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Phosphorelay as the Sole Physiological Route of Signal Transmission by the Arc Two-Component System of *Escherichia coli*

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The Arc two-component system, comprising a tripartite sensor kinase (ArcB) and a response regulator (ArcA), modulates the expression of numerous genes involved in respiratory functions. In this study, the steps of phosphoryl group transfer from phosphorylated ArcB to ArcA were examined in vivo by using single copies of wild-type and mutant *arcB* alleles. The results indicate that the signal transmission occurs solely by His-Asp-His-Asp phosphorelay.

The ArcB-ArcA two-component signal transduction system of *Escherichia coli* regulates the expression of more than 30 operons depending on the redox conditions of growth (12, 17, 18). This system comprises ArcB as the membrane-bound sensor kinase and ArcA as the cognate response regulator (Fig. 1). The ArcB protein has three cytoplasmic domains: a primary transmitter domain (H1) containing a conserved His292, a receiver domain (D1) containing a con-

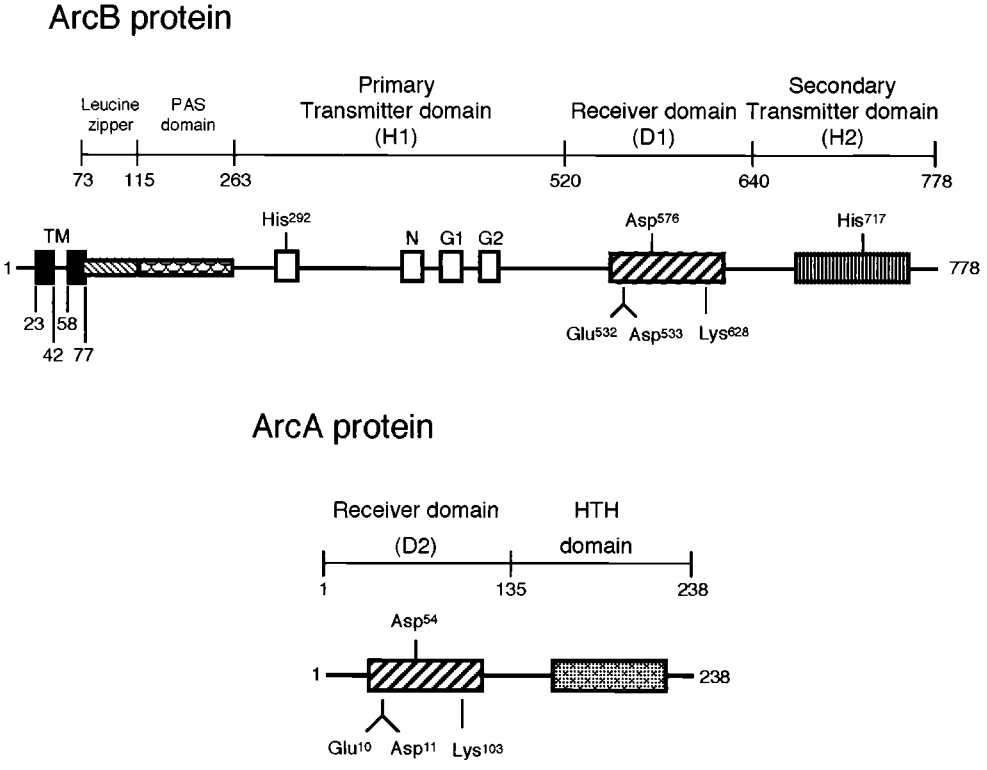

FIG. 1. Schematic representation of ArcB and ArcA. (Top) The N-terminal transmembrane domain of ArcB was determined by alkaline phosphatase fusions (16). A putative leucine zipper (6) and a PAS domain (26) were predicted on the basis of amino acid sequence homology. The primary transmitter domain (H1) contains the conserved His292 and the catalytic determinants N, G1, and G2. The G1 and G2 sequences typify nucleotide-binding motifs. The receiver domain (D1) contains the conserved Asp576, and the secondary transmitter domain (H2) contains the conserved His717 (13, 27). (Bottom) ArcA consists of an N-terminal receiver domain (D2) containing the conserved Asp54 and a C-terminal helix-turn-helix (HTH) domain (12).

