PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Phil. Trans. R. Soc. Lond. B 1990 **326**, doi: 10.1098/rstb.1990.0024, published 30 January 1990

Phil. Trans. R. Soc. Lond. B **326**, 455–463 (1990)
Printed in Great Britain

Molecular analysis of an ATP-dependent anion pump

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The plasmid-borne arsenical resistance operon encodes an ATP-driven oxyanion pump for the extrusion of the oxyanions arsenite, antimonite and arsenate from bacterial cells. The catalytic component of the pump, the 63 kDa ArsA protein, hydrolyses ATP in the presence of its anionic substrate antimonite (SbO_2^-). The ATP analogue 5′-*p*-fluorosulphonylbenzoyladenosine was used to modify the ATP binding site(s) of the ArsA protein. From sequence analysis there are two potential nucleotide binding sites. Mutations were introduced into the N-terminal site. Purified mutant proteins were catalytically inactive and incapable of binding nucleotides. Conformational changes produced upon binding of substrates to the ArsA protein were investigated by measuring the effects of substrates on trypsin inactivation.

The hydrophobic 45.5 kDa ArsB protein forms the membrane anchor for the ArsA protein. The presence of the ArsA protein on purified inner membrane can be detected immunologically. In the absence of the *arsB* gene no ArsA is found on the membrane. Synthesis of the ArsB protein is limiting for formation of the pump. Analysis of mRNA structure suggests a potential translational block to synthesis of the ArsB protein. Northern analysis of the *ars* message demonstrates rapid degradation of the mRNA in the *arsB* region.

1. Introduction

From protein sequence analyses all presently characterized ion-translocating ATPases can be classified as members of one of two families. The F_0F_1 family consists of proton pumps that are found in the bacterial, mitochondrial and chloroplast membrane (Pedersen & Carafoli 1987). A related class of proton pumps is found in plant (see Zimniak *et al.* (1988)) and fungal (Bowman *et al.* 1988) tonoplasts and in the plasma membrane of the archaebacterium *Sulfolobus acidocaldarius* (Denda *et al.* 1988). The other major family is the E_1E_2 cation-translocating ATPases (Pedersen & Carafoli 1987).

We have recently described the existence of a new family of ion-translocating ATPases (Mobley & Rosen 1982; Chen *et al.* 1986). The arsenical resistance operon of resistance plasmid R773 encodes an anion-translocating ATPase. In *Escherichia coli* this pump catalyses extrusion of arsenite, antimonite, and arsenate, thus conferring resistance to these toxic oxyanions (Mobley & Rosen 1982; Silver & Keach 1982). From the nucleotide sequence of the operon (Chen *et al.* 1986) this enzyme is evolutionarily unrelated to the other two classes of ion-translocating ATPases, and this family is unique in transporting anions rather than cations.

The operon has four genes, one regulatory and three structural (Chen *et al.* 1986; M. J. D. San Francisco & B. P. Rosen, unpublished results). The products of these genes have each been identified (table 1). The *arsR* gene is a regulatory gene (M. J. D. San Francisco & B. P. Rosen, unpublished results). The *arsA* and *arsB* genes are sufficient for resistance to arsenite and

Typescript received – 23 *February* 1989.

TABLE 1. GENE PRODUCTS OF THE *ars* OPERON

protein	ArsR	ArsA	ArsB	ArsC
total residues	117	583	429	141
molecular mass (Daltons)	13213	63169	45577	15811
Estimated pI	9.8	6.1	9.8	5.9

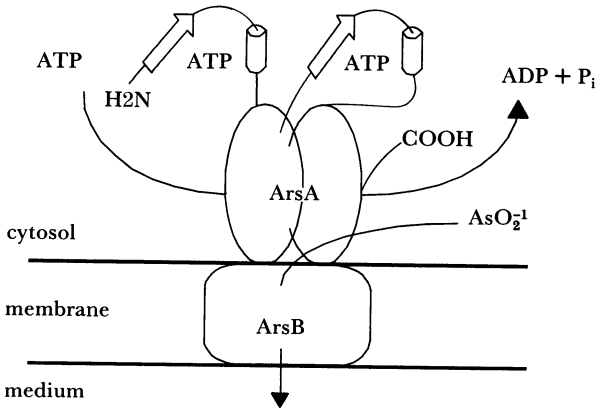

FIGURE 1. Model of the arsenical pump. The integral membrane protein ArsB functions as an anion channel. The ArsA protein is an extrinsic membrane protein with oxyanion-stimulated ATPase activity. The complex of the ArsA and ArsB proteins is an ATP-coupled pump for arsenite or antimonite. Interaction of a third subunit, the ArsC protein, (not shown) with the complex allows recognition and extrusion of arsenate (Rosen & Borbolla 1984; Mobley *et al.* 1985, Chen *et al.* 1986).

