Respiratory electron transfer in Escherichia coli: components, energetics and regulation

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The ArcBA two-component system of *Escherichia coli* is regulated by the redox state of both the ubiquinone and the menaquinone pool

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This chapter is under review
Summary

ArcBA is the major two component regulatory system of *Escherichia coli* involved in sensing oxygen availability and the concomitant regulation of oxidative and fermentative catabolism. Based on in vitro data, it has been postulated that the redox state of the ubiquinone pool is the major determinant for ArcB kinase activity. Here we report on the in vivo regulation of ArcB activation, using a *lacZ* reporter specifically responsive to phosphorylated ArcA. This reporter indicates that upon deletion of a ubiquinone biosynthetic enzyme, regulation of ArcB in the anaerobic/aerobic transition is not affected. In contrast, interference with menaquinone biosynthesis does lead to inactivation of ArcB during anaerobic growth, and this phenotype can be rescued by addition of a menaquinone precursor. Therefore, the menaquinones also contribute to ArcB activation.

ArcB shows a complex pattern of regulation when *E. coli* is titrated through the entire aerobiosis range: ArcB is activated under anaerobic and sub-aerobic conditions and much less active under fully aerobic and in micro-aerobic conditions. Furthermore, no unique correlation exists between ArcB activation and the redox state of the ubiquinone pool, but a restricted correlation is observed between the total cellular ubiquinone content and ArcB activity, because of the considerable increase of the ubiquinone pool size with increasing degrees of aerobiosis. These results lead to the working hypothesis that the redox state of both quinones, i.e. ubiquinone and menaquinone, in *E. coli* modulates the activity of ArcB in vivo.

Introduction

Two-component systems are employed by prokaryotes to allow them to respond to changing environmental and intracellular conditions. The ArcBA (anoxic redox control) system is the major two component system in *E. coli* and functions as the aerobiosis sensing device to tune catabolic pathways to variations in oxygen availability. Its first component, ArcB, functions as the sensor that relays a signal via signal-dependent kinase activity to the second component, the response regulator ArcA (75, 79). Upon signal perception ArcB is phosphorylated at the conserved His-292 residue. Subsequently the phosphoryl group is transferred intramolecularly to the conserved Asp-576 to be transferred once more intramolecularly to His-717 in the phosphoryl transfer domain. Then the latter transfers the phosphoryl group to ArcA (56, 103). 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 conditions of growth (5, 61, 114). Thus, the ArcBA system is paramount in the organism’s ability to distribute energy generation over fermentation and respiration.

ArcB can form dimers via two disulfide bonds, involving Cysteine-180 and -241, which are located in the PAS domain of the protein. Kinase activity of ArcB is highly dependent on this dimerization: A disulfide bond formed between the two Cys-180 residues results in a 85% reduction of kinase activity, and a bond between the two Cys-241 residues in a 15% reduction (120). It has been shown in vitro that the cysteine residues can be oxidized by ubiquinone (120) and hence this latter redox carrier was postulated to regulate the kinase activity of ArcB. Consistently, maximal kinase activity of ArcB towards ArcA has been shown to occur under anaerobic conditions (117); nevertheless it has also been suggested that significant levels of ArcA~P are present in aerobic cells (76, 77).

In this study, a PcydA-176+1-lacZ reporter is used that is not responsive to FNR, but retains dependence on ArcA~P (2), to characterize the factors that are involved in the in vivo regulation of ArcB activation in relation to (decreasing) oxygen availability (2). Our findings indicate that in addition to the regulation by the ubiquinone pool, the menaquinone pool also plays an important role in ArcB activation.

The ArcBA system exhibits maximal activity under slightly sub-aerobic- and fully anaerobic conditions and much lower activity under micro-aerobic- and fully aerobic conditions. Furthermore, regulation by none of the catabolites known so far to affect ArcB activity in vitro (D-lactate, acetate and pyruvate), nor of the ArcB specific phosphatase SixA could be detected in vivo (56, 75, 77). Here we show that additional regulation of ArcB kinase activity by the redox state of the menaquinone pool is prevalent under micro-aerobic to anaerobic conditions.

