} 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 OD_{600} 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. OD_{600} 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.

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 Q_{10} 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 C_{18} 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 Q_{10} solution were injected under the same conditions to generate a standard curve which was used for Q_8 quantification. The signal of the absorbance at 210 nm was used to estimate the content in DMK_8 and MK_8 by integrating the respective peaks. Possible sample loss during the organic extraction was corrected on the basis of the recovery of the Q_{10} internal standard.

Western blot analysis. p*ubiJ*, p*ubiJ* 50C, p*ubiJ* 50C+1, and p*ubiJ* 50C+2 carrying a His_6 tag at the C terminus were transformed in a MG1655 *E. coli* strain. The resulting strains were grown in LB until an OD_{600} of 4 was reached and then diluted 1:100 in LB supplemented with

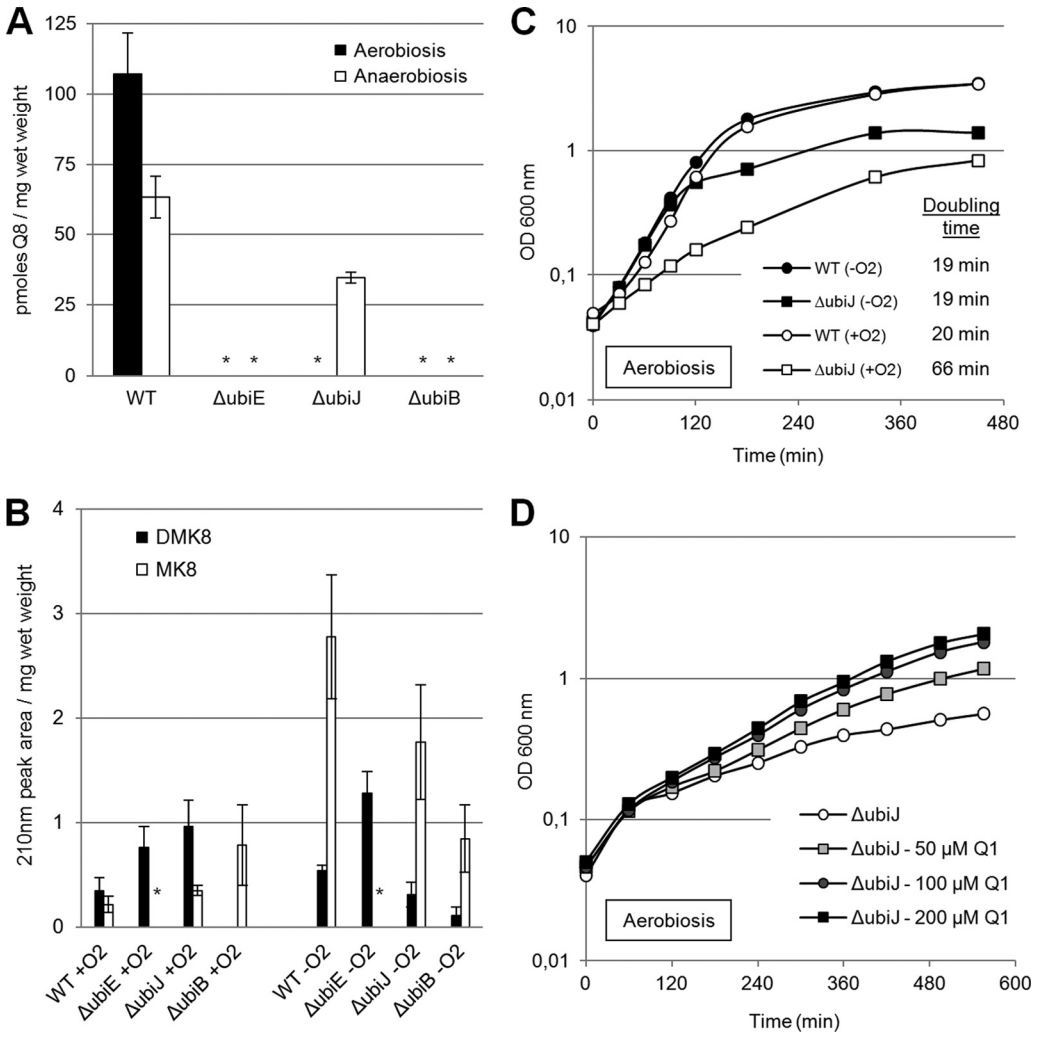

FIG 3 UbiJ is required for Q_8 biosynthesis under aerobiosis. (A) Quantification of cellular Q_8 content in lipid extracts from WT, Δ*ubiE*, Δ*ubiJ*, and Δ*ubiB* cells grown under aerobiosis (black bars) or anaerobiosis (white bars). Error bars show standard deviations. *, not detected. (B) Demethylmenaquinone (DMK_8, black bars) and menaquinone (MK_8, white bars) content in lipid extracts from WT, Δ*ubiE*, Δ*ubiJ*, and Δ*ubiB* 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 Q_1 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.

0.02% arabinose. The cultures were incubated 4 h 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 Miniprotean 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 4×10^5 cells per well in 6-well tissue culture plates containing Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) (HyClone). Bacteria were cultured overnight at 37°C with shaking and opsonized in DMEM containing FCS and normal mouse serum (10%; Perbio) for 30 min. Bacteria were added to the monolayers at a multiplicity of infection of 10:1, centrifuged at 400 × *g* for 5 min at 4°C, and incubated for 30 min at 37°C in 5% CO_2. The macrophages were washed three times and incubated with DMEM containing FCS and 100 μg/ml gentamicin for 60 min, after which the gentamicin concentration was decreased to 10 μg/ml for the remainder of the experiment.

