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AN ABSTRACT OF THE THESIS OF Terry M. Coons for the Master of Science in Biology presented July 29, 1986.

Title: Restriction Mapping and Expression of Recombinant Plasmids Containing the Arsenic Resistance Genes of the Plasmid R45.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

John W. Myers, Chairman

Mary L. Taylor

Deborah A. Duffield

John H. Golbeck

The trivalent (arsenite) and pentavalent (arsenate) forms of arsenic are introduced into the environment through the use of arsenic in herbicides, pesticides,

fertilizers, and the smelting of arsenic-bearing ores. Bacteria resistant to arsenic are readily isolated from surface waters, sewage, and clinical infections. Although some bacterial resistance is provided by inducible phosphate transport systems that discriminate against arsenate, marked resistance is carried on bacterial plasmids.

A 6.9 kilobase fragment previously derived from one such plasmid, R45, and containing the genes for inducible resistance to arsenite and arsenate was ligated into the cloning vectors pUC8 and pUC9 in opposite orientations and transformed into Escherichia coli JM 105. Insertion into the multiple cloning site of the pUC vectors places the inserted fragment under the inducible control of the lac operon promoter. An attempt was made to determine the direction of transcription in the fragment by growth in $10^{-3}M$ isopropyl- β -D-thiogalactoside prior to challenge with arsenite.

The minimal inhibitory concentrations of arsenite for strains with and without recombinant plasmids were determined. Recombinant plasmids conferred up to five-fold greater resistance to arsenite than was observed in strains containing intact pUC vectors or no plasmid at all.

A restriction map of the fragment was constructed. The fragment contained six restriction sites for the enzymes Pvu I, Bgl II, Sph I and Sal I.

The recombinant plasmids were transformed into the maxicell strain E. coli CSR 603 to facilitate identification of the polypeptides encoded by the cloned fragment. Polypeptides of apparent molecular weights 62, 16.5 and 13.5 kilodaltons were identified.

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
Terry M. Coons presented July 29, 1986.

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RESTRICTION MAPPING AND EXPRESSION
of RECOMBINANT PLASMIDS CONTAINING
THE ARSENIC RESISTANCE GENES
OF THE PLASMID R45

by
TERRY M. COONS

A thesis submitted in partial fulfillment of the
requirements for the degree of

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in
BIOLOGY

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1986

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This thesis is dedicated to Jack Myers, my advisor and friend. Special thanks to Kathleen Sampson for sharing her knowledge and material support, and to Bonnie R. Lee, for her dedication and production skills.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
MATERIALS AND METHODS	9
Bacterial Strains and Sources.....	9
Growth Media.....	11
Isolation of Plasmid DNA.....	12
Isolation of Bacteriophage λ DNA.....	13
Digestion of DNA with Restriction Endo- nucleases.....	15
Electrophoresis of DNA Fragments.....	16
Isolation of DNA Fragments.....	16
Construction of Recombinant Plasmids...	17
Transformation.....	18
Minimal Inhibitory Concentration of Arsenite.....	20
Induction of Arsenite Resistance by IPTG.....	20
Restriction Mapping of the Arsenite Resistance Fragment.....	21
Identification of Plasmid-Determined Proteins.....	22

RESULTS	28
Isolation and Ligation of Arsenic Resistant Fragment DNA.....	28
Transformation of Recombinant Plasmids.	28
Confirmation of Recombinant Plasmids...	29
Restriction Map.....	36
Minimal Inhibitory Concentrations.....	41
Arsenite Resistance Induction Assays...	41
Identification of Plasmid-Encoded Proteins.....	49
DISCUSSION.....	55
LIST OF REFERENCES.....	60

LIST OF TABLES

TABLE		PAGE
I.	List of Abbreviations.....	10
II.	Minimal Inhibitory Concentration of Arsenite.....	42

LIST OF FIGURES

FIGURE		PAGE
1.	Electrophoretic Analysis of Restriction Digests of Recombinant Plasmids.....	30
2.	Opposite Orientation of Insertions in Recombinant Plasmids.....	32
3.	Pvu I Restriction Map of Recombinant Plasmids.....	34
4.	Double Digests of the R45 Fragment....	38
5.	Single Digests and Restriction Map of the R45 Fragment.....	40
6.	Optimal Arsenite Concentration for Induction of Resistance.....	44
7.	Induction of Arsenite Resistance with IPTG.....	47
8.	Plasmid-Determined Proteins.....	51
9.	β -Lactamase and 36 Kdal Protein.....	53

INTRODUCTION

Elemental arsenic is found in the earth's crust, and oxides of arsenic are produced as by-products from the smelting of ores and the burning of fossil fuels in power plants (7). Arsenic is also added to the environment as an ingredient of many insecticides, herbicides and phosphate fertilizers (36). Practices causing increased erosion, as well as the use of arsenic in sheep dips to control ticks and fleas, also contribute to the incidence of arsenic in the environment. Major hazards to public health arise from local contamination of drinking water in areas surrounding mining or agricultural operations or from job-related inhalation of particles (7). In one study, 7% of 1500 freshwater samples exceeded the limit for arsenic recommended by the U.S. Public Health Service (7).

The two most common forms of inorganic arsenic are the pentavalent arsenate and the trivalent arsenite salts. Their mechanisms of toxicity differ. Arsenate toxicity arises from its similarity to phosphate both in geometry and reactivity (20). Arsenate can substitute for phosphate in all phosphorolytic reactions; however, the resulting compounds are much less stable and are hydrolyzed as soon as they dissociate from the surface of an enzyme. An

important example is the oxidation of glyceraldehyde 3-phosphate in the presence of phosphate to form 1,3-diphosphoglycerate. Normally, the 1-phosphoryl group is then transferred to ADP to generate ATP. If arsenate is present and substitutes for phosphate, the resulting compound, 1-arseno-3 phosphoglycerate, is unstable and is quickly hydrolyzed to 3-phosphoglycerate. Thus, even though glyceraldehyde 3-phosphate continues to be oxidized, synthesis of ATP is blocked, and the cell is gradually depleted of energy. The energy used to form the arsenate intermediates is also lost to the cells.

Arsenite is a sulfhydryl agent which reacts rapidly with the thiol groups of proteins, resulting in enzyme inactivation (20). It is particularly active against dithiol groups, such as lipoic acid:

Lipoic acid is a component of the α -ketoglutarate and pyruvate dehydrogenase complexes. Normally, these enzymes catalyze the oxidative decarboxylation of pyruvate and α -ketoglutarate to form acetyl CoA and succinyl CoA, respectively. An acetyl group from pyruvate is transferred to a thiol group on lipoic acid. By preventing this reaction from occurring, arsenite causes the accumulation

in the cell of pyruvate and other α -keto acids and blocks complete oxidation of pyruvate via the tricarboxylic acid cycle.

