# MOLECULAR APPROACHES TO IDENTIFY GENETICALLY PROGRAMMED RESPONSES TO TOXIC METAL EXPOSURE

by Angelina Guzzo

Department of Microbiology and Immunology

McGill University

Montreal, Quebec, Canada

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This thesis is dedicated to my grandfather, Costantino Guzzo, who passed away on August 8, 1994. His strength, honesty, courage and love will always be a part of me. He devoted his whole life to the happiness and well being of his family. We love and miss him, and will always remember the important role he had in our lives.

Questa thesi é dedicata a mio nonno Costantino Guzzo, morto l'8 agosto 1994. La sua forza, la sua onestà, il suo coraggio e amore sarà sempre presente in me. Ha dedicato la sua vita al benessere e alla felicità della sua famiglia. Tuttora ci manca molto, ricordermo sempre il suo ruolo che ha impresso alla nostra vita.

## ABSTRACT

Elevated environmental concentrations of toxic metals can occur due to leaching, anthropogenic sources and industrial activity. Organisms can survive elevated exposure to toxic metals by reorienting their cellular physiology to cope with metal-induced stress, and this often includes changes in gene expression. In order to identify metal-induced changes in *Escherichia coli* chromosomal gene expression, a library of 3000 single-copy *luxAB* gene fusions was prepared and screened for increased luminescence in the presence of aluminum and nickel. Two clones were found which displayed increased luminescence in the presence of aluminum (strains LF20110 and LF20111), and one was found to increase luminescence in the presence of nickel (strain LF20112). Strain LF20111 has the *luxAB* genes inserted 97 base pairs downstream of the *E. coli fliC* translational start site and strain LF20112 contains the *luxAB* genes inserted 3204 base pairs downstream of the *cel* operon transcriptional start site, in the *celF* gene. DNA sequencing downstream of *celF* revealed the presence of a gene that encodes an expressible 28-kDa polypeptide which may play a role in nickel metabolism. Strain LF20110 contains the *luxAB* genes fused to a previously uncharacterized gene, designated *ais* (aluminum and iron stimulated), located at 2 378 450 bp on the *E. coli* genetic map. An mRNA, 800 nucleotides in length, was produced from this gene in the presence of aluminum and iron, and a polypeptide of 22-kDa was expressed *in vivo*. Genes in human cells induced in the presence of arsenite were also sought. A subtraction was performed between cDNA synthesized from HeLa cell poly(A)$^+$ RNA extracted from unexposed and arsenite-exposed cells. Cloning and sequencing of three cDNAs that showed a higher hybridization signal to RNA from HeLa cells in the presence versus the absence of arsenite showed that two of the cDNAs coded for human ferritin H chain, while the other coded for metallothionein-II.

## RESUME

De fortes concentrations de métaux toxiques peuvent être retrouvées dans l'environnement dû au phénomène de lessivage et aux activités tant humaines qu'industrielles. En cas d'exposition à de fortes concentrations de métaux toxiques, les organismes peuvent survivre en réorientant leur physiologie cellulaire afin de surmonter le stress causé par les métaux. Cette réorientation physiologique implique souvent des changements dans l'expression des gènes chromosomiques chez *Escherichia coli*. Une banque de clones comptant 3000 gènes de fusion, entre les gènes *luxAB* et l'ADN de *E. coli*, a été préparée. Chaque gène de fusion est retrouvé en une seule copie. Cette banque de clones a été testée pour détecter une augmentation de la luminescence, résultant de l'expression de *luxAB*, en présence d'aluminium et de nickel. Deux clones présentant une luminescence accrue en présence d'aluminium ont été trouvés (souches LF20110 et LF20111), ainsi qu'un clone répondant en présence de nickel (souche LF20112). Dans la souche LF20111 les gènes *luxAB* ont été insérés 97 paires de bases en aval du site d'initiation de la traduction du gène *fliC* de *E. coli*. Pour ce qui est de la souche LF20112, les gènes *luxAB* ont été insérés 3204 paires de bases en aval du site d'initiation de la transcription de l'opéron *celF*. Le séquençage de l'ADN en aval de celF a révélé la présence d'un gène codant pour un polypeptide de 28-kDa, polypeptide qui peut être exprimé et jouer un rôle dans le métabolisme du nickel. Dans la souche LF20110 les gènes *luxAB* ont fusionné à un gène qui n'avait jamais été caractérisé auparavant. Ce gène désigné "*ais*" (aluminum and iron stimulated) est localisé à la position 2 378 450 pb sur la carte génétique de *E. coli*. En présence d'aluminium et de fer, un ARNm de 800 nucléotides a été produit à partir de ce gène, et un polypeptide de 22-kDa a été exprimé *in vivo*. Il a également été observé que l'arsenic peut induire l'expression de certains gènes dans les cellules humaines. L'ARN poly(A)$^+$ de cellules HeLa exposées

et non exposées à l'arsenic a été extrait, et les ADN complémentaires synthétisés à partir de l'ARN. Une expérience de soustraction a été réalisée en mettant en présence les ADNc des cellules exposées et non exposées.Trois ADNc présentaient un plus haut taux d'hybridation à l'ARN des cellules HeLa exposées, qu'à celui des cellules non exposées. Le clonage et le séquençage de ces trois ADNc ont montré que deux des trois ADNc codaient pour la chaine H de la ferritine humaine, alors que le troisième codait pour la métallothionéine-II.

## ACKNOWLEDGEMENTS

One of the greatest influences for this thesis comes from Michael DuBow, who is my mentor and friend and has always been a source of inspiration. It was through his wonderful teaching that I first got interested in Science. However, it was his undying passion for Science that got me hooked and I haven't been able to turn away since.

My greatest fans and supporters are my parents, Tony Guzzo and Annette Henrikso. There isn't enough space in this thesis to describe what they mean to me. Through them, I have learned how to survive in this crazy world. They taught me to believe in myself and that with hard work and perseverance, everything is possible. Their unconditional love and acceptance is what got me through the rough times and made it all worth while during the good times. My sister, Julie Guzzo, is my best friend who I grew up with and had the honor of working with in the last year. She is kind, helpful, smart and has a heart of gold. She typed, did experiments and always had time to talk. I also had the great fortune of knowing my grandparents, Laura Shelton, Arthur Henrikso, and Costantino and Maria Stella Guzzo. It is by their strength and support that I have such a wonderful family. They are very special people who contributed in a major way to my success.

There are some very special people who always had time to help me out and to whom I have grown very close to over the last couple of years. From the first day I met Caroline Diorio, a bond developed. She has helped with every aspect of this thesis, and we have shared many memories and secrets. Gina Macintyre is also a great friend and devoted a lot of time to helping me out, and whose company I always enjoy. I have become a little wiser by listening to her. Josée Brisebois, my lab neighbour, had an adjoining desk. We shared many great discussions and a little insight about life. Peter Ulycznyj has dug me out of some rough spots and a

friendship developed that extends past lab hours.

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Through McGill, I was part of the hockey team legacy called the Mother Puckers. I had a great time with all my fellow teammates. I also participated in Kung Fu which kept me sane much of the time. Close friendships developed with Ari Papas, Linda Dion, Claude Bédard and David Blustein. I would also like to thank Masters Bunn and Kin Hong for being such splendid instructors.

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## PREFACE TO THE THESIS

In accordance with the guidelines concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, I have opted to present the experimental portion of this thesis (Chapters 2 to 7, inclusive) in the form of original papers. A provision in the guidelines concerning thesis preparation reads as follows:

> Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
>
> - If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory.
> - The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general overview of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.
> - Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
> - In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

With regard to these conditions, I have included as chapters of this thesis, six original papers, of which three have been published, and three are being submitted for publication. Chapters 2 to 7, inclusive, each contain an Abstract, Introduction, Materials and Methods, Results and Discussion section. Chapters 3 to 7 contain prefaces that serve as the connecting text to bridge the manuscripts. A Literature Review (Chapter 1) and a Summary (Chapter 8) have also been included, and

references for all chapters are collated alphabetically at the end of the thesis. Abbreviations, other than those accepted by the Journal of Bacteriology, are listed at the beginning of the thesis.

The manuscripts, in order of their appearance, in the thesis are:

1. Guzzo, A. and DuBow, M.S. 1991. Construction of stable, single-copy luciferase gene fusions in *Escherichia coli*. Arch. Microbiol. 156:444-448.

2. Guzzo, A., Karatzios, C., and DuBow, M.S. Time-dependent Tn*5* transposition after transformation and antibiotic selection in *Escherichia coli*. Submitted to Plasmid.

3. Guzzo, A., Diorio, C., and DuBow, M.S. 1991. Transcription of the *Escherichia coli fliC* gene is regulated by metal ions. Appl. Environ. Microbiol. 57:2255-2259.

4. Guzzo, A., and DuBow, M.S. 1994. A *luxAB* transcriptional fusion to the cryptic *celF* gene of *Escherichia coli* displays increased luminescence in the presence of nickel. Mol. Gen. Genet. 242:455-460.

5. Guzzo, A. and DuBow, M.S. Identification, sequencing and characterization of an aluminum-inducible *Escherichia coli* gene. Submitted to Journal of Bacteriology.

6. Guzzo, A., Karatzios, C., Diorio, C., and DuBow, M.S. Metallothionein-II and ferritin H mRNA levels are increased in arsenite exposed cells. Submitted to Biochemical and Biophysical Research Communications.

I was responsible for all the research described in Chapter 2 to 7 inclusively, with the following exceptions:

1. In Chapter 2, Christos Karatzios did the transformations.

2. In Chapter 4, Caroline Diorio prepared the phage λ library of the *E. coli* chromosome.

3. In Chapter 7, Christos Karatzios and I contributed equally to the cDNA preparation, subtraction, and cloning and preparation of the northern blots. Caroline Diorio prepared the RNA dot blots and performed the hybridization.

## CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. I developed a method to isolate single-copy *luxAB* gene fusions in the *Escherichia coli* chromosome by introducing the ColE1 plasmid pFUSLUX which contains a left end-truncated Tn*5* element flanking the promoterless *Vibrio harveyi luxAB* genes and a *tet* gene (tetracycline resistant), into a strain containing the p15A RNA1-overproducing plasmid, pTF421 (ampicillin resistant), which inhibits the replication of ColE1 plasmids.

2. I showed that Tn*5* transposition events could occur for several days post-transformation and that 20% of stable Tn*5*-*luxAB* containing clones also had a change in IS*1*, IS*2* or IS*5* chromosomal distribution. I also showed that colonies appeared that would not regrow on double antibiotic plates, and suggested that ampicillin degradation and pTF421 segregation may be responsible for these results.

3. I constructed a library of 3000 single-copy, chromosomal *E. coli luxAB* gene fusions and used it to find two clones that displayed increased luminescence in the presence of 1 and 10 µg/ml aluminum (strains LF20110 and LF20111) and to find one clone that displayed increased luminescence in the presence of 1 and 10 µg/ml nickel (strain LF20112).

4. I showed that the luminescence of strain LF20111 was also induced in the presence of copper, iron and nickel on LB agar petri dishes. I determined that strain LF20111 had the *luxAB* genes inserted 97 bp downstream of the the *E. coli fliC* translational start site.

5. I showed that the luminescence of strain LF20112 was specifically induced in the presence of nickel and cobalt. I determined that strain LF20112 had an insertion of the *luxAB* genes in the *E. coli celF* gene, the distal most gene of the *cel* operon. By DNA sequencing the region downstream of *celF*, I connected the sequence between the *cel* operon and the convergently transcribed *katE* gene. I also showed by cloning,

sequencing, and *in vivo* expression of a DNA segment downstream of *celF*, the presence of an expressible polypeptide, designated ORF 28.5, that may play a role in nickel metabolism.

6. I showed that the luminescence of strain LF20110 was specifically augmented in the presence of aluminum and iron. I determined that the *luxAB* genes were inserted at 2 378 450 bp on the *E. coli* genetic map, in a region that had not been previously characterized. I sequenced 1286 bp of the genomic region spanning the site of *luxAB* insertion. By northern blotting I showed that a transcript of 800 nucleotides is induced from this DNA region in the presence of aluminum and iron. By performing promoter resection, I delineated the aluminum-mediated transcriptional DNA sequences to 370 bp. By S1 nuclease analysis, I located two putative transcriptional start sites. Translation of the DNA sequence downstream of the transcriptional start site revealed the presence of a potential open reading frame coding for a polypeptide of 22-kDa, which I subsequently showed was expressed *in vivo*. I designated this new *E. coli* gene as *ais* ( aluminum and iron stimulated).

7. I showed that the human ferritin H and metallothionein-II mRNAs are augmented 2- and 6-fold, respectively, after HeLa cells are exposed to 5 μM arsenite for 24 hours, using a magnet-assisted cDNA subtraction technique.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ap: | ampicillin |
| Cb: | carbenicillin |
| Cm: | chloramphenicol |
| DMEM: | Dulbelcco's Modified Eagle's Medium |
| DTT: | dithiothreitol |
| dNTP: | dinucleotide triphosphate |
| HEPES: | N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) |
| IPTG: | isopropyl-β-D-thiogalactoside |
| IS: | insertion sequence |
| Kn: | kanamycin |
| MMS: | methyl methanesulfonate |
| MNNG: | N-methyl-N'-nitro-N-nitrosoguanidine |
| MnSOD: | manganese-containing superoxide dismutase |
| MOPS: | 3-(N-morpholino)propanesulfonic acid |
| ORF: | open reading frame |
| ppm: | parts per million |
| PU: | palindromic unit |
| R: | resistant/resistance |
| REP: | repetitive extragenic palindromic sequences |
| RLU: | relative light units |
| TG: | 1-thioglycerol |
| Tc: | tetracycline |
| Tn: | transposon |
| Xgal: | 5-bromo-4-chloro-3-indoyl-β-D-galactoside |

# CHAPTER 1

## The Use of Gene Fusions to Identify and Characterize Genetically Programmed Responses of *Escherichia coli* to Environmental Stress

## 1.1 INTRODUCTION

Organisms extract carbon, phosphate, nitrogen, sulfur, essential trace elements and energy sources from their environment. Sometimes, an essential nutrient is lacking or a harmful substance is present, posing a stress to the organism. Organisms have evolved genetically programmed mechanisms to allow them to survive exposure to environmental stresses. Frequently, these involve changes in gene expression in order to increase (or decrease) the synthesis of proteins to cope with their altered physiology. In order to elucidate genes that are affected, as well as their function and signal transduction pathways, many genetic techniques have been developed or employed. The first part of this review discusses the technology used to elucidate genetically programmed responses to environmental stresses in *Escherichia coli*, starting with mutations and two-dimensional protein gel electrophoresis (section 1.2). Another technique, called gene fusions, involves the fusion of genes to a gene that codes for an easily assayable protein product. This technology allows the identification of environmentally regulated proteins without knowing their function a priori. The second part of this review focuses on gene fusion systems (section 1.3). The third part of this review deals with chromosomal *E. coli* genes and genetic networks, discovered by gene fusion technology, that have been found to play a role in, or whose expression is regulated by, particular environmental stresses (section 1.4). Lastly, the use of chromosomal gene fusions to create living biosensors to detect toxic substances, and their use to study the transposition of bacteriophage Mu, will be discussed (section 1.5). Transposable elements, such as Mu DNA, contribute an important mechanism of mediating rapid evolutionary changes. The study of physiological mechanisms that cause changes in transposition frequencies can provide insight into environmental regulation of transposition.

## 1.2 METHODS TO CHARACTERIZE THE FUNCTION OF GENES RESPONSIVE TO ENVIRONMENTAL STRESS

Many genetic techniques have been developed to define genes and the function of their products. The first part of this section discusses how the isolation of mutants and their selection can be used to determine *E. coli* genes that are responsive to environmental stress. Two-dimensional protein gel electrophoresis, the focus of the last part of this section, has proven invaluable for determining the sets of genes that are induced by specific environmental stimuli.

### 1.2.1 The Use of Mutations to Study Genes Involved in Environmental Stress

Mutations can be created in genes whose expression is affected by, or function is involved in, environmental stress. In this case, loss or gain of a response to the environmental stress is selected. It was Muller (1927) who first showed that treatment of *Drosophila* germ cells with X-Rays increased the number of mutations 15 000%. Since then, it has been established that mutations alter DNA sequences, including the coding sequence of genes, and that many environmental agents can induce mutations. When a gene's DNA sequence is altered, the coding sequence of its protein product is in turn affected. This can result in altered protein structures or enzymatic activities and can be used to study biological processes. For instance, if a mutation results in the complete inactivation of an enzyme that is part of a biosynthetic pathway, the function of the enzyme can be determined by defining which step in the biosynthetic pathway is blocked. Furthermore, the gene that codes for the enzyme can be determined if the mutation is localized.