antimonite (Chen *et al.* 1985). Cells expressing only those two genes extrude arsenite but not arsenate (Rosen & Borbolla 1984). The *arsC* gene is required for arsenate resistance and transport. The 16 kDa ArsC protein is postulated to be a modifier subunit that allows the oxyanion pump to transport arsenate (Rosen *et al.* 1988*b*). Thus the minimal composition of the pump is two types of subunit, the catalytic ArsA protein, and the inner membrane ArsB protein; the latter is postulated to be both the anion-conducting subunit and the membrane anchor for the ArsA protein. A cartoon of the oxyanion pump is shown in figure 1.

2. STRUCTURE AND FUNCTION OF THE ArsA PROTEIN

The ArsA protein has been purified and shown to be an oxyanion-stimulated ATPase (Rosen *et al.* 1988*a*). From sequence information there are two potential adenylate binding sites (Chen *et al.* 1986), as suggested from sequence homology with nucleotide binding proteins (Walker *et al.* 1982). Purified ArsA was specifically photocrosslinked with α-[^{32}P]ATP (Rosen *et al.* 1988*a*) and shown to bind the fluorescent ATP analogue 2′,3′-*o*-(2,4,6)trinitrophenyl adenosine triphosphate (Rosen *et al.* 1988*b*). The only protein to which the ArsA protein shows even distant relatedness is the *nifH* gene product of the nitrogenase complex (Mevarech *et al.* 1980); dinitrogen reductase is a 64 kDa homodimer of two 32 kDa *nifH* gene products. Two dinitrogen reductases exhibit a striking resemblance to ArsA when aligned as a head-to-tail dimer with the 63.2 kDa ArsA protein (Hsu & Rosen 1989). Both proteins are energy transducing ATPases with similar nucleotide binding domains. A common ancestor of both the *arsA* and *nifH* genes could have encoded a 30 kDa polypeptide with ATPase activity. By gene

duplication and fusion this 900 base pair (b.p.) ancestor may have evolved into the 1749 b.p. *arsA* gene.

The ArsA protein was purified from the cytosol by a modification (Hsu & Rosen 1989) of the previously described method (Rosen *et al.* 1988*a*), where in the final step Superose 12 chromatography was replaced by hydrophobic interaction chromatography by using phenyl Sepharose. The specific activity of the purified enzyme was 1.2 μmol ATP hydrolysed per minute per milligram protein.

Reaction of the ArsA protein with the ATP analogue 5′-fluorosulphonylbenzoyladenosine (FSBA) inhibited oxyanion-dependent ATPase activity (figure 2). Inhibition was non-first order, indicating the participation of more than one reactive site. The presence of SbO_2^- enhanced reaction with FSBA; enzymatic activity was almost totally inhibited when the protein was incubated with FSBA and SbO_2^- together (table 2). When ATP was added with