Based on these observations we conclude that regulation of the ArcBA system in vivo is a multi-factorial process, more complicated than presumed so far on the basis of in vitro data that suggested a dominant role for the redox state of the ubiquinone pool.
Methods

Strains and plasmids used in this study.
To create pSal1, a 176-bp DNA fragment from the upstream region (Fig. 1a) of the cydAB operon (corresponding to bases 512 to 687 in GenBank entry J03939; bases +1 to −176 relative to the start site of cydAB P1 transcription) was amplified by PCR with primers Cyd(E)-174 (5’ T ATG AAT TCT TTT TAT CTT TAA TTG CCA ACC G) and Cyd(Bam)+1 (5’ ATA GGA TCC CGA GAA CAA TTT ATC TCT TTT TGA TGC C), using *E. coli* chromosomal DNA from strain MC4100 as a template. The PCR product was digested with BamHI and EcoRI and cloned in the corresponding sites of pQE30.
To create pSal2, PCR-directed (QuikChange Site-directed mutagenesis kit, Stratagene) mutagenesis using the primers cydmut1 (5’ CAT AAT TTG TAG GAA ATT AAT TTT AAC AAT GTA TAA GTC TTG G) and cydmut2 (5’ CCA AGA CTT ATA CAT TGT TAA AAT TTT CCT ACA AAT TAT G) was performed, exactly according to the manufacturer’s instructions. Plasmid pSal1 was used as a template, which resulted in point mutations in the essential base pairs of the consensus sequence of the FNR-binding site (GGAATTGATATTTATCAATGTA to GGAAATTAATTTTAACAATGTA). Through these mutations a VspI restriction site was introduced. Successful mutagenesis was confirmed by restriction analysis and sequencing using the semi-automated DNA sequencing technique.
To construct the operon fusions, the 176-bp EcoRI-BamHI fragment of plasmid pSal2 was ligated into EcoRI-BamHI-digested *lacZ* operon fusion vector pRS551 (Simons et al., 1987), resulting in pRSS2. The fusion (Fig. 1b) was then transferred to the λ transducing phage λRS45 (the MC1061 strain, bearing pRSS2 was infected with λRS45) as described earlier (Sawers & Böck, 1988), yielding λRSS2. Lysate containing λRSS2 was used to lysogenise strains MC4100, BW25113, JW5713, JW2237, JW5536, RM101 and RM3133, yielding ASA12, JA001, JA023, JA029, JA032, ASA22 and ASA32, respectively.

Measurement of enzyme activity.
β-Galactosidase activity was measured in permeabilised cells as originally described by Miller (1972) and modified by Giacomini et al (1992).
Overproduction and purification of His6-ArcA. For overproduction of His6-ArcA, 500 ml cultures of *E. coli* strain BL21 transformed with pETArcA-1 were grown in 2 l conical flasks with vigorous shaking (200 rpm) on a rotary shaker at 37°C in PB medium (20 g l⁻¹ tryptone, 10 g l⁻¹ yeast extract, 5 g l⁻¹ glucose, 5 g l⁻¹ NaCl and 8.7 g l⁻¹ K2HPO4, pH 7). Kanamycin was routinely
included at a final concentration of 50 μg×ml⁻¹ for plasmid maintenance. When the culture attained an OD₆₀₀₀nm of approximately 0.4, induction of arcA expression was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM. After growth for 2.5 hours, the cells were harvested by centrifugation 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 (0.5M NaCl, 20mM Tris-HCl, pH 7.9), and 1.3 mg×ml⁻¹ lysozyme and 30 μg×ml⁻¹ DNase and RNase were added. After 30 min 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. Phenylmethylsulphonyl 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 (Qiagen), equilibrated with buffer A. After washing the column with 10 ml of 10 mM imidazole (in buffer A, see above), the protein was eluted with 50 mM imidazole (in buffer A) and collected in five 2 ml fractions. These fractions were immediately dialysed against 50 mM Tris-HCl, pH 7.5, 0.1 mM DTT and 0.1 mM EDTA. After dialysis glycerol was added to a final concentration of 10% (v/v) and the fractions were analysed on SDS-PAGE. Immediate dialysis, addition of 0.1 mM DTT, plus 0.1 mM EDTA to the dialysis buffer and addition of 10% (w/v) glycerol after dialysis was essential to prevent precipitation of ArcA after storage at –20°C and thawing. Protein concentrations were measured by the microbiuret method (Goa, 1953), after precipitation with trichloroacetic acid (Bensadoun & Weinstein, 1976). Bovine serum albumin was used as a standard.

In vitro phosphorylation of His6-ArcA.
His6-ArcA protein was phosphorylated by incubating the protein in TEGD buffer (50 mM Tris-HCl, pH 7.5, 0.5mM EDTA and 10% glycerol) to which MgCl₂ was added to 5 mM and carbamoyl phosphate to a final concentration of 50 mM, as described by Drapal & Sawers (1995). The mixtures were incubated for 90 min at 25°C and the phosphorylated ArcA was used immediately for DNA-binding assays. For the purpose of calculations it was assumed that this procedure yielded 100% phosphorylation of the protein. In parallel, similar incubations lacking carbamoyl phosphate were used for DNA-binding reactions with unphosphorylated protein.

