Polyamine Stimulation of *arcA* Expression in *Escherichia coli*

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The effects of two natural polyamines (putrescine and spermidine) on the synthesis of ArcA, a response regulator of the Arc two-component signal transduction system, were studied using an *E. coli* mutant deficient in polyamine biosynthesis. Endogenous polyamine deficiency of the mutant resulted in marked reduction in the ArcA level determined by Western blot analysis. Putrescine supplement to the growth medium effectively increased the ArcA level of the mutant in a concentration-dependent manner. Spermidine also stimulated the ArcA level in the mutant to a greater degree than putrescine. Expression of *arcA'::lacZ* operon fusion in the mutant was stimulated 6-fold and 10-fold by putrescine and spermidine at a 1mM concentration, respectively, indicating that the stimulatory effect of the polyamines on ArcA synthesis is due to transcriptional induction, and that spermidine is a more potent *arcA* inducer than putrescine. The polyamine-dependent *arcA'::lacZ* induction was growth-phase-dependent and independent of either *arcA* or *fnr* which are two regulators involved in anaerobic stimulation of the ArcA level. These results suggested that putrescine and spermidine polyamines may be potential intracellular signal molecules in the control of *arcA* expression, and thereby may play an important role in cellular metabolism.

Key words: polyamine, putrescine, spermidine, transcriptional regulation, *arcA*, *Escherichia coli*

The Arc two-component signal transduction system is a major regulatory system mediating adaptive responses of *Escherichia coli* in changing respiratory conditions of growth (Iuchi and Lin, 1992, 1993; Lynch and Lin, 1996). The Arc system, comprised of the ArcB transmembrane sensor kinase and cytoplasmic ArcA response regulator, modulates expression of some 30 operons (Arc regulon) involved in respiratory or fermentative metabolism in response to changes in redox conditions of growth (Hoch and Shihavy, 1995). The intracellular level of the functional ArcA regulator, phosphorylated ArcA (ArcA-P), is sensitively regulated by the oxidation/reduction status of the cell. Under anaerobic conditions, ArcB autophosphorylates and then transphosphorylates ArcA, which then binds to the target promoters (Iuchi and Lin, 1992; Compan and Touati, 1994), thereby exerting regulatory effect on the target genes. Under aerobic conditions, ArcB autophosphorylation is inhibited by the oxidized quinone pool (Georgellis *et al.*, 1997, 2001), thus preventing ArcA-P-dependent regulation of the Arc regulon. Expression of *arcA* is also regulated by the redox condition of growth; *arcA* expression increases in anaerobiosis about 4-fold and both Fnr and

ArcA are required for full expression (Compan and Touati, 1994).

Polyamines (putrescine, spermidine, and cadaverine), polycationic compounds ubiquitous in all living organisms, have been implicated in many physiological functions including nucleic acid biosynthesis, cell growth, and differentiation (Tabor and Tabor, 1985; Koski and Vaara, 1991). However, their specific physiological role at the molecular level is still largely unknown. During the course of our effort to investigate the *in vivo* role of polyamine, we found that *ycjL* of *Escherichia coli* is inducible by putrescine under aerobic, but not anaerobic conditions. The polyamine-dependent expression of *ycjL* is, in part, mediated through ArcA (Rhee *et al.*, 2001). These data suggested that polyamine may play a role as a modulator of the Arc signal transduction system and prompted us to assess the effect of polyamine on ArcA expression. We found that ArcA synthesis is markedly stimulated by two major natural polyamines (putrescine and spermidine), which is due to the transcriptional induction of *arcA* by polyamines. These results suggest that the polyamines may play a role as endogenous signal molecules modulating induction of *arcA*, and thereby may control numerous operons involved in respiratory or fermentative metabolism of *Escherichia coli*.

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Materials and Methods

Bacterial strain and media

Bacterial strains used are listed in Table 1. The rich medium was Luria Bertani (LB: tryptone 10 g, yeast extract 5 g, NaCl 10 g per liter) and minimal medium 56 (M56) (10.6g KH_2PO_4, 17.4g Na_2HPO_4, 4 mL 10% $MgSO_4$, 2 mL 1% $(NH_4)_2SO_4$, 2 mL 0.05% $FeSO_4$ per liter) supplemented with glucose at 1.0% was used as the minimal medium. Where required, 50 µg of proline was added per ml. Appropriate antibiotics were added for culturing the cells carrying plasmids. Cells were grown at 37°C with vigorous shaking.

