Two-component systems are employed by prokaryotes to respond to changing environmental and intracellular conditions. The ArcBA (anoxic redox control) system is a two-component system in *Escherichia coli* that functions as the aerobiciosis-sensing device that tunes the activity of catabolic pathways to variations in oxygen availability. Its first component, ArcB, functions as the sensor that relays a signal via a signal-dependent kinase activity to the second component, the response regulator ArcA (17, 20). Upon signal perception, ArcB is phosphorylated at the conserved His-292 residue. The phosphoryl group is subsequently transferred intramolecularly to the conserved Asp-576 residue, after which it is transferred once more intramolecularly to His-717, which is located in the phosphoryl transfer domain. Then the latter residue transfers the phosphoryl group to ArcA (12, 23). The extent of phosphorylation of ArcA determines the expression of operons involved in a wide variety of mostly catabolic pathways that are operative under different redox growth conditions (3, 14, 24). Thus, the ArcBA system is important for the organism’s ability to distribute energy generation for fermentation and respiration (3).

ArcB can form intermolecular disulfide bonds via Cys-180 and Cys241, which are located in the PAS domain of the protein. The kinase activity of ArcB is highly dependent on this covalent linkage. A disulfide bond formed between two Cys-180 residues results in a 85% reduction in kinase activity, and a bond between two Cys-241 residues results in a 15% reduction (26). It has been shown *in vitro* that the cysteine residues can be oxidized by ubiquinone (26), and hence the latter redox carrier was postulated to regulate the kinase activity of ArcB *in vivo*. Consistent with this hypothesis, maximal kinase activity of ArcB with ArcA has been shown to occur under anaerobic conditions (25); nevertheless, it has also been suggested that significant levels of ArcA−P are present in aerobic cells (18, 19).

Previous studies on ArcB have used *lacZ* reporters that could be or are known to be subject to regulation by additional factors (e.g., FNR for the *cydA-lacZ* reporter used by Georgelis et al. [11, 26, 27]). In this study, a *P*$_{cydA1−176+15}^{lacZ}$ reporter that is not responsive to FNR but is dependent on ArcA−P (1) was used to characterize the factors that are involved in the *in vivo* regulation of ArcB activation in relation to (decreasing) oxygen availability (1). Our findings indicate that the menaquinone pool plays an important role in ArcB activation.
The ArcBA system exhibits maximal activity under high-microaerobic conditions (equivalent to 80% aerobicis according to the quantitative definition formulated by Alexeeva et al. [1]) and fully anaerobic conditions and much lower levels of activity under low-microaerobiosis conditions (20% aerobicis) and fully aerobic conditions. We show that additional regulation of ArcB kinase activity by the redox state of the menaquinone and the ubiquinone pools is prevalent under microaerobic conditions.

Based on these observations we concluded that regulation of the ArcBA system in vivo in our microaerobic conditions is controlled by both the menaquinone and the ubiquinone pools.

MATERIALS AND METHODS

Strains and plasmids used in this study. To create pSal1 (Table 1), a 176-bp DNA fragment from the upstream region (Fig. 1A) of the cydAB operon (cydA-176 +1 ) (corresponding to bases 512 to 687 in the GenBank accession number J03939 sequence; bases 1 to −176 relative to the start site of cydAB P1 transcription) was amplified by PCR with primers Cyd(E)-174 (5' TCT TTT TAT CTT TAA TTG CCA ACC G) and Cyd(Bam) (5' TCC CGA GAA CAA TTT ATC TCT TTT TGA TGC C) using E. coli K-12 (not to scale). The PCR product was digested with BamHI and EcoRI and cloned in the corresponding sites of the T7 RNA polymerase gene (not to scale).

Based on these observations we concluded that regulation of the ArcBA system in vivo in our microaerobic conditions is controlled by both the menaquinone and the ubiquinone pools.
To create plasmid pSal2, PCR-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene) was performed using primers cydmut1 (5′-CAT AAT TTG TAG GAA ATT TTT AAC AAT GTA TAA TTC CCT ACA AAT TAT G-3′) and cydmut2 (5′-CCA AGA CTT ATA CAT TGT TAA AAT TTT CCT ACA AAT TAG-3′) and plasmid pSal1 as the template according to the manufacturer’s instructions. This resulted in the introduction of point mutations (underlined) in the essential base pairs of the consensus sequence of the FNR-binding site (GAAGATTATTTATCAATGTTA to GGAAGATTATTTATCAATGTTA). These mutations vs a Spel restriction site was confirmed. Successful mutagenesis was confirmed by restriction analysis and sequencing using the semiautomated DNA sequencing technique.

