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AN ABSTRACT OF THE THESIS OF Joshua T. Clark for the Master of Science in Biology presented May 6, 1988.

Title: Determination of Homology Between the Arsenic Resistance Plasmids R45 and R773 in *Escherichia coli*.

Approved by members of the thesis committee:

Mary L. Taylor, Chair

Robert Millette

Richard Petersen

Gordon Kilgour

The resistance transfer factor R45 from *Escherichia coli* confers inducible arsenate and arsenite resistance in that bacterium. The

genes for these resistances were cloned into the EcoR1 - Sph1 multiple cloning site of PGEM3 Blue vector (Promega) to produce a 4.9 kilobase plasmid, pJC1. This recombinant plasmid, pJC1, conferred IPTG induced resistance to arsenite and arsenate. In addition, pJC1 was tested for homology with the *E. coli* plasmid R773, which encodes for arsenic resistance in that bacterium as well.

Through DNA-DNA hybridization the arsenic resistance determinants of R45 and R773 were compared. Under stringent hybridization conditions, R45 demonstrated DNA sequence homology to the ArsB and Ars C genes of R773 but not to the ArsA gene of R773.

DETERMINATION OF HOMOLGY BETWEEN THE ARSENIC
RESISTANCE PLASMIDS R45 AND R773 IN
ESCHERICHIA COLI

by

JOSHUA T. CLARK

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

MASTER OF SCIENCE
IN
BIOLOGY

Portland State University

1988

TO THE OFFICE OF GRADUATE STUDIES :

The members of the Committee approve the thesis of
Joshua T. Clark presented May 6, 1988.

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INTRODUCTION

Arsenicals are toxic to most living organisms. Ionized arsenic exists in several forms. Arsenate (5+ oxidation state) is the least toxic form and competes with phosphate in phosphorylation reactions to produce unstable arsenylated derivatives. Arsenite (3+ oxidation state) is more toxic and reacts directly with the sulfhydryl groups of proteins. In mammals, detoxification of arsenicals is accomplished by the liver or kidney via reduction of the arsenicals to the inorganic arsonous acids (1+ oxidation state) followed by excretion. In some marine algae, arsenicals are incorporated into 1-5-deoxyribosylarsine oxides, that may be further converted to arsenobetaine or arsenylated phospholipids and then excreted. An arsenate resistant strain of bacteria *Alcaligenes faecalis*, produces an arsenite oxidase, a cytochrome chain which oxidizes arsenite, AsO_2^- , to the less toxic arsenate, AsO_4^{3-} (14).

In bacterial cells, genes which code for resistance to many toxic metals and antibiotic drugs can be carried on plasmids, which are also called R (resistance) factors. Many R-factors have been shown to confer resistance to arsenicals in *Escherichia coli*. This resistance may be mediated by arsenical extrusion systems (exit pumps) or, in

Alcaligenes, by conversion of the highly toxic arsenite to the less toxic arsenate (12).

The arsenical resistance (ars) operon of plasmid resistance factor R773 confers inducible resistance to arsenate, arsenite, and antimony (12). The plasmid is a member of the F1 compatibility group and is easily transferred between strains of *E. coli*. Resistance to arsenate results from an energy-dependent efflux of arsenate from cells, thus preventing the accumulation of lethal amounts of the toxic metal. Arsenate, arsenite and antimonate resistance are encoded by separate genes on the Ars operon (2).

The energetics of the arsenate resistance system of R773 have been studied by introducing the plasmid into *E. coli* strain AN120, a mutant deficient in the H⁺ translocating ATPase of oxidative phosphorylation. Starved AN120 cultures depleted of endogenous energy reserves were loaded with arsenate. When glucose was added, rapid extrusion of arsenate occurred. When oxidation was inhibited by cyanide, glucose metabolism still produced arsenate efflux. Energy sources such as succinate, which provides a protonmotive force (PMF), but not ATP did not result in efflux. Measurement of intracellular ATP levels demonstrated a direct correlation of ATP concentration and rate of efflux. These results indicate that the arsenate efflux pump is driven by hydrolysis of ATP and not by the PMF (12).

Further work with R773 involved the cloning of the 4.3 kilobase pair ars operon into the Hind III site of plasmid pBR322. On the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) fractionation of cells and radiolabeled minicells that contain the recombinant plasmids, at least two polypeptides of 64,000 daltons and 16,000 daltons are produced when the plasmid-bearing strains are grown in the presence of arsenite (10).