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*, *ubiJ*, and *ubiB* deletion mutants in *Salmonella*. During aerobic growth in LB at 37°C, the *ubiJ* and *ubiE* 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 *ubiJ* mutation did not affect *ubiB* expression, the *ubiJ* mutant was transformed with plasmids carrying either *ubiJ* or *ubiB*. Whereas the *ubiJ* mutant was fully comple-

mented by its wild-type allele, a plasmid carrying *ubiB* did not restore the *ubiJ* 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 *ubiJ* mutant was found to grow as well as its wild-type parent in the absence of oxygen in LB, whereas growth of the *ubiB* and *ubiE* mutants was still affected (Fig. 2D). Moreover, the *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, *ubiB* and *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 *Salmonella* growth under aerobic conditions but is dispensable under anaerobic conditions.

Coenzyme Q synthesis is abolished in an *ubiJ* mutant under aerobic conditions. UbiE and UbiB were previously shown to be involved in Q_8 biosynthesis in *E. coli*. Therefore, we tested if *Salmonella* paralogs as well as UbiJ were also involved in this biosynthetic pathway. The cellular Q_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_8, whereas wild-type cells reached a level of 107 pmol/mg (wet weight) (Fig. 3A). Surprisingly, under anaerobic conditions, the Q_8 content in the *ubiJ* mutant accounted for 55% of that in the wild-type (35 and 63 pmol, respectively), whereas *ubiB* and *ubiE* mutants did not present any detectable level of Q_8 (Fig. 3A). As previously shown in *E. coli*, an electroactive compound was detected in the *ubiE* mutant grown aerobically with a retention time and UV spectrum similar to those of demethyldemethoxyQ_8 ($DDMQ_8$) (see Fig. S1 in the supplemental material) (17). We also measured the cellular content of menaquinone (MK_8) and demethylmenaquinone (DMK_8) in the different strains. No MK_8 was detected in the *ubiE* mutant, as UbiE methyltransferase converts DMK_8 into MK_8 (Fig. 3B) (19). In contrast, the levels of MK_8 were not significantly different in the wild type and the *ubiJ* mutant (Fig. 3B), ruling out any role for UbiJ in menaquinone biosynthesis.