To construct the operon fusions, the 176-bp EcoRI-BamH1 fragment of plasmid pSal2 was ligated into EcoRI-BamH1-digested lacZ operon fusion vector pRS551 (38). Resulting plasmid pRS22. The fusion (Fig. 1B) was then transferred to the Δ transducing phage λRS45 (the MC1061 strain bearing pRS22 was infected with λRS45) as described previously (35), yielding λRS22. Lysate containing λRS22 was used to lysogenize strains MC4100, yielding ASA12.

Measurement of enzyme activity. β-Galactosidase activity in permeabilized cells was measured by a method originally described by Miller (30) and modified by Giaconi et al. (31).

Overproduction and purification of His6-ArcA. For overproduction of His6-ArcA, 500-mL cultures of E. coli strain BL21 transformed with pET23c-A1 were grown in 2-L conical flasks with vigorous shaking (200 rpm) on a rotary shaker at 37°C in LB medium (20 g liter−1 tryptone, 10 g liter−1 yeast extract, 5 g liter−1 NaCl). After growth for 4 h, the culture was harvested by centrifugation at 9,000 × g, and the cell pellet was stored at −20°C until use. All subsequent steps were performed at 4°C. Next, the cell pellet was resuspended in 4 mL of buffer A (0.5 M NaCl, 20 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, pH 7.9), containing 1.3 mg · mL−1 lysozyme, 30 μg · mL−1 phenylmethylsulfonyl fluoride (PMSF), and 1% (vol/vol) Triton X-100. After 30 min of incubation at room temperature, the cells were disrupted by sonication. The resulting cell lysate was clarified by centrifugation at 15,000 rpm for 30 min. Phenylmethylsulfonyl fluoride (PMSF) was added to the lysate to a final concentration of 0.1 mM in order to prevent protein degradation. The cell lysate was then applied to a 1.5-ml Ni-nitrilotriacetic acid–agarose column (Qia- gen) equilibrated with buffer A. After the column was washed with 10 mL of 10 mM imidazole (in buffer A) and collected in five 2-ml fractions. These fractions were routinely analyzed for β-galactosidase activity by a color reaction. Amounts of ArcA (with the purified ArcA protein) resolved on a 12.5% (wt/vol) SDS-PAGE gel were blotted onto nitrocellulose in a Trans-Blot semidyem cell (Bio-Rad) and subsequently immunolabeled as described by Towbin et al. (39). The rabbit anti-ArcA antibody was used at a dilution of 1:10,000. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), was used at a dilution of 1:3,000 for subsequent visualization by a color reaction. Amounts of ArcA (with the purified ArcA protein used as a reference) were quantified using densitometric analysis (Image Master ID Prime, version 2.0; Pharmacia Biotech).

Gel retardation assays. Gel retardation assays were performed essentially as described by Ramanujam et al. (32) with 8 to 25% (wt/vol) gradient polyacrylamide gels (Pharmacia), using a PhastSystem at 4°C and the following separation method: sample application 1.2 using 0 V · h; sample application up 1.2 using 2 V · h; separation 1.1 using 400 V, 10 mA, 5 W, 4°C, and 10 V · h; separation 1.2 using 400 V, 1 mA, 5 W, 4°C, and 2 V · h; and separation 1.3 using 400 V, 1 mA, 2.5 W, 4°C, and 268 V · h. After completion of the run, the lower part of the gel, containing unincorporated [α-32P]dATP from the DNA labeling reactions, was removed to prevent the signal from the nucleotides from interfering with the signal from the DNA fragments. The results were visualized by exposing the gel to film with an intensifying screen overnight at −70°C. Quantification of bound and unbound DNA fragments was performed by densitometric analysis using ImageMaster 1D Prime, version 2.0 (Pharmacia Biotech).