Construction of arcA'::lacZ fusion strain

Construction of *arcA'::lacZ* fusion strain was performed as described by Sylhavy (Raivio *et al.*, 1999). The *arcA* promoter was amplified by PCR from chromosomes of MG1655 (primer 1: CATCGTGTAAGAATTCAGCC; primer 2: CGCGGATCCATGATCACCAGGTTGAT). The 1013 bp PCR product was digested by restriction enzymes *Eco*RI and *Bam*HI, and then ligated with promoterless *lacZ* gene of pRS415, resulting in plasmid pJH1. The *arcA::lacZ* fusion was transferred from plasmid pJH1 to phage λRZ5 by *in vivo* recombination as described before (Raivio *et al.*, 1999). The resulting phage λJH1 was transduced into strain SN101 and single lysogens were selected for further study.

β-galactosidase assay

Single colonies of each bacterial strain were inoculated in 10 ml LB broth and grown overnight at 37°C with aeration. The next day, the cultures were diluted at an initial A_{600} of 0.05. Aliquots of the culture were taken at various time intervals, and cells were kept with chloramphenicol (300 µg/ml) in ice until assay. β-galactosidase levels were measured as described by Miller (Miller, 1972) with modification. The β-galactosidase activity is expressed as follows: $1{,}000 \times (O.D_{420}/[O.D_{600}$ culture $\times$ reaction time $\times$ volume]). Shown in the figures are data from a single representative experiment, but all experiments were repeated several times to ensure the reproducibility of results.

Protein expression and purification

The whole open reading frame of *arcA* was amplified by PCR from chromosomes of MG1655 (primer 1: AGGTAG-CACATATGCAGACC; primer 2: TTGGGATCCAGTGT-GCTGGTGG). The 910 bp PCR product was placed in the MBP open reading frame in pMal-P2X (New England Biolab), and the resulting plasmid pJL911 was transformed into XL1-Blue. Strain JL911, carrying plasmid pJL911, was inoculated in 10ml LB broth and grown overnight at 37°C with aeration. The next day, the cultures were diluted 1:200 into the same medium and grown at 37°C with aeration. 0.3 mM IPTG was added at A_{600} of 0.5, and harvested at 4 h. Cell pellets were suspended in 10mM Tris-Cl (pH 7.4) containing 50 mM NaCl and then sonicated (Ulsso). ArcA protein was purified by FPLC®(Amersham Bioscience) using columns, HiTrap Q, HiTrap Heparin, and HiPrep Sephacryl HR (Amersham Bioscience).

Table 1. Bacterial strains, plasmids, and phages used in this study

Strain	Relevant genotype	Source/references
E. coli K-12 strains		
MG1655	F⁻ λ⁻	Geiger *et al.* (1978)
KL527	F⁻ Δ(*speA⁻ speB*)97 (*speC-glc*)63 *gyrA96* λ⁻	Panagiotidis *et al.* (1987)
EG333	*car96::Tn10 met⁻* Δ(*pro-lac*)XIII *cysG-303*	Golub and Low (1983)
SN101	F⁻ Δ(*speA⁻ speB*)97 Δ(*speC-glc*)63 *gyrA96* Δ(*lac-pro*) λ⁻	This study
ECL618	MC4100, *arcA2 zji::Tn10*	Georgellis *et al.* (1997)
SE1932	BW545 *fnr zcj::Tn10*	Laboratory collection
JL911	XL1 Blue, pJL911	This study
JL921	SN101 λJH1	This study
JL922	JL921, *arcA2 zji::Tn10*	This study
JL923	JL921, *fnr⁻ zcj::Tn10*	This study
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻*	Laboratory collection
Plasmid		
pRS415	*LacZ⁺ lacY⁻ lacA⁺ Amp^R*	Simon *et al.* (1987)
pJH1	*arcA::lacZ⁺ lacY⁻ lacA⁺ Amp^R*	This study
pMal-P2X	*malE-lacZá⁺ lacI Amp^R*	New England Biolab.
pJL911	*arcA⁺ Amp^R*	This study
Bacteriophages		
P1_vir		Laboratory collection
λRZ5	'*bla* '*lacZ lacY⁺*	Iuchi *et al.* (1990)
λJH1	Φ(*arcA'::lacZ⁺*) *bla⁺*	This study

