Portland State University

PDXScholar

Dissertations and Theses

Dissertations and Theses

1981

Plasmid-mediated resistance to arsenite and arsenate in *Escherichia coli*

Thomas Patrick Trezona
Portland State University

Follow this and additional works at: https://pdxscholar.library.pdx.edu/open_access_etds

Part of the [Bacteriology Commons](#), and the [Biology Commons](#)

Let us know how access to this document benefits you.

Recommended Citation

Trezona, Thomas Patrick, "Plasmid-mediated resistance to arsenite and arsenate in *Escherichia coli*" (1981). *Dissertations and Theses*. Paper 3131.
<https://doi.org/10.15760/etd.3125>

This Thesis is brought to you for free and open access. It has been accepted for inclusion in Dissertations and Theses by an authorized administrator of PDXScholar. Please contact us if we can make this document more accessible: pdxscholar@pdx.edu.

AN ABSTRACT OF THE THESIS OF Thomas Patrick Trezona for the Master of Science in Biology presented July 28, 1981.

Title: Plasmid-mediated Resistance to Arsenite and Arsenate in Escherichia coli.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

[REDACTED]
Dr. W. Herman Taylor, Chairman

[REDACTED]
Dr. Mary E. Taylor

[REDACTED]
Dr. John W. Myers

[REDACTED]
Dr. Stanley S. Hillman

[REDACTED]
Dr. Gordon L. Kilgour

ABSTRACT

Although arsenic compounds are prevalent in the environment, the most toxic forms are added intentionally in vast quantities as pesticides and herbicides, or liberated as by-products of industry. The quantity of the trivalent inorganic form (arsenite) introduced to the environment poses a serious health hazard because the toxicity of this form is explicable in terms of its ability to form stable bonds with cellular constituents. It is accumulated in tissues and it is known to be mutagenic. In the environment, arsenite is transformed into other arsenic compounds, mostly arsenate (the pentavalent inorganic form). Arsenate is also highly toxic, but less so than arsenite.

Bacterial strains resistant to arsenite, arsenate (or both) are readily isolated from nature. The resistance, in many cases, can be shown to be determined by conjugal plasmids. The mechanisms by which these plasmids confer resistance to arsenic compounds are entirely unknown. This study was undertaken to shed light on some of the physiological and genetic aspects of this resistance using plasmid-bearing strains of Escherichia coli which are highly resistant to both arsenite and arsenate. Most studies were carried out using E. coli J53 (R773) or the plasmid-free strain J53.

A minimal, defined medium was used in most aspects of this study. A system for separating and detecting arsenite, arsenate and phosphate, by thin-layer electrophoresis, was developed and used to show that

plasmid-mediated resistance to arsenite is not due to oxidation to arsenate. Nor is it due to excretion of a detoxifying thiol reagent, as shown by growth experiments. The tolerance of plasmid-free and plasmid-bearing strains of E. coli to arsenite and arsenate was examined by determining the minimal inhibitory concentration (MIC) of these compounds. Growth responses to arsenite and arsenate, as well as MIC determinations, indicate that a probable target site for arsenite toxicity lies within respiratory metabolism. Growth inhibition by arsenate toxicity is shown to be dependent on arsenate accumulation by phosphate uptake systems. Studies on the reversibility of arsenite and arsenate toxicity using growth experiments show that these compounds are bacteriostatic. Growth experiments were used to examine for plasmid-mediated protection of the phosphate uptake systems (Pst, Pit, GlpT and UhpT) from arsenate accumulation. The constitutive Pit system was shown to be functional in J53 (R773), and along with GlpT and UhpT, shown to be protected by R773. Growth experiments were used to study the induction and regulation of the arsenic resistance system in R773. Arsenite was shown to induce arsenite resistance alone, while arsenate was shown to induce both arsenate and arsenite resistance. Non-inhibiting levels of arsenite were shown to induce arsenite resistance to high levels within 15 minutes. The process was shown, by amino acid starvation experiments, to require protein synthesis. Finally, arsenic sensitive variants of R773 and R45 were isolated by transposon insertion mutagenesis. The phenotypes of the variants isolated indicate that the arsenite and arsenate resistance systems are genetically separate in these plasmids. A model

for the organization and regulation of the arsenic resistance genes of R773 is proposed and possible mechanisms of resistance are discussed in the Conclusions.

PLASMID-MEDIATED RESISTANCE TO ARSENITE AND
ARSENATE IN ESCHERICHIA COLI

by

THOMAS PATRICK TREZONA

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

MASTER OF SCIENCE
in
BIOLOGY

Portland State University

1981

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of Thomas
Patrick Trezona presented July 28, 1981.

[REDACTED]
Dr. W. Herman Taylor, Chairman

[REDACTED]
Dr. Mary L. Taylor

[REDACTED]
Dr. John W. Myers

[REDACTED]
Dr. Stanley S. Hillman

[REDACTED]
Dr. Gordon L. Kilgour

APPROVED:

[REDACTED]
W. Herman Taylor, Head, Department of Biology

[REDACTED]
Stanley E. Rauch, Dean of Graduate Studies and Research

This thesis work is
dedicated to my finest teachers:

DR. JAMES HERBERT TREZONA

and

MONICA ALICE TREZONA

for helping me develop the tools with which to pursue knowledge

and

for their support and encouragement while doing so.

ACKNOWLEDGMENTS

I would like to express my deep appreciation and thanks to Herman Taylor and Mary Taylor, whose support and assistance made my tenure at Portland State much more fruitful and enjoyable than it otherwise would have been. I am particularly grateful for the support they provided which enabled me to present this research at the Annual Meetings of the American Society for Microbiology in 1980 and 1981.

I would like to thank Jack Myers for his continual guidance, suggestions and constructive criticisms during the course of this research, as well as his assistance in the preparation of this thesis.

I would like to thank the other members of my thesis committee for their helpful suggestions during the preparation of this thesis as well.

I would also like to express my gratitude and thanks to Jack Rosoff for his assistance in the preparation of figures, and to Reitha Weeks for her assistance in the examination of membrane proteins.

TABLE OF CONTENTS

	PAGE
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
 INTRODUCTION	 1
MATERIALS AND METHODS	8
Bacterial Strains and Sources	8
Growth Media	11
Strain and Plasmid Verification	15
Strain Purification and Stocking	17
Cell Growth and Estimation of Concentration	18
Separation and Detection of Arsenite, Arsenate and Phosphate	21
Minimal Inhibitory Concentrations of Arsenite and Arsenate	22
Conjugation	24
Construction of Antibiotic and Arsenate Resistant Mutants of <u>E. coli</u>	26
Assay for Alkaline Phosphatase Activity	28
Methods of Obtaining Arsenic Sensitive Variants of R773 and R45 by Transposon Insertion Mutagenesis	30

	PAGE
RESULTS AND DISCUSSION	34
Examination for Arsenite Detoxification	34
Minimal Inhibitory Concentrations of Arsenite and Arsenate	46
Growth Responses to Arsenite and Arsenate	56
Reversibility of Arsenite and Arsenate Toxicity	75
Studies on the Induction and Regulation of Arsenite and Arsenate Resistance	79
Protection of Pit, GIpT and UhpT from Arsenate Uptake by R773	99
Studies on the Organization of the Arsenic Resistance Genes of R773 and R45	110
CONCLUSIONS	117
REFERENCES	122

LIST OF TABLES

TABLE		PAGE
I.	Abbreviations and Symbols.	10
II.	Resistance and Sensitivities of Plasmid-Free and Plasmid-Bearing Strains of <u>E. coli</u> to Various Antibiotics.	16
III.	Minimal Inhibitory Concentrations of Arsenite and Arsenate for Plasmid-Free and Plasmid-Bearing Strains of <u>E. coli</u>	49
IV.	Alkaline Phosphatase Activity in Wild-type and Arsenate Resistant Strains of <u>E. coli</u>	101
V.	Classes of Tn5 Insertion Mutants.	115

LIST OF FIGURES

FIGURE	PAGE
1. Estimation of Cell Concentration as a Function of Turbidity	19
2. Thin Layer Electropherogram of Arsenite, Arsenate and Phosphate Controls	36
3. Thin Layer Electropherogram of Growth Medium Supernates form Arsenite Oxidizing <u>Alcaligenes faecalis</u> YE56	38
4. Thin Layer Electropherogram of Growth Medium Supernates from Arsenite Grown <u>E. coli</u> J53 (R773) . . .	40
5. Examination for Arsenite Detoxification by <u>E. coli</u> J53 (R773)	43
6. Minimal Inhibitory Concentration of Arsenite for <u>E. coli</u> J53	47
7. Minimal Inhibitory Concentration of Arsenite for <u>E. coli</u> J53 (R773)	51
8. Minimal Inhibitory Concentration of Arsenate for <u>E. coli</u> J53	54
9. The Effect of Arsenite Concentration on Glucose Grown <u>E. coli</u> J53	57

FIGURE	PAGE
10. The Effect of Arsenite Concentration on Glycerol Grown <u>E. coli</u> J53.	59
11. The Effect of Arsenite Concentration on Glucose Grown <u>E. coli</u> J53 (R773)	62
12. The Effect of Arsenite Concentration on Glycerol Grown <u>E. coli</u> J53 (R773).	64
13. The Effect of Arsenate Concentration on Glucose Grown <u>E. coli</u> J53	66
14. The Effect of Arsenate Concentration on Glycerol Grown <u>E. coli</u> J53.	68
15. The Effect of Arsenate Concentration on Glucose Grown <u>E. coli</u> J53 (R773).	70
16. The Effect of Arsenate Concentration on Glycerol Grown <u>E. coli</u> J53 (R773).	72
17. The Reversibility of Arsenate and Arsenite Toxicity in Arsenic Sensitive <u>E. coli</u> J53.	76
18. The Effect of Arsenite on the Expression of Arsenite and Arsenate Resistance in Glycerol Grown <u>E. coli</u> J53 (R773).	80
19. The Effect of Arsenate on the Expression of Arsenate and Arsenite Resistance in Glycerol Grown <u>E. coli</u> J53 (R773).	82
20. The Effect of Arsenite on the Expression of Arsenite and Arsenate Resistance in Glucose Grown <u>E. coli</u> J53 (R773).	84

FIGURE	PAGE
21. The Effect of Arsenate on the Expression of Arsenate and Arsenite Resistance in Glucose Grown <u>E. coli</u> J53 (R773)	86
22. The Effect of Tetracycline Concentration on Glucose Grown <u>E. coli</u> J53 (R773).	90
23. The Effect of Arsenite on the Expression of Tetracycline Resistance in Glucose Grown <u>E. coli</u> J53 (R773).	92
24. Time Course for the Expression of Arsenite Resistance in Glucose Grown <u>E. coli</u> J53 (R773).	94
25. Amino Acid Requirement for Expression of Arsenite Resistance in <u>E. coli</u> J53 (R773).	97
26. The Effect of Arsenate on Induced GltP and UhpT Systems in <u>E. coli</u> J53 Spc ^r Asa ^r	103
27. The Effect of the Plasmid R773 on the Arsenate Sensitive GltP and UhpT Systems in <u>E. coli</u> J53 Spc ^r Asa ^r	107
28. Summary of Tn5 Insertion Mutagenesis of R773.	112
29. Model for the Organization and Regulation of the Arsenic Resistance Genes of R773.	118

INTRODUCTION

Arsenic exists in nature in many different biologically active forms. It is stable in four oxidation states (+5, +3, 0, -3) under the redox conditions normally found in aquatic systems (9). The most important of these are As^{+3} as arsenite and As^{+5} as arsenate. Arsenic metal and As^{-3} occur rarely.

Arsenic enters aquatic environments primarily by two mechanisms. It is leached from the earth's crust, where it exists in several forms, e.g., metal ores such as arsenopyrite (FeAsS), and it is introduced by human activities both unintentionally and intentionally.

Arsenic trioxide (As_2O_3) enters the environment in massive quantities, liberated as a by-product of the smelting of ores and the combustion of fossil fuels in power plants. In 1971, 50,000 tons of arsenic trioxide were intentionally produced by smelters around the world. The bulk of this was used in the United States as herbicides in fresh water lakes and streams, as soil sterilants, and as insecticides (9,26,30,33). In 1977, it was found that one power plant burning brown coal as the fuel liberated 1/2 ton of arsenic trioxide into the atmosphere daily (4).

When arsenic trioxide is dissolved in water, it is hydrolyzed to arsenous acid. At physiological pH this acid probably exists predominantly as HASO_2 ($\text{pK}_a = 9.2$), although hydrates of this also occur (32). Arsenous acid is customarily referred to as arsenite even though

relatively little of the anion AsO_2^- exists in solution.

