Molec. gen. Genet. 154, 185–189 (1977)

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Arsenate-Resistant Alkaline Phosphatase-Constitutive Mutants of *Escherichia coli*

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Summary. When arsenate-resistant mutants are selected approximately 50 per cent of them are also consitutive for the synthesis of alkaline phosphatase and the Pi-binding protein. Some of these mutants are linked to *ilv* (*phoS*$^-$ or *phoT*$^-$), others are linked to *proC* (*phoR*$^-$). One of the mutant strains linked to *ilv* lost the Pi-binding protein (the *phoS* gene product). Resistance to arsenate, constitutivty for alkaline phosphatase synthesis and loss of the Pi-binding protein occurred pleiotropically by the same *phoS*$^-$ mutation.

Introduction

The arsenate ion (AsO_4^{-3}, abbreviated as Ars) is a toxic growth inhibitor of *Escherichia coli*. Being an analog of inorganic phosphate (Pi) it acts as a substrate of the Pi-transport system (Rae and Strickland, 1976; Rosenberg and La Nauze, 1968). Thus, Ars-resistant strains can be isolated which are impaired in Pi-transport (Bennett and Malamy, 1970). In *E. coli* four Pi-transport systems were postulated; the relationships and interactions between these systems is not yet clear (Rosenberg, Gerdes and Chegwidden, 1977; Sprague, Bell and Cronan, 1976; Willsky and Malamy, 1973; Willsky and Malamy, 1974). One of these Pi-transport systems includes the periplasmic Pi-binding protein, coded by the *phoS* gene which is located at position 82 on the *E. coli* genetic map (Bachman, Low and Taylor, 1976; Gerdes and Rosenberg, 1974; Willsky and Malamy, 1976; Yagil, Silberstein and Gerdes, 1976). *phoS*$^-$ mutants are impaired in Pi-transport (Kida, 1974; Willsky and Malamy, 1974) and they are also constitutive for alkaline phosphatase (AP) synthesis. In the wild type *phoS*$^+$ cells AP is strongly repressed by Pi (Torriani, 1974). From this information we have reasoned that among Ars-resistant mutants, if impaired in the Pi-uptake system, some ought to be simultaneously constitutive for the synthesis of AP. Such mutants were isolated, mapped genetically and partially characterized.

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Materials and Methods

Bacterial Strains. Strain CSH57A has the following genotype: F$^-$ *ara, leu, lacY, proC, purE, gal, trp, his argG, malA, xyl, mtl ilv, metA* or *thi* (Miller, 1972). K10 is an Hfr wild type strain and KL14-1 is Hfr *thi, thy*. The *thy*$^-$ mutation was induced to strain KL14 by nitrosoguanidine mutagenesis (see below) and selection for resistance to trimetoprim (Stacey and Simson, 1965). The origin and direction of transfer of the Hfr strainsis shown in Figure 1 (see also Bachman, 1972).

Media and Growth Conditions. Tris buffered minimal medium was as described by Torriani (1968). This was supplemented with 0.2% glucose, 20 μg/ml of the appropriate amino acid 1 μg/ml thiamine and 100 μg/ml streptomycin. Pi-source was either KH_2PO_4 or α-glycerol phosphate as indicated in each case. For solid media 2% agar was added. *Medium A* is tris minimal medium enriched with 0.5% bactopeptone and 0.5% glucose. The bactopeptone includes traces of Pi ("Low Pi") such that an overnight culture undergoes strong derepression of AP (Levinthal, Signer and Fetherolf, 1962). To prevent AP-derepression 1 mM KH_2PO_4 was added ("High Pi").

Tryptone broth was previously described (Bracha and Yagil, 1969)

Asay of Alkaline Phosphatase. The substrate was p-nitrophenyl phosphate (1 mg/ml) as previously described (Yagil, Bracha and Lifshitz, 1975). For a colorimetic detection of AP activity in colonies the plates were sprayed with a mixture of α-naphtyl phosphate and Fast Blue B as previously described (Bracha and Yagil, 1969).

Mutant Isolation. Approximately 10^8 cells of strain CSH57A were spread on plates containing supplemented tris minimal medium, containing 0.01 M sodium and arsenate and 0.5 mM α-glycerophosphate as sole source of Pi (Bennett and Malamy, 1970). A paper

disc soaked in 1 mg/ml N-methyl-N′-nitro-N-nitrosoguainidine was placed in the center of the plate. The colonies grown after 2–3 days were sprayed for AP, those showing activity were purified on the same plates. An AP-inducible revertant was selected from strain ARS82-14 as follows: Cell growing logarithmically in tryptone broth were treated for 2 h at room temperature with 15 µl/ml ethylmethane sulfonate. The cells were centrifuged, diluted and plated for single colonies on rich Tryptone plates. The colonies were sprayed for AP activity. The rich plates contained ample Pi to repress AP thus colonies which did not show activity were isolated and purified. One of them (ARS82-14R) was analyzed.