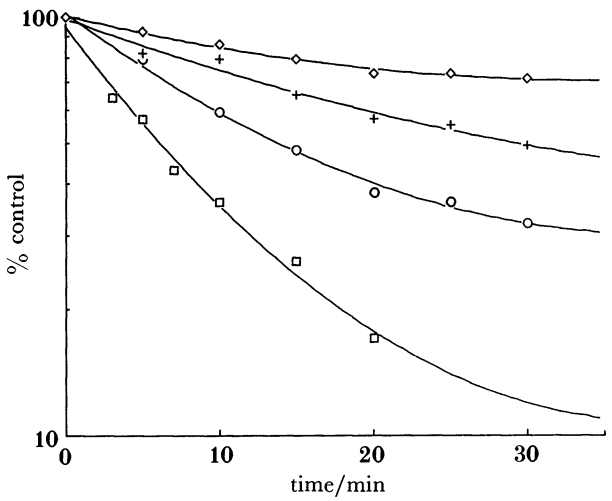

FIGURE 2. Inhibition of oxyanion-stimulated ATPase activity by FSBA. ArsA protein (0.1 to 0.2 mg ml^{-1}) was incubated with FSBA at 37 °C in 50 mM MOPS–KOH buffer (pH 7.5) containing 0.25 mM Na_2EDTA. FSBA was added to the indicated concentration of FSBA in 5% final [dimethylsulphoxide]. Portions were removed at the indicated time intervals and assayed for SbO_2^--stimulated ATPase activity. ATPase activity was assayed spectrophotometrically at 340 nm and 37 °C by coupling the production of ADP to the oxidation of NADH. The assay mixture contained in 1 ml 50 mM MOPS–KOH buffer (pH 7.5), 0.25 mM Na_2EDTA, 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 10 units of pyruvate kinase and lactate dehydrogenase with or without 0.1 mM SbO_2^-. FSBA concentrations (mM) of 0.1 (◇), 0.25 (+), 0.5 (○), 1.0 (□).

TABLE 2. EFFECT OF SUBSTRATES ON FSBA INHIBITION

Addition	ATPase activity[a]
	% control
control[b]	100
no addition	32
5 mM ATP	58
0.5 mM SbO_2^-	5
5 mM ATP + 0.5 mM SbO_2^-	98

[a] FSBA inactivation and ATPase activity assays were done as described in the legend to figure 2. ArsA protein was preincubated with the indicated substrates for 20 min at 25 °C, followed by addition of 0.5 mM FSBA and incubation at 37 °C for an additional 30 min before initiation of the assay.

[b] Not FSBA treated.

FSBA only partial inhibition was observed. ATP and SbO_2^- together fully protected the activity from FSBA inhibition. These results suggest that the affinity of the enzyme for nucleotide, whether ATP or FSBA, appears to be increased by binding of SbO_2^-. Purified enzyme was reacted with [^{14}C]FSBA in the presence and absence of SbO_2^- (figure 3). The amount of FSBA

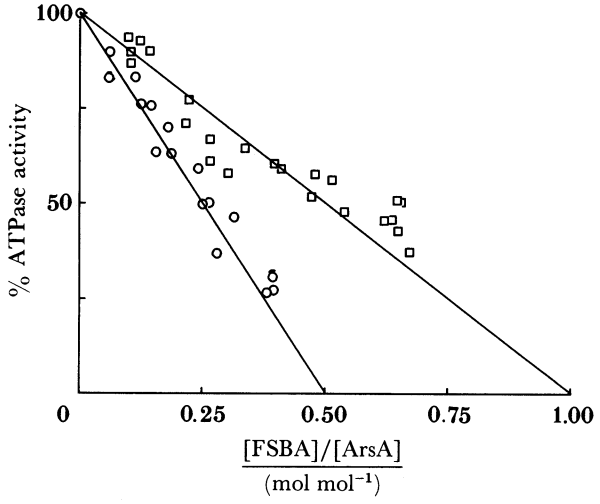

FIGURE 3. Stoichiometry of FSBA incorporation. Reaction of the ArsA protein with [^{14}C]FSBA was carried out as described in the legend to figure 2. [^{14}C]FSBA (1000 c.p.m. nmol^{-1}) was added to a final concentration of 1 mM to 1.2 ml of 50 mM MOPS–KOH buffer (pH 7.5) containing 0.25 mM Na_2EDTA containing 0.8–1.0 mg purified ArsA protein with (○) or without (□) 0.5 mM SbO_2^-. FSBA was added in ethanol, and all reactions were adjusted to 5% (by volume) ethanol. At various time intervals samples were removed for determination of SbO_2^--stimulated ATPase activity and radioactivity.