Pentavalent arsenic enters the environment in several forms. It is used as an insecticide in the form of lead arsenate (PbHAsO_4) (9,21) and exists as a contaminant in presoaks and household detergents (1), and phosphate fertilizers as HAsO_4^{-2} . The latter compound also results from microbial action on both organic (e.g., cacodylic acid) (38) and inorganic (e.g., As_2O_3) (19,22) pesticides.

Arsenic compounds have been known through the ages both for their toxic and medicinal properties. Throughout the Middle Ages and until the nineteenth century arsenicals were the preferred poison for homicidal practitioners. Arsenicals were used in Oriental medicine 2,000-3,000 years ago. Therapeutic applications have continued ever since. In the nineteenth century, arsenicals were commonly used against trypanosomal and spirochetal infections. Even today, Fowler's solution (KAsO_2) continues to be used for the treatment of asthma in some regions (3).

The toxic properties of arsenicals are known mostly through the action of arsenite. Its toxicity is invariably explained (15) by its action as a sulfhydryl reagent (however, see Results). It is particularly active against vicinal dithiols such as those present in the lipoic acid moiety of α -ketoglutarate and pyruvate dehydrogenases. By blocking these oxidative enzymes arsenite causes the accumulation of α -ketoglutarate, pyruvate and other α -keto acids.

Arsenite may also react directly with the sulfhydryl groups of enzymes or their substrates by a number of possible mechanisms (32).

Arsenite accumulates in the tissues of humans and other organisms mostly because of its action as a sulfhydryl reagent. It can be found bound to the keratin of skin, nails and hair long after exposure (23). Poisoning is usually of a chronic rather than acute nature. Many routes of chronic poisoning are known, including drinking contaminated well water, long term inhalation of arsenic particulates and medicinal applications. Some authors are convinced arsenite is a potent carcinogen (10), while others cite numerous studies showing strong linkage between arsenite and cancer (30). Evidence has been obtained which suggests arsenite is mutagenic and may interfere with DNA repair mechanisms (10,24).

The toxicity of arsenate is roughly 60-fold less than arsenite (32). This difference is due primarily to the ability of arsenite to form more stable bonds with cellular constituents. Arsenate does not accumulate in tissues and does not react with sulfhydryl groups. It is rapidly excreted by the kidneys. Its toxicity is due to its

similarity to phosphate, both in size and geometry. It is thought to be able to substitute for phosphate in virtually all phosphorylation reactions (32). However, the resulting esters are very unstable.

Once a substrate which has been enzymatically esterified with arsenate dissociates from the enzyme, it spontaneously undergoes hydrolysis.

The overall process, which reforms arsenate and the original substrate (or product), is called arsenolysis.

There are two particularly important reactions which seem to be sensitive to uncoupling by arsenate. Both involve the synthesis of ATP from ADP and inorganic phosphate, namely, the reactions of substrate level and oxidative phosphorylations. The first involves the oxidation of 3-phosphoglyceraldehyde to 1,3-diphosphoglycerate. The inorganic phosphate incorporated in the reaction is subsequently transferred to ADP forming ATP and 3-phosphoglycerate. Arsenate may substitute for the phosphate forming 1-arseno-3-phosphoglycerate. This structure then undergoes spontaneous arsenolysis to reform arsenate and 3-phosphoglycerate.

The second important reaction involves the synthesis of ATP from ADP and inorganic phosphate by membrane-bound adenosine triphosphatase (ATPase), an enzyme complex energized by the proton motive force generated during electron transport. Again arsenate may substitute forming

γ -arseno-ADP, which then spontaneously undergoes arsenolysis to ADP and arsenate.

In both of these examples the energy consumed during the reactions forming the arseno compounds is lost. Hence, arsenate appears to act by a general mechanism which depletes a cell of metabolic energy.

In light of the mechanisms of toxicity and a few of the routes of environmental contamination just described, the need for the maximum safe limits for arsenic which have been set by the U.S. Public Health Service are obvious (9). That pollution of our natural waters is indeed a potentially serious problem was shown in 1971 when a study demonstrated that 21% of 727 fresh water lakes and streams examined were contaminated with arsenic at levels exceeding the standards set by the U.S. Public Health Service (9). These standards were exceeded far more frequently for arsenic than for any other element potentially hazardous to human health.

Bacterial strains resistant to arsenite and arsenate (or both) are readily isolated from nature and represent a broad range of species (12,18,22,29). The resistance, in many cases, can be shown to be determined by a plasmid. Often these plasmids can be transferred by conjugation among the Enterobacteriaceae (12,29).

The mechanisms of arsenate resistance in plasmid-free strains of *E. coli* have been extensively studied (5,35,37). These strains possess three uptake systems which are able to transport phosphate and which fail to effectively discriminate between phosphate and arsenate. These are the constitutive Pit system, which is the primary phosphate transport system, and two inducible systems, the glycerol phosphate

transport system (GlpT) and the hexose phosphate transport system (UhpT). The latter two systems also transport phosphate and arsenate when induced by growth on glycerol phosphate (or glycerol) or hexose phosphates (e.g., glucose-6-phosphate), respectively.

Chromosomal mutations which confer arsenate resistance (*pit*⁻) invariably result in the loss of the constitutive phosphate uptake system. These mutants are resistant to arsenate when grown using glucose as the carbon source because a fourth phosphate uptake system, the inducible Pst system, is used under these conditions. The Pst system is able to effectively discriminate between phosphate and arsenate. Thus *pit*⁻ mutants are resistant to arsenate when grown using glucose as the carbon source, but are sensitive to arsenate when grown using glycerol or glucose-6-phosphate as carbon sources (5,35). Chromosomal mutations which confer resistance to arsenate when grown using glycerol (*glpT*⁻) or glucose-6-phosphate (*uhpT*⁻) as the carbon source can also be isolated (5,35).

The mechanisms of arsenite resistance in plasmid-free strains of E. coli are less well characterized. Chromosomal mutations which confer arsenite resistance have been isolated (28) and seem to result in the alteration of the membrane-bound ATPase used for ATP synthesis during oxidative phosphorylation. These uncoupled (*unc*⁻) mutants lack a functional ATPase and as such require a fermentable carbon source (e.g., glucose) from which they obtain energy through substrate level phosphorylation. They are unable to grow on respiration dependent carbon sources such as succinate.

Although these findings suggest that the ATPase may either be a target site for arsenite toxicity or a site by which arsenite might gain entrance to the interior of cells, neither of these possibilities has as yet been verified.

The mechanisms of plasmid-mediated resistance to arsenite and arsenate are entirely unknown. This study was undertaken to shed light on these mechanisms using plasmid-bearing strains of E. coli which are highly resistant to both arsenite and arsenate. The original bacterial strains which possessed these plasmids were isolated from nature. Of particular importance in this investigation is the question of whether or not plasmid-bearing arsenic resistant strains of E. coli carry out some transformation of arsenite to a less toxic form. A method of separating and detecting arsenite, arsenate and phosphate was developed in order to determine this. Other methods of detecting arsenite detoxification were also used. Several methods were used to determine the tolerance and growth responses to arsenic. Studies on the reversibility of arsenite and arsenate toxicity were carried out. Each of the uptake systems known to transport phosphate and arsenate was examined to determine which were protected by the presence of the plasmid. Studies on the regulation of the arsenic genes were carried out using induction experiments under various conditions. Finally, in order to learn something about the organization of the arsenic resistance genes, plasmid mutants were isolated which had lost functional arsenic resistance genes.

MATERIALS AND METHODS

Bacterial Strains and Sources

Each of the strains of Escherichia coli used in the course of this study was a derivative of E. coli K-12 (2). Plasmid-free and plasmid-bearing strains of E. coli J53 were obtained from Dr. Ann Summers, University of Georgia, Department of Microbiology. Escherichia coli WA802 was obtained from the E. coli Genetic Stock Center, Yale University, School of Medicine, Department of Human Genetics. Alcaligenes faecalis YE56 was obtained from Dr. Mary Taylor, Portland State University, Department of Biology. Bacteriophage lambda (λ ::Tn5) was obtained from Dr. Douglas Berg, Washington University, School of Medicine, Department of Microbiology and Immunology. Those strains marked with an asterisk (*) were constructed in the course of this study.

STRAIN

RELEVANT PHENOTYPES^{a,b}

Escherichia coli

J53

Pro⁻Met⁻F⁻ λ ⁺

J53(R45)

(Asi^rAsa^rTet^rAmp^rSul^r)

J53(R773)

(Asi^rAsa^rTet^rStr^r)

WA802

Met⁻F⁻ λ ⁻

*J53Spc^r

Spc^r

c*J53Spc^rAsa^r

Spc^rAsa^r

c*J53Spc^rAsa^r(R773)

Spc^rAsa^r(Asi^rAsa^rTet^rStr^r)

*WA802 Rif ^r	Rif ^r
c*WA802 Rif ^r Asa ^r	Rif ^r Asa ^r
*J53Spc ^r (R773 A::Tn5)	Spc ^r (Asi ^r Asa ^s Kan ^r)
*J53Spc ^r (R773 B::Tn5)	Spc ^r (Asi ^s Asa ^r Kan ^r)
*J53Spc ^r (R773 C::Tn5)	Spc ^r (Asi ^s Asa ^s Kan ^r)
*J53Spc ^r (R45 A::Tn5)	Spc ^r (Asi ^r Asa ^s Kan ^r)
*J53Spc ^r (R45 B::Tn5)	Spc ^r (Asi ^s Asa ^r Kan ^r)
*J53Spc ^r (R45 C::Tn5)	Spc ^r (Asi ^s Asa ^s Kan ^r)

Alcaligenes faecalis

YE56 Asi^rAsa^r

Bacteriophage lambda λ b221 cI857 rex::Tn5 (Kan)

- a. Abbreviations and symbols used are defined in Table I.
- b. Phenotypes which are determined by plasmid genes are shown in parentheses. Those not shown in parentheses are chromosomally determined.
- c. The chromosomally determined spontaneous mutation to arsenate resistance was selected for on growth medium containing glucose as the carbon source. These strains, as well as wild type strains, show arsenate sensitivity when grown on other carbon sources. Genetic techniques were not utilized to map this mutation; however, it is most likely *pit*⁻. Strains with this mutation have been shown by Willsky (35) to possess an altered inorganic phosphate transport system (Pit). These strains are discussed in more detail in the results.

TABLE I.

Abbreviations and Symbols

MOPS		morpholinopropane sulfonic acid
Pro ⁻		requirement for L-proline
Met ⁻		requirement for L-methionine
Pit		inorganic phosphate transport system
Pst		phosphate specific transport system
GlpT		glycerol phosphate transport system
UhpT		uptake for hexose phosphates transport system
F ⁻		lacking the <u>E. coli</u> sex factor F
λ		bacteriophage lambda
Asi		arsenite
Asa		arsenate
::Tn5		carrying the transposable genetic element Tn5
Tet		tetracycline
Amp		ampicillin
Str		streptomycin
Sul		sulfanilamide
Spc		spectinomycin
Kan		kanamycin
Rif		rifampicin
r		resistant
s		sensitive

Growth Media

I. Coli-MOPS. The standard medium used for most studies is Neidhardt's minimal medium for Enterobacteria (17), with slight modification. The abbreviations employed for this medium are based on the different carbon sources utilized as follows: glucose (CM-Glu), glycerol (CM-Gly), acetate (CM-Ace), and glucose-6-phosphate (CM-G6P). The final concentrations and sources of chemicals are as follows:

A.	MOPS buffer	40.00mM	Sigma
B.	Tricine	4.00mM	Mallinckrodt - AR
	FeSO ₄ ·7H ₂ O	0.01mM	"
	(NH ₄) ₂ SO ₄	9.52mM	"
	K ₂ SO ₄	0.28mM	"
	MgSO ₄ ·7H ₂ O	0.52mM	"
	NaCl	50.00mM	"
C.	(NH ₄) ₆ (Mo ₇) ₂₄ ·4H ₂ O	3 X 10 ⁻⁶ mM	Baker & Adamson - RG
	H ₃ BO ₃	4 X 10 ⁻⁴ mM	Mallinckrodt - AR
	CoCl ₂ ·6H ₂ O	3 X 10 ⁻⁵ mM	"
	CuSO ₄ ·5H ₂ O	1 X 10 ⁻⁵ mM	"
	MnCl ₂ ·4H ₂ O	8 X 10 ⁻⁵ mM	Baker & Adamson - RG
	ZnSO ₄ ·7H ₂ O	1 X 10 ⁻⁵ mM	Mallinckrodt - AR
	CaCl ₂ ·2H ₂ O	5 X 10 ⁻³ mM	"
D.	One of the following:		
	glucose	0.5%	"
	glycerol	0.5%	"
	Na-acetate	1.0%	Baker Analyzed Reagent
	Na-glucose-6-phosphate	0.5%	Sigma
E.	L-proline	100μg/ml	"
	L-methionine	100μg/ml	"
	thiamine-HCl	2μg/ml	"

F.	K_2HPO_4	1.32mM	Mallinckrodt - AR
G.	$NaAsO_2$	(as indicated)	Mallinckrodt - AR
	$Na_2HAsO_4 \cdot 7H_2O$	"	"
	spectinomycin	"	Upjohn
	tetracycline	"	Sigma
	streptomycin	"	"
	kanomycin	"	"
	rifampicin	"	"
	sulfanilamide	"	"
	ampicillin	"	Bristol

Preparation. MOPS buffer, glucose-6-phosphate, antibiotics, and arsenic were weighed out immediately prior to the preparation of media. Components B, C, D, E, and F were prepared as 50, 10,000, 100, 100, and 100-fold concentrates respectively, using glass distilled water and stored at room temperature. The medium preparation was carried out by successive addition of the components, in order, to approximately 80% of the final volume of distilled water, titration to pH 7.4 with sodium hydroxide, followed by adjustment of the final volume. Arsenite and arsenate were always added before titration since these compounds cause significant alterations in pH. Antibiotics were usually added before titration, however, on occasion they were added to complete medium immediately before use without sterilization. Stock solutions and media were sterilized by filtration using a glass Millipore support with either Millipore or Sartorius 0.45 μ m cellulose acetate filters pre-washed with 250ml of hot distilled water. Prepared media was stored at 5°C and used no later than one month after preparation.