In addition, the nucleotide sequence of the 4.3 kilobase pair Hind III fragment has been determined. Three open reading frames for genes arsA, arsB, and arsC were found. The arsA gene encodes a hydrophilic protein of 63,169 daltons, with two potential adenylate-binding sites. The arsB gene encodes a potential membrane protein of 45,557 daltons. The arsC gene encodes a 15,811 dalton hydrophilic protein. It appears that the arsA and arsC gene products correspond to cytosolic proteins identified from the minicell experiments. A model has been proposed in which the gene products of the ars operon comprise an anion translocating ATPase. The arsA protein is the energy-transducing ATPase subunit, with specific sites for ATP and arsenite. Binding of arsC protein to the complex either changes the specificity to arsenate or increases the range of substrates to allow recognition of both arsenate and arsenite (3).

Other R factors carrying arsenate, arsenite, and antimony resistance have also been reported in other organisms (15, 5). It was of interest to determine how widespread the arsenate-arsenite

resistance determinant of R773 was, with respect to plasmid DNA derived from human isolates of arsenate- and arsenite resistant enterobacteria. Of the 15 isolates, 7 showed strong homology as demonstrated by colony blot hybridization. It may be concluded that there is at least one more genetically distinct plasmid-borne arsenate resistance determinant in addition to that represented by R773 (11). The determinants that did not show homology may represent a heterogeneous group of gene sequences, as has been found for plasmid-borne tetracycline resistance (9)

The subject of this thesis is the arsenic resistance gene(s) of R45. This R factor is a 45 kilobase pair plasmid first found in enteric bacteria isolated from sewage (6). The original plasmid confers resistance to arsenite, arsenate, ampicillin, tetracycline, sulfonamides, and streptomycin and is a member of the N Incompatibility group (Anne O. Summers, personal communication). The arsenite and arsenate resistance genes of R45 were localized to a 6.9 kb region of plasmid DNA (13). This fragment was cut from the R45 plasmid and ligated into the pBR322 cloning vector to produce the plasmid pEH (J.W. Myers, personal communication).

The objectives of the present study were to: 1) further localize the genes coding for arsenical resistance in R45, 2) construct a restriction map of the arsenic resistance determinant, and 3) determine if there was homology between the arsenic resistance determinants of R45 and R773.

MATERIALS AND METHODS

TABLE I

TABLE OF ABBREVIATIONS

amp	ampicillin
asi	arsenite
asa	arsenate
BCIP	5-bromo-4-chloro-3-indolyphosphate
dATP	deoxy adenosinetriphosphate
F'	carrying the <i>E. coli</i> sex factor F', with a chromosomal insertion
IPTG	isopropyl- <i>B</i> - D-thiogalactosidase
k b	kilobases
LA	Luria agar
lac	lactose operon
LB	Luria broth
X-gal	5-bromo-4-chloro-3-indoyl- <i>B</i> - D-galactoside

Culture Media

The culture media used were Luria Broth, Minimal Media, Modified MOPS Media, and Plasmid Media. For solid media, 15 g of agar was added to one liter of liquid media just prior to autoclaving.

Luria Broth

Per liter:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
K ₂ HPO ₄	1 g

Minimal Media

Arsenite resistance was assayed in Davis Minimal Medium (Difco) containing the appropriate arsenite concentration (usually 10 mM Na Arsenite), 0.5% glucose and the required growth factors of the bacterial strain under study.

Plasmid Media

Plasmid media for large scale extraction of plasmid DNA from bacterial cultures was prepared by adding 100 ml of a sterile solution of 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ to 900 ml of a sterile solution containing 12 g Bacto-tryptone, 24 g of Bacto-yeast extract, and 4.0 ml glycerol. After autoclaving, the media was supplemented with ampicillin to a final concentration of 25 mg/liter.

Modified MOPS Media

Arsenate resistance was assayed in a modified (J. Myers, personal communication) morpholinopropane sulfonic acid (MOPS) medium:

Solution A.:

To 400 ml of water add 8.4 g of MOPS;
add 1 M Na-arsenate solution to achieve
desired concentration. Adjust pH to 7.2
with KOH. Autoclave and let cool to 55°C.

To the above solution A add the following amounts of filter sterilized solutions:

10 ml of solution 1.

1. 5M NaCl, 0.05M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

10 ml of solution 2.

2. 10% $(\text{NH}_4)_2\text{SO}_4$
0.1 M K_2HPO_4

0.5 ml of solution 3.