Possible mutagenic effects of arsenite are suggested by the observation that low levels of arsenite inhibit excision repair in Escherichia coli following ultraviolet radiation by inhibiting formation of single-strand breaks in the DNA (9). Increased incidences of respiratory cancers have been reported in metallurgical, chemical and agricultural workers who are exposed to increased levels of arsenic (36). Although respiratory cancers are most common, cancers of the skin and internal organs have also been reported. Confirmatory carcinogenesis in laboratory animals is lacking, however (7). Based on the observation that arsenite reduces the ability of E. coli to repair ultraviolet-induced damage, it may act as a co-carcinogen (34).

Bacteria resistant to arsenate and arsenite are commonly isolated from sewage, surface water and clinical infections. In one study of river and sewage-isolated enteric bacteria, 38.4% were found to be resistant to arsenite (35).

Different possible mechanisms of resistance include:

1. alterations of target sites
2. enzymatic degradation
3. enzymatic alteration

4. altered transport

In E. coli, chromosomal mutations to arsenate resistance are known. This resistance reflects the fourth mechanism -- altered transport -- and involves two systems responsible for the uptake of phosphate (34, 40, 42). The Pit system is the primary inorganic phosphate transport system in E. coli. It is constitutive and does not discriminate between phosphate and arsenate. Under limiting phosphate conditions, however, the phosphate-specific Pst system is induced, which more effectively discriminates between phosphate and arsenate. Chromosomal mutations that result in the loss of a functional Pit system (pit⁻) confer the arsenate-resistant phenotype (33) since, under this condition, only the phosphate-specific Pst system operates. Chromosomal resistance to arsenite has been less extensively studied, but alteration of the membrane-bound ATPase used for ATP synthesis has been suggested. This was based on the existence of an arsenite resistant mutant which was also uncoupled and therefore unable to grow on non-fermentable carbon sources (34).

The resistance to high concentrations of arsenite and arsenate observed in natural isolates, however, is associated with bacterial plasmids (3, 22, 26, 35). Plasmids conferring antibiotic resistances are often called

R factors and are frequently found in human coliform isolates. Resistances to other compounds, such as arsenite and arsenate, are often carried on these plasmids. In one study of 775 enteric plasmids isolated from various sources, over half exhibited resistance to various antibiotics, and 38.4% also carried resistance to arsenite (35).

Mechanisms of plasmid-determined resistance have only recently been explored. Evidence suggests that resistance derives from separate plasmid-mediated efflux pumps for arsenate and arsenite. In one study, the R factor R773 (10) was introduced into E. coli strain AN 120 (23), an uncoupled mutant strain that has a defective H⁺ translocating ATPase. Thus, oxidative phosphorylation is blocked, and ATP is produced only from substrate-level phosphorylation. These cells were starved of endogenous energy reserves and passively loaded with arsenate. When glucose was added, arsenate was extruded. The rate of efflux was not inhibited by the addition of cyanide, which inhibits electron flow from NADH to O₂, preventing formation of the proton motive force (PMF), but does not affect substrate-level phosphorylation. The addition of fluoride, which prevents substrate-level phosphorylation, reduced the rate of efflux. These observations suggest that the arsenate efflux pump is driven by the phosphate-bond energy of ATP and not by the PMF.

Using the same strains and methods, a similar system was suggested for arsenite resistance (30). The two systems were found to be independent, since inactivation of one by mutation did not affect the activity of the other (3). Whether arsenite and arsenate are pumped out by the same efflux pump or two separate ones, as well as the components and their location, is unknown.

The fact that two plasmids confer resistance to a given substance is not proof of identical resistance mechanisms or of significant DNA homology. For example, using restriction enzyme analysis and DNA-DNA hybridization techniques, four genetically distinct tetracycline resistance determinants have been identified in a broad range of bacterial plasmids (18). These differences result in variations in expression of resistance to tetracycline and its analogs. Recognition sequences for four restriction enzymes were missing in some of the resistance markers studied, suggesting genetic dissimilarity. Colony blot DNA hybridization was performed against the tetracycline resistance marker in the transposon Tn10. The resistance determinants were divided into four genetically distinct classes based on DNA-DNA hybridization. These classes also show phenotypic differences in resistance to tetracycline and its analogs. This multiplicity of genetically distinct determinants specifying a common resistance suggests the possibility of parallel yet

distinct biochemical pathways leading to the same phenotypic result.

Hybridization has also been carried out between the arsenic resistant plasmid R773 and plasmid DNA from 15 other Gram-negative arsenite-resistant human isolates (22). Eight were highly homologous, while seven showed no evidence of hybridization. A few of the plasmids conferred resistance to one arsenic species but not the other. A conjugative plasmid in the Gram-positive bacterium Corynebacterium flaccumfaciens shows no genetic homology to R773 but also encodes inducible protection from arsenite and arsenate and codes for two proteins of the same size as those of R773 (3). These results show that there are other, genetically distinct, plasmid-borne arsenite resistance determinants in addition to those represented by R773.

The present study is an investigation of the R factor, R45, a 45 kb plasmid originally obtained from an enteric bacteria isolated from sewage (13). It confers resistance to both arsenite and arsenate, as well as to ampicillin, tetracycline, sulfonamides and low levels of streptomycin. The genes conferring resistances to both arsenite and arsenate have been localized to a 6.9 kb region of the plasmid DNA (24). This region was excised from the plasmid and ligated to the cloning vector, pBR322 (J.W. Myers, personal communication).

The goals of the present study were 1) to construct a restriction map of the region of the R45 plasmid containing the arsenite-resistance determinant; 2) to determine the direction of transcription of the cloned fragment containing this region; and 3) to identify the proteins determined by the fragment.