Preparation of radioactively labelled DNA fragments. Plasmid pSal2 (see: Construction of plasmids and strains) contains a 121 bp EcoRI-VspI fragment encoding the ArcA binding site II of the cydAB promoter region (Lynch & Lin, 1996a; Cotter et al., 1997) from position –59 to –175, relative to the start
of *cydAB* P1 (see Fig. 1a), which also corresponds to bases 512 to 628 in the GenBank entry J03939. EcoRI-VspI-digested pSal2 was separated on a 12.5% (w/v) polyacrylamide gel. The 121 bp DNA fragment was eluted overnight at 65°C (1 mM sodium acetate, 10 mM magnesium acetate, 0.5 mM EDTA pH 8.0, 0.1% (w/v) SDS) and recovered by ethanol precipitation in the presence of 10 mM MgCl$_2$ and 0.3 M sodium acetate. DNA concentrations were determined spectrophotometrically. End-labelling of the fragment was performed with the Klenow fragment of *E. coli* DNA polymerase and [$\gamma$-32P] dATP (3000 Ci/mmol; Amersham) in OPA buffer (10 mM Tris-acetate pH 7.5, 10 mM MgCl$_2$ and 50 mM potassium acetate). The labelled DNA fragments were not separated from unincorporated nucleotides but used directly in mobility shift experiments.

**Gel retardation assays.**
The labelled 121 bp DNA fragment was used in all retardation assays at a concentration of 0.32 nM. Protein samples were combined with 32P end-labelled DNA substrates in 10 μl reaction volumes containing 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% (w/v) glycerol, 300 μg/ml BSA and 1 μg poly(dI-dC)-poly(dI-dC) (Sigma). After incubation for 15 min at 30°C, loading buffer (50% (w/v) glycerol, 0.2% (w/v) bromphenol blue and 0.2% (w/v) xylene cyanol) was added to 10% (w/v) of the final volume and the mixture was directly applied to the gel using PhastSystemTM sample applicators (Pharmacia).

Gel retardation assays were performed essentially as described by (Ramanujam et al., 1990) on 8-25% (w/v) gradient polyacrylamide gels (Pharmacia), using the PhastSystemTM at 4°C, applying the following separation method:

<table>
<thead>
<tr>
<th>Sample appl. down at</th>
<th>1.2</th>
<th>0 Vh</th>
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<tr>
<td>Sample appl. up at</td>
<td>1.2</td>
<td>2 Vh</td>
</tr>
<tr>
<td>Sep. 1.1</td>
<td>400 V</td>
<td>10 mA</td>
</tr>
<tr>
<td>Sep. 1.2</td>
<td>400 V</td>
<td>1 mA</td>
</tr>
<tr>
<td>Sep. 1.3</td>
<td>400 V</td>
<td>1 mA</td>
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After completion of the run, the lower part of the gel, containing unincorporated [$\gamma$-32P] dATP was removed to prevent the signal of these nucleotides from interfering with the signal from the DNA fragments. The results were visualised 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).
Western blotting.
A rabbit anti-ArcA polyclonal antiserum was produced for this study by immunisation of a rabbit with highly purified His6-ArcA protein. The antiserum was checked for cross-reactions and its titre was determined by Western blot analysis using cell free extracts of the MC4100 and RM3133 (\DeltaarcA) E. coli strains. Equal amounts of cell-free extract (20 μg total protein per lane) resolved on 12.5% (w/v) SDS-PAGE were blotted onto nitrocellulose in a Trans-Blot semi-dry cell (Bio-Rad) and subsequently immuno-labelled according to (Towbin et al., 1979). The rabbit anti-ArcA antibody was used at a dilution of 1:10000. The secondary antibody, horseradish peroxidase-conjugated goat-anti-rabbit IgG (Bio-Rad), was used at a dilution of 1:3000 for subsequent visualisation by a colour reaction. Amounts of ArcA (with the purified ArcA protein used as a reference) were quantified using densitometric analysis (Image Master 1D prime, version 2.0, Pharmacia Biotech).

Continuous cultures.
Cells were grown in Applicon type Fermentors (7) at a dilution rate (D) of 0.15 ± 0.01 h⁻¹ under glucose-limited conditions. A simple salts medium, as described by Evans et al.(48) was used, but instead of citrate, nitriloacetic acid (2 mM) was used as chelator. Selenite (30 μg/l) and thiamine (15 mg/l) were added to the medium. Glucose was used as the single carbon- and energy source at 45 mM final concentration in the feed. pH was maintained at 7.0 ± 0.1 by titrating with sterile 4 M NaOH and the temperature was set to 35°C. The oxygen supply was varied as described previously (5). In addition to β-galactosidase activities, in all cultures the steady state specific rates of fermentation product formation, glucose and O₂ consumption were measured as described in Alexeeva et al.(5) to determine the percentage of aerobiosis. In previous experiments the chemostat cultures were calibrated to obtain quantification of oxygen availability (3). Essentially, the 0 % value of aerobiosis reflects fully anaerobic conditions and 100% equals the minimal oxygen input rate required for completely aerobic catabolism (see Alexeeva et al.(3) for a quantitative definition of microaerobiosis).