As we showed that Q_8 was still produced anaerobically in an *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 *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 *ubiJ* mutant during the first cycles of cell division, suggesting that accumulation of Q_8 sustains aerobic growth for a few generations. To confirm that Q_8 deficiency was the cause of the aerobic growth defect in the *ubiJ* mutant, we added exogenous ubiquinone 1 (Q_1) in the culture medium, since this component was shown to partially rescue the growth defect of an *ubiCA* mutant (28). Under aerobic conditions, increasing amounts of Q_1 were correlated with an improved growth rate of

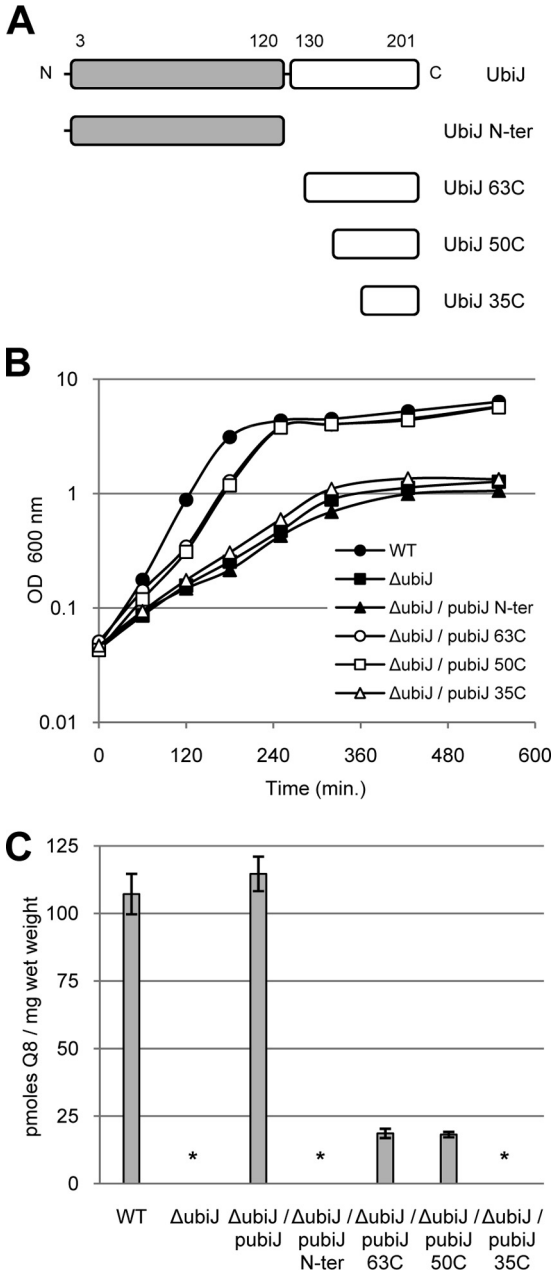

FIG 4 The 50 C-terminal amino acids of UbiJ are sufficient to sustain Q_8 biosynthesis and aerobic growth of the *ubiJ* mutant. (A) Schematic representation of the *ubiJ* plasmids used in this study. UbiJ N-ter represents the plasmid encoding the 120 amino acids at the N terminus, and UbiJ 63C, 50C, and 35C are the plasmids encoding the 63, 50, and 35 amino acids (respectively) at the C terminus. These constructs were cloned in a pBAD24 vector under the control of the pBAD promoter. (B) The wild type (filled circles), the Δ*ubiJ* mutant (filled squares), and the Δ*ubiJ* mutant transformed with p*ubiJ* N-ter (filled triangles), p*ubiJ* 63C (empty circles), p*ubiJ* 50C (empty squares), and p*ubiJ* 35C (empty triangles) were grown overnight and then diluted at an OD_{600} of 0.05 in LB under aerobic conditions. Growth was monitored at 600 nm. This experiment was performed in triplicate, identical patterns were obtained, and results of a representative experiment are shown. (C) Quantification of cellular Q_8 content in lipid extracts from the strains previously described and grown in LB under aerobic conditions. Error bars show standard deviations. *, not detected.

