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Ubiquinone and Menaquinone Electron Carriers Represent the Yin and Yang in the Redox Regulation of the ArcB Sensor Kinase

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The Arc two-component system, comprising the ArcB sensor kinase and the ArcA response regulator, modulates the expression of numerous genes in response to respiratory growth conditions. Under aerobic growth conditions, the ubiquinone electron carriers were proposed to silence the kinase activity of ArcB by oxidizing two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation. Here, we confirm the role of the ubiquinone electron carriers as the silencing signal of ArcB *in vivo*, we show that the redox potential of ArcB is about −41 mV, and we demonstrate that the menaquinols are required for proper ArcB activation upon a shift from aerobic to anaerobic growth conditions. Thus, an essential link in the Arc signal transduction pathway connecting the redox state of the quinone pool to the transcriptional apparatus is elucidated.

The Arc (anoxic redox control) two-component system (TCS) is a key element in the transcriptional regulatory network that allows facultative anaerobic bacteria to sense various respiratory growth conditions and adapt their gene expression accordingly (1–4). This system comprises the cytoplasmic response regulator ArcA and the membrane-anchored sensor kinase ArcB (5, 6). ArcA is a typical response regulator possessing an N-terminal receiver domain with a conserved Asp residue at position 54 and a C-terminal helix-turn-helix DNA binding domain. In contrast, ArcB is an unorthodox sensor kinase, having a very short periplasmic sequence of only 16 amino acid residues delimited by two canonical transmembrane segments. Interestingly, these segments of ArcB do not directly participate in signal sensing but, rather, serve as an anchor that keeps the protein close to the source of the signal (7). Moreover, ArcB contains three catalytic domains: an N-terminal transmitter domain with a conserved His292 residue, a central receiver domain with a conserved Asp576 residue, and a C-terminal phosphotransfer domain with a conserved His717 residue (6, 8). Finally, in the linker, that is, the region connecting the catalytic domains with the transmembrane domain, there are a functional leucine zipper (9) and a PAS domain (10).

Under reducing conditions, ArcB autophosphorylates through an intramolecular reaction (11), a process shown to be enhanced by certain anaerobic metabolites, such as D-lactate, acetate, and pyruvate (12, 13), and transphosphorylates ArcA via a His292 → Asp576 → His717 → Asp54 phosphorelay (14, 15). Phosphorylated ArcA (ArcA-P), in turn, represses the expression of many operons involved in respiratory metabolism and activates others encoding proteins involved in fermentative metabolism (16–19). Under nonstimulating conditions, ArcB acts as a specific ArcA-P phosphatase that catalyzes the dephosphorylation of ArcA-P by a reverse Asp54 → His717 → Asp576 → Pi phosphorelay (20, 21). Previously, it was reported that regulation of the catalytic activity of ArcB is set by rotational movements that alter the orientation of the cytosolic portion of ArcB (22) and that the molecular event for ArcB regulation involves the oxidation of two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation, a reaction in which ubiquinones (UQ) act as direct oxidants (23, 24). However, in a recent study, it was reported that the aerobic and anaerobic levels of an ArcA-P-specific reporter (*cydA*$^{-176+1}$*-lacZ*) in a mutant blocked in ubiquinone biosynthesis were similar to the ones of the isogenic wild-type (wt) strain, leading the authors to conclude that aerobic inhibition of the ArcB kinase *in vivo* can be due to other effectors and thus is regulated not only by ubiquinone (25). On the other hand, a mutant strain blocked in the menaquinone (MQ) biosynthetic pathway resulted in the loss of anaerobic activation of reporter expression, suggesting that the menaquinone pool plays a major role in ArcB redox regulation (25). Moreover, in another recent study, it was reported that no correlation between ArcA activity/phosphorylation and the ubiquinone/ubiquinol redox profile was found (26). It was therefore suggested that ArcB integrates the response to multiple signals (e.g., ubiquinone, menaquinone, acetate, and other fermentation products) and that the rate of fermentation product synthesis exerts a greater influence on ArcA activity than the redox state of the quinone pools (26).

Here we present experiments addressing the above-mentioned questions and discrepancies. Our results confirm our previous findings that ubiquinones ($E'^{\circ} = +100$ mV) are necessary for silencing the kinase activity of ArcB during aerobic growth or upon a shift from anaerobiosis to aerobiosis and also extend these studies by determining the redox potential of ArcB to be about −41 mV and demonstrating that the menaquinone electron carriers ($E'^{\circ} = -74$ mV) are required for activation of ArcB upon a shift to anoxic conditions. A simple model for the ArcB redox signaling which differs significantly from those previously suggested (25, 26) is presented and discussed.