MATERIALS AND METHODS

Bacterial Strains and Sources

<u>Strain or Plasmid</u>	<u>Relevant Properties</u>	<u>Source or Reference</u>
<u>E. coli</u> K-12 Strains		
JM 105	<u>thi</u> , <u>rpsL</u> , <u>endA</u> , <u>sbcB15</u> , <u>hspR4</u> ▲(<u>lac-proAB</u>), [F', <u>traD36</u> , <u>proAB</u> , <u>lacI</u> ⁺ , <u>lacZ</u> ▲M15]	44
C600	<u>thi-1</u> , <u>supE44</u> , F ⁻ , <u>leuB6</u> , <u>tonA21</u> , <u>thr-1</u>	S. Falkow
CSR 603	<u>recA1</u> , <u>uvrA6</u> , <u>phr-1</u> , <u>rpsL31</u> , <u>thi-1</u> <u>supE44</u> , F ⁻ , <u>thr-1</u> , <u>leuB6</u> , <u>proA2</u>	31
CSH 45	▲(<u>lac</u>), <u>thi</u> , <u>trpR</u> , (λ cI857S7)	21
Plasmids		
pEH	Amp ^r Asa ^r Asi ^r	J. Myers
pUC8	Amp ^r	41
pUC9	Amp ^r	41
pUC84	Amp ^r Asa ^r Asi ^r	This work
pUC96	Amp ^r Asa ^r Asi ^r	This work

TABLE I

TABLE OF ABBREVIATIONS

amp	ampicillin
asi	arsenite
asa	arsenate
LB	Luria broth
LA	Luria agar
lac	lactose operon
IPTG	isopropyl- β -D-thiogalactoside
MOPS	morpholinopropane sulfonic acid
F'	carrying the <u>E. coli</u> sex factor F, with a chromosomal insertion
r	resistant
s	sensitive
MCS	multiple cloning site
SDS	sodium dodecyl sulfate
kb	kilobases
Kdal	kilodaltons
MIC	minimal inhibitory concentration
RNAP	RNA polymerase
PMF	proton motive force
X-gal	5-bromo-4-chloro-3-indolyl- β -D- galactoside

Glycerol stocks and strains constructed in this study were verified on appropriate selective media prior to use. Short-term working stocks were maintained on these media at 4°C. Long-term stocks were stored in 50% glycerol at -20°C.

Growth Media

All chemicals were of analytical grade and were purchased from various sources. The standard liquid medium used for growth studies was Luria broth (21), supplemented as indicated. For solid media, 15 g agar was added before autoclaving. Since phosphate competes with arsenate for uptake, arsenate resistance was assayed on morpholinopropane sulfonic acid (MOPS) medium:

MOPS buffer (pH 7.2)	40.0	mM
Na_2HAsO_4	50.0	mM
NaCl	50.0	mM
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5	mM
$(\text{NH}_4)_2\text{SO}_4$	0.1%	
K_2HPO_4	1.0	mM
glucose	0.5%	
required growth factors for each strain		

Arsenite resistance was assayed on Davis minimal medium (Difco) containing 10 mM sodium arsenite, 0.5% glucose and the required growth factors of the strain being assayed. Top agar was prepared as for Luria agar, with the agar concentration reduced to 0.7%.

Isolation of Plasmid DNA

Plasmid DNA was extracted from verified strains by the method of Birnboim and Doly (1). The centrifuged cell paste was re-suspended in 10 ml 25 mM Tris-Cl (pH 8), 10 mM Na₂EDTA and 1% (w/v) glucose. To lyse the cells, 20 ml 0.2 M NaOH containing 1% SDS was added. At high pH, plasmid DNA remains covalently closed, while the linear chromosomal fragments become single-stranded. A 5 M acidified potassium acetate solution (15) was added to lower the pH and precipitate proteins, high molecular weight RNA and single-stranded DNA. The plasmid DNA renatured to the supercoiled form and remained in the supernatant. The precipitate was removed by centrifugation, and the plasmid DNA was precipitated with two volumes of 95% ethanol. After centrifugation the pellet was dissolved in 6 ml TE buffer (15).

To remove contaminating chromosomal DNA, RNA, and proteins, the solution was centrifuged in a cesium chloride density gradient containing 0.6 ml ethidium bromide (EtBr) (10 mg/ml) to purify the plasmid DNA (2). Ethidium bromide intercalates into the DNA, reducing its density. Since linear and open circular DNA are not as physically constrained as the covalently closed circular plasmid DNA, the first two forms bind more ethidium bromide molecules than the third, become less dense, and band with a different buoyant density. The DNA was centrifuged in the

Ti 50 rotor of a Beckman preparative ultracentrifuge for 36 hours at 45,000 rpm. DNA bands were visualized with an ultraviolet lamp, and the lower plasmid DNA band was extracted. Ethidium bromide was removed from the plasmid DNA solution by extraction with isopropanol saturated with 5 M NaCl (4). Remaining impurities were removed by ethanol precipitation of the DNA in two volumes of ethanol in the presence of 2.5 M ammonium acetate, and the pellet was suspended in 100 μ l TE buffer containing 1 unit/ml RNase T₁ (ternase) (4).

Isolation of Bacteriophage λ DNA

E. coli CSH 45 contains the prophage λ cI857S7 (4). This viral strain has two mutations, cI857 and S7. The cI repressor gene is responsible for inhibiting the expression and replication of λ and keeping it in the lysogenic state. The cI857 mutation results in production of a temperature sensitive repressor. The S7 mutation renders the phage lysis-deficient, and it continues to multiply inside the cell. E. coli CSH 45 was grown on agar at 30°C and 42°C (4). Cells picked from colonies showing no growth at the non-permissive temperature were grown in LB at 32°C for one hour, and the temperature was increased to 43°C for 20 minutes to inactivate the repressor and induce the lytic cycle.

The cultures were allowed to grow for an additional

three hours. They were then centrifuged and suspended in 10 ml λ diluent (4). One ml chloroform was added to lyse the bacterial cells. The phage was separated from cellular debris by passage through a CsCl density block gradient (4). A lysate was laid over two CsCl solutions having densities of 1.6 and 1.4, placed in a Beckman SW 65 swinging bucket rotor, and centrifuged in the Beckman preparative ultracentrifuge for two hours at 30,000 rpm. The phage, with a density of about 1.5, collected at the interface between the two CsCl solutions and was removed.

The DNA was isolated by treating the lysate with SDS, followed by 5M potassium acetate. Proteins were removed by centrifugation for 30 minutes in a microfuge. The λ DNA was precipitated with ethanol and suspended in 1 ml ternaase. Absorbancy readings were taken in a Beckman spectrophotomer at 260nm to determine the concentration of DNA (1 O.D.₂₆₀=50 ug/ml). Portions of the DNA were diluted with ternaase to give standards of 10, 5, 2.5, 1.25, and 0.625 ug/ml. These were used to estimate DNA concentration based on EtBr fluorescence.

Saran Wrap was placed across a Chromato-vue[®] transilluminator in a darkroom (15). The prepared standards and DNA samples were pipetted onto the Saran Wrap and mixed with 5 ul of EtBr (2 mg/ml). Ultraviolet light was transmitted through the samples, and photographs were taken with a Polaroid MP-4 Land camera using a yellow

filter and Polaroid Type 57 film. The concentration of DNA was then determined by comparing the fluorescence of the samples with that of the known standards.