Continuous cultures. Cells were grown in Applikon-type fermentors (1–3, 10) at a dilution rate of 0.15 ± 0.01 h−1 under glucose-limited conditions. A simple salts medium described by Evans et al. (10) was used, but instead of citrate, nitrosoacetic acid (2 mM) was used as the chelator. Selenite (30 μg liter−1) and thiamine (15 μg liter−1) were added to the medium. Glucose was used as the single carbon and energy source at a final concentration of 45 mM in the feed. The pH was maintained at 7.0 ± 0.1 by titration with sterile 4 M NaOH, and the feed nitrogen supply was varied as described previously (3). In addition to β-galactosidase activities, in all cultures the steady-state specific rates of fermentation product formation and glucose and O2 consumption were measured as described by Alexeeva et al. (3) to determine the percentage of aerobicity. In previous experiments the chemostat cultures were calibrated to quantify oxygen availability (2). Essentially, 0% aerobicity reflects fully anaerobic conditions and 100% aerobicity is the minimal oxygen input rate required for completely aerobic catalysis (Alexeeva et al. [2] provide a quantitative definition of microaerobiosis).

Batch culture. In batch cultures, the composition of the medium was similar to the composition of the medium described above, except that sodium phosphate (pH 7) was used at a concentration of 100 mM instead of 10 mM to increase the buffering capacity of the medium. Glucose (final concentration, 1% [wt/vol]) was sterilized separately. High levels of aeration of cultures during aerobic growth were obtained by shaking 10-mL cultures in 100-mL Erlenmeyer flasks at 180 rpm. For anaerobic growth 15-mL cultures in sealed Greiner tubes (15 mL) were used. Inoculated cells were obtained from cultures pregrown under aerobic or anaerobic conditions (after dilution to an OD600nm of ~5 × 10−3) and allowed to double about seven times to mid-log exponential phase (final OD600nm for aerobic conditions, 0.4 to 0.6; final OD600nm for aerobic conditions, 0.8 to 1.2) prior to measurement of β-galactosidase enzyme activity. Batch cultures (100 mL) were used for measurement of the redox activity of the ubiquinone pool with the cydA1 Δ196-1, lacZ expression level (Fig. 2) were grown in 1-liter Erlenmeyer flasks stirred at a rate of 250 rpm. Other conditions were similar to those described above. The strains were maintained in vials in LB medium with 30% (wt/vol) glycerol at −70°C.
Analysis of carbon fluxes. Steady-state bacterial dry weight was measured as described previously (2). Glucose, pyruvate, lactate, formate, acetate, succinate, and ethanol contents were determined by high-performance liquid chromatography (HPLC) (LKB) with a REZEX organic acid analysis column (Phenomenex) at a temperature of 45°C with 7.2 mM H₂SO₄ as the eluent, using an RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration. The carbon balance for all data is ≥92% (mean, 96%), as calculated from the glucose consumption and product formation rates.

Quinone extraction. Two-milliliter samples from a culture were quenched with 6 ml of ice-cold 0.2 M HClO₄ in methanol or with only methanol. Next, 6 ml of petroleum ether (boiling point, 40 to 60°C) was added rapidly to the mixture, which was vortexed for 1 min. After the mixture was centrifuged (900 × g, 2 min), the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of nitrogen. Another 3 ml of petroleum ether was added to the lower phase, and the vortexing and centrifugation steps were repeated. The upper phases were combined. After evaporation to dryness, extracts could be stored for at least 7 days under nitrogen at −20°C. Immediately before use, the extracted quinone-quinol mixture was resuspended with a glass rod in 80 μl ethanol and fractionated by HPLC (Pharmacia LKB 2249 gradient pump system with an LKB 2151 variable-wavelength monitor) using a reversed-phase Lichrosorb (Chrompack, Bergen op Zoom, The Netherlands) 10 RP 18 column (size, 250 mm; internal diameter, 4.6 mm). The column was equilibrated with ethanol-methanol (1:1, vol/vol) or pure methanol (HPLC grade), and the ethanol-methanol mixture was used as the mobile phase. The flow rate was 2 ml/min at 50°C. Detection of the quinones was performed at 290 nm for ubiquinones and at 248 nm for menaquinones. The amount of each quinone species was calculated from the relevant peak area, using ubiquinone-10 (UQ₁₀) and menaquinone-4 as standards and the method described by Shestopalov et al. (37). The methanol, ethanol, and petroleum ether used were analytical grade.

