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Mutational Analysis of Signal Transduction by ArcB, a Membrane Sensor Protein Responsible for Anaerobic Repression of Operons Involved in the Central Aerobic Pathways in *Escherichia coli*

S. IUCHI AND E. C. C. LIN

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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In *Escherichia coli*, the expression of a group of operons involved in aerobic metabolism is regulated by a two-component signal transduction system in which the *arcB* gene specifies the membrane sensor protein and the *arcA* gene specifies the cytoplasmic regulator protein. ArcB is a large protein belonging to a subclass of sensors that have both a transmitter domain (on the N-terminal side) and a receiver domain (on the C-terminal side). In this study, we explored the essential structural features of ArcB by using mutant analysis. The conserved His-292 in the transmitter domain is indispensable, indicating that this residue is the autophosphorylation site, as shown for other homologous sensor proteins. Compression of the range of respiratory control resulting from deletion of the receiver domain and the importance of the conserved Asp-533 and Asp-576 therein suggest that the domain has a kinetic regulatory role in ArcB. There is no evidence that the receiver domain enhances the specificity of signal transduction by ArcB. The defective phenotype of all *arcB* mutants was corrected by the presence of the wild-type gene. We also showed that the expression of the gene itself is not under respiratory regulation.

Escherichia coli regulates the synthesis of a number of enzymes according to the respiratory conditions of growth (15, 43). For instance, numerous genes encoding enzymes of aerobic metabolism are expressed at higher levels aerobically than anaerobically. The enzymes include several dehydrogenases of the flavoprotein class; the cytochrome *o* complex; and members of the tricarboxylate acid cycle, the glyoxylate shunt, and the pathways for fatty acid degradation. Transcriptional control is exerted by anaerobic repression. Two regulatory genes are necessary: *arcA* at min 0 and *arcB* at min 69.5 (9, 14). The role of the Arc system, however, is not limited to repression. It appears that transcription of the *cydAB* operon encoding the O_2-scavenging cytochrome *d* terminal oxidase complex is under joint activation by the Arc system and Fnr (5, 10). Fnr generally functions as a pleiotropic activator of genes involved in anaerobic respiration (42).

It turned out that *arcA* is the gene previously known as *dye*, *sfrA*, *fexA*, *msp*, or *seg*, depending on the nature of the study conducted (for references, see reference 14). Homology comparisons of the deduced amino acid sequence of the gene revealed it to be the regulator element of a family of two-component regulatory systems that respond to specific environmental changes in different bacterial species. Typically, such a system comprises a sensor protein with a conserved transmitter domain and a regulator protein with a conserved receiver domain. In almost every case, the sensor is a transmembrane protein, whereas the regulator is a cytoplasmic protein (3, 16, 19, 33, 36, 45). In several systems, it was shown that upon stimulation, the sensor undergoes autophosphorylation at a conserved His residue. The phosphoryl group is then transferred to a conserved Asp residue of the cognate regulator, whereupon it becomes functionally active (1, 4, 7, 8, 17, 18, 25, 27, 30, 31, 37, 45, 52). ArcA is unusual in that it appears to receive signals from two different sensor elements, ArcB and CpxA (16, 36, 51), and behaves in different manners depending on whether it is activated by one sensor or the other. This complex interaction is inferred from mutant analysis. Mutations in *arcB* affect the synthesis of enzymes of aerobic function but not the expression of the F plasmid; the opposite holds for mutations in *cpxA*. Mutations in *arcA* can result in both defects (11). Recently, the *cpxA* gene was also reported to be identical to a gene previously known under several other names on the basis of phenotypical changes observed in the mutants (35).

ArcB belongs to the subclass of sensors that contains both a transmitter and a receiver domain (16, 45, 46). This large sensor of 778 amino acid residues appears to have only two transmembrane segments with a periplasmic domain of about 7 amino acid residues near the N terminus. It is thus probable that this small periplasmic structure of ArcB merely reinforces the anchorage of ArcB so that the stimulus reception site remains within or near the inner face of the plasma membrane (16). So far, the stimulus itself has not been identified, although there is evidence that a compound in the membrane-associated respiration network plays the role when chemically reduced (10).

In this study, we used site-directed mutagenesis of *arcB* to test (i) the possibility that the only two Cys residues (at positions 180 and 240) in the protein play a role in redox sensing, (ii) the consequence of replacing the highly conserved His-292 residue in the transmitter domain, and (iii) the possible role of the receiver domain in the activity of signal transduction. Finally, we asked whether the expression of *arcB* itself is subject to respiratory regulation.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. All strains used were derivatives of *E. coli* K-12. The origins and genotypes of the bacteria, bacteriophages, and plasmids are given in Table 1. P1 *vir* phage was used for transductions. The *pcnB* mutation, which reduces the copy number of pBR322 in cells to about 3 or 4 (23), was cotransduced with a closely linked Tn*10* (90%). Each Tet^r transductant was purified. The inheritance of the *pcnB* mutation was scored by an indirect method. Cells of each transductant were transformed with pBR322 by selection on Luria-Bertani (LB) agar containing 100 μg of ampicillin per ml on a lawn of strain ECL525 (a derivative of MC4100). A transformant colony retaining *pcnB*$^+$ supported the growth of ECL525 satellites, whereas a colony inheriting *pcnB* did not because of the lowered level of β-lactamase. (ECL525 cells are slightly more resistant to ampicillin than typical K-12 strains.) The *arcA131* allele was cotransduced with the *thr*$^+$ gene. First, *thr-43*::Tn*10* in strain BW6164 was transduced into a desired recipient by selecting for Tet^r on LB agar. The transductants were purified on the same medium, and a threonine auxotroph was identified by failure to grow on glucose mineral agar without the supplement. The auxotroph was infected with P1 particles prepared on an *arcA131 thr*$^+$ strain (ECL981), and the transductants were selected and purified on glucose mineral medium. Coinheritance of the *arcA131* allele was scored for sensitivity to toluidine blue *o* (16). The *arcB1* allele was cotransduced with the *glnF*$^+$ gene. First, *glnF*::Tn*10* in strain YMC22 was transduced into a desired recipient by selecting for Tet^r on LB agar supplemented with glutamine (400 μg/ml). The transductants were purified on the same medium, and a glutamine auxotroph was identified by failure to grow on glucose mineral agar without the supplement. The auxotroph was infected with P1 particles prepared on an *arcB1 glnF*$^+$ strain (ECL590), and the transductants were selected and purified on glucose mineral medium. Coinheritance of the *arcB1* was scored for sensitivity to toluidine blue *o*. To transduce Δ*cpxA*, P1 particles grown on strain ECL358 bearing Δ(*rha-pfkA*)*15* and *zig-1*::Tn*10* (80% linked to the deletion) were used to infect the desired recipient. The Tet^r transductants were scored for loss of ability to ferment rhamnose. Since *cpxA* lies between the *rha* and *pfkA* genes, the loss of rhamnose-utilizing ability also indicates the deletion of *cpxA*. To transduce a Φ(*ompC-lac*) or Φ(*ompF-lac*) fusion, P1 particles grown respectively on strain MH225 or MH513 were used. The Φ(*ompC-lac*) transductants were selected for growth on lactose in a medium of high osmolarity (resulting from the addition of 0.3 M NaCl), and the Φ(ompF-lac) transductants were selected for growth on lactose of low osmolarity.