II. Overnight Coli-MOPS. The medium used for culturing cells overnight was prepared in the same fashion as standard CM except that the carbon source was limited to 0.05%. This medium will only support a limited amount of growth before the source of carbon is exhausted. The following morning these cells are subcultured into fresh CM medium. The advantage of using this technique is that the cells subcultured the following morning after carbon source limitation enter exponential growth phase rapidly.

Arsenic and antibiotics were added where indicated. The abbreviations used for this medium are CM-Glu (0.05%), CM-Gly (0.05%), CM-Ace (0.05%), or CM-G6P (0.05%).

III. Coli-MOPS Agar. Coli-MOPS agar was prepared by the addition of filter sterilized double strength CM to autoclave sterilized double strength agar (Difco Purified Agar - 2% final concentration). Arsenic and antibiotics were added where indicated.

IV. Coli-MOPS with Limiting Phosphate. This medium was prepared in the same fashion as standard CM-Glu except that the phosphate concentration was reduced to 5×10^{-5} M. It is abbreviated CM-Glu (lim.Pi).

V. Davis Minimal Broth. Davis minimal (DM) broth was prepared from Minimal Broth Davis (Difco). The salts component was autoclave sterilized. Glucose, amino acids (components D and E from CM medium), and antibiotics (as indicated) were added after sterilization.

VI. Davis Minimal Agar. Davis minimal (DM) agar was prepared by the addition of double strength DM broth to autoclave sterilized double strength agar (2% final concentration). Antibiotics were added as indicated.

VII. Nutrient Broth plus Yeast Extract. Each liter of NBYE contained 8g Bacto Nutrient Broth (Difco) and 3g Bacto Yeast Extract. This medium was sterilized by autoclaving.

VIII. H-Broth and H-Agar. Each liter of H-broth contained 10 g Bacto-tryptone (Difco) and 8g NaCl (Mallinckrodt - AR). Each liter of H-agar contained the same plus 2% agar (Bacto Agar-Difco). These media were sterilized by autoclaving.

IX. H-Top Agar. This medium was prepared in the same fashion as H-agar except that the agar concentration was reduced to 0.8%.

X. Soft Agar. Soft agar tubes were prepared by delivering 3ml of molten 0.8% agar to capped test tubes followed by autoclave sterilization.

Strain and Plasmid Verification

All strains received from other sources were tested for nutritional requirements by streaking for single colonies on minimal agar medium with and without the required supplements. Sensitivities to antibiotics, and plasmid determined resistances, were confirmed by streaking on minimal agar medium containing the required growth supplements and a single antibiotic at the concentrations shown in Table II. Growth was scored as heavy (+++), moderate (++), light (+), or no growth (-) relative to the growth of antibiotic free controls (+++) after 48 hours incubation at 37°C.

Methods used for verification of strains constructed in the course of this study are described elsewhere.

Table II.

Resistance and Sensitivities of Plasmid-Free and Plasmid-Bearing
Strains of E.coli to Various Antibiotics.

Antibiotic	J53	J53(R45)	J53(R773)
Control	+++	+++	+++
Tet ($\mu\text{g/ml}$)			
3	+	+++	+++
5	-	+++	+++
50	-	+++	+++
Sul (mg/ml)			
1	+	+++	+
5	-	+++	-
Str ($\mu\text{g/ml}$)			
2	-	-	+++
30	-	-	+++
60	-	-	+++
Spc ($\mu\text{g/ml}$)			
5	++	+	++
10	-	-	-
50	-	-	-
Amp ($\mu\text{g/ml}$)			
10	+	+++	
20	-	+++	-
60	-	+++	-
Kan ($\mu\text{g/ml}$)			
2	+	+	+
5	-	-	-
60	-	-	-

Strain Purification and Stocking

Each strain received or constructed in this study was purified by serial streaking for isolated colonies on minimal medium containing appropriate selective agents and growth requirements to achieve uniformity. Following purification a single colony was suspended in 50% glycerol in 0.1M phosphate buffer (pH 7.1) and stored in vials at -20°C . Cells used for daily work were maintained on minimal agar streak plates containing appropriate selective agents at 37°C . These plates were restreaked regularly from glycerol freezer stocks.

Cell Growth and Estimation of Concentration

Cell Growth. Cell growth in liquid media was carried out in volumes no greater than 10ml in 125ml side arm flasks. Aeration was achieved by shaking the flasks mounted on 30° Erl-angle supports at 200rpm in a New Brunswick Gyrotory water bath shaker (model G-77). Cell growth was carried out at 37°C except where otherwise indicated.

Cell growth was monitored turbidimetrically with a Klett-Summerson photoelectric colorimeter (model 800-3) using a #66 filter. Turbidity is recorded in Klett units.

Cell Concentration. Cell concentration was estimated by determining the number of viable cells which corresponds to a given number of Klett units. This calibration was carried out using E. coli J53 (R773) grown in CM-Glu. Aliquots from an exponential growth phase culture at 20, 50, 75, and 100 Klett units were diluted 10^{-6} . Soft agar inoculated with 0.1ml of the 10^{-6} dilution was used to overlay each of three CM-Glu agar plates. The plates were incubated at 37°C for 24 hours and the number of colonies was counted.

These bacteria seldom form chains or aggregates while growing, nor does the average size of the cells change appreciably during exponential growth phase. Because of these factors a plot of turbidity vs. number of viable cells per ml (Figure 1) shows that turbidity is proportional to the number of viable cells per ml over the range employed.

Figure 1. Estimation of Cell Concentration as a Function of Turbidity

E. coli J53 (R773) was grown in CM-Glu at 37⁰C. See Materials and Methods for methodology.

Separation and Detection of Arsenite, Arsenate, and Phosphate

Arsenite, arsenate and phosphate were separated by thin-layer electrophoresis. A slurry of silica gel G (0.6g/ml H_2O - type 60 from E.M. Laboratories, Inc.) was spread 0.3 mm thick on 20 cm X 20 cm glass plates and allowed to air dry overnight. Samples (8ul each) were spotted 5 cm from one edge. The plate was sprayed with electrophoresis buffer (0.1 M Na-acetate pH 8.1) and placed in a flat bed, tap water cooled, Desaga-Heidelberg electrophoresis apparatus. Buffer tanks were filled with 0.1 M Na-acetate. Electrophoresis wicks were prepared from Whatman filter paper # 1, washed several times with distilled water and soaked with buffer before bridging between the silica gel and the buffer tanks. Electrophoresis was carried out for 45 minutes at 500 Volts using a constant voltage mode power source. Following electrophoresis, the plates were quickly dried using a hot blow dryer.

After drying, the plates were exposed to iodine vapors in a closed chamber for 3 minutes. Arsenite reacts with the iodine to give a yellow-brown spot on a light yellow background. After removal, the plates were left in the air until both the brown spots and the background color disappeared. The plates were then sprayed with a 1% solution of ammonium molybdate, blown dry at room temperature, sprayed with a 1% solution of stannous chloride in 25% concentrated HCl, and finally blown dry at room temperature. Arsenite (oxidized to arsenate by I_2), arsenate, and phosphate appear as bright blue spots against a light blue background.

Minimal Inhibitory Concentrations of Arsenite and Arsenate

Plate method. Minimal inhibitory concentrations (MIC) of arsenite and arsenate were determined by spreading approximately 100-150 cells on the surface of 3 CM agar plates containing a given concentration of sodium arsenite or sodium arsenate, or on 2 CM agar plates without arsenic as a control. The colony forming units were counted and the fraction of survivors relative to the unexposed controls (N/No) was determined. The MIC is taken as the concentration of arsenic at which N/No equals 0.5.

Cells were grown overnight in CM (0.05% carbon source) harvested by centrifugation and resuspended in fresh CM at 20 Klett units. After exponential growth had resumed, at 60 Klett units, an aliquot was removed and diluted 10^{-6} in the same medium. A 0.18ml volume from the 10^{-6} dilution was delivered to the surface of all 5 plates before spreading with a sterile glass rod. The plates were incubated at 37°C and the colony forming units were counted no later than 4 days after inoculation. The various carbon sources and arsenic concentrations used are indicated in the results.

Liquid method. The minimal inhibitory concentration of arsenate for plasmid bearing cells grown in glucose or glycerol containing media is exceedingly high. The plate method for determining the MIC proved inappropriate for these conditions.

In this liquid method the cells were cultured in the same fashion as that described for the plate method. Approximately 10^6 cells were inoculated into a series of tubes containing 5ml of CM-Glu or CM-Gly

along with various concentrations of arsenate. The tubes were incubated with shaking at 37°C for 3 days. Growth was scored as heavy (+++), moderate (++) , light (+), or no growth (-) relative to the growth of unexposed controls (+++).

Conjugation

Liquid method. The method used was a modification of Miller (16). Single colonies of donor and recipient were removed from selective CM-Glu agar plates and grown overnight to saturation in NBYE. The following morning each was diluted 10 fold and allowed to enter exponential growth phase. Both were grown in 10ml volumes with vigorous aeration at 37°C. When exponential phase resumed each was diluted to a cell density of approximately 10^8 cells/ml. A 2ml volume of the donor was placed in a 100ml flask and incubated without shaking at 37°C. The recipient was maintained with shaking. Following 1 hour incubation, 2ml of the recipient was gently added to the donor and the mixture was allowed to remain at 37°C without shaking. Following a 2 hour mating period, selective plates were streaked and incubated at 37°C for 3 days. Exconjugates were screened for the appropriate plasmid determined phenotype, purified, and stocked as described.

Plate method. The method used was a modification of Bradley (8). Single colonies of donor and recipient were removed from selective CM-Glu agar plates and grown overnight to saturation in NBYE. The following morning each was diluted 10 fold and allowed to enter exponential growth phase. When the cell concentrations reached 4×10^9 /ml an equal volume of the donor and recipient cultures were mixed. A 0.3ml volume of this suspension was spread on the surface of a dry NBYE agar plate and incubated for 2 hours at 37°C. Following the mating period, the surface of the plate was washed 3 times with 1ml NBYE to collect the cells. The cells collected were then used to

inoculate selective media as indicated. Exconjugates were screened for the appropriate plasmid determined phenotype, purified and stocked as described.

Construction of Antibiotic and Arsenate Resistant Mutants of *E. coli*

Selection of J53 Spc^r. A culture of *E. coli* J53 was grown to late exponential phase (280 Klett units) in DM broth, harvested by centrifugation, and then resuspended to about 400 Klett units. One ml volumes of the concentrated suspension were plated on each of two DM agar plates containing 200 µg/ml Spc using soft agar overlays. The plates were incubated at 37°C for 72 hours. Typical large colonies were picked, purified, and stocked as previously described.

Selection of J53 Spc^rAsa^r. A culture of *E. coli* J53 Spc^r was grown to mid-exponential phase (100 Klett units) in CM-Glu. One and 0.1ml volumes of the culture were plated on CM-Glu agar plates containing 50mM sodium arsenate plus 50 µg/ml-Spc using soft agar overlays. Typical large colonies were picked and treated as described for J53 Spc^r.