Peaks were identified by UV/visible and mass spectral analysis. A UV/visible spectrum of demethylmenaquinone-8 (DMK₈) was kindly provided by A. V. Bogachev (Moscow University, Moscow, Russia). For mass spectral analysis frac-
ions collected from the HPLC were evaporated under nitrogen and redissolved in 90% (wt/vol) acetonitrile, 1% (vol/vol) formic acid (LC grade; Merck, Franklin, Germany). Then fractions were analyzed by off-line electrospray mass spectrometry using coated Picotips (Econo12; New Objective, Woburn, MA) with an electrospray ionization quantitative time of flight mass spectrometer (Micromass; Waters, Manchester, United Kingdom). Ions selected for tandem mass spectrometry collided with argon in the hexapole collision cell.

RESULTS

Specific interaction of His$_6$-ArcA–P with the regulatory region of the cydAB operon. In order to construct an ArcA–P-dependent reporter system that responds exclusively to this regulator, we amplified a 176-bp DNA fragment from the cydAB regulatory region of E. coli (cydA$^{-176+1}$). This DNA fragment contains an ArcA-binding site (site II), an FNR-binding site, and the cydAB promoter P1 (8). The FNR-binding site in this regulatory region was selectively inactivated by site-directed mutagenesis (Fig. 1; also see Materials and Methods). This fragment was fused with the lacZ gene and introduced into E. coli strain MC4100 to create strain ASA12 (see Materials and Methods).

To verify that the reporter construct indeed responds solely to the phosphorylation state of ArcA in vivo and that FNR does not interfere with this response, strain ASA12 (wild type with cydA$^{-176+1}$-lacZ) and strain ASA22 (Δfnr cydA$^{-176+1}$, lacZ) (see Materials and Methods) were grown in anaerobic and aerobic batch conditions, respectively. The expression of the reporter was found to be twofold higher in anaerobic conditions than in aerobic conditions for both strains (data not shown). In contrast, the change in expression of the reporter in strain ASA11, which expresses FNR and contains the reporter with the FNR-binding site, was less than 1.5-fold (data not shown).

The interaction of the ArcA protein with the mutated cydAB regulatory region was quantified in vitro by performing gel retardation assays. Relative binding affinities of ArcA and ArcA–P were determined using a $^{32}$P-end-labeled fragment derived from pSal2 (Fig. 1 and 2A). ArcA was phosphorylated by incubation with carbamoyl phosphate. A single retarded complex was observed for each binding reaction. Fractions of DNA retarded by ArcA or ArcA–P were quantified by densitometric analysis and plotted (Fig. 2B) as a function of protein concentration. For ArcA–P, an apparent $K_{d_{	ext{obs}}}$ [the ArcA–P concentration at which one-half of the amount of the DNA fragment is bound, designated $K_{\text{ArcA-P}}(0.5)$] was estimated to be approximately 0.25 μM, whereas the corresponding value for ArcA [$K_{\text{ArcA}}(0.5)$] was estimated to be approximately 4.5 μM. The sigmoid shapes of the binding curves suggest that binding of neither ArcA nor ArcA–P to the regulatory DNA fragment obeys simple binding kinetics (i.e., a hyperbolic [bound fraction]/[substrate] function). Analysis of the data essentially as described by Keleti (22) and Segel (36) by fitting a double-reciprocal plot of $1/Y$ versus $1/[S]$, where $Y$ is the fraction of bound DNA and $[S]$ is the ArcA–P concentration, to the equation $Y = Y_{\text{max}}[S]^{n}/(K_{m} + [S]^{n})$ provided the best fits ($R^2$), with a value of 2 for ArcA and a value of 6 for ArcA–P (Fig. 2C), strongly suggesting that ArcA and ArcA–P bind to the DNA fragment with different stoichiometries. The $K_{m}$ values for binding derived from the double-reciprocal plot with the coordinates $(1/Y; 1/[S]^{n})$ when $n$ is 2 for ArcA and 6 for ArcA–P were 2.44 μM and 0.26 μM, respectively. Comparable stoichiometries (when $n$ is 3 for ArcA and 6 for ArcA–P) and $K_{m}$ values (4.3 μM for ArcA and 0.27 μM for ArcA–P) were obtained by analysis of the data using a Hill plot (not shown). Together, these results suggest that ArcA–P binds to the DNA fragment as a hexamer, which is in accordance with the observations of Jeon et al. (21).

