J. Microbiol. Biotechnol. (2001), **11**(5), 825–830

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Effect of ArsA, Arsenite-Specific ATPase, on Inhibition of Cell Division in *Escherichia coli*

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Received: April 24, 2001
Accepted: August 20, 2001

Abstract *Escherichia coli*, which harbored the *ars* operon from a plasmid pMH12 of *Klebsiella oxytoca* D12, showed filamentation due to the expression of *ars* genes in the presence of arsenite. The continued DNA replication in the absence of cell division was revealed, since nucleoids bound with DAPI appeared to be arranged in chains. In contrast to overexpression of *arsA*, its frame-shift mutant and knock-out mutant lost filamentation in the presence of arsenite, which suggested that *ars*-induced division block was dependent on expression of *arsA*. ArsA-induced division inhibition was not a consequence of an inhibition of DNA replication, and the inability of arsenite to induce an SOS response indicated that *arsA*-mediated division inhibition was dependent on the expression of the gene product encoded by the *minB* operon. ArsA is a peripheral membrane protein with an ATP-binding domain, which is homologous to MinD that requires ATP-dependent efflux. These results suggested that ArsA could possibly recruit MinC to the membrane and modulate cytoplasmic FtsZ to block assembly at the middle of the cell.

Key words: Arsenite resistance, arsenite-specific ATPase, *asrA*-mediated division inhibition

The *ars* operon of the *E. coli* plasmid R773 [6, 18] and R46 [4] consists of five genes, *arsR, arsD, arsA, arsB,* and *ars*C. The ArsR protein is a cytoplasmic polypeptide that binds to an operator region as a dimer and operates to repress the functioning of the *ars* operon with its own synthesis [22, 25]. The ArsD protein is known as a cytoplasmic, secondary regulator which has a weak affinity to the promoter, and is a *trans*-acting negative repressor. Unlike the ArsR repressor protein, the down-regulation produced by the ArsD is inducer-independent and its expression has a little effect on the level of resistance set by the ArsR [24]. ArsA and ArsB are necessary and sufficient for the ATP-coupled oxyanion pump, which catalyzes extrusion of arsenite and antimonite, producing resistance to these toxic anions [6, 18]. The ArsA is a catalytic subunit of the oxyanion-translocating ATPase, which is stimulated by arsenite and antimonate. It is peripherally associated with the cytoplasmic surface of the inner membrane through interacting with the ArsB protein [23]. The ArsA functions as a homodimer on binding of the anions, which is required for achieving hydrolysis of ATP [12]. The ArsB is an inner membrane protein that serves as a membrane anchor for the catalytic ArsA component [2]. It mediates the electrochemical energy-dependent arsenite efflux in the absence of the ArsA protein, while the ArsA-ArsB complex catalyzes the ATP-dependent transport [10]. The ArsC is a soluble polypeptide and functions as an arsenate reductase which converts arsenate to arsenite. In addition to ArsA and ArsB, it is required to alter the substrate specificity of the pump and to transport the arsenate [21].

A determination was made regarding the complete nucleotide sequence of the 5.6 kb *Eco*RI fragment containing the *ars* operon from the 67-kb cryptic plasmid (pMH12) of *Klebsiella oxytoca* D12, which was isolated from the Jungrang stream of the Han river in Seoul (GenBank access No. AF168737) [5, 15]. A search of the protein databases revealed that the organization of the *ars* locus was the same and the amino acid sequence was similar to the corresponding operon in *E. coli* plasmid R773. It was also shown that ArsA was 32% identical to the sequence of MinD. MinD is a membrane-associated protein that is capable of binding and hydrolyzing ATP [9]. MinD functions to activate the division inhibition activity of MinC, presumably by mediating the membrane attachment of MinC [20]. MinCD acts similarly to SulA by blocking the polymerization of FtsZ, thereby preventing formation of the FtsZ ring [14]. In

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this study, an overexpression of *arsA* was first demonstrated in the presence of arsenite that leads to an inhibition of septation at all potential division sites(PDSs), which resulted in the formation of long nonseptate filaments.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Table 1 lists the genotypes and origins of the strains of *E. coli* and the plasmids which were used in this study. Luria-Bertani (LB) and M9 minimal media were prepared as previously described [17]. The following antibiotics were used at the indicated concentration in microgram per milliliter: ampicillin (100), kanamycin (50), and spectinomycin (150). When cells were grown in the presence of sodium arsenite, an overnight culture in LB was diluted 100-fold in LB; sodium arsenite was added, and growth was continued until harvesting.