Selection of WA802 Rif^r. A culture of *E. coli* WA802 was grown to mid-exponential phase (250 Klett units) in DM broth. One and 0.1ml volumes of the culture were plated on DM agar plates containing 100 µg/ml Rif using soft agar overlays. Further treatment was as described for J53 Spc^r.

Selection of WA802 Rif^rAsa^r. The method used was the same as that used for selection of J53Spc^rAsa^r except that 50 µg/ml Rif was included instead of Spc.

Construction of J53 Spc^rAsa^r (R773). *Escherichia coli* J53

(R773) and J53 Spc^rAsa^r were used as donor and recipient respectively. Conjugation was carried out according to the liquid method previously described. The mating mixture was streaked on CM-Glu agar plates which contained 10mM sodium arsenite to select for cells receiving the plasmid (exconjugates) and 50 µg/ml Spc to select against the donor. Several representative clones of the exconjugates were picked, purified, and screened for retention of the plasmid determined phenotypes before stocking as previously described.

Assay for Alkaline Phosphatase Activity

Cells for this assay were grown in standard CM-Glu, CM-G6P, or in CM-Glu with the phosphate concentration reduced to 5×10^{-5} M (CM-Glu (lim.Pi)). Standard CM medium contains a phosphate source in excess of that required for growth to reach stationary phase. CM-Glu (lim.Pi), on the other hand, will only support a limited amount of growth before the source of phosphate is exhausted and growth ceases.

Cells were grown overnight in CM-Glu (0.05%), CM-G6P (0.05%), or in CM-Glu (lim.Pi). The first two cultures were harvested by centrifugation and resuspended in fresh CM-Glu or CM-G6P at 40 Klett units. Exponential growth was allowed to resume and the cells were harvested at 75 Klett units. The third culture was harvested after overnight growth without subculturing. These cells had undergone several generations before growth ceased at 75 Klett units due to phosphate limitation.

Harvested cells were washed once with an equal volume of 0.5 M Tris-HCl, (pH 8.0) (Sigma) and resuspended in the same buffer at 40 Klett units (#66 filter). Six ml of the suspension was treated with 0.2ml of toluene, shaken for 5 minutes, and then allowed to stand for 15 minutes. The bottom 5ml of the aqueous phase was removed and mixed with 1ml of a solution containing 12 mg/ml of p-nitrophenylphosphate (PNPP) (Sigma). Alkaline phosphatase hydrolyzes PNPP to p-nitrophenol, a brightly yellow colored compound. The absorbance of the resulting p-nitrophenol was measured with a Klett-Summerson photoelectric

colorimeter using a #42 filter blanked with toluenized cells at the same concentration without PNPP. Absorbance measurements were taken at 10 and 20 minutes following addition of the cell suspension to the PNPP. All manipulations during the assay were performed at room temperature.

Methods of Obtaining Arsenic Sensitive Variants of R773 and R45 by Transposon Insertion Mutagenesis

Preparation of λ ::Tn5 Lysate. Phage lysates were prepared essentially by the method of Miller (16) for phage ϕ 80. A culture of *E. coli* WA802 was grown to exponential phase in H-broth, harvested by centrifugation, and resuspended in 10mM MgSO_4 at 60 Klett units (corresponding to a cell density of $5 \times 10^8/\text{ml}$). A 0.1ml volume of a phage lysate containing $1-2 \times 10^5$ total phage was mixed with 0.2ml of the cell suspension in a sterile test tube and incubated at 37°C for 10 minutes to allow adsorption. A 3ml volume of molten H-top agar at 45°C was added, the suspension was lightly mixed and then poured on the surface of H-agar plates. The plates were incubated at 42°C for 8-10 hours.

Following cell lysis the soft top agar was scraped from the surface of the plates with a glass loop, and then transferred to a centrifuge tube. Several drops of chloroform were added and the suspension was shaken vigorously for 30 seconds. The cellular debris was removed by centrifugation and the supernate was retained. A few drops of chloroform were added to the suspension, now termed the phage lysate, before storage at 5°C . Phage lysates prepared in this fashion contained approximately 7.5×10^{10} mature infective phage per ml.

Filtering the λ ::Tn5 Lysate. The method used was a modification of Miller (16). A culture of *E. coli* WA802 was grown overnight to saturation in H-broth, harvested by centrifugation, and resuspended in the same volume of 10mM MgSO_4 . Dilutions of 10^{-7} , 10^{-8} , and 10^{-9} of

the phage lysate were prepared in 10mM MgSO_4 . A 0.1ml volume of each phage dilution was mixed with 0.1ml of the cell suspension in a sterile test tube and incubated at 37°C for 10 minutes to allow adsorption. A 3ml volume of molten H-top agar at 45°C was added, the suspension was lightly mixed, and then poured on the surface of H-agar plates. Each phage dilution was plated in triplicate. The plates were incubated at 42°C overnight. The following morning the plaques were counted. The titer was determined by averaging the number of plaques on that set of plates containing from 20 to 200.

Plasmid Mutagenesis.

I. Phage infection. Infection of plasmid bearing E. coli was essentially by the methods of Berg (7). A culture of E. coli J53 (R773) was grown overnight to saturation in NBYE plus 0.25% maltose at 37°C, subcultured the following morning and allowed to enter exponential growth phase. The cells were harvested by centrifugation and resuspended at a cell density of $4 \times 10^8/\text{ml}$ in 10mM MgSO_4 . A 4ml volume of the cell suspension was infected with $\lambda::\text{Tn5}$ at a multiplicity-of-infection of 5 and allowed to incubate at 30°C for 30 minutes to allow adsorption.

Following adsorption, the cells were harvested by centrifugation, resuspended in 10ml NBYE without maltose, and incubated at 30°C for 30 minutes to allow for the expression of kanamycin resistance in those cells receiving Tn5 insertions. Kanamycin was then added to 20 $\mu\text{g}/\text{ml}$ and the culture was allowed to grow overnight at 30°C to saturation. The method used for the plasmid R45 was the same.

II. Selection of plasmids with Tn5 insertions. Following infection, the cells from the saturated overnight culture enriched for cells with Tn5 insertions was harvested by centrifugation, resuspended in 20ml NBYE (2-fold dilution) and allowed to enter exponential growth phase. When exponential phase had resumed the cells were harvested and concentrated to a cell density of about 4×10^9 /ml. These cells were then conjugated with E. coli J53 Spc^r using the plate method previously described.

The cells collected from the conjugation plates were mixed to disrupt mating aggregates, harvested by centrifugation and resuspended in 10ml NBYE containing 20 µg/ml Kan to select for those exconjugates receiving Tn5 insertions, and 100 µg/ml Spc to select against the donor. The culture was incubated at 37°C and allowed to grow overnight to saturation. The method used for the plasmid R45 was the same.

III. Isolation of arsenic sensitive variants. The saturated culture enriched for plasmids containing Tn5 insertions was used to streak CM-Glu agar plates containing Kan @ 20 µg/ml, Spc @ 100 µg/ml, Tet @ 10 µg/ml, and Str @ 10 µg/ml. When R45 was used for the mutagenesis the Str was replaced by Amp @ 20 µg/ml. The plates were incubated at 37°C for 48 hours.

Clones from these plates were picked, patched onto master plates containing the same medium, and then replica plated onto the same medium as a control, the same medium plus 10mM sodium arsenite, and the same medium plus 50mM sodium arsenate. Those clones showing arsenic sensitive phenotypes were retained, purified, and again examined for their phenotypes. Those clones which maintained their

arsenic sensitive phenotypes throughout the purification were retained and stocked as previously described.

RESULTS AND DISCUSSION

Examination for Arsenite Detoxification

There are several possible mechanisms by which bacteria could protect themselves from the highly toxic arsenite. Those known to occur include the oxidation of arsenite to arsenate by Alcaligenes (19,22), and the reductive methylation of arsenite by Methanobacterium (14). It has been suggested that the ability to oxidize arsenite might be determined by plasmids in Alcaligenes faecalis YE56 (31). This was hypothesized when plasmids possessed by YE56 were observed to undergo rearrangements following exposure of the cells to arsenite. Because of this observation in Alcaligenes I postulated that arsenite resistance in E. coli might be due to the synthesis of a plasmid determined enzyme system which could oxidize arsenite to arsenate.

In order to test this hypothesis, I developed a system for separating and detecting arsenite, arsenate, and phosphate. Until this investigation there have been no adequate means of separating these compounds. The difficulty stems from the similarity of phosphate and arsenate. In order to overcome this difficulty the three compounds were separated by thin-layer electrophoresis. The silica gel chosen for the preparation of electrophoresis plates (see Materials and Methods) contained CaSO_4 as the binding agent. Phosphate forms an insoluble precipitate with calcium and thus does not migrate in an electric field. Arsenate, which remains soluble, migrates towards

the anode. Arsenite, which remains uncharged as arsenous acid, migrates towards the cathode as a result of electroendosmosis.

A typical control plate showing the behavior of arsenite, arsenate, and phosphate using this system is shown in Figure 2 (lanes 1, 2, 3, and 4). In order to determine whether or not components of the growth medium interact with these compounds, controls of CM-Glu plus arsenite or arsenate were included (Figure 2, lanes 5 and 6). This method is sufficiently sensitive to detect spontaneous air oxidation of arsenite after incubation of growth medium for 3 days (Figure 2, lane 7). As a means of confirming that the system functioned as desired a positive control was included. The results from a typical positive control showing arsenite oxidizing activity in Alcaligenes faecalis YE56 is shown in Figure 3. This organism oxidizes arsenite throughout exponential growth phase so that little, if any, remains by the time the cells reach stationary phase.

In order to assess the ability of plasmid-bearing strains of E. coli to oxidize arsenite, several different growth conditions were employed. The growth medium used (CM) contained either catabolite repressing (glucose) or non-catabolite repressing (lactose or succinate) carbon sources. In addition, since the bacterial strains which originally possessed these plasmids were isolated from nature, the cells were grown at 30°C as well as 37°C. Several plasmid-bearing strains of E. coli were tested for arsenite oxidizing activity, including J53 (R773) and J53 (R45) as well as three arsenic resistant strains not included in the strain list; J53 (R46), J53 (R826-2), and J53 (R828).

Figure 4 shows the results of an experiment which was typical

Figure 2 . Thin Layer Electropherogram of Arsenite, Arsenate and Phosphate Controls

A reproduction of a typical control plate. See materials and methods for methodology.

Lane #

1. 10mM sodium arsenite
2. 10mM sodium arsenate
3. 10mM potassium phosphate
4. 10mM arsenite, arsenate, and phosphate
5. CM-Glu plus 10mM sodium arsenite
6. CM-Glu plus 10mM sodium arsenate
7. CM-Glu plus 10mM sodium arsenite following 3 days incubation at 37⁰ C.

1 2 3 4 5 6 7

Figure 3. Thin Layer Electropherogram of Growth Medium Supernates from Arsenite Oxidizing Alcaligenes faecalis YE56

A reproduction of a typical control electropherogram showing arsenite oxidizing activity in Alcaligenes faecalis YE56. The cells were grown in CM-Glu at 30°C. See Materials and Methods for methodology.

Lane #

1. 10mM arsenite, arsenate, and phosphate
2. CM-Glu plus 10mM arsenite - mid exponential phase control
3. CM-Glu plus 10mM arsenite - stationary phase control
4. CM-Glu plus 10mM arsenite - 3 day control
5. YE56 in CM-Glu plus 10mM arsenite - mid exponential phase
6. YE56 in CM-Glu plus 10mM arsenite - stationary phase
7. YE56 in CM-Glu plus 10mM arsenite - 3 days

1 2 3 4 5 6 7

Figure 4. Thin Layer Electropherogram of Growth Medium Supernates
from Arsenite Grown E. coli J53 (R773)

A reproduction of a typical electropherogram from an examination for arsenite oxidizing activity in E. coli J53 (R773). The cells were grown in CM-Glu at 30°C. See Materials and Methods for methodology.

Lane #

1. 10mM arsenite, arsenate, and phosphate
2. CM-Glu plus 10mM arsenite - mid exponential phase control
3. CM-Glu plus 10mM arsenite - stationary phase control
4. CM-Glu plus 10mM arsenite - 3 day control
5. J53 (R773) in CM-Glu plus 10mM arsenite - mid exponential phase
6. J53 (R773) in CM-Glu plus 10mM arsenite - stationary phase
7. J53 (R773) in CM-Glu plus 10mM arsenite - 3 days

1 2 3 4 5 6 7

of all conditions employed. In no case was plasmid mediated oxidation of arsenite to arsenate detected. Although the results indicated that arsenite had not been transformed by J53 (R773), it should be pointed out that the system is not sufficiently sensitive to detect the loss of small amounts of arsenite through reduction to arsine gas or methylated arsines. This possibility is, however, unlikely.