Batch cultures.
In batch cultures, the composition of the medium was similar to that 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 (added to 1% w/v final concentration) was sterilised separately. High aeration of cultures during aerobic growth was accomplished by shaking 10 ml culture volumes in 100 ml Erlenmeyer flasks at 180 RPM. For anaerobic
growth 15 ml culture volumes in sealed Greiner tubes (15 ml) were used. Cells were inoculated from cultures pre-grown under aerobic- or anaerobic conditions (after dilution to OD600 = ~5*10^-3) and allowed to double ~7 times to mid-log exponential phase (final OD600 = 0.4 - 0.6 and OD600 = 0.8 – 1.2 for anaerobic- and aerobic conditions, respectively) prior to β-galactosidase enzyme activity measurement. Batch cultures for the comparison of the redox state of the ubiquinone pool with the cydA-176+1-lacZ expression level (see Fig. 2) were grown in a volume of 100 ml, in a 1 liter Erlenmeyer at a stirring rate of 250 RPM. Other conditions were similar to those described above. The strains were maintained in vials in LB medium with 30% (w/v) glycerol at -70°C.

**Analysis of carbon fluxes**
Steady state bacterial dry weight was measured as described previously (3). Glucose, pyruvate, lactate, formate, acetate, succinate and ethanol were determined by HPLC (LKB) with a REZEX organic acid analysis column (Phenomenex) at a temperature of 45°C with 7.2 mM H₂SO₄ as eluent, using a RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration. All data present a carbon balance of >92% (mean 96%) as calculated from the glucose consumption- and product formation rates.

**Quinone extractions.**
2 ml samples from a culture were quenched with 6 ml of ice-cold 0.2 M HClO₄ in methanol or methanol only. Next, 6 ml of petroleum ether was added rapidly to the mixture (boiling point 40-60 °C) and it 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 gradient pump 2249 system with a LKB 2151 Variable wavelength monitor) using a reversed phase Lichrosorb (Chrompack, Bergen op Zoom, The Netherlands) 10 RP 18 column (size 4.6 mm, internal diameter, 250 mm). The column was equilibrated with ethanol:methanol (1:1, v/v) or pure methanol (HPLC grade) and this mixture was used as the mobile phase. The flow rate was set at 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 and menaquinone-4 as standards,
according to the method applied by Shestapolov et al (167). Methanol, ethanol, and petroleum ether were of analytical grade. Peaks were identified by UV/Vis and mass spectral analysis. A UV/Vis spectrum of DMK8 was kindly provided by Dr Bogachev (Moscow University, Russia). For mass spectral analysis fractions collected from the HPLC were evaporated under nitrogen and re-dissolved into 90% (w/v) acetonitrile, 1% (v/v) formic acid (LC-Grade, Merck, Frankfurt, Germany). Then fractions were analysed by off-line electrospray-mass spectrometry using coated Picotips [Econo12] (New Objective, Woburn USA) on an ESI QTOF mass spectrometer (Micromass, Waters, Manchester UK). Ions selected for MSMS collided with Argon in the hexapole collision cell.

**Results**

In order to construct an ArcA~P-dependent reporter system that is exclusively responsive to this regulator, we amplified a 176 bp DNA fragment from the cydAB regulatory region of *E. coli*. This DNA fragment contains an ArcA-binding site (site II), an FNR-binding site and the cydAB promoter P1 (40). The FNR-binding site within this regulatory region was subsequently selectively inactivated by site-directed mutagenesis (see Fig. 1 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 + *cydA*~176+1-*lacZ*) and strain ASA22 (*Δfnr* + *cydA*~176+1-*lacZ*, see Methods) were grown in anaerobic and aerobic batch conditions, respectively. The expression of the reporter was found to be two-fold higher in anaerobic conditions versus aerobic conditions for both strains (data not shown). In contrast, the change in expression of the reporter in strain ASA11, that contains the reporter with the FNR binding site, was less than 1.5 fold (data not shown).