Preparation of ArcA antibody

One ml of ArcA antigen solution containing 150 μg of purified ArcA in PBS (0.14M NaCl, buffered to pH 7.3 with 0.02M sodium phosphate) was emulsified with an equal volume of complete Freunds adjuvant (Difco) and then injected into a 1.5 kg rabbit. One week after the first injection, a booster injection was given with ArcA antigen solution emulsified in incomplete Freunds adjuvant (Difco). One week after the initial booster, the booster immunization was repeated twice at one week intervals. After final injection, serum was collected by heart puncture.

Western blotting assay

ArcA antibody was obtained by injection of purified ArcA into the rabbit. Western blotting assay was performed as described before (Ausubel *et al.*, 1999). Preparative protein samples were boiled for 10min and then assayed by the Lowry method (Lowry *et al.*, 1951). Equal amounts of proteins were separated by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and transferred onto n3itrocellulose membrane (Msi). For detection of ArcA, a rabbit arcA antiserum was used at a dilution of 1:1000. Antigen-antibody complexes were reacted with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) at a dilution of 1:2500 and visualized by using a chemiluminescence kit (Amersham Bioscience). Blots were exposed to AGFA X-ray film. The relative unit of band intensity was detected by densitometer (Molecular Dynamics).

Results

Polyamine stimulation of ArcA synthesis

To assess the effect of polyamine on Arc-mediated transcriptional regulation, we tested the effect of polyamines on the synthesis of ArcA by Western blot analysis. For this purpose, we constructed an ArcA expression vector, overexpressed ArcA, and purified ArcA protein (Fig. 1). Polyclonal antibody was prepared against the purified ArcA and used for Western blot analysis. The effect of polyamine on the synthesis of ArcA was first examined in both a polyamine synthesizing wild type control strain, MG1655, and a polyamine-deficient mutant, KL527, which lacks biosynthesis of putrescine by carrying three deletion mutations in the genes encoding the biosynthetic arginine decarboxylase (*speA*), the biosynthetic ornithine decarboxylase (*speC*), and agmatine ureohydrolase (*speB*). The levels of ArcA in MG1655 and KL527 cultured in the presence or absence of exogenous putrescine (3 mM) supplement to the growth medium were determined by Western blot analysis. Under the conditions of no putrescine supplement, the polyamine-deficient strain KL527 produced a markedly reduced level (approximately 18-fold reduction) of ArcA compared with that of MG1655 (Fig.
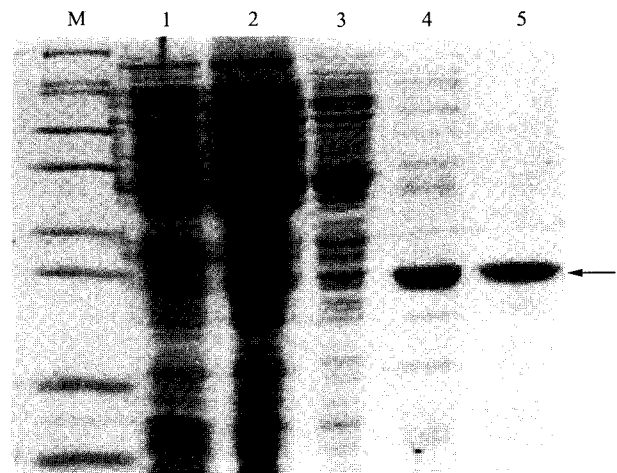