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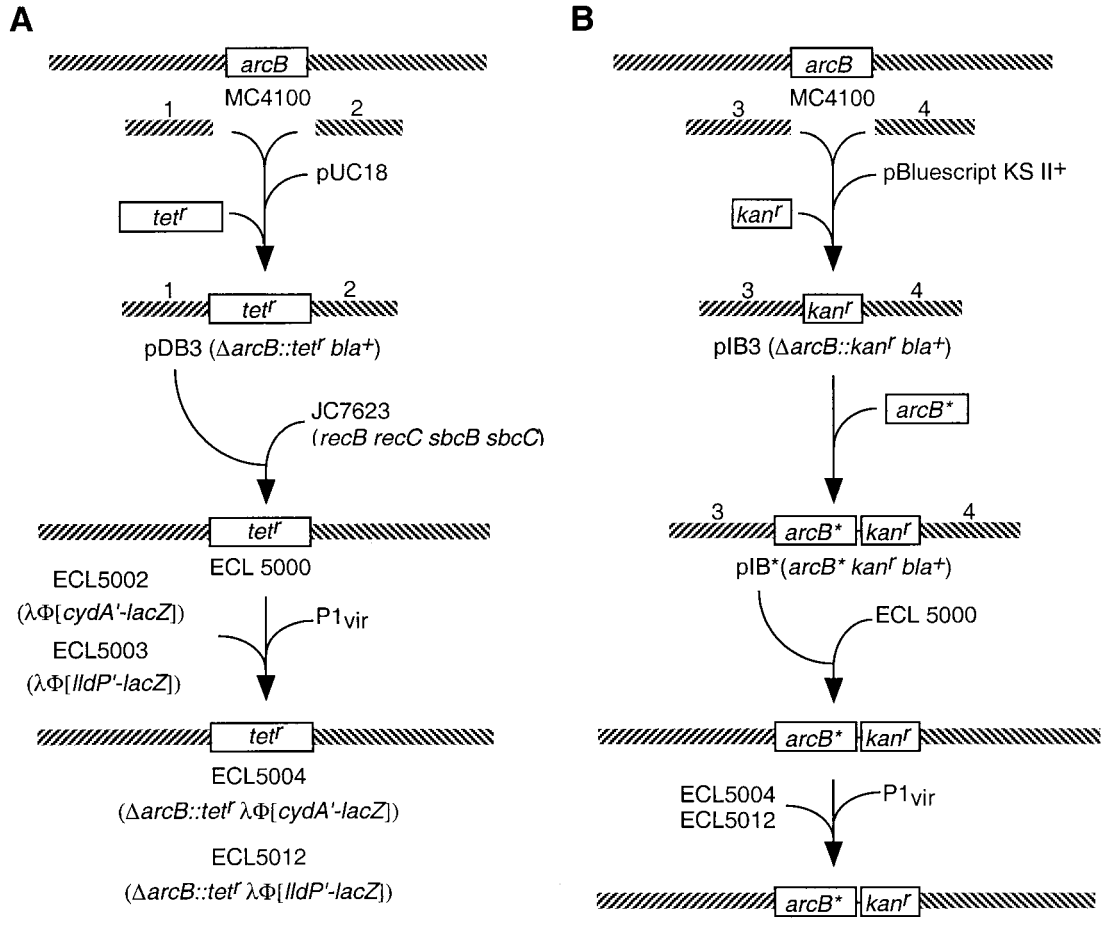

