Effect of d-Lactate on the Physiological Activity of the ArcB Sensor Kinase in *Escherichia coli*

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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 the respiratory growth conditions. Under anaerobic growth conditions ArcB autophosphorylates and transphosphorylates ArcA, which in turn represses or activates its target operons. The anaerobic metabolite d-lactate has been shown to stimulate the in vitro autophosphorylating activity of ArcB. In this study, we investigated the in vivo effect of d-lactate on the kinase activity of ArcB. The results demonstrate that d-lactate does not act as a direct signal for activation of ArcB, as previously proposed, but acts as a physiologically significant effector that amplifies ArcB kinase activity.

The Arc (anoxic redox control) two-component system is an important element in the complex transcriptional regulatory network that allows facultative anaerobic bacteria, such as *Escherichia coli*, to sense various respiratory growth conditions and adapt their gene expression accordingly (13, 15, 22). This system consists of the transmembrane sensor kinase ArcB and the cognate response regulator ArcA. The ArcB protein belongs to a subfamily of tripartite hybrid kinases, because it contains three catalytic domains: an N-terminal transmitter domain (H1) with a conserved His717 residue, a central receiver domain (D1) with a conserved Asp576 residue, and a C-terminal phosphotransfer domain (H2) with a conserved His717 residue (10, 15). Under reducing conditions, ArcB autophosphorylates at the expense of ATP and transphosphorylates ArcA via a His292→Asp576→His717→Asp54 phosphorelay (8, 19). Phosphorylated ArcA (ArcA-P), in turn, represses the expression of many operons involved in respiratory metabolism and activates a few operons encoding proteins involved in fermentative metabolism (22, 23). Under oxidizing conditions ArcB autophosphorylation is inhibited by the quinone electron carriers (7), and ArcA-P dephosphorylates ArcA via a reverse Asp54→His717→Asp576 phosphorelay (8). Signal transduction by phosphorelay has also been reported for the Kin/Spo system of *Bacillus subtilis* (4), the BvgS/BvgA system of *Escherichia coli* (4), the BvgS/BvgA system of *Salmonella typhimurium* (19), the TorS/TorR system of *Saccharomyces cerevisiae* (28), and the Sln1p/Ypd1p/Ssk1p system of *Saccharomyces cerevisiae* (29). This complex phosphotransfer mechanism is believed to allow multiple levels of control for fine-tuning.

It has been reported previously that the presence of certain fermentation intermediates, such as d-lactate, acetate, and pyruvate, accelerates the autophosphorylation activity of ArcB and enhances the subsequent transphosphorylation of ArcA (6, 11). However, no in vivo evidence has been obtained to support the physiological significance of such an effect. Furthermore, because the cellular metabolites mentioned above accumulate during anaerobiosis, it has been proposed that these compounds might act as the actual signals through which ArcB senses anaerobic environments in vivo (2, 11).

Here we present the results of experiments designed to probe the in vivo effect of d-lactate on Arc signaling and to test whether this compound acts as a primary signal or as an allosteric effector. We limited our study to d-lactate, which was reported previously to have the strongest effect on the activity of ArcB (6).

**MATERIALS AND METHODS**

**Construction of strains.** To construct a Δ*dld* strain, a 5′ flanking sequence (0.8 kb) of *dld* was PCR amplified from the chromosomal DNA of strain MC4100 with primers *ldh*-5F (′-CCAGCGGCCTGGACAGACATCCGGTC-3′) and *ldh*-5R (′-CCAGCGGCCGCTCCCTGGTGC-3′). The PCR product was digested with NotI and BamHI and cloned between the corresponding sites of *lac*Z. The construct was then transformed into strain ECL 5001 (Table 1) to delete the *dld* gene by homologous recombination as described previously (21), yielding strain ECL 5201. Plasmid pDld2 (27) was then transformed into strain ECL 5201 to delete the *dll* gene by homologues recombination, yielding strain ECL 5202. Deletion of the *dll* and *dll* genes was confirmed by PCR. Subsequently, a Δ*arc*:Tn10 (′-Cmr′) allele was PI transduced into strain ECL 5202 from ECL 5003, yielding strain ECL 5203. To construct strain ECL 5204, a *arc*:Kan′ allele was PI transduced into strain ECL 5203 from ECL 5005.