Digestion of DNA with Restriction Endonucleases

Lyophilized restriction endonucleases were reconstituted according to manufacturer's instructions and kept at -20°C . Restriction digests were carried out at 37°C for two hours. Different restriction enzymes require different salt concentrations for maximum activity. When sequential restriction digests were performed, the enzyme requiring lower salt conditions was used first. Then the salt concentration was increased according to the following table. This is based on a volume of 15 μl following the first restriction, to be increased to 20 μl with ternase following buffer adjustment:

<u>Stock Solution</u>	<u>Low to Medium</u>	<u>Low to High</u>	<u>Medium to High</u>
4 M NaCl	0.25 μl	0.5 μl	0.3125 μl
1 M Tris-Cl, pH 7.5	0.05 μl	0.85 μl	0.85 μl

Electrophoresis of DNA Fragments

Agarose gels ranging from 0.7 to 1.2% (w/v) were prepared in tris-acetate electrophoresis buffer (15) containing 0.25 ug/ml of ethidium bromide (39). Samples containing 100-200 ng DNA and one-quarter volume of DNA tracking dye (1) were run in a horizontal slab gel apparatus (16) at 3 V/cm. The running time varied with the specific procedure.

Isolation of DNA Fragments

Digested DNA was separated by electrophoresis, and the gel was placed on an ultraviolet transilluminator to visualize the bands. The gel was sliced, and a strip of Whatman DE 81 DEAE cellulose paper was inserted in front of the desired band (5). The DNA was moved onto the paper by electrophoresis at 100V for 30 minutes. The paper was then removed, packed into the barrel of a disposable plastic 1 ml syringe and placed in a 15 ml Corex centrifuge tube. The paper was first washed by pipetting 200 ul of 0.1 M NaCl, 0.1 mM EDTA, 10 mM Tris-Cl (pH 8) into each syringe and centrifuging at 2000 rpm for 30 seconds in a Sorval GLC-1 centrifuge. This procedure was repeated twice and the eluates discarded. To remove the DNA from the paper, a 1.5 ml polypropylene tube was placed in the Corex tube to catch the eluate, and the paper was washed by adding 100 ul of a 1.0 M NaCl, 0.1 M EDTA, 10 mM

Tris-Cl (pH 8.0) solution, followed by centrifugation for 30 seconds at 2000 rpm. This was repeated three times, for a total DNA eluate of 400 ul. Two volumes of 95% ethanol were added to the eluate, the tubes were placed in a dry ice-ethanol bath for five minutes to precipitate the DNA and then centrifuged in a microfuge for 30 minutes.

The pellet was suspended in 100 ul ternaase and mixed with an equal volume of a 1:1 (v/v) mixture of phenol equilibrated with TE buffer and chloroform mixed 24:1 (v/v) with isoamyl alcohol (15) and then centrifuged 15 seconds in a microfuge to separate the organic and aqueous layers. The upper aqueous layer was removed to a new tube. To maximize DNA recovery, the remaining organic layer was re-extracted with 100 ul TE buffer. The upper aqueous layer was again removed and mixed with the first aqueous portion. Residual phenol was then removed by mixing with an equal volume of chloroform and centrifuging as before. The aqueous layer was removed, and the DNA was precipitated with ammonium acetate and ethanol, as previously described.

Construction of Recombinant Plasmids

Vector DNA was digested with Hind III, followed by Eco RI, using buffers and conditions previously described. The enzymes were then removed by phenol extraction. Ligations were carried out using 200 ng of

each vector DNA with an equimolar amount of isolated fragment DNA. A tube containing 400 ng of vector DNA digested with Hind III alone was included as a ligase control.

Two units of T4 DNA ligase were added to each reaction mixture, along with 10X ligation buffer (15):

0.5 M Tris-Cl, pH 7.4
0.1 M MgSO_4
10.0 mM spermidine
0.1 M dithiothreitol
10.0 mM adenosine triphosphate
1.0 mg/ml bovine serum albumin

The reaction mixtures were incubated at 4°C for 12 hours. Ligated plasmids were then used to transform host cells.

Transformation

E. coli JM 105 and CSR 603 were prepared for transformation (14) by growing cultures to a density of 5×10^7 cells/ml. Cell density was determined using a Klett-Summerson Photoelectric Colorimeter, Model 800-3, with a red #66 filter. The culture was chilled on ice for 10 minutes and then centrifuged at 4000 rpm at 0°C for five minutes. The pellets were suspended in one-half the original culture volume in a cold (4°C) 50 mM CaCl_2 solution, centrifuged again and resuspended in CaCl_2 at one-fifteenth the original volume. Aliquots of 0.2 ml were stored at 4°C for 24 hours. Up to 40 ng of plasmid DNA was added to each tube. The tubes were kept on ice for 30

minutes and then transferred to a 42°C water bath for two minutes. Then 1.0 ml LB was added and the cells were incubated for two hours at 37°C to allow for expression of plasmid-carried ampicillin resistance. Successful transformation was detected by plating the cells on Luria agar containing ampicillin at a final concentration of 50 ug/ml.

When transformation was carried out with pUC vectors, amp^r transformants containing vectors with inserts could be distinguished from those without inserts by the following procedure. Approximately 10⁸ cells from each transformation tube were plated on LA containing amp, IPTG (4X10⁻⁴ M) and X-gal (0.004%) (32). The pUC vector codes only for the promoter-proximal region of β -galactosidase (α donor) and the JM105 F' plasmid specifies a β -galactosidase functional only in the promoter-distal region (ω donor); the two complementary polypeptide chains can associate to produce active β -galactosidase (intracistronic complementation). Such activity can be detected by the production of the insoluble blue dye dibromodichloroindigo (19) when X-gal is hydrolyzed by the enzyme. If the transformant has an insert in the MCS, the coding region for the promoter-proximal β -galactosidase is interrupted and no α donor polypeptide is made. Transformants containing pUC vector inserts produce white colonies under these conditions.

Three ml of molten top L agar was mixed with 0.2 ml of transformed cells, and 100 ul of 0.1 M IPTG was added, along with 50 ul of 2% X-gal (dissolved in N,N-dimethyl-formamide) (37). The top agar was poured over LA previously spread with ampicillin. The plates were incubated for 12 hours at 37°C.

Minimal Inhibitory Concentration

The two E. coli host strains used, JM 105 and CSR 603, and two recombinant-plasmid-bearing strains, JM 105(pUC84) and CSR 603(pUC84), were grown overnight in 10 ml LB, with and without the addition of 1 mM arsenite. These were subcultured 1:100 into side-arm flasks containing the same media and shaken at 37°C until they reached a density of 2.5×10^8 cells/ml. From each flask, 50 ul was spread over the surface of LA plates containing the following concentrations of arsenite: 2, 4, 6, 8, 10, 12, 15 and 20 mM. The plates were incubated for 48 hours at 37°C, and the colonies were counted. The lowest concentration of arsenite on which no growth was observed was taken to be the minimal inhibitory concentration of arsenite for that strain (32, 34).