Growth conditions. For routine cultures, LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) was used. To prevent the loss of plasmid vectors bearing a drug resistance marker, ampicillin (100 μg/ml) or chloramphenicol (34 μg/ml) was added to the growth medium. For L-lactate dehydrogenase assays, the cells were grown at 37°C in a standard mineral medium at pH 7.6 buffered by 0.1 M 3-(*N*-morpholino)-propanesulfonate (13) and supplemented with 20 mM xylose, 0.5% casein acid hydrolysate, and 2 mM L-lactate. For β-galactosidase assays of strains bearing Φ(*sdh-lac*) or Φ(*arcB-lac*), cells were grown in a similar medium without L-lactate. For assaying the expression of Φ(*narC-lac*), 10 mM nitrate was added to the xylose-casein hydrolysate growth medium. For assaying the expression of Φ(*ompF-lac*) and Φ(*ompC-lac*) fusions, cells were grown in nutrient broth respectively in the presence or absence of 0.3 M NaCl. Cultures were grown aerobically with vigorous shaking to mid-exponential growth phase or anaerobically in screw-cap bottles filled to the top with the medium and left undisturbed overnight. To screen colonies for β-galactosidase activity, cells were plated on LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) at 40 μg/ml.

Test for sensitivity to MS2 phage. Cells were grown in LB medium to early stationary phase, and 0.2 ml of the culture was mixed with 2.5 ml of top LB agar. The mixture was layered over LB agar and left until solidified. The surface was then spotted with 5 μl of serially 100-fold-diluted MS2 phage and examined for plaques after overnight aerobic incubation.

DNA manipulation and transformation. λ DNA was prepared as described previously (16, 26). Plasmid DNA was prepared on a small scale by the boiling method. The cells were transformed by the $CaCl_2$ method (26).

Nucleotide-directed mutagenesis. Nucleotide-specific mutagenesis was carried out according to the method of Kunkel et al. (21) using a Muta-Gene mutagenesis kit (Bio-Rad). To obtain the desired single-stranded DNA, the following procedures were carried out. Strain CJ236 was transformed with plasmid pBB12 containing an *arcB*$^+$ gene. The transformants were then infected by a helper phage, VCS-M13 (multiplicity of infection of about 20) and grown overnight on LB medium containing chloramphenicol and ampicillin. The synthesized single-stranded pBB12 plasmids containing uracil and bearing the complementary *arc*$^+$ sequence were collected from the supernatant fraction of the centrifuged culture. The single-stranded DNA in the supernatant fraction was precipitated by the addition of 0.25 volume of a solution containing 20% polyethylene glycol and 3.75 M ammonium acetate. The precipitate was dissolved with 10 mM Tris and 1 mM EDTA at pH 8 and extracted twice with an equal volume of chloroform and twice with an equal volume of phenol. Ammonium acetate (2.5 M final concentration) was then added to the remaining aqueous phase, and two volumes of ethanol were added. The precipitate was washed with 70% ethanol and finally dissolved in water.

PCR. Chromosomal DNA was extracted from cells in 1.5 ml of overnight LB cultures by the lysozyme-sodium dodecyl sulfate (SDS) method (28). The mixture for the polymerase chain reaction (PCR) contained chromosomal DNA (0.5 μg), two primers (final concentration, 20 nM for each), and the necessary reagents (provided by Perkin-Elmer Cetus) in a final volume of 100 μl. The reaction was carried out for 30 cycles under conditions in which each cycle consisted of 1 min of denaturing at 94°C, 2 min of annealing at 55°C, and 3 min of polymerization at 72°C. The products were extracted once by chloroform prior to analysis or cloning.

DNA sequencing. Double-stranded plasmids were denatured for 5 min at 85°C in 0.2 N NaOH–4 mM EDTA (50). Oligomers containing 17 to 30 nucleotides were used as primers for sequencing the regions expected to contain the directed mutation. The priming sites were about 130 to 300 bases 5′ to the expected mutation site. Nucleotide sequences of the region of interest were determined by the dideoxynucleotide method with the modified T7 DNA polymerase system (Sequenase from United States Biochemical) employing [^{35}S]ATP.

Computer analysis. Program CALTECH was used for analyzing coding regions. Program PRSTRC and program BLAST in the computer facility of the Molecular Biology Computer Research Resource (Dana-Farber Institute) were