To construct strain ECL 5205, a mutant *arcB* allele expressing cytosolic protein was created by deleting sequences encoding the transmembrane region of ArcB (amino acid residues of 23 to 77) by a two-step fusion PCR. Briefly, a DNA fragment encoding amino acid residues 1 to 22 of ArcB was amplified by PCR by using primers BSNDE (′-CAGCGATCCATGATAAGGAAATTCTGCC-3′) and BDT (′-GTCGACTTCTCCATGATTGGTCG-3′). The PCR product was digested with BglII and SalI and cloned between the corresponding sites of *lac*Z. The reaction was purified and used as a megaprimmer for PCR in combination with primers BINRU (′-GTAATGCGGCCGACCGACAACTACCCATCAAACC-3′) and with pABS (20) as the template. The product of this reaction was amplified and used as a megaprimmer for PCR in combination with primers BINRU and pABS (20) as the template. Finally, the product of the second PCR was digested with NdeI and SalI and cloned between the corresponding sites of *lac*Z. This construct was then transformed into strain ECL 5205 (amino acid residues of 23 to 77) by a two-step fusion PCR. Briefly, a DNA fragment encoding amino acid residues 1 to 22 of ArcB was amplified by PCR by using primers BSNDE (′-CCAGCGATCCATGATAAGGAAATTCTGCC-3′) and BDT (′-GTCGACTTCTCCATGATTGGTCG-3′). The PCR product was digested with BglII and SalI and cloned between the corresponding sites of *lac*Z. This construct was then transformed into strain ECL 5205 (amino acid residues of 23 to 77) by a two-step fusion PCR.

**Growth conditions.** Luria-Bertani (LB) broth and LB agar (15 g/liter) were used for routine growth. Ampicillin, tetracycline, kanamycin, and chloramphenicol were provided at final concentrations of 50, 12, 40, and 20 μg/ml, respec-
tration of 4/H9262/pansulfonic acid (pH 7.4) and 20 mM D-xylose. 

sayed with exponentially growing cultures as described previously (26). 

small magnetic bar.

plastic vials, and counted in the presence of Emulsi
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2086 RODRIGUEZ ET AL. J. BACTERIOL.

Plasmids

P1vir Laboratory stock

C. The Coomassie Plus protein assay reagent (Pierce) was

D-lactate dehydrogenase was expressed in nanomoles per minute per milligram

14C-labeled substrate 10-fold with the cell suspension to obtain a

1 mM D-lactate as a substrate. The speci

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conditions, we constructed a mutant strain in which the intra-
cellular concentration of D-lactate depended solely on the ex-
ogenus supply. The following aspects of D-lactate metabolism

were considered in the design of the mutant strain. During 
glucose fermentation, D-lactate is formed by an NAD-linked 
oxidoreductase encoded by the monocistronic operon ldhA at 
min 30.5 (3). Under aerobic conditions, however, D-lactate can

be scavenged from the medium by the membrane transport carriers LldP (L-lactate permease) and GlcA (glycolate permease) (27). The captured D-lactate is then converted back to pyruvate by a flavin adenine dinucleotide-dependent dehydro-
genase encoded by the dld monocistronic operon at min 47.9. Hence, strain ECL 5203 with the genotype Δldd ΔldhA was constructed as described in Materials and Methods. This strain also harbors a Φ(cyaA-lacZ) operon fusion as the reporter and a Δfnr::Tn9(Cmr) allele to avoid repression of the reporter by Fnr (5).