Nucleoids Staining

The Hiraga's fluo-phase combining method was used for nucleoid staining [11]. Cells were collected by centrifugation (19,000 ×g for 5 min) and washed once with saline. After the centrifugation, cells were resuspended in an appropriate volume of saline. A portion of the sample (5–10 μl) was spread on a clean glass slide, dried at room temperature, and fixed completely with drops of methanol for 5 min. The slide was then washed six times with tap water in a large beaker, dried at room temperature, and spread with 10 μl of poly-L-lysine (5 mg/ml). Then, it was dried again at room temperature and covered with 10 μl of DAPI (4,6-diamino-2-phenylindole) (5 mg/ml), which binds specifically to DNA. The observed nucleoids were photographed by using simultaneous phase contrast and fluorescent microscopy. Magnification of the microscope was ×400 for all panels.

β-Galactosidase Assays

β-Galactosidase assays were performed as previously described [17, 19]. The β-galactosidase activity of the culture are expressed as nmoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute. All assays were performed in duplicate, at least on two independent cultures.

Introduction of a Frame-Shift Mutation into *arsA*

The wild type *arsA* gene was amplified with Taq DNA polymerase by using the pAE48 as a template and the following primers: 5'-ATGAAATTCTTACAGAATATC-3' and 5'-TTAACTCATCAACTCTCTGAG-3'. Frame-shift mutation was constructed by amplifying *arsA* using the primers: 5'-ATGCAAATTCTTACAGAATATC-3' and 5'-TTAACTCATCAACTCTCTGAG-3' [13]. These products were introduced into a commercial pGEM-T Easy vector tailed with thymidine, as indicated by the manufacturer (promega). The 1.7-kb *Eco*RI fragments of wild-type and frame-shift alleles from the resulting plasmids were introduced into the pUC18 to generate pAE300 and pAE400.

RESULTS AND DISCUSSION

Ars-mediated Division Inhibition in the Presence of Arsenite

Evidence that Ars was capable of blocking cell division in the presence of arsenite came from studies conducted on the effects of high level of *ars* expression in *E. coli* DH5α. To vary the expression of *ars*, DNA fragment containing the complete *ars* operon was placed in the high-copy-number plasmid named pAE48, and the low-copy-number plasmid pLG339, and the division phenotype was determined microscopically.

As shown in Fig. 1, an overexpression of Ars from the high-copy-number pUC18-derivative, pAE48, in host *E.*

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or description	Reference or source
Bacteria		
E. coli		
DH5α	*supE*44 Δ*lacU*169(ϕ80*lacZ*M15) *hsdR*17 *recA*1 *endA*1 *relA*1	Lab collection
BL21(λDE3)	F⁻*dcm ompT hsd*(rB⁻mB⁻)*gal*	Lab collection
NK8027	*thi strA*Δ(*lac-pro*)Δ(*gal*-λG)Δ(*bio-uvrB*)λRSam7*imm*^{434}pL:: *lacZ*	N. Kleckner
MG1655	*E. coli* K12 Wild type	Lab collection
Plasmid		
pUC18	Cloning vector: Ap^r	Lab collection
pLG339	Cloning vector: Tc^r and Km^r	Lab collection
pAE48	*ars* operon cloned into pUC18	Ref. 5
pAE300	*arsA* gene under control of *lacZ* promoter	This study
pAE400	Mutant *arsA* gene under control of *lacZ* promoter	This study
pFT41	*ars* operon under control of T7 promoter from pT7-7	This study
pDB164	*minD* gene under control of *lacZ* promoter	L. I. Rothfield