A second hypothesis concerning resistance to arsenite is that the cells might synthesize a plasmid determined system, or modify a chromosomally determined system, which could affect excretion of a thiol reagent that would form stable bonds with arsenite (e.g., lipoic acid as discussed in the introduction), hence eliminating its toxic properties. Dithiol compounds such as 2,3-dimercaptopropanol (British anti-lewisite) have long been known to reverse the toxic effects of arsenite poisoning in humans (3).

Although no alteration of arsenite was detected in the experiments discussed above, this thiol excretion hypothesis was tested directly. In this experiment, exponential growth phase J53 (R773) was suspended in medium containing 10mM arsenite. The cells underwent an 6-8 hour lag phase before entering exponential growth phase. It was hypothesized that, during the lag phase, the cells "conditioned" the medium by the excretion of a thiol, following which growth could occur. At the end of the lag phase, the cells were removed from the medium and replaced with exponential phase cells which had not previously been exposed to arsenite.

The results of this experiment (Figure 5) indicate that the "conditioned" growth medium retained the ability to inhibit the growth of sensitive cells.

Figure 5. Examination for Arsenite Detoxification by E. coli
J53 (R773)

- A. Cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium (○), or in medium containing 10mM sodium arsenite (□). Following 8 hours exposure to the arsenite, at the end of the lag phase (indicated by the arrow), the culture was harvested by centrifugation and the culture supernate was filter sterilized.
- B. Aliquots from a second exponential phase CM-Glu culture of J53 (R773) were removed, the cells were harvested by centrifugation, and resuspended in either the same medium (○) or in the supernate from the first arsenite exposed culture (△).

These results suggest that plasmid determined arsenite resistance in E. coli is not due to the transformation of arsenite to a less toxic form of arsenic, but rather it is due to the modification or protection of sensitive target sites within the cells.

Minimal Inhibitory Concentrations of Arsenite and Arsenate

The tolerance of plasmid-free arsenic sensitive and plasmid-bearing arsenic resistant strains of E. coli to arsenite and arsenate were determined quantitatively by measuring the minimal inhibitory concentrations (MIC) of these compounds. The MIC is defined as that concentration of arsenic which reduces the survival of colony forming units to 50% of unexposed controls. The method used is particularly rigorous for determining the tolerance of cells to inhibitory agents, because it measures the ability to grow and divide without the protective influence of surrounding cells. The usual disk diffusion or liquid culture methods invariably result in higher values for MIC determinations.

Arsenite MIC. In order to gain insight as to possible target sites for arsenite toxicity the MIC was determined using different carbon sources, both fermentable (glucose) and non-fermentable (glycerol and acetate). In addition, in order to determine whether or not sensitive or resistant cells were able to become adapted or induced to tolerate higher concentrations of arsenite, the MIC determinations were carried out with and without prior growth in sub-inhibitory levels of arsenite.

The MIC for J53 (Figure 6 and Table III) varied considerably depending on whether the carbon source being utilized was fermentable or strictly respiratory. The MIC for glucose grown cells was 3 fold greater than for acetate grown cells, and nearly 9 fold greater than for glycerol grown cells. Also, there was essentially no difference

Figure 6. Minimal Inhibitory Concentration of Arsenite for
E. coli J53

The cells used for MIC determinations were pregrown^a in CM-Glu (○), CM-Glu plus 1mM sodium arsenite (●), CM-Gly (△) , CM-Gly plus 0.5mM sodium arsenite (▲), CM-Ace (□), or CM-Ace plus 0.1mM sodium arsenite (■), and then plated on the same medium containing the concentrations of sodium arsenite indicate.

^a Cells used for pregrowth in medium without arsenic were streaked repeatedly on plate medium without arsenic before MIC determination.

J53
MIC – mM Asi

TABLE III.

Minimal Inhibitory Concentrations of Arsenite and Arsenate for Plasmid-free and Plasmid-bearing Strains of E. coli

Strain	Growth Medium	Asi MIC w/o and w/pregrowth in Asi	Asa MIC w/o and w/pregrowth in Asa
J53	CM-Glu	3.5	3.6
			12.4
			--
J53	CM-Gly	0.4	0.4
			1.4
			1.4
J53	CM-Ace	1.2	1.2
			--
			--
J53 (R773)	CM-Glu	13.8	18.7
			>300
			--
J53 (R773)	CM-Gly	--	--
			~220
			--
J53 (R773)	CM-Ace	13.8	16.0
			--
			--
J53 (R45)	CM-Glu	15.0	18.8
			>300
			--

in the MIC whether the cells were pregrown in subinhibitory levels of arsenite or not.

These results suggest two important things. First, J53 is much more sensitive to arsenite when grown utilizing carbon sources that require oxidative phosphorylation as a means of obtaining energy, as opposed to carbon sources which allow the cells to ferment. It would seem therefore, that a component of respiratory metabolism would be a likely target site for arsenite toxicity. This is consistent with what is reported in the literature (32) as well as the data obtained by Smiley (28) suggesting that the ATPase is a target site for arsenite toxicity. Second, pregrowth of the cells in subinhibitory levels of arsenite does not enable the cells, by an adaptive or inductive process, to tolerate elevated concentrations.

These results are in sharp contrast to those obtained following the introduction of the plasmid R773 into sensitive cells. The tolerance of J53 (R773) to arsenite was not affected appreciably by the carbon source being utilized (Figure 7 and Table III). In addition, pregrowth in subinhibitory concentrations of arsenite did enable these cells to tolerate elevated levels of arsenite, an indication that the arsenite resistance system is inducible.

The presence of the plasmid R773 provides the cells with a significant increase in resistance to arsenite relative to the plasmid-free strain, roughly 5 fold for glucose grown cells and over 10 fold for acetate grown cells.

Figure 7. Minimal Inhibitory Concentration of Arsenite for E. coli
J53 (R773)

The cells used for MIC determinations were pregrown^a in CM-Glu (●), CM-Glu plus 5mM sodium arsenite (○), CM-Ace (▲), or CM-Ace plus 5mM sodium arsenite (△), and then plated on the same medium containing the concentrations of sodium arsenite indicated.

^a See footnote to Figure 6.

J53(R773)
MIC – mM Asi

Arsenate MIC. The MIC for arsenate was determined in a manner similar to that for arsenite, except that only glucose and glycerol were used as carbon sources. The arsenate MIC for glucose grown J53, like that for the arsenite MIC, was roughly 9 fold greater than for glycerol grown cells (Figure 8 and Table III). In addition, pregrowth in subinhibitory levels of arsenate, like that for the arsenite MIC, did not enable the cells to tolerate elevated concentrations.

The reason for the 9 fold difference between the arsenate MIC for glucose and glycerol grown cells is probably not the same as that for the arsenite MIC. With arsenate, the difference is probably due to the induction, by glycerol, of an additional transport system capable of actively accumulating arsenate (see Introduction).

The tolerance of J53 (R773) to arsenate is extraordinary. The methods used for the MIC results just described proved inadequate for measuring the arsenate MIC for this strain. In this case the MIC was obtained from growth in liquid culture, and as such, is only a rough approximation of that obtainable by the plate method. Cells from a culture of J53 (R773) grew without significant inhibition when transferred to glucose medium containing 300mM arsenate. Those cells transferred to glycerol medium were moderately inhibited by 200mM arsenate and completely inhibited by 240mM arsenate (Table III).

These results indicate the presence of a plasmid determined system which is highly effective in preventing arsenate from penetrating to the cell's interior.

Figure 8. Minimal Inhibitory Concentration of Arsenate for E. coli
J53

The cells used for MIC determinations were pregrown^a in CM-Gly (●), CM-Gly plus 0.1mM sodium arsenate (○), or CM-Glu (■), and then plated on the same medium containing the concentrations of sodium arsenate indicated.

^a See footnote to Figure 6.

J53
MIC – mM Asa

Growth Responses to Arsenite and Arsenate

These experiments were carried out in order to determine the behavior of exponential phase *E. coli* J53 and J53 (R773) when challenged with various concentrations of arsenic.

Arsenite. The response of J53 to arsenite when grown using glucose or glycerol as the carbon source is shown in Figures 9 and 10. In arsenite concentrations less than 2mM, glucose grown cells (Figure 9) experience a lag phase followed by exponential growth. Under these conditions the cells appear to recover after the lag period as if they turn on a second, less sensitive energy producing system. That this is due to the availability of a fermentable carbon source is not clear. The results are in contrast to the response of glycerol grown cells to arsenite (Figure 10). Under these conditions, the cells simply experience a reduced growth rate, regardless of the arsenite concentration. The cells do not experience a lag phase. The inhibition by arsenite is roughly linear with respect to concentration, i.e., doubling the arsenite concentration appears to double the generation time. These findings are somewhat similar to those obtained in the MIC studies. Again they suggest the most sensitive target site for arsenite toxicity is a system involved in respiratory metabolism rather than fermentation. They also suggest that when cells are grown using a fermentable carbon source, it is necessary that two systems be inhibited before growth ceases, namely, respiration and fermentation.

Figure 9 . The Effect of Arsenite Concentration on Glucose Grown
E. coli J53

Cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenite indicated.

Figure 10 . The Effect of Arsenite Concentration on Glycerol Grown
E. coli J53

Cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenite indicated.

The response of J53 (R773) to arsenite when grown using glucose or glycerol as the carbon source is shown in Figures 11 and 12. In both cases, although the growth rates, the final yields and the tolerance of arsenite are greater for glucose grown cells, the general response to arsenite appears similar. The cells experience a lag phase followed by exponential growth. This type of response to an inhibitory agent is often taken to mean that the resistance system is inducible, i.e., during the lag phase the cells channel available metabolic energy into the synthesis of components of the resistance system. Following sufficient synthesis of these components, the cells are able to grow exponentially.

Arsenate. The response of J53 to arsenate when grown using glucose or glycerol as the carbon source is shown in Figures 13 and 14. In both cases the cells behave in a manner similar to glucose grown J53 in arsenite (Figure 9). The cells experience a lag phase followed by exponential growth. The reason for this behavior is not clear. In light of the mechanism of arsenate toxicity (see Introduction), the reason is not likely to be the same as that responsible for the behavior following exposure to arsenite. Again the cells show a greatly increased sensitivity to arsenate when grown using glycerol as the carbon source.

The response of J53 (R773) to arsenate when grown using glucose or glycerol as the carbon source is shown in Figures 15 and 16. In contrast to the behavior of glucose grown J53 (R773) to arsenite, exposure of glucose grown cells to arsenate has little inhibitory

Figure 11 . The Effect of Arsenite Concentration on Glucose Grown
E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenite indicated.

Figure 12. The Effect of Arsenite Concentration on Glycerol Grown
E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenite indicated.

Figure 13. The Effect of Arsenate Concentrations on Glucose Grown
E. coli J53

Cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenate indicated.

Figure 14. The Effect of Arsenate Concentration on Glycerol Grown
E. coli J53

Cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation, and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation, and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenate indicated.

Figure 15. The Effect of Arsenate Concentration on Glucose Grown
E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenate indicated.

Figure 16. The Effect of Arsenate Concentration on Glycerol Grown
E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in the same medium containing the concentrations of sodium arsenate indicated.

effect (Figure 15). No lag phase is observed in concentrations up to 100mM. In fact, as will be shown in later experiments, only a 30 minute lag is observed when these cells are exposed to 200mM arsenate.

The response of glycerol grown J53 (R773) to arsenate is quite different (Figure 16). Under these conditions, the cells experience a lag of roughly 4 hours when exposed to as little as 5mM arsenate. It is important to point out, however, that these cells recover from the lag phase in arsenate concentrations up to 220mM.

These results demonstrate that this plasmid determines a resistance system which is highly effective in protecting the cells from the toxic effects of arsenate. The system is functional with a high degree of efficiency in glycerol grown cells only after an apparent inductive process has occurred. Glucose grown cells, however, are able to grow exponentially in very high concentrations of arsenate without this inductive effect having occurred.

Reversibility of Arsenite and Arsenate Toxicity

In order to test the mechanisms reported in the literature for arsenite and arsenate toxicity, I decided to determine if these compounds were bacteriostatic or bacteriocidal. Because arsenite is reported to accumulate in tissues by forming stable bonds with cellular constituents, one would expect it to be highly bacteriocidal in concentrations that completely inhibited growth. Arsenate, on the other hand, might be expected to be bacteriostatic because it does not form stable bonds with cellular constituents (32), and has been shown to be in dynamic equilibrium with extracellular phosphate (5).