D-Lactate uptake by the Δldd ΔldhA mutant strain. 
The ability of the constructed Δldd ΔldhA mutant strain to accumu-
late but not metabolize D-lactate was verified. ECL 5203 was grown aerobically or anaerobically, and at mid-exponential phase the cells were incubated with D-[14C]lactate. After 0.5 and 5 min of incubation the intracellular amount of D-lactate was determined (Table 2). It was found that the mutant strain was able to accumulate radiolabeled D-lactate to a concentra-
tion gradient (intracellular concentration/ extracellular concentra-
tion) greater than 16. The calculations were based on the 
intracellular volume of water determined by the method of 
Maloney et al. (24). It was also observed that the D-lactate 
uptake by anaerobically growing cells was about three times 
lower than that by aerobically growing cells. This might be 
explained by the fact that the expression of the l-lactate per-
mease gene (lldP) is inhibited by the ArcA/ArcB system under

RESULTS

Construction of a mutant strain able to accumulate but not metabo-
lize D-lactate. D-Lactate has been shown to accelerate 
the transphosphorylation of ArcA in vitro (6, 11). To test the effect of D-lactate on the kinase activity of ArcB under physiological conditions, we constructed a mutant strain in which the intra-
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TABLE 1. E. coli K-12 strains, bacteriophage, and plasmids used in this study

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<td>or pIvir</td>
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<td>Relevant genotype</td>
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<table>
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<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
</thead>
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<tr>
<td>MC4100</td>
<td>F′ anaD139 Δ(argF-lac) U169 relA1 trp501 deoC ptsF25 rbsR</td>
<td>20</td>
</tr>
<tr>
<td>JM109</td>
<td>recA endA1 gyrA96 thi hsdRI7 supE44 relA1 Δ(lac-proAB) F′ traD36 proAB lacF lacZ ΔM15</td>
<td>Promega</td>
</tr>
<tr>
<td>ECL 5001</td>
<td>MC4100 but Φ(cyaA-lacZ)</td>
<td>20</td>
</tr>
<tr>
<td>ECL 5003</td>
<td>MC4100 but Δfru::Tn9(Cmr) Φ(cyaA-lacZ)</td>
<td>20</td>
</tr>
<tr>
<td>ECL 5005</td>
<td>Φ(cyaA-lacZ) Δfru::Tn9(Cmr) ΔarcB::Kan'</td>
<td>20</td>
</tr>
<tr>
<td>ECL 5201</td>
<td>Φ(cyaA-lacZ) ΔldhA Δfru::Tn9(Cmr)</td>
<td>This study</td>
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<tr>
<td>ECL 5202</td>
<td>Φ(cyaA-lacZ) ΔldhA Δfru::Tn9(Cmr)</td>
<td>This study</td>
</tr>
<tr>
<td>ECL 5203</td>
<td>Φ(cyaA-lacZ) ΔldhA Δfru::Tn9(Cmr) ΔarcB::Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>ECL 5204</td>
<td>Φ(cyaA-lacZ) ΔldhA Δfru::Tn9(Cmr) ΔarcB::Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>ECL 5205</td>
<td>Φ(cyaA-lacZ) ΔldhA Δfru::Tn9(Cmr) ΔarcB::Kan'</td>
<td>This study</td>
</tr>
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<td>pK03</td>
<td>ΔldhA in pK03</td>
<td>21</td>
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<tr>
<td>pLDH2</td>
<td>ΔldhA in pK03</td>
<td>This study</td>
</tr>
<tr>
<td>pLDLD2</td>
<td>Δldl in pK03</td>
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<tr>
<td>pABW</td>
<td>arcB in pBluescript KS II (+)</td>
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<tr>
<td>pABS</td>
<td>arcB in pBluescript KS II (+)</td>
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<tr>
<td>pBW</td>
<td>arcB in pBluescript KS II (+)</td>
<td>20</td>
</tr>
<tr>
<td>pBI8</td>
<td>arcB in pBluescript KS II (+)</td>
<td>20</td>
</tr>
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</table>

Permease assays. Exponentially growing cells were collected by centrifugation at 4,000 × g and washed twice with buffer A (0.1 M MOPS, 0.5 mM MgCl₂, pH 7.0). The bacterial pellet was suspended in buffer A at a final density of 0.5 mg (dry weight)/ml and kept at 15°C. The rate of uptake was assayed by diluting the

14C-labeled substrate 10-fold with the cell suspension to obtain a final concentration of 4 μM. 14C-D-lactate (56 mCi/mmol) was purchased from ICN. Since