The interaction of the ArcA protein with this mutated *cydAB* regulatory region was quantified in vitro by gel retardation assays. Relative binding affinities of ArcA and ArcA~P were determined using 0.32 nM concentrations of a [32P] end-labelled 121-bp EcoRI-VspI fragment, derived from pSal2 (see Figs. 1 and 2A). ArcA was phosphorylated through incubation with carbamoyl phosphate. A single retarded complex was observed in 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 Kd(a) (ArcA-P concentration at which half of the amount of the DNA fragment is bound, designated KArcA~P(0.5)) was estimated to be approximately 0.25 μM, whereas for ArcA (KArcA(0.5)) the corresponding value is 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 according to Keleti (89) and Segel (166), i.e. by fitting a double reciprocal plot of 1/Y against 1/[S] (with Y as the fraction of bound DNA and [S] as the ArcA(~P) concentration) to the equation:

\[ Y = \frac{Y_{\text{max}} [S]^n}{K_M + [S]^n} \]  

provides the best fits (R2) with a numerical value of 2 for ArcA and 6 for ArcA~P (Fig. 2C), strongly suggesting that ArcA and ArcA~P bind to the DNA fragment with different stoichiometry. The Km values for binding, derived from the double reciprocal plot in coordinates \{1/Y; 1/[S]^n\}, with n = 2 for ArcA and n = 6 for ArcA~P, were 2.44 μM and 0.26 μM, respectively. Comparable stoichiometries (n=3 for ArcA, n=6 for ArcA~P) and Km values (4.3 μM for ArcA and 0.27 μM for ArcA~P) were obtained by analysis of the data in a Hill-plot (not shown). Taken together, these results suggest that ArcA~P binds to this DNA fragment as a hexamer, which is in close accordance with the observations of Jeon et al. (82).

Only when ArcA was phosphorylated a sequence-specific interaction with the DNA fragment containing the ArcA-binding site II of the cydAB regulatory region was observed. This was concluded from the absence of an effect of addition of a molar excess of competing fragments with a random sequence.
Fig. 2. Concentration dependence of binding of ArcA and phosphorylated ArcA (ArcA~P) to the DNA fragment containing the ArcA-binding site II (from position –59 to –175, relative to the start of cydAB P1). A) Radiolabelled DNA fragment (0.32 nM) was incubated with increasing amounts of either phosphorylated or unphosphorylated ArcA. Protein concentrations are indicated above each lane. B) Graphic representation of quantitative evaluation of the results of the gel retardation assays, shown in Fig 2A. Each point represents the mean of 4 to 8 independent experiments. C) Doubly reciprocal plot for binding of ArcA and ArcA~P to the DNA fragment with n=2 for ArcA and n=6 for ArcA~P.

Addition of a 50-fold molar excess of a competitor DNA [poly(dI-dC)-poly(dI-dC) or pQE30] in the binding assay with non-phosphorylated ArcA did prevent sequence specific complex formation. These competing fragments had no observable effect when phosphorylated 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 & Lin (117). The specific ArcA~P-dependent-lacZ reporter construct (see (2) and above) was transduced with phage P1 to strain JW5536 (8), lacking the ArcB histidine kinase, and JW5713 (ΔubiC) generating strains JA032 and JA023, respectively.
Fig. 3. Activity of the ArcA~P-dependent-$\lambda$-lacZ reporter construct ($\lambda$.RSS2). The construct was tested in the wild-type (JA001), ΔubiC (JA023) and ΔarcB (JA032) background after batch culture growth in mineral medium supplemented with glucose. Cultures were grown aerobically (white bars) and anaerobically (black bars).

The resulting strains were grown in batch culture in minimal medium, supplemented with glucose as the sole carbon and energy source. In the wild type derivative, JA001, cydA$^{-176+1}$-lacZ expression increased two-fold under anaerobic conditions, as compared to aerobic conditions (see Fig. 3) whereas for strain JA032, which lacks arcB, expression levels were not significantly different under anaerobic and aerobic conditions. This finding confirms that the anaerobic regulation mediated by ArcA is dependent upon the histidine kinase ArcB, and therefore presumably on ArcA~P.

Georgellis et al. (55) have reported a substantial effect of ubiquinone on the histidine kinase activity of ArcB in vitro. However, in the experiments with strain JA023, which lacks ubiC, the difference between the aerobic and anaerobic expression levels of the cydA$^{-176+1}$-lacZ reporter was similar to that of the wild type. This result is incompatible with the assumption that ubiquinone would be the sole regulator of ArcB kinase activity. Although the ubiquinol/ubiquinone ratio is expected to be significantly different under aerobic and anaerobic conditions in batch culture, the ΔubiC strain does not contain detectable amounts of quinone.
Fig. 4. Activity of the ArcA~P-dependent-lacZ reporter construct (ΔRSS2). The construct was tested in the wild-type (JA001) and the ΔmenB (JA022) background, after either growth in anaerobic batch culture in medium supplemented with (right slanted line bars) and without 2 μM naphthoic acid (black bars), or aerobically without naphthoic acid (white bars).

