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 (H1) with a conserved His717 residue (10, 15). Under reducing 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, the in vivo effect of d-lactate on the kinase activity of ArcB was assessed. 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.
tively. For the β-galactosidase activity assay, the Φ(cydA-lacZ)-bearing strains were cultured in buffered LB broth containing 0.1 M MOPS (morpholinepropanesulfonic acid) (pH 7.4) and 20 mM d-xylose.

**β-Galactosidase activity assay.** Aerobic cultures (5 ml) were grown in 250-ml baffled flasks at 37°C with shaking (300 rpm), whereas anaerobic cultures were grown in closed 5-ml test tubes which were filled to the brim and stirred with a small magnetic bar. β-Galactosidase activity, expressed in Miller units, was assayed with exponentially growing cultures as described previously (26).

**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. d-[14C]Lactate (56 mCi/mmol) was purchased from ICN. Since it has been reported previously that d-lactate uptake by E. coli cell suspensions reaches the steady state within 2 min of the start of incubation (17, 27), samples (100 ml) were withdrawn at 0.5 and 5 min and filtered through 0.65-μm-pore-size cellulose nitrate filters. The filters were washed with 4 ml of buffer A, placed in plastic vials, and counted in the presence of Emulsifier-safe (Packard, Meriden, Conn.). To calculate the concentration of d-lactate inside the cells, the following conversion factor was used: 0.63 μl of intracellular H₂O per ml of cell suspension at an optical density at 600 nm (OD₆₀₀) of 1.

**Enzyme assays.** For d-lactate dehydrogenase assays, cells were harvested by centrifugation at 5,000 × g for 15 min and washed once in cold 10 mM potassium phosphate buffer (pH 7.0). The pellet was weighed and suspended in 4 volumes of the same buffer. The suspended cells were lysed for 1 min/ml with a model 60W ultrasonic disintegrator at 1.5 A while they were chilled in a dry ice-ethanol bath. Lysates were cleared by centrifugation for 30 min at 10,000 × g. Enzyme assays were performed by a method similar to the method used for glycerol-3-phosphate dehydrogenase assays by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide mediated by phenazine methosulfate (18), but with 0.1 M d-xylose as a substrate. The specific activity of d-lactate dehydrogenase was expressed in nanomoles per minute per milligram of protein at 30°C. The Coomassie Plus protein assay reagent (Pierce) was employed to estimate protein concentrations, and bovine serum albumin was used as the standard.

**RESULTS**

**Construction of a mutant strain able to accumulate but not metabolize 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 intracellular concentration of d-lactate depended solely on the exogenous 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 dehydrogenase encoded by the dld monocistronic operon at min 47.9. Hence, strain ECL 5203 with the genotype Δlld ΔldhA was constructed as described in Materials and Methods. This strain also harbors a λΦ(cydA-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 Δlld ΔldhA mutant strain.** The ability of the constructed Δlld ΔldhA mutant strain to accumulate 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 concentration gradient (intracellular concentration/extracellular concentration) 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 permease gene (lldP) is inhibited by the ArcA/ArcB system under
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 Δdld ΔldhA 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 λΦ(cydA− lacZ) operon fusion. To do this, strains ECL 5203 [Δdld ΔldhA Φ(cydA− lacZ) Δfor::Tn9(Cm′)] and ECL 5204, the isogenic ΔarcB strain, were cultured aerobically or anaerobically in buffered LB medium containing 0.1 M MOPS (pH 7.4), 20 mM D-xylose, and 10 mM D-lactate. D-lactate dehydrogenase activity was measured as described in Materials and Methods with about 60 μg of protein. The reaction was allowed to proceed for 2 min, and the OD_{570} was monitored. Open bars, reaction mixture without substrate (control); solid bars, reaction mixture with substrate.

**TABLE 2. Accumulation of D-lactate against concentration gradient by the ΔldhA Δdld mutant strain ECL 5203**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Incubation time (min)</th>
<th>Conc (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+O₂</td>
<td>0.5</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>67.5</td>
</tr>
<tr>
<td>−O₂</td>
<td>0.5</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>23.7</td>
</tr>
</tbody>
</table>

* Cells were grown in xylose minimal media to an OD_{600} of ~0.3.
* D-lactate uptake was assayed with 4 μM tritiated D-lactate as the substrate as previously described (27).

In an independent study, we noticed that the reduction of NAD in the cytoplasm may be an important step in the metabolism of D-lactate. However, the initial reduction of NAD in the cytoplasm is not well understood. Therefore, it is important to further investigate the role of NAD in the metabolism of D-lactate.

**FIG. 2. Effect of D-lactate on expression of λΦ(cydA− lacZ).** (A) Strain ECL 5203 (arcB') and the isogenic strain ECL 5204 (ΔarcB) were grown aerobically or anaerobically in LB medium containing 0.1 M MOPS (pH 7.4) and 20 mM D-xylose with or without 10 mM D-lactate. (B) Strain ECL 5003 and the isogenic strain ECL 5203 (ΔldhA Δdld) were grown anaerobically in the medium described above with or without 10 mM D-lactate. β-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).
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 DTT-dependent ArcB activity, mimicking the effect exerted during anaerobiosis. To test this hypothesis, strains ECL 5203 (arcB hypothet) and ECL 5204 (ΔarcB) were grown aerobically in four parallel cultures for each strain to an OD 600 of 0.3. One of the cultures served as the control, whereas either DTT or D-lactate or both DTT and 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 λΦ(cydA’-lacZ) expression, in agreement with our previous observation. Moreover, simultaneous addition of DTT and D-lactate to the culture medium had an even stronger effect on the expression of the reporter, which reached a level significantly higher than the level in the culture with only DTT (Fig. 4). On the other hand, addition of D-lactate alone had no effect on expression of the reporter. Finally, addition of DTT alone or in combination with D-lactate to the growth medium of the ΔarcB strain had no effect on the expression level of the reporter. Thus, D-lactate is able to amplify the in vivo kinase activity of ArcB under aerobic growth conditions when ArcB is active as a kinase.

**Effect of D-lactate on the aerobic activity of ArcB**. To obtain independent support for our conclusion, we took advantage of the previous finding that liberation of ArcB from the plasma membrane results in a partially constitutive kinase activity in vivo (20). We therefore tested the effect of D-lactate on the aerobic signaling activity of a truncated version of ArcB, ArcBcyt, 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 Δ) and ECL 5203 (arcB hypothet), both harboring the λΦ(cydA’-lacZ) operon fusion, were grown aerobically in buffer LB medium (Fig. 5). At an OD 600 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 ArcBΔ 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>C57</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 ϕ(αcA<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 accelerates the in vivo kinase activity of the active ArcB protein.

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>C57</sup> mutant gene, which encodes a partially active ArcB kinase protein, results in an immediate increase in the level of the ϕ(αcA<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., D<sup>A</sup>Cyst576→Ala<sup>+</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 $V_{\text{max}}$ of this reaction. If H1 auto-phosphorylation 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 $V_{\text{max}}$ 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 into 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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