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anaerobic growth conditions (12) and therefore D-lactate uptake relies solely on the glycolate permease (GlcA). The inability of the constructed strain to metabolize D-lactate was verified by comparing the D-lactate dehydrogenase activities in the cellular extracts of strain ECL 5203 and the isogenic wild-type strain (Fig. 1). It was found that the specific D-lactate dehydrogenase activity for the wild-type strain was 57 ± 5 nmol/mg of protein per min, in agreement with a previously reported value (17). In contrast, no specific activity was detected for the mutant strain. Moreover, the growth of ECL 5203 on M9 minimal media with D-lactate as the sole carbon source was monitored. No growth was detected (data not shown). Taken together, these results suggest that strain ECL 5203 is unable to metabolize D-lactate.

**Effect of exogenous D-lactate on the aerobic and anaerobic activities of ArcB.** The constructed dld dldhA mutant strain was used to test the effect of D-lactate on the in vivo signaling activity of ArcB by monitoring changes in the levels of phosphorylated ArcA, as indicated by the expression pattern of the \( \Phi(cydA'::lacZ) \) operon fusion. To do this, strains ECL 5203 [dld dldhA \( \Phi(cydA'::lacZ) \Delta(for::Tn9(Cm')) \)] and ECL 5204, the isogenic \( \DeltaarcB \) strain, were cultured aerobically or anaerobically in the medium described above with or without 10 mM D-lactate. \( \beta \)-Galactosidase activity was assayed and was expressed in Miller units (26). Open bars, growth with no D-lactate; solid bars, growth with D-lactate. The data are averages from four experiments (the variations were less than 10% of the means).

The mid-exponential growth phase the \( \beta \)-galactosidase activity levels were determined (Fig. 2A). It was found that during aerobic growth D-lactate had no effect on the expression of the reporter in both the \( \text{arcB}^+ \) strain and the \( \DeltaarcB \) strain. In contrast, during anaerobic growth the presence of D-lactate in the growth medium resulted in a significant increase in \( \Phi(cydA'::lacZ) \) expression in the \( \text{arcB}^+ \) strain but not in the \( \DeltaarcB \) strain. Thus, D-lactate appears to amplify the ArcB kinase activity under anaerobic growth conditions, as judged by the increased level of reporter expression.

To exclude the possibility that deletion of dld and or ldhA has a direct influence on the expression of \( \Phi(cydA'::lacZ) \), the expression levels in the dld dldhA mutant strain and the isogenic \( \text{ldhA}^- \text{ldhA}^- \) strain (ECL 5003) were compared. It was found that the aerobic expression levels of the \( \Phi(cydA'::lacZ) \) reporter were very low and nearly indistinguishable in the two strains. In contrast, the anaerobic expression level of the reporter in the dld dldhA mutant strain was only half of that in the wild-type strain (Fig. 2B), indicating that ArcB was less active in the mutant strain. Since no D-lactate is formed in this strain because of the ldhA deletion, a less active ArcB kinase would expected only if D-lactate is a physiological modulator of ArcB. In such a case, addition of D-lactate to the growth medium should restore the kinase activity of ArcB. To test this reasoning, 10 mM D-lactate was added to the growth medium of the mutant strain. It was found that the level of reporter expression was restored to almost wild-type levels (Fig. 2B).

Thus, the results described above provide strong support for the conclusion that D-lactate does not act as a direct signal, because it has no influence on the activity of ArcB under aerobic growth conditions, but it is required for full activity under anaerobic conditions.

**Enhancement of the DTT-dependent activity of ArcB by D-lactate.** In an independent study, we noticed that the reducing agent dithiothreitol (DTT) activates ArcB under aerobic growth conditions. Because DTT is a strong reductant, we
argued that it might affect the redox state of the quinone pool and thereby result in aerobic activation of ArcB. We therefore tested whether the quinone electron carriers can be reduced by DTT. To do this, we incubated ubiquinone 0 (Q0), a soluble analog of ubiquinone 8, with DTT and monitored its spectroscopic characteristics. As shown in Fig. 3, the maximum absorbance for the oxidized Q0 was ~265 nm. However, upon incubation with DTT the maximum absorbance was shifted to ~295 nm, which is characteristic for the reduced form of Q0. Thus, it seems likely that DTT exerts its effect on ArcB through reduction of the quinone electron carriers.