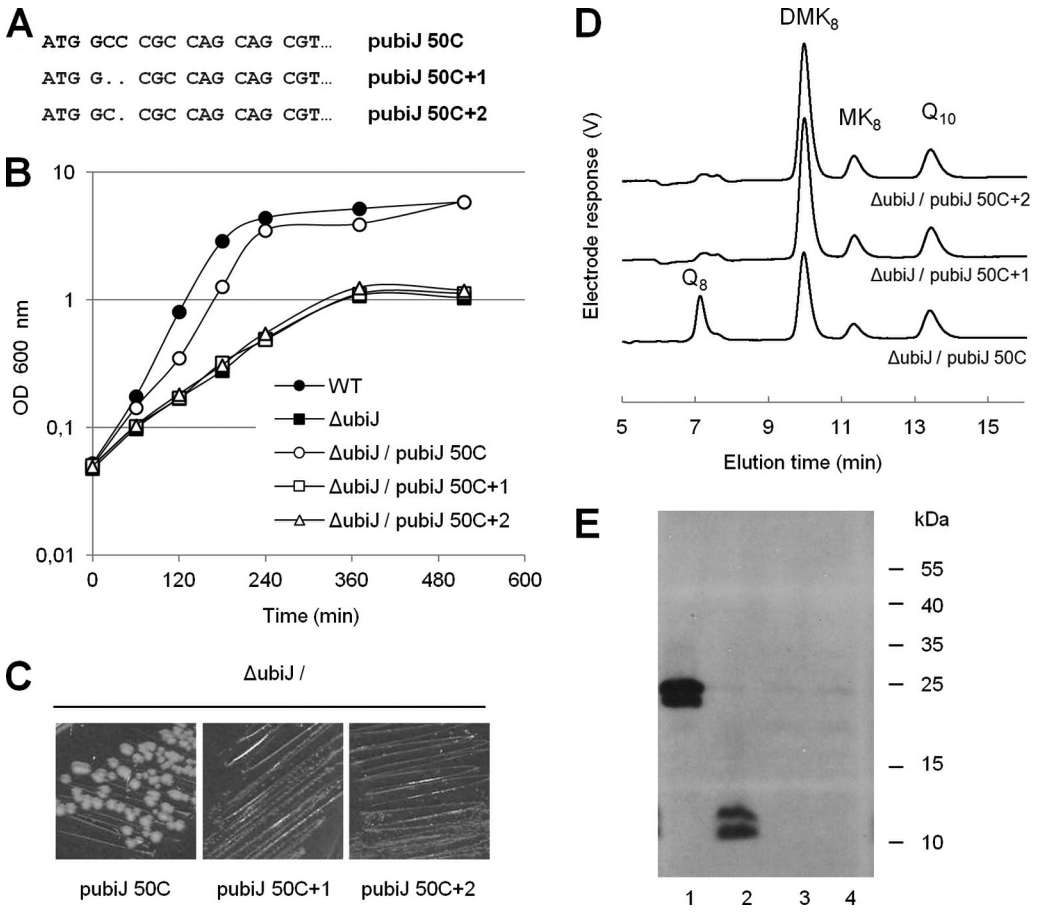

FIG 5 *Salmonella* UbiJ activity relies on protein synthesis. (A) Schematic representation of the 5′ ends of the p*ubiJ* 50C derivative plasmids. Frameshift mutations were introduced after the start codon of p*ubiJ* 50C, yielding p*ubiJ* 50C+1 (frameshift of one nucleotide) and p*ubiJ* 50C+2 (frameshift of two nucleotides). (B) The wild type (filled circles), the Δ*ubiJ* mutant (filled squares), and the Δ*ubiJ* mutant transformed with p*ubiJ* 50C (empty circles), p*ubiJ* 50C+1 (empty squares), and p*ubiJ* 50C+2 (empty triangles) were grown overnight and then diluted at an OD_{600} 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 Δ*ubiJ* strain transformed with p*ubiJ* 50C (left), p*ubiJ* 50C+1 (middle), and p*ubiJ* 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 Q_8, demethylmenaquinone (DMK_8), and menaquinone (MK_8) and to the Q_{10} standard are marked. (E) Western blot analysis of p*ubiJ* (lane 1), p*ubiJ* 50C (lane 2), p*ubiJ* 50C+1 (lane 3), and p*ubiJ* 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 His_6 tag antibody.