In the experiment shown in Figure 17, arsenic sensitive E. coli J53 was grown using glycerol as the carbon source. This carbon source was chosen because the cells experience greater sensitivity to both arsenite and arsenate than when grown using any other carbon source tested. The cells were challenged with arsenite and arsenate at concentrations roughly 20 fold higher than the MIC. Following exposure to the arsenic for various times the cells were tested for viability using two methods. First, at 2, 4, 6 and 24 hour intervals cells were removed from the culture and plated on the same medium without arsenic. The viable cell density in the culture was then determined for each of the times. Second, at 1 and 24 hour intervals, cells were removed from the culture, washed, and then the time required for the culture to resume growth in fresh medium without arsenic was determined.

As shown in Figure 17B, the viability of individual cells following exposure to these extreme concentrations of arsenite and arsenate for as long as 24 hours, is not affected. The toxicity of

Figure 17. The Reversibility of Arsenate and Arsenite Toxicity in
Arsenic Sensitive E. coli J53

A) Cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same medium (▲), in medium containing 25mM sodium arsenate (○), or in medium containing 7.5mM sodium arsenite (□).

(Hours on ordinate are 0 - 24)

B) An aliquot from the unexposed culture in "A" at time = 0 was removed and the cells were spread on 2 CM-Gly agar plates from a 10^{-7} dilution. The colony forming units were counted and the viable cell density in the unexposed culture at time = 0 was taken as the control. Aliquots from the arsenite (□), and arsenate (○) exposed cultures were removed at 0, 2, 4, 6 and 24 hours, and the cells were spread on CM-Gly agar plates from 10^{-3} , 10^{-5} and 10^{-6} dilutions. The colony forming units were counted and the viable cell density is plotted as a per cent of the unexposed control.

(Hours on ordinate are 0 - 24)

C) Aliquots from the arsenic exposed cultures were removed at 1 (arsenite (□), and arsenate (○)) and 24 (arsenite (◇), and arsenate (△)), hours, the cells were harvested by centrifugation, and washed once in an equal volume before resuspending in fresh CM-Gly.

(Hours on ordinate 0 - 9)

both arsenite and arsenate is readily reversible. In fact, Figure 17C shows that after 1 hours exposure, both arsenite and arsenate may easily be washed from the cells, and that there is still sufficient energy reserves to enable the cells to immediately enter exponential growth phase. After 24 hours, the cells appear to behave as though their energy reserves have been substantially depleted; however, they are still able to recover and enter exponential growth phase in about 6 hours.

It is quite suprising that the behavior of the cells following removal from the toxic conditions (Figure 17C) is the same, whether exposed to arsenite or arsenate. This indicates that, although the specific target sites for toxicity may be different, the end result is probably the same; a generalized depletion of cellular energy. If arsenite does react with cellular constituents, forming covalent bonds with sulfhydryl groups as reported in the literature (15,32), they must be, in contrast to the literature, very unstable. They must easily undergo hydrolysis and the resultant arsenite should be rapidly washed from the cells.

Studies on the Induction and Regulation of Arsenite and Arsenate Resistance

In order to study the inductive effect of arsenite and arsenate on both arsenite and arsenate resistance in *E. coli* J53 (R773), cells not previously exposed to arsenic were exposed to arsenite for a period, removed from the arsenite containing medium, and then exposed to fresh arsenite or arsenate containing medium. The same experiment was also carried out using cells first exposed to arsenate. Both glucose and glycerol were used as carbon sources.

Figure 18 shows the inductive effect of arsenite on the expression of arsenite and arsenate resistance in glycerol grown cells. Exposure of J53 (R773) to 4mM arsenite or arsenate causes the cells to experience a 3 to 4 hour lag phase. This lag period is eliminated upon subculturing of the arsenite exposed cells into fresh arsenite containing medium but not arsenate containing medium. This indicates that arsenite acts as an inducer of arsenite resistance but not of arsenate resistance.

These results are in contrast to those obtained when arsenate exposed glycerol grown J53 (R773) is subcultured into fresh arsenate and arsenite containing medium (Figure 19). Under these conditions, the lag phase is eliminated for both arsenate and arsenite. These results indicate that arsenate acts as an inducer of both arsenate and arsenite resistance.

The inductive effect of pregrowth in arsenite or arsenate can also be shown for glucose grown cells (Figure 20 and 21), although this is somewhat more difficult for arsenate due to the extreme resistance. Figure 20 shows that pregrowth in arsenite induces the cells for growth

Figure 18. The Effect of Arsenite on the Expression of Arsenite and Arsenate Resistance in Glycerol Grown E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same medium (◇), in medium containing 4mM sodium arsenite (◆), or in medium containing 4mM sodium arsenate (△). Following 6½ hours, aliquots from the arsenite exposed culture were removed, the cells were harvested by centrifugation and resuspended in either the same medium (◆), or in medium containing 4mM sodium arsenate (△).

Figure 19. The Effect of Arsenate on the Expression of Arsenate and Arsenite Resistance in Glycerol Grown E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Gly (0.05%), harvested by centrifugation and resuspended in fresh CM-Gly at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same medium ($\diamond$), in medium containing 4mM sodium arsenate ($\blacklozenge$), or in medium containing 4mM sodium arsenite ($\triangle$). Following 6½ hours, aliquots from the arsenate exposed culture were removed, the cells were harvested by centrifugation and resuspended in either the same medium ($\blacklozenge$) or in medium containing 4mM sodium arsenite ($\triangle$).

Figure 20. The Effect of Arsenite on the Expression of Arsenite and Arsenate Resistance in Glucose Grown E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same medium ($\triangle$), in medium containing 5mM sodium arsenite ($\blacktriangle$), or in medium containing 200mM sodium arsenate ($\blacksquare$). Following $3\frac{1}{2}$ hours, aliquots from the arsenite exposed culture were removed, the cells were harvested by centrifugation and resuspended in medium containing either 10mM sodium arsenite ($\square$), or 200mM sodium arsenate ($\blacksquare$).

Figure 21. The Effect of Arsenate on the Expression of Arsenate and Arsenite Resistance in Glucose Grown E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same medium ($\triangle$), in medium containing 10mM sodium arsenate ($\blacktriangle$), or in medium containing 6mM sodium arsenite ($\square$). Following $3\frac{1}{2}$ hours, aliquots from the arsenate exposed culture were removed; the cells were harvested by centrifugation and resuspended in either the same medium ($\blacktriangle$), in medium containing 200mM sodium arsenate ($\diamond$), or in medium containing 10mM sodium arsenite ($\blacksquare$).

in arsenite, even at higher levels, but not for growth in arsenate. Figure 21 shows that pregrowth in arsenate induces the cells for growth in arsenate, again at higher levels, and for growth in arsenite as well.

The results of these induction experiments suggest that the mechanisms of regulation and resistance to arsenite and arsenate are different.

A possible mechanism by which induction of arsenic resistance could occur is by an increase in gene copy number through plasmid amplification. This is known to occur during the induction of chloramphenicol resistance in the Proteus plasmid NR1 (20). In this system, following challenge with inhibiting concentrations of chloramphenicol, that portion of the plasmid which carries the resistance determinants excises from the remainder of the plasmid (the resistance transfer factor), replicates numerous times, and then reintegrates forming several, larger plasmids. The process, called "transitioning", may increase the gene copy number to as many as 50 per cell.

I hypothesized that this process might occur in the arsenic resistance plasmid R773. In order to detect transitioning, I chose to examine the induction of tetracycline resistance, also determined by the plasmid R773, following exposure of the cells to arsenite. I hypothesized that if the plasmid resistance determinants increased in copy number, the lag phase, known to occur following exposure to tetracycline, would be reduced. A similar phenomenon can be demonstrated for streptomycin following exposure of the Proteus plasmid NR1 to chloramphenicol (11).

Figure 22 shows the lag phases experienced by J53 (R773) upon exposure to various concentrations of tetracycline. The behavior of the cells during induction of resistance to 50 $\mu\text{g/ml}$ tetracycline following the complete induction of the arsenite resistance system is shown in Figure 23. The results indicate that induction of arsenite resistance has no effect on the induction of tetracycline resistance, and thus probably does not involve an increase in gene copy number.

While studying the effects of arsenite and arsenate on the induction of these resistance systems, I observed that exposure of J53 (R773) to a non-inhibiting inducing stimulus (1mM arsenite) would eliminate the lag phase when subcultured in medium containing 10mM arsenite. Because of this, I decided to examine the kinetics of the induction process.

In this experiment, uninduced cells were exposed to 1mM arsenite for various times followed by subculturing into medium containing 10mM arsenite. It was assumed that in order to grow exponentially in medium containing 10mM arsenite, induction of the resistance system must be complete, or at least near completion. As shown in Figure 24, exposure of J53 (R773) to 10mM arsenite without prior exposure to arsenite causes the cells to experience a lag phase of 6 to 8 hours. However, exposure of the cells to 1mM arsenite for 10 minutes shortened the lag phase to 3 hours, and exposure for 15 minutes eliminated the lag phase when subcultured into medium containing 10mM arsenite.

The time period required for induction of arsenite resistance seemed somewhat short for the process to involve the synthesis of new proteins. A different mechanism which might account for this rapid inductive process would be some sort of conformational changes or modifications of pre-existing proteins.

Figure 22. The Effect of Tetracycline Concentration on Glucose Grown
E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in the same medium containing the concentrations of tetracycline indicated.

Figure 23. The Effect of Arsenite on the Expression of Tetracycline Resistance in Glucose Grown E. coli J53 (R773)

Cells were grown overnight in CM-Glu (0.05%) plus either 10mM sodium arsenite or 50 μ g/ml tetracycline. The cells were harvested by centrifugation and resuspended in similar media at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots from both cultures were removed, and the cells were harvested by centrifugation. The arsenite induced cells were resuspended at 20 Klett units in either the same medium ($\circ$), or in medium containing 50 μ g/ml tetracycline ($\bullet$). The tetracycline induced cells were resuspended at 20 Klett units in either the same medium ($\square$), or in medium containing 10mM sodium arsenite ($\blacksquare$).

Figure 24. Time Course for the Expression of Arsenite Resistance in
Glucose Grown E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, the culture was split into two parts. One part was made 1mM sodium arsenite at 20 Klett units (□), while the other was made 10mM sodium arsenite at 20 Klett units (▲). Following exposure to the non-inhibiting inducing stimulus (1mM arsenite) for the times indicated, aliquots were removed and resuspended in 10mM sodium arsenite.

In order to determine whether or not protein synthesis was required for the induction of arsenite resistance, advantage was taken of the fact that J53 (R773) is auxotrophic for proline and methionine. In this experiment (Figure 25), exponential phase cells were exposed to a non-inhibiting inducing stimulus (1mM arsenite) both in the presence and the absence of the required amino acids for 1/2 hour. Figure 24 shows that the cells are completely induced for resistance to 10mM arsenite by this time. Following exposure to the inducing stimulus, the cells are challenged with 10mM arsenite. As shown in Figure 25, starvation of the required amino acids prevents the induction of the arsenite resistance system. These results indicate that amino acids, and presumably protein synthesis, are required for the induction of arsenite resistance.

Figure 25. Amino Acid Requirement for Expression of Arsenite Resistance in E. coli J53 (R773)

Uninduced cells were grown overnight in CM-Glu (0.05%), harvested by centrifugation and resuspended in fresh CM-Glu at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended in either the same complete medium plus 10mM sodium arsenite (◆), or in medium lacking the required amino acids. After 15 minutes of amino acid starvation, the latter culture was split into two parts and the cells were harvested by centrifugation. The first part was resuspended in complete medium plus 1mM sodium arsenite (▲). The second part was resuspended in medium containing 1mM sodium arsenite, but lacking the required amino acids (△). Following 30 minutes incubation, at the times indicated by arrow #'s 1 and 2 respectively, the cells from both cultures in the presence of 1mM sodium arsenite were harvested by centrifugation and resuspended in complete medium plus 10mM sodium arsenite.

Protection of Pit, GlpT and UhpT from Arsenate Uptake by R773

As discussed in the Introduction there are four active uptake systems (Pst, Pit, GlpT and UhpT) which transport phosphate in E. coli. Three of these systems (Pit, GlpT and UhpT) fail to discriminate between phosphate and its toxic analog, arsenate. One of these systems (Pit) operates constitutively and is the primary phosphate uptake system. The Pst system, which effectively discriminates between phosphate and arsenate, is induced by limiting phosphate conditions. The GlpT and UhpT systems are inducible by growth utilizing glycerol and glucose - 6 - phosphate as carbon sources, respectively.