TABLE 1. *E. coli* K-12 strains, phages, and plasmids

Bacterium, phage, or plasmid	Genotype and relevant characteristics[a]	Derivation	Source or reference
E. coli strain			
AE1019	Hfr *cpxA2 arcB6 cpxB1 thyA argG6 fhuA2 gal-6 galP63? hisG1 lacY1 leuB6 mtlA2 malT1*(λ^r) *rfbD1? rpsL104 supE44 tonA2*		P. Silverman
AE1031	*cpxA*$^+$ *cpxB*$^+$ *metB1 zdb-1*::Tn*10*; otherwise as in AE1019		P. Silverman
BW6164	HfrRa-2(PO48) *thr-43*::Tn*10 mal-28 sfa-4 snpE42*		B. L. Wanner
MH225	F$^-$ Φ(*ompC-lac*) *10–25 araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 ptsF25 deoCl*		T. J. Silhavy
MH513	F$^-$ Φ(*ompF-lac*)*15–13* Δ(*argF-lac*)*U169 rpsL150 relAl flb-5301 pstF25 deoCl*		T. J. Silhavy
CJ236	*dut ung thi relA*/pCJ105(Cm^r)		21
JM109(DE3)	*endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lac-proAB*) DE3/[F′ *proA*$^+$*B*$^+$ *lacI*^{q}ZΔ*M15*]		Promega
XL1-Blue	*endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* (*lac-proAB*)/[F′ *proA*$^+$*B*$^+$ *lacI*^{q}ZΔ*M15* Tn*10*]		Stratagene
YMC22	*glnF*::Tn*10* Δ(*argF-lac*)*U169 endA hsr thi hut*$^+_{Klebs}$ *hutC*$_{Klebs}$		B. Magasanik
ECL358	F$^-$ Δ(*rha-pfkA*)*15 zig-1*::Tn*10 endA hsdR* Δ(*araF-lac*)*U169* Φ(*fucO-lac*) *thi*		D. F. Fraenkel 2
ECL525	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 ptsF25 deoCl* Δ*frd-101*		14
ECL547	*sdh*$^+$ Φ(*sdh-lac*); otherwise as in ECL525		14
ECL561	Φ(*narC218-lac*) Δ*zcg-622*::Tn*10 araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 pstF25 deoCl gyrA non*		12, 44
ECL590	*sdh*$^+$ Φ(*sdh-lac*) *arcB1*; otherwise as in ECL525		9
ECL932	*pcnB80 zad*::Tn*10 arcB1* Δ*zgi*::Tn*10 endA hsdR* Δ(*argF-lac*)*U169 thi*		16
ECL964	*sdh*$^+$ Φ(*sdh-lac*) *pcnB80 zad*::Tn*10*	P1(ECL932) × ECL547	This study
ECL965	*sdh*$^+$ Φ(*sdh-lac*) *arcB1 pcnB80 zad*::Tn*10*	P1(ECL932) × ECL590	This study
ECL967	sdh$^+$ Φ(*sdh-lac*) *thr-43*::Tn*10*	P1(BW6164) × ECL547	This study
ECL968	*sdh*$^+$ Φ(*sdh-lac*) *arcA131*	P1(ECL981) × ECL967	This study
ECL969	*sdh*$^+$ Φ(*sdh-lac*) *arcB1 thr-43*::Tn*10*	P1(BW6164) × ECL590	This study
ECL970	*sdh*$^+$ Φ(*sdh-lac*) *arcB1 arcA131*	P1(ECL981) × ECL969	This study
ECL971	*glnF*::Tn*10*	P1(YMC22) × ECL525	This study
ECL972	*arcB1*	P1(ECL590) × ECL971	This study
ECL973	*arcB1 pcnB80 zad*::Tn*10*	P1(ECL932) × ECL972	This study
ECL974	Φ(*ompC-lac*)*10–25 arcB1 pcnB80 zad*::Tn*10*	P1(MH225) × ECL973	This study
ECL975	Φ(*ompF-lac*)*15–13 arcB1 pcnB80 zad*::Tn*10*	P1(MH513) × ECL973	This study
ECL976	Φ(*ompC-lac*)*10–25 pcnB80 zad*::Tn*10*	P1(ECL932) × MH225	This study
ECL977	Φ(*ompF-lac*)*15–13 pcnB80 zad*::Tn*10*	P1(ECL932) × MH513	This study
ECL978	Φ(*narC218-lac*) *pcnB80 zad*::Tn*10*	p1(ECL932) × ECL561	This study
ECL979	Φ(*arcB-lac*)	Insertion of λ p*lac*Mu9 into ECL525 and P1 transduction of fusion into ECL525	This study
ECL980	Φ(*arcB-lac*) *pcnB80 zad*::Tn*10*	P1(ECL932) × ECL979	This study
ECL981	*sdh*$^+$ Φ(*sdh-lac*) *arcA131*	Spontaneous mutation	This study
ECL982	*sdh*$^+$ Φ(*sdh-lac*) *arcA131 pcnB80 zad*::Tn*10*	P1(ECL932) × ECL968	This study
ECL983	*sdh*$^+$ Φ(*sdh-lac*) *arcB1 arcA131 pcnB80 zad*::Tn*10*	P1(ECL932) × ECL970	This study
Phage and plasmids			
DE3	λ *lacI* Φ(*lacUV5-T7 gene1*) *i21 nin5*		47
MS2	Male-specific phage		
P1 *vir*			
λ3G10	Encoding *arcB* gene region		20
VCS-M13	Kn^r in IG region (helper phage)		Stratagene
pBR322			
pBluescript SK−	f1(−) Ap^r		Stratagene
pCJ105(Cm^r)	F′ Cm^r		21
pBB12	$arcB_{\Delta p}$ in pBluescript KS+		16
pBB25	*arcB*$^+$ in pBluescript SK−	See text	This study
pBB31	*arcB*$^+$ in pBR322	See text	This study
pBB32	$arcB^{Cys\text{-}241\text{-}Gly}$ in pBR322	See text	This study
pBB33	$arcB^{His\text{-}292\text{-}Gln}$ in pBR322	See text	This study
pBB34	$arcB^{Asp\text{-}533\text{-}Ala}$ in pBR322	See text	This study
pBB35	$arcB^{Asp\text{-}576\text{-}Ala}$ in pBR322	See text	This study
pBB36	$arcB^{S517}$ in pBR322	See text	This study
pBB41	$arcB^{S517\ \Delta H}$ in pBR322	See text	This study
pBB42	$arcB^{S516}$ in pBR322	See text	This study
pBB43	$arcB^{+\ \Delta E}$ in pBluescript SK−	See text	This study
pBB44	$arcB^{His\text{-}292\text{-}Gln\ \Delta E}$ in pBluescript SK−	See text	This study
pBB46	$arcB^{Asp\text{-}576\text{-}Ala\ \Delta E}$ in pBluescript SK−	See text	This study
pBB47	$arcB^{S517\ \Delta E}$ in pBluescript SK−	See text	This study
pBB49	$arcB^{S517\ \Delta H\ \Delta E}$ in pBluescript SK−	See text	This study
pBB50	$arcB^{S516\ \Delta E}$ in pBluescript SK−	See text	This study

[a] Symbols: ΔE, *Eco*RI-*Eco*RI deletion within the promoter of *arcB*; ΔH, *Hin*cII-*Hin*cII deletion within the coding region of *arcB*; *arcB*^{S}, *arcB* encoding a segment without the receiver domain (Fig. 1 and 3).