Bacterial Conjugation. The parental strains growing exponentially in tryptone borth were mixed at a ratio of approximately 1 Hfr:10F$^-$ cells. After 90 minutes at 37° C (without shaking) the culture of conjugants was diluted and plated onto the selective plates.

Extraction of Periplasmic Protein, SDS Gels Electrophoresis and Immunoelectrophoresis were as described previously (Yagil et al., 1976).

Results

1. Isolation of Ars-resistant Mutant Strains

Ars-resistant mutant strains were obtained when approximately 10^8 mutagen-treated cells were plated on 0.01 M sodium arsenate (see Materials and Methods). The phosphate source on these plates was α-glycerol-phosphate which has a specific transport system (Bennett and Malamy, 1970; Hayashi, Koch and Lin, 1965). When the Ars-resistant colonies which grew on the selective plates were sprayed with substrate for AP (see Materials and Methods) approximately half of them showed enzyme activity. The α-glycerol phosphate present in the plates represses AP (probably because a part of it is hydrolyzed to Pi) thus the colonies which showed AP activity were constitutive. Table 1 shows a quantitative AP assay of three such mutants and their parental strain each grown in excess Pi or under Pi-starvation. All three mutant strains show AP activity in the "High Pi" medium and are therefore constitutitive.

Table 1. Alkaline phosphatase activity in Ars-resistant AP-constitutive strains

Strain	Enzyme activity		Genotype[a]
	High Pi	Low Pi	
CSH57A	< 0.003	9.4	wild type
ARS82-1	6.5	5.3	*phoS*$^-$ or *phoT*$^-$
ARS82-14	4.4	11.9	*phoS*$^-$
ARS9-8	7.9	9.0	*phoR*$^-$

Cells were grown overnight in medium A (Low Pi) or in medium A supplemented with 1 mM KH_2PO_4 (High Pi). One ml of the culture was treated with 50 µl toluene and a sample was assayed for AP activity. One enzyme unit was defined as ΔA_{410} per minute. Specific activity is expressed as enzyme units divided by the trubidity of the culture (A_{540})

[a] See below for determination of genotype

2. Genetic Mapping of the Ars-resistant AP-constitutive Mutants

Two distinct loci are known on the *E. coli* genetic map mutations in which render the cell constitutive for the synthesis of AP (Fig. 1). These are *phoR* (position 9) and *phoS* (position 82, this locus is subdivided to two *phoS* and *phoT*). *phoR* is closely linked to *proC* (0.2 minutes apart) and *phoS* is linked to *ilv* (0.8 minutes apart) (Fig. 1). The Ars-resistant AP-constitutive mutants were isolated from an *F*$^-$, *proC*$^-$, *ilv*$^-$, *strA*$^-$ strain; to determine the linkage relation of the AP-constitutive character they were