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TABLE 1 *E. coli* strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source
Strains		
MC4100	*F⁻ araD139* (*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR*	29
BW25113	K-12 wild type	30
JW5713	BW25113 Δ*ubiC*::Kan^r	30
ECL5001	MC4100 λΦ(*cydA′-lacZ*)	7
ECL5039	ECL5001 Δ*ubiCA*::Kan^r	23
ECL5003	MC4100 Δ*fnr*::Tn*9*(Chl^r) λΦ(*cydA′-lacZ*)	7
IFC5006	ECL5003 Δ*menFDHB*::Kan^r	This work
ECL594	MC4100 *sdh*$^+$ Φ(*sdh-lacZ*) *arcBl zgi*::Tn*10*	28
IFC5007	MC4100 Φ(*sdh-lacZ*) from ECL594	This work
IFC5008	IFC5007 Δ*ubiCA*::Kan^r from ECL5039	This work
IFC5009	ECL5001 Δ*ubiC*::Kan^r from JW5713	This work
Plasmids		
pEXT21	Low-copy-no. vector; Spt^r	32
pBR322	Cloning vector; Amp^r Tet^r	31
pMX517	Full-length *arcB* under the control of the *ara* promoter; Amp^r	9
pQE30ArcB$^{78-778}$	ArcB$^{78-778}$ in pQE30 under an IPTG-inducible promoter	15
pMX537	pEXT21::*ubiCA*; Spt^r	This work
pMX538	pBR322::*menFDHBCE*; Amp^r	This work

[a] Kan^r, kanamycin resistant; Chl, chloramphenicol; Spt, spectinomycin; Amp, ampicillin; Tet, tetracycline.

MATERIALS AND METHODS

Strain and plasmid constructions. The strains and plasmids used in this work are listed in Table 1. Strain IFC5006 (Δ*menFDHB*::Kan^r Δ*fnr*::Tn*9*-Chl^r *cydA′-lacZ*) was constructed by homologous recombination using the lambda red recombinase system (27). Briefly, a PCR-amplified fragment, using primers menF-up-pKD-Fw and menB-down-pKD-Rv (Table 2) and plasmid pKD4 (27) as the template, was used to replace the *menFDHB* operon with a kanamycin (Kan) cassette in strain ECL5003 (Δ*fnr*::Tn*9*-Chl^r *cydA′-lacZ*) (7). Strain IFC5007 (*sdh′-lacZ*) was constructed by P1*vir* transduction of the Φ(*sdh′-lacZ*) allele from strain ECL594 (28) into strain MC4100 (29). Strain IFC5008 (Δ*ubiCA*::Kan^r *sdh′-lacZ*) was constructed by P1*vir* transduction of the Δ*ubiCA*::Kan^r allele from strain ECL5039 (Δ*ubiCA*::Kan^r *cydA′-lacZ*) (23) into strain IFC5007. Strain IFC5009 (Δ*ubiC*::Kan^r) was constructed by P1*vir* transduction of the Δ*ubiC*::Kan^r allele from strain JW5713 (30) into strain ECL5001. The relevant genotype of all mutants was verified by PCR. To construct plasmid pMX538, the complete *men* operon (*menFDHBCE*), including its promoter region, was PCR amplified, using primers menF-Fw and menE-Rv (Table 2) and chromosomal DNA from MC4100 as the template, and cloned into the BamHI and HindIII sites of plasmid pBR322 (31). To construct plasmid pMX537, the *ubiCA* operon and its promoter region were PCR amplified, using primers ubiC-Fw and ubiA-Rv (Table 2) and chromosomal DNA from MC4100 as the template, and cloned into the EcoRI and HindIII sites of the low-copy-number plasmid pEXT21 (32). Construction of plasmids pQE30ArcB$^{78-778}$ and pMX517 has been described earlier (9, 15).

Growth conditions. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) medium at 37°C. For β-galactosidase activity assays, either LB broth buffered with 0.1 M MOPS (morpholinepropanesulfonic acid; pH 7.4) and supplemented with 20 mM D-xylose or defined minimal medium [1 mM KH_2PO_4, 40 mM KCl, 34 mM NaCl, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 0.3 mM $MgSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, and 0.1 M MOPS; pH 7.4] supplemented with 0.4% (wt/vol) glucose and 15 mg/liter of thiamine was used. When necessary, ampicillin, kanamycin, tetracycline, or spectinomycin was used at a final concentration of 100, 50, 20, or 50 μg ml^{-1}, respectively. L-Arabinose and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at final concentrations of 0.13 and 1 mM to induce expression of full-length ArcB and ArcB$^{78-778}$, respectively. For complementation assays, 1,4-dihydroxy-2-naphtoic acid (DHNA) was added to the medium at a final concentration of 5 μM.

ArcB-enriched inverted vesicle preparation, purification of His$_6$-tagged proteins, and phosphorylation assays. ArcB-enriched inverted membrane vesicles and His$_6$-tagged ArcB$^{78-778}$, used in phosphorylation assays, were prepared as described previously (9, 15, 33). Phosphorylation assays were carried out at room temperature in the presence of 40 mM [γ-^{32}P]ATP (specific activity, 2 Ci mmol^{-1}; New England Nuclear), 33 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol. For the estimation of the ArcB redox potential, quinone analogues, including 1,4-benzoquinone, 1,2-naphtoquinone, 1,4-naphtoquinone, juglone, menadione, plumbagin, lawsone, and anthraquinone-2-sulfonate, were used at 1 mM. The phosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP and terminated 2.5 min later by addition of an equal volume of 4× SDS sample buffer, and the mixtures were immediately subjected to SDS-PAGE on 10% polyacrylamide gels. The radioactivity of proteins resolved in the gels was analyzed by using a PhosphorImager (Molecular Dynamics).

