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Characterization of Three Putative Sub-Domains in the Signal-Input Domain of the ArcB Hybrid Sensor in *Escherichia coli*[1]

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The ArcB sensor plays a crucial role in the histidine to aspartate (His-to-Asp) phosphorelay signal transduction, which is involved in the transcriptional regulatory network that allows *Escherichia coli* cells to sense various respiratory growth conditions. ArcB is one of the best-studied hybrid His-kinases involved in the multi-step His-to-Asp phosphorelay. However, a major question that remains to be elucidated is: how does ArcB sense an anoxic signal? The N-terminal region of ArcB is considered to be a signal-input domain, which probably plays a role in such signal-perception. In this study, this N-terminal region of ArcB was dissected into three putative sub-domains, a "transmembrane domain," a "leucine-zipper-like domain," and a "PAS-like domain." The importance of these structural domains was assessed *in vivo* and *in vitro* by systematically analyzing a number of *arcB* mutants, each of which encodes a mutant ArcB protein having an amino acid substitution or a deletion within one of these sub-domains. The results are discussed with special reference to the nature of the ArcB anaerobic sensor.

Key words: ArcB His-kinase, *Escherichia coli*, PAS domain, phosphorelay signal transduction.

The ArcB and ArcA components play a crucial role in the multi-step histidine-to-aspartate (His-to-Asp) phosphorelay signal transduction, which is implicated in the complex transcriptional regulatory network that allows *Escherichia coli* cells to sense various aerobic and anaerobic respiratory growth conditions (*1*, *2*). ArcB is a hybrid His-kinase, which has multiple (at least three) phosphorelay domains in its primary amino acid sequence, including a His-kinase, a receiver, and a HPt domain at its C-terminus (*3–6*), as schematically shown in Fig. 1. Based on recent intensive *in vitro* studies, a plausible schema of the complex circuitry of the multi-step ArcB-to-ArcA phosphotransfer circuit was proposed (*7*, *8*). First, His-292 in the ArcB His-kinase acquires the γ-phosphoryl group from ATP through its own catalytic activity. This reaction is essential for the subsequent phosphotransfer, and in fact the phosphoryl group on His-292 moves onto its intrinsic phospho-accepting aspartate (Asp-576) in the ArcB receiver. The His-717 in the HPt domain can also be modified by phosphorylation, in which His-292 and Asp-576 play a crucial role. The final destination of the phosphoryl group on His-717 is Asp-54 in the ArcA receiver. ArcA can also receive the phosphoryl group directly from His-292. In other words, ArcA acquires the phosphoryl group from either His-292 or His-717, presumably at the same aspartate site, Asp-54. Several models to explain to the physiological relevance of such a complicated dual-signaling mechanism were also proposed (*2*, *4*).

As to the ArcB sensor, two of the major questions that remain to be answered are: how does ArcB sense an anoxic signal, and what is the primary stimulus (stimuli)? It has been proposed that a redox state, perhaps an element of the electron-transport chain or proton motive force, may be a primary stimulus (both physiological and genetic experiments excluded O_2 itself as the signal) (*1*, *2*, *9*). It has also been proposed that a certain set of cytoplasmic metabolites, such as D-lactate, acetate, pyruvate, and NADH, may directly affect the activity of ArcB (*10*). In any event, the N-terminal portion of ArcB, which is generally referred to as "a signal input domain," probably plays a role in such a signal-perception (or signal-propagation). Nonetheless, the structure and function of this particular N-terminal region of ArcB have received little attention. In this study, we addressed this particular issue by dissecting the N-terminal region into three putative sub-domains, a "transmembrane domain," a "leucine-zipper domain," and a "PAS domain," although the latter two are solely hypothetical. Through intensive analyses employing a number of *arcB* mutants, the importance of these sub-domains of ArcB was assessed *in vivo* and *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Media—*E. coli* K-12 strain DAC-903 was mainly used: this was constructed previously (*4*, *5*) and is a derivative of MC4100 (F⁻*Δlac169 araD139 rpsL relA thiA flbB*). DAC903 carries an *arcB*::Cm^r allele and an *sdh-lacZ* transcriptional fusion gene on the chromosome at the λ att site. OG903 is an isogenic parental strain of DAC-903 and carries the wild-type *arcB* gene. These cells were grown in Luria-Bertani broth unless otherwise noted.