Only when ArcA was phosphorylated was a sequence-specific interaction with the DNA fragment containing ArcA-binding site II of the cydAB regulatory region observed. This was concluded from the lack of an effect of addition of a large excess of competing fragments [poly(dI-dC)-poly(dI-dC) or pQE30] with a random sequence to the binding assays with ArcA–P. In contrast, addition of a 50-fold molar excess of either competitor DNA in the binding assay with nonphosphorylated ArcA did prevent sequence-specific complex formation. These competing fragments did not have an effect when phosphorilated ArcA was used (data not shown). Similar observations with respect to the specificity of ArcA and ArcA–P binding to the same binding site have been made previously by Lynch and Lin (25).

ArcB activation by various quinone species. The specific ArcA–P-dependent lacZ reporter construct (1) (see above) was transduced with phage P1 into strains BW25113 (wild type), JW5536 (ΔarcB) (4), and JW5713 (ΔubiC), generating strains JA001, JA032, and JA023, respectively. The resulting strains were grown in batch culture in minimal medium supplemented with glucose. Cultures were grown aerobically (open bars) and anaerobically (filled bars).

FIG. 3. Activity of the ArcA–P-dependent-lacZ reporter construct (ARSS2). The construct was tested in the wild-type (JA001), ΔubiC (JA023), and ΔarcB (JA032) backgrounds after batch culture growth in mineral medium supplemented with glucose. Cultures were grown aerobically (open bars) and anaerobically (filled bars).
in regulation of ArcB, we constructed strain JA023, which lacks ubiC and is therefore unable to synthesize ubiquinone. The difference between the aerobic and anaerobic levels of expression of the cydA\(^{-176+1}\)-lacZ reporter was similar to the difference found for the wild-type strain. This result strongly suggests that aerobic inhibition of the ArcB kinase \textit{in vivo} can be due to other effectors and thus is regulated not only by ubiquinone.

Previously, we showed (5) that the ubiquinone pool of \textit{E. coli} is gradually reduced during growth in batch culture when the OD\(_{600}\) reaches values higher than 1.0. One would therefore expect that an increase in cydA\(^{-176+1}\)-lacZ expression would parallel the increase in the ratio of ubiquinol concentration to ubiquinone concentration if this expression is governed solely by this ratio. To test this hypothesis, strain ASA12 was grown in aerobic batch culture, as described previously (5). As anticipated, a considerable increase in the ubiquinol/ubiquinone ratio was observed at the later stages of growth; however, this increase was not accompanied by a significant increase in the level of cydA\(^{-176+1}\)-lacZ expression (see Fig. S1 in the supplemental material). Together, these observations indicate that there was no apparent regulation by the ubiquinol/ubiquinone ratio under the conditions used in these experiments.

Besides addition of ubiquinone, addition of menadione has been reported to influence the \textit{in vitro} autophosphorylation rate of ArcB, although higher concentrations of the latter quinone were required to obtain half-maximal inhibition (50 \(\mu\)M, compared to 5 \(\mu\)M for ubiquinone). To investigate whether demethylmenaquinone plays a role in the regulation of ArcB in \textit{vivo}, strain JW2257 (ΔmenB) was lysogenised with the P1 phage lysate obtained from strain ASA12, resulting in strain JA022. The latter strain’s inability to synthesize demethylmenaquinones yielded a surprising phenotype: loss of the activation response of cydA\(^{-176+1}\)-lacZ expression to anaerobiosis (Fig. 4). Moreover, addition of 2 \(\mu\)M 1,4-dihydroxy-2-naphthoic acid, an intermediate in the menaquinone biosynthetic pathway that is synthesized downstream of the block in this pathway introduced by deletion of \textit{menB} (encoding naphthoate synthase), to anaerobic batch cultures restored the wild-type phenotype. Independent analyses of cellular quinone levels verified that addition of naphthoic acid resulted in significant restoration of the intracellular demethylmenaquinone and menaquinone pools (see Fig. S2 in the supplemental material) but had no effect on the redox state of the ubiquinone pool (data not shown). This clearly shows that menaquinone (or the menaquinone pool) also has a role in the regulation of ArcB activation.