Induction of Arsenite Resistance by IPTG

The E. coli strains JM 105, JM 105(pUC8), JM

105(pUC84) and JM 105(pUC96) were grown overnight in 10 ml LB, with the following additions for each strain:

1. None
2. 1 mM arsenite
3. 10^{-3} M IPTG (25)

Cultures were diluted 1:100 into the same media in side-arm flasks and shaken at 37°C until they reached a density of 1.5×10^8 cells/ml. This dilution and subsequent growth period was repeated. The cultures were challenged by the addition of arsenite to a final concentration of 15 mM, and the growth of each was monitored over a period of several hours.

Restriction Mapping of the Arsenite Resistance Fragment

Aliquots of 0.5 ug of the isolated R45 fragment DNA were digested with the restriction enzymes Pvu I, Bgl II, Sph I and Sal I. Resulting fragments were separated by electrophoresis through 1.0% agarose for two hours at 100 V. The distance from the origin of each separated band was measured in millimeters, and compared with a standard curve of the log molecular weight against the distance traveled by the λ DNA Hind III-digested fragments. By this means, the molecular weights of the digestion products were estimated (16).

A portion of each single digest was then subjected to a separate, second digestion with each of the remaining three enzymes under analysis. The digestion products of

the second digestion were separated by electrophoresis and the fragment sizes estimated as described for the first.

Identification of Plasmid-Encoded Proteins

The recA⁻ uvr⁻ genotype of the E. coli strain CSR 603 (31) was confirmed by spreading 10^8 cells of an overnight culture grown from a single CSR 603 colony on LA and irradiating with a total UV dose of 0.5 J/m^2 . The plates were incubated for 24 hours at 37°C in the dark (A. Sancar and G. Sancar, personal communication). Plates having fewer than 100 survivors were used in the following procedures.

Recombinant plasmids and intact pUC vectors were transformed into E. coli CSR 603. Cultures were grown overnight in LB with the following additions. Ampicillin was included to select against variants that may have lost the plasmid.

<u>Culture</u>	<u>Additions</u>
603	none
603(pUC8)	50 ug/ml amp
603(pUC84), uninduced	50 ug/ml amp
603(pUC84), induced	50 ug/ml amp + 10 mM asi
603(pUC96), uninduced	50 ug/ml amp
603(pUC96), induced	50 ug/ml amp + 10 mM asi

The cultures were diluted 1:100 into 10 ml of the same media without ampicillin in side-arm flasks and grown to a

density of 2×10^8 cells/ml. The cultures were diluted 1:100 and grown to the same cell density to insure that cells were in log phase before proceeding. All cultures were centrifuged at 4000 rpm for five minutes, and the pellets were suspended in 10 ml Davis Minimal broth. They were then irradiated with swirling in a petri dish, for a total UV dose of 5 J/m^2 , to inactivate chromosomal DNA. The suspensions were centrifuged and the pellets resuspended in 10 ml LB with the additions previously described. The cultures were incubated for three hours, 0.1 ml of a freshly made cycloserine solution (200 ug/ml) was added, and the cultures were incubated for 12 hours. From each culture, 1.5 ml was centrifuged in a microfuge for five minutes, and the cells were resuspended in 40 ul of lysing buffer (8):

0.05 M Tris-Cl, pH 6.8
2% SDS
15% glycerol
2% 2-mercaptoethanol
0.005% bromophenol blue

These mixtures were boiled for three minutes to lyse the cells and then stored at 4°C until needed.

Polyacrylamide gels were used for the separation of proteins by the method of Laemmli (12). Stock solutions were as follows:

1. 30% acrylamide
2. 4X running gel buffer (1.5 M Tris-Cl, pH 8.8).
3. 4X stacking gel buffer (0.5 M Tris-Cl, pH 6.8).
4. 10% (w/v) sodium dodecyl sulfate (SDS)
5. 10% (w/v) ammonium persulfate (APS)
6. Commercial stock of TEMED
(N,N,N',N'-tetramethylethylenediamine)
7. Tank buffer (0.025 M Tris-Cl, pH 8.3, 0.192 M glycine and 0.1% SDS).
8. 1% Coomassie blue R-250 stain stock. This stock was used to make a 0.125% Coomassie blue, 50% methanol, 10% acetic acid stain for gels.
9. 50% methanol, 10% acetic acid (primary destaining solution).
10. 5% methanol, 7% acetic acid (secondary destaining solution).

Glass plates with 1.0 mm spacer bars were sealed with 1.0% agarose to form a sandwich 16.5 X 19 cm. A 12.5% separating gel solution was prepared:

12.5 ml monomer solution
7.5 ml 4 X running gel buffer
0.3 ml 10% SDS
9.7 ml H₂O

To this, 10 μ l TEMED and 0.75 ml APS were added. This solution was quickly delivered into the glass sandwich to within 5 cm of the top, and 1.0 ml isopropanol was layered

over the gel to form a level surface. The gel was allowed to polymerize two hours. A stacking gel solution was prepared:

1.33 ml monomer solution
 2.5 ml 4 X stacking gel buffer
 0.1 ml 10% SDS
 6.1 ml H₂O

Five ul TEMED and 0.25 ml APS were added, the sandwich was filled with this solution, and a 16-well comb was inserted. The stacking gel was allowed to polymerize at least one hour. The sandwich was sealed in place in a Watson vertical gel apparatus with 1.0% agarose. Ten ul of each sample (1.5 ug protein) and protein standard (1.0 ug protein) were pipetted into the wells, and the gel was electrophoresed at 70 V constant voltage until the dye front began to enter the separating gel. The voltage was then increased to 170 V, and the gel was run 4-5 hours (39). Protein standards used were:

<u>Mixture</u>	<u>Containing</u>	<u>Molecular weight</u>
A	lactalbumin	14.2 Kdal
	carbonic anhydrase	29.0 Kdal
B	Trypsin inhibitor	20.1 Kdal
	glyceraldehyde-3-phosphate dehydrogenase	36.0 Kdal
	bovine serum albumin	66.0 Kdal
C	trypsinogen	24.0 Kdal
	egg albumin	45.0 Kdal

Gels were stained by placing them in a glass dish and covering them with 100 ml Coomassie blue stain. The pan was shaken on a Kraft rotary shaken at 60 rpm for one hour. The gel was drained, immersed in 100 ml of primary destaining solution and shaken for one hour. The gel was again drained, covered with 100 ml secondary destaining solution and shaken overnight.

