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The SixA phospho-histidine phosphatase modulates the ArcB phosphorelay signal transduction in *Escherichia coli*

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Abstract The *Escherichia coli* SixA protein is the first discovered prokaryotic phospho-histidine phosphatase, which was implicated in a His-to-Asp phosphorelay. The *sixA* gene was originally identified as the one that interferes with, at its multi-copy state, the cross-phosphorelay between the histidine-containing phosphotransmitter (HPt) domain of the ArcB anaerobic sensor and its non-cognate OmpR response regulator. Nevertheless, no evidence has been provided that the SixA phosphatase is indeed involved in a signaling circuitry of the authentic ArcB-to-ArcA phosphorelay in a physiologically meaningful manner. In this study, a SixA-deficient mutant was characterized with special reference to the ArcB signaling, which allows *E. coli* cells to respond to not only external oxygen, but also certain anaerobic respiratory conditions. Here evidence is provided for the first time that the SixA phosphatase is a crucial regulatory factor that is involved in the ArcB signaling, particularly, under certain anaerobic respiratory growth conditions. We propose a novel mechanism, involving an HPt domain and a phospho-histidine phosphatase, by which a given multi-step His-to-Asp signaling can be modulated.

Key words: SixA phosphatase; ArcB His-kinase; Anaerobic regulation; Phosphorelay in *Escherichia coli*

1. Introduction

Widespread bacterial signal transduction circuits are generally referred to as 'two component systems' or 'phosphorelay systems' [1–5]. Such a phosphorelay signaling system is made up of two or more multidomain signal transducers, generally referred to as 'sensors' and 'response regulators', each of which contains one or more common phosphorelay signaling domains [6,7]. They are histidine (His)-kinases, phosphoaccepting-receivers, and histidine-containing phosphotransmitters (HPt). Such a histidine to aspartate (His-to-Asp) phosphorelay appears to be an evolutionary-conserved common tactics for intracellular signaling in response to an external or internal stimulus, both in prokaryotes and eukaryotes [6–9].

In *Escherichia coli*, as many as 30 distinct His-to-Asp phosphorelay pathways operate in response to a wide variety of environmental stimuli, thereby regulating a large variety of cellular responses, including bacterial chemotaxis, osmoregulation, and pathogenesis [10]. Amongst them, the ArcB-to-ArcA signal transduction is one of the best-studied model systems for multi-step phosphorelay (see Fig. 1A) [11–24]. This particular phosphorelay system is involved in the complex transcriptional regulatory network that allows *E. coli* cells to sense certain aerobic and anaerobic growth conditions (termed generally as 'anaerobiosis') [16]. ArcB is a hybrid His-kinase, which has multiple (at least three) phosphorylation domains, including a His-kinase, a receiver, and a HPt domain [25,26]. This ArcB sensor is located in the cytoplasmic membrane. On the basis of recent intensive in vitro studies, a scheme as to the complex circuitry of the multi-step ArcB-to-ArcA phosphorelay was proposed (see Fig. 1) [14,20,21,25,26]. First of all, His-292 in the ArcB His-kinase acquires the γ-phosphoryl group from ATP through its own catalytic activity. Then, the phosphoryl group on His-292 moves onto its intrinsic phospho-accepting aspartate site (Asp-576) in the ArcB receiver. The His-717 in the HPt domain can also be modified by phosphorylation through His-292 and Asp-576. The final destination of the phosphoryl group on His-717 is Asp-54 in the ArcA receiver. It should be emphasized that 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 at the same aspartate site.

The physiological relevance of such a complicated multi-step phosphorelay through ArcB was recently discussed with regard to *E. coli* anaerobiosis [16,18,21]. Nonetheless, clarification of the function of the HPt domain that serves as an intermediate for the multi-step phosphorelay is at a very early stage. In particular, the mechanistic advantage of such a multi-step phosphorelay is the subject of general debate [5,27]. In this respect, we recently uncovered an *E. coli* protein (named SixA) that appears to exhibit a phospho-histidine phosphatase activity towards the HPt domain of ArcB [28]. This is in fact the first phospho-histidine phosphatase that is implicated in a given His-to-Asp phosphorelay, as far as we know. However, clarification of the physiological significance of the SixA phosphatase must await further examination.

To this end, in this study a SixA-deficient mutant was examined in terms of *E. coli* anaerobiosis with special reference to the ArcB-dependent His-to-Asp phosphorelay. Here we provide evidence for that the SixA phosphatase plays a crucial physiological role by modulating the HPt domain-dependent phosphorelay, particularly, under certain anaerobic respiratory growth conditions.

2. Materials and methods

2.1. Bacteria and plasmids

E. coli K-12 strains, used in this study, are derivatives of CSH26 [*thi ara* Δ(*pro-lac*)]. Strain OG910 carries an *sdh-lacZ* fusion gene in the λ *att* site. Strains TK12 and DAC910 are its Δ*sixA* (*sixA*::Tet^r) and

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Δ*arcB* (*arcB*::Cm^r) derivatives, respectively [26–28]. Plasmid pIA021 is a single-copy-number plasmid, which carries the wild-type *arcB* gene, whereas plasmid pIA022 is its derivative, which carries the mutant *arcB* gene encoding the mutant ArcB protein containing the amino acid substitution, His-717 to Leu [26]. Plasmid pSTN6-2-D86 is a multi-copy-number plasmid, which carries the *sixA* gene [28].