DNA nucleotide changes occur in the range of $10^{-8}$ to $10^{-9}$ per base per replication cycle (Moses and Summers, 1988) and, with an appropriate selection procedure, can be used to isolate mutations in desired genes. Methods to increase muta-

tion rates are available. These include the use of *E. coli* strains that are mutant in DNA repair pathways, and the use of UV, ionizing radiation, or mutagenic chemicals (Table 1) (Foster, 1991; Kushner, 1987).

Another technique for the isolation of mutations uses transposable elements. Transposable elements can move from one DNA molecule to another or from one chromosomal location to another, insertionally disrupting the DNA coding sequence that was inserted into. The smallest are the IS elements that carry only the proteins and *cis*-acting sequences required for transposition. Transposons are bigger and usually carry, in addition to the genes required for transposition, antibiotic or heavy metal resistance genes. Some transposons are composed of two IS elements flanking auxiliary genes. The largest are the transposable bacteriophages, which also contain genes required for phage growth, maturation and lysogenization. The discovery of Mu and its capacity to insert at random allowed a more versatile approach to mutagenizing chromosomal DNA. The use of transposons carrying selectable markers facilitated selection. Many specialized transposable element derivatives have been constructed (for reviews see Berg and Berg, 1987; Berg *et al.*, 1989; Kleckner *et al.*, 1991).

### 1.2.2 Phenotype Selection

The first RNAse I (Gesteland, 1966) and DNA polymerase I (De Lucia and Cairns, 1969) mutants were found by screening individual clones for their lack of activity. To aid in the isolation of particular mutants, methods can be devised to select for their phenotype. Mutants can be selected for the inability to grow by replica plating onto petri dishes in the absence and presence of a compound that the cells become sensitive to. Alternatively, selecting the ability to grow in the presence of a potentially toxic compound that mutant cells become resistant to can be used to

## Table 1

## Selected Mutagenesis Approaches

### Mutations in DNA Repair Pathways

| Gene | DNA Damage Affected |
|---|---|
| *uvrABC* | bulky lesions |
| *ada, alkA* | alkylated residues |
| *phr* | pyrimidine dimers |
| *xth, nfo* | abasic sites |
| *ung* | uracil residues |
| *mutH,L,S, uvrD* | mismatches |
| *dnaQ, mutD* | proofreading |

### Chemical Mutagenesis

| Chemical | Type of DNA Damage |
|---|---|
| ultraviolet light | pyrimidine dimers |
| mitomycin C | DNA strand cross-links |
| ethylmethane sulfonate, MNNG | alkylation |
| hydroxylamine | cleavage of phosphodiester backbone |
| hypoxanthine | deamination |
| X- or $\gamma$-Rays | strand nicks or breaks |

### Other

| | |
|---|---|
| transposable elements | insertion (or rearrangement) mutagenesis |

identify gene(s) that encode products involved in an environmental stress response. It is also possible to select for the gained ability to catabolize substrates. Suppressors of mutant phenotypes can also be selected. For example, second site mutations of a *lon*$^-$ *E. coli* strain were selected by resistance to nitrofurantoin (Gayda *et al.*, 1976). Some examples are listed in Table 2 (Vinopal, 1987).

A mutation can either affect the function of a single gene product or the expression of many gene products by affecting a regulator, or a key intermediate, in a biochemical pathway. Genes coordinately regulated by a single protein are termed a regulon (Neidhardt, 1987). For instance, the two mutants *recA*(Def) and *lexA*(Ind$^-$) prevented the induction of the SOS response (Walker, 1987). In the heat shock response, a mutant was isolated due to its inability to grow at high temperatures. It was assigned as a regulator of the heat shock proteins because two-dimensional protein gels (see section 1.2.3) revealed the inability to induce these proteins at high temperature in a strain carrying the mutation, designated *htpR* (Neidhardt and VanBogelen, 1987).

Some proteins are essential for growth, thus rendering isolation of a mutant unlikely. In this case, conditional lethal mutations, which are expressed only under certain growth conditions, can be used (Beckwith, 1991). When grown under certain conditions, the cell behaves similarly to wild type, but when the cells are grown at elevated (or lowered) temperatures, called heat (or cold) sensitive, the mutation is expressed.

### 1.2.3 Two-dimensional Protein Gels

Two-dimensional protein gels (O'Farrell, 1975) are useful for identifying proteins whose expression is altered by environmental stresses. A gene-protein index of *E. coli* is being prepared to assign individual proteins to the *E. coli*

## Table 2

## Phenotype Selection for Environmental Toxicants

## (Selected Examples)

### Resistance

| Selection | Gene/Operon Affected | Reference |
|---|---|---|
| arsenate | *pit* | Bennett and Malamy, 1970 |
| nickel | *cor* | Webb, 1970 |
| selenate | *cys* | Springer and Huber, 1973 |
| streptomycin | *cmlA* | Baughman and Fahnestock, 1979 |
| manganese | *mng* | Silver *et al.*, 1972 |

### Sensitivity

| Selection | Gene/Operon Affected | Reference |
|---|---|---|
| radiation | *ras* | Walker, 1969 |
| novobiocin | *rfa* | Coleman and Leive, 1979 |
| low levels of potassium | *kdp* | Epstein and Davies, 1970 |

### Metabolism

| Selection | Gene/Operon Affected | Reference |
|---|---|---|
| salicin | *bgl* | Prasad and Schaefler, 1974 |
| cellobiose | *cel* | Kricker and Hall, 1984 |

genetic map (Phillips *et al.*, 1987). The heat shock response has been extensively studied with two-dimensional protein gels, and each protein has been assigned a number based upon its position of migration on the gel (Neidhardt and VanBogelen, 1987). Two-dimensional gels can also be used to identify proteins induced by a particular environmental agent by analyzing extracts prepared from cells in the absence and presence of the environmental agent. Analysis of two-dimensional protein gels after exposure of *E. coli* to cadmium chloride, monochlorophenol or pentachlorophenol revealed that the synthesis of the OmpF and aspartate transcarbamoylase proteins was repressed (Faber *et al.*, 1993). Two-dimensional gel analysis of *E. coli* exposed to benzene, cadmium chloride, chlorpyrivos, 2,4-dichloroaniline, dioctylphtalate, hexachlorobenzene, pentachlorophenol, trichloroethylene and tetrapropylbenzosulfonate revealed the induction of thirteen to thirty-nine proteins, some of which overlapped with heat shock and carbon starvation proteins (Blom *et al.* 1992). Two-dimensional gel electrophoresis of proteins extracted from cells grown in the absence of nitrogen, carbon and phosphorus revealed the increased synthesis of proteins which were either unique or shared by these stresses (Matin, 1991). Furthermore, as exemplified by *htpR* (see section 1.2.2), key regulators can be defined.

## 1.3 GENE FUSIONS

It is difficult to identify and study the expression of genes that are involved in a cell's response to environmental stress if no known method to measure the expression and/or activity of its product is available. However, gene fusions can be employed such that the transcriptional or translational signals of a gene, whose expression is regulated by an environmental stress, controls the expression of another gene which produces a product that is easily assayable. The gene that produces a

measurable product acts as a "reporter" for the expression of the gene it is fused to. Transcriptional, or operon, fusions place an intact reporter gene downstream from the transcriptional start signals of a target gene (Figure 1B). Transcriptional-translational fusions fuse the promoter and translational signals of a target gene to a reporter gene lacking its own signals and results in the formation of hybrid proteins (Figure 1C) (Groisman, 1991).

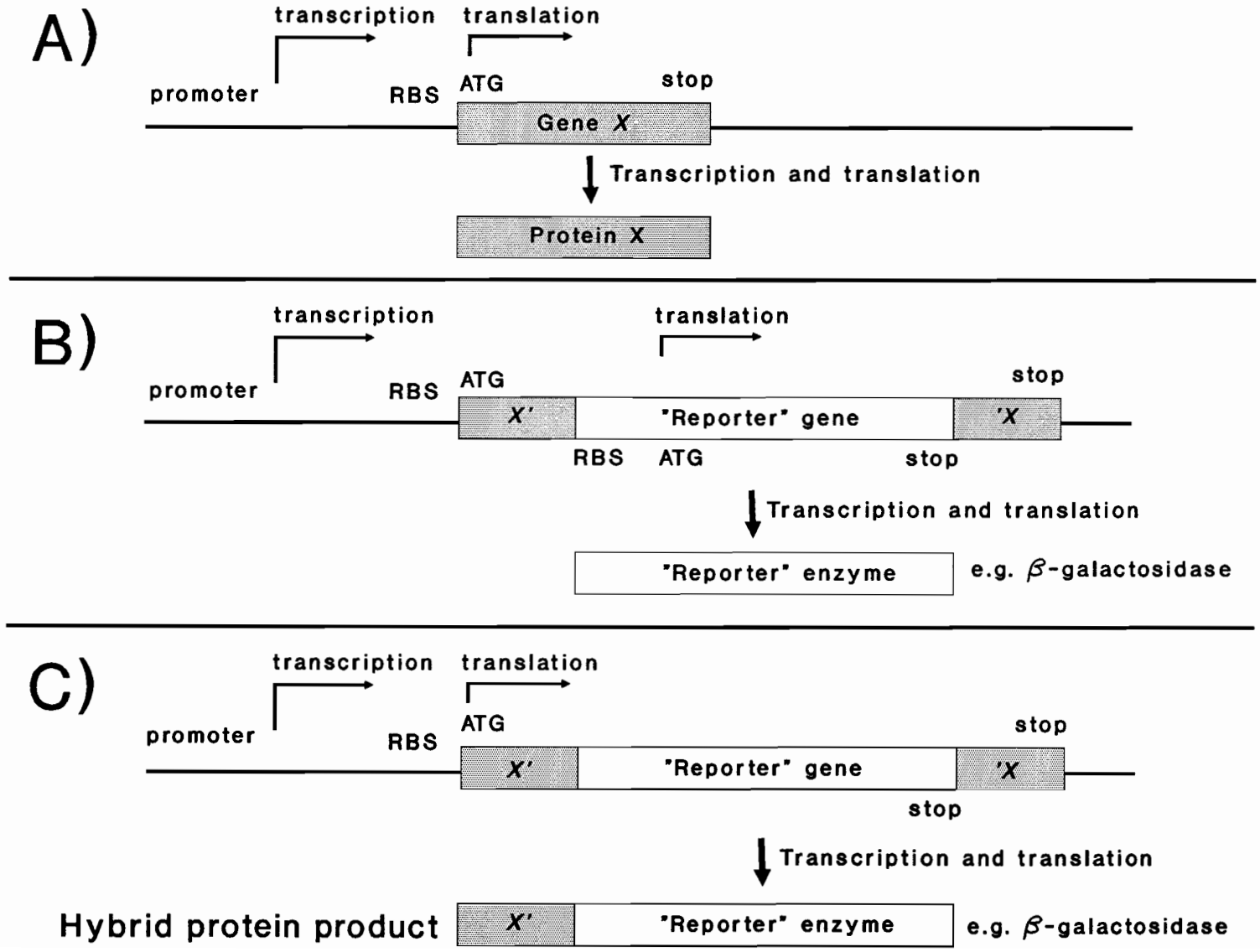
Gene fusions can be constructed either by using recombinant DNA techniques to insert the target gene into a vector containing a reporter gene or to insert the reporter gene into a vector containing the target gene (*in vitro* gene fusions). Alternatively, non-homologous recombination, or transposable elements, can be used to deliver the reporter gene to the target gene (*in vivo* gene fusions) (Casadaban *et al.*, 1983). This section concentrates on gene fusion technology, emphasizing methods employing transposable elements to create *in vivo* gene fusions, and includes a brief account of *in vitro* gene fusion vectors. Only nonessential genes can be fused to reporter genes in the chromosome, since insertion usually causes inactivation of the target gene. This can be overcome by creating the gene fusion on an extrachromosomal, self-replicating DNA molecule such as a plasmid.

### 1.3.1 History

Strong polar mutations early in *lacZ* eliminate *lacY* expression due to translational coupling with transcription (Newton *et al.*, 1965). When these mutants were selected on melibiose, which requires a functional *lacY* gene product for transport, revertants were isolated that were $lacZ^{-}Y^{+}$ and found to contain chromosomal deletions that fused *lacZ* to an upstream region (Beckwith, 1964). These fusions were used to localize the controlling region of the *lac* operon.

Phage $\phi$80 *lac* transducing particles were isolated and used to lysogenize the

Figure 1: Schematic of transcriptional/operon and translational gene fusions. A) The transcriptional and translational start sites of a hypothetical gene are indicated by arrows. B) The reporter gene contains its own ribosome binding site sequence and translational start site, hence a functional reporter gene product is made as long as the site of insertion occurs downstream of a transcriptional start site and in the appropriate orientation. C) The reporter gene lacks signals for translation, hence a functional, hybrid protein product is only produced when it is fused in frame with respect to the start site of the target gene. ATG= translational start site; RBS = ribosome binding site; stop = translational stop site.


A)
transcription
translation
promoter
RBS
ATG
stop
Gene X
Transcription and translation
Protein X
B)
transcription
translation
promoter
RBS
ATG
stop
X'
"Reporter" gene
'X
RBS
ATG
stop
Transcription and translation
"Reporter" enzyme
e.g. β-galactosidase
C)
transcription
translation
promoter
RBS
ATG
stop
X'
"Reporter" gene
'X
stop
Transcription and translation
Hybrid protein product
X'
"Reporter" enzyme
e.g. β-galactosidase

*lac* genes at the $\phi$80 attachment site near the *trp* operon, placing *tonB* between *trp* and *lac* (Beckwith *et al.*, 1966). The *tonB* gene, located between *trp* and *lac*, confers sensitivity to phages T1, $\phi$80, and to certain colicins. Selection for T1$^R$ cells yielded, at a low frequency, deletions which brought the *lacZY* genes under control of the *trp* promoter/operator (Miller *et al.*, 1970). This provided an early example of the fusion of the *lac* operon to an exogeneous gene. The utility of the *lac* operon to measure gene expression stems from the many techniques available for selection of different lactose phenotypes, and the ease of assay of the *lacZ* gene product, $\beta$-galactosidase.

### 1.3.2 *lacZ* as a Reporter Gene

The study of the *lac* genes of *E. coli* led to the operon theory of genetic regulation, involving an operator-repressor controlling element (Jacob *et al.*, 1960; Pardee *et al.*, 1959). When *E. coli* is growing on a carbon source other than lactose, the expression of the genes coding for products involved in lactose metabolism, *lacZY* and *A*, is repressed by the lactose repressor, product of *lacI*. Upon the addition of lactose to the medium, the repressor is inactivated and *lacZYA* is cotranscribed. Due to their coordinate regulation and expression, *lacZYA* was termed an operon. The *lacZ* gene codes for $\beta$-galactosidase. Lactose is a disaccharide that is cleaved by $\beta$-galactosidase to produce galactose and glucose. Lactose is transported into the cell by the permease, encoded by *lacY*. The *lacA* gene codes for a thiogalactoside transacetylase, the function of which is unclear. One hypothesis is that it is acts to detoxify certain toxic analogs of lactose (Andrews and Lin, 1976).

Due to its extensive biochemical characterization, several useful assays for $\beta$-galactosidase activity have been developed (Beckwith, 1987; Silhavy and Beckwith, 1985; for protocols refer to Miller, 1992). Because lactose is a sugar, it can be used

as a carbon source for selection. Alternatively, rich media with indicator dyes are available that produce red colonies when the cells are Lac$^+$ (Lactose-MacConkey agar) or red colonies when the cells are Lac$^-$ (tetrazolium agar). Lac$^+$ cells grown on eosin-methylene blue plates produce dark purple colonies which display a green sheen, while Lac$^-$ cells are pink or white. Lactose-MacConkey agar plates are particularly useful for selecting Lac$^+$ bacteria arising from a Lac$^-$ population. Lactose-MacConkey is a rich media that allows growth of Lac$^-$ cells, but once the nutrients are exhausted, the cells stop growing. However, lactose is also present in the media, so that Lac$^+$ revertants can still grow and will appear as red microcolonies, called papillae, which outgrow the rest of the white colony. Many rare Lac$^+$ revertants ($<1$ in $1.0 \times 10^{10}$) can be isolated on a single plate (Silhavy and Beckwith, 1985).