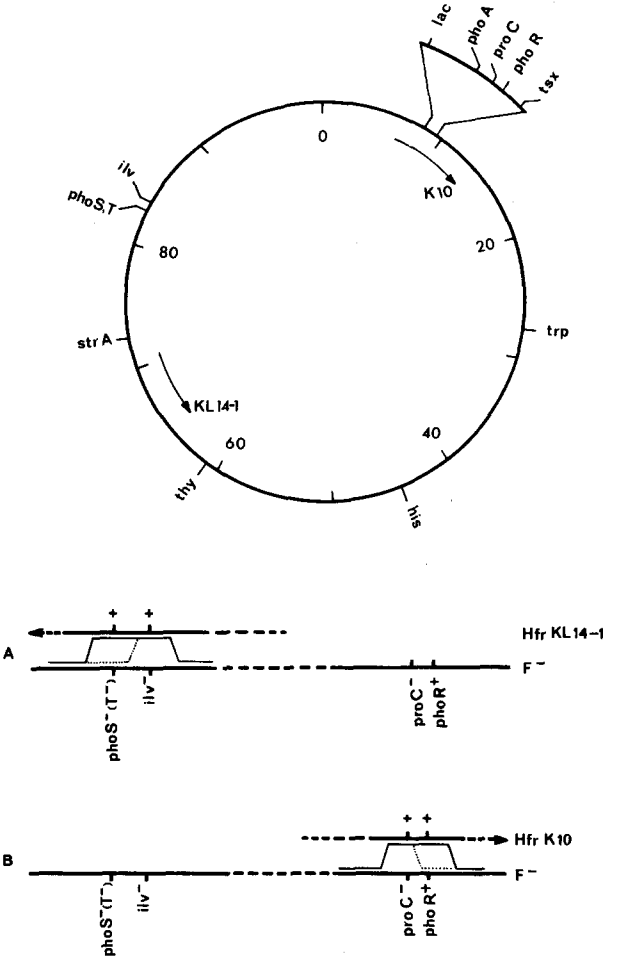

Fig. 1. Genetic map of *E. coli* (Bachman et al., 1976) showing the genes relevant to this study. The arrows indicate point of entry and direction of chromosome transfer of the two Hfr strains used. (A) shows the cross between HfrKL14-1 and a *phoS*$^-$ (or *phoT*$^-$) strain (B) shows the cross between HfrK10 and *phoS*$^-$ (or *phoT*$^-$)

Table 2. Mapping of Ars-resistant AP-constitutive mutant strains

Ars-resistant AP-constitutive F^- strain	Hfr strain	Type of recombinant selected	No. of recombinants tested	Percent of AP-constitutive segregants	genotype
ARS82-1	KL14-1	Ilv^+Thy^+	217	21.2	*phoS*$^-$ or
	K10	Pro^+Str^r	74	100	*phoT*$^-$
ARS82-14	KL14-1	Ilv^+Thy^+	152	17.4	*phoS*$^-$[a]
	K10	Pro^+Str^r	107	100	
ARS9-8	KL14-1	Ilv^+Thy^+	60	93.2	*phoR*$^-$
	K10	Pro^+Str^r	171	2.3	

The selective plates consisted of appropriately supplemented tris-minimal medium and 1 mM KH_2PO_4 as source of Pi

[a] See section 3

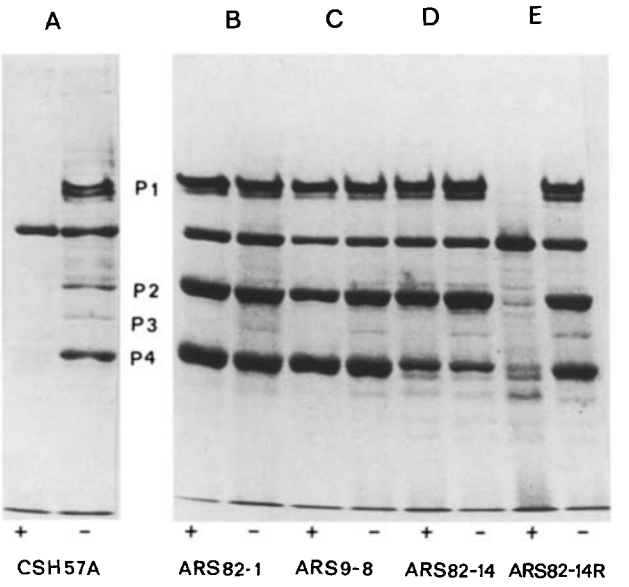

Fig. 2A–E. Gels of periplasmic proteins from strains grown in excess (+) or in low (−) concentrations of Pi. See text for further explanations