2.2. Media and growth conditions

Under both aerobic (+O_2) and anaerobic (−O_2) conditions, *E. coli* cells were grown at 37°C in a certain medium (named Medium-B), unless otherwise noted. Medium-B contains 7 g nutrient broth (Difco), 1 g yeast extract (Difco), 3.7 g K_2PO_4, 1.3 g KH_2PO_4 in 1 l of H_2O. Plasmid-bearing cells were grown with appropriate antibiotics such as ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml). Our anaerobic growth conditions are the following. Cells were first cultivated in Luria–Bertani broth overnight under aerobic growth conditions, and then, an aliquot was inoculated into a screw-capped tube, previously filled with air-free Medium-B. The tube was incubated without shaking for 24 h. An aliquot of the anaerobically grown pre-culture was again inoculated into the same fresh medium, and it was incubated under the same conditions as those described above.

2.3. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to the method of Laemmli [30].

2.4. Enzyme assay

β-Galactosidase activity was measured by Miller's method with slight modification [29]. The cells were grown as described above, and they were suspended in a volume of 250 mM sodium phosphate (pH 7.1) for accurate measurement of the cell density. A portion of the cell suspension was used for β-galactosidase assay, after it was permeabilized with toluene.

2.5. In vitro phosphorylation experiments

In vitro phosphorylation experiments were carried out, as follows. The HPt domain of ArcB, the SixA phosphatase, and the cytoplasmic membrane containing the overexpressed ArcB protein were purified, as described previously [25,28,31]. Radioactively-phosphorylated HPt domain of ArcB was prepared, as follows [28]. The isolated cytoplasmic membrane (25 μg) was incubated with the purified HPt domain (5 μg) at 37°C for 30 min in the presence of 0.05 mM [γ-^{32}P]ATP (10 000 cpm/pmol), 200 mM KCl, and 5 mM $MgCl_2$ in TEDG buffer (Tris–HCl, EDTA, dithiothreitol, and glycerol) [32]. The radiolabelled HPt domain was isolated with Sephadex G-75 column previously equilibrated with TEDG buffer. This purified phospho-HPt (0.5 μg) was incubated with the purified SixA proteins (2 μg) in TEDG buffer containing 50 mM KCl and 5 mM $MgCl_2$ at 37°C. The samples were immediately subjected to SDS–PAGE, followed by autoradiography. To prepare the radioactively autophosphorylated ArcB, the cytoplasmic membrane containing ArcB was incubated at 37°C for 10 min with 0.05 mM [γ-^{32}P]ATP (10 000 cpm/pmol) in TEDG buffer containing 200 mM KCl, 5 mM $CaCl_2$. The samples were further incubated in the presence of the purified SixA protein (2 μg) in the same buffer supplemented with an excess amount of cold ATP (final 0.25 mM). The cytoplasmic membrane containing the ArcB-ΔH2 mutant protein was also radioactively phosphorylated. These reaction mixtures were analyzed by SDS–PAGE, followed by autoradiography.

2.6. Immunoblot analysis

Protein samples were separated by SDS–PAGE (15% acrylamide). Proteins on the gels were transferred onto nitrocellulose filters. Detection with an anti-SixA antiserum was carried out with the ELC™ Western blotting analysis system (Amersham Pharmacia Biotech).

3. Results

3.1. SixA exhibits a phospho-histidine phosphatase activity towards the HPt domain of ArcB

We previously purified the SixA phosphatase, and then, demonstrated that it exhibits an in vitro ability to stimulate dephosphorylation of the phospho-HPt domain of ArcB [28].