FIG. 2. Allele replacement strategy. (A) Construction of the Δ*arcB*::Tet^r strains. The 5′- and 3′-flanking DNA fragments of *arcB* (fragments 1 and 2) were prepared by PCR, using chromosomal DNA from strain MC4100 as the template and, respectively, the primer pairs DAB-5N–DAB-5C and DAB-3N–DAB-3C (16). The PCR products were cloned into pUC18. A Tet^r cassette, isolated from pNK81 (30), was then inserted between the two *arcB*-flanking fragments to generate pDB3. This plasmid was transformed into strain JC7623 (22) to create a Δ*arcB*::Tet^r strain (ECL5000) by homologous recombination. The Δ*arcB*::Tet^r allele was then P1 transduced into strains ECL5002 and ECL5003, resulting in ECL5004 and ECL5012, respectively. (B) Introduction of modified *arcB* sequences into the Δ*arcB*::Tet^r strain. The 5′- and 3′-flanking DNA fragments of *arcB* (fragments 3 and 4) were prepared by PCR, using chromosomal DNA from strain MC4100 as the template and, respectively, the primer pairs IAB-5N–IAB-5C and IAB-3N–IAB-3C (16). The PCR products were cloned into pBluescript II KS (+). A Kan^r cassette, isolated from pUC4-KIXX (2), was then inserted between the two *arcB*-flanking fragments to generate pIB3. Fragment 3 includes the *arcB* promoter, the ribosome-binding site, and an introduced *Nde*I site that includes the initiation codon of *arcB* followed by a *Hin*dIII site. A modified *arcB* sequence (*arcB**) was cloned into the pIB3 between the *Nde*I site and *Hin*dIII site, generating pIB*. This plasmid was transformed into strain ECL5000 to replace the Δ*arcB*::Tet^r allele with *arcB** Kan^r by homologous recombination. Recombinants were selected by their Tet^s Kan^r Amp^s phenotypes and confirmed by PCR. The *arcB** Kan^r construct was then P1 transduced into strains ECL5004 and ECL5012.

served Asp576, and a secondary transmitter domain (H2) containing a conserved His717 (10, 13, 15, 27). ArcB thus belongs to the tripartite hybrid sensor kinase subfamily (23), which also includes BarA (21), EvgS (29), and TorS (14) of *E. coli*, BvgS of *Bordetella pertussis* (1), LemA of *Pseudomonas syringae* pv. syringae (9), and RteA of *Bacteroides thetaiotaomicron* (25).

In vitro studies showed that the primary transmitter domain of ArcB is autophosphorylated at His292 at the expense of ATP (8, 11). The phosphoryl group is then sequentially transferred to Asp576 and His717 and from there to Asp54 of ArcA. However, the phosphoryl group on His292 could also be directly transferred in vitro to ArcA at a very low rate (8). An in vivo study utilizing ArcB domains borne by a low-copy-number plasmid led to the conclusion that the phosphoryl group from His292 could be transferred to ArcA and that this transfer was regulated by the nature of the carbon source. On the other hand, the phosphoryl group from His717 could also be transferred to ArcA, but this transfer was regulated by redox conditions (19). Possible misleading results caused by multiple gene dosage effect and different degrees of catabolite repression during utilization of various carbon sources, however, were not discussed. In yet another study, it was suggested that His717 received the phosphoryl group from an unknown sensor kinase (10). Here, we address the questions of whether ArcA can be phosphorylated by both H1 and H2 and whether H2 can be phosphorylated by a noncognate sensor kinase, under in vivo conditions in cells bearing a single copy of an *arcB* allele on the chromosome.

Strategy for the in vivo study with modified *arcB*. The strains, phage, and plasmids used in this study are listed in Table 1. To determine the sequence of phosphotransfer in the Arc system, the *arcB*$^+$ allele on the chromosome was replaced by various mutant sequences (Fig. 2). The strategy of single-copy replacement circumvents possible complementation, epi-