Determination of the redox potential of the disulfide bonds of ArcB. The redox potential value of ArcB was determined using cysteine/cystine

TABLE 2 DNA primers used in this work

Name	Sequence (5′–3′)[a]	Use
menF-up-pKD-Fw	CCCCGTATAATGTGAGGCTTTTAACAGGGAG AGGTCCGCGTGTAGGCTGGAGCTGCTTC	Forward primer for *men* operon deletion
menB-down-pKD-Rv	CGTCCATGGGGATCTGCCAGCGGTATACCTGC GCGCTACGCATATGAATATCCTCCTTAG	Reverse primer for *men* operon deletion
menF-Fw	CCCGGATCCGTGATACACGTATCGATG (BamHI)	Forward primer for *men* operon cloning and *men* mutant confirmation
menE-Rv	CCCAAGCTTGACCAGCCATTCCATTGC (HindIII)	Reverse primer for *men* operon cloning and *men* mutant confirmation
ubiC-Fw	GATACCCAACAGATGATCG	Forward primer for *ubi* operon cloning and *ubi* mutant confirmation
ubiA-Rv	CCCAAGCTTCGCACGCTACAGCTGC (HindIII)	Reverse primer for *ubi* operon cloning and *ubi* mutant confirmation
k1-pKD4	GTCATAGCCGAATAGCC	Primer for mutant genotype confirmation
k2-pKD4	GTGCCCTGAATGAACTG	Primer for mutant genotype confirmation

[a] Restriction enzymes whose sites were introduced for subsequent cloning (underlined) are indicated in parentheses.

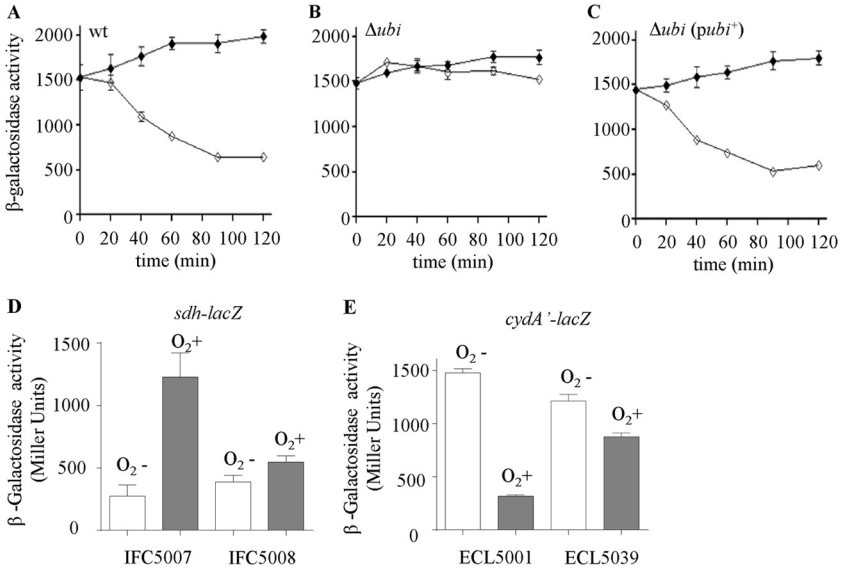

FIG 1 Requirement of ubiquinones for ArcB silencing *in vivo*. Cultures of strain ECL5001 (wt) (A), its isogenic ECL5039 strain (Δ*ubiCA*) (B), and ECL5039 harboring plasmid pMX537 (p*ubi*$^+$) (C), all of which carry the ArcB activatable *cydA′-lacZ* reporter, were grown anaerobically in Luria-Bertani broth containing 0.1 M MOPS (pH 7.4) and 20 mM D-xylose. At an OD_{600} of 0.2, one aliquot was withdrawn for measuring the β-galactosidase activity (depicted as 0 min) and the rest of the culture was divided in two. One part was kept under anaerobic conditions (filled diamonds) as a control, whereas the other was shifted to aerobiosis (empty diamonds), and the time course of the β-galactosidase activity was followed. (D) Strain IFC5007 (wt) and its isogenic IFC5008 strain (Δ*ubiCA*), both carrying the ArcB repressible *sdh-lacZ* reporter, were grown anaerobically in the above-described medium. At an OD_{600} of 0.2, the culture was divided in two. One part was kept under anaerobic conditions (empty bars), whereas the other was shifted to aerobiosis (solid bars), and after 2 h of incubation, the β-galactosidase activity was assayed and expressed in Miller units. (E) Strain ECL5001 (wt) and its isogenic ECL5039 strain (Δ*ubiCA*), both carrying the ArcB activatable *cydA′-lacZ* reporter, were grown anaerobically (empty bars) or aerobically (solid bars) in defined mineral medium with glucose as the sole carbon source. The cells were harvested at mid-exponential growth phase, and the β-galactosidase activity was assayed and expressed in Miller units. The data represent the averages from four independent experiments, and the standard deviation values are indicated.