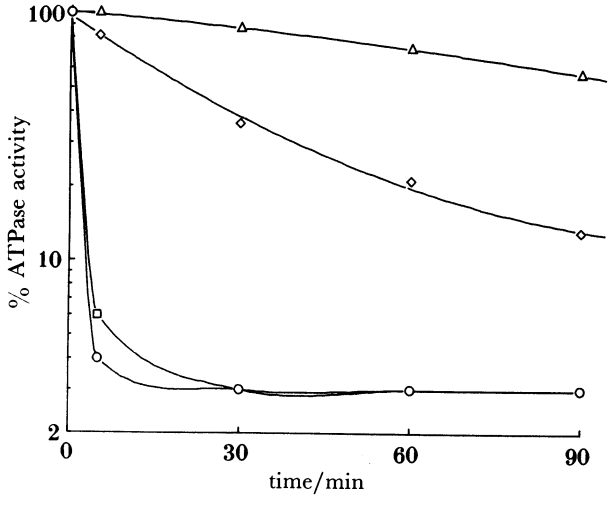

FIGURE 4. Trypsin inactivation of oxyanion-stimulated ATPase activity. Proteolysis was performed at 25 °C in 50 mM MOPS–KOH buffer (pH 7.5) at an ArsA concentration of 0.2 mg ml.$^{-1}$ The ratio of trypsin:ArsA was 1:100 (by mass). At the indicated times, samples were withdrawn, the reaction terminated by addition of a twofold excess of soybean trypsin inhibitor, and the SbO_2^--stimulated ATPase activity determined as described in the legend to figure 2. Where indicated the ArsA protein was preincubated with substrates for 10 min before addition of trypsin; (○), no addition; (□), 0.5 mM SbO_s^-; (◇), 5 mM ATP; (△), 5 mM ATP and 0.5 mM SbO_2^-.

required to completely inhibit oxyanion-stimulated ATPase activity was 1 mol mol^{-1} ArsA in the absence of oxyanion and 0.5 mol mol^{-1} in the presence of SbO_2^-. This result suggests that binding of the anionic substrate produces dimerization of the protein, with the dimer having higher affinity for nucleotide than the monomer.

Interaction of the anion and nucleotide binding sites could also be deduced from the effect of substrates on trypsin inactivation of oxyanion-stimulated ATPase activity (figure 4). Trypsin treatment in the absence of substrates resulted in rapid inactivation of enzymatic activity. Addition of SbO_2^- had no effect on trypsin inactivation. Addition of ATP partially protected. Addition of both substrates together resulted in substantial protection. Thus accessibility of a trypsin site or sites was not affected by anion and was partially occluded by nucleotide binding. Binding of anion and nucleotide produced a conformational change, which was different from the state of the protein in the presence of either substrate alone.

3. Mutagenesis of the ArsA gene

The conservation of glycine-rich consensus sequences in nucleotide binding proteins suggests that flexibility of this region is important. Four mutations were introduced into the first putative glycine-rich nucleotide binding loop of the ArsA protein. The sequence from the N-terminus of the protein is $MQFLQNIPPYLFFTGKGG_{18}VG_{20}KT_{22}S\ldots.$ Gly_{18} was changed to arg (mutant R_{18}) and asp (mutant D_{18}). In mutant S_{20}, gly_{20} was changed to ser. Finally, thr_{22} was changed to ile (mutant I_{22}). Cells with plasmids or phage having altered *arsA* genes were sensitive to arsenite or antimonite. In each case the ArsA proteins were produced in