Lactose analogs have been synthesized that substitute different moieties for glucose. One of these, orthonitrophenyl-$\beta$-D-galactoside, when cleaved, produces a yellow colour that can be measured spectrophotometrically. A quantitative assay has been devised using this substrate that can measure a $10^6$-fold range of enzyme activity. Another indicator, Xgal, produces a blue colour upon cleavage. It is active in minimal and rich media plates and does not require the *lacY* gene product to be transported intracellularly. Cleavage of orthonitrophenyl-thiogalactopyranoside, transported via the lactose permease, produces a compound that is bacteriostatic, hence allowing selection for Lac$^-$ clones. Some problems for selection can occur if a mutation is leaky, and although mutant, there is enough expression or activity to allow growth. The analog phenylethyl-$\beta$-D-thiogalactoside is an inhibitor of $\beta$-galactosidase and thus can be added to the medium at concentrations which inhibit leaky expression of $\beta$-galactosidase, hence mitigating growth of the mutant.

To be able to analyze the expression of any *E. coli* gene using *lacZ* as a reporter gene, it is necessary to be able to fuse the *lacZ* gene to chromosomal genes.

### 1.3.3 *lac* Gene Fusions in Two Steps

The first method devised to systematically obtain *lac* fusions to promoters in the *E. coli* genome involves two steps. The first is to obtain a Δ*lac* lysogen of Mu into the target gene by selecting for loss/gain of function (e.g. *trp*⁻). This strain is then infected with a specialized λ transducing phage, λ p1(209). λ p1(209) contains the Mu left end and the *lacZ* and *Y* genes in the *b* region of λ, causing deletion of its attachment site. Hence the phage can form stable lysogens after recombination between homologous Mu sequences, placing the left end of Mu between the target gene and the *lacZY* genes. Because the Mu phage contains a temperature-sensitive repressor, growth of the lysogen at elevated temperatures selects for strains that have a deletion of Mu, due to illegitimate recombination, placing *lacZY* in proximity of the target gene's promoter (Casadaban, 1976).

### 1.3.4 *lac* Gene Fusions in One Step

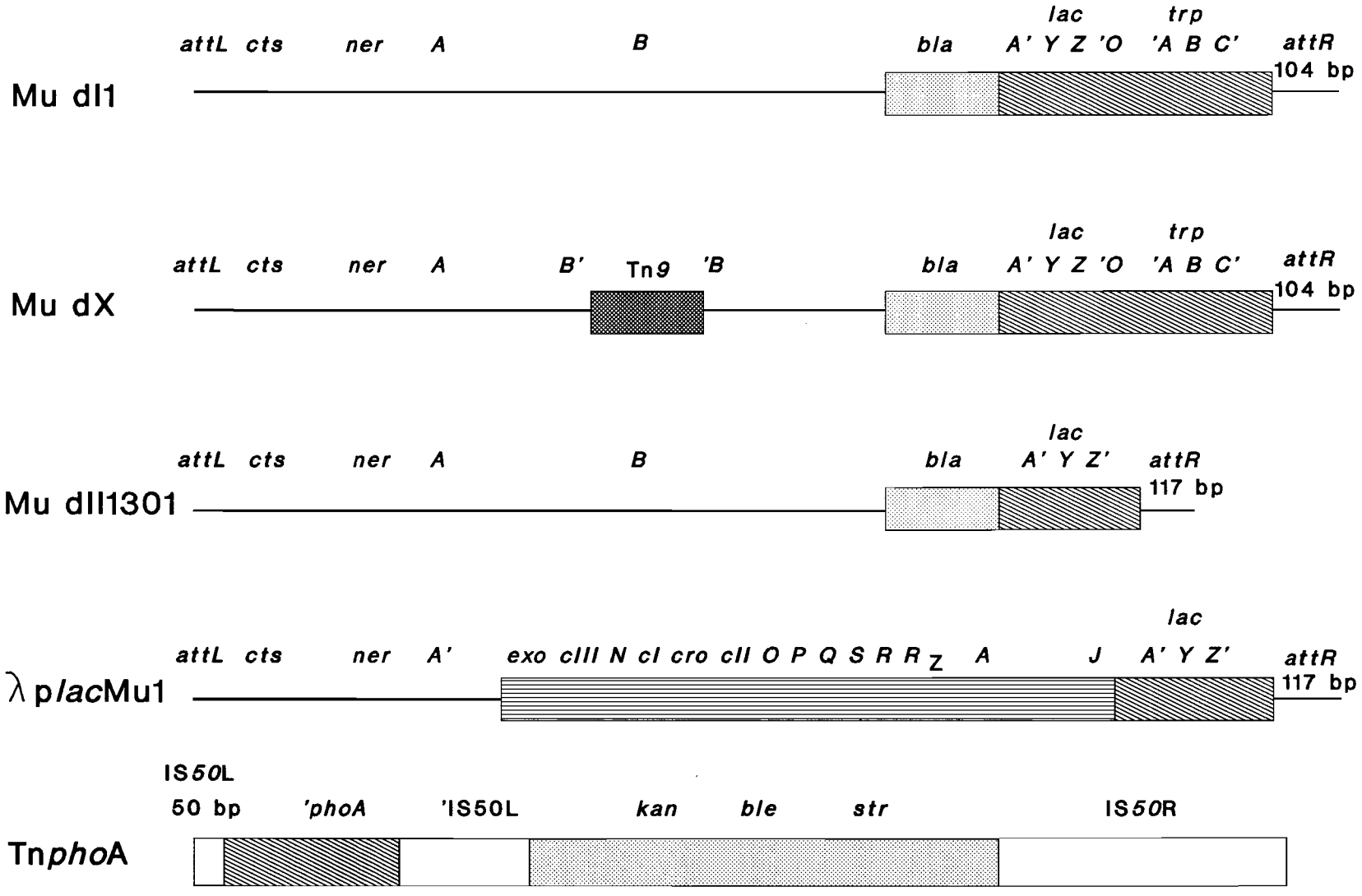
The Mu phage derivative, Mu dI1, contains the *lacZYA'* genes and the *bla* gene in between its left and right ends. Also, the Mu *A* and *B* genes, essential for transposition, remains present. Hence, this truncated and defective Mu phage is competent for transposition, but because no transcriptional start or stop sites are present within the 1.5 kb preceeding the *lacZ* coding region, transcription can proceed through the right end into the *lacZ* gene. Mu dI1 requires a helper phage for packaging (Figure 2) (Casadaban and Cohen, 1979). Recently the right end of Mu dI1 was sequenced and shown to contain a large inverted repeat, called Mu d1-R, with only the first 48 bp corresponding to the Mu *attR* sequence of the 104 bp that are not part of the *trp-lac* fusion joint (Metcalf *et al.*, 1990). Another Mu phage derivative, Mu dII1301, constructed using *in vivo* and *in vitro* methods, that shortened the 1.5 kb right end preceeding *lacZ* to 117 bp, contains no translational start

or stop codons, but is still able to transpose (Figure 2) (Casadaban and Chou, 1984; Bremer *et al.*, 1984). If transposition of Mu dII1301 occurs in the correct reading frame with respect to an upstream translational start site, an active hybrid β-galactosidase protein can be formed. Mu d transcriptional and translational fusion generating vectors are given a I and II designation, respectively.

### 1.3.5 Other Useful Reporter Genes

Since the initial use of the *lac* genes to create gene fusions to study transcription and translation, many other genes, expressing products that are easily assayable, have been described. The *phoA* gene codes for alkaline phosphatase, which is assayed colorimetrically using the substrate 5-bromo-4-chloro-3-indoyl phosphate, or quantitated using paranitrophenyl phosphate (Michaelis *et al.*, 1983). Alkaline phosphatase is active only in the periplasm and can thus be used to measure the regulation of membrane, or secreted proteins, if a translational fusion is prepared. The *luxAB* genes from *Vibrio harveyi* or *V. fischeri* code for luciferase, an enzyme that catalyzes a reaction which ends in light emission (for review, refer to Meighen, 1991). Hence, fusions to *luxAB* can be quantitated simply using luminescence as the endpoint. The Lumi-Gal $^{Tm}$ substrate for *lacZ* is available which when cleaved produces a chemiluminescent product (Beale *et al.*, 1992). Another substrate for *lacZ*, methylumbelliferyl-β-D-galactoside, yields a fluorescent product. The *uidA* gene codes for an acid hydrolase that cleaves β-glucunorides. Colorimetric assays have been described that make this an attractive complement to *lacZ* (Jefferson *et al.*, 1986). The *cat* gene codes for chloramphenicol transacetylase which can be selected either as resistance to chloramphenicol or assayed using a color substrate (Shaw, 1966). Other genes used for selection code for resistance to the antibiotics kanamycin, tetracycline, ampicillin, streptomycin and bleomycin (summarized in

Figure 2: The physical structures of several gene fusion vectors. The vectors are not drawn to scale. Symbols used for different DNA derivatives are: Mu (-----), lambda (  ), Tn*5* (  ), antibiotic resistance genes (  ), reporter genes (  ).


attL cts ner A B bla lac A' Y Z 'O trp 'A B C' attR 104 bp
Mu dI1
attL cts ner A B' Tn9 'B bla lac A' Y Z 'O trp 'A B C' attR 104 bp
Mu dX
attL cts ner A B bla lac A' Y Z' attR 117 bp
Mu dII1301
attL cts ner A' exo cIII N cI cro cII O P Q S R R Z A J lac A' Y Z' attR 117 bp
λ placMu1
IS50L
50 bp 'phoA 'IS50L kan ble str IS50R
TnphoA

Berg and Berg, 1987). However, quantitation is limited to determining the minimal inhibitory concentration or the dose at which 50% of the cells die. These genes afford the advantage that antibiotics can be used to select for promoters induced by environmental agents via selection for antibiotic resistance in the presence of the environmental agent.

### 1.3.6 *In Vivo* Gene Fusion Vectors

Since the initial construction of Mu dI1 and Mu dII1301, close to 100 different Mu derivatives have been described (Berg and Berg, 1987; Berg *et al.*, 1989; Faelen, 1987; Groisman, 1991; Slauch and Silhavy, 1991; Van Gijsegem *et al.*, 1987). The Mu dI1 phage contains a temperature sensitive repressor, and is thus unstable at high temperatures. The Mu *A* and *B* genes, required for transposition, are intact, and thus secondary transpositions occur at high enough frequencies to make isolation of second site mutants affecting expression of the gene fusion difficult (Groisman, 1991). To increase the stability of gene fusions, Komeda and Iino (1979) devised techniques to convert the resident transposition competent Mu d prophage to one that is no longer capable of transposition, or to delete one end of Mu. However, a Mu d prophage that is enfeebled in transposition can also be employed. Mu dX contains Tn*9* transposed into the Mu *B* gene, rendering it nonfunctional (Figure 2) (Baker *et al.*, 1983). Mu dI1681 is a Mu dI1 derivative in which the Tn*5* *kan* gene replaces the *bla* gene (Castilho *et al.*, 1984). Deletion of the Mu *A* and *B* genes in Mu dI1681 resulted in phage Mu dI1781 that is stable and temperature resistant, but requires the transposition functions to be provided *in trans* (Castilho *et al.*, 1984). Another Mu dI1 derivative, Mu dI-8, contains an amber mutation in the Mu *A* gene. Thus, fusions are stable in a suppressor-minus background, but can be generated either with a helper phage or in a suppressor-plus background (Hughes and Roth,

1984). Another vector, called λ p*lac*Mu1, is a derivative of Mu dII1301 which has the *bla* gene and part of Mu replaced by the λ genome, but has the λ attachment site deleted (Figure 2) (Bremer *et al.*, 1984). Hence, insertions are generated at random using the ends of Mu and a helper phage. However, specialized transducing phages can be isolated by selecting for excision via illegitimate recombination, allowing the chromosomal DNA adjacent to the fusion to be cloned. The *kan* gene was introduced into λ p*lac*Mu1 generating λ p*lac*Mu9 (Bremer *et al.*, 1985). The λ p*lac*Mu50 phage is analogous to λ p*lac*Mu1 but was derived from Mu dI1, generating type I fusions (Bremer *et al.*, 1985). A derivative of the λ p*lac*Mu50 phage which contains the *kan* gene and an amber mutation in Mu *A* is called λ p*lac*Mu55 (Bremer *et al.*, 1988).

Many other gene fusion vectors have been constructed that each use a transposable element and a selectable marker and/or reporter gene (reviewed in Berg and Berg, 1987; Berg *et al.*, 1989; Slauch and Silhavy, 1991). One useful protein fusion generating vector is a Tn*5* that contains the *phoA* gene, called Tn*phoA* (Figure 2). The *phoA* gene in Tn*phoA* lacks its own signal sequence and activity is dependent upon fusion to the signal sequences of other periplasmic or transmembrane proteins (Manoil and Beckwith, 1985). It has also been used to distinguish between the cytoplasmic versus periplasmic domains in proteins (Manoil and Beckwith, 1986). *PhoA* and *lacZ* translational fusions can be used as complementary reporter genes for secreted versus cytosolic protein activity, and vectors have been created that allow *lacZ* and *phoA* to be interchanged by recombination (Manoil, 1990; Wilmes-Riesenberg and Wanner, 1992). A Mini-Mu-*lux* was created, and screening of Lac$^-$ cells containing this transposon showed that some had an IPTG-inducible luminescent phenotype due to insertion of *luxAB* into the *lac* operon (Engebrecht *et al.*, 1985). A mini-Tn*5* series was recently described that allows the

isolation of gene fusions to *lacZ*, *phoA*, *xylE* and *luxAB* (de Lorenzo *et al.*, 1990). The most popular transposon for creating gene fusions is Mu, but others include Tn*3*, Tn*5*, Tn*10* and gamma-delta, to mention but a few (Berg and Berg, 1987; Berg *et al.*, 1989; Slauch and Silhavy, 1991).

### 1.3.7 Delivery of *In Vivo* Gene Fusion Vectors

Gene fusion vectors can be introduced into *E. coli* either from a phage by transduction, or from plasmids using transformation or conjugation. To isolate gene fusions to study the expression of a single chromosomal gene, it is necessary to limit the replication of the donor molecule containing the gene fusion vector. The most popular phages are λ and Mu. For λ vehicles, either the replication genes or the attachment site is deleted. Temperate, transposable phage Mu can form lysogens, hence most Mu vectors are defective but require a helper phage for packaging. Plasmids are used under conditions in which replication is inhibited (Table 3) (Berg *et al.*, 1989; Kleckner *et al.*, 1991).

### 1.3.8 *In Vitro* Gene Fusion Vectors

Plasmid vectors have been designed that contain restriction enzyme sites upstream of a reporter gene such that a DNA fragment containing its own transcriptional or transcriptional-translational signals can be cloned into it. Alternatively, vectors are available that allow the isolation of the reporter gene with restriction enzymes which can subsequently be cloned into a vector downstream of the target gene (Casadaban *et al.*, 1983; Slauch and Silhavy, 1991). These are particularly useful for studying the expression of essential genes or for mutagenizing the target gene. Transcriptional fusion vectors have been problematic because of the high level of readthrough from plasmid promoters. This is overcome by placing transcrip-

## Table 3

## Delivery of Gene Fusion Vectors

### Phage Delivery Vehicles and Strategies

Strategy

Defective for replication
Defective for site-specific recombination
Restriction of infecting phage DNA

### Delivery of Plasmids by Conjugation or Transformation

Strategy

Narrow host range
Host or plasmid mutation required for replication
Plasmid-plasmid incompatibility
Plasmid-host incompatibility
Inhibition of plasmid replication or stability *in trans*
Restriction of vector DNA

tional terminators upstream of the cloning site of the target gene (Slauch and Silhavy, 1991). In addition to the numerous vectors described which contain reporter genes such as *lacZ*, *galK*, *phoA*, *bla* and *cat* on different types of plasmid replicons and phages (Slauch and Silhavy, 1991), a set of promoter-probe vehicles containing a pair of promoterless *lacZ*, *luxAB* or *phoA* genes were constructed to study transcriptional control of divergent promoters (Ronald *et al.*, 1990).

## 1.4. USE OF GENE FUSIONS TO IDENTIFY GENETIC PROGRAMS

One of the great benefits of gene fusions has been in their ability to identify genes that are regulated by an environmental stimulus. To find genes that are regulated by environmental stresses, a library of gene fusions can be constructed and screened in the absence and the presence of the environmental agent to look for differences in the activity of the reporter gene's product. Once a gene fusion has been isolated, the activity of the environmentally responsive gene can be studied. Furthermore, the reporter gene can act as a tag to genetically map or isolate the gene, so that the function of its product can be determined. In this section, the uses of gene fusions to identify genes regulated by stressful enviromental stimuli are investigated. A summary of this section is presented in Table 4.