couple redox buffers as previously described (34) but with some modifications. Briefly, ArcB-enriched inverted membrane vesicles containing ~1 μM concentrations of the full-length ArcB were incubated for 3 h at 25°C in the redox buffers (33 mM HEPES [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 μM UQ0, 40 μM cystine, and different concentrations of L-cysteine, ranging from 0.04 μM to 1 mM). All solutions were flushed with nitrogen to eliminate oxygen and minimize air oxidation. After incubation, samples were subjected to phosphorylation by addition of 40 mM [γ-^{32}P]ATP and analyzed as described above. The results were expressed as the fractions of reduced (active) protein, and the equilibrium constant (K_{eq}) was calculated according to the equation $R = ([Cys]^2/[cystine])/\{K_{eq} + ([Cys]^2/[cystine])\}$, where R is a fraction of the reduced proteins at equilibrium. The standard redox potential of ArcB was calculated from the Nernst equation, $E'^{\circ}_{ArcB} = E'^{\circ}_{Cys/Cystine} - (RT/2F) \ln K_{eq}$, where F and R are the Faraday constant and the gas constant, respectively, using a value of −200 mV for the Cys/cystine redox couple ($E'^{\circ}_{Cys/Cystine}$) (35, 36).

β-Galactosidase activity assay. For the aerobiosis-to-anaerobiosis shift, cells were cultured in 50 ml of medium in 250-ml baffled flasks at 37°C with shaking (300 rpm). At an optical density at 600 nm (OD_{600}) of 0.2, part of the culture was transferred to five prewarmed screw-cap tubes, filled up to the rim, and stirred by a magnet. The rest of the aerobic culture was further incubated with shaking at the same temperature. The time course of the experiment was followed by taking a sample from a filled screw-cap tube (anaerobiosis) and from the baffled flasks (aerobiosis) at each chosen time. For the anaerobiosis-to-aerobiosis shift, cells were cultured in seven screw-cap tubes filled with medium up to the rim at 37°C and stirred by a magnet. At an OD_{600} of 0.2, the content of a tube was passed to a 250-ml prewarmed baffled flask and incubated with shaking at the same temperature. Samples from aerobic and anaerobic cultures (the rest of the screw-cap tubes) were withdrawn at 0, 20, 40, 60, 90, and 120 min after the shift. β-Galactosidase activity was assayed and expressed in Miller units as described previously (37).

RESULTS

The ubiquinone electron carriers are required for ArcB silencing upon a shift from anaerobic to aerobic growth conditions. It has been previously reported that under aerobic conditions, the ubiquinone electron carriers act as negative signals that silence the kinase activity of ArcB (23) by oxidizing two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation (24). However, in a recent study, the role of ubiquinones as the specific silencing signal for ArcB was disputed, as it was found that the aerobic and anaerobic levels of an ArcA-P-specific reporter in a *ubiC* mutant (blocked in the ubiquinone biosynthesis) were similar to the ones of the isogenic wild-type strain (25). To address this discrepancy of whether or not ubiquinones are required for ArcB regulation *in vivo*, strain ECL5039, a *ubiCA* mutant carrying the ArcA-P activatable *cydA′-lacZ* operon fusion (23), and its isogenic wild type were grown anaerobically in buffered Luria broth, and at an OD_{600} of ~0.2, the culture was divided in two. One part was shifted to aerobiosis, whereas the other half was kept under anaerobiosis as a control, and the time course of the β-galactosidase activity was followed (Fig. 1). Monitoring the Arc activity during a shift from stimulatory to nonstimulatory growth conditions in buffered LB was decided because ArcB has been shown to respond rapidly to the presence or absence of the specific stimulus (21) and because the *ubiCA* mutant grows poorly aerobically (38–40). It was found that

shifting the anaerobic culture to aerobiosis caused a rapid decrease in reporter expression in the wild-type strain, indicative of ArcB/A silencing (Fig. 1A). However, no decrease in reporter expression was observed in the *ubiCA* mutant during the course of the experiment (Fig. 1B), indicating a defect in the inhibition of the ArcB kinase activity and thereby no silencing of the system. On the other hand, complementation of the mutant strain with a low-copy-number plasmid carrying the *ubiCA* operon (pMX537) fully restored reporter expression to wild-type levels (Fig. 1C). To ensure that the above-described result is due to the lack of inhibition of the ArcB kinase activity and not specific to the *cydA′-lacZ* reporter, we pursued a similar experiment using strains IFC5007 (wt) and IFC5008 (*ubiCA*), carrying the ArcA-P repressible *sdh-lacZ* operon fusion. The two isogenic strains were grown anaerobically to an OD_{600} of ~0.2, and half of the cultures were shifted to aerobiosis for 2 h. It was found that shifting the anaerobic cultures to aerobiosis resulted in release of the ArcB-dependent repression of *sdh-lacZ* expression in the wild-type strain but not in the *ubiCA* mutant (Fig. 1D), confirming the above-mentioned result. Therefore, it can be concluded that the ubiquinone electron carriers are required for rapid silencing of the ArcB kinase activity upon a shift from anoxic to oxic growth conditions.