3. 0.02 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
(neutralized with 1N HCl)

Mix the solutions and add to 600 ml of sterile H_2O . (for solid media, autoclave the water with 15 g of agar) Supplement the above mixture with:

2 ml of 0.1% sterile B_1 solution

10 ml of 20% (w/v) glucose solution

10 ml of 1% (w/v) sterile proline solution

10 ml of 1% (w/v) sterile methionine solution

10 ml of 1% (w/v) sterile thiamine solution

Isolation of Plasmid DNA

Plasmid DNA was extracted from verified strains by the method of Birnboim and Doly (1). The centrifuged cell paste from 100 ml of Plasmid Broth culture was resuspended in TE (10 ml of 25 mM Tris-Cl (pH 8), 10 mM Na₂EDTA) supplemented with glucose 1% (w/v). Lysis of the cells was accomplished by adding 20 ml of 0.2 M NaOH, 1% SDS. At high pH, plasmid DNA remains covalently closed and circular, while chromosomal linear fragments become single-stranded. A 15 ml solution of 5 M potassium acetate, 3 M acetic acid solution was added to precipitate proteins, high molecular weight RNA, and single-stranded DNA. The precipitate was removed by two successive centrifugations and the plasmid DNA in the supernatant was precipitated by 0.6 volumes of isopropanol. After centrifugation for 30 minutes, the DNA pellet was dissolved in approximately 7 ml of TE buffer (7).

To remove contaminating chromosomal DNA, RNA, and proteins the solution was centrifuged in a cesium chloride, ethidium bromide density gradient. Before ultracentrifugation, a cotton tipped swab was used to pick up any protein/cell debris flocculant from along the rim of the polyallomer tube containing the plasmid DNA solution. The solution was centrifuged at room temperature in a Ti 50 rotor of a Beckman L ultracentrifuge at 45,000 RPM for 36 hours (7).

Since closed circular plasmid DNA is more tightly coiled than linear or open circular DNA, plasmid DNA is not able to bind as much ethidium bromide (EtBr) and is consequently more dense in the cesium chloride EtBr gradient. Thus, separation of plasmid DNA from chromosomal DNA can be achieved by differences in their respective buoyant densities. After centrifugation, DNA bands were visualized with an ultraviolet lamp (280 nm) and the lower (more dense) plasmid DNA band was extracted. The EtBr was removed from the plasmid DNA by extraction in an isopropanol solution saturated with 5 M NaCl, 10 mM Tris HCl, 1 mM Na₃EDTA (pH 8.5). The DNA was further purified by ethanol precipitation and the pellet was resuspended in 200 ul of TE.

Digestion of DNA with Restriction Endonucleases

Restriction endonucleases were obtained from BRL (Bethesda Research Laboratories) or USB (United States Biochemical). Digests were performed at 37°C for two hours under reaction conditions specified by the manufacturer.

Electrophoresis of Plasmids and Restriction Fragments

Plasmids and restriction fragments were characterized by electrophoresis through agarose gels whose concentrations ranged from 0.7% to 1.5% (w/v) in a tris-acetate electrophoresis buffer (7).

Samples containing 50 to 200 ng of DNA were mixed with 0.25 volumes of DNA tracking dye (1) and loaded on a horizontal slab gel apparatus (8) and run at 3V/cm.

Isolation of DNA Fragments

Digested DNA was separated by gel electrophoresis, stained with EtBr, and visualized by UV light. A slit was made in front of the desired DNA band to be extracted, and a strip of Whatman DE 81 DEAE cellulose paper was inserted into the gel. Electrophoresis was resumed at 100 V for 20 minutes until the DNA migrated onto the paper. The paper was removed and packed into a 400 ul microfuge tube in which a hole had been pierced in the bottom with a red hot needle. The 400 ul tube containing the paper was nested inside a 1.5 ml Eppendorf tube. The paper was treated by the following protocol:

Step:

- A. 200 ul of 0.1 M NaCl, 0.1 mM Na₃EDTA, 10 mM Tris HCl was added to the paper and centrifuged 15 sec.. The eluates were discarded and the procedure was repeated 3 times.
- B. 200 ul of 1.0 M NaCl, 0.1 M Na₃EDTA, 10 mM Tris HCl was added to the paper and centrifuged 15 sec.. The eluates were retained and the procedure was repeated 3 times.