\textbf{ArcB activation under microaerobic conditions.} Previously, we have described a controlled aerobiosis system based on glucose-limited chemostat cultures (see reference 2 for details concerning the experimental setup) that allows growth of steady-state cultures at any ratio of aerobic catabolism to anaerobic fermentation. To monitor the degree of ArcA phosphorylation \textit{in vivo} in relation to quantified and steady-state oxygen availability, strain ASA12, an MC4100 derivative which contains the reporter system described above for strain JA001 and its relatives, was grown in glucose-limited chemostat cultures at a growth rate of 0.15 h\(^{-1}\) with controlled oxygen input rates ranging from 0 to 110% aerobiosis (see Materials and Methods). Under these conditions a rather complex pattern of cydA\(^{-176+1}\)-lacZ expression was observed, with maximal values at 0% and 85% aerobiosis. The maximal values were approximately 2.5-fold higher than the minimal values measured at 20% aerobiosis and under fully aerobic conditions (Fig. 5). The expression levels of the cydA\(^{-176+1}\)-lacZ reporter under the latter two conditions were not significantly different. In contrast, in similar experiments with strain ASA32, which lacks ArcA, the level of cydA\(^{-176+1}\)-lacZ expression increased linearly with increasing oxygen availability, and there was an approximately 1.5-fold increase when anaerobic growth conditions were compared to fully aerobic growth conditions (see Fig. S3 in the supplemental material).

In view of the \textit{in vitro} results of Georgellis et al. (11) and Malpica et al. (26), the cellular content of the ubiquinol-ubiquinone pools was measured for all steady-state chemostat cultures by HPLC-UV analysis that allowed separation of the reduced and oxidized quinone species (5). As expected, the ubiquinol/ubiquinone ratio gradually increased toward anaerobiosis (Fig. 5) and exhibited a pattern that does not resemble that of the changes observed for cydA\(^{-176+1}\)-lacZ expression. Furthermore, in accordance with previous reports for aerobic

\begin{figure}
\centering
\includegraphics[width=\textwidth]{activity_of_the_arca_p-dependent-lacz_reporter_construct.png}
\caption{Activity of the ArcA\(^{-P}\)-dependent-lacz reporter construct (λRSS2). The construct was tested in the wild type (JA001) and ΔmenB (JA022) backgrounds after growth in anaerobic batch cultures in medium with (bars with diagonal lines) or without (filled bars) 2 \(\mu\)M 1,4-dihydroxy-2-naphthoic acid or after aerobic growth without 1,4-dihydroxy-2-naphthoic acid (open bars).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{activity_of_the_arca_p_dependent_lacz_reporter_construct.png}
\caption{Activity of the ArcA\(^{-P}\)-dependent-lacz reporter construct (ASA12) (filled squares) and redox state of the UQ\(_8\) pool (open circles) under various aerobiosis conditions. The construct was tested in the wild-type ASA12 background after glucose-limited continuous growth in mineral medium supplemented with various amounts of oxygen.}
\end{figure}
FIG. 6. Contents of total UQ8 (black triangles), DMK8 (open diamonds), and MK8 pool (open squares) under various aerobiosis conditions. Strain ASA12 was tested in glucose-limited continuous growth conditions in mineral medium supplemented with various amounts of oxygen. The values are data from single experiments.

and anaerobic batch conditions (37), the absolute cellular content of ubiquinol and ubiquinone combined increased gradually with increasing oxygen availability up to 80% aerobiosis (Fig. 6), which was followed by a decrease from 80 to 100% aerobiosis. Thus, it appears that the size of the ubiquinol pool correlates with cydA-176-lacZ expression throughout the range from 20 to 100% aerobiosis but not at aerobiosis levels below 20% (i.e., at very low oxygen supply rates).