We next attempted to discern whether the differences between the above-mentioned results and the ones reported by Bekker et al. (25) are due to the distinct experimental settings. Strain ECL5039 (*ubiCA*) and its isogenic wild-type strain ECL5001, both carrying the *cydA′-lacZ* reporter, were grown aerobically and anaerobically in defined minimal medium with glucose as the sole carbon source, and at mid-exponential growth, the β-galactosidase activity was measured (Fig. 1E). As expected, the anaerobic reporter expression was 5-fold higher than the aerobic expression in the wild-type strain. On the other hand, the aerobic reporter expression in the *ubiCA* mutant strain was 2.7-fold higher than that of the wt, indicative of diminished inhibition of ArcB kinase activity, in agreement with the above-presented results and our previous report (23). Moreover, the anaerobic reporter expression in the *ubiCA* mutant strain was only 1.3-fold higher than the aerobic expression, revealing a significant loss of regulation due to the elevated ArcB kinase activity under aerobic conditions. Thus, further support for the role of ubiquinones in the inhibition of the ArcB kinase activity, in agreement with our previous observations (23), is provided.

Finally, we tested whether the above-mentioned discrepancy is due to the different strains used in the two studies. It has been previously demonstrated that ubiquinone-deficient mutants grow poorly aerobically and that they fail to grow on succinate-mineral salt medium (38). Therefore, we compared the aerobic growth of ECL5039 (the *ubiCA* mutant used in this study), JW5713 (the *ubiC* mutant strain used by Bekker et al.), and their isogenic wild-type strains (ECL5001 and BW25113, respectively) on glucose- and succinate-mineral salt medium-agar plates. Curiously, although ECL5039 grew poorly on glucose and failed to grow on succinate, strain JW5713 grew almost equally as well as the wild-type strain on both substrates (see Fig. S1 in the supplemental material), indicating that a possible mutation suppresses the *ubi* mutant growth phenotype. Moreover, we found that this trait of JW5713 was cotransducible with the *ubi*::*kan* mutation by P1*vir* (see Fig. S1 in the supplemental material). Therefore, it is possible that the different strains used in the two studies might contribute to differences in ArcB activity and subsequent control of reporter expression. However, no further efforts were made to map this mutation because this was outside the scope of the present study.

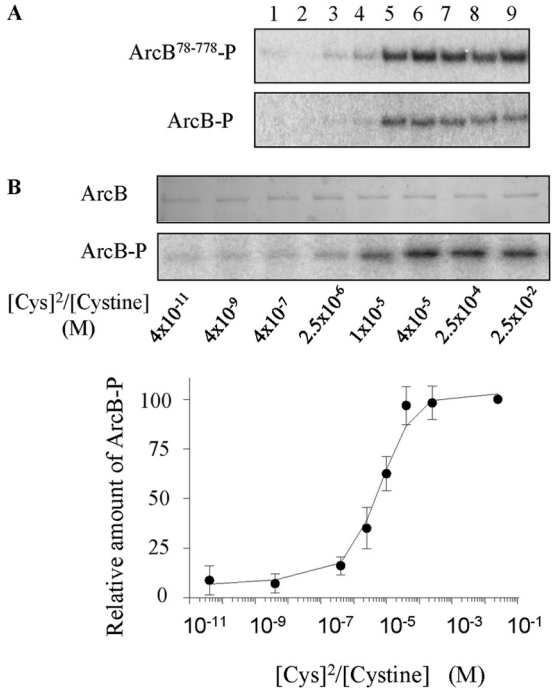

FIG 2 Determination of the redox potential of ArcB. (A) Purified $ArcB^{78-778}$ (top) or full-length ArcB-enriched inverted membrane vesicles (bottom) were incubated with [γ^{-32}P]ATP in the presence of 1 mM: lane 1, 1,4-benzoquinone (E'° = +274 mV); lane 2, 1,2-naphtoquinone (E'° = +134 mV); lane 3, 1,4-naphtoquinone (E'° = +69 mV); lane 4, juglone (E'° = +30 mV); lane 5, menadione (E'° = ±0 mV); lane 6, plumbagin (E'° = −29 mV); lane 7, lawsone (E'° = −137 mV); lane 8, anthraquinone-2-sulfonate (E'° = −225mV); and lane 9, nothing. After 2.5 min, the reactions were terminated by addition of an equal volume of 4× SDS sample buffer and the mixtures were immediately subjected to SDS-PAGE. Representative autoradiograms of the dried gels are presented. (B) A concentration of ~1 μM full-length ArcB embedded in inverted membrane vesicles was incubated with buffers containing various cysteine and cystine concentrations for 3 h at 25°C. Subsequently, the protein was incubated with [γ^{-32}P]ATP for 2.5 min, the reactions were terminated by addition of an equal volume of 4× SDS sample buffer, and the mixtures were immediately subjected to SDS-PAGE. Gel stained with Coomassie blue (top), an autoradiogram of phosphorylated proteins on the dried gel (middle), and a plot of ArcB net phosphorylation versus $[Cys]^2/[Cystine]$ (bottom) are shown. Data represent the averages from seven independent experiments, and the standard deviation values are indicated. The plot was interpreted by the equations given in Materials and Methods, and the K_{eq} and E'° values for ArcB were determined to be 4.2×10^{-6} M and −41 mV, respectively.