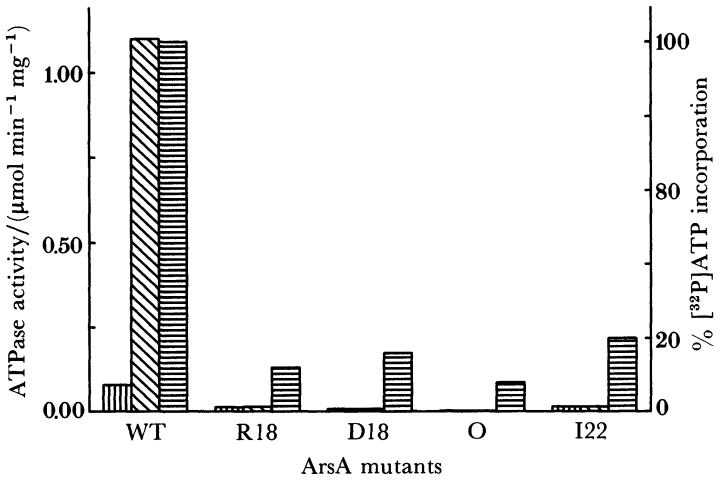

Figure 5. ATP photolabelling of wild-type and mutant ArsA proteins. The proteins were prepared from cells of *E. coli* bearing plasmids or M13 phage with single mutations in the *arsA* gene (C. E. Karkaria, C. M. Chen & B. P. Rosen, unpublished results). The ArsA proteins were purified by chromatography on Q-Sepharose and Reactive Red Agarose (Hsu & Rosen 1989). ATPase activity was determined in the presence or absence of 0.5 mM SbO_2^- as described in the legend to figure 2. Photolabelling was done as described previously (Rosen *et al.* 1988*a*) by using 5 μM α-[^{32}P]ATP. Samples were analysed by using autoradiography after electrophoresis on 15% SDS polyacrylamide gels. Incorporation of ^{32}P was quantified by densitometry; (▥), ATPase ($-SbO_2^-$); (▧), ATPase ($+SbO_2^-$);(▤), photo-crosslinking.

normal amounts and were of normal size. Immunoblots of cells with altered ArsA proteins did not indicate abnormal degradation of the proteins. The altered proteins were partially purified. None exhibited antimonite-stimulated ATPase activity (figure 5). Binding of ATP was examined by using a photolabelling assay with α-[^{32}P]ATP. Binding was proportional to protein concentration with wild type ArsA protein but was practically absent in all four mutant proteins (figure 5).

4. The ArsB protein is the membrane anchor for the ArsA protein

The ArsB protein was first identified by creation of an in-frame gene fusion of the *arsB* gene with *lacZ* (San Francisco *et al.* 1989) and localized in the inner membrane. It can be visualised as an [^{35}S]methionine labelled membrane protein when expressed by using a T7 polymerase vector (Tabor & Richardson 1986) but is not visible as a stained band by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). To demonstrate that the ArsA and ArsB proteins form a membrane-bound complex, purified inner membranes were washed with 0.5 M KCl, electrophoresed on SDS gels and electrophoretically transferred to nitrocellulose. The nitrocellulose filter was probed with antiserum against the ArsA protein. In cells with both the *arsA* and *arsB* genes, significant amounts of ArsA protein were membrane bound (figure 6).

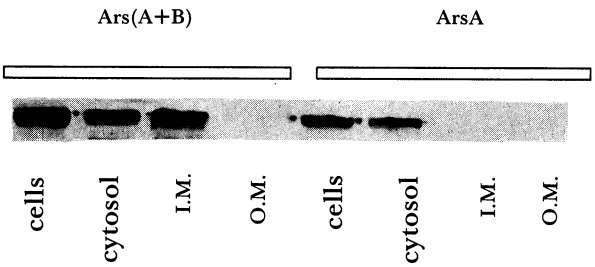

FIGURE 6. Cellular localization of the ArsA protein. Cells of *E. coli* strain K38 (pGP 1.2), which carries the T7 phage RNA polymerase under control of the temperature sensitive cI repressor, were transformed with the plasmids in which the *ars* genes were inserted behind the T7 promoter. The cells were fractionated into cytosol, inner and outer membranes by sucrose gradient centrifugation. Samples were boiled in SDS sample buffer and electrophoresed on a 12% SDS polyacrylamide gel, electrophoretically transferred to nitrocellulose, and reacted with ArsA antiserum. To visualise the antigen–antibody complex the filter was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG, hydrogen peroxide and 4-chloro-1-naphthol. Lanes (1 at left, 8 right) 1–4: cells with plasmid pT7-5-1538 (*arsABC*); lanes 5–8: cells with plasmid pT7-5-91 (*arsA*). Lanes 1 and 4: whole cells; lanes 2 and 6: cytosol; lanes 3 and 7: inner membrane; lanes 4 and 8: outer membrane.

In the absence of the *arsB* gene no ArsA protein was bound to the membrane. Inner membranes with the ArsA and ArsB proteins exhibited SbO_2^--stimulated ATPase activity (table 4). Although the specific activity is only about 5% of that of purified ArsA protein, the amount of ArsA on purified inner membrane is less than 5% of the total membrane protein. Precise quantification is difficult, but the data suggest that the specific activity of the membrane-bound form of the ArsA protein is at least the same and probably higher than that of the soluble form. No oxyanion-stimulated activity was found in membranes from cells bearing plasmids without the *arsB* gene. These results indicate that the two polypeptides form a membrane complex predicted to be the functional oxyanion-translocating ATPase.