### 1.4.1 DNA Damaging Agents

The first example of the use of gene fusions to dissect genetic regulatory networks in response to environmental stimuli utilized phage Mu dI1. It was known that after *E. coli* was exposed to DNA damaging agents, physiological changes, such as the onset of filamentous growth, ensued and that lysogenic prophages such as λ were induced to grow lytically. This has come to be known as the SOS response, which was abolished in *lexA*$^-$ or *recA*$^-$ cells. Kenyon and Walker (1980) wanted to

**Table 4: Examples of Genes Responsive to an Environmental Stress Discovered by Gene Fusion Approaches**

| Gene Fusion | Vector | Inducing Agent | Gene/Map Position* |
|---|---|---|---|
| *dinA* | Mu dI1 | mitomycin C, UV | *polB* |
| *dinB* | Mu dI1 | mitomycin C, UV | near *lac* |
| *dinD* | Mu dI1 | mitomycin C, UV | between *mtl* and *argE* |
| *dinE* | Mu dI1 | mitomycin C, UV | *uvrA* |
| *dinF* | Mu dI1 | mitomycin C, UV | near *lexA* |
| *dinGop*::*lacZ* | pLKL5 | mitomycin C | 17.8 min |
| *dinHop*::*lacZ* | pLKL5 | mitomycin C | 19.8 min |
| *dinY* | Mu dX | mitomycin C, UV in *lexA*(Def) | 41-41.5 min |
| *aidA*::*lacZ* | Mu dI1 | MNNG, MMS | *alkA* |
| *aidB*::*lacZ* | Mu dI1 | MNNG, MMS | 95 min, near *mutL* |
| *aidC*::*lacZ* | Mu dI1 | MNNG | 92 min, near *uvrA* |
| *aidD*::*lacZ* | Mu dI1 | MNNG, MMS | *alkB* |
| *aidI*::*lacZ* | Mu dI1 | MMS | 13 to 23 min |
| *soi-17*::*lacZ*/*soi-19*::*lacZ* | Mu dX | paraquat, plumbagin | 45 to 61 min |
| *soi-28*::*lacZ* | Mu dX | paraquat, plumbagin | 47 min |
| *soi-5* | Mu dI1 | paraquat, plumbagin, menadione | 6 to 26 min |
| *soi-7* | Mu dI1 | paraquat, plumbagin, menadione | 6 to 26 min |
| *soi-8* | Mu dI1 | paraquat, plumbagin, menadione | 6 to 26 min |
| *soi-11* | Mu dI1 | paraquat, plumbagin, menadione | 6 to 26 min |
| *soi-13* | Mu dI1 | paraquat, plumbagin, menadione | 6 to 26 min |
| *soi-9* | Mu dI1 | paraquat, plumbagin, menadione | 26 to 36 min |
| *soi-10* | Mu dI1 | paraquat, plumbagin, menadione | 26 to 36 min |
| *soi-12* | Mu dI1 | paraquat, plumbagin, menadione | 26 to 36 min |
| *cst-2*::*lacZ* | λ p*lac*Mu9 | carbon starvation in *cya*$^+$ | 10 to 15 min |
| *cst-4*::*lacZ* | Mu dX | carbon starvation in *cya*$^+$ | ND |
| *cst-8*::*lacZ* | λ p*lac*Mu9 | carbon starvation in *cya*$^+$ | ND |
| *csi2*::*lacZ* | λ p*lac*Mu55 | carbon starvation in *cya*$^-$ | *katF* |
| *psiA*::*lacZ* | Mu dI1 | phosphate starvation | *phoA* |
| *psiB*::*lacZ* | Mu dI1 | phosphate starvation | *ugp* |
| *psiC*::*lacZ* | Mu dI1 | phosphate starvation | *ugpB* |
| *psiD*::*lacZ* | Mu dI1 | phosphate starvation | *phnD* |
| *psiE*::*lacZ* | Mu dI1 | phosphate starvation | between *malG* and *xylE* |
| *psiF*::*lacZ* | Mu dI1 | phosphate starvation | downstream of *phoA* |
| *psiH*::*lacZ* | Mu dI1 | phosphate starvation | *phoH* |
| *psiP*::*lacZ* | Mu dI1 | phosphate starvation | *himA* |
| *psiQ35*::*lacZ* | Mu dI1 | phosphate starvation | *gltB* |
| *psiQ39*::*lacZ* | Mu dI1 | phosphate starvation | *gltD* |
| *psi-51*::*lacZ* | Mu dI1 | phosphate starvation | *glpB* |
| *exa* | Mu dI1734 | low external pH | *cadA* |
| *ina*-1 | Mu dI1734 | low internal pH | ND |
| *ina*-2 | Mu dI1734 | low internal pH | ND |
| *alx*-1 to -4 | Mu dI1734 | high external pH | 67.5 min |
| *phmA* | Tn*phoA* | high external pH | 12 min |
| *phmB* | Tn*phoA* | high external pH | 18 min |
| *phmC* | Tn*phoA* | high external pH | 41 min |
| *phmD* | Tn*phoA* | high external pH | 45 min |
| *phmE* | Tn*phoA* | high external pH | 75 min |

**Table 4 (continued):**

| Gene Fusion | Vector | Inducing Agent | Gene/Map Position |
|---|---|---|---|
| *phmF* | Tn*phoA* | high external pH | 84 min |
| *osr-2* | Mu dI1 | high osmolarity | *proU* |
| *osr-3* | Mu dI1 | high osmolarity | *kdp* |
| *osr-4* | Mu dI1 | high osmolarity | about 20 min |
| *osr-5* | Mu dI1 | high osmolarity | *lamB* |
| *osmA* | Tn*phoA* | high osmolarity | 26 min |
| *osmB* | Tn*phoA* | high osmolarity | 28 min |
| *osmC* | Tn*phoA* | high osmolarity | 32.5 min |
| *osmD* | Tn*phoA* | high osmolarity | 34.5 min |
| *osmE* | Tn*phoA* | high osmolarity | 38 min |
| *osmF* | Tn*phoA* | high osmolarity | 46 min |
| *osmG* | Tn*phoA* | high osmolarity | *proU* |
| *osmH* | Tn*phoA* | high osmolarity | 77.5 min |
| *osmI* | Tn*phoA* | high osmolarity | 77.5 min |

Symbols:

ND, not determined

*, See text for References.

assess whether there was a set of genes whose expression was induced by DNA damaging agents. The only genes known to be induced by DNA damage were *recA* (Gudas and Mount, 1977; McEntee, 1977) and those of a λ prophage (Witkin, 1976). A collection of 35 000 *E. coli* colonies containing the Mu dI1 prophage was screened in the absence and presence of 1 μg/ml of the DNA damaging agent mitomycin C. Twenty-two clones were identified that displayed increased β-galactosidase synthesis with mitomycin C, ten of which were of independent origin. The sites of Mu insertion in these isolates were designated *din* (damage-inducible loci). The induction ratio, defined as the β-galactosidase activity in the presence of mitomycin C versus its absence, ranged from 2.7 to 24 for the *din*::Mu dI1 strains. All of the Mu dI1 fusion strains were also induced by UV irradiation. Induction by mitomycin C was abolished when either a *recA*$^-$ or *lexA*$^-$ mutation was transduced into the *din*::Mu dI1 fusion strain. Interrupted mating experiments and P1 transductions assigned the Mu dI1 insertions to at least five different loci, designated *dinA, B, D, E* and *F*. This led them to postulate that the SOS functions may be encoded by *din* genes that are coordinately expressed by promoter-regulatory regions. But it was also realized that expression of each gene may be individualized due to differences in the observed basal and final levels, and the time of induction after exposure.

Today, it is known that under normal conditions, SOS-inducible *E. coli* DNA repair enzymes are not expressed because their promoters are blocked by the LexA repressor. In the presence of excess single-stranded DNA (caused by DNA damage or stalled replication forks), RecA protein becomes activated as a protease and cleaves LexA, reducing its ability to bind to DNA as an effective repressor. RNA polymerase is thus able to transcribe genes encoding the repair enzymes, ultimately resulting in their expression, and thus in repair of the damaged DNA. A consensus DNA sequence, to which LexA binds, was delineated from a comparison of the

DNA sequences from regions upstream of LexA regulated genes. Mu dI1 fusions to genes thought to be induced by DNA damaging agents has shown that *uvrA*, *uvrB*, *sulA*, *umuDC*, *himA*, *uvrD*, *ruv*, *recA* and *recN* are all part of the SOS regulon (Walker, 1987). The *dinE* gene was shown to be *uvrA* (Kenyon and Walker, 1980) and *dinA* to be the *polB* gene (Bonner *et al.*, 1990). The *dinB* gene mapped near *lac*, the *dinD* gene was located between *mtl* and *argE*, while the *dinF* gene mapped near *lexA* (Kenyon and Walker, 1980). Recently, two new *din* genes have been isolated by ligating *E. coli* chromosomal DNA hydrolyzed with *Sau*3A into a multicopy vector containing the *galK* gene as a reporter gene (Lewis *et al.*, 1992). The *dinG* gene was localized to 17.8 minutes and the *dinH* gene to 19.8 minutes on the *E. coli* genetic map using the Kohara library (Kohara *et al.* 1987). The Mu dX phage was used to isolate a gene, called *dinY*, that is UV- or mitomycin-inducible in a strain that is *lexA*(Def) (Petit *et al.*, 1993). Induction required activated RecA protease. The *lacZ* reporter gene was localized to between 41 and 41.5 minutes on the *E. coli* genetic map close to the *ruv* operon. Hence, *lacZ* gene fusions are currently being used to define new promoters and genes that are inducible by DNA damage.

Another DNA repair pathway, different than the SOS response, was defined and called the adaptive response. Exposure to sublethal levels of alkylating agents protects *E. coli* from the mutagenic and lethal levels of higher exposure to these agents (Jeggo *et al.*, 1977; Samson and Cairns, 1977). Two induced enzymes were identified. The *alkA* gene codes for a glycosylase that removes alkylated bases leaving an abasic site, and the *ada* gene codes for a methyl transferase which has two methyl accepting sites. Methyl transfer to one of the sites activates Ada as a positive transcriptional regulator (Lindahl *et al.*, 1988; Volkert, 1988). Using the Mu dI1 phage, sixty-five gene fusions, inducible after treatment with either MMS or MNNG, were found (Volkert and Nguyen, 1984; Volkert *et al.*, 1986), of which fifty-

one were also UV-inducible. The fourteen fusions, specifically MMS- and/or MNNG-inducible, were named *aid* (alkylation inducible genes), and mapped to five different loci. The *aidA*::*lacZ* genes mapped to *alkA* at 45 to 45.5 minutes on the *E. coli* genetic map. The *aidD* fusions mapped to within 0.01 minutes of the *ada* gene, but did not inhibit alkylating agent inducibility, and is thus probably a fusion to the *alkB* gene, the gene immediately downstream of *ada*. The *aidB* gene maps near *mutL* at 95 minutes and *aidC* maps close to *uvrA* at 92 minutes. The *aidI* gene mapped between 13 and 23 minutes. The *alkA*, *aidB* and *aidD* genes were inducible by both MNNG and MMS, whereas *aidC* is only induced by MNNG and *aidI* is induced by MMS. Introduction of an *ada10*::Tn*10* mutation into the *aid*::*lacZ* fusions abolished the ability of MNNG to induce *alkA* and *aidB*. However, alkylation inducibility remained intact for *aidC* and *aidI*. It was impossible to isolate an *ada10*::Tn*10* *aidD*::*lacZ* strain, due to the proximity of *ada* to *aidD*. This demonstrates that there is more than one alkylation-induced response which depends on the agent used, of which at least four genes were defined to be part of the adaptive response. The effects of different methylating agents was further explored and shown to be heterogeneous (Fram *et al.*, 1988). An *alkAalkB* double mutant displays a greater sensitivity to MMS or MNNG than either of the single mutants suggesting that these act on independent lesions or in different pathways of repair (Volkert and Hajec, 1991).

### 1.4.2 Oxygen Damage

The ability of organisms to use oxygen necessitated the evolution of systems to deal with the toxic by-products of aerobic metabolism. These by-products, which include the superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical, cause damage to DNA, RNA, proteins and membranes (Demple, 1991; Farr

and Kogoma, 1991). In *E. coli*, two enzymes catalyze the reduction of the superoxide anion radical to hydrogen peroxide and oxygen: MnSOD, encoded by *sodA* (Touati, 1983), and the iron-containing superoxide dismutase, encoded by *sodB* (Sakamoto and Touati, 1984). Two catalase enzymes catalyze the reduction of hydrogen peroxide to oxygen and water, encoded by *katG* (Loewen *et al.*, 1985b) and *katE* (Loewen, 1984). Treatment of cells with low concentrations of the superoxide radical generator, paraquat, protected the cells from higher doses (Hassan and Fridovich, 1977), and was found to be due to increased levels of MnSOD. Two other chemicals that generate superoxide radicals are menadione and plumbagin.

The Mu dX phage was used to isolate three *lacZ* gene fusions that were induced in the presence of paraquat and designated *soi*::*lacZ* (superoxide inducible) (Kogoma *et al.*, 1988). The three fusions were also induced in the presence of plumbagin, and when oxygen was bubbled through the media. Hydrogen peroxide induced β-galactosidase only at very high concentrations. OxyR is a positive regulator of nine hydrogen peroxide-inducible genes (Christman *et al.*, 1985). The *htpR* gene encodes the $\sigma^{32}$ subunit of RNA polymerase responsible for the transcription of heat shock proteins (Neidhardt and VanBogelen, 1987). RecA is the protease responsible for induction of the SOS response (Walker, 1987). When either an *oxyR*$^-$, *htpR*$^-$ or *recA*$^-$ mutation was introduced into the fusion-containing strains, there was no loss of induction in the presence of paraquat. The *soi-19*::*lacZ* and the *soi-28*::*lacZ* strains showed increased sensitivity to paraquat compared to wild-type, whereas the *soi-17*::*lacZ* strain did not. Genetic mapping of these three strains, using Hfr-mediated conjugation, showed that they were unlinked. However, the possibility that *soi-17*::*lacZ* and *soi-19*::*lacZ* were in the same gene at different locations was not ruled out. *Soi-28*::*lacZ* was localized to 47 minutes and *soi-17*::*lacZ*/*soi-19*::*lacZ* was localized to between 45 and 61 minutes. These fusions were later found to be

under *soxRS* control (see below) (Farr and Kogoma, 1991).

Two-dimensional protein gels revealed that approximately forty proteins are induced upon exposure to a superoxide generating agent, in addition to the other thirty to forty proteins induced in the presence of hydrogen peroxide (Greenberg and Demple, 1989; Walkup and Kogoma, 1989). Nine of the forty proteins inducible by superoxide-generating compounds are under the control of two regulatory genes, *soxR* and *soxS* (Greenberg *et al.*, 1990; Tsaneva and Weiss; 1990). Proteins known to be under the control of *soxRS* include: (1) MnSOD, (2) glucose-6-phosphate dehydrogenase, (3) NADH diaphorase, (4) an enzyme that adds glutamic acid residues to the carboxy terminus of ribosomal protein S6, (5) endonuclease IV, and (6) an antisense mRNA of *ompF* encoded by *micF* (see below) (Demple, 1991; Farr and Kogoma, 1991). Several other superoxide inducible gene fusions, different than those previously found by Kogoma *et al.* (1988), were isolated using Mu dI1 (Mito *et al.*, 1993). Approximately 20 000 Mu dI1 phage gene fusions were screened in the presence of paraquat. Of these, twelve were induced as shown by *in vitro* β-galactosidase assays, and eight were further studied. Plumbagin and menadione also induced the *soi*::*lacZ* gene fusions in the presence of oxygen. This induction was abolished when the cells were treated anaerobically. Hydrogen peroxide did not induce β-galactosidase, even at high concentrations. The induction of the eight *soi*::*lacZ* fusions in the presence of paraquat was abolished when *soxS*$^-$ was introduced into the strains, but not in an *oxyR*$^-$ or *recA*$^-$ background. At least five of the strains were found to be more sensitive to paraquat and menadione. Five of the *soi*::*lacZ* strains mapped to 6 to 26 minutes and the other three were localized to 26 to 36 minutes on the *E. coli* genetic map. None of these locations corresponded to previously mapped genes under the control of *soxRS*. These genes are currently being cloned and characterized.