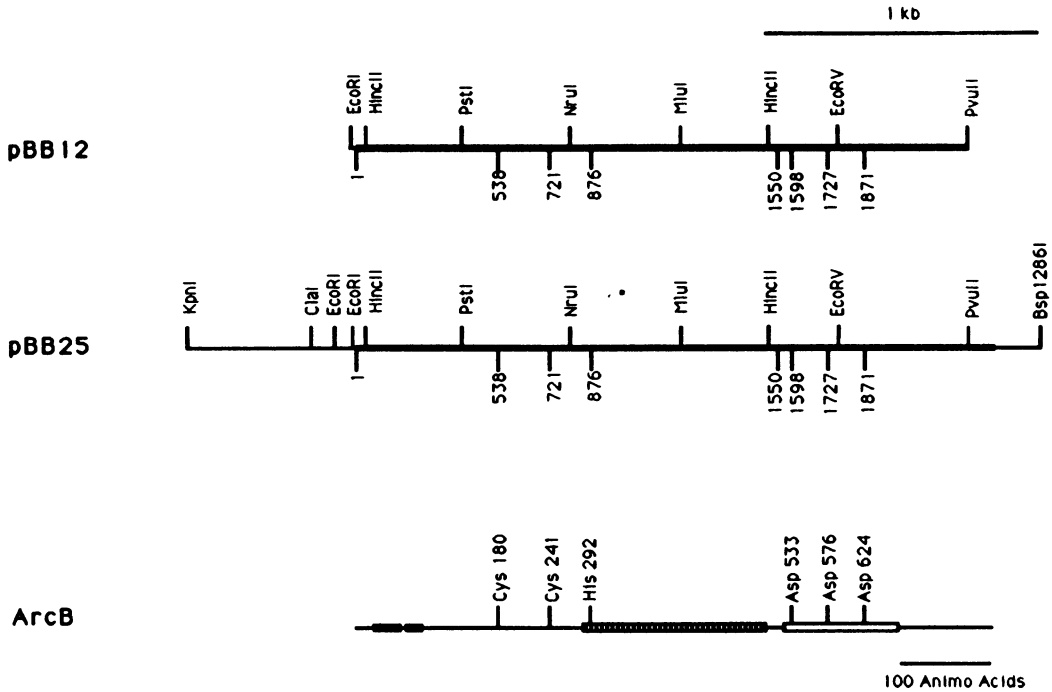

FIG. 1. Physical map of *arcB*$^+$ in plasmids and the ArcB product. Numbers on thick line indicate base pair numbers counted from A of the translation start site ATG. The coding region of the *arcB* gene in pBB12 is inserted between the *Eco*RI and *Eco*RV sites of pBluescript KS+. The complete *arcB*$^+$ gene in pBB25 is inserted between the *Kpn*I and *Sma*I sites in pBluescript SK− (see Results).

used for protein secondary-structure analysis and a homology search against GenBank date base.

Analysis of proteins encoded by plasmids. The structural gene *arcB* used in this analysis lies downstream of the T7 promoter. Transcription for the structural gene could therefore be greatly increased when the T7 polymerase encoded by DE3 [λ *lacI* Φ(*lacUV5-T7 gene1*)] in the host strain JM109 DE3 was induced by isopropyl β-D-thiogalactopyranoside (IPTG). Strain JM109 DE3 transformed with the plasmid vector or plasmid containing *arcB* was grown to about 150 Klett (no. 42 filter) units with shaking in 10 ml of mineral medium (49) supplemented with 1% casein acid hydrolysate and ampicillin. Cells from 1 ml of each culture were collected by centrifugation, washed once with 5 ml of the mineral medium, suspended in the same volume of the medium containing ampicillin and a mixture of 18 amino acids (each added at 0.02%) without methionine and cysteine, and shaken at 37°C. After 1 h, IPTG was added at 1 mM for 30 min to enhance the synthesis of the *arcB* product. A sample of each culture (0.5 ml) was then treated with rifampin at 200 μg/ml for 20 min, mixed with 10 μCi of [^{35}S]methionine (1 Ci/μmol), and incubated for 5 min (48). The labeled cells were collected by brief centrifugation, suspended in 50 μl of loading buffer (39), and boiled for 5 min. Samples (10 μl) were analyzed by SDS-acrylamide gel (10%) electrophoresis (22) with a minigel apparatus (Bio-Rad MINI 2-D). The dried gel was exposed to X-OMAT AR Kodak diagnostic film.

Enzyme assays. For flavin-linked L-lactate dehydrogenase assay, the harvested cells were washed once with 30 ml of 10 mM potassium phosphate (pH 7.0) and suspended in the same buffer (4 volumes cell wet weight) for sonic disruption. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min. The crude extract was used to determine the enzyme activity with phenazine methosulfate as electron carrier and 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide as terminal electron acceptor at 30°C (14). Protein concentration was estimated with bovine serum albumin as a standard (24). Specific activity units of the enzyme were expressed in nanomoles per minute per milligram of protein. β-Galactosidase activity was assayed in whole cells (rendered permeable by the addition of 0.1% SDS and 2 drops of chloroform to the cell suspension) at 30°C by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactoside at 420 nm. The specific activity was expressed in units by the method of Miller (29).

Nucleotide sequence accession number. The promoter sequence of *arcB* has been assigned the accession number M90703 by GenBank.

RESULTS

Cloning of the entire *arcB* gene and sequencing of its promoter region. In a previous study, we used plasmid pBB12 to sequence the coding region of the *arcB* gene and subsequently determined the cellular distribution of the protein. Although the *arcB* insert lacked the natural promoter region, a low level of expression was made possible by a vector promoter (16). For the present study, we recloned the entire gene by starting with λ3G10 containing the 69.5-min region on the chromosome (20). First, a 4.8-kb fragment in λ3G10 was excised by *Kpn*I and *Hin*dIII and ligated to the *Kpn*I-*Hin*dIII site of pBluescript SK− vector. The unnecessary 2.4-kb sequence downstream of *arcB* was removed with *Bsp* 1286 and *Sma*I. After blunting the *Bsp* 1286 site with the Klenow fragment, the shortened DNA was religated to give plasmid pBB25 (Fig. 1). Nucleotide sequence analysis of the upstream *arcB* coding region indicated putative −10 and −35

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CACTGCCGGAGAAATGATTCGCCATACGCCACCGGAAGTGATCTACCACCGCATTTCCGC  -238

                                                    ClaI
CAGCGCCCGTCGCCCGACGCTGCTTGCTCCGCTGTGGTGCGAAAATCGATGGACGGGAAT  -178

GGTCGAGCTGGATCGCTATCTGAATGAACATGGCGTACAGGGATCGGCGCTGGGACGTCC  -118

                                  ECoRI
GTGGCTACCTCCAACGGAGTAGGTCGTTGAGGGGAATTCCGCATTTCTCACACAATTTAT   -58

                                                        EcoRI
AACGTAACTGTCAGAATTGGGTATTATTGGGGCAGGTTGTCGTGAAGGAATTCCCTAATG     3
-35                  -10                  SD
```

FIG. 2. DNA sequence of *arcB* promoter region. The predicted −35 and −10 sequences, ribosome-binding site, and translation start site ATG are underlined. The A of the start codon is designated number 1 in the sequence. SD, Shine-Dalgarno sequence.