This study represents a series of experiments designed to answer three questions with respect to arsenate resistance in J53 (R773). The first question is whether it is the Pit or the Pst system which is in operation in glucose grown cells during exposure to arsenate. This experiment was undertaken in order to eliminate a possible mechanism of arsenate resistance. If the plasmid were to effect repression of the Pit system, glucose grown cells would be resistant to arsenate by virtue of the fact that the Pst system would be in operation under these conditions. As mentioned above, this system effectively discriminates between phosphate and arsenate. The second question is whether or not the plasmid provides protection from arsenate accumulation through the GlpT system during growth utilizing glycerol as the carbon source. The third question is whether or not the plasmid provides protection from arsenate accumulation through the UhpT system during growth utilizing glucose - 6 - phosphate as the carbon source. Before these last two questions can be answered, however, it must be demonstrated

that the GlpT and UhpT systems will accumulate sufficient arsenate to inhibit the growth of a plasmid-free, *pit*⁻ strain when grown utilizing glycerol and glucose - 6 - phosphate as carbon sources.

I. Identification of Phosphate Uptake System Operating in Glucose Grown J53 (R773). In order to determine whether it is the Pit or the Pst system which is functioning in glucose grown J53 (R773), advantage was taken of the fact that the Pst system and the periplasmic protein, alkaline phosphatase, are co-regulated (34). This enzyme is able to provide cells with a source of phosphate by hydrolyzing organic monophosphate esters (if available) during conditions of inorganic phosphate starvation. Strains of E. coli which are dependent on the Pst system for phosphate uptake are starved for phosphate when grown in medium containing arsenate at a concentration 10 fold greater than phosphate (37). Under these conditions, the Pst system and alkaline phosphatase are derepressed above basal levels.

Cultures of glucose grown J53 (R773) were examined for their relative alkaline phosphatase activities under conditions of excess phosphate, excess phosphate plus 10mM arsenate (ratio of arsenate to phosphate = roughly 10/1), and limiting phosphate. As a control for wild type alkaline phosphatase activity, cultures of glucose grown J53 Spc^r were examined under conditions of excess and limiting phosphate.

As shown in Table IV, the activity of alkaline phosphatase in J53 (R773) under conditions of excess or limiting phosphate are comparable to J53 Spc^r. In addition, the presence of 10mM arsenate in cultures of J53 (R773), which results in complete induction of the arsenate resistance system, has no effect on the regulation of alkaline

TABLE IV.

Alkaline Phosphatase Activity in Wild-type and Arsenate Resistant Strains of E. coli

Strain	Growth Medium	p-nitrophenol (absorbance in Klett units)	
		@ 10 min.	@ 20 min.
J53 Spc ^r	CM-Glu	22	27
J53 Spc ^r	CM-G6P	21	26
J53 Spc ^r	CM-Glu (lim. Pi)	390	620
J53 Spc ^r Asa ^r	CM-Glu	27	35
J53 Spc ^r Asa ^r	CM-G6P	25	31
J53 Spc ^r Asa ^r	CM-Glu (lim. Pi)	260	350
J53 Spc ^r Asa ^r	CM-G6P plus 10mM Asa	26	32
J53 (R773)	CM-Glu	21	27
J53 (R773)	CM-Glu plus 10mM Asa	23	28
J53 (R773)	CM-Glu (lim. Pi)	375	600

phosphatase activity.

Although these results do not provide direct evidence of the uptake system functioning in glucose grown J53 (R773), they do provide strong indirect evidence that the Pit uptake system is functional both in the presence and absence of arsenate.

II. Selection and Characterization of J53 Spc^rAsa^r . In order to demonstrate accumulation of arsenate by the GlpT and UhpT systems, the constitutive phosphate uptake system, Pit, must be rendered inactive. In order to accomplish this, an arsenate resistant mutant of a Spc^r derivative of J53, J53 Spc^rAsa^r , was selected on minimal glucose medium containing 50mM arsenate. Because this mutation was selected for on glucose medium, the mutation which confers arsenate resistance is probably pit^- . Although this mutation was not verified using genetic mapping techniques, it has been shown repeatedly that it is the mutation most likely to be isolated under these conditions (35,37). Indirect proof that the mutation is indeed pit^- in J53 Spc^rAsa^r is provided by the fact that in this variant, alkaline phosphatase is synthesized only under phosphate starvation conditions (Table IV). Mutations of the Pst system which render it inactive to phosphate uptake are known to result in alkaline phosphatase constitutivity (34).

III. Demonstration of Arsenate Accumulation by GlpT. The effect of 10mM arsenate on glycerol grown J53 Spc^rAsa^r is shown in Figure 26B. Following exposure to 10mM arsenate, growth of the cells is completely inhibited. This is strong evidence that arsenate is accumulated by this uptake system.

Figure 26 . The Effect of Arsenate on Induced GlpT and UhpT Systems
in E. coli J53 spc^r Asa^r

Cells were grown overnight in CM (0.05% carbon source), harvested by centrifugation and resuspended in fresh CM at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in either the same medium (●), in medium containing 10mM sodium arsenate (▲), or in medium containing 50mM sodium arsenate (■) as indicated. Carbon sources utilized are:

- A. Glucose
- B. Glycerol
- C. Glucose - 6 - Phosphate

IV. Demonstration of Arsenate Accumulation by UhpT. The effect of arsenate on glucose - 6 - phosphate grown J53 Spc^rAsa^r is shown in Figure 26C. Following exposure to 10mM arsenate, the growth rate of the cells is reduced by about 10%, and exposure to 50mM arsenate reduces the growth rate by 60%.

Because the growth rate reduction of glucose - 6 - phosphate grown J53 Spc^rAsa^r following exposure to arsenate is not consistent with data reported in the literature for a *pit*⁻ strain under similar conditions (5), three explanations were examined. First, it is possible that the UhpT system in J53 Spc^rAsa^r is able to more effectively discriminate between phosphate and arsenate, or it may take up both these compounds at significantly reduces rates compared to other wild type strains of E. coli (5). To examine this possibility, an arsenate resistant mutant of another wild strain of E. coli, WA802, was selected on glucose medium. This mutant (WA802 Rif^rAsa^r) was tested for arsenate sensitivity when grown utilizing glucose - 6 - phosphate as the carbon source using the same method as that described for J53 Spc^rAsa^r, with similar results (data not shown). Second, the selection of J53 Spc^rAsa^r may have resulted in a strain altered for alkaline phosphatase regulation, synthesizing the enzyme in response to growth utilizing glucose - 6 - phosphate as the carbon source.^a To eliminate this hypothesis, J53 Spc^rAsa^r was tested for

^aThe rationale for this hypothesis is as follows: if alkaline phosphatase were synthesized during growth on G6P, the enzyme would hydrolyze the G6P to glucose and inorganic phosphate. The resulting glucose would, by catabolite repression, repress the synthesis of the UhpT system. Therefore, under these conditions, the cells in reality would be utilizing glucose as the carbon source and not G6P.

alkaline phosphatase activity during growth utilizing glucose - 6 - phosphate as the carbon source, both with and without arsenate present. The results (Table IV) indicate that alkaline phosphatase activity under these conditions is normal. The third possibility examined was the contamination of the reagent, glucose - 6 - phosphate, with glucose. The use of reagents from several chemical sources, however, produced the same results. In all probability, the resistance to arsenate of the UhpT system in J53 Spc^rAsa^r is due to a greater specificity towards the normal substrates (hexose phosphates) than the strain examined by Bennett (5). The results do, however, strongly suggest that arsenate is accumulated by this uptake system when it is present in the extracellular environment in high concentrations.

V. Construction of J53 Spc^rAsa^r (R773). In order to demonstrate whether or not the plasmid provides protection from arsenate accumulation through GlpT and UhpT the plasmid must be introduced into the *pit*⁻ mutant, J53 Spc^rAsa^r . This was carried out by conjugation of this strain with J53 (R773). Exconjugates were selected with arsenite and spectinomycin was used to select against the donor.

VI. Demonstration of Protection from Arsenate Accumulation through GlpT by R773. The effect of 10mM arsenate on glycerol grown J53 Spc^rAsa^r (R773) is shown in Figure 27B. Although these cells are somewhat inhibited initially following exposure to the arsenate, they recover rapidly (compare to Figure 16) and enter exponential growth phase in about 6 hours.

Figure 27. The Effect of the Plasmid R773 on the Arsenate Sensitive
GlpT and UhpT Systems in E. coli J53 Spc^r Asa^r

Uninduced J53 Spc^r Asa^r (R773) was grown overnight in CM-Glu (0.05% carbon source), harvested by centrifugation and resuspended in fresh CM at 40 Klett units. After exponential growth had resumed, at 80 Klett units, aliquots were removed, the cells were harvested by centrifugation and resuspended at 20 Klett units in either the same medium (●), in medium containing 10mM sodium arsenate (▲), or in medium containing 50mM sodium arsenate (■) as indicated. Carbon sources utilized are:

- A. Glucose
- B. Glycerol
- C. Glucose - 6 - Phosphate

VIII. Demonstration of Protection from Arsenate Accumulation through UhpT by R773. The effect of 10mM and 50mM arsenate on glucose - 6 - phosphate grown J53 Spc^rAsa^r (R773) is shown in Figure 27C. Although higher concentrations of arsenate are required to inhibit glucose - 6 - phosphate grown J53 Spc^rAsa^r than glycerol grown cells, it can be seen from Figure 27C that the introduction of the plasmid R773 into this strain increases the growth rate in 50mM arsenate by nearly 100%.

The results of these experiments indicate that each of the uptake systems capable of accumulating arsenate (Pit, GlpT and UhpT) in sensitive cells, albeit at varying rates, are protected from arsenate uptake by the presence of the plasmid R773.

Studies on the Organization of the Arsenic Resistance Genes of R773 and R45

In order to gain insight into the organization of the arsenic resistance genes in the plasmids R773 and R45, arsenic sensitive variants were isolated. In order to do this, a method had to be devised to increase the frequency of mutation over that which occurs spontaneously. The method used was transposon insertion mutagenesis using the translocatable genetic element Tn5.

Transposons are unique sequences of DNA which, as discrete genetic and physical entities, can bring about their own translocation from one site in the bacterial genome to another, or to a different genome. These translocations are promoted by recombinational systems other than those required for generalized homologous recombination in bacteria. This "illegitimate" recombination enables transposons to insert at random within a target DNA sequence, i.e., within regions which are not homologous in base-pair sequence with the transposon. When transposon insertions occur within genes, the result is a complete loss of function of the genes. Insertion mutants can be recovered at high frequency because transposons typically determine resistance to an antibiotic. For example, Tn5 determines resistance to kanamycin. The phenotype of insertion mutations is completely linked to the drug resistance.

In order to introduce the transposon into plasmid-bearing cells of E. coli, advantage was taken of a specially constructed bacteriophage; λ b221 cI857 rex::Tn5 (Kan) (6). This phage has several important

properties which make it particularly suitable as a vector for the introduction of Tn5 into E. coli cells. First, infection techniques can be utilized to ensure that nearly all cells receive the phage genome. Second, the deletion b221 in this phage, removes the phage integration site, thus rendering the phage genome incapable of integrating into the bacterial chromosome by λ - specific mechanisms. Most kanamycin resistant transductants ($\geq 98\%$) do not contain a λ prophage (7). They arise, therefore, by the transposition of the Tn5 segment from its λ vector to either the bacterial chromosome or to plasmids carried by the bacteria. This transposition occurs at high frequencies, about 10^{-2} to 10^{-3} per infected cell. Third, the temperature sensitive mutation cI857 renders the phage thermally inducible for its lytic cycle. Thus infected cells lyse when grown at 45°C but phage replication is prevented when grown at 30°C (6). The phage genome is rapidly lost from a growing culture of infected E. coli at 30°C . The possibility of obtaining double insertions is very remote. It is estimated to be approximately 10^{-6} for chromosomal insertions (27). Since these plasmids are roughly 50 fold smaller than the chromosome, the chance of obtaining double insertions within a plasmid is negligible.