Fig. 1. Purification of ArcA protein. Strain JL911 harboring ArcA expression vector, pJL911, was induced at A$_{600}$ of 0.5 with 0.3 mM IPTG. After 4 h, cell was harvested and sonicated. Crude extract was loaded in FPLC column for purification. Lane: 1, cell crude extract - IPTG; 2, cell crude extract +IPTG; 3, Ion exchange Q column fraction; 4, Affinity Heparin column fraction; 5, Sephacryl column fraction.
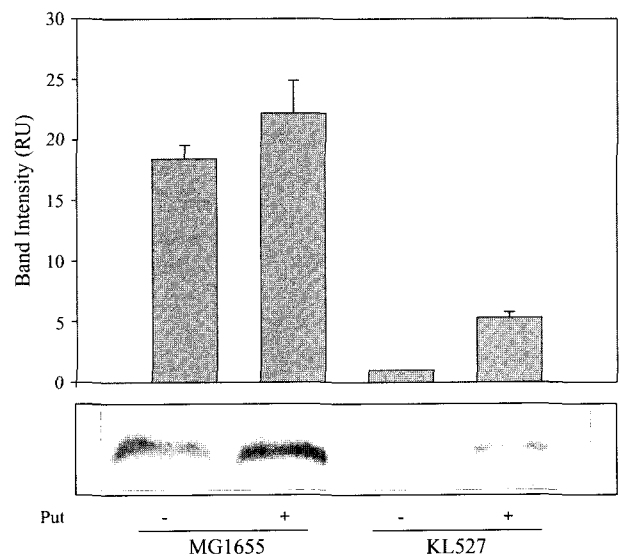

Fig. 2. Effect of putrescine on ArcA synthesis in MG1655 and KL527. Western blot of ArcA was performed using 15 μg protein of cell lysate from the wild type strain (MG1655) and polyamine-deficient mutant (KL527) grown in M56 broth with or without putrescine supplement at a 3 mM final concentration. Strains MG1655 and KL527 were precultured in polyamine-free M56 broth, as described in Materials and Methods, for depletion of endogenous polyamine carry-over from previous LB culture and then were used as inoculum. M56 broth was inoculated with the polyamine-starved culture at an initial A$_{600}$ of 0.05 and incubated at 37°C with shaking. Samples were harvested at 9 h.

2). Addition of putrescine (3 mM) stimulated ArcA level significantly (about 5-fold) in KL527. The stimulatory effect of putrescine on the ArcA synthesis in KL527 was observed in a concentration-dependent manner with a maximum stimulatory effect at 5mM putrescine (Fig. 3).
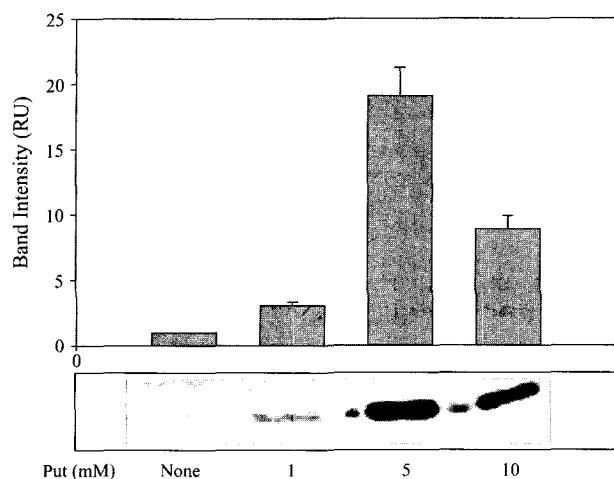
Fig. 3. Concentration effect of putrescine on ArcA synthesis in KL527. Western blot of ArcA was performed using 15 μg cellular protein of strain KL527 grown in M56 broth supplemented with putrescine at a 0, 1, 5, or 10 mM final concentration. Culture and sampling conditions were the same as described in the legend to Fig. 2.
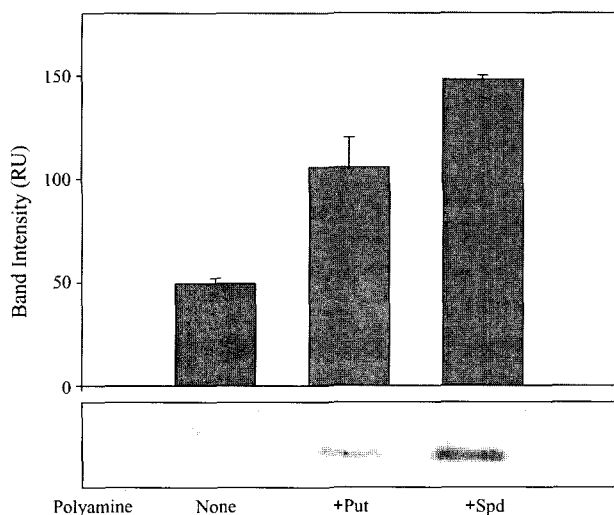

Fig. 4. The effects of polyamines in ArcA expression. Western blot of ArcA was performed using 20 μg cellular protein of strain KL527 grown in M56 broth supplemented with putrescine and spermidine at a 0 or 3 mM final concentration. Culture and sampling conditions were the same as described in the legend to Fig. 2.