```
                                    1550
                                     |
arcB        ... GAT GAT GCG TTT GAT GAA GAC GAT ATG CCT TTA CCG ...
                Asp Asp Ala Phe Asp Glu Asp Asp Met Pro Leu Pro
                           516 517

                                    1550
                                     |
arcB^S516   ... GAT GAT GCG TTT_TGA AGACGATATGCCTTTACCG ...
                Asp Asp Ala Phe END
                           516

                                    1550
                                     |
arcB^S517   ... GAT GAT GCG TTT GGA TGA AGACGATATGCCTTTACCG ...
                Asp Asp Ala Phe Gly END
                           516 517
```

FIG. 3. Site-directed mutagenesis of *arcB*, resulting in a truncated protein.

sequences. The consensus is well conserved at the −10 region but less so at the −35 region (Fig. 2).

Construction of *arcB* mutant plasmids. Six plasmids bearing a point mutation in *arcB* were generated by nucleotide-directed mutagenesis of pBB12 (Fig. 1), and the mutation was verified by DNA sequencing of the appropriate region. Each plasmid was transformed into strain ECL932 (*arcB1*) to test the effect of the altered ArcB on anaerobic induction of L-lactate dehydrogenase. Among the six amino acid substitution mutations, the $arcB^{Cys\text{-}180\text{–}Gly}$ and $arcB^{Asp\text{-}624\text{–}Ala}$ changes gave no mutant phenotype. Therefore, only the other four mutant alleles with single amino acid substitutions, $arcB^{Cys\text{-}241\text{–}Gly}$, $arcB^{His\text{-}292\text{–}Gln}$, $arcB^{Asp\text{-}533\text{–}Ala}$, and $arcB^{Asp\text{-}576\text{–}Ala}$, were studied further. To avoid possible occurrence of accidental mutations outside the sequenced region during the mutagenic procedure, each piece of DNA (several hundred base pairs long) containing the desired mutation was excised from the plasmid vector by suitable restriction enzymes. The segments between *Pst*I and *Nru*I including nucleotide 721 (Cys-241), that between *Nru*I and *Mlu*I including nucleotide 876 (His-292), and those between *Mlu*I and *Eco*RV including nucleotide 1598 (Asp-533) or 1871 (Asp-576) were used to replace the corresponding region of the wild-type *arcB* gene in plasmid pBB25 (Fig. 1). The reconstituted *arcB* mutant genes were sequenced from junction to junction, and no undesired base change was found. Finally, in order to exclude the remnant *lacZ* in pBluescript SK−, the mutant *arcB* alleles in pBB25 were excised between the *Cla*I site upstream of the gene and the *Bam*HI site of the vector and ligated to vector pBR322.

Two plasmids bearing *arcB* alleles ($arcB^{S516}$ and $arcB^{S517}$), which produce proteins that contain the transmitter domain ($ArcB^{S}$) on the amino-terminal side but not the receiver domain on the carboxyl-terminal side, were constructed by a method similar to that described above. The $arcB^{S516}$ mutation created a TGA stop codon by loss of GA at nucleotide positions 1499 and 1550 (Fig. 3). The $arcB^{S517}$ mutation created a TGA stop codon by insertion of a G at 1550. Therefore, $arcB^{S517}$ differed from $arcB^{S516}$ only by the additional presence of codon 517 specifying a Gly residue.

Products of *arcB* genes. Multicopy plasmids bearing $arcB^{+}$ or its mutant allele retarded host cell growth, probably because of oversynthesis of the membrane protein. Removal of the *Eco*RI-*Eco*RI site in the promoter region overcame the growth impairment (Fig. 2) but still allowed the host to produce abundant [^{35}S]methionine-labeled ArcB for molecular weight determination by SDS-polyacrylamide gel electrophoresis (Fig. 4). $ArcB^{+}$, $ArcB^{His\text{-}292\text{–}Gln}$, and $ArcB^{Asp\text{-}576\text{–}Ala}$ migrated at the rate expected of a 80-kDa protein. By the same criterion, $ArcB^{S516}$ and $ArcB^{S517}$ were about 50 kDa. No polypeptide product was detected when a *Hin*cII-*Hin*cII deletion of $arcB^{S517}$ was made.

Characterization of *arcB* and *arcA* mutations in the chromosome. The chromosomal alleles $arcB^{+}$ (in strain ECL547) and *arcB1* (in strain ECL590) were amplified by PCR, with one primer spanning nucleotides 169 to 185 and the other spanning nucleotides 2420 to 2436. Agarose gel electrophoresis of the amplified product revealed that whereas the wild-type chromosome provided a 2.4-kb DNA fragment, the *arcB1* chromosome provided a 3.7-kb product. Comparison of digestion of products derived from $arcB^{+}$ and *arcB1* chromosomes with *Pst*I, *Xho*I, *Mlu*I, or *Pvu*II indicated that an extra sequence was inserted between *Xho*I and *Mlu*I in *arcB1* (Fig. 5). *Eco*RV digestion of the $arcB^{+}$ product

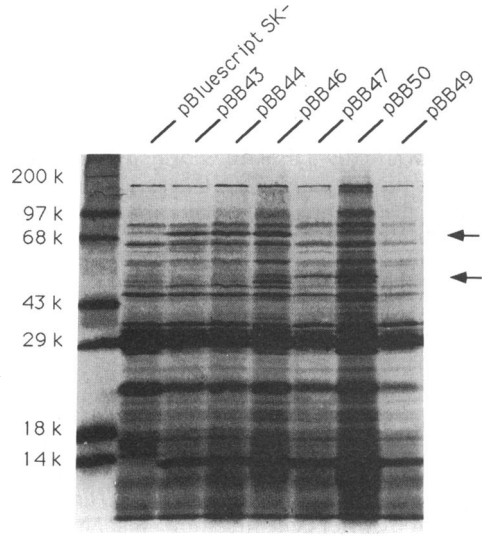

FIG. 4. Identification of *arcB* products by electrophoresis of [^{35}S]methionine-labeled SDS cell lysates. IPTG was used to induce synthesis of the *arc* proteins. For details, see Materials and Methods. Numbers on the left are molecular sizes of marker proteins in kilodaltons. Inserts in the vector pBluescript SK− are $arcB^{+\Delta E}$ (pBB43), $arcB^{His\text{-}292\text{–}Gln\ \Delta E}$(pBB44), $arcB^{Asp\text{-}576\text{–}Ala\ \Delta E}$(pBB46), $arcB^{S517\ \Delta E}$(pBB47), $arcB^{S516\ \Delta H}$(pBB50), and $arcB^{S517\ \Delta H\ \Delta E}$(pBB49) (ΔE indicates an *Eco*RI-*Eco*RI deletion within the promoter of *arcB*, ΔH indicates a *Hin*cII-*Hin*cII deletion within the coding region of *arcB*, and $arcB^{S}$ indicates segment without the region encoding the receiver domain) (see also Fig. 1 and 3). Arrow indicates product of wild-type or mutant *arcB* gene.