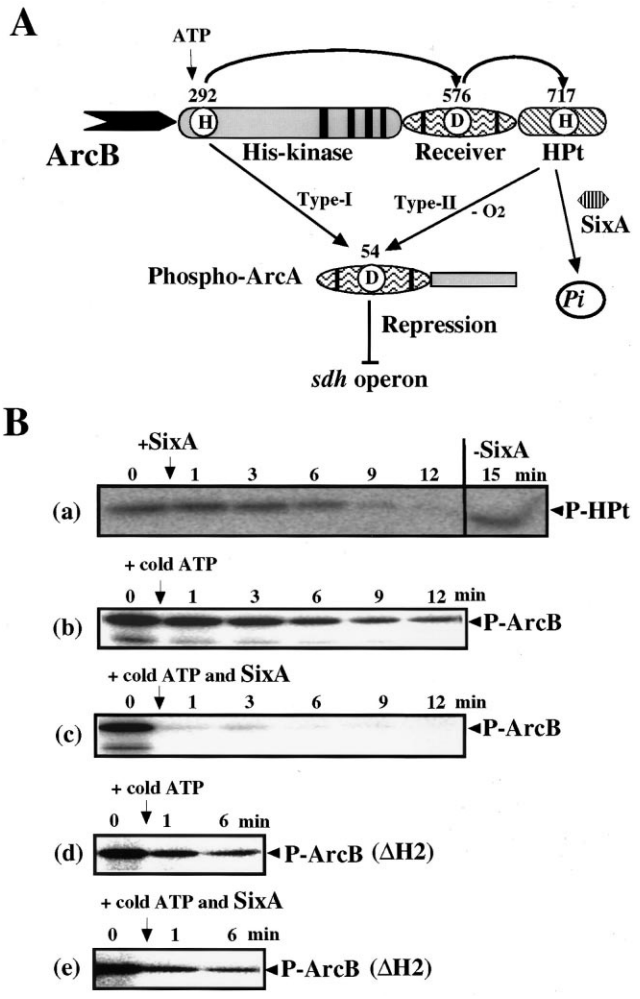

Fig. 1. Schematic representation of the multi-step His-to-Asp phosphorelay, mediated by the ArcB-ArcA signal transducers and the SixA phospho-histidine phosphatase. A: This schematic model is based on the in vivo and in vitro findings of this study, together with those of previous ones [20,21]. Other details are given in the text. B: In vitro phosphorylation experiments showing the phosphatase activity of SixA. Radioactively phosphorylated HPt domain of ArcB was purified, and then, it was incubated with the purified SixA proteins for the times indicated (a). Phospho-HPt was incubated in the absence of SixA for 15 min, as a reference. Radioactively phosphorylated ArcB in the cytoplasmic membrane was prepared. The samples were further incubated, together with an excess amount of cold ATP, in the absence (b) and presence (c) of the purified SixA protein for the times indicated. The cytoplasmic membrane containing the ArcB-ΔH2 mutant were also radioactively phosphorylated. This was also used as substrate of SixA (d and e). These reaction mixtures were analyzed by SDS–PAGE, followed by autoradiography.

Such an in vitro result was reproduced here as a reference (Fig. 1B, panel a). However, this substrate is the isolated HPt domain encompassing only the C-terminal amino acid residues (Ile-639 to Lys-778). Therefore, the above observation did not show whether or not SixA exhibits its phosphatase activity towards the intact form of ArcB in the cytoplasmic membrane. This issue is crucial to understand the physiological role of SixA in the ArcB signaling system. Thus, we first addressed this issue in vitro.

The cytoplasmic membrane containing the intact form of ArcB was purified [26]. This sample was incubated in the

presence of ^{32}P-ATP for 10 min under appropriate in vitro conditions. The result of the autoradiogram showed that ArcB was autophosphorylated, presumably, either at His-292, Asp-576, or His-717 (Fig. 1B, panel b, 0 min). Then, a large excess amount of cold ATP was added. The radioactive phosphoryl group was slowly released from ArcB in a spontaneous manner. When the same chase-experiment was carried out in the presence of the purified SixA protein, the dephosphorylation process was markedly accelerated (Fig. 1B, panel c). Such a dephosphorylation experiment was carried out also with a mutant ArcB protein that has the amino acid substitution of His-717 to Leu (Fig. 1B, panel d and panel e). This mutant ArcB protein (designated as ΔH2) was also efficiently autophosphorylated, most likely, at both His-292 and Asp-576 (but not His-717). SixA showed no ability to stimulate dephosphorylation of this particular substrate (Fig. 1B, panel e). These results together supported the view that SixA exhibits a phospho-histidine phosphatase activity specifically towards phospho-His-717 in the HPt domain of ArcB (see Fig. 1A).

3.2. Experimental design to examine the SixA function with special reference to the ArcB signaling

In vivo experimental design to assess the ArcB phosphorelay signaling with special reference to the SixA phosphatase is following. We employed an *E. coli* strain (named OG910) carrying an *sdh-lacZ* transcriptional fusion gene on the chromosome [21,24]. This allowed us to appropriately explore the in vivo ArcB-to-ArcA signaling, based on the fact that the expression of the *sdhCDAB* operon (encoding the succinate dehydrogenase complex) is severely repressed under certain anaerobic growth conditions in a manner dependent on the ArcB signaling [15,17]. Under such anaerobic conditions, ArcB phosphorylates ArcA, consequently, phospho-ArcA functions as the DNA-binding repressor for the *sdhCDAB* operon (see Fig. 1) [14]. A Δ*sixA* derivative of OG910 was also constructed to yield TK12, in which the *sixA*-coding region on the chromosome was replaced by a tetracycline-resistant (Tet^r) cassette [28]. A Δ*arcB* derivative (named DAC910) was also used, which carries an *arcB* null allele (*arcB*::Cm^r) [25]. Based on these genetic backgrounds, in this study we asked the question of whether or not the Δ*sixA* mutant exhibits any particular phenotype with respect to the ArcB signaling under certain physiological conditions.