Plasmids—A set of pLIA-series of plasmids, listed in Table I, were constructed from pLIA001. The low-copy-number plasmid pLIA001 was constructed previously (*8*), and carries the wild-type *arcB* gene. This parental plasmid

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TABLE I **List of plasmids relevant to this study.**

Plasmids[a]	Amino acid substitutions in the ArcB sensor[b]
pLIA001	None
pLIA-ΔTM	Deletion (Asp-13 to Val-70)
pLIA-L80A	Leu-80 to Ala
pLIA-L87A	Leu-87 to Ala
pLIA-L94A	Leu-94 to Ala
pLIA-ΔPAS	Deletion (Glu-151 to Arg-264) Insertion (ASP)
pLIA-N181A	Asn-181 to Ala
pLIA-G189A	Gly-189 to Ala
pLIA-Q194A	Gly-194 to Ala
pLIA-G237A	Gly-237 to Ala
pLIA-C241G	Gly-241 to Gly

[a]These are low-copy-number plasmids, each of which carries the *arcB* gene. [b]On these plamids, the *arcB* gene was mutated by site-direction mutagenesis.

was subjected to extensive site-directed mutagenesis to construct the pLIA-series of plasmids. These constructs were all confirmed by DNA sequencing.

Enzyme Assay—β-Galactosidase activity was determined by Miller's method with slight modification (*11*) Cells were grown to the mid-logarithmic phase in Luria-Bertani broth under both standard aerobic and anaerobic conditions. The anaerobic growth conditions are as follows. Cells were first cultivated overnight under aerobic conditions, then an aliquot was inoculated into a screw-capped tube, previously filled with air-free medium. The tube was incubated without shaking at 37°C. For β-galactosidase assay, cells were collected and suspended in one volume of 250 mM sodium phosphate (pH 7.1) for accurate determination of the cell density. A portion of the cell suspension was subjected to β-galactosidase assay after permeabilization with toluene. Each figure represents triplicate cultures (for clarity, error bars are omitted).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was carried out according to the method of Laemmli (*12*).

Immunoblotting Analysis—Protein samples were separated by SDS-PAGE (12.5% acrylamide). Proteins on the gels were transferred onto nitrocellulose filters. Detection with anti-ArcB antiserum was carried out with the ELC Western blotting analysis system (Amersham Pharmacia Biotech), according to the supplier's instructions.

In Vitro Phosphorylation Experiment—*In vitro* phosphorylation experiments were carried out as described previously (*13*). Briefly, the cytoplasmic membranes containing ArcB and the ArcA response regulator were isolated as described previously (*8*, *14*). These samples were incubated together at 37°C for 20 min with 0.05 mM [γ-^{32}P]ATP (10,000 cpm/pmol) in TEDG buffer containing 200 mM KCl and 5 mM $CaCl_2$. The samples were immediately subjected to SDS-PAGE, followed by autoradiography

RESULTS AND DISCUSSION

Inspection of Structural Features of the N-Terminal Domain of ArcB—As schematically illustrated in Fig. 1, ArcB is a hybrid sensor kinase consisting of 776 amino acids, which contains three common His-to-Asp phosphorelay domains in its C-terminal part (His-kinase domain, receiver domain, HPt domain). The characteristic function of each of these domains has previously been well characterized *in vivo* and *in vitro*, in terms of the underlying mechanism of