The redox potential of ArcB is −41 mV. The fact that the molecular event for ArcB regulation involves the oxidation/reduction of cysteine residues C^{180} and C^{241} renders the assessment of the ArcB redox potential of foremost importance. Therefore, in an effort to approximate the ArcB redox potential, the effect of several quinone analogs, covering a broad range of redox potential values, on the [γ^{32}P] ATP-dependent phosphorylation of ArcB was tested (Fig. 2A). The analogues tested were 1,4-benzoquinone (E'° = +274 mV), 1,2-naphtoquinone (E'° = +134 mV), 1,4-naphtoquinone (E'° = +69 mV), juglone (E'° = +30 mV), menadione (E'° = ±0 mV), plumbagin (E'° = −29 mV), lawsone (E'° = −137 mV), and anthraquinone-2-sulfonate (E'° = −225mV). It was found that quinone analogs having a positive

redox potential were effective inhibitors of the kinase activity of ArcB whereas analogs with negative redox potentials were without effect. The same effect was obtained with the quinone analogs for both purified ArcB$^{78\text{-}778}$ and ArcB-enriched inverted membrane vesicles (Fig. 2A).

To determine a more accurate redox potential for ArcB, we took advantage of the fact that the exact redox potential of a cysteine pair in a protein can be achieved by quantifying the steady-state ratios of their reduced and oxidized forms in the presence of excess of a composition-defined redox buffer. Therefore, we determined the redox potential of full-length ArcB embedded in inverted membrane vesicles from its redox equilibrium constant (K_{eq}) with a cysteine/cystine redox buffer at 25°C and using the ArcB phosphorylation ability as the indicator for the reduced/oxidized state of ArcB (Fig. 2B). The percentage of net phosphorylation as a function of the [cysteine]^2/[cystine] relation was used for the calculations of the K_{eq} value and the standard redox potential of ArcB, which were found to be 4.2×10^{-6} M and −41 mV, respectively (Fig. 2B).

Thus, the ubiquinones, having a redox potential of +100 mV, may serve perfectly as the ArcB oxidizing agent, in accordance with the above-presented *in vivo* results.

The menaquinone electron carriers are required for reactivation of ArcB upon a shift from aerobic to anaerobic growth conditions. Given that the redox potential of ArcB was found to be −41 mV, significantly higher than the redox potential of menaquinone (−74 mV), we considered that menaquinols may serve as the specific electron donors for ArcB. In such a scenario, the electrons would flow from menaquinols to ArcB, resulting in the reduction of the cysteine residues and activation of the kinase activity of ArcB. We therefore argued that if menaquinones were indeed involved in the regulation of ArcB, no activation of *cydA′-lacZ* expression should be observed in a menaquinone-deficient mutant after a shift from aerobic to anaerobic growth conditions. To test this, a mutant strain blocked in the menaquinone biosynthetic pathway was constructed (IFC5006) by deleting part of the *men* operon (*menF*, *menD*, *menH*, and *menB*) in a *cydA′-lacZ*-carrying strain. The choice of blocking the first steps of this pathway was in order to prevent the undesirable accumulation of intermediates. The mutant and its isogenic wild-type strain were grown aerobically in buffered Luria broth, and at an OD_{600} of ~0.2, the culture was divided in two. One part was kept under aerobic conditions as a control, whereas the other was shifted to anaerobiosis, and the time course of the β-galactosidase activity was followed (Fig. 3). It was found that shifting the aerobic culture to anaerobiosis caused a rapid increase in reporter expression in the wild-type strain (Fig. 3A), indicative of proper activation of the ArcB kinase activity. In contrast, no increase in reporter expression was observed in the *menFDHB* mutant (Fig. 3B), indicating a failure in the activation of ArcB kinase activity. Reporter expression was restored to wild-type levels when this mutant strain was complemented with a low-copy-number plasmid carrying the *menFDHBCE* operon (pMX538) (Fig. 3C). Likewise, addition of 1,4-dihydroxy-2-naphtoic acid (DHNA), which is the product of MenB and substrate of MenA to produce demethylmenaquinone, which is converted to menaquinone by UbiE, to the growth medium of the *menFDHB* mutant did not affect the aerobic expression of *cydA′-lacZ* but fully restored reporter expression after a shift to anaerobiosis (Fig. 3D), in agreement with a previous report (25). Therefore, it can be concluded that the menaquinone electron carriers are required for proper reactivation of the ArcB kinase activity after a shift from aerobic to anaerobic growth conditions.