3.3. SixA has apparently nothing to do with the ArcB signaling in response to external oxygen

When OG910 carrying the *sdh-lacZ* fusion gene was grown under fully aerobic (+O2) conditions in Medium-B, the *sdh* operon was highly expressed, as judged by the level of β-galactosidase activity (Fig. 2A). When the cells were grown in the same medium under anaerobic ($-O_2$, or microaerobic) conditions, the expression of *sdh* was severely repressed by phospho-ArcA repressor in a manner dependent on the ArcB signaling. When the Δ*sixA* mutant cells were examined, essentially the same regulatory profile in response to external oxygen was observed (Fig. 2B). This suggested that SixA has apparently nothing to do with the ArcB signaling, as far as the anoxic ($-O_2$) regulation of *sdh* is concerned. To confirm such a conclusion, we conducted a kinetic analysis of expression of *sdh* in response to external oxygen (Fig. 2C). Both the wild-type and Δ*sixA* cells were grown anaerobically to fully

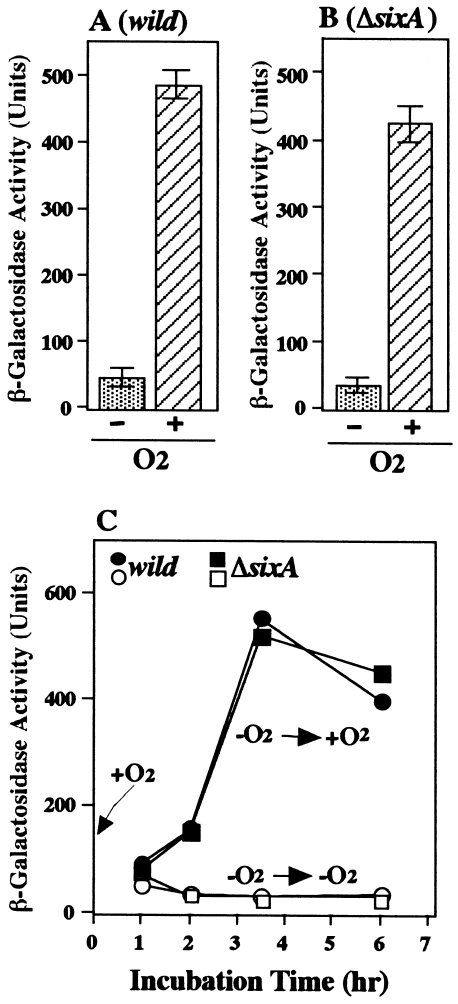

Fig. 2. β-Galactosidase activity expressed by the *sdh-lacZ* transcriptional fusion gene. *E. coli* strain OG910 carrying an *sdh-lacZ* fusion gene, and its Δ*sixA* (*sixA*::Tet^r) derivative (TK12) were used. Cells were grown in Medium-B under both aerobic ($+O_2$) and anaerobic ($-O_2$) conditions. The cells were harvested at the mid-logarithmic growth phase. The harvested cells were assayed for β-galactosidase (A for the wild-type cells, and B for the Δ*sixA* cells). C: Both the wild-type and mutant cells were grown in Medium-B overnight under anaerobic conditions. Then, the cells were inoculated into the same fresh medium in an open culture-tube, and they were incubated under fully aerobic conditions. At intervals, the cells were harvested and subjected to β-galactosidase assay. Appropriate control experiments were also carried out, as also shown in C.

repress the expression of *sdh*, and then, external oxygen was supplied by vigorously shaking in an open tube. The kinetic results showed that the derepression profiles of *sdh* are indistinguishable between the wild-type and Δ*sixA* backgrounds. This result supported the above notion.

3.4. The ArcB signaling under anaerobic respiratory conditions

The above observation was disappointing. From previous literature, however, we learnt that the ArcB signaling pathway functions in a more complex regulatory network that allows *E. coli* cells to respond to not only external oxygen, but also certain anaerobic respiratory conditions [15,17]. In particular, expression of the *sdh* operon, typically regarded to be involved in aerobic metabolism, can also be recruited for anaerobic respiration in the absence of oxygen, which instead is medi-

ated by anaerobic electron acceptors, such as nitrate, trimethylamine-*N*-oxide (TMAO), dimethyl sulfoxide (DMSO), and fumarate. Several groups suggested previously that this process is also directly regulated, at least partly, through the ArcB signaling [15,17]. These facts prompted us to examine the function of SixA with special reference to such an anaerobic respiratory regulation of *sdh*.