Previously, we showed (13) that the ubiquinone pool of *E. coli* becomes gradually more and more reduced during growth in batch culture when the OD600 reaches values higher than 1.0. One would therefore expect that an increase in *cydA176+l-lacZ* expression would parallel this increase in the [UQH2]/[UQ] ratio, if its expression is governed solely by this ratio. To test this hypothesis strain ASA12 was grown in aerobic batch culture, as described previously (13). As anticipated, a strong increase in the ubiquinol/ubiquinone ratio was observed at the later stages of growth; however, this was not paralleled by a significant increase in the level of *cydA176+l-lacZ* expression (See Supplemental Fig. 1). These combined observations indicate that it is not solely the ubiquinol/ubiquinone ratio that regulates ArcB activation.

Besides ubiquinone, addition of menadione also influenced the in vitro autophosphorylation rate of ArcB, although higher concentrations of the latter quinone were needed to reach half-maximal inhibition (50 μM, as compared to 5 μM for ubiquinone). To investigate whether (demethyl)menaquinone could have a role in the regulation of ArcB, strain JW2257 (ΔmenB) was lysogenised with the phage lysate obtained from strain ASA12, resulting in strain JA022. The inability to synthesize demethylmenaquinones resulted in the surprising phenotype that the activation response of *cydA176+l-lacZ* expression upon anaerobiosis was abolished (Fig. 4). Moreover, the addition of 2 μM
naphthoic acid, an intermediate in the menaquinone biosynthetic pathway that is synthesized downstream of the block in this pathway by deletion of menB (encoding naphthoate synthase), to anaerobic batch cultures, restored the wild-type phenotype. Independent quinone analyses verified that this addition of naphthoic acid resulted in significant restoration of the intracellular demethylmenaquinone and menaquinone pools (see Supplemental Fig. 2), but had no effect on the redox state of the ubiquinone-pool (data not shown). This clearly shows that (the) menaquinone (pool) also has a role in the regulation of ArcB activation.

Previously we have reported on a controlled aerobiosis system, based on glucose-limited chemostat cultures (see Alexeeva et al. (3) for details of the experimental set up), which allows to set steady-state cultures at any value of the ratio of aerobic catabolism over anaerobic fermentation. To monitor the degree of ArcA phosphorylation in vivo in relation to quantified and steady-state oxygen availability, strain ASA12, an MC4100 derivative which contains the same reporter system as outlined 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 – 110 % aerobiosis (see Methods). Under these conditions a rather complex pattern of cydA⁻¹⁷⁶⁺¹-lacZ expression was observed with maximum values at 0 % and 85 % of aerobiosis. These maxima were approximately 2.5 fold higher than the minimum values measured at 20 % aerobiosis and under fully aerobic conditions (Fig. 5). The expression level of the cydA⁻¹⁷⁶⁺¹-lacZ reporter under the latter two conditions was not significantly different. In contrast, in similar experiments with strain ASA32, that lacks ArcA, the level of cydA⁻¹⁷⁶⁺¹-lacZ expression increased linearly with increasing oxygen availability, with an approximately 1.5-fold increase when going from anaerobic to fully aerobic growth conditions (See supplemental Fig. 3).

In view of the in vitro results of (55, 120), the cellular content of the ubiquinol/ubiquinone pools was measured for all steady-state chemostat cultures. It was observed that, as expected, the ubiquinol/ubiquinone ratio gradually increases towards anaerobiosis (see Fig. 5), following a pattern that does not resemble that of the changes observed for cydA⁻¹⁷⁶⁺¹-lacZ expression. Furthermore, the absolute cellular content of ubiquinol plus ubiquinone showed a gradual increase with increasing oxygen availability up to 80% aerobiosis (Fig. 6), followed by a decrease from 80 to 100 % aerobiosis. Because of this, the size of the ubiquinol pool correlates with the cydA⁻¹⁷⁶⁺¹-lacZ expression throughout the range of 20 to 100 % aerobiosis, but not in the aerobiosis range below that, i.e. with very small oxygen supply rates.
Mizuno and colleagues (124) have identified a phosphatase (SixA) that selectively dephosphorylates ArcB-P. One possible explanation for the decreased ArcB-dependent activation of the cydA<sup>-176+1</sup>-lacZ reporter in the region from 0 to 80 % aerobiosis might be the SixA-mediated dephosphorylation of ArcB. Therefore, the ArcA~P reporter system was inserted by P1 phage transduction into strains JW2337, lacking sixA, and strain BW25113 (the corresponding wild-type), resulting in strains JA029 and JA001, respectively. These two strains were grown in glucose-limited chemostat culture at a constant growth rate (D = 0.15 h<sup>-1</sup>) with variable, but controlled, oxygen-input rates, through the entire aerobiosis range. No phenotypic differences were observed between these two strains with respect to cydA<sup>-176+1</sup>-lacZ expression (see Supplemental Fig. 4) and a possible role for SixA in the regulation of ArcB activity between different aerobiosis conditions therefore was excluded. Catabolic intermediates, such as pyruvate, acetate and lactate, have been shown in vitro to modulate ArcBA activity (79, 157). The experimental set-up used varied the degree of aerobiosis allowing for the calculation of metabolic fluxes. The acetate flux showed a linear decrease towards aerobiosis, as was shown before by Alexeeva et al. (2). Furthermore, fluxes towards lactate and pyruvate were virtually absent (data not shown). We therefore conclude that these metabolites do not affect ArcB activation under our aerobiosis conditions.