Alternatively, gels were stained with silver (43), either alone or following Coomassie blue (9). The gel was soaked overnight in 50% methanol to fix the proteins, rehydrated under running water for one hour and drained. The silver staining solution was prepared by mixing 21 ml of 0.36% NaOH with 1.4 ml of 14.8 M NH_4OH . The solution was stirred while 4.0 ml of stock AgNO_3 solution (4.0 g AgNO_3 dissolved in 20 ml H_2O) was added dropwise, to prevent precipitation. The solution was poured over the gel, and the gel was then agitated on the rotary shaker for 15 minutes. The gel was rinsed for 30 minutes under running water and then drained. A developing solution containing 0.005% (w/v) citric acid and 0.002% (v/v) 37% formaldehyde was freshly prepared and poured over the drained gel. The gel was then agitated over a white background until protein bands began to appear. The process was stopped by pouring 50% methanol over the drained gel and agitating for 15 minutes. Protein size was estimated from a plot of the log of the size of the protein

standards in kilodaltons against the distance traveled from the origin in millimeters.

Stained gels were photographed with a Polaroid 4 X 5 Land camera using Polaroid Type 55 positive/negative film. The gels were rinsed in running water overnight to remove all traces of methanol, soaked in an aqueous solution of 1% glycerol and 10% acetic acid for 45 minutes, placed on either Whatman 3 MM filter paper or cellophane sheets, and dried at 80°C for two hours in a BioRad Model 224 Gel Slab Dryer.

RESULTS

Isolation and Ligation of Arsenic-Resistant Fragment DNA

The source of the insert DNA used to construct recombinant plasmids in this study was the previously constructed plasmid, pEH, containing the arsenic-resistance region from R45 ligated into the cloning vector, pBR (John Myers, personal communication). Plasmid DNA was isolated by methods previously described. Plasmid DNA yield was routinely 1.0-2.0 mg per liter of starting culture. The R45-derived fragment was then separated from pBR by double digestion with Hind III and Eco RI. Each cloning vector was subjected to a double digest with Hind III and Eco RI and 200 ng was mixed with 500 ng of the fragment and ligated with T4 ligase. After ligation, the DNA was used to transform E. coli strain JM 105.

Transformation of Recombinant Plasmids

Transformants were selected by mixing 0.3 ml transformed cultures with IPTG and X-gal in top agar and pouring this over LA spread with ampicillin. Blue colonies revealing a functional β -galactosidase and thus an intact vector were rejected. White colonies reflect an insert in the MCS of the vector. These constituted up to 20% of

observed colonies. White colonies were picked and spread on LA containing ampicillin and 10 mM arsenite, to confirm the *asi^r* phenotype. Short-term working stocks were maintained on this medium at 4°C. Since the pUC vectors differ only in their orientation of the MCS, the fragment insertion in pUC8 should have the opposite orientation of the insert in pUC9.

Confirmation of Recombinant Plasmids

To confirm that the resistance was due to the presence of a plasmid with a molecular weight equal to the sum of those of the inserted fragment (6.9 kb) and the pUC vector (2.7 kb), plasmid DNA was extracted from several separately maintained clones of both supposed orientations. This DNA was digested with Hind III and Eco RI, and samples containing 100-200 ng DNA were electrophoresed through a 0.7% agarose gel. Double-digested pUC and pEH plasmid DNA were included in the gel as size markers. Bacteriophage λ DNA was digested with Hind III, producing seven fragments of known size -- 23.67, 9.67, 6.66, 4.26, 2.30, 1.96, and 0.59 kb respectively. This mixture was used as size markers in this and other agarose gels. Fig. 1 shows that all recombinant plasmids contained an insert of the expected size.

To confirm that the recombinant plasmids derived from pUC8 and pUC9 did indeed contain the R45 fragment in

Figure 1. Electrophoretic Analysis of Restriction Digests of Recombinant Plasmids

Plasmid DNA was removed from transformed *E. coli* JM 105 strains and sequentially digested with EcoR I and Hind III. Lane 1, pEH; lane 2, λ H; lane 3, 105(pUC83); lane 4, 105(pUC84); lane 5, 105(pUC92); lane 6, 105(pUC96); lane 7, pUC8. Size standards are shown on the left in kilobases.

opposite orientation, plasmid DNA was digested with Pvu I. The results of this digest are shown in Fig. 2. There are two known Pvu I sites in the pUC vectors outside the MCS at nucleotides 280 and 2070, and none inside the MCS. If a pUC vector is linearized by deletion of the MCS, Pvu I cuts either vector into three fragments of 0.12, 0.9 and 1.6 kb (personal communication, Bethesda Research Laboratories):

Figure 2. Opposite Orientation of Insertions in Recombinant Plasmids

Recombinant plasmid DNA from two clones in each supposed insertion orientation were digested with Pvu I. Lane 1, λ H; lane 2, 105(pUC83); lane 3, 105(pUC84); lane 4, 105(pUC92); lane 5, 105(pUC96). In lane 3, there appears to be a small deletion in the smallest fragment of the vector. In lanes 4 and 5, the 2.2 and 2.12 kb fragments are seen as a doublet. Size standards are indicated on the left in kilobases.

It can be seen that, with any insert, the 0.9 kb fragment will be retained, and the remaining two fragments will vary in size. As shown in Fig. 2, this is indeed the case. In both orientations, the 0.9 kb fragment is conserved, along with a 4.3 kb fragment, indicating that segment is internal to any Pvu I sites within the insertion. Moving in either direction from this 4.3 kb segment, one finds two remaining fragments that will vary in size, representing the remaining termini of the inserted fragment plus any pUC DNA between the MCS borders and the first Pvu I site encountered in the vector. The fragment sizes are:

<u>pUC8 clones</u>	<u>pUC9 clones</u>
0.6 kb	0.9 kb
0.9	2.1
3.72	2.12
4.3	4.3

These results are illustrated in Fig. 3.

Figure 3. Pvu I Restriction Map of Recombinant Plasmids

The Pvu I restriction sites (P) are shown on a recombinant plasmid in each orientation. The Eco RI (E) and Hind III (H) termini of the vector (thick line) are also indicated. The fragment sizes are shown in kilobases.

From these data the following Pvu I map of the fragment was constructed:

Restriction Map

The location of the Pvu I restriction sites within the R45 fragment provided a convenient starting point for constructing a restriction map of the fragment itself. Four restriction enzymes were found that cut the fragment into 2-3 smaller fragments. These were Bgl II, Pvu I, Sph I and Sal I. The following enzymes have no restriction sites within the R45 fragment:

Pvu II	Bam HI	Sst II
Hind III	Cla I	Xba I
Eco RI	Xho I	Kpn I
		Bcl I

The following enzymes were found to cut the fragment into an unmanageable number of smaller fragments:

Pst I	Hinc II
Hae III	Hpa I
Ava II	Taq I

Aliquots of isolated R45 fragment DNA were restricted with each of the four selected enzymes, and the resulting fragment sizes estimated by electrophoresis with DNA standards. A portion of each single digest was then separately restricted with each of the remaining enzymes, resulting in a series of double digests. Each of these was analyzed on a 1.2% agarose gel. The results are given in Fig. 4.