the *ubiJ* mutant in LB (Fig. 3D). Collectively, these data show that UbiJ is required for Q_8 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 Q_8 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 P_{BAD} 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 Q_8, whereas the full-length UbiJ rescued the wild-type level of Q_8 (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* was a small RNA or a polypeptide, we introduced frameshift mutations after the start codon of the p*ubiJ* 50C plasmid, yielding p*ubiJ* 50C+1 (frameshift of one nucleotide) and p*ubiJ* 50C+2 (frameshift of two nucleotides) (Fig. 5A). Whereas the p*ubiJ* 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

ubiJ mutant transformed with p*ubiJ* 50C formed individual colonies in 16 h at 37°C, whereas the *ubiJ* mutant transformed with p*ubiJ* 50C+1 and p*ubiJ* 50C+2 did not (Fig. 5C). The two plasmids carrying the frameshift mutations did not restore Q_8 production in an *ubiJ* mutant grown aerobically, whereas Q_8 was still produced in an *ubiJ* mutant transformed with p*ubiJ* 50C (Fig. 5D). Western blot analysis confirmed that p*ubiJ* 50C encodes a 7-kDa peptide, whereas p*ubiJ* encodes the 23-kDa full-length protein (Fig. 5E). As expected, p*ubiJ* 50C+1 and p*ubiJ* 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 Q_8 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 p*ubiJ*. 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 Q_8 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 (5.1 ± 0.6) (Fig. 6B). Interestingly, the ratio of the proliferation index of the wild type (WT) to that of the Δ*ubiJ* mutant was found to be about 10 times lower in macrophages infected with an anaerobic inoculum (6.6) than in macrophages infected with an aerobic 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 *ubiE* mutant to range from 2.3 to 4.2 (Fig. 6B). In addition, whereas anaerobic precultures modestly improved the proliferation index of the *ubiE* 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 Q_8 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 Q_8 production for bacterial virulence.

The *E. coli ubiJ* gene is also required for Q_8 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