Here we employed DAC910 carrying the Δ*arcB* allele (Fig. 3). This strain was transformed by a single-copy-number plasmid pIA021 containing the wild-type *arcB* gene (designated as *arcB*-wild). The same strain was transformed also by pIA022, which contains the mutant *arcB* gene encoding the mutant protein lacking His-717 (designated as *arcB*-ΔH2). These cells were grown under anaerobic ($-O_2$) growth conditions in Medium-B containing each one of the anaerobic electron acceptors (nitrate, TMAO, DMSO, fumarate), and then, expression of *sdh* was monitored by measuring β-galactosidase activity. In the wild-type background, it was found that each electron acceptor lifted the level of expression of *sdh*, even in the absence of oxygen, to each varied extent (Fig. 3A). Nitrate exerted the most striking effect on the derepression of *sdh*, the level of which was comparable with that in the case of oxygen. When the strain carrying the *arcB*-ΔH2 allele was characterized in this particular aspect, such a regulatory profile was abolished (Fig. 3B). These results were explained by the view that the expression of *sdh* is regulated under anaerobic growth conditions, apparently, in response to certain anaerobic electron acceptors as stimuli. In this particular anaerobic respiratory regulation of *sdh*, it was suggested that the HPt-mediated ArcB-to-ArcA phosphorelay is crucial.

Then, we examined the phenotype of the Δ*sixA* mutant with special reference to this anaerobic respiratory regulation of *sdh* (Fig. 4A). It was found that the level of derepression of *sdh*, caused by the addition of each anaerobic electron acceptor, was considerably lower in the Δ*sixA* background, as compared with that in the wild-type background. This phenomenon (or phenotype) of the Δ*six A* mutant can be reasonably explained, if it is assumed that SixA plays a role in down-regulation of the ArcB-to-ArcA phosphorelay by exhibiting its phosphatase activity towards the HPt domain, under such anaerobic respiratory conditions (see Fig. 6). Consequently, the ArcB-to-ArcA phosphorelay (i.e. resulting pool of phospho-ArcA) may be down-regulated. This event should result in derepression of *sdh*. The important finding is that, in the SixA-deficient cells, such a regulation was impaired.

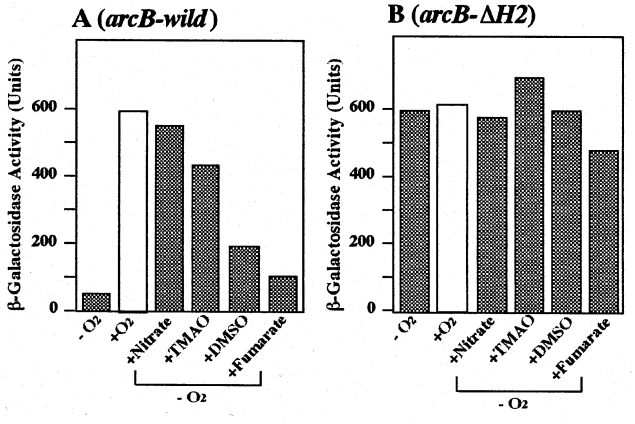

Fig. 3. β-Galactosidase activity expressed by the *sdh-lacZ* transcriptional fusion gene. *E. coli* strain DAC910 (a Δ*arcB* derivative of OG910) were used. This strain was transformed either by pIA021 carrying the wild-type *arcB* gene or pIA022 carrying the mutant *arcB*-ΔH2 gene. These transformants were grown under aerobic conditions ($+O_2$) and anaerobic conditions in Medium-B (see Fig. 2). When grown under anaerobic condition, each following anaerobic electron acceptor was added at its concentration of 40 mM (nitrate, TMAO, DMSO, and fumarate), as indicated. The cells were harvested at the mid-logarithmic growth phase, and then, they were subjected to β-galactosidase assay.

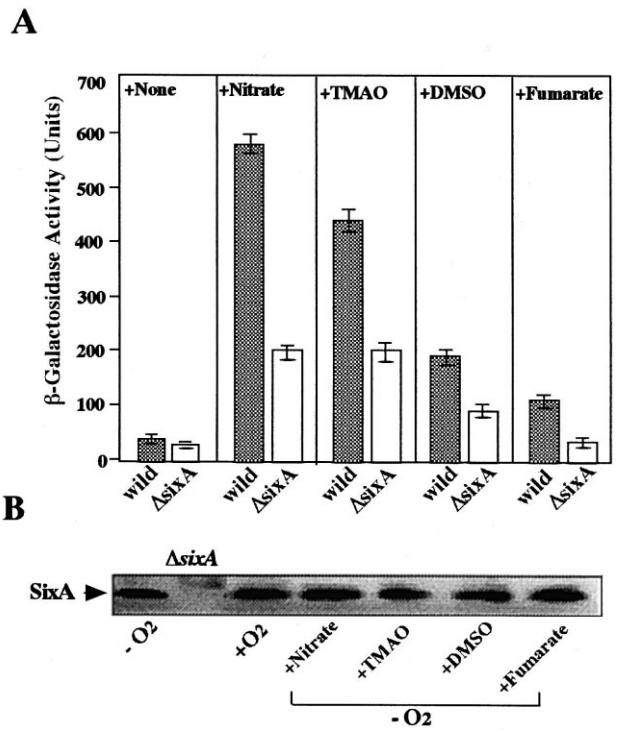

Fig. 4. β-Galactosidase activity expressed by the *sdh-lacZ* transcriptional fusion gene, and immunoblotting with anti-SixA antiserum. A: β-Galactosidase activity expressed by both the wild-type and Δ*sixA* strains (DAC910 and TK12, respectively) were assayed for the cells grown in Medium-B, into which each indicated anaerobic electron acceptor was added (see Fig. 3). The cells were harvested at the mid-logarithmic growth phase, and then, they were subjected to β-galactosidase assay. B: Total cellular proteins were prepared from the wild-type and Δ*sixA* mutant cells, grown in each medium indicated. The protein samples were analyzed by SDS–PAGE, followed by immunoblotting with an anti-SixA antiserum.