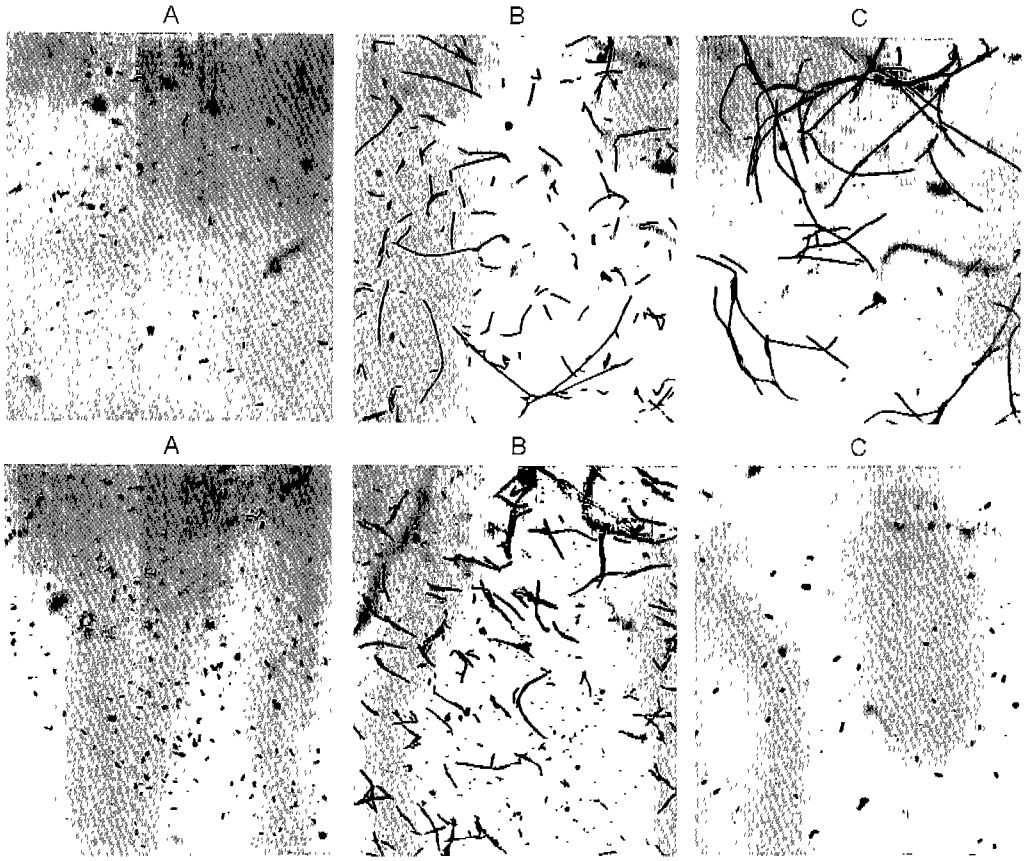

Fig. 1. Phase micrographs showing cell division phenotypes.
Top panel: Effect of arsenite on filamentation. *E. coli* DH5α containing pAE48 was grown in the presence of 100 μg/ml ampicillin (A), 3 mM arsenite (B), and 5 mM arsenite (C). Bottom panel: Effect of *ars* expression on filamentation. *E. coli* containing pFT41 was grown in the absence of IPTG (A), in the presence of 1 mM IPTG (B), and in the presence of 1 mM IPTG prior to the addition of 150 μg/ml spectinomycin (C).

coli DH5α caused a general inhibition of cell division, leading to large numbers of long filamentous cells as the arsenite concentration increased to 5 mM. In contrast, division was moderately affected when *ars* was induced from a low-copy-number plasmid in a medium containing 5 mM arsenite (data not shown). The continued DNA