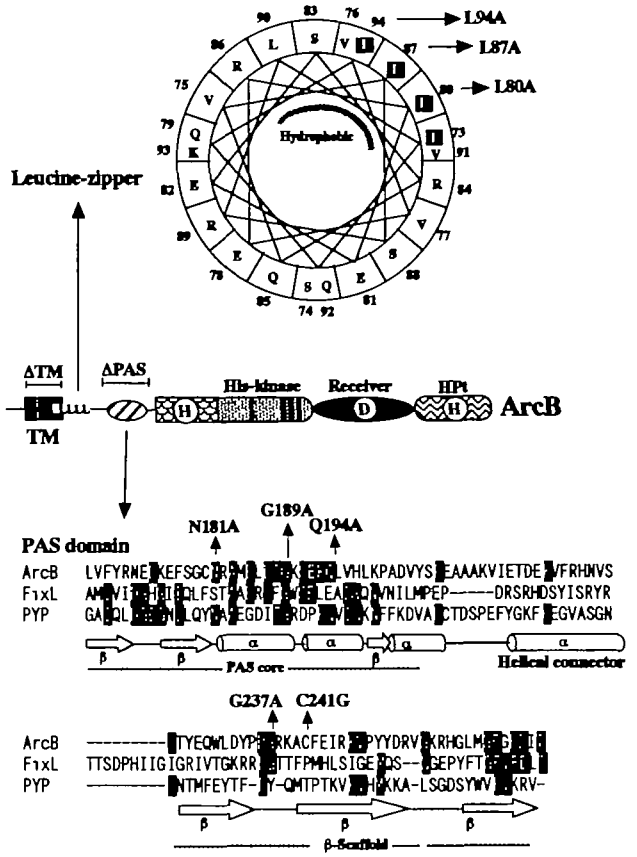

Fig. 1 **Schematic representation of the structural features of ArcB.** The structural features of ArcB are shown, namely, transmembrane domain (denoted by TM), leucine-zipper (denoted by LLLL), PAS domain, His-kinase domain, receiver domain, and HPt domain (from its N-terminal to C-terminal ends) The amino acid sequence of the putative leucine-zipper domain is plotted in an α-helical wheel The amino acid sequence of the putative PAS domain is aligned with those of FixL (*29*) and PYP (*30*), along with the secondary structure determined previously for PYP (*30*). The conserved amino acids among them are highlighted. Also shown schematically are the set of amino acid substitution and deletion mutants of ArcB constructed and characterized in this study (for details, see Table I).

His-to-Asp phosphorelay signal transduction (*1*, *2*, *4–8*, *10*, *15–17*). In addition, ArcB has an N-terminal extension, whose function has not yet been elucidated. This N-terminal region extends to the amino acid position of about 270. To gain insight into the nature of this N-terminal region, we examired its structural features. This revealed the occurrence of three intriguing types of sub-domains, referred to hereafter as the "transmembrane domain (TM)," the "leucine-zipper domain," and the "PAS domain" (Fig. 1).

In general, a typical His-kinase has two membrane-spanning sequences, which may be buried in the cytoplasmic membrane with a loop in the periplasmic space (*18*). ArcB also has two such stretches of hydrophobic amino acids (Phe-23 to Val-50, and Ser-58 to Val-77), each of which appears to be sufficient to traverse a lipid bilayer (*15*). However, ArcB is atypical in the sense that the membrane-spanning segments do not delimit a large periplasmic domain. In any case, the ArcB protein was recovered in the cytoplasmic membrane when *E. coli* cellular components were fractionated (*8*).

We found that the membrane-spanning regions are immediately followed by an amino acid stretch (Leu-73 to Leu-94), which shows a structural feature similar to a leucine-zipper. In this amino acid stretch, leucine is the first residue in each of the four contiguous heptad repeats (Fig. 1). If this particular sequence adopts an α-helical structure, it may create an amphipathic helix, as illustrated. In general, leucine-zippers are typically found in eukaryotic proteins (*19–22*). Several instances of bacterial leucine-zipper-like motifs are also known (*23*), and some of them are found in His-kinases (*24, 25*). These leucine-zipperes are generally considered to be important for homo- or heterodimer formation of proteins through a coiled-coil structure.