Roles for some *soxRS* regulated proteins, induced during the superoxide stress response, are known. MnSOD is directly responsible for reducing the level of $O_2^-$. Glucose-6-phosphate dehydrogenase produces NADPH which provides electrons for thioredoxin reductase and glutathione reductase, two intracellular reducing agents. NADPH may also be depleted by chemicals that cause redox-cycling. DNA damaged by oxidation is repaired by endonuclease IV. The function of other proteins is not as clear. Increased synthesis of *micF*, an antisense *ompF* mRNA, reduces synthesis of OmpF, a porin involved in solute uptake. This causes exclusion of antibiotics and some other compounds. However its protective role in oxidative stress has yet to be demonstrated, since *ompF*$^-$ strains are as resistant to menadione as wild-type. It is still possible that other agents that cause oxidative damage are excluded by OmpF (Demple, 1991; Farr and Kogoma, 1991).

### 1.4.3 Carbon Starvation

When *E. coli* enters stationary phase, the cells become smaller and more spherical (Lange and Hengge-Aronis, 1991a) and the endogeneous metabolic rate, including bulk protein synthesis, decreases. Approximately thirty to fifty protein spots are induced 4 to 5 hours after carbon starvation (Groat *et al.*, 1986). These confer a general resistance to carbon starvation, oxidation, heat and osmotic stress (Jenkins *et al.*, 1988; 1990). Protein degradation was shown to be required for prolonged survival using peptidase mutants (Reeve *et al.*, 1984). This probably provides amino acids for the synthesis of starvation-induced proteins. The synthesis and excretion of cAMP is increased when cells are glucose starved (Buettner *et al.*, 1973). Analysis of two-dimensional protein gels in Δ*cya* or *crp E. coli* strains, that do not synthesize either cAMP or the cAMP receptor protein respectively, revealed that nineteen of thirty proteins required cAMP for expression. These were termed

Cst proteins (carbon starvation response proteins). The other eleven were designated Pex proteins (postexponential proteins). It was also found that the Δ*cya* strain was not more sensitive to carbon starvation than the wild-type strain.

To identify the genes involved in carbon starvation and to determine their regulation, *lacZ* fusions to the *cst* genes were isolated using Mu dX (Groat *et al.*, 1986) or λ p*lac*Mu9 phages (Schultz *et al.*, 1988). Analysis of three of the *cst*::*lacZ* fusions revealed different modes of regulation (Blum *et al.*, 1990). A Δ*cya* mutation abolished carbon starvation induction in all of the *cst*::*lacZ* strains. However, exogenous addition of cAMP restored the inducibility of the *cst*-2::*lacZ* fusion, but not that of *cst*-4::*lacZ* or *cst*-8::*lacZ*. Removal of glucose from exponentially growing cells induced *cst*-2::*lacZ* and *cst*-8::*lacZ*. All three fusions were dependent upon $\sigma^{70}$, the 'house-keeping' sigma subunit of RNA polymerase, for basal levels of transcription. However, only *cst*-2::*lacZ* showed increased transcription when cAMP was added. Hence, the other two *lacZ* fusions required some other factor produced in postexponential phase. The *cst*-2 gene fusion was mapped to between 10 to 15 minutes on the *E. coli* genetic map. The *cst*-4 and *cst*-8 gene fusions have not been mapped yet (Matin *et al.*, 1989).

Using the λ p*lac*Mu55 phage, chromosomal fusions to genes induced upon carbon starvation in a Δ*cya* strain were isolated. One of the gene fusions, *csi* 2::*lacZ* was analyzed (Lange and Hengge-Aronis, 1991b). Transition into stationary phase induced β-galactosidase 5-fold. When succinate was exchanged for glucose, expression was induced 2-fold. When the fusion was transduced into a *cya*$^+$ strain, β-galactosidase expression decreased, indicating that cAMP negatively regulates expression of this gene. When comparing wild-type or the Δ*cya* strain to the *csi* 2::*lacZ* strain, the half-life of survival during prolonged starvation conditions was reduced from 6 days to 1 day. At least sixteen proteins were shown to be missing in

the *csi* 2::*lacZ* strain when compared to the isogenic Δ*cya* strain after growth to late exponential phase. This strain was also unable to develop $H_2O_2$ resistance or stationary-phase thermotolerance. Cloning and sequencing of *csi* 2 revealed that it is allelic to the *katF* gene, which was known to positively activate catalase HPII and exonuclease III, essential enzymes for $H_2O_2$ resistance. The *katF* gene displays strong homology to *rpoD*, which encodes $\sigma^{70}$, and was thus renamed *rpoS*. It appears that *katF* is an alternative sigma factor required for the expression of stationary-specific functions.

The regulation of the carbon-starvation response is now known not to be a simple induction of an alternative sigma factor. There is a lack of a consensus sequence common to the promoter regions of all *rpoS*-controlled genes. Some *rpoS*-controlled genes have regulatory roles themselves. As well, the histone-like protein H-NS is involved in the regulation of some *rpoS* regulated genes. It has been suggested that additional regulatory proteins may be involved in transcription (Hengge-Aronis, 1993). The signal for increased synthesis of *rpoS* was shown to be homoserine lactone, a metabolite synthesized from intermediates in threonine biosynthesis (Huisman and Kolter, 1994).

Understanding the regulation of the starvation response is important to defining how the response occurs. The Cst proteins, not involved in starvation resistance, were postulated to enhance the cell's metabolic rate. The Pex proteins play a major role in the defense of the cell to carbon starvation. However, many of them remain to be identified (Matin *et al.*, 1989). One clue as to the role of some starvation proteins is that three of the Pex proteins were recognized to be DnaK, GroEL and HtpG, which are expressed during the heat shock response and are dependent upon *rpoH* rather than *rpoS* for expression (Jenkins *et al.*, 1991). These proteins are involved in the folding and macromolecular assembly of multi-subunit proteins. It is

possible that cells under stress tend to synthesize abnormally folded proteins and that these stress proteins may have a protective role (Matin, 1991). The *katF* gene product is involved in the expression of *katE*, a catalase, which would contribute to protection against oxidative damage (Loewen *et al.*, 1985a). Increased synthesis of trehalose, an osmolyte, occurs upon starvation and could contribute to increased thermotolerance and osmoprotection (Hengge-Aronis *et al.*, 1991).

### 1.4.4 Phosphate Starvation

The primary energy source in cells is ATP, which is formed from ADP and inorganic phosphate. ATP is also the primary phosphoryl donor for membrane lipids, complex carbohydrates, and nucleic acids. *E. coli* preferentially uses inorganic phosphate as a phosphate source, but can also use organophosphates and phosphonates. Two inorganic phosphate transport systems exist. The Pit system is low affinity and expressed constitutively. The Pst system is high affinity and inducible. The organophosphate *sn*-glycerol-3-phosphate can act as a phosphate donor, and is transported by the Ugp transporter system. Phosphonates, containing a C-P bond, may also act as a donor of phosphate in *E. coli* (Wanner, 1993).

Phage Mu dI1 was used to isolate fifty-five gene fusions that showed β-galactosidase induction when starved for phosphate, termed *psi* genes (phosphate starvation inducible). These were shown to be in at least twenty different genes and were placed into six different classes based upon inducibility in response to carbon or nitrogen limitation, UV, anaerobiosis and growth in tryptone-yeast extract medium. Seven *psiA*::*lacZ* fusions were localized to *phoA*, coding for alkaline phosphatase. Three *psiD*::*lacZ* fusions were localized to *phnD*, involved in phosphonate transport (Wanner and McSharry, 1982). The *psiB* and *psiC* genes were postulated to lie in the *ugp* operon, involved in *sn*-glycerol-3-phosphate transport. The *psiP* gene is thought

to be *himA*, which codes for the $\alpha$ subunit of integration host factor (Wanner, 1987). The effects of other regulatory mutations were also tested (summarized in Wanner, 1987). In order to identify the other *psi::lacZ* gene fusions, Metcalf *et al.* (1990) cloned and sequenced the junction at the site of Mu R insertion. Twenty base pairs of each sequence were compared to known DNA sequences. The *psiC28::lacZ* insertion was localized to *ugpB*. The *psiQ35::lacZ* and *psiQ39::lacZ* were mapped to *gltB* and *gltD*, respectively, which encode the two subunits of glutamine synthase. The *psi-51* fusion contains *lacZ* in *glpB*, coding for anaerobic glycerol-3-phosphate dehydrogenase. Three unknown genes were also localized to the genome: two *psiE* genes are in an open reading frame between *malG* and *xylE*, the *psiF* gene was localized to an open reading frame downstream of *phoA*, and three *psiH* insertions were in a new gene called *phoH* (Metcalf *et al.*, 1990; Wanner, 1993).

The study of phosphate starvation-inducible genes exemplifies how gene fusions were employed to identify a large number of genes involved in the transport and metabolism of different forms of phosphate and define the phosphate regulon. New genes were also found, the function of which remains to be elucidated. Furthermore, genes involved in nitrogen metabolism, the *gltBDF* operon, and carbon metabolism, the *glpABC* operon, were also identified and provides examples of the response of an operon to more than one environmental stimuli. Two distinct promoters have been defined in the *ugpBAECQ* response to phosphate and carbon limitation (Kasahara *et al.*, 1991).

### 1.4.5 Changes in pH

*E. coli* can grow in a pH range from 5.0 to 8.5. The internal pH remains relatively constant, between 7.4 to 7.8, independent of the external pH (Padan *et al.*, 1976; Slonczewski *et al.*, 1981; Zilberstein *et al.*, 1984). The study of enzyme activity

showed that decarboxylases were induced at low external pH and deaminases were induced at high external pH (Gale and Epps, 1942).

A search for genes induced in the presence of low external pH involved two screening methods (Slonczewski *et al.*, 1987). To screen for genes induced by a lower external pH, 82 000 Mu dI1734-*lacZ* gene fusions were grown on LB containing 100 mM MOPS (pH 7.4), which rose to pH 7.9 after growth, a range known to be optimal for *E. coli* internal pH, and should thus represent a low rate of gene expression. These were then replica plated onto LB plates containing 100 mM MES [2-(N-morpholino)ethanesulfonic acid] (pH 5.6) and Xgal. Two clones were found that were induced in the pH range of 5.0 to 6.3 and called *exa* (external-acid-inducible). Furthermore, they were induced to a higher level when grown anaerobically under low pH. Anaerobic induction did not occur at pH 7.6. A search for internal acid-inducible genes involved screening in the presence of membrane-permeable weak acids, which are protonated until they enter the cell, where the protons dissociate causing a decreased internal pH. Screening of 49 000 colonies on LB plates containing 100 mM PIPES [piperazine-N-N'-bis(2-ethanesulfonic acid)] (pH 6.8) and Xgal without and with 20 mM benzoate did not yield any differences in $\beta$-galactosidase activity. However, when 3 800 colonies were screened on Lactose-MacConkey media containing 20 mM benzoate, two clones were found that were induced. Lactose-MacConkey media is a less sensitive indicator than Xgal, and can hence be used to detect $\beta$-galactosidase changes in genes that display a higher basal level of expression. The two clones were called *ina* (internal-acid-inducible). Induction of the *ina*-1 and *ina*-2 genes varied in proportion to the strength of three weak acids that were tested (salicylate > benzoate > dimethyloxazoledinedione) suggesting that they are regulated directly by changes in internal pH. The *exa* genes have now been shown to be alleles of *cadA* which encodes lysine decarboxylase (Slonczewski,

1992). Construction of *lacZ* fusions to arginine decarboxylase, *adi*, have also shown this gene to be acid-inducible (Auger *et al.*, 1989). Amino acid decarboxylases produce basic amines which could act to neutralize an increase in internal pH. The *cadA* gene lies in the *cadBA* operon. CadC is postulated to be a membrane protein that can also bind to the operator of *cadBA* to induce transcription under acidic conditions (Watson *et al.*, 1992).

To search for genes induced by high external pH, 91 000 Mu dI1734 colonies were screened using LB containing either 100 mM PIPES (pH 6.5) or 100 mM TAPS [N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid] (pH 8.5) and Xgal (Bingham *et al.*, 1990). Four clones showed a 100-fold increase in $\beta$-galactosidase at the alkaline pH, and were designated *alx*-1 to -4. All were found to map at about 67.5 minutes on the *E. coli* genetic map. Induction occurred over the pH range 5.0 to 8.7, with a sharp increase between pH 8.0 to 8.7. No induction occurred in the presence of the weak membrane-permeable base, diethanolamine. Thus these genes were specifically induced by changes in external pH. Using Tn*phoA*, Heyde *et al.* (1991) identified genes that were induced over the pH range of 5.0 to 8.5. Mapping of the Tn*phoA* insertions revealed that in addition to *ompF* and *lamB*, insertions had occurred in six different genetic loci designated *phmA* to *phmF* (pH modulated) and mapped to 12, 18, 41, 45, 75 and 84 minutes on the *E. coli* genome. Induction ranged from 2.5-fold to 20-fold. A decrease in pH had previously been shown to cause a decrease in the amount of outer membrane porins OmpF and LamB, and an increase in the amount of OmpC (Heyde and Portalier, 1987). OmpR and EnvZ modulate the expression of OmpC and OmpF, and *envZ*$^-$ cells have an altered ability to modulate OmpF and OmpC as a function of pH (Heyde and Portalier, 1987). The only effect of *ompR*$^-$ or *envZ*$^-$ was on *phmA*::*phoA* where expression was increased at pH 8.5. Addition of sodium benzoate led to 5- and 2-

fold decreases in *phmA* and *ompF* gene expression respectively, and a 2-fold increase in *ompC*. Tn*phoA* gene fusions to both *malB* operons were isolated. It was shown that in the maltose regulon, only genes coding for components of the transport system are pH regulated. These results suggest that the expression of individual membrane proteins have evolved to cope with changes in pH. The *phm* genes may include $H^+$ transporters that aid to maintain internal homeostasis. The sodium-proton antiporter, NhaA, is induced at high external pH in the presence of increased sodium (Karpel *et al.*, 1991). The *alx* gene maps to a different position than the *phm* loci and thus might display a different protective mechanism.

### 1.4.6 Osmotic Stress

*E. coli* can grow in the osmolarity range of 0 to 1 200 mOsm, approximately 0.7 M NaCl. At high osmolarity, water diffuses out of the cell, decreasing the turgor pressure, resulting in plasmolysis. Restoration of the turgor pressure occurs after increasing the cytoplasmic osmolarity by accumulating intracellular $K^+$ and synthesizing glutamate (Ingraham, 1987). Some of the potassium glutamate is replaced by other compatible solutes, the organic osmolytes proline, glycine betaine and trehalose, to relieve the harmful effects of high intracellular $K^+$ concentrations (Csonka, 1989).

Mu dI1 was used to find genes that are induced in the presence of 0.4 M NaCl when grown on $K^+$ medium (Gowrishankar, 1985). Of 3 000 *lacZ* gene fusions, three were shown to be induced and one was shown to be repressed upon an increase in osmolarity and were called *osr*-2 to -5 (osmoresponsive genes). Mapping of *osr*-2, a gene induced 400-fold, to 57.5 minutes suggested that it could be similar to *proU* in *Salmonella typhimurium*, and subsequently confirmed by further functional studies. The *proU* gene encodes a glycine-betaine transport protein also capable of

transporting proline. The *osr*-3::*lacZ* fusion was localized to the *kdp* gene, which encodes a repressible, high-affinity $K^+$ transporter. The β-galactosidase activity of *osr*-3 was increased 12-fold in high osmolarity, but only when $K^+$ was limiting. The repressible *osr*-5::*lacZ* strain was localized to *lamB*, a porin involved in maltodextrin transport and λ adsorption. The *osr*-4::*lacZ* gene fusion mapped to approximately 20 minutes on the *E. coli* genetic map.

Another search for genes regulated by osmolarity employed Tn*phoA* to specifically find genes encoding cell envelope proteins. From among 30 000 Tn*phoA* fusions, thirty-seven were induced after growth in high osmolarity and fell into ten linkage groups (Gutierrez *et al.*, 1987) of which one group was found to contain *phoA* in the *ompC* gene, as shown by their resistance to phage hy2, and was not given an *osm* designation. The rest were designated *osmA* to *osmI* and mapped to 26, 28, 32.5, 34.5, 38, 46, 57.5, 77.5 and 77.5 minutes on the *E. coli* genome, respectively. One gene fusion, *osmG*, was identified as the *proU* gene. The rest of the *osm* genes were previously uncharacterized. The induction of all the *osm* genes was by solutes that could not cross the cytoplasmic membrane, and hence unable to decrease the turgor pressure. The single exception, *osmI*, was also induced by glycerol which can freely diffuse through the cytoplasmic membrane.