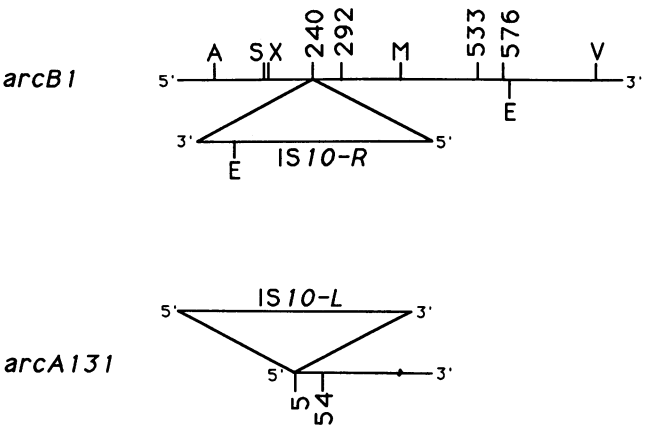

FIG. 5. *arcB1* and *arcA131* mutations. Numbers represent positions of deduced amino acid residues. Abbreviations: A, *Acc*I; S, *Pst*I; X, *Xho*I; M, *Mlu*I; E, *Eco*RV; V, *Pvu*II; and IS, insertion sequence element.

yielded two fragments, whereas digestion of the *arcB1* product yielded at least three fragments. This showed that the insertion sequence introduced an extra *Eco*RV site(s) in *arcB1*. To identify the exact location of the insertion, a fragment from the *arcB1* product was excised by *Pst*I and *Eco*RV and cloned into the pBluescript KS+ vector. Partial DNA sequencing pinpointed the insertion at the third position of codon 240, converting the following codon from TGC (Cys) to the stop codon TGA (Fig. 4). A homology search of the nucleotide sequence in the GenBank data base showed the 30 bp of the 5′ end of the insertion sequence to be identical to the 5′ end of the complementary strand of IS*10*-R (6).

The *arcA131* (in strain ECL981) and wild-type (in strain ECL547) alleles were amplified with one primer spanning nucleotides 43 to 81 (modified to create an *Eco*RI site) and the other spanning nucleotides 795 to 836 (modified to create a *Hin*dIII site). Digestion by *Eco*RI and *Hin*dIII showed that the PCR product of the *arcA131* allele was about 1.3 kb longer than that of the *arcA*$^+$ allele. Cloning and partial sequencing of the *arcA131* product showed that the insertion was at the third position of the fifth codon, converting the following codon from ATT (Ile) to the stop codon TGA. The 60 bp at the 5′ end of the insertion is identical to the 5′ end of IS*10*-L (Fig. 5).

Effects of plasmid-borne *arcB*$^+$ and mutant alleles on expression of a target operon. In strain ECL964 (*arcB*$^+$ *arcA*$^+$), anaerobic repression of Φ(*sdh-lac*) was 26-fold. In strain ECL965 (*arcB1 arcA*$^+$), both the aerobic and the anaerobic expression levels exceeded the wild-type aerobic level. Control experiments showed that the presence of the pBR322 vector itself did not have any significant effect on aerobic and anaerobic expressions in the hosts. When an *arcB*$^+$-bearing plasmid was introduced into the *arcB1 arcA*$^+$ background, the wild-type expression pattern was restored (Table 2).

When the plasmid bearing *arcB*$^{Cys\text{-}241\text{-}Gly}$ was introduced in the *arcB1 arcA*$^+$ host, both aerobic and anaerobic expression of Φ(*sdh-lac*) exceeded the corresponding levels observed with the plasmid bearing *arcB*$^+$, but the degree of anaerobic repression was not significantly altered. In contrast, when the plasmid bearing *arcB*$^{His\text{-}292\text{-}Gln}$ was introduced in the *arcB1 arcA*$^+$ host, anaerobic repression of Φ(*sdh-lac*) failed to occur. Moreover, both aerobic and anaerobic expression levels exceeded the aerobic level of the same host transformed with the plasmid bearing *arcB*$^+$ (Table 2).

The functional importance of the receiver domain was demonstrated by genetically truncating ArcB. When the plasmid bearing the *arcB*^{S516} allele was introduced, surprisingly, aerobic expression of Φ(*sdh-lac*) was down modulated but the anaerobic expression was up modulated. Consequently, the aerobic/anaerobic expression ratio was decreased to 2. Tests with the plasmid bearing the *arcB*^{S517} allele containing the transmitter but not the receiver domain gave similar results (Table 2). The results suggest that the receiver domain is necessary for proper regulation of the transmitter activity. The plasmid harboring *arcB*$^{Asp\text{-}533\text{-}Ala}$ or *arcB*$^{Asp\text{-}576\text{-}Ala}$ in the *arcB1* host did not confer any regulatory effect on Φ(*sdh-lac*). In the *arcB*$^+$ background, the wild-type pattern of Φ(*sdh-lac*) expression was observed with plasmids bearing any of the mutant *arcB* alleles (data not shown).

***arcA131* mutation is epistatic over the *arcB* mutations.** Assuming that the respiratory signal from ArcB is mediated only by ArcA, all *arcB* mutations, whether plasmid borne or

TABLE 2. Effect of *arcB* and *arcA* alleles on Φ(*sdh-lac*) expression

arcB in plasmid[a]	*arcB* on chromosome[b]	β-Galactosidase activity (U)					
		arcA$^+$ on chromosome			*arcA131* on chromosome		
		$+O_2$	$-O_2$	$+O_2/-O_2$	$+O_2$	$-O_2$	$+O_2/-O_2$
None[c]	*arcB*$^+$	600	23	26	1,200	2,300	0.5
None[c]	*arcB1*	730	1,000	0.7	1,500	2,300	0.7
arcB$^+$	*arcB1*	540	21	26	1,300	2,100	0.6
arcB$^{Cys\text{-}241\text{-}Gly}$	*arcB1*	1,100	55	20	1,500	2,400	0.6
arcB$^{His\text{-}292\text{-}Gln}$	*arcB1*	1,200	1,200	1	1,400	2,100	0.6
arcB^{S516}	*arcB1*	150	77	1.9	1,300	2,100	0.7
arcB^{S517}	*arcB1*	270	77	3.5	1,400	2,100	0.6
arcB$^{S517\ \Delta H}$	*arcB1*	810	1,100	0.7	1,400	2,300	0.6
arcB$^{Asp\text{-}533\text{-}Ala}$	*arcB1*	630	810	0.8	1,500	2,200	0.7
arcB$^{Asp\text{-}576\text{-}Ala}$	*arcB1*	690	920	0.8	1,400	2,100	0.7

[a] All plasmids are listed in Table 1.
[b] Strain ECL964 was used as *arcB*$^+$ *arcA*$^+$, strain ECL965 was used as *arcB1 arcA*$^+$, strain ECL982 was used as *arcB*$^+$ *arcA131*, and strain ECL983 was used as *arcB1 arcA131* host.