We also tested the effect of spermidine on the synthesis of ArcA in the mutant background (Fig. 4). Spermidine also enhanced ArcA synthesis with even a greater stimulatory effect than putrescine.

Transcriptional stimulation of *arcA* by putrescine and spermidine

Since polyamine (putrescine and spermidine) stimulated ArcA protein synthesis (Fig. 2, 3, and 4), we speculated that the stimulatory effect of the polyamines on the ArcA synthesis might be due to the transcriptional expression of *arcA*. To evaluate this possibility, the effect of polyamine

on the transcriptional expression of *arcA* was tested using an *arcA'::lacZ* operon fusion in an isogenic polyamine-deficient mutant background. For this purpose, we first constructed *arcA'::lacZ* operon fusion *in vitro* by ligation PCR amplified *arcA* promoter region (from -846 bp to +167 bp of translational start site) (Fig. 4A) with plasmid pRS415. A *Δ(pro-lac)* derivative strain of KL527, SN101, was also constructed by Hfr-mediated conjugation using EG333 and KL527, as a donor and a recipient strain, respectively. The *arcA'::lacZ* was then introduced into SN101 chromosome as described in Materials and Methods.

We determined the effect of polyamine on *arcA'::lacZ* expression by monitoring β-galactosidase activity in the polyamine-deficient *arcA'::lacZ* operon fusion strain JL921 during growth in the absence or presence of exogenous polyamine (putrescine or spermidine) at a 1mM concentration. As shown in Fig. 5B, both polyamines induced *arcA'::lacZ* markedly; spermidine had a higher (10-fold) stimulatory effect on *arcA'::lacZ* expression than putrescine (6-fold). The stimulatory effects of putrescine and spermidine on *arcA'::lacZ* expression (Fig. 5B) were consistent with those on ArcA synthesis determined by Western blot analysis, though the degree of the stimulation was different, possibly due to the limitations of Western blotting in determining protein concentrations as described by others (Shellhorn *et al.*, 1998; Yoshida *et al.*, 2001; Iglesias-De *et al.*, 2002). These results indicated that enhancement of ArcA level by putrescine and spermidine was due to the transcriptional induction of *arcA* by the polyamines.

Growth-phase-dependent stimulation of ArcA synthesis and *arcA'::lacZ* expression by putrescine and spermidine

The β-galactosidase activity of the *arcA'::lacZ* fusion strain JL921 during the growth with putrescine or spermidine was induced after the early-stationary phase or late-log phase, respectively (Fig. 5). The polyamine-dependent *arcA'::lacZ* expression was induced at reproducibly different times with putrescine and spermidine. Spermidine induced *arcA'::lacZ* about 2 hours earlier than the growth of putrescine. In both polyamines, however, *arcA'::lacZ* fusion was maximally induced at the stationary phase. These results demonstrate that spermidine and putrescine induce *arcA* transriptional expression in a growth-phase-dependent manner.