Besides the *proU* and *kdp* genes, the only other known genes/operons to be osmoregulated are *bet* (Styrvold *et al.*, 1986), coding for a choline transporter and enzymes required for its oxidation to glycine betaine, *phoA* (Villarejo *et al.*, 1983) and *ompC*/*ompF* (Hall and Silhavy, 1981a), coding for the major outer membrane porins. Osmoregulation of OmpC and OmpF depends upon the *ompB* locus, which encodes two proteins, OmpR and EnvZ (Hall and Silhavy, 1981b). Transduction of either an *ompR*$^-$ or *envZ*$^-$ mutation into the *osm* fusion strains did not decrease alkaline phosphatase activity at high osmolarity. Activity in the *ompC*::*phoA* strain

was decreased. Thus, there are at least two mechanisms for regulating gene expression by osmolarity.

The study of *proU* regulation has revealed conflicting reports. It was shown that at high osmolarity, increased expression of *proU* from a plasmid correlated with an increase in supercoiling of DNA (Higgins *et al.*, 1988). Others showed that potassium glutamate is responsible for activating $\sigma^{70}$-dependent transcription of the *proU* gene at high osmolarity (Prince and Villarejo, 1990; Ramirez and Villarejo, 1991). Two promoters and a negative regulatory element were shown to be responsible for full scale osmo-induction of *proU* (Dattananda *et al.*, 1991). The negative regulatory element caused a reduction in plasmid linking number when cells containing it were grown under high osmotic conditions. An *osmZ* mutation was shown to increase expression of *proU* under low osmolarity (Higgins *et al.*, 1988). The *osmZ* gene product was shown to be the histone-like DNA-binding protein H-NS (May *et al.*, 1990). Studies of the effects of *osmZ*$^-$ on each of the regulatory elements fused to *lacZ* showed that all were derepressed under low osmolarity except for P1, which was further repressed. Growth in high osmolarity showed an increase in $\beta$-galactosidase activity of both promoters and the negative regulatory element, independent of *osmZ*. This led Dattananda *et al.* (1991) to state that *osmZ* affects all three regulatory elements. It has recently been shown that downstream of *proU* in *Salmonella typhimurium*, there is a curved DNA element which binds H-NS *in vitro* and is responsible for osmotic induction and osmotic changes in plasmid linking number (Owen-Hughes *et al.*, 1992). H-NS has been proposed to be a negative regulator that controls expression by affecting local changes in topology. H-NS regulation of the negative regulatory element in *E. coli* is probably analogous, but the role of potassium glutamate, if any, remains to be elucidated.

The *osmB* (Jung *et al.*, 1989) and *osmC* (Gutierrez and Devedjian, 1991)

genes have been cloned and sequenced. The *osmB* gene codes for a lipoprotein and is also induced during stationary phase (Jung *et al.*, 1990). The function of the *osmC* gene product is unknown, but mutations that affected the osmotic induction of *osmC* were localized to the gene coding for H-NS (Gutierrez and Devedjian, 1991).

### 1.4.7 Thiol Agents

Thiol agents block the growth of *E. coli* at high concentrations. However, at intermediate concentrations of TG, in the range of 2 to 90 mM, there is a slow down of growth, and *S*-adenosylmethionine metabolism (Javor, 1983a) and aerobic respiration (Javor, 1983b) are affected. Upon entry into stationary phase, thiol-treated *E. coli* cultures secrete higher amounts of riboflavin and porphyrin (Javor *et al.*, 1988). A screening of 5 000 Mu dX gene fusions, in the absence and presence of 70 mM TG, resulted in the isolation of eighteen strains that were induced and thirty-one strains that were repressed by thiol (Javor *et al.*, 1988). Twenty strains were tested. Thiol effects occurred in six strains only during logarithmic growth, and in fourteen during stationary-phase growth. All of the logarithmically affected *lacZ* gene fusions were repressed by DTT, except for one which was induced in the presence of both TG and DTT. Anaerobiosis only induced *lacZ* expression in one strain. TG and DTT either induced or repressed the stationary-phase responsive *lacZ* gene fusions in the same manner, and all except one were inducible by anaerobiosis to an extent intermediate between the induction seen in the presence of DTT and TG. Two-dimensional gels revealed that over one hundred proteins are either induced or repressed after TG treatment, suggesting that thiols invoke a reductive stress with global consequences. This study proved the existence of thiol-responsive promoters and is being followed up by protein gel analysis to determine if the proteins induced overlap with those of other stress responses.

## 1.5 OTHER USES OF GENE FUSIONS

Gene fusions have not only provided information about genes that are coordinately regulated by environmental agents, but they have also provided tests for measuring the presence of these agents. Thus, clones which contain reporter genes induced by a toxic environmental stress will only produce the reporter gene's product in the presence of the toxic stresses. The ease, rapidity, sensitivity and low cost of a reporter gene product assay makes it an attractive tool as a sensor for the monitoring of toxic agents in the environment (Blaise, 1991). Furthermore, the amount of toxic agent measured can be proportional to the amount available to the organism. Gene fusions have also provided a means to study how the transposition of bacteriophage Mu is affected by environmental agents in *E. coli.* Transposition is an interesting phenomena since it provides a means to mutate genes that result in rapid evolutionary changes (Shapiro, 1992). Understanding the cellular mechanisms of what induces transposition may provide clues as to how evolution can be induced to occur.

### 1.5.1 Gene Fusion-Based Biosensors to Measure the Presence of Particular Genotoxic Agents

The Ames test to measure the mutagenic potential of compounds was based on the ability of chemicals to cause reversion of histidine auxotrophic mutations in *S. typhimurium.* The mutagenic potency of some compounds occurs after they are modified by metabolism, hence the mutagenicity of the test compounds was also assessed after incubation with liver extracts. This led to the understanding that many carcinogens are mutagens and vice versa (Ames *et al.*, 1973).

When exposed to a DNA damaging agent, *E. coli* displays filamentous growth, because cell division continues without septum formation. A suppressor of

filamentation was isolated that had a mutation in *sfiA* (George *et al.*, 1975). A *sfiA*::*lacZ* fusion, created using phage Mu dI1, was shown to be induced during the SOS response and under the control of LexA, and postulated to be an inhibitor of cell division (Huisman and D'Ari, 1981). Using the *sfiA*::*lacZ* fusion strain, the SOS Chromotest was developed to test the mutagenicity of compounds (Quillardet *et al.*, 1982). This test is based upon the ability of compounds to induce a *sfiA*::*lacZ* gene fusion. This strain was engineered to be *uvrA*$^-$, so that repair of DNA lesions was impaired and the SOS response was prolonged, and *rfa*$^-$, causing a lipopolysaccharide deficiency and making the cells more permeable. Synthesis of alkaline phosphatase was constitutive, providing a means to measure the non-specific inhibition of gene expression. The mutagenicity of compounds correlates well between the Ames test and the SOS Chromotest. This has recently been the subject of a review (Quillardet and Hofnung, 1993). Transcription of the λ *cI* gene, encoding repressor, is also under SOS control. A lysogenic λ $P_LO_L$::*lacZ* fusion, controlled by cI (Elespuru and Yarmolinsky, 1979), can be used in a similar manner to the SOS Chromotest.

Two other tests based upon *lacZ* gene fusions have been developed to measure the mutagenicity of compounds. The Umu test is based upon a transcriptional fusion to the *umuC* gene in *S. typhimurium* (Oda *et al.*, 1985). Another test was developed as a *lacZ* translational fusion to the *cea* gene on a plasmid, that can be used in *E. coli* and *S. typhimurium* (Schumann *et al.*, 1991). A lambda *cI* fusion to the *E. coli gal* operon was used to detect compounds that cause mutagenesis of cI or induce the SOS response. Furthermore, the induction of genetic recombination between two *lacZ* genes can be detected in this strain. Hence, this strain provides a measure of multiple effects of DNA damaging agents (Toman *et al.*, 1985). It has been proposed that the most effective way to determine mutagenic activity is to use a battery of bacterial tests (Blaise, 1991; Quillardet and Hofnung, 1988). A fusion of

the two heat shock regulated genes, *dnaK* and *grpE*, to the *V. fischeri lux* genes on plasmids has provided a test that produces increased luminescence in the presence of various heat-shock inducing agents, including metals, solvents and crop protection chemicals (Van Dyk *et al.*, 1994).

### 1.5.2 Gene Fusions to Study Mu Transposition in Colonies and to Determine Host Proteins that Affect Transposition

Unicellular organisms such as bacteria can form structured multicellular communities (Shapiro, 1991). *E. coli* colonies display varying zones of biochemical activity, cell morphology and aggregation (Shapiro, 1987). Phage Mu dII1681 is the same as Mu dI1681 (section 1.3.6) except that it forms translational *lacZ* fusions (Castilho *et al.*, 1984). When a Mu dII1681 lysogen is grown on minimal plates containing Xgal for several days, sectored colonies or concentric rings arise that display reproducible patterns of blue and white (Shapiro and Higgins, 1988). Areas of the colony that had a blue colour showed increased frequencies of Mu dII1681 transposition as determined by Southern blotting and colony hybridization (Shapiro and Higgins, 1989). Selection for Tn*10* insertions that altered $\beta$-galactosidase activity also abolished the capacity of Mu dII1681 to transpose. It was proposed that concentric patterns occur because similar physiological factors that affect Mu transposition occur periodically in cells with similar positions in the colony.

Another study of gene fusions led to the questioning of the tenet that mutations are random. Shapiro (1984) was studying the fusion of *araB*, which is positively regulated by arabinose, to the *lacZY* genes separated by a defective Mu prophage. It was shown that fusions by deletion of Mu, to generate a Lac(Ara) $^{+}$ phenotype, appeared 5 to 6 days after the cells were plated onto minimal media containing lactose and arabinose. Gene fusion formation did not appear at a detectable fre-

quency in rich media, nor during exponential growth. Lac(Ara) $^{+}$ cells did not appear in rich media lacking either lactose or arabinose (Cairns *et al.*, 1988), suggesting that the selective agent directed their appearance. Mittler and Lenski (1990) showed that Lac(Ara) $^{+}$ cells did form after cells were grown in a glucose-limited liquid media for 9 days and plated on selective media. Furthermore, the frequency of Lac(Ara) $^{+}$ formation was constant, independent of the growth period in unselected media. This was taken as evidence that the mutations preexisted, but did not rule out the possibility that fusion formation occurred during selection (refer to Foster, 1993 for discussion).

When a Mu phage carrying a wild-type repressor is introduced into the Mu dII1681 lysogen strain, $\beta$-galactosidase expression is blocked because Mu dII1681 transposition is inhibited (Shapiro and Higgins, 1988). Furthermore, the formation of Lac(Ara) $^{+}$ fusions is also abolished (Shapiro, 1984). The *E. coli* ATP-dependent Clp protease is formed by the association between the ClpP protease subunit and an ATPase subunit, one of which is called ClpX. The ClpX protein was shown to be necessary for normal Mu replicative transposition to go to completion (Mhammedi-Aloui *et al.*, 1994). Geuskens *et al.* (1992) showed that the Mu repressor was more stable in a strain that was *clpP*$^{-}$. Shapiro (1993) studied the role of the host protein ClpP on Mu-mediated *lac* fusion formation. A mutation in *clpP* was shown to completely abolish sector production and severely inhibit Lac(Ara) $^{+}$ formation. Thus, ClpP is thought to be a protease that cleaves the Mu repressor. Introduction of an additional Mu phage increases the intracellular amount of repressor. Hence, the frequency of Mu transposition is decreased because repressor titrates out ClpP. ClpP mutants are unable to degrade carbon starvation proteins when the cells are subsequently exposed to glucose (Damerau and St. John, 1993). Hence, ClpP could connect the host's physiological status to Mu transposition. Increased rates of muta-

tion under starvation conditions (Cairns *et al.*, 1988; Foster, 1993; Lenski and Mittler, 1993; Shapiro, 1984) may also be due to the change in expression of proteins. The isolation of mutants that decrease fusion formation may aid in the elucidation of what those proteins are.

## 1.6 CONCLUSION

Since the initial construction and use of the first Mu dI1 phage, a great deal of knowledge has been generated about transcriptional and translational regulation. The use of gene fusions is a powerful method to define genes regulated by a specific stimulus. However, determining what the genes are and the role of their products in the response is sometimes difficult. For example, some of the *din* genes, first uncovered in 1980, have yet to be identified. Thus, it is important to complement these studies with biochemical information generated by the study of mutational effects. The direct sequencing of the gene fusion junction, as was done for the *psi::lacZ* strains, will speed up the identification of genes. Furthermore, the combination of the study of gene fusions and the analysis of two-dimensional protein gels has helped to define key regulators of responses, such as the role of the *rpoS* product during carbon starvation, and to separate proteins induced by particular stresses into classes such as the Cst and Pex proteins.

The information that has been gained by using gene fusions to study the regulation of genetically programmed responses to environmental stresses was illustrated. The vast array of regulatory mechanisms is contrasted by the repressor LexA which binds to a specific DNA sequence, the negative osmotic regulator H-NS which binds to a structural DNA motif, and the RNA polymerase sigma factor coded by *rpoS*, which unlike $\sigma^{32}$, may interact with the promoter in conjunction with other proteins. Regulatory factors are responsible for specific responses, such as

SoxR and SoxS to superoxide generating agents and OxyR to hydrogen peroxide. However a single gene can be regulated by more than one response such as the increased amounts of OmpC by low pH or high osmolarity, or the involvement of some *psi* genes in the carbon and nitrogen starvation responses. Furthermore, a particular compound can invoke more than one response, such as the induction of both the SOS and adaptive responses to the alkylating agents MMS and MNNG. The genes induced by a particular stimulus may be subject to different modes of regulation such as that illustrated during osmotic upshift. The gene *proU* is regulated by H-NS, and possibly potassium glutamate, whereas *ompC* and *ompF* expression are controlled by OmpR and EnvZ. In the case of genes induced by thiols, it is likely that instead of a particular thiol-responsive factor, thiols cause a general reductive stress that affects factors in other responses. This illustrates the caution that must be exercised when defining a regulon, a set of genes controlled by a regulatory factor, versus a stimulon, a set of genes regulated by a particular stimulus or compound (Neidhardt, 1987).

In *E. coli*, *lac* gene fusions have also provided microbiotests for genotoxic agents and yielded interesting information about host cellular physiology and transposition. Other examples, such as anaerobically induced genes, identified using *lacZ* gene fusions techniques (Choe and Reznikoff, 1991; Winkelman and Clark, 1986), were not discussed.

Gene fusion studies to identify genetic networks controlled by environmental stresses have relied on *lacZ* or *phoA* as reporter genes. However, transposable vectors containing *luxAB* are also available (de Lorenzo *et al.*, 1990; Engebrecht *et al.*, 1985). We have used a Tn*5-luxAB* (Guzzo and DuBow, 1991) gene fusion generating system to find genes specifically induced by the toxic metals aluminum and nickel (Guzzo *et al.*, 1991; Guzzo and DuBow, 1994a,b).

## PLAN OF THE THESIS

The goal of this thesis was to identify and characterize genetically programmed responses in *E. coli* to toxic metals. Chapter 1 is a historical overview of gene fusion vectors and their use to identify genes regulated by environmental stresses. Chapter 2 describes the construction of a library of 3000 *E. coli* random *luxAB* gene fusions using a truncated Tn*5* transposon. The Tn*5-luxAB* element was introduced into *E. coli* from a multicopy ColE1 plasmid vector. In order to obtain single copy gene fusions, the replication of the plasmid was inhibited by a second plasmid which overproduced RNA1. It was found that the number of *E. coli* Tn*5-luxAB* insertions obtained increased over time. Chapter 3 is an investigation into the increased numbers of *E. coli* clones, containing Tn*5-luxAB* transpositions, over time.