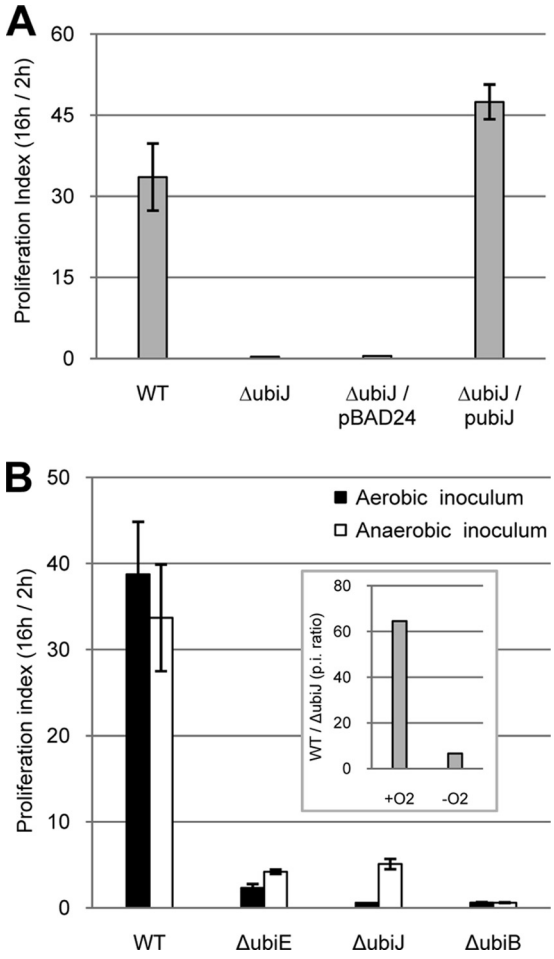

FIG 6 UbiJ is necessary for *Salmonella* intracellular proliferation in macrophages. Opsonized bacteria were phagocytosed by RAW 264.7 cells. At 2 and 16 h postinfection, mouse macrophages were lysed for enumeration of intracellular bacteria (gentamicin protected), as determined by CFU counts. The values are proliferation indexes, calculated as the ratio of the number of intracellular bacteria at 16 h to that at 2 h postinfection. (A) Wild-type, Δ*ubiJ*, Δ*ubiJ*/pBAD24, and Δ*ubiJ*/p*ubiJ* strains were grown overnight in LB under aerobic conditions before opsonization. (B) Wild-type, Δ*ubiE*, Δ*ubiJ*, and Δ*ubiB* strains were grown overnight in LB under aerobic (black bars) or anaerobic (white bars) conditions before opsonization. The inset shows the proliferation index ratios (WT/Δ*ubiJ* mutant) calculated from the aerobic ($+O_2$) and the anaerobic ($-O_2$) inocula. Results are the means ± standard deviations from at least three independent experiments, each in triplicate.

E. coli (22). This was clearly at odds with the result obtained with *Salmonella* described above, and we wished to know whether the *ubiJ* gene had differential importance in the two species. Therefore, mutation experiments were undertaken in *E. coli* by deletion and replacement of *ubiJ* by a kanamycin-resistant cassette using the Datsenko and Wanner method (23). Transformants resistant to kanamycin were obtained anaerobically and subsequently tested aerobically. Although we noticed a small-size phenotype for colonies grown under aerobiosis, no difference in viability was observed between the two growth conditions. In liquid cultures, the *ubiJ* strain reached an OD value slightly lower than that of the wild type yet was viable (Fig. 7A). Together, these observations showed that like in *Salmonella*, an *ubiJ* mutation affects the aerobic growth rate, but the mutant is perfectly viable. Moreover, het-