[c] pBR322 was used as the control plasmid.

in the chromosome, should give the same phenotype in an *arcA* null (*arcA131*) background. Table 2 shows that this is essentially the case. It might also be noted that the *arcA131* mutation raised both aerobic and anaerobic expression levels of Φ(*sdh-lac*) twofold over the corresponding levels in the *arcA*$^+$ *arcB1* background. Why these elevations occurred is not clear. To explore whether CpxA might be involved, the Δ*cpxA* allele was transduced into strains ECL547 (*arcB*$^+$ *arcA*$^+$), ECL590 (*arcB1 arcA*$^+$), and ECL968 (*arcB*$^+$ *arcA131*). When aerobic and anaerobic levels of Φ(*sdh-lac*) expression were assayed, the increase of Φ(*sdh-lac*) expression by the *arcA131* was still observed in the Δ*cpxA* background (data not shown).

Lack of helix-turn-helix structure in ArcB receiver domain. Many transcriptional regulatory proteins, including regulator elements of the two-component systems, possess in their C-terminal portions a helix-turn-helix structure for DNA sequence recognition. Using the program PRSTRC, we identified a helix-turn-helix motif in ArcA. The motif was well aligned with a typical regulator protein, the λ repressor (34). In contrast, no such motif was identified in the ArcB receiver domain by the same program.

Effect of deletion of the ArcB receiver domain on sensitivity to F-pilus-specific phage MS2. Because the mutations in *arcB*^{S} (*arcB* encoding a segment without the receiver domain) modified the expression of Φ(*sdh-lac*), we also looked for a possible effect of *arcB*^{S516} on F-pilus synthesis, which appears to be controlled by ArcA via CpxA. The synthesis was examined by sensitivity of the cells to an RNA phage for which the F pilus serves as a receptor (40). When transformed with the multicopy vector pBR322, cells of strain AE1019 (*cpxA2 arcB6*) gave no visible plaques, whereas cells of strain AE1031 (*cpxA*$^+$ *arcB6*) gave clear plaques. When transformed with pBB31 (*arcB*$^+$) or pBB42 (*arcB*^{S516}), AE1019 cells gave very turbid plaques, whereas AE1031 cells gave clearly visible plaques. Thus, the deletion of the receiver domain did not affect pilus synthesis.

***ArcB*^{S516} does not communicate with other two-component systems.** Since even without the receiver domain, Arc^S retained some regulatory properties for Φ(*sdh-lac*) expression, we wondered whether the shortened protein has also altered its signal transduction specificity so that it could communicate with regulator elements of the two-component system other than ArcA. A good candidate for such "cross-talk" would be OmpR, one of the proteins most homologous to ArcA, which regulates the synthesis of the outer membrane porins OmpF and OmpC. We therefore compared plasmids bearing *arcB*$^+$ or *arcB*^{S516} for their effects on aerobic expression of Φ(*ompF-lac*) and Φ(*ompC-lac*) under either hyper- or hypotonic growth conditions both in *arcB1* chromosomal (strain ECL974 or ECL975) and *arcB*$^+$ chromosomal (strain ECL976 or ECL977) backgrounds. No significant effect was observed with either *arcB1* or *arcB*$^+$ (data not shown). We also examined the expression of the nitrate reductase operon, which is controlled by another two-component system with *narX* as the sensor and *narL* as the regulator. No effect on aerobic or anaerobic expression of Φ(*narC-lac*) in the presence or absence of the inducer nitrate in strain ECL978 was observed with plasmids bearing *arcB*$^+$ or *arcB*^{S} (data not shown).

ArcB is synthesized both aerobically and anaerobically. We constructed an operon, Φ(*arcB-lac*), to test whether its expression is under respiratory control. From a population of cells with *lacZ* fusions resulting from random λ p*lac*Mu*9* insertions into the chromosome (14), clones sensitive to toluidine blue were isolated and genetically purified by transduction into strain ECL525 by selection of kanamycin resistance and scored for blue color on X-Gal agar. Possible candidates with Φ(*arcB-lac*) were screened by transduction with P1 particles grown on cells containing *zgi*::Tn*10* near *arcB*$^+$ (85% linkage). A clone (ECL979) that showed 90% linkage of Tc^r to the toluidine blue sensitivity was verified for the expected Φ(*arcB-lac*) fusion by PCR analysis (Fig. 6). When primer 5 (spanning nucleotides 165 to 149 of the *lacZ* structural gene) was used together with primer 1 (spanning nucleotides −249 to −237), primer 2 (spanning nucleotides 1135 to 1154), or primer 3 (spanning nucleotides 1470 to 1487), the sizes of the PCR products were respectively 3.9, 2.6, and 2.3 kb. In contrast, when primer 5 was used together with primer 4 (spanning nucleotides 2130 to 2147 of *arcB*), there was no PCR product. Primer 4, however, did give a PCR when used with primer 6 (spanning nucleotides 2420 to 2436), indicating that primer 4 was capable of annealing to the DNA. The results together show that *lacZ* was inserted roughly between the positions of primers 3 and 4.

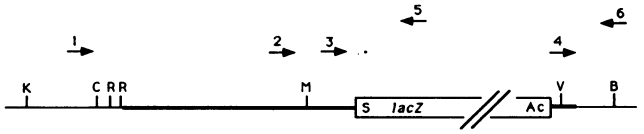

FIG. 6. PCR strategy. Arrows indicate the rough positions of primers used. The corresponding positions in *arcB* are as follows: 1, −249 to −237; 2, 1135 to 1142; 3, 1470 to 1487; 4, 2130 to 2147; and 6, 2420 to 2436. Primer 5 corresponds to *lacZ* structural gene bp 165 to 149 in λ p*lac*Mu*9*. Abbreviations: S, S end; c, C end; A, gene A; K, *Kpn*I; C, *Cla*I; R, *Eco*RI; M, *Mlu*I; V, *Pvu*II; B, *Bst* 1286.