TABLE 4. OXYANION-STIMULATED ATPASE ACTIVITY OF INNER MEMBRANES

plasmid[a] (relevant *ars* genes)	ATPase activity[b] $-SbO_2^-$	$+SbO_2^-$	SbO_2^--stimulated
	μmol min^{-1} mg^{-1} protein		
pT7-5 (no insert)	0.046	0.034	0.000
pT7-5-1538 (*arsABC*)	0.048	0.107	0.059
pT7-5-91 (*arsA*)	0.036	0.036	0.000
pT7-5-16 (*arsBC*)	0.044	0.033	0.000

[a] Inner membranes were prepared from cells of *E. coli* strain AN120 (*uncA*) carrying the indicated plasmids (San Francisco *et al.* 1989).

[b] ATPase activity was determined in the presence or absence of 0.1 mM SbO_2^- as described in the legend to figure 2. NaCN (40 mM) was added to the membrane preparations to inhibit NADH oxidase activity. All measurements were corrected for cyanide-insensitive oxidase activity.

5. mRNA DEGRADATION LIMITS SYNTHESIS OF THE ArsB PROTEIN

The synthesis of the ArsA and ArsC proteins is proportional to the number of plasmid copies of the operon. However, resistance and transport do not increase with plasmid copy number because synthesis of the ArsB protein is limiting for assembly of the complex. Analysis of the *arsB* translational initiation region indicates that secondary structure in the mRNA may limit translation of this gene (figure 7). There is a potential hairpin structure immediately upstream of the ribosome binding site and a stem–loop structure beginning with the third codon. In addition, the second codon, UUA, is the most inefficiently utilized leucine codon in *E. coli* (Sharp *et al.* 1988). We would predict that the combination of these factors would result in uncoupling of transcription and translation, preventing ribosome movement past the initiation codon of the *arsB* message. If transcription continued, the RNA would be exposed to endonucleolytic attack. Northern analysis of the *ars* mRNA demonstrates that the operon is transcribed initially as a full-length 4500 base RNA (figure 8). This transcript disappears quickly. This is not unexpected as expression of the pump would produce rapid expulsion of the inducer arsenite, shutting down transcription. However, the full-length transcript is rapidly converted into smaller species. When an *arsA* probe is used, the major species is 2700 bases long, which corresponds to the *arsR* and *arsA* messages. The same results were found by using an *arsR*

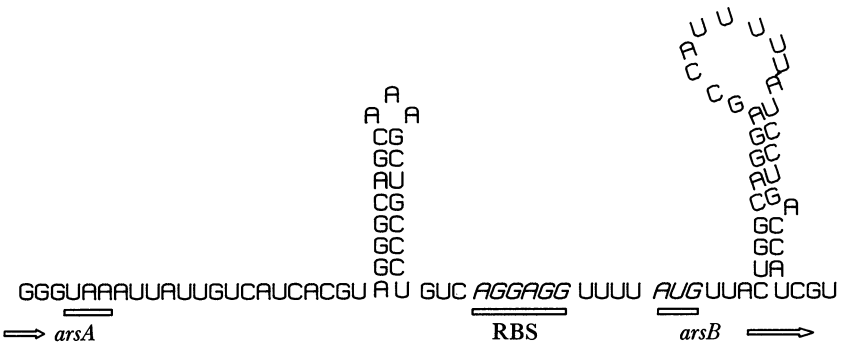

FIGURE 7. Translational initiation region of the *arsB* gene. The portion of the *ars* mRNA containing the start of the *arsB* sequence is shown. The termination codon of *arsA*, putative ribosome binding site and initiation codon of *arsB* are underlined. Potential secondary structure is indicated.