We then hypothesized that if D-lactate is a physiologically significant modulator of ArcB, it may have an augmenting effect on the aerobic DTDependent ArcB activity, mimicking the effect exerted during anaerobiosis. To test this hypothesis, strains ECL 5203 (arcB) and ECL 5204 (ΔarcB) were grown aerobically in four parallel cultures for each strain to an OD600 of 0.3. One of the cultures served as the control, whereas either DTT or D-lactate were added to the other cultures (Fig. 4). It was found that addition of DTT to the growth medium of strain ECL 5203 led to immediate activation of β-galactosidase expression (λΦ(cydA'-lacZ)), four parallel cultures of strain ECL 5203 (arcB') and ECL 5204 (ΔarcB) were grown aerobically in LB medium containing 0.1 M MOPS (pH 7.4) and 20 mM D-lactate. At an OD600 of 0.3 one aliquot was withdrawn from each culture and used to measure the β-galactosidase activity (~15 min). At time zero either 5 mM DTT (●), 20 mM D-lactate ( ●), or both DTT and D-lactate ( ●), or nothing ( ○) was added to the cultures, and the β-galactosidase activity was monitored for 1 h at 15-min intervals. Left panel, ECL 5203; right panel, ECL 5204 (ΔarcB). The data are the averages from four experiments (the variations were less than 10% of the means).

ArcB<sup>95</sup>, which was devoid of the transmembrane segments (amino acids 23 to 77) and therefore was not anchored to the membrane. For this experiment, strains ECL 5205 (in which the chromosomal wild-type arcB allele was replaced with arcB<sup>95</sup>) and ECL 5203 (arcB'), both harboring the λΦ(cydA'-lacZ) operon fusion, were grown aerobically in buffered LB medium (Fig. 5). At an OD<sub>600</sub> of ~0.3 a sample for the β-galactosidase assay was withdrawn (at ~15 min in Fig. 5). As expected, the expression of the reporter was significantly higher in the mutant strain than in the wild-type strain, confirming that ArcB<sup>95</sup> is a partially active kinase under aerobic growth conditions. After an additional 15 min of growth, the
cultures were each divided into two aliquots; for each pair, one aliquot served as the control, while 10 mM d-lactate was added to the other, and the β-galactosidase activity was monitored (Fig. 5). It was found that addition of d-lactate to the growth medium of the wild-type strain had no effect. However, addition of d-lactate to the growth medium of the strain carrying ArcB<sup>35</sup> resulted in an immediate increase in the expression level of the reporter, supporting our conclusion that d-lactate accelerates the in vivo kinase activity of the active ArcB protein.

Therefore, it seems reasonable to conclude that d-lactate acts as an allosteric effector, which plays a significant role in modulating the activity of ArcB under physiological conditions.

**DISCUSSION**

In a previous in vitro study, it was reported that the level of autophosphorylation of the primary transmitter domain of ArcB was up to three times greater than the basal level of autophosphorylation in the presence of the fermentative metabolite d-lactate (6). In this paper, we present results of in vivo experiments demonstrating that the enhancement of the kinase activity of ArcB by this metabolic intermediate also occurs under physiological conditions. Moreover, our results rule out the previously proposed possibility that d-lactate might serve as an actual signal through which ArcB senses anaerobic environments in vivo (2, 11).

Our conclusions are based on the following findings. First, under nonstimulating (aerobic) growth conditions the presence of d-lactate has no influence on the expression of the ψ(cydA-<sup>−</sup>lacZ) reporter, suggesting that ArcB remains inactive. Because the kinase activity of ArcB does not respond to d-lactate under aerobic growth conditions, despite the fact that d-lactate reaches an intracellular concentration that is threefold higher than that under anaerobic conditions (Table 2), it is not plausible that this metabolite serves as a direct signal that activates ArcB. In contrast, under stimulating conditions (anaerobiosis) the presence of d-lactate causes a significant increase in reporter expression, indicating that ArcB operates with enhanced activity. Therefore, it appears that d-lactate serves as an allosteric effector that acts on the active ArcB.