This intriguing phenotype of the Δ*sixA* mutant should be confirmed more closely. Before doing so, it was needed to examine the existence of SixA under these growth conditions tested. To this end, a polyclonal antiserum was raised against the purified SixA protein [28]. By immunoblotting analysis with the anti-SixA antiserum, the cellular content of SixA was examined for the cells grown under each different condition (Fig. 4B). The results indicated that the cellular content of SixA is constant, regardless of the growth conditions tested.

3.5. *SixA is involved in regulation of the ArcB-ArcA phosphorelay under anaerobic respiratory growth conditions*

To demonstrate that SixA is indeed involved in modulation of the ArcB-to-ArcA phosphorelay under anaerobic respiratory growth conditions, a kinetic experiment was carried out (Fig. 5). Both the wild-type and Δ*sixA* strains were first grown

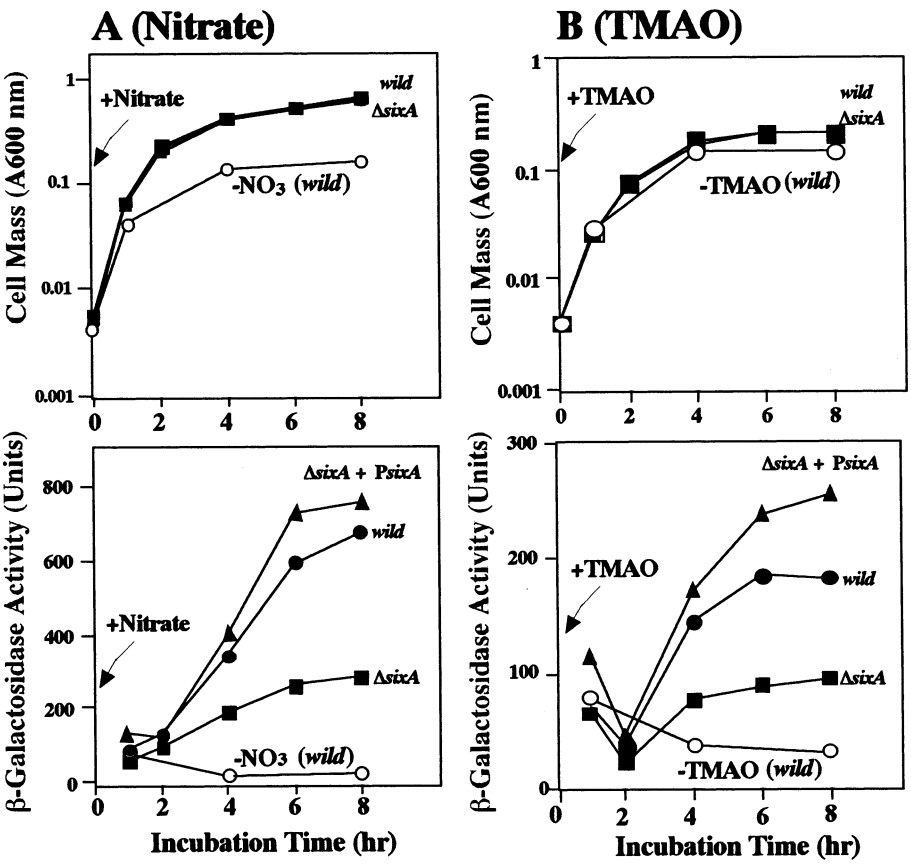

Fig. 5. β-Galactosidase activity expressed by the *sdh-lacZ* transcriptional fusion gene. Both the wild-type and Δ*sixA* strains (OG910 and TK12, respectively) were grown in Medium-B under anaerobic conditions overnight. TK12 carrying plasmid pSTN6-2-D86 encompassing the *sixA* gene was also grown (denoted by Δ*sixA*+P*sixA* in the figure). The cells were inoculated into fresh Medium-B supplemented either with nitrate (40 mM) or TMAO (40 mM), and then, they were further incubated under anaerobic growth conditions (upper panels are growth curves). The cells were harvested at the times indicated, and then, they were subjected to β-galactosidase assay (lower panels). An appropriate control experiment was also carried out, in which cells were grown in Medium-B without any anaerobic electron acceptor.

under anaerobic growth conditions in the absence of any anaerobic electron acceptor. The Δ*sixA* strain carrying the plasmid-born *sixA* gene was also grown. These cells were transferred into the fresh medium supplemented with either nitrate or TMAO, and then, the time course of derepression of *sdh* was followed, by measuring β-galactosidase activity (Fig. 5, upper panels for growth curve, and lower panels for β-galactosidase activity). These kinetic data clearly displayed the phenotype of the Δ*sixA* mutant, which could be suppressed by introducing the plasmid-born *sixA* gene. The results showed that the rapid derepression of *sdh*, induced by these anaerobic electron acceptors, is largely dependent on the function of SixA, although not absolutely. This phenotypic event can be explained by the view that the SixA phosphatase is responsible, at least partly, for modulation of the ArcB-to-ArcA phosphorelay under anaerobic respiratory growth conditions.