Figure 4. Double Digests of the R45 Fragment

Aliquots of R45 fragment DNA were digested with each of four restriction enzymes. Portions of each restriction were subjected to a second digest with each of the remaining enzymes. Lane 1, Bgl II + Pvu I; lane 2, Bgl II + Sal I; lane 3, Bgl II; lane 4, Bgl II + Sph I; lane 5, λ H; lane 6, Sph I + Pvu I. Size standards are indicated on the right in kilobases.

The single digests resulted in fragments of the following sizes:

<u>Pvu I</u>	<u>Bgl II</u>	<u>Sph I</u>	<u>Sal I</u>
4.3 kb	5.6 kb	4.6 kb	6.1 kb
2.12	1.4	2.3	0.6
0.48		0.2	

Fragment sizes resulting from the double digests are as follows:

<u>Bgl II+Pvu I</u>	<u>Bgl II+Sph I</u>	<u>Bgl II+Sal I</u>	<u>Sph I+Pvu I</u>
2.12 kb	4.7 kb	4.75 kb	2.5 kb
0.48	1.4	1.4	2.12
0.92	0.8	0.6	1.8
3.38		0.2	0.5

The location of the single Bgl II site can be determined when the double digest reveals that the 2.12 kb Pvu I fragment at the Eco RI terminus is conserved. The Bgl II site can only lie 1.4 kb in from the Hind III terminus. Such a location yields the double-digest fragment sizes observed. Building on this information, single and double digests resulted in the final map, as given in Fig. 5. Because the two Sal I sites do not overlap with any other sites, their locations relative to each other cannot be determined. The two Sal I sites are given dotted lines to indicate this fact.

Figure 5. Single Digest Maps and Restriction Map of the R45 Fragment

Minimal Inhibitory Concentration of Arsenite

The minimal inhibitory concentration of arsenite for each strain used, with or without recombinant plasmids, was determined by plating the strains on LA containing increasing amounts of arsenite. This allowed the selection of an arsenite concentration that would clearly distinguish between plasmid and non-plasmid-bearing strains. The results are given in Table II.

The minimal inhibitory concentration is taken to be that concentration of arsenite at which no growth is observed for a given strain. Minimal inhibitory concentrations were:

<u>JM 105</u>	<u>JM 105(pUC84)</u>	<u>CSR 603</u>	<u>CSR 603(pUC84)</u>
6mM	20 mM	4 mM	20 mM

Although MIC's determined by this method are approximate, a sharp distinction can be seen between the resistances of plasmid and non-plasmid-bearing strains.

Arsenite Resistance Induction Assays

Since the arsenite resistance fragment has been ligated into the pUC vectors next to the lac promoter, arsenite resistance may be induced from the lac promoter using IPTG. Since resistance would be induced only in clones with the fragment in the proper orientation with regard to the promoter, it should be possible to determine in which direction -- from the Eco RI site to the Hind III

TABLE II

MINIMAL INHIBITORY CONCENTRATION
OF ARSENITE

<u>E.coli strain</u>	ARSENITE, mM									
	<u>0</u>	<u>2</u>	<u>4</u>	<u>6</u>	<u>8</u>	<u>10</u>	<u>12</u>	<u>15</u>	<u>20</u>	
JM 105	TNTC ^a	40 ^b	20	0	0	0	0	0	0	
JM 105(pUC84)	TNTC	ND ^c	ND	ND	ND	160	30	20	0	
CSR 603	TNTC	2	0	0	0	0	0	0	0	
CSR 603(pUC84)	TNTC	ND	ND	ND	ND	100	42	15	0	

- a Too numerous to count
b Number of colonies counted per plate
c Not determined

site or vice versa -- the fragment is transcribed. When recombinant clones are pre-grown in IPTG, a lag in growth would be observed with one pUC recombinant plasmid upon addition of arsenite, because transcription must be induced from the arsenite promoter. In the other orientation, resistance will have been previously induced by IPTG from the lac promoter, and no such lag would be observed.

Experiments were conducted to find the optimum arsenite concentration that would consistently induce resistance and yet be subinhibitory to uninduced cultures. 1.0 mM arsenite was selected for this purpose (Fig. 6). One strain of each orientation -- JM 105(pUC84) and JM 105(pUC96) -- was chosen for study.

Figure 6. Optimal Arsenite Concentration for Induction
of Resistance

- A: Cultures of JM 105(pUC84) were grown overnight in LB, subcultured and grown to log phase. At the time indicated by the arrow, 0.5 mM, ○; 1.0 mM, □; 5 mM, ▲; or no arsenite, ◆, were added to the culture.
- B: Cultures of JM 105(pUC84) and JM 105(pUC8) were grown as above and challenged with 10 mM arsenite at the time indicated by the arrow. JM 105 (pUC 84) was induced with 0.5 mM, ○; 1.0 mM, □; or no arsenite, ▲. JM 105 (pUC 8) was induced with 1 mM arsenite, ◆.

Unfortunately, IPTG did not induce resistance in either orientation under any of the conditions used. Results from a clone in the pUC8 orientation are shown in Fig. 7. Results in clones of the opposite orientation were identical.

Figure 7. Induction of Resistance with IPTG

Cultures of JM 105(pUC84) were grown overnight in LB containing the following inducers: 1mM arsenite, ○; 2mM IPTG, ■; none, △. The cultures were subcultured and grown to log phase twice before being challenged with 15 mM arsenite at the time indicated by the arrow. A culture that contained no inducer and was not challenged, ◆, was included as a control.

Identification of Plasmid-Determined Proteins

The plasmids pUC8, pUC84 and pUC96 were transformed into the maxicell strain E. coli CSR 603. Uninduced and arsenite-induced strains were irradiated as previously described. This is a modification of the original maxicell procedure (31), since radioactive amino acids were not added. Due to the high copy number of the pUC vectors (approximately 200 per cell) (11), it was expected that recombinant plasmid proteins would be detected without the use of radioactivity.

Because the arsenite resistance genes are inducible, proteins from cultures grown with arsenite can be analyzed for differences on a gel beside those from uninduced cultures. In order to maximize these differences, cells were induced in 10 mM arsenite, rather than the subinhibitory 1 mM arsenite. The host and pUC-bearing strains are not viable at this concentration, so arsenite was not included in the growth media for these strains.