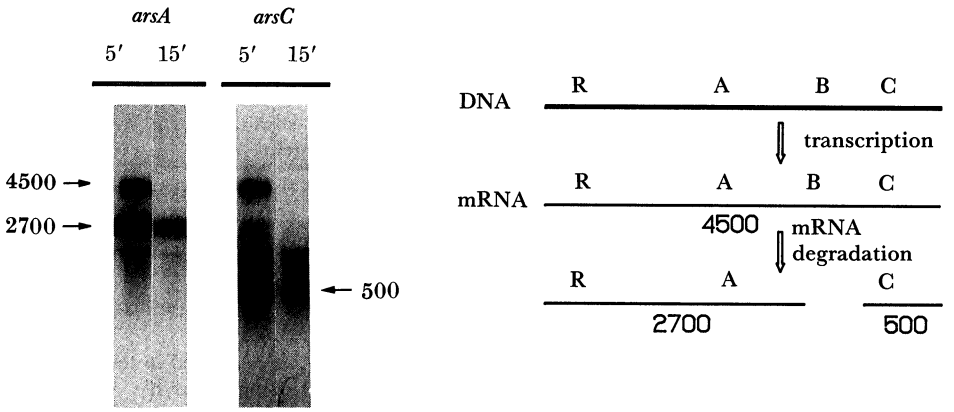

FIGURE 8. Analysis of transcription of the *ars* operon. Cells of *E. coli* strain HB101 bearing plasmid pUM2.1, which has the entire *ars* operon, were grown to mid-exponential phase. The operon was induced by addition of 0.2 mM AsO_2^-. Samples were withdrawn at 5 and 15 min, and total cellular RNA was isolated. The *ars* mRNA was visualised by Northern blot analysis by using strand-specific [^{32}P]-labelled *arsA* and *arsC* probes. End labelled denatured *Hind*III fragments of lambda phage DNA were used as molecular size markers.

probe (data not shown). However, when *arsC* was used as a probe, the major stable transcript was 500 bases in length, corresponding only to the *arsC* sequence. Transient intermediates ranging from 1800 to 1200 nucleotides were observed. The same species were seen by using an *arsBC* probe (data not shown). These results imply that ribosomal pausing at the beginning of the *arsB* message results in inefficient translation of *arsB*, and the uncoupling of transcription and translation exposes the *arsB* message to endonucleases and degradation. The polycistronic message is degraded to two stable forms, a 2700 nucleotide fragment containing the *arsR* and *arsA* sequences, and a 500 nucleotide *arsC* fragment. The ArsA and ArsC proteins are all produced in large amounts, but the inner membrane ArsB protein cannot be synthesized in more than limiting quantities. This may be a general mechanism by which cells prevent over-expression of membrane proteins.

We thank Dr J. E. G. McCarthy of the Gesellschaft für Biotechnologische Forschung mbH for analysis of the *arsB* translational initiation region.

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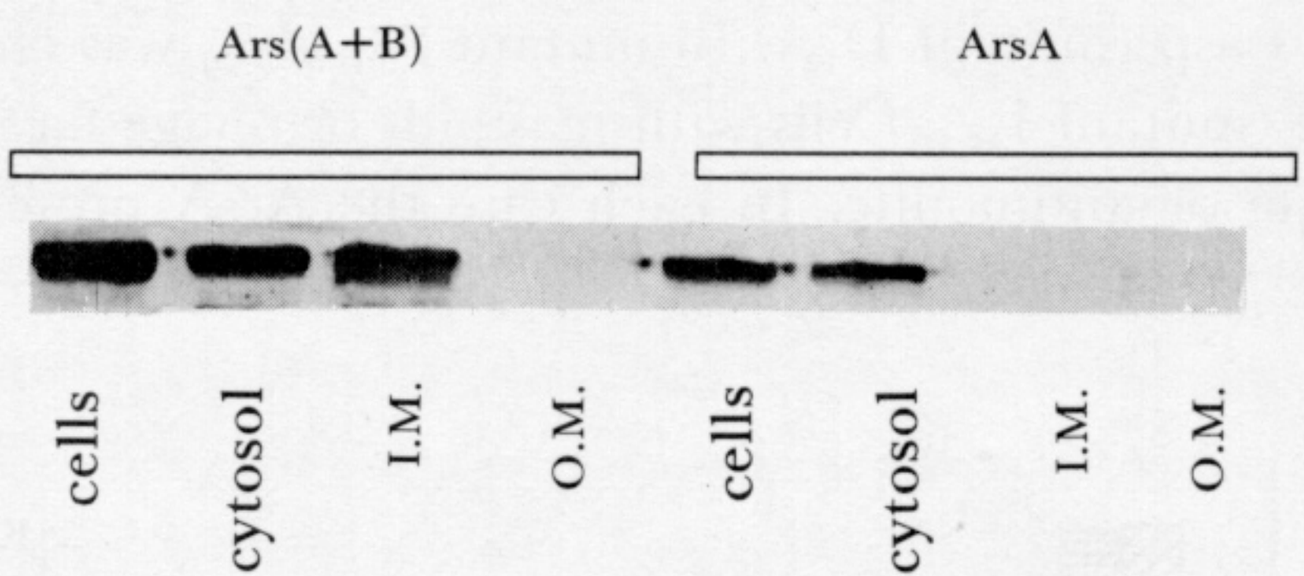