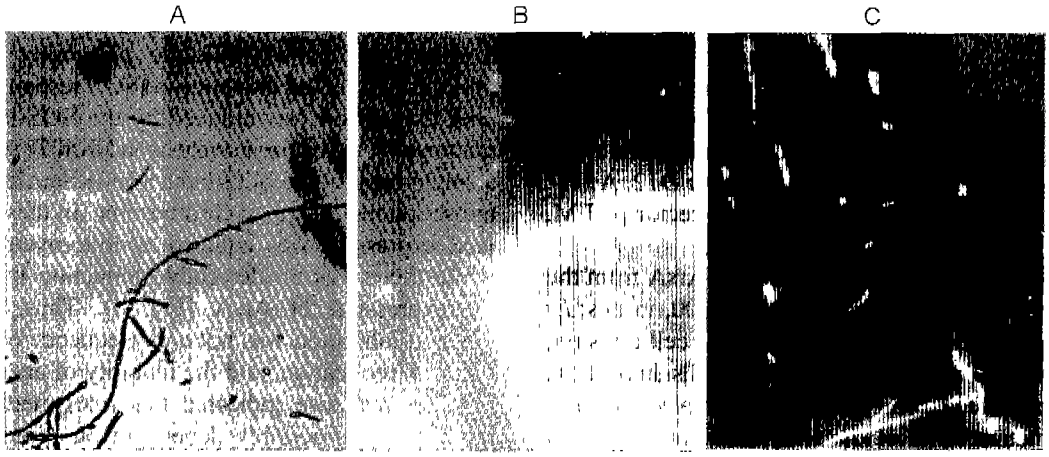

Fig. 2. Observation of fluorescent nucleoids in filamentous cell by staining with DAPI.
E. coli DH5α containing pAE48 was grown in the presence of 5 mM arsenite for 3 h and then stained with DAPI. They were visualized by phase microscopy (A) and by fluorescent microscopy (B, C). Arrowheads indicate nucleoids in chains.

replication in the absence of cell division was confirmed by demonstrating that nucleoids bound with DAPI appeared to be in chains (Fig. 2).

To further define the effects of Ars concentration on the division pattern, a P_{T7}::*ars* plasmid (pFT41) was constructed in which the T7 polymerase was induced by IPTG. Cells containing P_{T7}::*ars* plasmid were then grown in the presence of IPTG to induce *ars* expression. Expression of *ars* from the P_{T7}::*ars* plasmid in an *E. coli* BL21 strain resulted in the formation of long nonseptate filaments (Fig. 1). No filamentation was observed when the P_{T7}::*ars* plasmid was expressed in the absence of IPTG. In addition, the inhibition of cell division by Ars was readily reversible and filaments began to divide when spectinomycin was added to the culture two hours after Ars induction (Fig. 1) Thus, the reversibility was observed even when protein synthesis was blocked, indicating that the division machinery was undamaged by Ars action, and removal of Ars from the cell allowed cell division to proceed. This suggests that the *ars*-induced division block is dependent on expression of one or more of the gene products encoded by the *ars* operon.

ArsA-Mediated Division Inhibition

To determine which gene of the *ars* operon was required for the *ars*-mediated division inhibition, the effects of *arsA* overexpression and its mutations were studied. In addition, a complete nucleotide sequence of the 5.6-kb *Eco*RI fragment containing the *ars* operon from plasmid pMH12 of *Klebsiella oxytoca* D12 was determined [15]. A search of the protein databases revealed that ArsA was 32% identical to the sequence of MinD. The homologies were especially pronounced in the region including a putative ATP binding domain, GKGGVGKTS. MinD is a peripheral membrane protein with a capability of hydrolyzing ATP to activate the MinC-dependent septation inhibitor that is able to block septation at all potential division sites (PDSs). ArsA is also a peripheral membrane protein with an ATP-binding domain which is required for the ATP-dependent efflux [12]. Based on their nucleotide sequences, primers were designed for *arsA* and its frame-shift mutant, which were used in polymerase chain reactions with cloned plasmid DNA from *Klebsiella oxytoca* D12. A 1.7-kb band was amplified, inserted into the T-vector pGEM, and recloned into the pUC18.

As shown in Fig. 3, an overexpression of ArsA from the high-copy-number pUC18-derivative, pAE300, in host *E. coli* DH5α caused a general inhibition of the cell division, leading to large numbers of long filamentous cells. In contrast, division was not affected when *arsA* was disrupted, yielding a frame-shift mutant. An *arsA* knock-out mutation in plasmid was also created, containing the *ars* operon by introducing Tn5 through transpositon. The resulting *arsA* knock-out mutant had a phenotype that was consistent with a loss of filamentation in the presence of arsenite (data not shown). This suggests that the *ars*-induced division block is dependent on expression of *arsA*.