We checked whether the level of ArcA reflected the expression of *arcA'::lacZ* induced by putrescine. Strain KL527 was grown in the presence or absence of putrescine (3 mM), and the ArcA level was determined by immunoblotting experiments. ArcA was accumulated with putrescine only after the early stationary phase, but very little ArcA was present throughout growth when putrescine was not added to the medium (Fig. 6). Spermidine showed a similar stimulatory effect on the ArcA level as putrescine in the pattern of induction, but produced
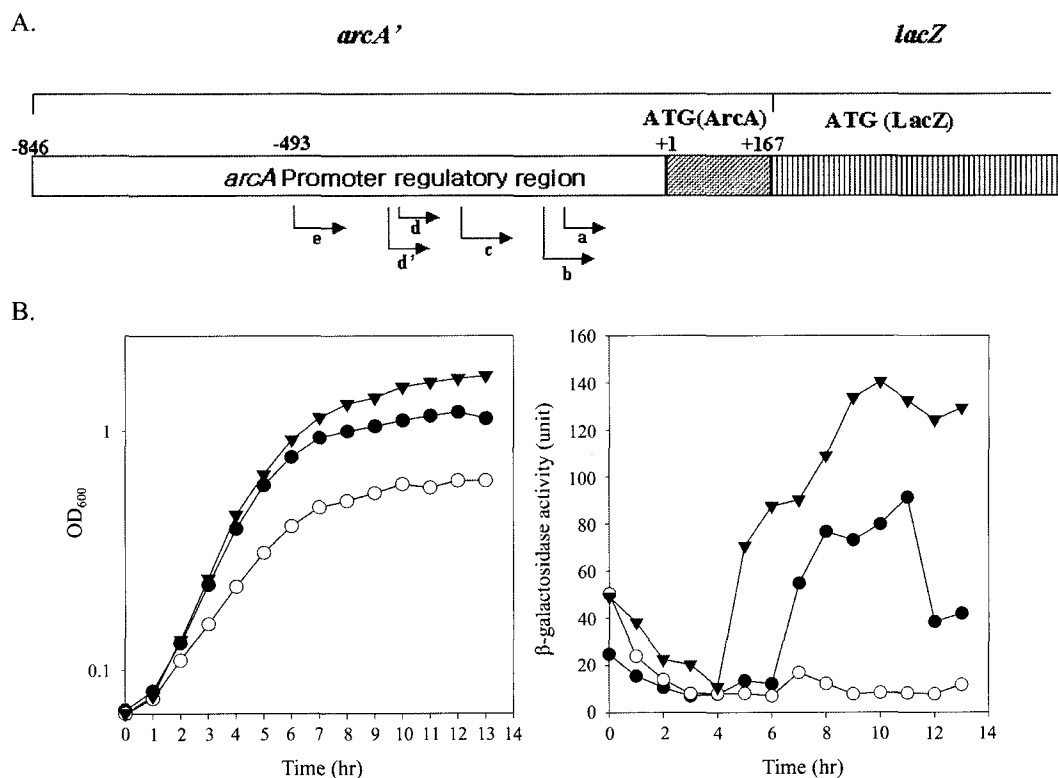
Fig. 5. Effect of polyamines on the expression of *arcA'::lacZ* operon fusion. (A) Schematic presentation of the promoter region of *arcA* (Compan and Touati, 1994) used for construction of *arcA::lacZ* operon fusion in this study. (B) Growth and β-galactosidase induction profiles of JL921 (*arcA'::lacZ*). Strain JL921 grown overnight in LB broth was inoculated in M56 broth, at an initial A_{600} of 0.05, with supplement of no polyamine (○), 1 mM putrescine (●), or 1 mM spermidine (▲), and incubated at 37°C with shaking. Samples were taken perodically and measured at A_{600} for growth (left panel) and β-galactosidase activity (right panel).

higher level of ArcA (data not shown). Overall, the patterns of ArcA level and the expression of the *arcA'::lacZ* induced by putrescine and spermidine were reasonably parallel.

Putrescine stimulation of arcA'::lacZ expression independent of ArcA autoregulation or Fnr-mediated induction

It was previously reported that *arcA* expression is regulated according to the availability of oxygen in the growth medium; expression of *arcA'::lacZ* was higher (about 4-fold) in anaerobic than aerobic conditions (Compan and Touati, 1994). The anaerobic induction of *arcA* is caused by two different regulatory mechanisms sensing oxygen availability in the environment: ArcA-mediated autoactivation and Fnr-mediated activation. We speculated that the stimulatory effect of the polyamines (Fig. 4) might be conveyed to the *arcA* induction via ArcA and/or Fnr. We examined this possibility first by measuring the putrescine-induced β-galactosidase activity of *arcA'::lacZ* in the presence or absence of an *arcA* null mutation (*arcA::Tn10*) on the chromosome. Introduction of *arcA::Tn10* to JL921, confirmed with both the presence of *arcA* phenotypes (Iuchi and Lin, 1988) and the absence of ArcA by immunoblotting (data not shown), did not significantly change the maximum level of β-galactosidase activity of *arcA'::lacZ*

induced by putrescine (Fig. 7), though there was a short time delay in β-galactosidase induction which is likely due to a reduction in the growth rate of the *arcA::Tn10* strain JL922. Under the qualitative plate assay conditions employed in this study with strain JL923, *fnr⁻* did not change, to any significant degree, the putrescine-dependent β-galactosidase activity of *arcA'::lacZ*, either (data not shown). These results indicate that the putrescine-dependent *arcA'::lacZ* induction is not mediated by either ArcA or Fnr.