To schematically explain how the function of SixA is crucial from a physiological viewpoint, a model experiment was finally carried out (Fig. 6). Both the wild-type and Δ*sixA* strains were exponentially grown under aerobic ($+O_2$) conditions. Then, the cells were grown under anaerobic ($-O_2$) conditions in the fresh medium supplemented with and without nitrate. The profiles of expression of *sdh* were followed (Fig. 6A–C, upper panels for β-galactosidase activity, lower panels for growth curve). The result showed that upon the onset of anoxic conditions, the expression of *sdh* is rapidly and severely repressed (A). However, even under such anoxic conditions, if nitrate is present in the environment, the once repressed expression of *sdh* is derepressed after a while (but not in a little while) (B). This regulation does make sense from the physiological viewpoint, because, whenever an exogenous electron acceptor is available in medium, *E. coli* cells tend to curtail its fermentation process in favor of respiration, even under anaerobic conditions (i.e. anaerobic respiration). The result for Δ*sixA* cells demonstrated that SixA is crucial in this important regulatory process (C).

4. Discussion

The SixA phospho-histidine phosphatase was originally identified through a complicated and artificial in vivo screening strategy [28]. Thus, no direct evidence has been provided that the SixA phosphatase is indeed involved in a signal transduction circuitry of the ArcB-to-ArcA phosphorelay system per se. Here the results of extensive studies provided the first and direct evidence that SixA is a crucial regulatory factor that is involved in the ArcB signaling, particularly, under certain anaerobic respiratory growth conditions. Based on these, we propose a novel mechanism, involving a phospho-histidine phosphatase, by which a certain His-to-Asp phos-

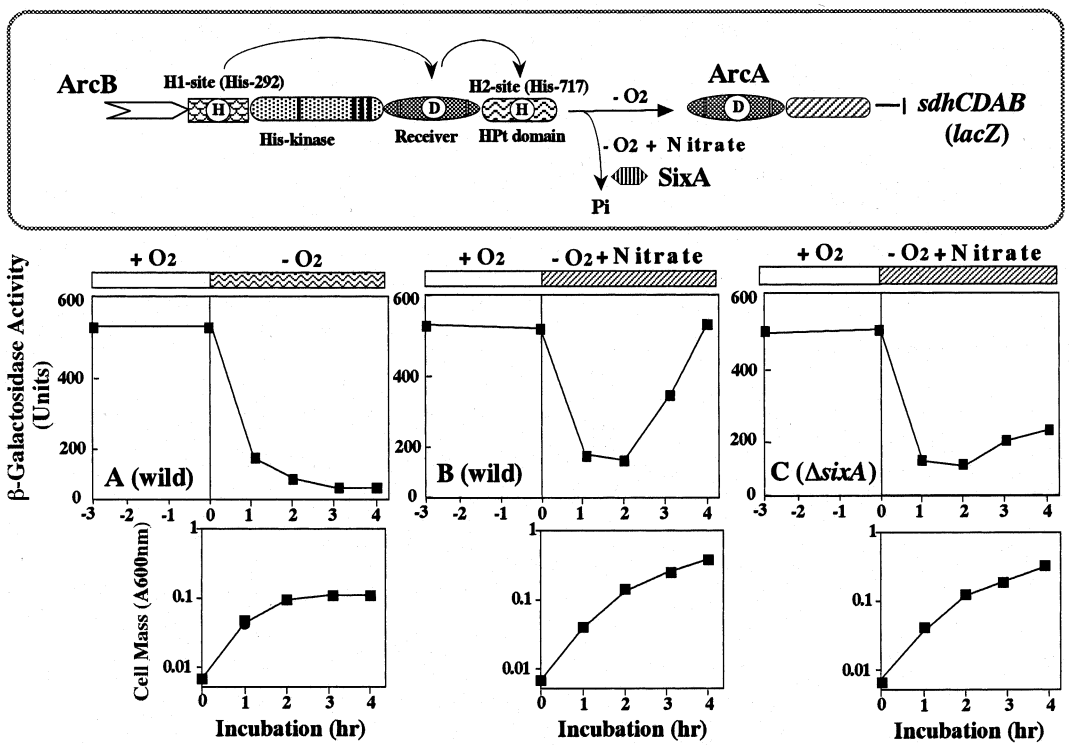

Fig. 6. A model experiment that explains the physiological function of SixA. Both the wild-type and Δ*sixA* strains (OG910 and TK12, respectively) were exponentially grown in Medium-B for 3 h in Medium-B under aerobic conditions. They were inoculated into fresh Medium-B supplemented with and without nitrate (40 mM), and then, they were further incubated under anaerobic growth conditions (lower panels are growth curves). The cells were harvested at the times indicated, and then, they were subjected to β-galactosidase assay (upper panels). As also shown schematically, to explain these events, a model was proposed, in which the physiological function of SixA was emphasized (details are given in the text).