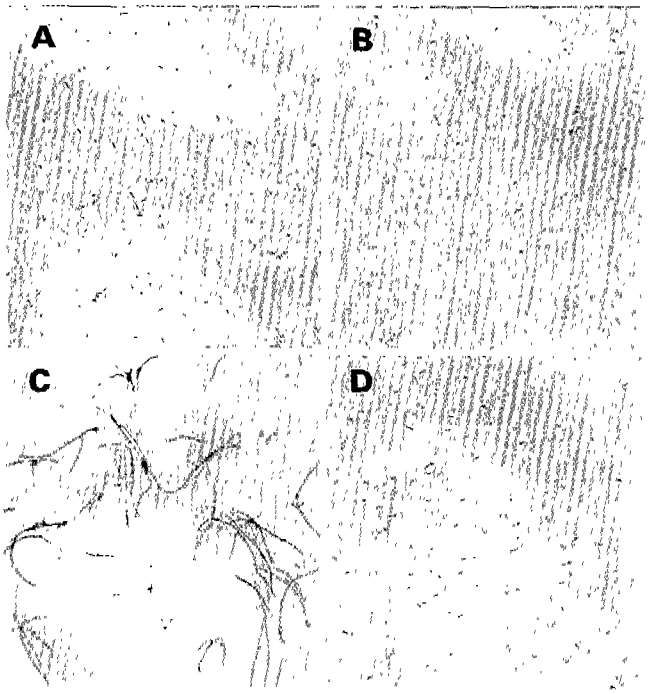

Fig. 3. Effect of wild-type and mutant *arsA* on division phenotype.
Phase micrographs were prepared from cultures of *E. coli* carrying pUC18 in the presence of 1 mM IPTG (A), pAE300[P_{lac}::*arsA*$^+$] in the absence of IPTG (B), pAE300[P_{lac}::*arsA*$^+$] in the presence of 1 mM IPTG (C), and pAE400[P_{lac}::*arsA*$^-$] in the presence of 1 mM IPTG (D).

Effect of *arsA* and *minD* Expression in *minBCD*$^+$ Cells

Induction of the cell division inhibitor SulA, a component of the SOS response, blocked the formation of the FtsZ ring that led to filamentation [2, 3]. A rapid increase in SulA occurred following DNA damage, leading to an interruption of DNA replication and a block of the cell division [3]. To exclude the possibility that division inhibition reflected induction of SOS response by arsenite, *lacZ* expression was induced by the SOS-inducing agent mitomycin C or by the arsenite of an NK8027 strain containing a single copy of the SOS-inducible λRSam7imm^{434}pL::*lacZ* transcriptional fusion in chromosome. In these experiments, *lacZ* expression was induced by mitomycin C treatment, while there was no detectable induction by arsenite and overexpression of ArsA (Fig. 4). Thus, *arsA*-induced division inhibition was not a consequence of an inhibition of DNA replication, and the inability of arsenite to induce an SOS response indicated that *arsA*-mediated division inhibition was possibly dependent on expression of gene products encoded by the *minB* operon.

The gene products of the *minB* operon of *Escherichia coli*, MinC, MinD, and MinE, are required to regulate the