When strain ECL980 bearing this Φ(*arcB-lac*) fusion was assayed for its activity levels of β-galactosidase, gene expression appeared to be a little higher under aerobic than anaerobic growth conditions (data not shown). To restore ArcB function, the fusion strain was transformed by an *arcB*$^+$-bearing plasmid. The pattern of β-galactosidase activity levels remained essentially the same. Thus, *arcB* is expressed both aerobically and anaerobically.

DISCUSSION

This exploratory study of a function-to-structure relationship allowed us to make several conclusions about the sensor ArcB, which is a transmembrane protein. Its two Cys residues are not implicated in the detection of redox changes in the environment, since either residue could be substituted for by Gly without significant changes in anaerobic repression of a target operon. The highly conserved His-292 in the transmitter domain, however, is important. Replacement of the residue by Gln abolished the repressive function. By analogy with other two-component systems, the result suggests that upon stimulation, His-292 undergoes autophosphorylation. The phosphoryl group should then be transferred to a conserved Asp residue in ArcA, the regulator protein. This hypothesis was recently confirmed in vitro by showing phosphoryl group transfer from [γ-^{32}P]ATP to ArcB (truncated from the N terminus to solubilize the protein) and from ArcB to ArcA (unpublished data). The importance of the conserved Asp-533 and Asp-576 in the receiver domain of ArcB for signal transduction suggests that at least one of these residues can also accept the phosphoryl group from His-292. Such intramolecular transphosphorylation was suggested for a homologous protein, FrzE (27). We also ob-

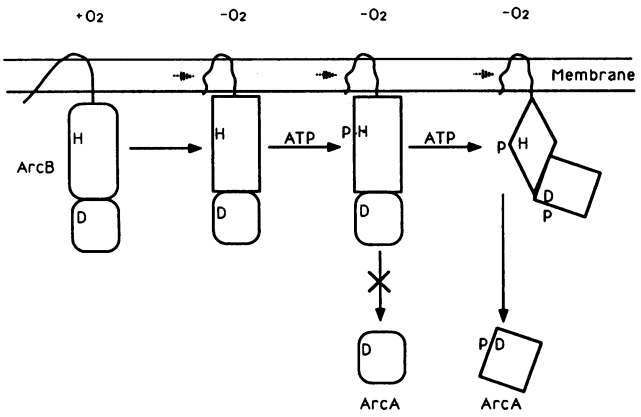

FIG. 7. A model of the signaling process of ArcB. Abbreviations: H, conserved His-292; D, conserved Asp-533 or Asp-576; P, phosphoryl group. Arrow drawn with broken line indicates stimulus. X indicates no signal transduction.

tained chemical evidence that ArcB can undergo phosphorylation at both a His and an Asp residue (unpublished data).

The lack of a helix-turn-helix motif in the ArcB receiver domain makes a direct role in target promoter recognition unlikely. The retention of some ability of ArcB^S (the ArcB polypeptide deprived of the receiver domain) to communicate with ArcA and the absence of its ability to influence the expression patterns of target operons under the control of OmpR or NarL indicate that the receiver domain is not there to prevent cross-talk between ArcB and noncognate regulators (32). The lack of difference between ArcB and ArcB^S in allowing F-pilus synthesis indicates that the receiver domain is not responsible for allowing ArcB to interact with ArcA in a way which is distinct from that of the other cognate sensor, CpxA (16, 36, 51). A possible function of the receiver domain is to play a role in modulating the signal transduction activity of ArcB to ArcA. Indeed, removal of the receiver domain still allowed some respiratory control of a target operon but narrowed the range. A simple model for signal transduction process by the Arc system is schematized in Fig. 7. Under highly oxidative environments, the nonphosphorylated form of the receiver domain of ArcB interferes with autophosphorylation and/or transphosphorylation to ArcA. Upon stimulation (possible accumulation of a reduced component of the electron transport system [15]), the resultant change in conformation of ArcB permits autophosphorylation at the His residue in the transmitter domain. The phosphoryl group is preferentially transferred to the Asp residue in the receiver domain. The histidyl residue then undergoes autophosphorylation again, and now the protein actively phosphorylates ArcA. Accordingly, the rate of signal transduction is modulated by the phosphorylation state of the receiver domain of ArcB. Since the regulatory machine has to constantly respond to environmental conditions, it can be predicted that dephosphorylation reactions of both ArcB and ArcA also occur. The model will be further tested in vitro with different mutant *arcB* products.

The synthesis of ArcB both aerobically and anaerobically allows the cell to respond immediately to the respiratory stimulus under any growth condition. There are two reasons for believing that ArcA is present at significant levels not only anaerobically but also aerobically. First, aerobic expression of the target operon, Φ(*sdh-lac*), was increased over the normal level by the *arcA131* mutation, indicating that the operon was under partial repression by ArcA in wild-type cells even during aerobiosis. Second, F-pilus synthesis and *traY* gene expression of the synthesis, which requires ArcA for activation, occur in wild-type cells both aerobically and anaerobically (11, 41). Thus, if there were respiratory regulation for the synthesis of ArcA, the range would be small. The lack of respiratory regulation of the Arc system contrasts with that of the FrzE and the Bvg systems, which are strongly regulated by the environmental stimulus (27, 38). The Arc system, therefore, should adapt more rapidly to the stimulus than the FrzE and Bvg systems.

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