The retained eluates of step B (total volume 800 ul) containing the DNA were precipitated in ethanol and centrifuged for 30 minutes. The DNA pellet was resuspended in TE and extracted with an equal volume of a 1:1 (v/v) mixture of phenol and CIA (chloroform mixed 24:1 with isoamyl alcohol), followed by CIA extraction to remove residual phenol. After extraction the DNA was again precipitated by 2 volumes of ethanol and 0.5 total volumes of 7.5 M ammonium acetate.

Construction of Recombinant Plasmids

Vector DNA (pGEM-Blue, a pUC derivative from Promega) was digested with the appropriate restriction endonucleases, cleaned by phenol-CIA, and precipitated as described above. Ligations were performed using a vector : insert molar ratio of 1:3. Two units of T4 DNA ligase were added to each reaction mixture along with 5X ligase reaction buffer (4).

5X ligase buffer

250 mM Tris HCl (pH 7.6)
50 mM MgCl₂
25 % (w/v) polyethylene glycol
8000
5 mM ATP
5 mM dithiothreitol

The ligation reaction mixtures were incubated at 13°C for 15 hours, and diluted five-fold just prior to adding the DNA to the competent cells.

Transformation of *E. coli* JM105 Cells

Cells of *E. coli* JM 105 were prepared for transformation as described by Manniatis (7). Cell aliquots of 200 μ l were allowed to become competent at 4°C overnight. Plasmid DNA (up to 40 ng) was added to the cells, mixed gently, then incubated for 30 minutes on wet ice. The tube containing cells was placed at 42°C for 45 seconds and then immediately returned to the wet ice. After several minutes, 700 μ l of LB was added, and the tube was incubated at 37°C for two hours in a shaking incubator. A 100 μ l aliquot of the DNA/competent cell mixture was spread onto LA containing 100 μ g/ml ampicillin, 40 μ g/ml X-Gal and 0.5 mM IPTG.

Since transformation was performed with pGEM-3 Blue (Promega) vector DNA, which contains the ampicillin resistance gene from pBR322 and the *B*-galactosidase-alpha peptide sequence from pUC 19, ampicillin resistant transformants could be distinguished by the color of the bacterial colonies (16). The vector carries the lac alpha-peptide and multiple cloning site arrangement from pUC 19. This arrangement gives rise to a functional alpha-peptide which is capable of complementing the product of the lacZ-delta-M15 gene to produce functional *B*-galactosidase. Cells (i.e. JM 105) containing the lacZ-delta-M15 gene on an F' and also containing the pGEM-3 Blue vector will be blue in color when plated on indicator media containing IPTG and X-Gal. However, when the lac-alpha-peptide is

disrupted by cloning into the pGEM-3 Blue multiple cloning site, complementation does not occur and no *B*-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM constructs remain white in color.

DNA-DNA Hybridization

Hybridization of biotin-labeled DNA probes to target DNA was accomplished by the following procedure:

1. Apply a minimum of 30 ug of digested plasmid DNA per lane of an agarose gel (0.7 % to 2.0 %).
2. After electrophoresis, denature the DNA by soaking the gel for 45 minutes in 1.5 M NaCl, 0.5 M NaOH. Neutralize the gel for 45 minutes in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 0.001 M Na₂EDTA. Southern blot overnight to a nylon membrane (Hybond™, Amersham) in 20X SSC (3.0 M NaCl, 0.3 M Na₃ citrate).
3. Fix the DNA to the nylon membrane by a 2 minute exposure to shortwave (260 nm) UV light with the DNA side down.
4. Transfer the filters to a heat sealable bag (Seal-a-Meal™, Dazey Co.) and prehybridize for at least 4 hours to overnight, in 5X SSC, 5X

Denhardt's solution (5X Denhardt's: 0.1% (w/v) Ficoll; 0.1% (w/v) polyvinylpyrrolidone; 0.1% (w/v) BSA Fraction V) , 0.1% SDS, at 65°C in a shaking water bath.

5. Hybridize filters overnight (16 to 20 hours) in the above solution (step 4) following the addition of heat-denatured, biotin labeled DNA probe to a final concentration of 100 ng/ml.

6. Wash the filters, with agitation, after hybridization in the following solutions:

- a. 2X SSC, 0.1% SDS, do two 5 minute washes at room temperature.
- b. 2X SSC, 0.1% SDS, do two 30 minute washes at 65°C.
- c. 1X SSC, 0.1% SDS, do one 10 minute wash at room temperature.