Alternative effectors of ArcB activity under microaerobiosis conditions. Matsubara and Mizuno (27) have identified a phosphatase (SixA) that selectively dephosphorylates ArcB-P. One possible explanation for the decreased ArcB-dependent activation of the cydA-176-lacZ reporter in the range from 0 to 80% aerobiosis is SixA-mediated dephosphorylation of ArcB. Therefore, the ArcA−P reporter system was inserted by P1 phage transduction into strain JW2337, which lacks sixA, and strain BW25113 (the corresponding wild-type strain), resulting in strains JA029 and JA001, respectively. These two strains were grown in glucose-limited chemostat cultures at a constant growth rate (dilution rate, 0.15 h−1) with variable but controlled rates of oxygen input through the entire aerobicity range. No phenotypic differences were observed between these two strains with respect to cydA-176-lacZ expression (see Fig. S4 in the supplemental material), and therefore the possibility of a role for SixA in the regulation of ArcB activity under these aerobicity conditions was excluded.

Catabolic intermediates, such as pyruvate, acetate, and lactate, have been shown in vitro to modulate ArcBA activity (20, 34). The experimental setup used to vary the degree of aerobicity allows calculation of metabolic fluxes. The acetate flux showed a linear decrease toward aerobicity, as shown previously by Alexeeva (1). Furthermore, fluxes toward lactate and pyruvate were virtually absent (data not shown). We therefore concluded that these metabolites do not affect ArcB activation under these aerobicity conditions.

Due to the low maximal growth rate (<0.2 h−1) of strain JA022, no comparative study in chemostat cultures with different oxygen availabilities could be carried out. To assess the role of the demethylmenaquinones in the regulation of ArcB activation, the cellular menaquinone and demethylmenaquinone contents were determined (Fig. 6). Unfortunately, we could not isolate these menaquinone species in their reduced forms due to (auto)oxidation during sample processing (data not shown). The cellular menaquinone content increased substantially between 100% and 80% aerobiosis and then increased only slightly further toward anaerobiosis. The demethylmenaquinone pool exhibited a similar pattern in the high-aerobiosis region, but there was a gradual decrease toward anaerobiosis. Therefore, the aerobiosis ranges in which there was increasing ArcB activation (i.e., from 100 to 80% and from 20 to 0%) correlate with an increase in the size of the menaquinone pool.

DISCUSSION

The regulatory signals that activate and the mechanisms that underlie the function of the ArcBA two-component system have been the subject of numerous studies (17, 19–21, 24, 26, 27, 34). ArcBA is at the core of the catabolic network of E. coli, and hence a detailed understanding of its role should give us valuable insight into the regulation of energy conservation in bacteria. This not only is of fundamental scientific importance but also is essential to a range of biotechnological applications for obvious reasons.

The results presented here show that the ArcB regulation system is not a straightforward linear system and also show that there is a nonlinear response system with regard to oxygen availability. Indisputably, the redox state of the ubiquinone pool and/or the concentration of ubiquinone per se (11, 12, 26) plays an important role in transducing environmental signals and may be a key factor in the regulatory network that governs catabolism. However, the complexity of the regulatory network is illustrated by the fact that no correlation was found between ArcB activation and oxygen availability (Fig. 5). If a single redox-active compound regulates ArcB activation, a sigmoidal relationship with the degree of aerobiosis would be expected. This study used steady-state growth conditions with different, but controlled, changes in the rate of oxygen supply. This facilitated description of these conditions in terms of redox state, the size of the ubiquinone pool, and the total size of the menaquinone pools and allowed correlation of the information obtained with ArcB kinase activity by use of a quantifiable reporter system. Here we provide evidence that our reporter system does indeed function according to its design and monitors the degree of ArcB activation. This allowed us to conduct a more detailed in vivo analysis of this process than has been reported previously (26, 34). Unfortunately, we have not successfully resolved the relative concentrations of the oxidized and reduced forms of menaquinone. Unidentified components presumably cause a high and variable rate of (auto)oxidation of the menaquinols, even when the cell extracts are acidified. The considerably more negative midpoint potential of menaquinone than of ubiquinone (~80 mV versus 110 mV [40]) may contribute to this sensitivity to autoxidation. It is relevant to note in this respect that Grammel and Ghosh (15) recently described an analysis of the redox state of the two quinone pools of Rhodospirillum rubrum (i.e., rhodoquinone and ubiquinone). These workers were unable to detect significant amounts of rhodoquinol in vivo. Rhodoquinol has a midpoint potential that is close to that of menaquinol (i.e., −65 mV [29]). We noted, however, that the decreasing rate of succinate
production (1) at the lowest levels of aerobiosis is consistent with the assumption that oxidized menaquinone is formed at the lowest rate of oxygen supply (i.e., 20% aerobiosis).