The procedure used for isolating arsenic sensitive variants of R773 is summarized in Figure 28 (see Materials and Methods for details). The cells, J53 (R773), were infected with $\lambda::\text{Tn5}$ followed by overnight growth in kanamycin containing medium to enrich for cells expressing kanamycin resistance. This enrichment selects for cells which carry Tn5 either within the chromosome or within the plasmid R773. In order

Figure 28. Summary of Tn5 Insertion Mutagenesis of R773

SELECT ASI^RASA^S, ASI^SASA^R, AND ASI^SASA^S CLONES.

to isolate cells with Tn5 located within the plasmid, J53 (R773) (Tn5) was conjugated with an alternate J53 host previously selected for spectinomycin resistance. In order to ensure exconjugates received plasmids with Tn5 insertions, and to restrict the possible insertion sites, spectinomycin, kanamycin, streptomycin and tetracycline were added to the mating mixture. The culture was then enriched for exconjugates able to express resistance to these antibiotics. Following the isolation of clones which expressed resistance to the four antibiotics mentioned above, arsenic sensitive clones were isolated by replica plating master plates on to arsenite, arsenate or controls. When arsenic sensitive variants of R45 were isolated, ampicillin was substituted for streptomycin.

The results of this mutagenesis technique are shown in Table V. Three phenotypes of arsenic sensitive variants were isolated; arsenite resistant-arsenate sensitive, arsenite sensitive-arsenate resistant, and arsenite sensitive-arsenate sensitive. The mutants were often found to be unstable. In fact, only 4 of 40 arsenic sensitive variants of R773 and 17 of 35 arsenic sensitive variants of R45 remained stable upon repeated subculturing following isolation. The reason for this instability is not clear, however, it is probably due to the precise excision of the transposon followed by integration at an alternate site. The clones which reverted to arsenic resistance retained resistance to kanamycin.

These results indicate that the arsenite and arsenate resistance systems determined by R773 and R45 are entirely separate. Since transposon insertions are known to cause strongly polar mutations (7) , the arsenite and arsenate resistance genes probably are

TABLE V.

Classes of Tn5 Insertion Mutants

Strain	Phenotype	Number Isolated	Number Stable Upon Repeated Subculture
R773 A::Tn5	Asi ^r Asa ^s	13	4
R773 B::Tn5	Asi ^s Asa ^r	25	0
R773 C::Tn5	Asi ^s Asa ^s	2	0
R45 A::Tn5	Asi ^r Asa ^s	5	4
R45 B::Tn5	Asi ^s Asa ^r	13	4
R45 C::Tn5	Asi ^s Asa ^s	17	9

Approximately 3000 clones were examined for each plasmid.

transcribed from separate promoters. Since double sensitive variants could be isolated, and since double insertions are highly unlikely, the arsenic resistance genes are probably regulated by the action of a single activator protein (see Conclusions for a possible mechanism).

CONCLUSIONS

The results of this study suggest several important things with respect to the organization and regulation of the arsenic resistance genes, and with respect to the mechanisms of arsenic resistance in E. coli J53 (R773). I would like to propose the model shown in Figure 29 for the organization and regulation of the arsenic resistance genes of R773.

The plasmid determines resistance to both arsenite and arsenate. That these resistances represent entirely separate systems was suggested by two findings. First, resistance to arsenite could be separated from arsenate resistance by transposon insertion mutagenesis, i.e., both arsenite resistant - arsenate sensitive and arsenite sensitive - arsenate resistant phenotypes could be isolated using this technique. This finding also suggests that the arsenic resistance genes are transcribed from different promoters because transposon insertions result in strongly polar mutations. Second, growth experiments show that arsenite induces arsenite resistance alone, while arsenate induces both arsenate and arsenite resistance. Thus, these two systems have been shown to be separate both physiologically and genetically.

Transposon insertion mutagenesis has also shown that an arsenite sensitive - arsenate sensitive phenotype can be isolated. This suggests that the arsenic resistance genes are regulated by a single

Figure 29. Model for the Organization and Regulation of the
Arsenic Resistance Genes of R773

activator protein. The presence of arsenite, which binds to the activator, facilitates transcription of only the arsenite gene(s) because the resulting activator-arsenite complex only recognizes the arsenite gene(s) promoter. Arsenate, on the other hand, binds to the activator and facilitates transcription of both arsenite and arsenate genes because the activator-arsenate complex recognizes both the arsenate and arsenite gene promoters.

Physiological studies suggest that these genes are transcribed at basal levels sufficiently great to allow rapid completion of the inductive process upon exposure to arsenite or arsenate.

The mechanisms of plasmid-mediated resistance to arsenite and arsenate remain unknown, however, this study has shed some light on some possibilities and eliminated others. The least understood is the resistance to arsenite. Data collected in this study demonstrate that the plasmid does not determine a system for arsenite detoxification. The target site for arsenite toxicity appears to be a component of respiratory metabolism. The fact that arsenite uptake is unknown to occur, and the data collected by Smiley (28) on chromosomally determined resistance to arsenite suggest that a probable target site for arsenite toxicity lies within the cytoplasmic membrane, specifically the membrane-bound ATPase. Arsenite may either gain entrance to the interior of the cell through the ATPase, it may alter the structure of the ATPase so as to allow leakage of ions across the membrane, it may simply disrupt the ability of the ATPase to carry out oxidative phosphorylation, or combinations of these possibilities may occur depending on the concentration of arsenite which the cells are exposed to.

Two possible explanations may reasonably account for plasmid-mediated resistance to arsenite if the major target is the ATPase. First, the plasmid may determine the interposition of an element between the ATPase and the extracellular environment, either a periplasmic or outer membrane protein, which prevents arsenite from coming in contact with the ATPase. Second, the plasmid may determine the synthesis of an alternate, or an additional, component of the ATPase which renders it insensitive to arsenite toxicity.

The mechanism of plasmid-mediated resistance to arsenate is also unknown, however, the data collected in this study strongly suggests only one reasonable explanation. All uptake systems which transport phosphate and fail to discriminate between phosphate and arsenate (Pit, GlpT and UhpT) are protected from arsenate uptake by the plasmid. It is highly unlikely that the plasmid provides protection to each of these systems by a separate mechanism, nor does it seem likely that a single plasmid-determined element could interact with all of these uptake systems to provide the kind of specificity possessed by the Pst system. Rather, it seems necessary to postulate the interposition of a single plasmid-determined element which prevents arsenate from entering the periplasmic space. This element would exist within the outer membrane and function as a "gatekeeper" that would allow free passage of phosphate and other growth factors to the periplasmic space, but prevent the passage of arsenate. I have collected preliminary evidence (not included in this thesis work) from a study of membrane proteins which suggests that this hypothesis is correct.

REFERENCES

1. Angino, E.E., L.M. Magnuson, T.C. Waugh, O.K. Galle and J. Bredfeldt. 1970. Arsenic in detergents: Possible danger and pollution hazard. *Science* 168:389.
2. Bachman, B.J. 1972. Pedigrees of Some Mutant Strains of Escherichia coli K-12. *Bact. Rev.* 36:525.
3. Beeson, P.B., W. McDermott and J.B. Wyngaarden. 1979. Textbook of Medicine. Philadelphia, W.B. Saunders Co.
4. Benko, V. and K. Symon. 1977. Health Aspects of Burning Coal with a High Arsenic Content. *Environ. Res.* 13:378.
5. Bennett, R.L. 1973. Studies on the Utilization of Orthophosphate by Wild Type and Arsenate Resistant Strains of Escherichia coli. Tufts University, Ph.D. Dissertation.
6. Berg, D.E., J. Davis, B. Allet and J. Rochaix. 1975. Transposition of R factor genes to bacteriophage λ . *P.N.A.S.* 72:3628.
7. Berg, D.E. 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn5. In: DNA insertion elements, plasmids, and episomes. Eds. A.I. Bukarhi, J. Shapiro and S.L. Adhya. New York, Cold Spring Harbor Laboratory, Cold Spring Harbor.

8. Bradley, D.E., D.E. Taylor and D.R. Cohen. 1980. Specification of Surface Mating Systems Among Conjugative Drug Resistance Plasmids in Escherichia coli K-12. J. Bact. 143:1466.
9. Ferguson, J.F. and J. Garvis. 1972. A Review of the Arsenic Cycle in Natural Waters. Water Res. 6:1259.
10. Fong, K., F. Lee and R. Bockrath. 1980. Effects of Arsenite on Single-strand DNA Break Formation and Post-Replication Repair in E. coli Following UV Irradiation. Mut. Res. 70:151.
11. Hashimoto, H. and R.H. Rownd. 1975. Transition of the R factor NRI in Proteus mirabilis: level of drug resistance in non-transitioned and transitioned cells. J. Bact. 123:56.
12. Joly, B.H., R.A. Cluzel, S.M. Petit and M.E. Chanal. 1979. Transferable Sodium Arsenate Resistance in Enterobacteriaceae. Curr. Micro. 2:51.
13. Kleckner, N., J. Roth and D. Botstein. 1977. Genetic Engineering in Vivo Using Translocable Drug-Resistance Elements. J. Mol. Biol. 116:125.
14. McBride, B.C. and R.S. Wolfe. 1971. Biosynthesis of Dimethylarsine by Methanobacterium. Biochem. 10:4312.
15. Metzler, D.E. 1977. Biochemistry. New York, Academic Press.
16. Miller, J.H. 1972. Experiments in Molecular Genetics. New York, Cold Spring Harbor Laboratory, Cold Spring Harbor.

17. Neidhardt, F.C., P.L. Bloch and D.F. Smith. 1974. Culture Medium for Enterobacteria. J. Bact. 119:736.
18. Novick, R.P. and C. Roth. 1968. Plasmid-linked Resistance to inorganic Salts in Staphylococcus aureus. J. Bact. 95:1335.
19. Osborne, F.H. and H.L. Ehrlich. 1976. Oxidation of Asenite by a Soil Isolate of Alcaligenes. J. Appl. Bact. 41:295.
20. Perlman, D. and R. Stickgold. 1977. Selective amplification of genes on the R plasmid, NR1, in Proteus mirabilis: an example of the induction of selective gene amplification. P.N.A.S. 22:2518.
21. Phillips, S.E. 1973. A Study of the Physiological Factors Affecting the Biological Conversion of Arsenite to Arsenate. Portland State University, Ph.D. Dissertation.
22. Phillips, S.E. and M.L. Taylor. 1976. Oxidation of Arsenite to Arsenate by Alcaligenes faecalis. Appl. Envir. Micro. 32:392.
23. Robbins, S.L. and R.S. Cotran. 1979. Pathological Basis of Disease. Philadelphia, W.B. Saunders Co.
24. Rossman, T.G., M.S. Meyn and W. Troll. 1977. Effects of Arsenite on DNA Repair in Escherichia coli. Environ. Health. Prosp. 19:229.

25. Sachs, R.M., J.L. Michael, F.B. Anastasia and W.A. Wells. 1971. Determination of Arsenical Herbicide Residues in Plant Tissues. Weed Sci. 19:412.
26. Shapiro, J. 1971. Arsenic and Phosphate Measured by Various Techniques. Science 171:234.
27. Shaw, K.J. and C.M. Berg. 1979. Escherichia coli K-12 Auxotrophs Induced by Insertion of the Transposable Element Tn5. Genet. 92:741.
28. Smiley, D.G., M.L. Taylor, J. Myers and W.H. Taylor. 1981. An Arsenite-resistant Mutant of Escherichia coli with a Defective Membrane-bound Adenosine Triphosphatase. Abstr. Am. Meet. Am. Soc. Microbiol. p. 159.
29. Smith, H.W. 1978. Arsenic Resistance in Enterobacteria: its Transmission by Conjugation and by Phage. J. Gen. Micro. 109:49.
30. Sunderman, F.W. 1976. A Review of the Carcinogenicities of Nickel, Chromium and Arsenic Compounds in Man and Animals. Prev. Med. 5:279.
31. Taylor, M.L. 1980. Personal communication.
32. Webb, J.L. 1966. Enzyme and Metabolic Inhibitors, III. New York, Academic Press.

33. Wilkinson, R.E. and W.S. Hardcastle. 1969. Plant and soil arsenic analysis. *Weed Sci.* 17:536.
34. Willsky, G.R., R.L. Bennett and M.H. Malamy. 1973. Inorganic phosphate transport in Escherichia coli: involvement of two genes which play a role in alkaline phosphatase regulation. *J. Bact.* 113:529.
35. Willsky, G.R. 1976. Inorganic Phosphate Transport and Alkaline Phosphatase Regulation in E. coli. Tufts University, Ph.D. Dissertation.
36. Willsky, G.R. and M.H. Malamy. 1980. Characterization of Two Genetically Separable Inorganic Phosphate Transport Systems in Escherichia coli. *J. Bact.* 144:356.
37. Willsky, G.R. and M.H. Malamy. 1980. Effect of Arsenate on Inorganic Phosphate Transport in Escherichia coli. *J. Bact.* 144:366.
38. Woolson, E.A. and P.C. Kearney. 1973. Persistence and Reactions of ^{14}C -Cacodylic Acid in Soils. *Environ. Sci. Tech.* 7:47.