Discussion

In this study, using a polyamine-deficient mutant (KL527) background, we showed that lack of endogenous polyamine synthesis markedly reduced expressions of both ArcA and *arcA'::lacZ*, and exogenous addition of polyamine (putrescine and spermidine) resulted in an increease in both ArcA synthesis and *arcA'::lacZ* induction, suggesting that putrescine and spermidine (or metabolites derived from them) are signal molecules modulating transcriptional expression of *arcA*.

Although both putrescine and spermidine effectively induced ArcA synthesis and *arcA'::lacZ* expression, they
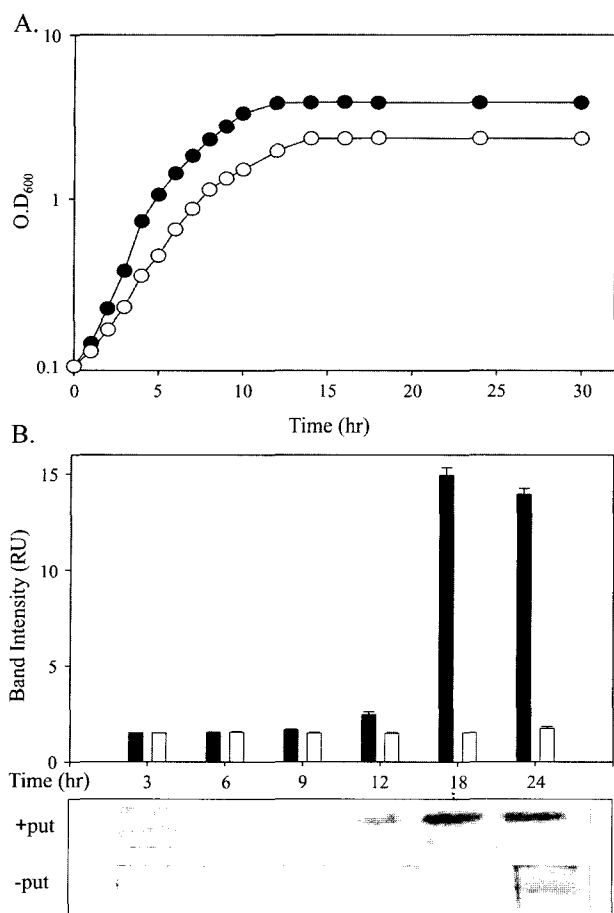
Fig. 6. Growth phase dependency of polyamine-dependent synthesis of ArcA in KL527. Conditions of culture and Western blot analysis were as described in the legend to Fig. 2. (A) Growth profiles of KL527. KL527 was cultured in the presence (●) and absence (○) of putrescine (3 mM). Sampling times were indicated with vertical arrows. (B) Western blot analysis of ArcA synthesis.

Fig. 7. Effect of *arcA⁻* mutant on the expression of *arcA'::lacZ* operon fusion. Strain JL921 (*arcA⁺*; ●,○) and JL922 (*arcA⁻*; ▲,△) were grown in the presence (●,▲) or absence (○,△) of putrescine as described in the legend to Fig. 5. Samples were taken periodically and measured growth (left panel) and β-galactosidase activity (right panel) were measured.

showed differences in the induction patterns. First, spermidine was a more potent inducer than putrescine; spermidine stimulated both the ArcA level (Fig. 4) and *arcA'::lacZ* expression (Fig. 5) at a greater degree compared with putrescine at an equal amount of supplement. Secondly, spermidine induced *arcA'::lacZ* at an earlier growth stage (late log-phase) than putrescine (early stationary-phase) (Fig. 3). A possible explanation for these differences is that the stimulatory effect of putrescine may be exerted via spermidine converted from exogenously added putrescine, since strain KL527 has the capability to convert putrescine to spermidine due to the presence of *speE* encoding spermidine synthetase (Tabor *et al.*, 1986; Tabor and Tabor, 1987; Xie *et al.*, 1989). This possibility can be tested using a *speE* derivative of the polyamine-deficient mutants (KL527 and SN101).