7. Blot membranes on 3 mm Whatman filter paper at 80°C in vacuum oven (bakes probe on).

8. Block membrane's nonspecific binding sites in 3% (w/v) bovine serum albumin (Fraction V), 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl for one hour to overnight at 65°C.

9. Remove membranes from blocking buffer (step 8) and incubate at room temperature, with gentle agitation, for 10 minutes in binding

buffer: 20 ul strepavidin-alkaline phosphate solution (BluGENE™, BRL) mixed with 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl.

10. Wash the membranes, with agitation, in the following solutions at room temperature:

a. 100 ml of 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, do three, 2 minute washes.

b. 50 ml of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, do two, 2 minute washes.

11. Place the slightly wet filters in a heat sealable bag for the colorimetric detection. Add freshly prepared BCIP dye solution (BluGENE™, BRL) to the bag and allow color development to continue for 30 minutes.

12. Wash membranes in 20 mM Tris-HCl (pH 7.5), 0.5 mM Na_2EDTA to stop dye reaction. Store membranes damp dry.

Labeling DNA with Biotin by Nick Translation

Incorporation of the biotin-labeled nucleotide (7-dATP) into the DNA was accomplished enzymatically via a standard nick translation reaction as described in the BluGENE™ kit, (BRL). One ug of sample DNA, biotin-7-dATP, nonbiotynilated nucleotides (dCTP, dGTP, and dTTP), DNA polymerase I and DNA Pol I/DNase were mixed and

allowed to incubate at 15°C for 90 minutes. The reaction was stopped by the addition of 300 mM Na₂EDTA (pH 8.0), 5% (w/v) SDS. The biotin-labeled DNA was separated from the unincorporated nucleotides by two ammonium acetate ethanol precipitations and resuspended in TE. Just prior to use the biotin-labeled DNA probe was denatured by heating at 95°C for 5 minutes, followed by chilling on wet ice for minutes.

RESULTS

Restriction Mapping of pEH

The recombinant plasmid, pEH, contains the 6.8 kb EcoRI-Hind III arsenic-resistance fragment of R45 ligated into pBR322 cloning vector (J. Myers, personal communication). Please see figure 1. The pEH plasmid is 11.1 kb in size. To construct a restriction map of the EcoRI-Hind III R45 fragment, whole pEH was digested with various endonucleases and the resulting fragment sizes were analyzed by electrophoresis. A portion of certain single digests were subjected to a new separate digest and the resultant fragment sizes analyzed by electrophoresis. In this manner, locations of restriction sites both in pBR322 and in the EcoRI-Hind III R45 insert were determined.

Figure 1 Simple Map of pEH.

Four restriction enzymes were found that cut the R45 insert into a manageable number of fragments. These were Bgl II, Pvu I, Sal I, and Sph I. Please see figure 2. The following enzymes have no restriction sites within the R45 fragment:

Pvu II	Cla I	Xba I	Nhe I	Sst II
Hind III	EcoRI	Kpn I	BamHI	Bcl I

The pEH digests resulted in fragments of the following kilobase sizes:

TABLE II
DIGESTS OF pEH

<u>Pvu I</u>	<u>Pvu I+EcoRI</u>	<u>Pvu I+Hind III</u>	
5.55	5.55	4.44	
4.44	4.44	3.71	
1.08	0.62	1.84	
	0.46	1.08	
<u>Sph I</u>	<u>Sph I+EcoRI</u>	<u>Sph I+Hind III</u>	<u>Sph I+BamHI</u>
6.01	4.49	6.01	6.01
4.49	3.80	4.49	4.49
0.61	2.21	0.54	0.39
	0.61		0.19
<u>Sal I</u>	<u>Sal I+EcoRI</u>	<u>Sal I+Hind III</u>	<u>Sal I+BamHI</u>
9.79	6.08	9.79	9.79
0.71	3.71	0.71	0.57
0.57	0.71	0.62	0.44
	0.57		0.28
	<u>Bgl II+EcoRI</u>	<u>Bgl II+Hind III</u>	
	9.69	5.77	
	1.41	5.33

Figure 2 Restriction Map of pEH, an R45 Derivative.

Construction of pJC1

In an attempt to further localize the arsenic resistance gene(s) of R45, construction of additional recombinant arsenic resistant plasmids was undertaken. Insert DNA from pEH was double digested with EcoRI and Sph I. The cloning vector pGEM-3-Blue (Promega) was also digested with EcoRI and Sph I. Digested vector and insert DNA was mixed and ligated using T4 DNA ligase. Please see figure 3. After ligation, the DNA was used to transform *E. coli* strain JM105.