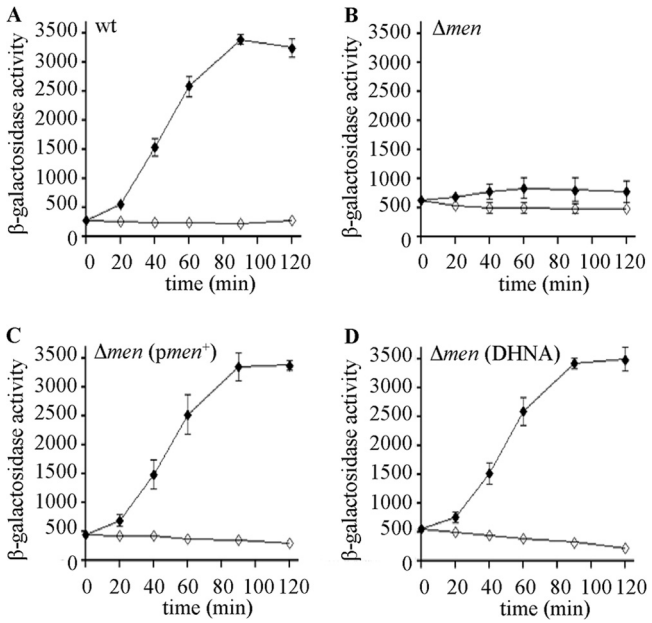

FIG 3 Requirement of menaquinones for ArcB activation *in vivo*. Cultures of strain ECL5003 (wt) (A), its isogenic strain IFC5006 (Δ*menFDHB*) (B), IFC5006 complemented with plasmid pMX538 (p*men*$^+$) (C), and IFC5006 in the presence of 1,4-dihydroxy-2-naphtoic acid (DHNA) (D), all bearing the ArcB activatable *cydA′-lacZ* reporter, were grown aerobically in LB broth containing 0.1 M MOPS (pH 7.4) and 20 mM D-xylose. At an OD_{600} of 0.2, one aliquot was withdrawn for measuring the β-galactosidase activity (depicted as 0 min) and the rest of the culture was divided in two. One part was kept under aerobic conditions (empty diamonds) as a control, whereas the other was shifted to anaerobiosis (filled diamonds), and the time course of the β-galactosidase activity was followed. Data represent the averages from four experiments, and the standard deviation values are indicated.

DISCUSSION

The Arc two-component system is a complex signal transduction system that plays a key role in regulating energy metabolism at the level of transcription in bacteria. Under reducing growth conditions, ArcB autophosphorylates and transphosphorylates ArcA, which, in turn, represses or activates the expression of its target operons. Under aerobic conditions, ArcB is inactivated as a kinase but acts as a phosphatase that catalyzes the dephosphorylation of ArcA-P, thereby releasing its transcriptional regulation. The molecular mechanism of ArcB kinase silencing was shown to require the ubiquinone-dependent oxidation of two cysteine residues, C^{180} and C^{241}, leading to intermolecular disulfide bond formation (23, 24). In this study, we demonstrate that ubiquinones (E'° = +100 mV) are required for ArcB silencing, in accordance with our previous report (23). In addition, we determine the midpoint redox potential of ArcB to be approximately −41 mV, and we demonstrate, in agreement with a previous study (25), that menaquinols (E'° = −74 mV) are required for proper ArcB reactivation upon a shift from aerobic to anaerobic growth conditions. Our results led us to propose a revised model for Arc redox signaling (Fig. 4) which differs significantly from those previously suggested (25, 26) but is in agreement with the model proposed by Portnoy

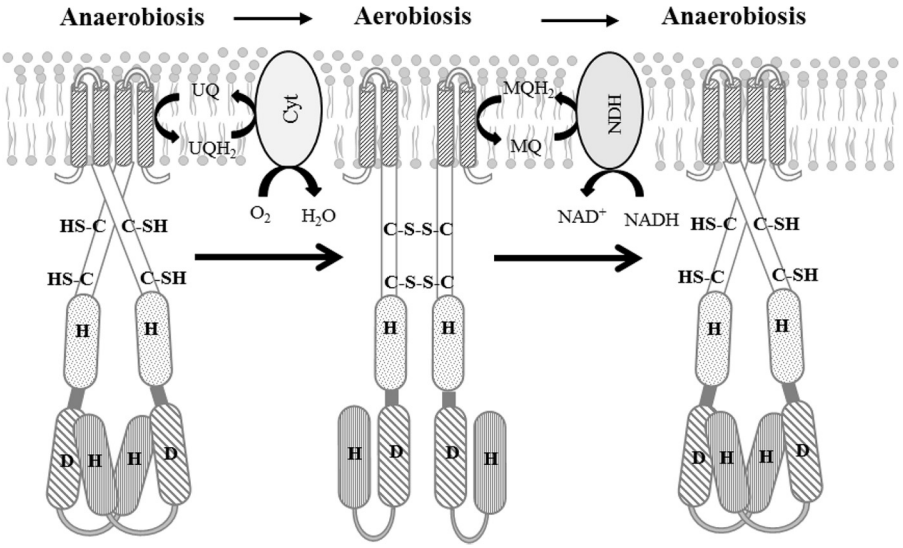

FIG 4 A simplified model for ArcB regulation. During aerobic growth conditions or upon a shift from anaerobic to aerobic growth, ubiquinones (UQ/UQH_2; $E'^{\circ} = +100$ mV) constitute the major quinones in the respiratory chain of *E. coli*. As the electrons flow toward O_2 via the cytochromes (Cyt), the ubiquinone pool maintains its oxidized state, allowing the electron transfer from the cysteine residues of ArcB ($E'^{\circ} = -41$ mV) to UQ. This results in disulfide bond formation and immediate silencing of the ArcB kinase activity. During microaerobic growth conditions or upon a shift from aerobic to anaerobic growth, the ubiquinone pool is gradually replaced by menaquinones (MQ/MQH_2; $E'^{\circ} = -74$ mV). The low redox potential of MQ/MQH_2 permits the electron transfer from menaquinol (MQH_2) to the cysteine residues of ArcB, resulting in disulfide bond breakage and activation of the ArcB kinase activity. The pool of menaquinol is restored by the electron flow from the NADH dehydrogenases (NDH).

and coworkers (41). Our results are also in accordance with the previous findings that despite the aerobic growth conditions, an ArcA-P expression profile was observed in mutant strains lacking both cytochrome *bo* and cytochrome *bd* terminal oxidases (42), the *ubiG* gene (43), or the three terminal cytochrome oxidase genes (*cydAB*, *cyoABCD*, and *cbdAB*) and a quinol monooxygenase gene (*ygiN*) (41). These observations are promptly explained by the demonstration that these mutations result in a shift in the content of the quinone pool from the ubiquinones to menaquinones (41). Such a shift should lead to the activation of ArcB, the rise of ArcA-P levels, and the adjustment of the expression of the target operons accordingly.