Finally, it was noted in a number of recent review articles that ArcB has a PAS domain (*26, 27*). PAS domains were first found in proteins associated with light and clock regulation in eukaryotes. They were named after the first three members to be identified, namely, the *Drosophila* clock protein (PER), the vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and the *Drosophila* single-minded protein (Sim) (*26*). More recently, PAS domains have been identified in a large family of prokaryotic proteins involved in sensing light, oxygen, redox status, and other signals (*26–28*). The proposed PAS domain in ArcB has been aligned extensively as one of them (*26, 27*). Such PAS domains are assumed to be important for protein–protein interaction and/or ligand binding. The amino acid sequence of the proposed PAS domain of ArcB (Phe-177 to Thr-267) was aligned with those of FixL (an oxygen sensor of *Bradyrhizobium japonicum*) (*29*) and PYP (a photoreceptor of *Ectothiorhodospira halophila*) (*30*), whose three-dimensional structures have recently been reported (Fig. 1). In fact, the putative PAS sequence of ArcB somewhat (but not strikingly) resembles those of FixL and PYP.

Thus, it was revealed that ArcB has a very complicated structural design, as summarized in Fig. 1, which should have functional relevance. It should be emphasized that the proposed leucine-zipper and PAS domains in ArcB are solely putative. However, it would be worth examining these sub-domains, together with the transmembrane domain, in terms of their structural and functional importance, because they are probably implicated in an as yet unknown mechanism underlying signal-propagation in response to an anoxic stimulus.

Construction of ArcB Mutants—To address the above issue, in this study we constructed a number of plasmids, each of which carries a certain mutant *arcB* gene. These low-copy-number plasmids were designated as members of pLIA-series (Table I). Each plasmid was designed to carry a mutant *arcB* gene, which encodes an altered ArcB protein with an amino acid substitution or a deletion either in the transmembrane domain, the leucine-zipper domain, or the PAS domain. Amino acids were selected that appeared to be crucial or highly conserved in each domain: for the transmembrane domain, pLIA-ΔTM (deletion of Asp-12 to Val-70); for the leucine-zipper, L80A (Leu-80 was changed to Ala), L87A, and L94A; and for the PAS domain, ΔPAS (deletion of Glu-151 to Arg-264), N181A, G189A, Q194A, G237A, and C241G (for details, see Table I). These ArcB mutants, also shown schematically in Fig. 1, were characterized, based on the following experimental rationales.

Experimental Rationale to Characterize the Set of ArcB Mutants In Vivo and In Vitro—Each mutant plasmid was transferred into an *arcB* null mutant (named DAC903), which carries an *arcB*::Cm^r allele and an *sdh-lacZ* fusion gene on the chromosome (*5*). This strain allowed us to appropriately explore the *in vivo* ArcB-to-ArcA signaling. This is based on the fact that expression of the *sdhCDAB* operon (encoding the succinate dehydrogenase complex) is severely repressed under anaerobic ($-O_2$) growth conditions in a manner fully dependent on the ArcB-to-ArcA phosphorelay (phospho-ArcA functions as a transcriptional repressor) (*5, 17, 31, 32*) On the other hand, the cytoplasmic membranes containing each mutant ArcB protein were purified from the transformed cells. They were subjected to the *in vitro* ArcB-to-ArcA phosphorelay assay, as established previously (*8*). It was also necessary to examine whether or not each mutant protein is synthesized at an appropriate level in