TABLE 1. *E. coli* K-12 strains, phage, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype or characteristics	Reference or source
Strains		
MC4100	*F⁻ araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR*	24
JC7623	*recB21 recC22 sbcB15 sbcC201*	22
ECL5000	Δ*arcB*::Tet^r *recB21 recC22 sbcB15 sbcC201*	16
ECL5001	MC4100 but λΦ(*cydA′-lacZ*)	16
ECL5002	MC4100 but λΦ(*lldP′-lacZ*)	16
ECL5003	MC4100 but Δ*fnr*::Tn*9* (Cm^r) λΦ(*cydA′-lacZ*)	16
ECL5004	Δ*arcB*::Tet^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	16
ECL5005	Δ*arcB*::Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	16
ECL5006	*arcB*$^+$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	16
ECL5012	Δ*arcB*::Tet^r λΦ(*lldP′-lacZ*)	16
ECL5013	Δ*arcB*::Kan^r λΦ(*lldP′-lacZ*)	16
ECL5014	*arcB*$^+$ Kan^r λΦ(*lldP′-lacZ*)	16
ECL5022	*arcB*^{H292Q} Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5023	*arcB*^{D576A} Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5024	*arcB*^{H717Q} Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5025	*arcB*$^{1-661}$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5026	*arcB*$^{1-661,\ D576A}$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5027	*arcB*$^{1-520}$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5028	*arcB*$^{D576A,\ H717Q}$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5029	*arcB*$^{H292Q,\ D576A}$ Kan^r λΦ(*cydA′-lacZ*) Δ*fnr*::Tn*9*(Cm^r)	This study
ECL5030	*arcB*^{H292Q} Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5031	*arcB*^{D576A} Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5032	*arcB*^{H717Q} Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5033	*arcB*$^{1-661}$ Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5034	*arcB*$^{1-661,\ D576A}$ Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5035	*arcB*$^{1-520}$ Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5036	*arcB*$^{D576A,\ H717Q}$ Kan^r λΦ(*lldP′-lacZ*)	This study
ECL5037	*arcB*$^{H292Q,\ D576A}$ Kan^r λΦ(*lldP′-lacZ*)	This study
Phage		
P1*vir*		Laboratory stock
Plasmids		
pDB3	Δ*arcB*::Tet^r *bla*$^+$	16
pIB3	Δ*arcB*::Kan^r *bla*$^+$	16

static, and dosage effects. The phenotypic consequences of ArcB modification were analyzed by changes in the in vivo levels of phosphorylated ArcA (ArcA-P), as indicated by expressions of target operons. We employed a λΦ(*cydA′-lacZ*) operon fusion as an ArcA-P-activable reporter and a λΦ(*lldP′-lacZ*) operon fusion as an ArcA-P-repressible reporter. A Δ*fnr*::Tn*9*(Cm^r) allele was incorporated into the λΦ(*cydA′-lacZ*)-harboring strains to avoid its repression by Fnr (3).

Requirement of all three conserved residues of ArcB for its ArcA-phosphorylating activity. To verify the importance of His292, Asp576, and His717 in the phosphotransfer pathway leading to the formation of ArcA-P, we replaced the chromosomal *arcB*$^+$ allele by *arcB*^{H292Q}, *arcB*^{D576A}, or *arcB*^{H717Q} in a reporter strain bearing λΦ(*cydA′-lacZ*) or λΦ(*lldP′-lacZ*). The cells were grown aerobically or anaerobically and their β-galactosidase activity levels were assayed. It was found that all three mutants exhibited phenotypes indistinguishable from that of the Δ*arcB* mutant, suggesting that ArcB phosphorylates ArcA exclusively by the relay pathway (Fig. 3). Western analysis showed that all point mutants did produce wild-type levels of ArcB (Fig. 4). To confirm that H1 cannot mediate the phosphorylation of ArcA without H2, we replaced *arcB*$^+$ by *arcB*$^{1-520}$, *arcB*$^{1-661}$, *arcB*$^{1-661,\ D576A}$, or *arcB*$^{D576A,\ H717Q}$. All of the tested alleles gave a null phenotype (Table 2).