Nevertheless, two recent studies (25, 26) put into question the importance of the ubiquinone pool in the regulation of the ArcB sensor kinase and our previously proposed model of ArcB regulation (3, 23, 24). In both studies, in which chemostat cultures were used, the challenge of the importance of ubiquinones in the regulation of ArcB kinase activity was based on the failure of establishing a correlation between reporter expression or the level of ArcA-P and the redox state of the ubiquinone pool (25, 26). Also, results showed that the aerobic and anaerobic expression levels of an ArcA-P-specific reporter (cyd^{-176+1}-*lacZ*) in a ubiquinone-deficient mutant strain were similar to the ones of the isogenic wild-type strain (25). Of note, the ArcA activity profiles and the quinol/quinone ratios differed significantly in these two studies, although both used steady-state chemostat cultures. Nevertheless, the correlation between reporter expression and ubiquinone/menaquinone levels prompted Bekker and coauthors to propose the following model (25). Upon a shift from anaerobiosis to low aerobiosis, the menaquinone pool oxidizes rapidly, resulting in oxidation of the regulatory cysteine residues and inactivation of ArcB. A further increase in aerobiosis leads to an increase in ubiquinol, resulting in the reduction of the cysteines and, thereby, reactivation of ArcB. Finally, in complete aerobiosis, the content of the quinone pool decreases, resulting in the oxidation of the cysteines and inactivation of ArcB. However, such a model implies that both menaquinones and ubiquinones are able to silence the ArcB kinase activity whereas both menaquinols and ubiquinols are able to activate it. This can be possible only if quinone/quinol binding was sufficient for regulation of the ArcB activity but not if electron transfer between the quinones/quinols and the two redox-active cysteines of ArcB were required. An alternative explanation might be that in JW5713, a *ubiC* mutant strain from the Keio collection (30), the amount of demethylmenaquinone ($E'^{\circ} = +36$ mV) increases. In such a case, demethylmenaquinone may, at least in part, compensate the absence of ubiquinone because its redox potential is considerably higher than that of ArcB ($E'^{\circ} = -41$ mV).

On the other hand, Rofle and coworkers, using transcriptomic analysis and direct measurement of ArcA-P across the aerobiosis range (0 to 100%), reported a linear decrease in the ArcB/A activity with increasing aerobiosis (26), in agreement with our results presented here and with previous reported studies (4, 5, 16). However, no correlation between ArcB/A activity and the redox state of the ubiquinone pool was found. Therefore, the authors concluded that inhibition of ArcB phosphatase activity mediated by fermentation products, such as acetate, may be the dominant mechanism for regulating ArcB and, thereby, ArcA activity instead of the redox state of the ubiquinone pool (26). However, the 2-fold difference in total ubiquinone between anaerobiosis and full aerobiosis contrasts the ~5-fold increase reported earlier (25, 39, 41, 44, 45) and weakens the above-mentioned conclusion. Moreover, this mode of ArcB regulation has been discarded in earlier *in vivo* and *in vitro* studies that demonstrate that fermentation products, such as acetate and lactate, have no effect on the ArcB phosphatase activity (12) and do not act as direct signals for activation of ArcB

but rather act as physiologically significant effectors that amplify the ArcB kinase activity (13).

In view of the data reported herein, a revised model is presented (Fig. 4). *E. coli* expresses branched electron transfer chains that can deal with various electron donors and several alternative electron acceptors. A central component in these networks is the quinone pool that acts as a redox mediator. During aerobic growth, the major quinone is ubiquinone ($E'^{\circ} = +100$ mV), which is replaced by menaquinone ($E'^{\circ} = -74$ mV) in anaerobic growth (25, 39, 41, 44, 45). Thus, during aerobic growth conditions or upon a shift from anaerobic to aerobic growth, the predominant ubiquinone pool acts to oxidize the cysteine residues of ArcB ($E'^{\circ} \approx -41$ mV), resulting in disulfide bond formation and immediate silencing of the ArcB kinase activity, leading to inactivation of ArcA. On the other hand, during microaerobic growth conditions or upon a shift from aerobic to anaerobic growth, the ubiquinone pool is gradually replaced by menaquinones that act to reduce the cysteine residues of ArcB, resulting in disulfide bond breakage, activation of the ArcB kinase activity, and consequent activation of ArcA. Based on the proposed model, we predict that both UQ and MQ are competing for a single quinone-binding pocket in ArcB that is modulated by the UQ/MQ ratio under aerobic, microaerobic, and anaerobic growth conditions. Such a mechanism allows rapid responses of bacterial cells to their environment, as the respiratory growth conditions are instantly sensed and transduced into the transcriptional apparatus.

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