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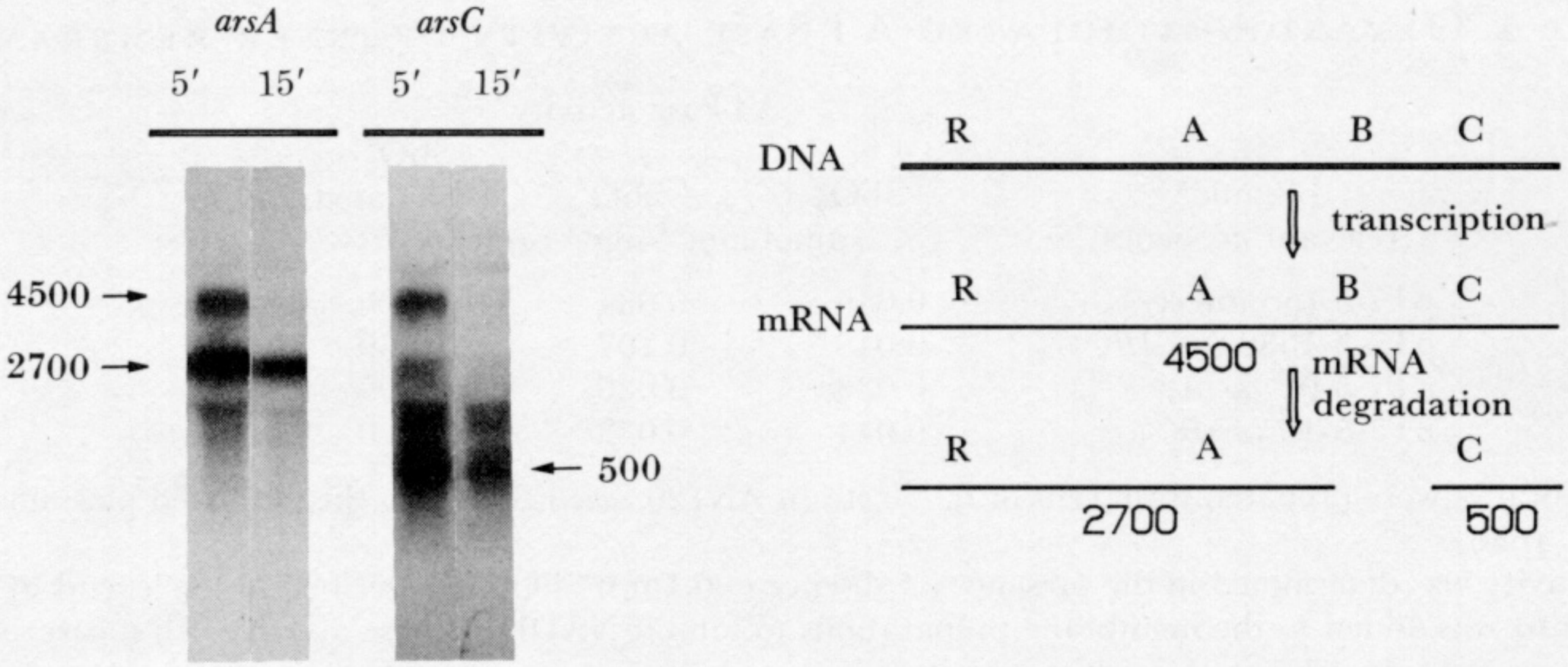

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