Second, although d-lactate has no influence on the activity of ArcB under aerobic growth conditions, it does enhance the aerobic DTT-dependent activity of ArcB. Thus, the d-lactate effect on ArcB can be observed even under aerobic growth conditions, with the prerequisite that ArcB be active. Interestingly, the DTT-dependent activation of ArcB under aerobic conditions seems to be a result of reduction of the quinone pool. In fact, because of its low redox potential (−380 mV), DTT should be able to readily reduce the pool of quinones, as the redox potentials are 45 mV for ubiquinone and −75 mV for menaquinone. An alternative might be that DTT, which is known to affect protein folding and in particular disulfide bond formation, acts by reducing either or both cysteine residues present in the linker region of ArcB. However, mutating Cys241 of ArcB to Ala has been shown to have no effect on the in vivo regulation of ArcB (14, 25).

Third, addition of d-lactate to an aerobic culture of a strain carrying the arcB<sup>35</sup> mutant gene, which encodes a partially active ArcB kinase protein, results in an immediate increase in the level of the ψ(cydA-<sup>−</sup>lacZ) reporter, supporting the conclusion that an active ArcB protein is required for the d-lactate effect to be observed. Finally, the function of this metabolic intermediate as an allosteric activator is further supported by the fact that its cellular level depends not only on the respiratory state of the cell but also on the oxygen/hydrogen ratio of the carbohydrate being fermented (1).

Thus, three independent lines of evidence support the conclusion that d-lactate has a physiological role as an allosteric effector that accelerates the kinase activity of ArcB under stimulating growth conditions.

Although the reaction mechanism by which d-lactate modulates the activity of ArcB remains unknown, it is worth mentioning that the ArcB receiver domain (D1), even when it is catalytically inactive (e.g., D1<sup>Asp576→Ala</sup>) is indispensable for d-lactate to exert its effect in vitro (6). It thus seems that either the effector binding site is located in D1 or the structural presence of D1 enables the effector to bind to the primary transmitter domain of ArcB (H1). Because Asp576 is the actual residue that receives the ~P from His292 (9), the site of autophosphorylation, and is also indispensable for the phosphatase activity of ArcB (8), d-lactate must act upon the first step of the Arc phosphorelay, which is the autophosphorylation reaction. It is therefore tempting to speculate that effector binding to ArcB may cause a conformational change that results in an increase in the V<sub>max</sub> of this reaction. If H1 autophosphorylation is the rate-limiting step in the Arc signal transduction cascade and the phosphorelay is the sole means of ArcA phosphorylation by ArcB, then increasing the V<sub>max</sub> of the autophosphorylation reaction with d-lactate would be sufficient to raise the level of ArcA-P. Such a process should be advantageous to the cell, because as d-lactate accumulates under anaerobic growth conditions, maximal activity of the ArcB kinase is ensured. Indeed, acceleration of the ArcB kinase activity by allosteric effectors such as d-lactate should result in a faster response to changes in redox conditions and thereby faster adaptation to the new environment. Also, a faster response should guarantee a more economic mode of growth, as the expression of many unneeded enzymes should be immediately repressed.

ArcB belongs to a subfamily of tripartite sensor kinases that possess three catalytic domains that participate in complex phosphorelay reactions for signal transmission and signal decay. It has been widely believed that the elaborate structure and complex phosphotransfer mechanism of such tripartite sensor kinases allow various inputs to be integrated in a multilevel control mechanism that fine-tunes their signaling activity. Here, ArcB provides the first example of an at least dual-level control mechanism, as illustrated by the orchestrated action of the quinone electron carriers and the fermentative metabolite d-lactate. The oxidized forms of ubiquinone and menaquinone have previously been shown to serve as direct negative signals that silence the kinase activity of ArcB and thereby act on the first level of control (7). Now, we show that d-lactate serves as an allosteric effector that amplifies the kinase activity of ArcB but is unable to override the quinone-dependent inhibition of ArcB and therefore acts on a secondary level of control.

Two challenges that remain are to pinpoint the structural requirement and reaction mechanism of the effectors and to
determine whether there are additional elements that influence signal transmission and signal decay in the Arc system.

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