Due to its low maximal growthrate (<0.2 h<sup>-1</sup>) no steady state growth of strain JA022 could be achieved in the above described micro-aerobiosis conditions. To assess the role of the (demethyl)menaquinones in the regulation of ArcB activation the content of menaquinone and the demethylmenaquinone pool was determined (Fig. 6). Unfortunately, we could not isolate these menaquinone species in their reduced form, due to (auto)oxidation during the sample processing (data not shown). The cellular menaquinone content increases strongly between 100% to 80% aerobiosis and then further increases only slightly towards anaerobiosis. The demethylmenaquinone pool follows a similar pattern in the high aerobiosis region, but shows a gradual decrease towards anaerobiosis. Therefore, for the menaquinones, aerobiosis ranges with increasing ArcB activation (i.e. from 100 to 80 and from 20 to 0 %) correlate with increasing menaquinone pool size.
Fig. 5. Activity of the ArcA~P-dependent-lacZ reporter construct (ASA12, filled squares) and the redox state of the UQ₈ pool (open circles) during various aerobiosis conditions. The construct was tested in the wild-type ASA12 background after glucose-limited continuous growth in mineral medium supplemented with varying amounts of oxygen.

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 (75, 77, 79, 82, 114, 120, 124, 157). ArcBA is at the core of the catabolic network of *E. coli* and hence a detailed understanding of its role will give us insight into the regulation of energy conservation in the living bacterial cell. This is not only of fundamental scientific importance but is essential to a range of biotechnological applications, for obvious reasons. The current view on ArcB regulation includes a principal role for the redox state of the ubiquinone pool, and/or the concentration of ubiquinone per se (see (55, 56, 120)). Indisputably, this cellular parameter plays a role in transducing environmental signals and may constitute a deciding feature in the regulatory network that governs catabolism. However, the results presented here illustrate that in the living cell not a straightforward, linear, regulatory system is at work.
but rather a multi-factorial, non-linear response system with regard to the oxygen availability and energy conservation occurs. This can be concluded from the assessment that if a single redox-active compound would regulate ArcB activation, a sigmoidal, or at least a first-order kinetic, relationship to the degree of aerobiosis would be expected. The actual relationship observed (Fig. 5) is more complex.

In this study use has been made of steady state growth conditions with varying, but controlled, changes in the rate of oxygen supply. This has made it possible to describe these conditions in terms of redox state and content of ubiquinone pool and the content of the two menaquinone pools, and relate this information to ArcB kinase activity via the use of the quantifiable reporter system. We provide evidence that our reporter system does function according to its design, and monitors the degree of ArcB activation. This has allowed us to carry out a more detailed in vivo analysis than has been reported previously (120, 157). Unfortunately, we have not succeeded in resolving the relative concentrations...

Fig. 6. Content of the 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 in mineral medium supplemented with varying amounts of oxygen. Values represent single experiments.
of the oxidized and reduced components of the two menaquinone pools. Unidentified components from the growth medium of the cells presumably cause a high and variable rate of (auto)oxidation of the reduced menaquinones, even when the extracts are acidified. The considerably more negative midpoint potential of menaquinone as compared to ubiquinone (-80 mV versus + 110 mV (188)) may contribute to menaquinone’s sensitivity towards auto-oxidation. It is relevant to note in this respect that Ghosh and co-workers (62) recently reported on the analysis of the redox state of both the rhodoquinone and the ubiquinone pool of Rhodospirillum rubrum. They were also unable to detect significant amounts of rhodoquinol in vivo. We note, however, that the observation of a decreasing rate of succinate production (2) already at the lowest degrees of aerobiosis is consistent with the assumption that oxidized menaquinone is formed already at the lowest rate of oxygen supply (i.e. <20% aerobiosis).