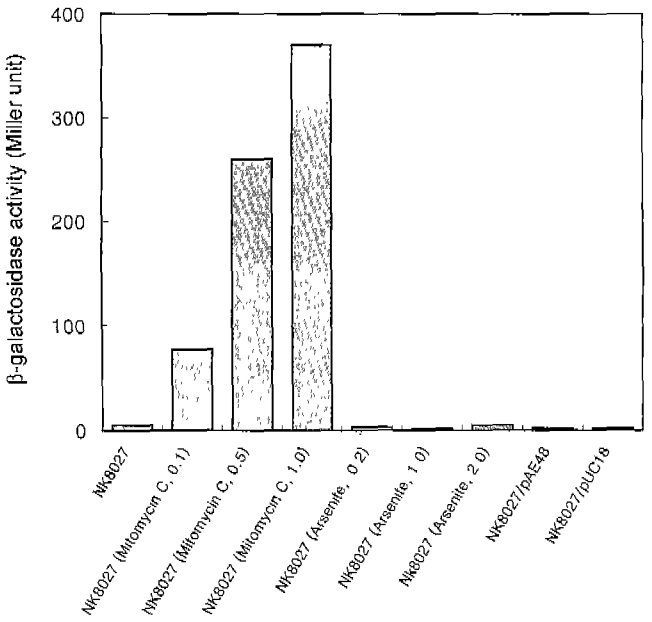

Fig. 4. Effect of mitomycin C and arsenite on the induction of SOS response.
E. coli NK8027 containing SOS-inducible λRSam7imm^{434}pL::*lacZ* was grown in the presence of mitomycin C (μg/ml) and arsenite (mM). β-Galactosidase activity was determined from culture samples obtained during the late exponential phase in LB at 37°C.

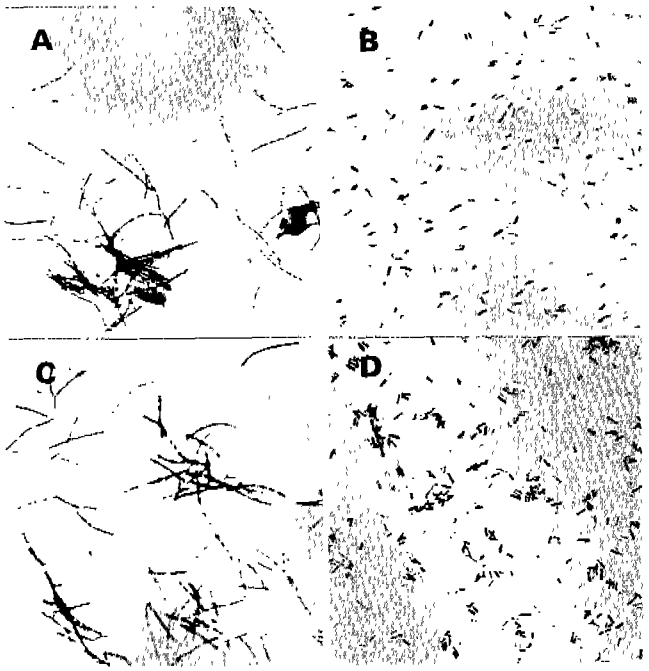

Fig. 5. Effect of *minD* and *arsA* on division phenotype.
Phase micrographs were prepared from cultures of *E. coli* MG1655 carrying pDB164[P_{lac}::*minD*] (A), pUC18 (B), pAE300[P_{lac}::*arsA*$^+$] (C), and *E. coli* MG1655 (D). Cells were grown in the presence of 1 mM IPTG.

placement of the division site [1, 16]. It is postulated that rod-shape cells have three PDSs; a new site at the middle of the cell and the old site near each of the cell poles [7]. MinC and MinD are believed to function in a complex, as division inhibitors, to block the formation of the FtsZ ring at all sites [8]. MinE relieves the MinCD cell division block at the midcell site, in which it allows for the binary fission. Previous work suggests that the ratio of MinE to the MinCD must be maintained within certain levels to avoid inhibition of division if the ratio is too low, or minicelling if the ratio is too high [7]. To determine whether the overexpression of *arsA* and *minD* had affected the ability of gene products to activate the MinC-dependent division inhibition, the multicopy plasmids containing *arsA* or *minD* were introduced into the minCDE$^-$ strain. As shown in Fig. 5, overexpression of ArsA and MinD caused an inhibition of cell division, leading to large numbers of long filamentous cells.

We, therefore, propose that the *arsA* prevented cell division at the middle of the cell in the following manner. MinC-MinD-mediated division inhibition is indeed a membrane-associated event [20]. One of the proteins with significant sequence similarity to MinD, ArsA, is also a peripheral membrane protein with ATPase activity, which possibly recruits MinC to the membrane [9]. ArsA could then directly modify the MinC protein, which modulates cytoplasmic FtsZ pools to a polymerization-incompetent state. Further work will be needed to elucidate the mechanism underlying the membrane complex of MinC-ArsA and to discover the role of MinE.

Acknowledgments

We sincerely thank Nancy Kleckner for providing the strain NK8027 and L. I. Rothfield for pDB164. This work was supported by a grant received from the Basic Science Research Institute Program, 1997, Project No. BSRI-97-4438 of Ministry of Education, Korea.

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