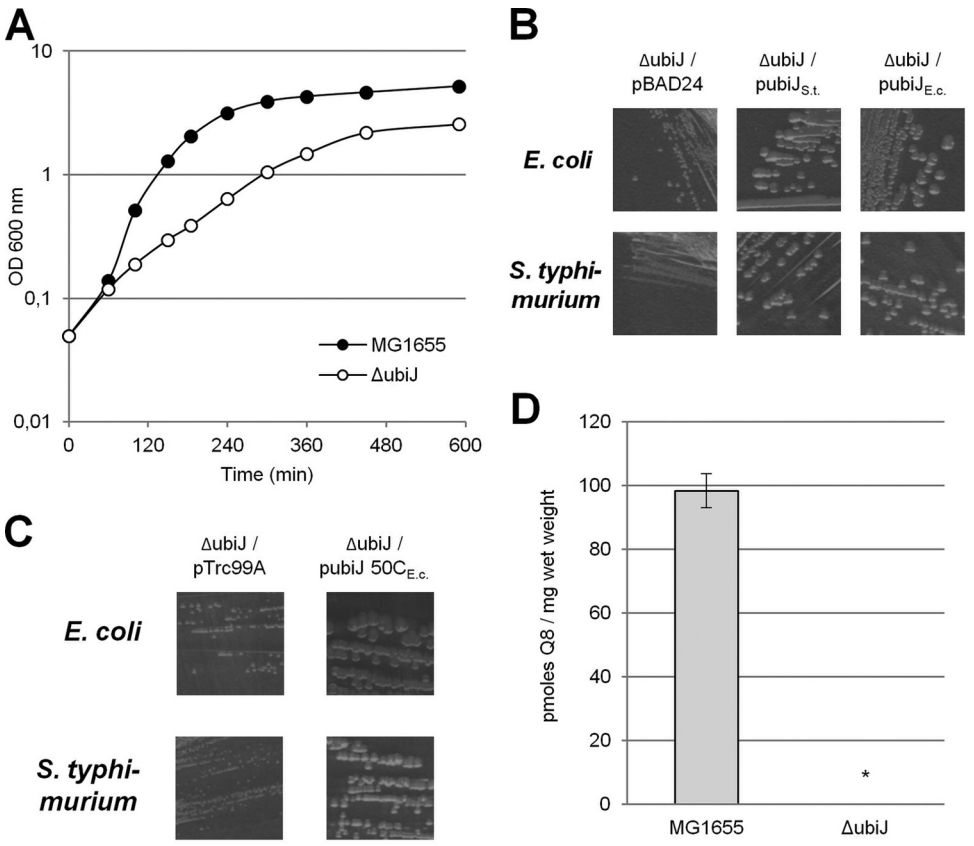

FIG 7 The *E. coli ubiJ* gene is also involved in Q_8 biosynthesis and required for aerobic growth. (A) The MG1655 *E. coli* wild-type strain (filled circles) and Δ*ubiJ* mutant (open circles) were grown overnight and then diluted at an OD_{600} of 0.05 in LB medium at 37°C under aerobic conditions. Growth was monitored at 600 nm. (B) The *E. coli* Δ*ubiJ* (top) and *S.* Typhimurium Δ*ubiJ* (bottom) mutants transformed with pBAD24, *E. coli ubiJ* (p*ubiJ*$_{E.c.}$), and *S.* Typhimurium *ubiJ* (p*ubiJ*$_{S.t.}$) were grown on LB plates with ampicillin 16 h at 37°C. (C) The *E. coli* Δ*ubiJ* (top) and *S.* Typhimurium Δ*ubiJ* (bottom) mutants transformed with pTrc99A or p*ubiJ* 50C$_{E.c.}$ were grown on LB plates with ampicillin 16 h at 37°C. (D) Quantification of cellular Q_8 content in lipid extracts from WT and Δ*ubiJ* cells grown under aerobic conditions. Error bars show standard deviations. *, not detected.

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 Q_8 biosynthesis and did not detect any Q_8 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 Q_8 biosynthesis, which is required for aerobic growth.

DISCUSSION

Owing to its electron transfer capacity, Q_8 is a key molecule for adaptation of bacteria to oxygenic growth conditions. Biosynthesis of Q_8 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 Q_8 biosynthesis in *E. coli* and *Salmonella* under aerobiosis. Under such conditions, the *ubiJ* mutants of both species were defective for Q_8 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 on plates. 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 Q_8, 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 Q_8 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 techniques 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 Q_8, but our recent study revealed this step to be catalyzed by a newly discovered monooxygenase called UbiI (9). *ubiE* and *ubiB* mutants did not accumulate Q_8 in *Salmonella* grown either aerobically or anaerobically. Conversely, Q_8 was still produced in the *ubiJ* mutant grown anaerobically. To date, the only mutants known to recover Q_8 biosynthesis under anaerobic conditions are *E. coli* strains in which the aerobic monooxygenase UbiI, UbiH, or UbiF is inactivated (9, 15). UbiJ is unlikely to be a monooxygenase, but it may assist the function of the aerobic monooxygenases. A clue of the role of UbiJ in Q_8 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 Q_8 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 Q_8 was still produced, yielded a proliferation index of >5. This clearly established the requirement for Q_8 for efficient intracellular proliferation. Several possibilities for connecting Q_8 defects and virulence attenuation can be envisioned. A first obvious possibility is that *Salmonella* uses aerobic respiration to grow intracellularly. Q_8 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 Q_8 as an antioxidant in reducing the production rate of $O_2{\cdot}^-$ and H_2O_2 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 Q_8 unbalanced the enzymatic defenses. A third possibility is that a defect in Q_8 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 Q_8 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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