Recombinant transformants were selected from LA plates containing: X-Gal, Amp, and IPTG (see page 11), and then picked onto Davis minimal medium plates containing 7 mM Asi and 0.5 mM IPTG, and also onto modified MOPS medium plates containing 70 mM Asa and 0.5 mM IPTG.

Confirmation of Recombinant Plasmids

Those transformants with the ability to grow on the above described selective media, were analyzed for the presence of a recombinant plasmid with a molecular weight equal to the sum of the inserted EcoRI-Sph I fragment (2.2 kb) and the pGEM vector (2.7 kb).

Plasmid DNA was extracted from transformant broth cultures and its size determined by electrophoresis. To further confirm the identity of the recombinant plasmid DNA, double digests with EcoRI and Sph I were performed, and the expected DNA bands (2.2 kb + 2.7 kb) were observed. On the basis of the above tests, a recombinant, arsenic

resistant plasmid: pJC1 containing the 2.2 kb EcoRI-Sph I fragment of R45 and the 2.7 kb pGEM-3-Blue vector was confirmed.

Construction of pJC2

In an attempt to further localize the arsenic resistance gene(s) of R45, construction of pJC2 was undertaken. The pJC2 clone consists of the 0.8 kb Bgl II-Sph I fragment from R45 ligated to the pGEM-4-Blue cloning vector. Please see figure 3. Unfortunately, none of the recombinants demonstrated the ability to grow on either arsenite or arsenate. However, pJC2 was of use in the hybridization experiments. Attempts to clone the 1.4 kb EcoRI-Bgl II fragment from R45 were unsuccessful.

Derivation of pJC1 and pJC2 from pEH

pEH

11.1 kbp

pJC1

4.913 kbp

pJC2

3.527 kbp

Figure 3 Construction of pJC1 and pJC2.

Restriction Mapping of pJC1

Additional restriction sites within the 2.2 kb EcoRI-Sph I fragment were mapped by the methods described on page 17. Please see figure 4. Two restriction enzymes: Pst I and Ava II were found that cut pJC1 into a manageable number of fragments. The digests of pJC1 resulted in fragments of the following kilobase sizes:

TABLE III
DIGESTS OF pJC1

<u>Pst I</u>	<u>Pst I+Pvu I</u>	<u>Pst I+Sph I</u>	<u>Pst I+Ava II</u>
2.82	1.69	2.78	1.46
1.49	1.08	1.49	1.14
0.60	0.90	0.56	0.88
	0.60		0.61
	0.41		0.22
	0.23		
	<u>Ava II+Bgl II</u>	<u>Ava II+Sph I</u>	
	2.22	2.02	
	2.02	1.42	
	0.45	1.25	
	0.22	0.22	

pJC 1

4.913 kbp

pJC 1

4.913 kbp

Figure 4. Restriction Map of pJC1, an R45 Derivative.

Carbon Source Requirements and Arsenic Resistance

The expression of arsenic resistance in pJC1-bearing JM105 cells was dependent on the carbon source present in the media. Plasmid-bearing cells were plated on Davis minimal media containing 7 mM Asi, 0.5 mM IPTG and one of three carbon sources: 0.5% glucose, 0.5% maltose, or 0.5% succinate. Growth was detected only on arsenite media containing glucose as a carbon source.

IPTG Induction of Arsenite and Arsenate Resistance

The expression of arsenite and arsenate resistance in pJC1-bearing JM105 cells is regulated by the presence of IPTG (0.5 mM) in the media. Without IPTG, induction of the arsenic resistance operon of pJC1 did not occur.

Homology of pJC1 and pJC2 to R773 Derivatives

To determine if the arsenic resistant determinants of pJC1 (R45) and R773 shared homology, hybridization studies with biotin labeled probes were undertaken. The recombinant plasmid pUM3 contains the 4.3 kb Hind III arsenic-resistant fragment of R773 ligated into the pBR322 cloning vector (10). Please see figure 5. This plasmid (pUM3, generously provided by B. Rosen) and constructed subclones: pUJ1 and pUJ2 (this work, please see figure 6) were used as probes

Plasmid pUJ1 was constructed by methods previously described and represents the 5.6 kb BamHI fragment of pUM3. Plasmid pUJ2 is comprised of the 1.1 kb BamHI fragment of pUM3 ligated into the pGEM-3-Blue cloning vector. Please see figure.6.

pUM3

8.71 kbp

Figure 5. Restriction Map of pUM3, an R773 Derivative.