The light emission of the 3000 *E. coli luxAB* gene fusion clones was screened in the absence and presence of 1 and 10 $\mu$g/ml of aluminum and nickel. Two clones were found that displayed increased luminescence in the presence of aluminum and one in the presence of nickel. Chapter 4 describes the cloning, mapping and sequencing of one of the aluminum-responsive clones, shown to contain *luxAB* in the *E. coli fliC* gene. Chapter 5 describes the localization of the *luxAB* genes of the nickel-inducible clone to *celF*, the distal-most gene of the cellobiose operon. Sequencing revealed the presence of other potential open reading frames downstream of *celF*. Cloning of this region, and its expression in *E. coli*, revealed that an open reading frame of 28.5-kDa was most likely expressed *in vivo*. Chapter 6 summarizes the cloning and sequencing of a previously unidentified gene that is induced in the presence of aluminum. The transcriptional start sites were identified using S1 nuclease analysis and promoter resection. A Northern analysis showed that the mRNA is specifically induced in the presence of aluminum and iron. A protein of

22-kDa was shown to be expressed from the aluminum-inducible gene.

Analysis of the effects of arsenite on HeLa gene expression was also pursued. In order to identify mRNAs which are increased in the presence of sodium arsenite, a cDNA subtraction was performed between cDNA synthesized from HeLa cell RNA isolated from unexposed and exposed cells. The cDNA that remained after subtraction was cloned, screened and sequenced. Chapter 7 describes these approaches and the results. Chapter 8 is a summary of the results and discusses possible avenues for further exploration.

# CHAPTER 2

## Construction of Stable, Single-Copy Luciferase Gene Fusions in *Escherichia coli*

## 2.1 ABSTRACT

A ColE1-based plasmid for transcriptional gene fusions was constructed that contains both the promoterless *luxAB* genes of *Vibrio harveyi* and a *tet* marker within the inverted repeats of a left end-truncated Tn*5* element. Introduction of this plasmid into an *Escherichia coli* strain containing a plasmid (pTF421) that overproduces ColE1 RNA1 (and thus inhibits replication of the ColE1 plasmid) allowed selection for cells that had a single copy of the *luxAB* operon transposed into the chromosome beginning 5 days post-transformation. The long latent period necessary for Tn*5* transposition is analogous to that found in other systems, where transposition frequencies and mutation rates increase in a time-dependent manner when selected for upon prolonged incubation on petri dishes under bacteriostatic conditions.

## 2.2 INTRODUCTION

The study of *in vivo* transcriptional control is important for the elucidation of a gene's function. Measurement of transcription is facilitated by fusing a promoterless reporter gene, whose product is easily measured, to the gene of interest. In *Escherichia coli*, the *luxAB* operon from *Vibrio harveyi*, encoding luciferase, is very useful for this purpose because its expression can result in the production of light from an exogenously added aldehyde (Baldwin *et al.*, 1984). The sensitivity of luciferase-mediated transcriptional activity (Engebrecht *et al.*, 1985) is estimated to be 5 to 6 orders of magnitude greater than that of a *lacZ* reporter gene (Carmi *et al.*, 1987).

Many systems have been developed that have the capacity to create transcriptional and translational gene fusions *in vivo* by delivering the reporter gene to cellular DNA (Silhavy *et al.*, 1984). In order to allow chromosomal *luxAB* fusions to be made in many gram-negative bacterial species, including *E. coli*, we employed the

transposable element Tn*5* on a ColE1 plasmid to target the *luxAB* operon to different, random sites. Tn*5* is a composite transposon comprised of the inverted repeats IS*50L* and IS*50R* (for a recent review, see Berg, 1989). These gene fusions, once isolated, are relatively stable because Tn*5* has a low rate of transposition, about $10^{-5}$ per cell per generation (Johnson and Reznikoff, 1983; Krebs and Reznikoff, 1988). The use of a plasmid-based transposable element bypasses the requirement for phage attachment (de Bruijn and Lupski, 1984).

By combining the extreme sensitivity of *luxAB* with the stability of Tn*5*, it was possible to create *in vivo* gene fusions that can potentially measure the transcription of any non-essential operon within the *E. coli* chromosome. Described here is a system which allowed the isolation of single, chromosomally-located *luxAB* gene fusions beginning 5 days post-transformation. The ability to use *luxAB* expression as a measure of gene transcription in this system is demonstrated using the previously characterized xylose operon (Briggs *et al.*, 1984; Davis and Henderson, 1987; Kurose *et al.*, 1987; Maleszka *et al.*, 1982; Rosenfeld *et al.*, 1984). Furthermore, the ability to observe Tn*5* transposition from a plasmid only after prolonged incubation under bacteriostatic conditions resembles the increased rate of transposition observed for a chromosomally-located bacteriophage Mu (Shapiro and Higgins, 1989).

## 2.3 MATERIALS AND METHODS

### 2.3.1 Bacterial Strains and Plasmids

*E. coli* strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd5* ($r_k^-$, $m_k^-$) (F' *proAB lacI*$^q$*ZΔM15*)] (Gough and Murray, 1983) was used for cloning of the pFUSLUX plasmid. Strain DH1 [F$^-$ *recA1 endA1 gyrA96 thi hsdR17* ($r_k^-$, $m_k^+$) *supE44 relA1*] (Hanahan, 1983) was the *E. coli* derivative used for transformation to create the gene fusion library. Strain LF20102 is DH1 containing the Ap$^R$ ColE1 RNA1-over-

producing plasmid pTF421 (Fitzwater *et al.*, 1984). Plasmid pRZ341-21::Tn*5lac* contains the *lac* structural genes, isolated from plasmid pMC903 (Casadaban *et al.*, 1980), inserted into the unique *Bam*HI site of plasmid pRZ341-21 (Johnson and Reznikoff, 1983).

### 2.3.2 Media

Bacterial strains were routinely propagated at 37°C, unless otherwise indicated, in LB broth or on LB plates containing 1.5% agar as described in Miller (1972), and supplemented with antibiotics as mentioned. Ap was used at a final concentration of 40 $\mu$g/ml. Tc was used at a final concentration of 10 $\mu$g/ml in broth and 20 $\mu$g/ml in plates. MacConkey agar base plates (Difco Laboratories, Detroit, USA) were prepared with 1% (w/v) xylose.

### 2.3.3 DNA Manipulations

Restriction endonuclease hydrolysis, DNA ligations and calf-intestinal alkaline phosphatase reactions were performed as previously described (Tolias and DuBow, 1987). Filling in of 3' recessed ends was performed by treatment of the DNA with the Klenow fragment of DNA polymerase I (Pharmacia Biotech, Montreal, Canada) plus 25 $\mu$M each of dATP, dCTP, dGTP and dTTP in nick-translation buffer (Maniatis *et al.*, 1982). Addition of *Bam*HI linkers was performed according to Maniatis *et al.* (1982). DNA transformations were performed according to Mandel and Higa (1970).

### 2.3.4 Construction of Plasmid pFUSLUX

The vector containing Tn*5* and *tet* was prepared by hydrolyzing plasmid pRZ341-21::Tn*5lac* with *Bam*HI, gel purifying the large DNA fragment, and treat-

ferred to a Hybond-N membrane (Amersham Ltd., Oakville, Canada) by the bidirectional method and hybridized as described by Smith and Summers (1980).

### 2.3.7 Assay for Light Production

The assay for light production was modified from Miyamoto *et al.* (1985). Briefly, cells present in a petri dish containing 12.5 ml of LB agar were placed upside down and exposed to Agfa CURIX RP1 film in the dark at 23°C after addition of 50 $\mu$l of dodecyl aldehyde (Aldrich, Milwaukee, USA) to the cover of the petri dish. The X-Ray films were developed after the times indicated.

## 2.4 RESULTS

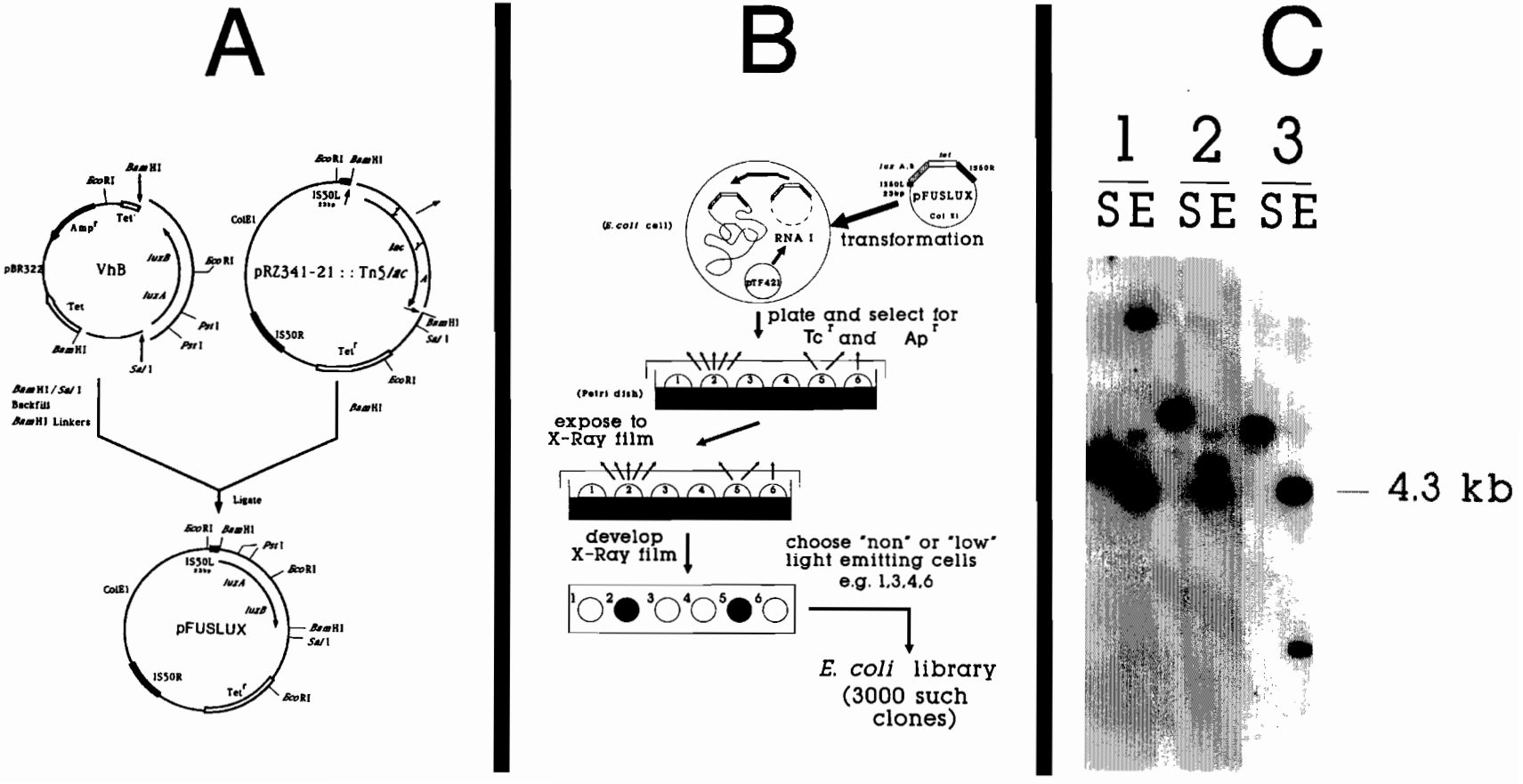
### 2.4.1 The Transposable *luxAB* Gene Fusion Vector

The *luxAB* genes were inserted between the inverted repeats of a modified Tn*5* element (Figure 1A). A truncated, but functional (Johnson and Reznikoff, 1983), version of IS*50L*, containing only its outer 23 bp, was used so that any potential transcriptional start and stop signals were eliminated. The *tet* gene, located between the inverted repeats, was used as a selectable marker for insertion of the Tn*5*-*luxAB* element into the *E. coli* chromosome as described below.

### 2.4.2 Rationale to Isolate Single Copy *luxAB* Insertions

The transposable *luxAB* operon is contained within a ColE1-based replicon that replicates extrachromosomally to about 15-20 copies per cell (for review, see Kües and Stahl, 1989). ColE1 replication can be inhibited by the plasmid pTF421 which overproduces an RNA, called RNA1, that inhibits correct RNA primer formation necessary for ColE1 replication (Fitzwater *et al.*, 1984) (Figure 1B). After transformation of pFUSLUX into an *E. coli* strain (LF20102) that already harbored

Figure 1. Construction and characterization of the *luxAB* fusion library. A) Construction of plasmid pFUSLUX. See Materials and Methods for details. B) Outline of the construction of the *E. coli luxAB* gene fusion library. Plasmid pFUSLUX was introduced into strain LF20102 and the transformants were selected on LB media containing Ap (resistance conferred by pTF421) and Tc (resistance conferred by the transposed Tn*5-luxAB* element); see Materials and Methods for details. For the purposes of later characterization in identifying operons that are induced by external stimuli (e.g. xylose), a total of approximately 3000 low or non-light emitting clones, as determined after a four hour assay for light emission with dodecyl aldehyde, were collected and placed into long-term storage in 25% (v/v) glycerol at -20°C. C) Southern blotting and hybridization results (see Materials and Methods) obtained for three different clones, labeled 1 to 3. Lanes S are digestions with *Sal* I and lanes E are digestions with *Eco*RI.


A
B
C
VhB
pBR322
Amp
Tet
luxB
luxA
EcoRI
BamHI
PstI
SalI
pRZ341-21 : : Tn5/lac
ColE1
IS50L
IS50R
lac
BamHI / SalI
Backfill
BamHI Linkers
Ligate
pFUSLUX
(E.coli cell)
RNA I
pTF421
transformation
plate and select for
Tc r and Ap r
(Petri dish)
expose to
X-Ray film
develop
X-Ray film
choose "non" or "low"
light emitting cells
e.g. 1,3,4,6
E. coli library
(3000 such
clones)
1 2 3
SE SE SE
4.3 kb

plasmid pTF421, clones that (theoretically) contain the Tn*5*(*tet*)-*luxAB* element transposed into the chromosome were selected on media containing Ap and Tc. One and one-half hours after transformation of pFUSLUX into strain LF20102, the cells were plated onto LB plates containing Ap and Tc and grown overnight at 37°C. The plates were then placed at 23°C for an additional 7 days. New transformants appeared throughout this incubation period and were analyzed to determine the optimal time for obtaining Tn*5* transposition to the chromosome and concomitant loss of pFUSLUX. When plasmid pFUSLUX is harbored by a cell, the *luxAB* cistrons are expressed from a promoter on the ColE1 segment of the plasmid and the cells emit a "medium" level of light (the amount of light that is detected as a dark spot on X-Ray film after a 4 hour exposure), as determined from the light emission of strain DH1 containing pFUSLUX alone (data not shown). Colonies began to first appear two days post-transformation. All of these were found to emit a "medium" level of light and contain plasmid pFUSLUX. However, transformants isolated five to eight days post-transformation displayed a variety of light emitting phenotypes but were found to contain only plasmid pTF421.

To ensure that replication of pFUSLUX had been successfully inhibited and that the Tn*5*-*luxAB* element had integrated in a single location without secondary transposition events, the number of chromosomal insertions was determined for several independent clones. The results (Figure 1C) showed that the transformants were single copy, as judged by the single dark band obtained with *Sal* I and the two dark bands obtained with *Eco*RI; one constant in size for each clone (representing the internal *Eco*RI fragment) and one that varied in size, representing the segment spanning the *Eco*RI site in *luxAB* to the next site in the chromosomal DNA, also indicating that the Tn*5*-*luxAB* element had integrated in different locations in the chromosome. The faint bands seen are due to a small amount of homology between

the *luxAB* probe and the plasmid pTF421 present in the preparation of total cellular DNA (data not shown).