phorelay is modulated in a sophisticated manner (Fig. 6). SixA appears to play a role in down-regulation of the ArcB-to-ArcA phosphorelay under certain anaerobic respiratory conditions, by exhibiting its phosphatase activity towards the HPt domain. Under such anaerobic respiratory conditions, SixA drains a phosphoryl group from the HPt domain. Consequently, the ArcB-to-ArcA phosphorelay (i.e. the resulting pool of phospho-ArcA) can be down-regulated. This event results in derepression of *sdh* even under anaerobic growth conditions, provided that an anaerobic electron acceptor is available.

We do not know how the SixA activity is regulated in response to anaerobic respiratory growth conditions. Our result ruled out the possibility that the cellular content of SixA is accordingly varied by an unknown mechanism. It is difficult to address this issue at present, because it is not yet known what is the primary anoxic stimulus that regulates the signaling activity of the ArcB sensor. Both physiological and genetic experiments excluded O_2 itself as the signal. Rather, a redox state, perhaps an element of the electron-transport chain or proton motive force, may be the one [18]. In any event, the functional state of ArcB, activated under such a primary anoxic stimulus, seems to be different from that modulated by an anaerobic respiratory stimulus. Because our model includes the idea that the former type of ArcB is resistant to SixA, but the latter is sensitive. Furthermore, it should be noted that the primary anoxic stimulus can be generated in a little while upon the onset of anaerobic growth conditions, whereas the anaerobic respiratory signal seems to be generated after a while upon the onset of anaerobic respiratory conditions (see Fig. 6B). Future studies on this intriguing issue should shed light on the mechanism underlying signal-perception through the ArcB sensor.

A well-defined scenario as to the molecular mechanism underlying the multi-step ArcB-to-ArcA phosphorelay has been proposed inductively from intensive studies of Lin and colleagues [12,20]. Recently, we proposed a refined model, which emphasized the functional importance of the HPt domain (see Fig. 1A) [21,26]. ArcA is phosphorylated through two distinct phosphotransfer pathways: one directly from His-292 and the other through the multi-step His-717-to-ArcA phosphorelay. In any case, the resulting phospho-ArcA functions as the transcriptional repressor for the *sdhCDAB* operon. The His-717-to-ArcA (type II signaling) pathway is primarily responsible for the adaptation under anoxic ($-O_2$) conditions and the shortcut His-292-to-ArcA (type I signaling) pathway appears to operate even under fully aerobic ($+O_2$) conditions in response to a presumed intracellular metabolic state. In this study, it was further proposed that the type II signaling pathway is responsible also for anaerobic respiratory regulation of *sdh* in response to certain anaerobic electron acceptors. In this particular signaling process, SixA plays a crucial role by draining phosphoryl group from the HPt domain, thereby accelerating the derepression of *sdh* in response to the presence of anaerobic electron acceptors (Fig. 6). This novel regulatory mechanism makes it possible for *E. coli* cells to derepress the expression of *sdh*, even under anoxic ($-O_2$) growth conditions, when an appropriate anaer-

obic electron acceptor is available. As a whole, the ArcB hybrid His-kinase can function, together with the SixA phosphatase, as a sophisticated device exhibiting the ability to propagate complicated physiological signals in its own right.

Finally, it should be noted that there are several precedents of regulatory mechanisms, by which a given His-to-Asp phosphorelay pathway is modulated by a phosphatase. Dephosphorylation of the phospho-CheY chemotactic response regulator is modulated by the CheZ phosphatase [33]. In *Bacillus subtilis*, the Spo0F response regulator, involved in the control of sporulation, is the target of the RapA and RapB phosphatases, which can modulate the phosphorelay [34]. In *E. coli*, it has been reported that the stress-responsive CpxR-CpxA pathway is modulated by the PrpA and PrpB phosphatase [35]. Note that each target of these known phosphatases is a given specific response regulator. One can also envisage that a phospho-histidine in an HPt domain would also be a potential and alternative target of a regulatory phosphatase. As far as we know, SixA is the first example of such phospho-histidine phosphatases, which play a physiological role, as described above. The recent discovery of the His-to-Asp phosphorelay raised the general question of what is the advantage of multistep signaling through the HPt domain [5,27]. One idea is that it may provide the potential for integration of multiple signals at its intermediate step [21]. Another is that the extra His-to-Asp phosphorelay component may serve as a regulatory checkpoint in the signaling pathway. The proposed ArcB-to-ArcA phosphorelay mechanism, involving the SixA phospho-histidine phosphatase, appears to be a novel example of the latter view.

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