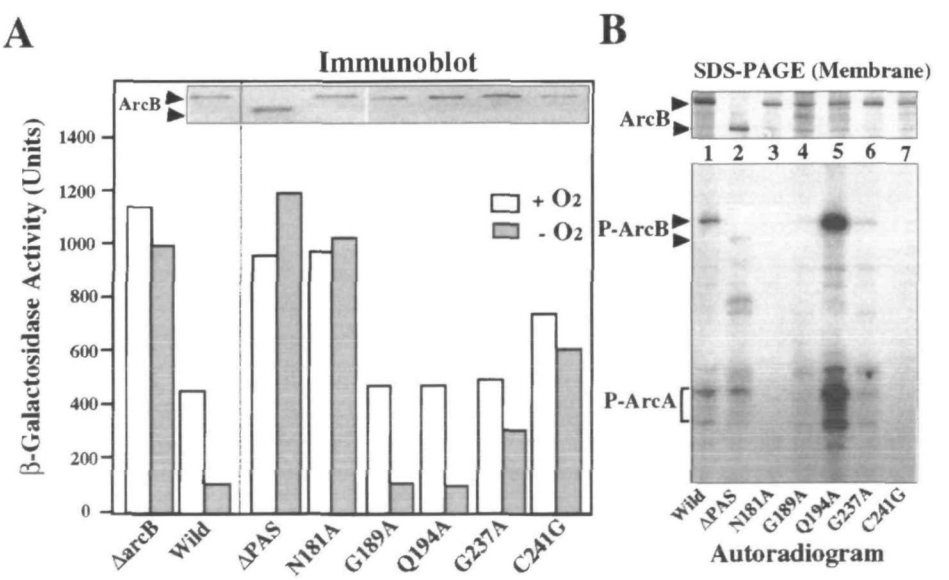

Fig 2. **Characterization of ArcB mutants of the PAS domain.** The *ΔarcB* strain carrying the *sdh-lacZ* fusion gene on its chromosome was transformed with a set of plasmids, each carrying a certain mutation in the PAS domain, as indicated. The transformants were grown in Luria-Bertani broth to the mid-logarithmic growth phase under both aerobic and anaerobic conditions. The harvested cells were subjected to β-galactosidase assay (A) From the cells, the total membrane fractions were isolated, then analyzed by immunoblotting with an anti-ArcB antiserum. The results are shown in the panel denoted by "Immunoblot." The cytoplasmic membranes were also purified, in which the ArcB protein was detected by SDS-PAGE followed by staining, as shown in the panel denoted by "SDS-PAGE." With use of these cytoplasmic membranes and the purified ArcA protein, *in vitro* phosphorylation experiments were carried out (B) The results are shown in the panel denoted by "Autoradiogram"

the transformed cells, and whether it is incorporated into the cytoplasmic membrane. This was accomplished by means of immunoblotting analysis with an anti-ArcB antiserum and by SDS-PAGE analysis. Based on these *in vivo* and *in vitro* experimental rationales, the set of ArcB mutants constructed in this study (Table I) were systematically characterized, as follows.

PAS Domain of ArcB—Certain amino acids in the PAS domain of ArcB, which appear to be conserved among the PAS family of domains, were each changed to Ala. They are Asn-181, Gly-189, Gln-194, and Gly-237 (see Fig. 1). Although Cys-241 is not conserved in the PAS domains, this was also changed to Gly as a reference, because the same ArcB-C241G mutant has previously been characterized by others (*17*). The entire PAS domain was also deleted (ArcB-ΔPAS). It was confirmed that each mutant protein was normally produced and incorporated into the cytolasmic membrane (Fig. 2, A and B). The *in vivo* phenotype of each mutant was assessed by examining the expression profile of *sdh-lacZ* in response to aerobic ($+O_2$) and anaerobic ($-O_2$) growth conditions (Fig. 2A). ArcB-N181A and ArcB-C241G were found to have lost the *in vivo* ability to repress the expression of *sdh* under the anaerobic growth conditions, while ArcB-G189A and ArcB-Q194A exhibited essentially the wild-type property. ArcB-G237A appeared to be partially defective. The result of ArcB-C241G was consistent with that reported previously (*17*). *In vitro*, ArcB-N181A and ArcB-C241G did not exhibit the autophosphorylation (His-kinase) activity. This fact simply explained the above *in vivo* result. It was then found that ArcB-ΔPAS lacking the entire PAS domain exhibited a null phenotype *in vivo*. Interestingly, however, this mutant exhibited the *in vitro* ability of the ArcB-to-ArcA phosphotransfer.

These results shed some light on the nature of the putative PAS domain of ArcB. On deletion of the PAS domain, the altered protein could no longer respond to an anoxic stimulus *in vivo*, thereby exhibiting a null phenotype. This mutant protein was shown to be located normally in the cytoplasmic membrane, and it maintains the catalytic activity, as far as its *in vitro* ability to phosphotransfer to the target response regulator is concerned. These facts suggest that the ArcB-ΔPAS protein is not just a dead protein, rather it lacks the ability to respond to an anoxic signal. Thus, the PAS domain is suggested to play a role in signal-perception *per se*. Asn-181 and Cys-241 may be deeply involved in such a presumed function of the PAS domain.