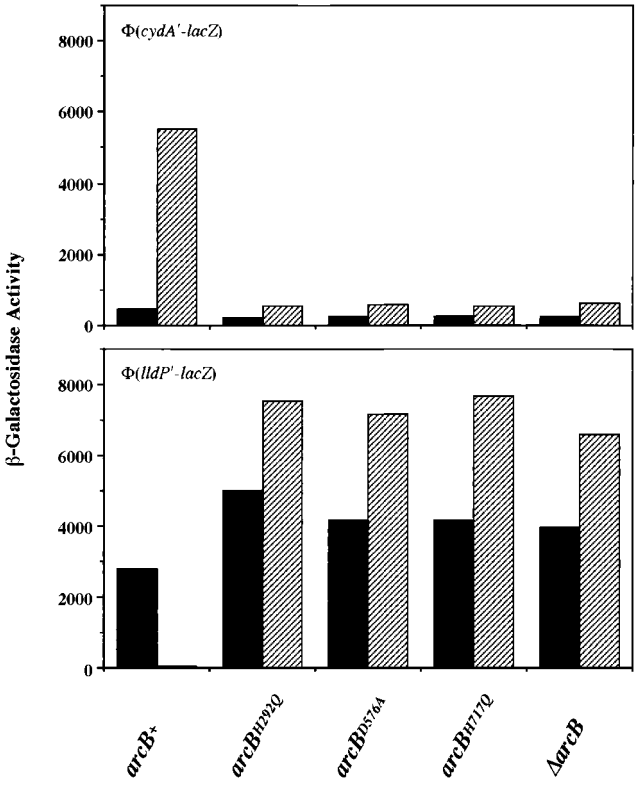

FIG. 3. Effects of mutations in the conserved amino acid residues of ArcB on the expressions of λΦ(*cydA′-lacZ*) or a λΦ(*lldP′-lacZ*). The *cydAB* operon encodes cytochrome *d* oxidase (5), and the *lldPRD* operon encodes proteins involved in L-lactate utilization (4). The Φ(*cydA′-lacZ*)-bearing strains were grown in Luria-Bertani broth containing 0.1 M MOPS (morpholinepropanesulfonic acid; pH 7.4) and 20 mM D-xylose. The Φ(*lldP′-lacZ*)-bearing strains were grown in the above medium supplemented with 20 mM L-lactate as an inducer (4). β-Galactosidase activity was assayed and expressed in Miller units (20). The data are averages from four experiments (variations were <10% from the mean). Solid bars, aerobically grown cells; hatched bars, anaerobically grown cells.

His717 of H2 derives its phosphoryl group exclusively from the relay. To test whether His717 can be phosphorylated by a noncognate sensor kinase(s), we replaced *arcB*$^+$ by *arcB*$^{H292Q,\ D576A}$. The mutant showed an *arcB*-null phe-

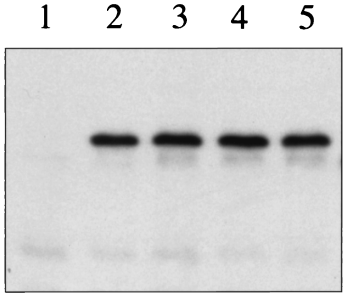

FIG. 4. Western blot analysis. A 1-ml sample of cultures grown aerobically in Luria-Bertani broth was harvested at an optical density at 600 nm of 0.5. The pelleted cells were washed with 1 ml of 10 mM Tris-HCl (pH 8.0) and solubilized by incubation at 95°C for 5 min in 100 μl of 2× sodium dodecyl sulfate sample buffer. Samples of 10 μl were subjected to electrophoresis in a sodium dodecyl sulfate–12% polyacrylamide gel, and the resolved proteins were electrotransferred to a Hybond-ECL filter (Amersham). Immunoblot analyses were subsequently performed, using ArcB polyclonal antibodies as previously described (16). Lane 1, Δ*arcB*; lane 2, *arcB*$^+$; lane 3, *arcB*^{H292Q}; lane 4, *arcB*^{D576A}; lane 5, *arcB*^{H717Q}.