Construction of pUJ1 and pUJ2 from pUM3

pUJ2

3.83 kbp

pUM3

8.71 kbp

pUJ1

5.647 kbp

Figure 6 Construction of pUJ1 and pUJ2 from pUM3.

DNA-DNA Hybridizations

Digests of pUM3, pUJ1, and pUJ2 were applied to each lane of an agarose gel. Following electrophoresis, the DNA bands were Southern blotted to a nylon membrane and probed with biotin labeled pJC1 or pJC2. Please see figures on the following pages. By convention, all bands in a given agarose lane were named alphabetically, with the 'A' band in a given lane representing the DNA of higher molecular weight than the 'B' band in a given lane.

From the above experiments, a map of DNA sequence homology between pUM3 and pJC1 was constructed. Please see figure 15.

Figure 7. DNA-DNA Hybridizations with pJC1 Probe*. When pJC1 was used as a probe against various digests of pUM3, pUJ1, and pUJ2, DNA sequence homology was not detected in the BamHI fragment (**fragment B**) of pUM3.

Lane 1: pUM3 digested with BamHI.

Lane 2: pUJ1 digested with BamHI.

Lane 3: pUJ2 digested with BamHI.

Lane 4: pUJ1 digested with Kpn I.

Asterisk * next to **fragment** indicates homology with probe.

pJC1 Probe *

pUM3 Lane 1

pUJ1 Lane 2

pUJ2 Lane 3

pUJ1 Lane 4

Figure 8. Fragment Homology with pJC1 Probe.

Figure 9. DNA-DNA Hybridizations with pJC2 Probe*. When pJC2 was used as a probe against various digests of pUJ1 and pUJ2, DNA sequence homology was not detected in the Kpn I fragment (**fragment B**) of pUJ2.

Lane 1: pUJ1 digested with Kpn I.

Lane 2: pUJ2 digested with BamHI.

Lane 3: pJC1 digested with EcoRI, Bgl II, and Sph I.

Asterisk * next to **fragment** indicates homology with probe.

pJC2 Probe *

pUJ1 Lane 1

pUJ2 Lane 2

pJC1 Lane 3

Figure 10. Fragment Homology with pJC2.

Figure 11. DNA-DNA Hybridizations with pUJ1 Probe^{*}. When pUJ1 was used as a probe against various digests of pJC1, DNA sequence homology was detected in all fragments of R45.

Lane 1: pJC1 digested with Pvu I, EcoRI, and Sph I.

Lane 2: pJC1 digested with Pvu I, Ava II, and Sph I.

Lane 3: pJC1 digested with Ava II, Bgl II, and Sph I.

Asterisk * next to **fragment** indicates homology with probe.

pUJ1 Probe *

pJC1 Lane 1

pJC1 Lane 2

pJC1 Lane 3

Figure 12. Fragment Homology with pUJ1 Probe.

Figure 13. DNA-DNA Hybridizations with pUJ2 Probe*. When pUJ2 was used as a probe against various digests of pJC1, DNA sequence homology was localized to the Bgl II-Sph I fragment (fragment N) of R45.

Lane 1: pJC1 digested with Pvu I, EcoRI, and Sph I.

Lane 2: pJC1 digested with Pvu I, EcoRI, and Sph I.

Lane 3: pJC1 digested with Ava II, Bgl II, and Sph I.

Diagram of the pGEM vector construct. The construct is a circular plasmid. The pBR322 origin of replication is located on the left. The BamHI restriction sites are marked. The vector sequence is between the first and second BamHI sites. The insert sequence is between the second and third BamHI sites. The R773 sequence is located on the right. The pGEM label is on the left.

Restriction map of the pR45 plasmid. The map shows a circular plasmid with the following features: Sph I site at the top left, Pvu I site, EcoRI site, another Pvu I site, and another Sph I site. Between the first Sph I and Pvu I sites is a box labeled 'B*'. Between the first Pvu I and EcoRI sites is a box labeled 'C*'. Between the EcoRI and second Pvu I sites are boxes labeled 'E' and 'D'. Between the second Pvu I and second Sph I sites is a box labeled 'A*'. The plasmid is labeled 'pR45' at the top right. Below the map, the 'pGEM vector' is indicated on the left and the 'insert' is indicated on the right.