In comparing induced recombinant-bearing strains against the uninduced strains, the following differences were apparent:

1. Three proteins were visible in induced lanes only, with apparent molecular weights of 62, 16.5 and 13.5 Kdal. Whereas the two smaller proteins were seen on gels stained with either silver or Coomassie blue, the 62 Kdal protein was seen only on Coomassie

blue-stained gels (Fig. 8).

2. When ampicillin was included in the medium, the mature 27 Kdal β -lactamase enzyme (37) encoded by the pUC vector was visible in all lanes, except that of the plasmid-less host strain (Fig. 9).
3. On most gels, a 36 Kdal protein was visible in all lanes except those of the induced strains (Fig. 9).

Figure 8. Plasmid-Determined Proteins

Various CSR 603 strains were irradiated and grown in LB without ampicillin for three hours. Cycloserine was added, and the cultures were grown eight hours before lysing. Cultures in lanes 2 and 5 also contained 10 mM arsenite. Lanes 1, 4 and 7, protein standards; lanes 2 and 3, 603(pUC96); lanes 5 and 6, 603(pUC84); lane 8, 603(pUC8); lane 9, CSR603. The sizes of plasmid-determined proteins are indicated on the left side of the figure. Protein standard sizes are indicated on the right. The gel was stained with silver, then with Coomassie blue (6).

Figure 9. β -Lactamase and 36 Kdal Protein

Various CSR 603 strains were irradiated and grown for 6 hours in LB with 50 ug/ml ampicillin before lysing. Cycloserine was not added. Cultures in lanes 2 and 5 also contained 10 mM arsenite. Lanes 1, 4 and 7, protein standards; lanes 2 and 3, 603(pUC96); lanes 5 and 6, 603(pUC84); lane 8, 603(pUC8); lane 9, 603. The β -lactamase protein (27 Kdal) encoded by the pUC vectors and the 36 Kdal protein absent in induced strains are indicated by arrows on the left; protein standard sizes are indicated on the right. The gel was stained with Coomassie blue.

DISCUSSION

Comparison of the minimal inhibitory concentrations of arsenite for strains growing on LA shows that the MIC's of the E. coli strains JM 105 and CSR 603 are similar -- 6 mM and 4 mM, respectively. Transformation of recombinant plasmids containing the Eco RI-Hind III fragment of R45 into these strains increases arsenite resistance by up to five-fold (the MIC was 20 mM in both cases), confirming successful transfer of the arsenite resistance region from R45.

Strains bearing recombinant plasmids were grown in liquid medium with and without arsenite and then challenged by the addition of arsenite. Pre-induced strains continued exponential growth while uninduced strains showed a growth lag of 1-2 hours. These results indicate that the inserted fragment contains the control elements for the resistance genes.

Resistance to arsenite could not, however, be induced in either pUC84 or pUC96 using IPTG. Hence, no conclusion can be drawn regarding the direction of transcription. In another study, a 3.5 kb fragment of DNA containing the gene for diadenosine tetraphosphatase was cloned into the pUC vectors (17). In one orientation, addition of IPTG produced an eight-fold increase in production of this

protein over the uninduced. In the other, no increase was observed. In a third study, however, cloning of a 2.5 kb region containing the gene for the activator protein of D-serine deaminase produced no such increase using IPTG in either orientation (Elizabeth McFall, personal communication).

Based on these observations, it was important to confirm that the plasmids pUC84 and pUC96 in fact contained the inserted fragment in opposite orientations. Accordingly, the plasmids were digested with the restriction enzyme Pvu I and, as seen in Fig. 2, this was confirmed.

A possible explanation for the inability to induce resistance from the lac promoter is that the RNA polymerase that binds there encounters a termination signal before reaching the structural genes for arsenite resistance. Using the restriction map of the fragment (Fig. 5), one could attempt to delete the termination signal using restriction digestion.

Another possibility exists. Since induction of arsenite resistance with IPTG was attempted in media lacking arsenite, a repressor gene may be present at the asi^r operator. This repressor may block passage of the RNAP initiating at the lac promoter.

Digestion might also be carried out using an exonuclease, such as Bal 31. This enzyme attaches to a

free end of DNA and continuously degrades it non-specifically. Alternatively, Tn5 insertion mutagenesis might be used to determine the relative locations of the arsenite and arsenate genes (3, 40). This transposon inserts randomly into DNA and carries a unique Bam HI restriction site. Insertion into a gene results in loss of function. Bam HI digestion, followed by electrophoretic analysis and resistance assays, can reveal gene location. Promising new expression vectors based on the bacteriophage T7 might also be employed (38). This dual plasmid system is specific for the T7 promoter and ignores bacterial promoters. It also ignores bacterial RNA termination signals, circumventing the necessity to locate and delete these signals.

The modified maxicell technique used in this study revealed three polypeptides of apparent molecular weights 62, 16.5 and 13.5 Kdal present in strains bearing recombinant plasmids derived from R45 after induction with 10 mM arsenite. These polypeptides were absent in cultures grown without arsenite. The β -lactamase gene in the pUC vectors is also inducible, and β -lactamase is seen only in gels from cultures containing ampicillin in the medium. This indicates that the modified procedure is capable of revealing specific plasmid-determined proteins, using appropriate media.

In addition, a polypeptide with an apparent molecular

weight of 36 Kdal present in all cultures, including the plasmid-less host strain, is absent only from recombinant plasmid bearing cells grown in arsenite. This interesting observation was possible only in this modified maxicell procedure. The original procedure, with the addition of radioactive amino acids to identify only plasmid-encoded proteins, would not have detected it. The mechanisms responsible for this observation are unknown.

The procedures used in this study do not, however, specifically determine whether these proteins are actually encoded by the plasmid itself or induced within the cell by regulatory genes carried on the plasmids. To answer this question, the maxicell procedure described previously must be carried out with the addition of radioactive amino acids following chromosomal inactivation, to specifically label plasmid-encoded proteins.

It is known that there exists more than one mechanism for resistance to arsenite and arsenate (27, 28). Much is still unknown regarding these resistances. Cell fractionation studies will be necessary to determine the location of the proteins within the cell. Footprinting, a technique for identifying protein binding sites on DNA by the fact that the protein protects the site from enzymatic digestion (25), can be used to ascertain whether the resistance is negatively or positively controlled. In addition, whether the resistances to arsenate and arsenite

are due to separate efflux pumps or one pump is modified to accommodate the separate arsenic compounds remains to be determined.

In this study, the proteins determined by the arsenic resistance region of the plasmid R45 have been identified. Comparison of these proteins with those of other arsenic resistance plasmids will help illuminate the differences and similarities between their mechanisms of resistance. In addition, a restriction map of the region has been prepared which can be used to further localize the arsenic resistance genes and study their organization and regulation.

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