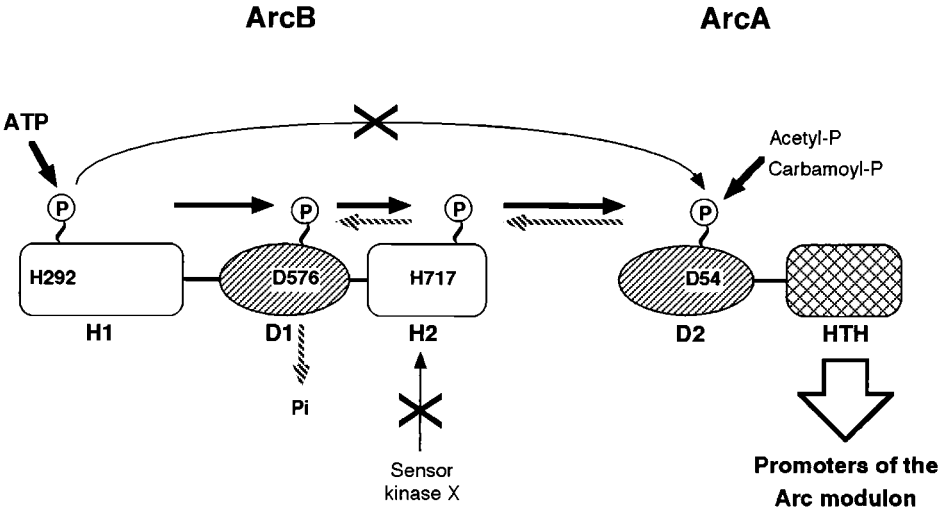

FIG. 5. Model for signal transduction by the Arc system. Heavy solid arrows indicate the forward phosphotransfer reactions leading to formation of ArcA-P. Hatched arrows indicate the reverse phosphotransfer reactions leading to signal decay (7). Arrows with crosses indicate phosphotransfer reactions not substantiated by this study.

notype, despite the presence of His717 in the ArcB protein (Table 2). Furthermore, when $arcB^{638–778}$ (H2) was expressed from a low-copy-number plasmid in an Δ*arcB* background, an *arcB*-null phenotype was also found. On the other hand, when the same plasmid was tested in an $arcB^{1–661}$ background, an $arcB^{+}$ phenotype was obtained (data not shown). This latter result indicated that the phosphorylation of ArcA via His717 depended on the presence of His292 and Asp576 and that the phosphorelay involved an intermolecular reaction between different ArcB domains, in agreement with the results of our previous in vitro study (8).

Discussion and conclusion. We were prompted to undertake this study not only because in vitro enzymatic data (8) and in vivo properties of cells with multiple gene dosage (19) may be misleading, but also because of certain conflicting results. For instance, in a study of purified proteins, the rate of ArcA phosphorylation catalyzed by $ArcB^{78–661}$ (H1-D1) was less than an order of magnitude smaller than that catalyzed by a mixture of $ArcB^{78–661}$ (H1-D1) and $ArcB^{638–778}$ (H2), indicating a predominant role of the phosphorelay (8). By contrast, in a study of everted vesicles, the rate of ArcA phosphorylation catalyzed by $ArcB^{D576Q}$ was almost as high as that catalyzed by wild-type ArcB, indicating a predominant role of His292 as a direct phosphoryl group donor to ArcA (27). However, in a third study, the same $ArcB^{D576Q}$ mutant protein (encoded by a low-copy-number plasmid) was inactive in vivo as a phosphoryl group donor to ArcA (19). Paradoxically, in that same study, $ArcB^{H717L}$ apparently was able to serve as a phosphoryl group donor to ArcA (19).

The results from the present study, based on single-copy *arcB* alleles, indicate that the sole route of phosphotransfer from ArcB to ArcA is by a relay involving His292, Asp576, and His717 of the sensor kinase (Fig. 5). In particular, there is no evidence for direct phosphoryl group transfer from His292 to ArcA or for the phosphorylation of ArcA by an unknown kinase via the H2 domain of ArcB. Thus, the mode of signal transmission in the Arc system seems to be no more elaborate than that proposed for the Bvg (28) and Tor (14) systems.

TABLE 2. Effects of various mutant *arcB* alleles on the expressions of λΦ(*cydA′-lacZ*) or λΦ (*lldP′-lacZ*)[a]

Relevant genotype	β-Galactosidase activity (U)[b]			
	Φ(*cydA′-lacZ*)		Φ(*lldP′-lacZ*)	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$
$arcB^{+}$	440	5,500	2,800	50
$arcB^{1–661}$	230	690	4,200	6,600
$arcB^{1–661,\ D576A}$	220	610	4,300	7,600
$arcB^{1–520}$	270	610	4,300	7,800
$arcB^{D576A,\ H717Q}$	240	570	3,900	7,100
$arcB^{H292Q,\ D576A}$	230	550	3,300	7,900
Δ*arcB*	260	630	3,900	6,600

[a] The Φ(*cydA′-lacZ*)-bearing strains were grown in Luria-Bertani broth containing 0.1 M MOPS (pH 7.4) and 20 mM D-xylose. The Φ(*lldP′-lacZ*)-bearing strains were grown in the same medium supplemented with 20 mM L-lactate as an inducer (4).

[b] β-Galactosidase activity was assayed and expressed in Miller units (20). The data are averages from four experiments (variations were <10% from the mean).

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