However, we were somewhat puzzled by the fact that the ArcB-ΔPAS protein exhibits the *in vitro* ability of phosphorylation. At present, it is difficult to fully account for this particular *in vitro* event. It is possible that our *in vitro* system for analyzing the ArcB-to-ArcA phosphotransfer may not perfectly reflect the *in vivo* situation. The *in vivo* functioning of ArcB may be controlled through an interaction with other, unknown cellular components, which are absent in our *in vitro* system. In any event, it will be interesting to lean how the PAS domain exerts its specific role in signal-perception *in vivo*. This region may be important for formation of the proper oligomeric state of ArcB, which is presumably crucial to signal propagation. Alternatively, the PAS domain may serve as a ligand-binding domain for putative intracellular effectors, such a D-lactate, acetate, pyruvate, and NADH.

Leucine-Zipper of ArcB—Three leucine residues in the putative leucine-zipper domain were each changed to Ala (Fig. 1). These ArcB mutants were characterized as described above (Fig. 3). The results suggested that Leu-87 is crucial, because ArcB-L87A showed a null phenotype *in vivo* (Fig. 3A). This mutant protein showed a considerable *in vitro* ability to phosphotransfer (Fig. 3B). The other two ArcB mutants exhibited essentially the wild-type properties, in terms of both the *in vivo* and *in vitro* abilities. Thus, our results did not support the idea that the putative leucine-zipper domain is important in the ArcB function. Rather, it is questionable whether ArcB does indeed contain a leucine-zipper structure in this particular region, because the leucine residues (Leu-80 and Leu-94) can be substituted with Ala without affecting the ArcB function. The observed nature of ArcB-L87A was similar to that of ArcB-ΔPAS, in the sense that both the mutants maintained their *in vitro* catalytic activities but could not function *in vivo*. This particular leucine residue may be somehow implicated in signal-propagation, as discussed in the case of ArcB-ΔPAS. As a whole, however our findings do not support the idea that ArcB contains a leucine-zipper in a general and strict sense.

Transmembrane Domain of ArcB—In ArcB-ΔTM, the

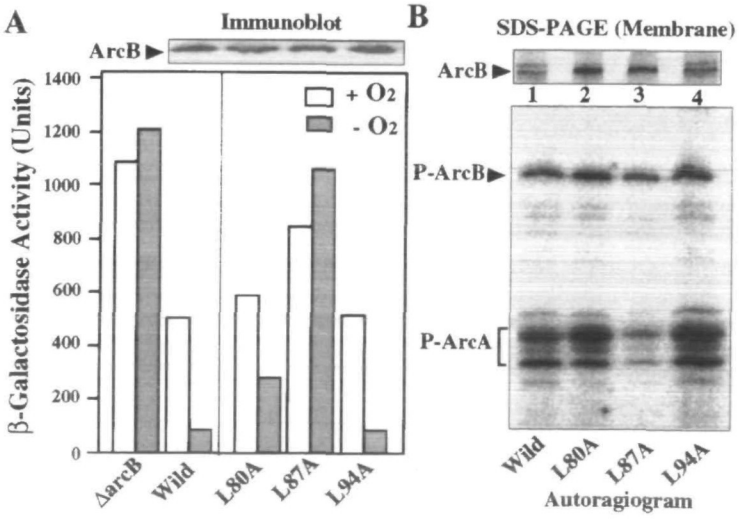

Fig 3 **Characterization of ArcB mutants of the leucine-zipper domain.** The *ΔarcB* strain carrying the *sdh-lacZ* fusion gene on its chromosome was transformed with a set of plasmids, each carrying a certain mutation in the leucine-zipper domain, as indicated. Other details are essentially the same as those given in the legend to Fig 2

entire hydrophobic region was deleted (Fig. 1). Surprisingly, the cells carrying pLIA-ΔTM showed essentially the wild-type phenotype, as far as the *in vivo* anaerobic regulation of *sdh-lacZ* was conserved (Fig. 4). When the cells were fractionated into the soluble and membrane fractions, the ArcB-ΔTM polypeptide was found in the soluble fraction (Fig. 4). In this particular experiment, it should be noted that the strain OG903 carrying the wild-type *arcB* gene on the chromosome was characterized as an appropriate reference, in order to examine and compare the localization of ArcB, both qualitatively and quantitatively. The wild-type ArcB protein was mainly localized in the membrane fraction, while the ArcB-ΔTM protein was recovered in the cytoplasmic fraction (note that the cellular content of ArcB-ΔTM is considerably higher than that in the wild-type situation).