The level of *arcA* expression varies by oxygen conditions during growth by ArcA and Fnr; *arcA* expression is 4 times higher in anaerobic than aerobic
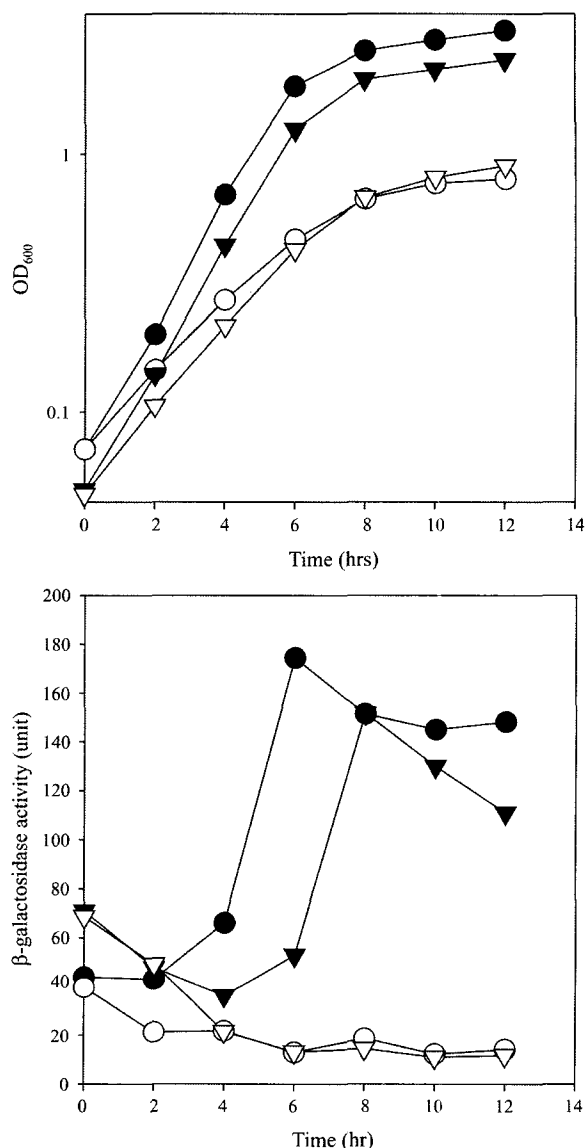
conditions (Compan and Touati, 1994). Recently, using DNA array analysis of the transcriptome of aerobically grown cultures, induction of *arcA* during growth arrests caused by diauxic growth and H_2O_2-treatment was observed with a maximum 4-fold *arcA* induction (Chang *et al.*, 2002) compared with the level during steady state growth. We concluded that the putrescine-dependent induction of *arcA* expression (Fig. 5) is independent of the anaerobic *arcA* induction for two reasons: aerobic growth conditions used, and being independent of either *arcA* or *fnr*. The polyamine-dependent *arcA* induction (Fig. 4 and 5), together with the stress-

induced *arcA* induction during growth arrest, suggests that polyamines play a role as a signaling molecule mediating the stress responses (such as growth arrests by carbon-starvation or H_2O_2-treatment).

Physiological relevance of the polyamine-dependent induction *arcA* is not clear, since phosphorylated ArcA, not ArcA, is required for regulation of Arc regulons. It is suggested that ArcA binds to its target promoter in an oligomeric form (Compan and Touati, 1993), and it has been recently reported that multimerization of phosphorylated and non-phosphorylated ArcA (in a 1:1 ratio) is required for binding to target promoters (Jeon et al., 2001). It is possible that both phosphorylation of ArcA mediated by ArcB and enhancement of ArcA levels by polyamine increase the level of functional ArcA multimers, thereby regulate ArcA regulon.

Although the mechanism underlying the stimulatory polyamine effect on *arcA':lacZ* transcriptional expression needs to be uncovered in future studies, the results obtained in this study indicate that polyamine can be a potential intracellular signal molecule in the control of *arcA*, and thereby may play an important role in cellular metabolism (such as respiratory and fermentative metabolism).

Acknowledgments

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