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

Previously, we have presented evidence that the ArcBA system is a subtle micro-aerobic sensory and regulatory system (5), rather than an aerobic/anaerobic detection device, and argued that such a system is needed, given the fact that key enzymes of the fermentative and the respiratory mode of energy conservation may be incompatible with the presence of traces of oxygen and with highly reducing conditions, respectively. Consequently, our physiological analyses prompt us to suggest that the current model of ArcB regulation is an oversimplification and that a more complex regulatory system is expected to be operative. Differential (with respect to oxygen availability) regulation of the SixA system would be an elegant means to achieve fine-tuning of ArcB kinase activity. However, our results show this not to be the case.

Our previous work (2) has shown that the steady-state cellular concentration of NADH shows a strong increase both in the upper (100 to 80 %) and in the lower (20 to 0 %) aerobiosis range, with a two-fold increase at both transitions. This NADH pattern coincides with the changes in the cydA<sup>176+1</sup>-lacZ expression in the same aerobiosis ranges. These data therefore do not exclude the interpretation that NADH would function as an additional signal input. The lack of high anaerobic cydA<sup>176+1</sup>-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 mutants is expected even to increase under anaerobic conditions as compared to the wild-type strain.
Demethylmenaquinone has not been tested here, nor by others (55), as an in vitro inhibiting or activating signal for ArcB autophosphorylation activity, due to the lack of commercially available demethylmenaquinone. Georgellis et al. (55) showed that both ubiquinone (UQ$_0$) and menadione (MK$_3$) influence the in vitro phosphorylation rate of the ArcB kinase. However, concentrations required for half-maximal inhibition are higher for menadione (50 μM) than for ubiquinone (5 μM). Strikingly, they 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 already 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, coming from anaerobic conditions, the MK-H2/MK ratio will decrease significantly, before this same transition occurs in the ubiquinone couple. One possibility would be that this menaquinone transition happens 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%) 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 mena- and ubiquinones, which is higher for the ubiquinones in vitro (55). This working hypothesis is presented in Fig. 7.

This model extends the view that the ubiquinone-ubiquinol couple interacts with the PAS domain of ArcB (120) to include an interaction of this domain also with the menaquinone-menaquinol couple. Accordingly, combined regulation of 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. Menaquinols would then be the dominant activators anaerobically, where the content of the ubiquinone pool is approximately five times lower than that of the menaquinones. Given the fact that menaquinones are involved in the transfer of electrons to alternative acceptors (24, 73, 150, 155), and 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 the total cellular respiratory capacity.
Fig 7. A simplified model for modulation of ArcB activity by the degree of aerobiosis. Upon a shift from anaerobic to low aerobiosis conditions of growth the menaquinone pool oxidizes rapidly resulting in an inactive ArcB kinase. Too little ubiquinol is present to prevent binding of the oxidized form of ubiquinone. A further increase in aerobiosis to sub-aerobic (80%) conditions results in an increase of the total ubiquinone pool and therefore of ubiquinol allowing for binding of ubiquinol that thereby brings back the cysteine in the reduced form. In complete aerobic conditions the content of the quinone pool decreases, which then results in oxidation of the key cysteines and in inactivation of ArcB. Adapted from Malpica et al (120).

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Supplemental Fig. 1. Absence of correlation between activity of the ArcA-P-dependent-lacZ reporter construct (open squares) in the wild-type strain ASA12 and the redox state of the UQ₈ pool (filled diamonds) during growth in batch culture in mineral medium supplemented with glucose under aerobic conditions.

Supplemental Fig. 2. Quinone content in ΔmenA strain (JA022) grown in Evan’s medium without (black bars) or with (grey bars) 2 μM naphthoic acid.
**Supplemental Fig. 3A.** Activity of the ArcA~p-dependent-*lacZ* reporter construct (ASA32, open triangles) during various aerobiosis conditions. The construct was tested in an arcA-deletion strain during glucose-limited continuous growth in mineral medium supplemented with varying amounts of oxygen (MU is Miller Units).

**Supplemental Fig. 3B.** Western blot analysis of the ArcA content in extracts from ASA12 cells, grown at various oxygen availabilities. A negative control (extract of ΔarcA strain) and a positive control (0.05 mg His6-ArcA) are included.
Supplemental Fig. 4. Activity of the ArcA~P-dependent-*lacZ* reporter construct in a wild-type (JA001, open squares) and a sixA deletion (JA029, filled diamonds) strain during various aerobiosis conditions. The line represents a curve fitted through data of the wild-type *cydA*-176+1-*lacZ* expression. The construct was tested in glucose-limited continuous growth in mineral medium supplemented with varying amounts of oxygen.