mated with two Hfr strains; strain KL14-1 transfers its chromosome clockwise from the point of origin at 66 min and strain K10 transfers its chromosome counter clockwise from the point of origin at 14 min (Fig. 1). In crosses using Hfr KL14-1 Ilv^+Thy^+ recombinants were selected (Fig. 1A) and with Hfr K10 the selected recombinants were Pro^+Str^r (Fig. 1B). The recombinant colonies obtained were sprayed for AP activity; since the plates contained excess Pi (1 mM Pi) only the segregating AP-constitutive colonies showed enzymatic activity. If the tested constitutive strain is of the *phoS, T* type (linked to *ilv*) the majority of the *ilv*$^+$ recombinant colonies (Fig. 1A) are expected to be AP-inducible (*phoS*$^+$ *T*$^+$), i.e. they will *not* show AP activity on the plates. Only those which have recombined between *ilv* and *phoS, T* (dotted line in Fig. 1A) will be constitutive. Among the *proC*$^+$ recombinants (Fig. 1B) the majority (or all) of them are expected to be constitutive. The opposite results is expected if the constitutive F^- strain is of the *phoR*$^-$ type (linked to *proC*), i.e. the majority of the *ilv*$^+$ recombinants should be constitutive whereas most of the *proC*$^+$ recombinants should be inducible. Table 2 shows the results obtained with the three Ars-resistant AP-constitutive strains listed in Table 1. It turned out that some of the constitutive strains tested were of the *phoS, T* type (these are represented in Table 2 by strains ARS82-1 and ARS82-14). Others were of the *phoR* type (strain ARS9-8). From 14 mutants thus analyzed 10 were of the *phoS, T* type (9–33 percent AP-constitutive segregants among *ilv*$^+$ recombinants) and the other 4 were of the *phoR* type (1–3 percent segregants among *proC*$^+$ recombinants). To emphasize that we are dealing with two genetic regions only (*phoS, T* and *phoR*) it should be pointed out that similar results are obtained with crosses using AP-constitutive strains isolated *independently* of Ars-resistance (Yagil et al., 1976). Segregants of these crosses (Table 2) were also tested on plates for their resistance to Ars. *All* AP constitutive segregatns were Ars-resistant and *all* AP-inducible ones were Ars-sensitive. We conclude that the resistance to Ars and AP-constitutivity are pleiotropic and have resulted from the same mutation (see also below).

3. SDS Electrophoresis and Immunoelectrophoresis of the Periplasmic Proteins

Pi starvation derepresses the synthesis of AP, that of the Pi-binding protein and several other periplasmic proteins. In AP-constitutive mutants the Pi-binding protein and a third unidentified band (P2) are constitutive as well (Morris, Schlesinger, Bracha and Yagil, 1975; Willsky and Malamy, 1976; Yagil et al., 1976). Figure 2 shows electrophoretic profiles of the periplasmic proteins extracted from cells of the strains shown in Tables 2 and 3, grown either in excess Pi or under Pi-starvation. The gel of the parental strain (CSH57A, Fig. 2A) shows the proteins derepressed by Pi-starvation. P1 includes the three isozymes of AP, P4 is the Pi-binding protein, P2 and P3 are as yet unidentified (Yagil et al., 1976). In the 3 Ars-resistant AP-constitutive mutant strains (Fig. 2B–D) P2 and P4 are synthesized constitutively as well but in strain ARS82-14 the Pi-binding protein (P4) is missing (the weaker band in position P4 is a different

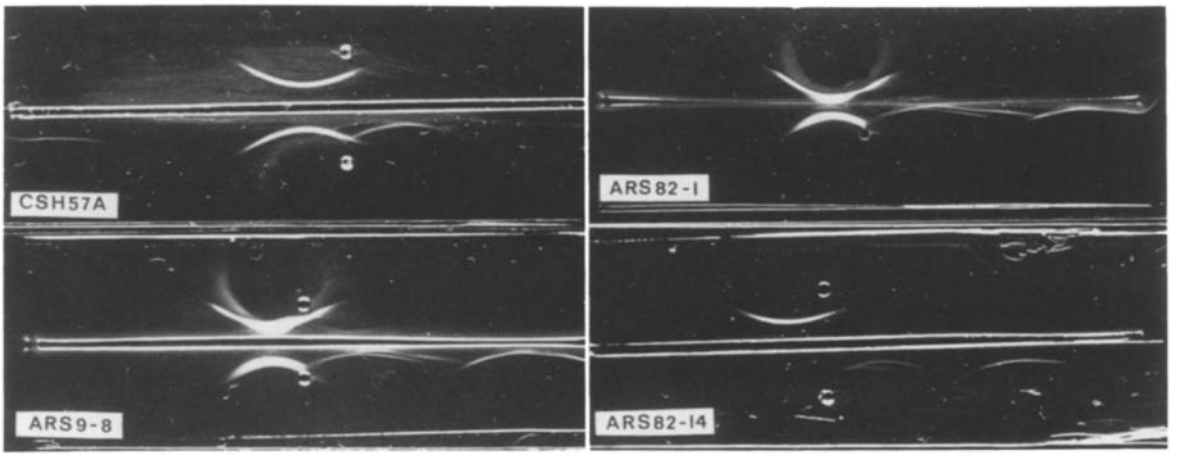

Fig. 3. Immunoelectrophoresis of periplasmic proteins. Concentrated periplasmic proteins of the strain indicated were applied to the lower well of each gel. The upper well in every case contained a standard sample of purified Pi-binding protein. Each strain grew overnight in medium A (Low Pi)