Restriction map of the pGEM vector and its insert. The map shows the pBR322 origin and various restriction sites. The insert is located between the PvuI and AvaII sites. The insert contains a start codon (ATG) and a stop codon (TAA). The insert is flanked by SphI sites. The map is labeled with 'pGEM vector' and 'insert'.

Restriction map of the pGEM vector and its insert. The map shows the pGEM vector (pBR322) with Sph I, Pst I, and Lcl sites. The insert (R45) contains two Ava II sites, a Bgl II site, and a Sph I site. The map is labeled with 'pGEM vector' and 'insert R45'.

Figure 14. Fragment Homology with pUJ2 Probe.

Homology Between pUM3 and pJC1

pUM3 (R773)

8.71 kbp

pJC1 (R45)

4.913 kbp

Figure 15 Homology Between pUM3 (R773) and pJC1 (R45). Lines connecting fragments indicate homology.

DISCUSSION

The results presented here demonstrate that the arsenic resistance determinant of two conjugative R factors, R45 and R773, have some homologous DNA sequences. Through DNA hybridization, it was shown that the R45 insert of pJC1 is homologous to the *ArsB* and *ArsC* structural genes of the *Ars* operon of R773. However, pJC1 does not hybridize with the *ArsA* structural gene of the *Ars* operon.

In the R773 system, a model has been proposed in which the gene products of the *Ars* operon comprise an anion pump. In this model, the *ArsA* and *ArsB* proteins form a complex with arsenite pumping activity (3). The *ArsB* protein is an integral membrane protein (it is a hydrophobic protein) which acts as an anion channel. Since the *ArsA* protein can be crosslinked specifically with ATP, it has been proposed that it is the energy-translocating ATPase subunit with specific sites for ATP and arsenite. Binding of the *ArsC* protein to the complex may change the specificity to arsenate or increase the range of substrates to allow recognition of arsenate and arsenite (3).

In the present work, subcloning experiments which resulted in the construction of pJC2 did not separate arsenite resistance from arsenate resistance. To further localize the arsenic resistance gene(s)

of R45, digestion of the EcoRI-Sph I fragment with the exonuclease Bal31 might be carried out. This enzyme attaches to the free end of DNA and continuously degrades it nonspecifically.

Since pJC1 confers resistance to both arsenite and arsenate, shares homology with ArsB and ArsC, and yet does not contain the ArsA gene of R773; two possibilities exist. Perhaps the above model needs to be modified so that all that is required for arsenic resistance are the gene products from ArsB and ArsC. On the other hand, perhaps R45 and R773 represent two genetically distinct arsenic resistance mechanisms. Sequencing the R45 fragment would provide the clearest answer to this question.

In the meantime, support for the latter view is provided by the fact that the restriction sites in the 2.2 kb EcoRI-Sph I R45 fragment do not exist in the 4.3 kb Hind III R773 fragment. The R45 fragment is homologous to the R773 fragment, but is not identical to it. A similar observation concerns the fully-sequenced arsenical resistance determinant of a *Staphylococcus aureus* plasmid. This *S. aureus* plasmid has three potential open reading frames, with the, middle reading frame translation product exhibiting 55% homology with the ArsB protein of R773. The other two reading frames were not homologous with R773 at either the nucleotide or protein sequence level (3).

Studies on the energetics of the arsenic resistance in R773 have suggested that ATP is the driving force and that an electrochemical proton gradient is neither necessary nor sufficient for extrusion of arsenicals (12). Energy sources such as glucose which supplies ATP resulted in arsenate efflux, while carbon sources such as succinate which supplies a protonmotive force, but not ATP, did not result in efflux. When pJC1 bearing cells were plated on arsenite or arsenate media containing either glucose, succinate or maltose as the sole carbon source (and IPTG), only those cells plated on glucose containing media were able to grow. This suggests that R773 and R45 may share a similar mechanism for the extrusion of arsenicals. Further work would be necessary to determine whether the resistances to arsenite and arsenate are due to separate efflux pumps, or one pump, modified to accomodate both compounds.

In this study, a restriction map of the pEH plasmid containing the EcoRI-Hind III insert of R45 was constructed. This map provided information to further localize the arsenic resistance determinant of R45 to a 2.2 kb EcoRI-Sph I fragment. This fragment was compared to the arsenic resistance determinant of R773. Through DNA hybridization studies, R45 was shown to share extensive homology with the *ArsB* and *ArsC* genes of R773, but not to the *ArsA* gene of R773.

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