These results shed some light on the nature of the transmembrane domain of ArcB. First, ArcB-ΔTM became soluble in the cytoplasm. This result supported the idea that the transmembrane domain is indeed needed for ArcB to be localized in the cytoplasmic membrane. However, we are not certain whether the ArcB-ΔTM protein can associate weakly with the membrane. In any case, the more interesting conclusion is that the membrane-spanning region appears to be dispensable for ArcB to function as a sensor for an anoxic signal. It was rather suggested that the anchoring of ArcB in the cytoplasmic membrane is not obligatory for ArcB to propagate signals. Our results are compatible with the idea that the transmembrane domain serves only to anchor the rest of the ArcB protein to the inner surface of the cytoplasmic membrane, where an anoxic signal may be generated (*15*).

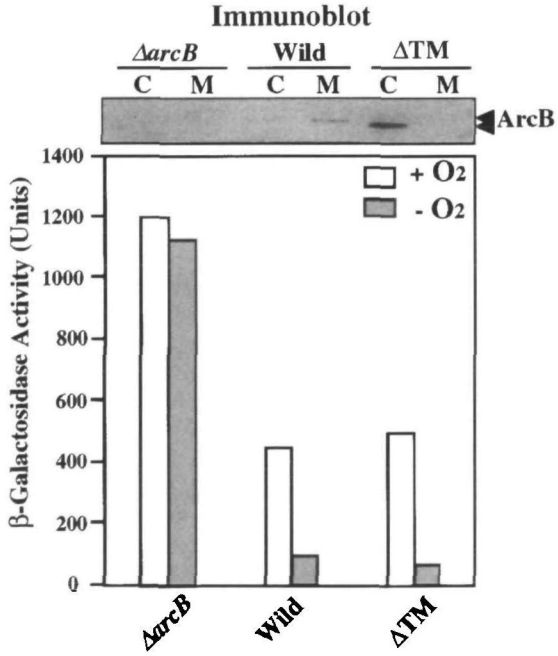

Fig 4. **Characterization of the ArcB mutant that lacks the transmembrane regions.** The *ΔarcB* strain carrying the *sdh-lacZ* fusion gene on its chromosome was transformed with pLIA-ΔTM Other details are essentially the same as those given in the legend to Fig. 2. In the immunoblotting analysis, the cytosolic (C) and membrane (M) fractions were isolated and analyzed. Note also that the *in vitro* phosphorylation ability of this particular mutant, ArcB-ΔTM, was not examined, because it is located in the cytosolic fraction and can reasonably be expected to maintain the *in vitro* catalytic activity.

Implication—As shown and discussed above, the results of this study provided insights into the structure and function of the N-terminal domain of ArcB, which presumably plays a role in signal-perception (or signal-propagation). It was first hypothesized that there exist three distinctive sub-domains in the N-terminal region, namely, a transmembrane domain, a leucine-zipper domain, and a PAS domain. As far as their amino acid sequences were concerned, each appeared to be present (Fig. 1). Nonetheless, their functional significance was not clear. In this study, we addressed these relevant issues, and our results from extensive characterization of each sub-domain led us to propose the following views. (i) The transmembrane domain is dispensable for ArcB to percept an anoxic signal. (ii) In a strict sense, it is questionable that ArcB contains a leucine-zipper structure. (iii) The proposed PAS domain appears to play a role in signal-propagation *per se*.

Clarification of the molecular mechanism by which ArcB senses an anoxic signal is still at a very early stage. Whatever the mechanism is, it should account for the intriguing findings presented here.

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