protein, see Willsky and Malamy, 1976 and Yagil et al., 1976). Immunodiffusion tests of the periplasmic proteins against antisera prepared from the purified Pi-binding proteins confirm the absence of P4 in strain ARS82-14 (Fig. 3). Only this strain failed to show cross reactivity. An AP-inducible revertant ARS82-14R) was isolated from strain ARS82-14 (see Materials and Methods). The gel of this revertant (Fig. 2E) shows that it had regained the Pi-binding protein and became inducible for the synthesis of Pi-binding protein as well as of P2. This revertant also became sensitive to Ars. We conclude that Ars-resistance. AP-constitutivity and the loss of the Pi-binding protein all occured pleiotropically in strain ARS82-14 as a result of *phoS*$^-$ mutation.

Discussion

We have shown that a mutation to Ars resistance can simultaneously make the cell constitutive for the synthesis of AP and two of the other co-regulated periplasmic proteins. Both heritable changes arise form the same mutation because they appear together frequently (approximately 50% of the selected Ars-resistant cells are AP-constitutive). In crosses the two characters did not segregrate from each other and both reverted simultaneously.

It has been shown previously that resistance to Ars may be associated with a defect in Pi-transport (Bennett and Malamy, 1970; Rosenberg and La Nauze, 1968; Rosenberg et al., 1977; Willsky and Malamy, 1974). Four genetic systems were postulated to control the transport of Pi. These are *glpT, uhp, pit* and the *phoS-phoT-pst* system (Rosenberg et al., 1977; Willsky and Malamy, 1976). *phoS*$^-$ and *phoT*$^-$ strains are constitutive for the synthesis of AP and hence it was not surprising to find AP-constitutive strains among the selected Ars-resistant mutants.

By gene-product analysis and immunoelectrophoresis we have found that one of the Ars-resistant mutants (ARS82-14) was *phoS*$^-$. This seems to contradict previous findings that *phoS*$^-$ mutants render the cell *sensitive* to arsenate (Willsky and Malamy, 1974). Our plates contained α-glycerol phosphate as the sole source of Pi; this compound induces the *glpT* system (Silhavy, Hartig-Beecken and Boos, 1976) which transports α-glycerol phosphate and possibly Pi and Ars as well (Willsky and Malamy, 1976; but see also Sprague et al., 1975). It was suggested (M. Malamy, personal communication) that the Ars-resistance of the *phoS*$^-$ mutation could have resulted from a secondary mutation in the *glpT* locus. However, all Ars-resistant strains grew well on plates containing α-glycerol phosphate as sole carbon source. Therefore it is unlikely these strains are *glpT*$^-$.

Genetic mapping has revealed that 10 out of 14 tested Ars-resistant AP-constitutive mutations mapped like *phoS-phoT* mutants (linked to *ilv*). Unexpectedly, the other four mapped as *phoR*$^-$ mutations (linked to *proC*). *phoR,* is linked to the structural gene of alkaline phosphatase synthesis (*phoA*, Fig. 1) and is considered to be its regulatroy gene (Kreuzer, Pratt and Torriani, 1975; Torriani, 1974). Our finding that also *phoR*$^-$ strains can be Ars-resistant can be interpreted in two ways: either that AP-constitutivity (*phoR*$^-$, *phoS*$^-$ or *phoT*$^-$) renders the cell resistant to Ars or that *phoR*, like *phoS* and *phoT* is not only involved in the regulation of AP synthesis but has a function in Pi-metabolism. The first possibility

is unlikely because we have tested some AP-constitutive strains isolated *independently* of Ars (Yagil et al., 1976); some proved to be resistant to Ars. other were sensitive. Thus, AP-constitutivity by itself is not sufficient to render the cell Ars resistant. A previous study has indicated that *phoR* may not be directly involved in the regulation of AP synthesis (Yagil and Hermoni, 1976). Our finding favours the second possibility.

In conclusion, there is, as yet, no a satisfactory explanation for the mechanism by which the cell can aquire resistance to Ars. Torriani and Rothman (1962) have reported a method for the direct selection of AP-constitutive strains. Resistance to Ars is another way by which such mutants can be obtained by direct selection.

Acknowledgements. We thank Dr. Robert G. Gerdes for doing the immunodiffusion tests and Ariella Herzberg and Nava Silberstein for technical assistance. The work was supported by a grant from the United-States – Israel Binational Science Foundation (BSF) Jerusalem, Israel.

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Communicated by O. Siddiqi

Received February 11, 1977