The observation made with both batch and chemostat cultures that significant variation in the ubiquinol/ubiquinone ratio is not reflected in alteration of ArcB kinase activity therefore justifies the conclusion that this protein histidine kinase must be under control of an additional factor.

Previously, we have presented evidence indicating that the ArcBA system is a subtle microaerobic sensory and regulatory system (3) rather than an aerobic-anaerobic detection device. We argued that such a system is needed, given the fact that key enzymes of the fermentative and respiratory modes of energy conservation may be inactivated by the presence of traces of oxygen and under highly reducing conditions, respectively. Consequently, our physiological analyses prompted us to suggest that the current model of ArcB regulation is an oversimplification and that a more complex regulatory system is expected to operate. Differential regulation of the SixA system (with respect to oxygen availability) would be an elegant means to fine-tune ArcB kinase activity. However, our results show that this is not the case (i.e., no difference in expression of cydA-lacZ was observed between strain JA029 [sixA] and JA001 [wild type]).

Previous work (1) has shown that the steady-state cellular concentration of NADH increases substantially in both the upper (100 to 80%) and lower (20 to 0%) aerobiosis ranges, with a twofold increase at both transitions. This NADH pattern coincides with the changes in the cydA-lacZ expression in the same aerobiosis ranges. These data therefore do not exclude the interpretation that NADH functions as an additional signal input. The lack of a high level of anaerobic cydA-lacZ expression in a mutant deficient in menaquinone biosynthesis does indicate, however, that NADH does not function as an activation signal as such, since the concentration of NADH in such a mutant is expected to even increase under anaerobic conditions compared to the concentration in the wild-type strain. Rather, alternative electron acceptors, like fumarate, may lower the level of ArcB activation under these conditions.

Demethylmenaquinone was not tested here or by other workers (11) as an in vitro regulatory signal for ArcB autophosphorylation activity due to the lack of commercially available dimethylmenaquinone. Georgellis et al. (11) showed that both ubiquinone (UQ0) and menadione (MK3) influence the in vitro phosphorylation rate of the ArcB kinase. However, the concentrations required for half-maximal inhibition are higher for menadione (50 μM) than for ubiquinone (5 μM). Strikingly, these authors observed that ubiquinol and menadiol did not activate ArcB autophosphorylation. Such an effect, however, may have been masked by isolation of the ArcB kinase in its activated form. The fact that the midpoint potential of the menaquinones is significantly more negative than that of ubiquinones (see above) makes it likely that in the range of aerobiosis, moving from anaerobic conditions, the menaquinone–H2/menaquinone ratio decreases significantly before the same transition occurs for the ubiquinone couple. One possibility is that this menaquinone transition takes place between 0 and 20% aerobiosis. The resulting increase in menaquinone concentration may then explain the corresponding decrease in ArcB activation. The increase in ArcB activation between 20 and 80%
aerobiosis (as well as the decrease between 80 and 100% aerobiosis) correlates with the cellular concentration of ubiquinol (while simultaneously the concentration of the menaquinone pool decreases), suggesting that in the higher aerobiosis ranges it is the ubiquinol couple that governs the activation level of ArcB. This complex mode of regulation of ArcB is supported by the relative affinities of ArcB for menaquinones and ubiquinones, which is higher for the ubiquinones in vitro (11). This working hypothesis is shown in Fig. 7, and it extends the view that the ubiquinone-ubiquinol couple interacts with the PAS domain of ArcB (26) to include an interaction of this domain with the menaquinone-menaquinol couple. Accordingly, combined regulation by the redox state of the ubiquinone pool and menaquinone pool provides a consistent hypothesis to explain the observed complex regulation of ArcB activation at variable rates of oxygen supply. Menaquinones would then be the dominant activators under anaerobic conditions, where the size of the ubiquinone pool is approximately five times less than the size of the menaquinone pool. Given the fact that menaquinones are involved in the transfer of electrons to alternative acceptors (7, 16, 31, 33) and the fact that the cell maintains a subtle balance between aerobic respiration, anaerobic respiration, and fermentation, this work shows that ArcB can be viewed as a master regulator that governs both anaerobic and aerobic respiration. In this respect, the system should be considered a redox-sensing system rather than an oxygen-sensing system.

REFERENCES