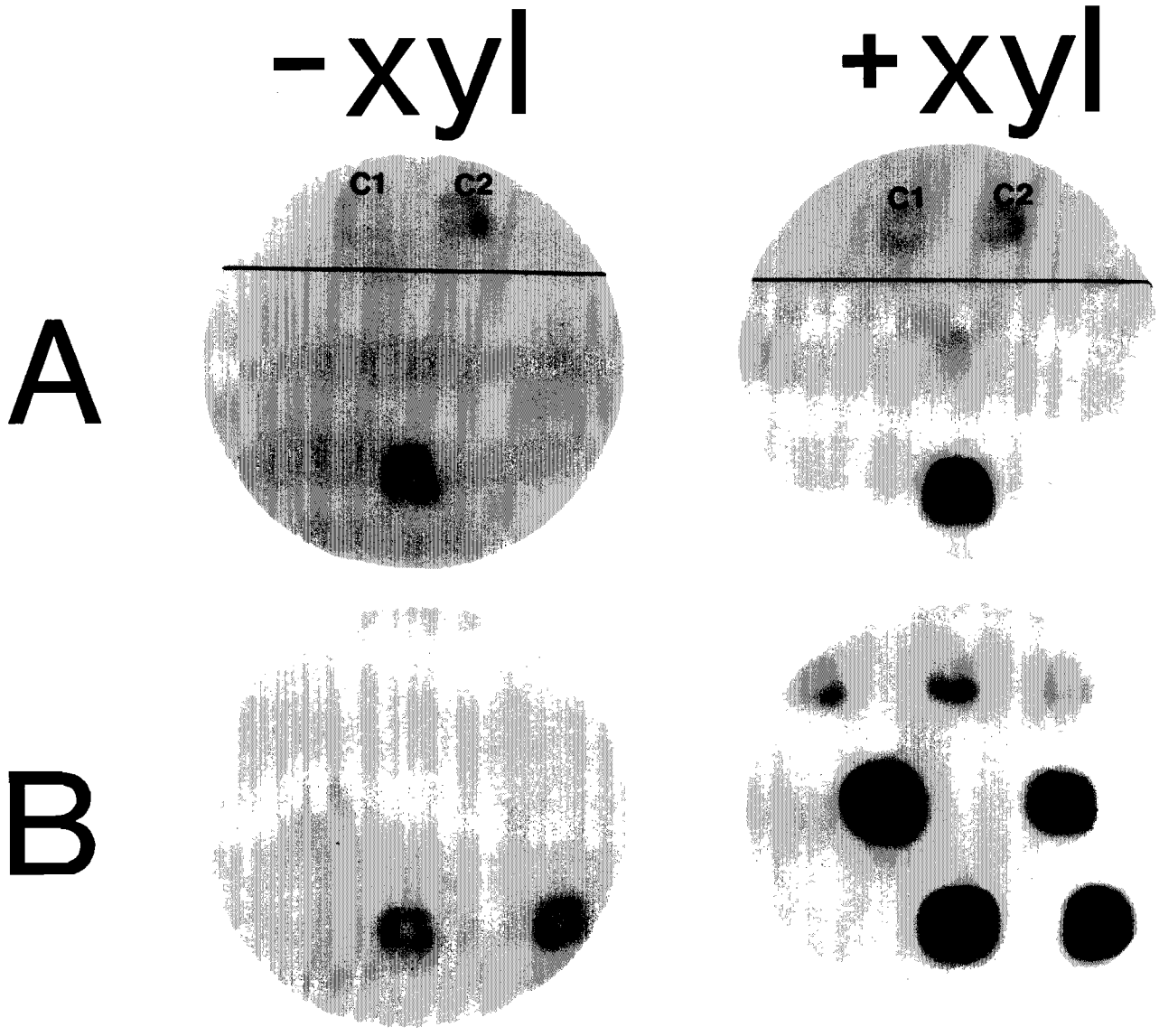
### 2.4.3 Characterization of the Tn*5-luxAB* Gene Fusions

To prove that the Tn*5-luxAB* element was fused to promoters, and that induction of transcription could be measured, fusions within the xylose operon were screened as *xyl*$^-$ mutants of strain DH1 on MacConkey agar plates containing xylose. Five genes have been identified that are involved in xylose metabolism (Briggs *et al.*, 1984; Davis and Henderson, 1987; Kurose *et al.*, 1987; Maleszka *et al.*, 1982; Rosenfeld *et al.*, 1984). The approximately 3000 clones containing Tn*5-luxAB* gene fusions (Figure 1B) were screened on MacConkey agar plates containing xylose, and 14 were found to be *xyl*$^-$. When a luminescence assay was performed on the mutants in the absence and presence of xylose, eleven of the fourteen *xyl*$^-$ clones showed an increase in luminescence in the presence of xylose, with different levels of luminescence detectable (Figure 2). The *xyl*$^+$ clones that emitted a low level of light (Figure 2A, labeled C1 and C2) were included as controls to show that the increase in transcription in the *xyl*$^-$ clones was specific for xylose. The three *xyl*$^-$ clones that did not increase their transcription in the presence of xylose may have contained the *luxAB* genes in the wrong orientation with respect to the *xyl* promoter/operator, an insertion into *xylR*, or a spontaneous mutation in one of the xylose genes in addition to a Tn*5-luxAB* insertion.

## 2.5 DISCUSSION

The system described here has been used to create *in vivo* transcriptional gene fusions of *E. coli* operons to the *luxAB* operon. The *luxAB* genes have great potential

Figure 2. Measurement of transcription as reflected by light emission of the *xyl*$^-$ clones. The light emission of these clones was assayed in the absence (left) and presence (right) of 0.2% (w/v) xylose on LB agar plates. The amount of light emission after the addition of xylose allowed the separation of these clones into two classes; those that emitted a low level (A) and those that emitted a high level (B) of light. Clones C1 and C2 are *xyl*$^+$ controls. All X-Ray film exposures were performed for 4 hours except for the clones that emitted a high level of light (B) in the presence of xylose, which was performed for 15 minutes.


-xyl
+xyl
A
B
C1
C2
C1
C2

as reporter genes due to the sensitivity and ease of measurement of the associated gene product (luciferase).

The transformants containing the Tn*5-luxAB* element in the chromosome, without any extrachromosomal copies of pFUSLUX, took longer to grow than those clones containing pFUSLUX. The ability to detect transposition events only after prolonged periods of incubation under bacteriostatic conditions has been studied in other systems. Shapiro and Higgins (1989) studied the transposition of a Mu d-*lac* gene fusion prophage (Mu dII1681) within colonies of *E. coli.* Mu dII1681 was resident as a prophage in a chromosomal location where the *lacZ* gene was not expressed. Sectored colonies were screened as Lac $^+$ cells that appeared from the Lac$^-$ parental strain. Detectable appearance of the Lac $^+$ sectors took a minimum of six days, and were shown to represent induction of duplicative transposition of the phage throughout the chromosome. Chromosomal mutations that are selected for on petri plates have also been observed to occur at an increased rate in a time-dependent manner when selected under bacteriostatic conditions (Cairns *et al.*, 1988). In our system and the others, the increase in transposition frequency (or mutation rate) was dependent upon the use of a bacteriostatic selective agent, and occurred in a time-dependent manner after a prolonged period of incubation on plates. It has been hypothesized that the cells, quiescent on plates under non-growth conditions, enter a hypermutable state allowing them to accumulate mutations which, when selected, would allow the cell to exit the hypermutable state and form a colony (Hall, 1991). In the case of transposition, it is possible that either the hypermutable state confers the ability to transpose, or the stress induced after prolonged incubation induces transposition. These results may also explain the bursts of DNA transposition of different mobile genetic elements that are observed in bacterial cells when selecting for single transposition events (Read and Jaskunas, 1980).

In our study, the long latent period prior to the appearance of Tn*5-luxAB* elements in the chromosome (causing $Tc^R$ and colony growth) could be explained using the above observations. Tc is a bacteriostatic agent (Maniatis *et al.*, 1982). Thus, transformants can remain on the plate in a quiescent state until either an event occurs that confers $Tc^R$ to the cell, or the Tc is inactivated. A minimum of 4 to 5 days was necessary in order to detect transposition of at least one Tn*5* element, which conferred $Tc^R$, into the chromosome. The clones that grew up to 4 days post-transformation contained a mixture of different events, but by the fifth day, $Tc^R$ clones contained only plasmid pTF421 and a chromosomally-located Tn*5* element.

In this study, a library of 3000 clones containing random, single-copy Tn*5-luxAB* insertions into an *E. coli* strain was created. These clones may now be screened (via replica plating) to detect genes whose transcription is regulated by many different physiological and environmental agents (Guzzo *et al.*, 1991; Kenyon and Walker, 1980; Kogoma *et al.*, 1988; Slonczewski *et al.*, 1987). These single copy gene fusions also facilitate further cloning to identify the gene of interest. To obtain downstream sequences of the operon fusion, total cellular DNA can be isolated, hydrolyzed with *Bam*HI or *Sal* I, ligated to a suitable vector and transformed into an appropriate *E. coli* strain with selection using Tc. The clones will thus contain the *tet* gene, IS*50*R and the host sequences between IS*50*R and the first *Bam*HI or *Sal* I site. Moreover, the conditions outlined here to obtain single copy insertions can be used in any bacterial species where Tn*5* can transpose, and luciferase is expressed. Tn*5* is functional in a wide variety of Gram-negative bacteria (Berg, 1989), while luciferase is functional in many bacterial species including nitrogen-fixing cyanobacteria, bacteria that are pathogens or symbionts in plants, and *Bacillus* species (Carmi *et al.*, 1987). The use of a plasmid borne transposable vehicle also overcomes the problems of limited phage host range for delivery of transposons in the case of

bacteriophage λ (Berg, 1989), or to the use of Mu phage as an insertion/delivery system (Van Gijsegem *et al.*, 1987). In theory, our system can be extrapolated to any replicon that can be inhibited *in trans* for DNA replication.

# CHAPTER 3

## Time-Dependent Tn*5* Transposition After Transformation and Antibiotic Selection in *Escherichia coli*

## PREFACE

In Chapter 2, a method to introduce a Tn*5-luxAB* element from the multicopy ColE1 plasmid, pFUSLUX, into the *Escherichia coli* chromosome was described. The replication of pFUSLUX was inhibited by providing RNA1 *in trans*. It was found that the colonies appearing within the first 4 days post-transformation contained pFUSLUX, however colonies that grew after 4 days post-transformation contained *Tn5-luxAB* inserted within the *E. coli* chromosome in single-copy. A more detailed investigation into the processes that occur after the transformation and inhibition of pFUSLUX replication is described in Chapter 3.

## 3.1 ABSTRACT

The ColE1-based plasmid pFUSLUX, containing a Tn*5* element flanking the *Vibrio harveyi luxAB* genes and a *tet* gene, was transformed into an *Escherichia coli* strain containing the ColE1 RNA1-overproducing p15A-based ($Ap^R$) plasmid pTF421. Selection for $Ap^R$ plus $Tc^R$ transformants yielded two types of colonies, whose appearance was time-dependent. The number of colonies containing a stable, single-copy chromosomal insertion of the Tn*5-luxAB* element ($Ap^R$ and $Tc^R$) was found to increase from 3 to 6 days post-transformation. Moreover, 20% of these stable transposition clones displayed changes in chromosomal IS*1*, IS*2* or IS*5* element distribution. However, colonies that would not grow upon restreaking ($Ap^R$ or $Tc^R$) also appeared, and were found to decrease in percentage during the same period. Analysis of the plasmid content of colonies that appeared post-transformation revealed that pFUSLUX was present in colonies that did not regrow after selecting for $Ap^R$ and $Tc^R$. Conversely, only plasmid pTF421 was present in colonies that can grow on Ap plus Tc plates. Transformation of a $Kn^R$ pTF421 derivative yielded a reduced number of total colonies post-transformation, but a vastly increased frequency of colonies containing a chromosomally-inserted Tn*5-luxAB* element ($Kn^R$ and $Tc^R$). These results suggest that $Ap^R$ or $Tc^R$ colonies arose as a consequence of Ap degradation around the cells, resulting in a detoxified zone, which may have enabled cells containing only pFUSLUX ($Tc^R$) to grow after segregation and loss of pTF421 ($Ap^R$). Our results also suggest that transposition frequencies, chromosomal DNA rearrangements and plasmid missegregation may occur at higher frequencies when cellular growth is limited by environmental selection or adverse conditions.

## 3.2 INTRODUCTION

Mutation and recombination are major forces in evolution. *Escherichia coli* contains many mobile genetic elements that vary in their sequence, structure and copy number (Galas and Chandler, 1987; Kleckner, 1981). Due to their repetitive nature, they can mediate chromosomal rearrangements via illegitimate recombination, as well as acting as mobile regions of homology (Arber, 1991). In addition, mobile genetic element insertions within a gene can cause insertional polar mutations, or can activate gene expression (Galas and Chandler, 1987; Kleckner, 1981; Syvanen, 1984).

The spread of genetic information through populations of bacteria is also a driving force in evolution (Syvanen, 1984) that can have serious consequences in microbial pathogenesis and ecology (Shapiro, 1992). For instance, the emergence of antibiotic resistant strains of bacteria continues to pose a challenge to human health (Davies, 1994). Transposons carrying antibiotic resistance genes are often found on plasmids, which are extrachromosomal DNA molecules capable of self replication and mobilization (Nordström and Austin, 1989). Hence, it has been proposed that transposable elements are basic tools for natural genetic engineering, including the evolution of plasmids (Shapiro, 1992).

We were interested in creating a library of *E. coli* clones that contained single-copy insertions of the *Vibrio harveyi luxAB* reporter genes in the *E. coli* chromosome, in order to screen for *luxAB* gene fusions that were inducible by toxic metals (Guzzo *et al.*, 1991; Guzzo and DuBow, 1994a). A Tn*5* element, flanking the promoterless *luxAB* genes and a $Tc^R$ marker, on a ColE1-based plasmid, was transformed into an *E. coli* strain harboring an $Ap^R$ p15A plasmid that overproduced RNA1, an inhibitor of ColE1 replication (Fitzwater *et al.*, 1984). By selecting for $Tc^R$ plus $Ap^R$ transformants, clones were obtained that contained the Tn*5-luxAB* element trans-

posed to random locations in the *E. coli* chromosome in single-copy (Guzzo and DuBow, 1991). These clones arose when the petri dishes were incubated at room temperature, beginning 1 day post-transformation, over a period between 3 and 7 days post-transformation. Here, we present an investigation into potential mechanisms underlying the appearance of these colonies over time by selecting with antibiotics which have different modes of action, and by analyzing the plasmid content of the colonies. Chromosomal rearrangements involving resident IS elements in the clones that acquired the Tn*5* in the chromosome were also found, and our results suggest that *E. coli* grown under adverse conditions may be enhanced to undergo gene rearrangements.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Bacterial Strains and Plasmids

All bacterial strains and plasmids are listed in Table 1. *E. coli* strain DH1 was used to harbor pTF421 and its derivatives, and for all subsequent transformations of pFUSLUX and its derivatives (Guzzo and DuBow, 1991). *E. coli* strain JM105 was used for large scale DNA purification of pFUSLUX (Sambrook *et al.*, 1989) and for the cloning and isolation of pFUSLUX derivatives.

### 3.3.2 Media

Bacterial strains were routinely propagated at 37°C, unless otherwise indicated, in LB broth or on LB plates containing 1.5% agar as described in Miller (1992). Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada), 40 μg/ml; Cb (Ayerst Laboratories, Montreal, Canada), 100 μg/ml; Cm (Sigma, St. Louis, USA), 50 μg/ml; Tc (Boehringer Mannheim,

**Table 1: Strains/Plasmids**

| Strain | Description | Source |
|---|---|---|
| DH1 | *supE44, hsdR17, recA1, endA1, gyrA96 thi-1, relA1* | Hanahan, 1983 |
| JM105 | *endA, sbcB15, hsdR4, rpsL, thi* Δ(*lac-proAB*), [F' *traD36, proAB, lacI*$^{q}$ZΔM15] | Yanisch-Perron *et al.*, 1985 |
| LF20102 | DH1 containing pTF421 | Guzzo and DuBow, 1991 |
| LF20150 | DH1 containing pAG42 | this work |

| Plasmid | Antibiotic $^{R}$ genes | Source |
|---|---|---|
| pJoA | Ap | Harel *et al.*, 1990 |
| pUC71K | Kn | Vieira and Messing, 1982 |
| pFUSLUX | Tc | Guzzo and DuBow, 1991 |
| pAG32 | Tc,Kn | this work |
| pAG34 | Tc,Ap | this work |
| pTF421 | Ap | Fitzwater *et al.*, 1984 |
| pAG42 | Kn | this work |
| pBRG36 | Cm,Ap | Biel *et al.*, 1984 |
| pBRK10 | Ap | Dykhuizen *et al.*, 1985 |
| pLX2 | Ap | Green *et al.*, 1984 |

Laval, Canada), 10 $\mu$g/ml in broth, 20 $\mu$g/ml in plates; Kn (Boehringer Mannheim, Laval, Canada), 50 $\mu$g/ml.

### 3.3.3 DNA Manipulations

Restriction endonuclease hydrolyses were performed in 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 75 mM NaCl, 6 mM 2-mercaptoethanol and 0.25 mg/ml bovine serum albumin at 37°C for 2 hours using 3 units of enzyme per $\mu$g DNA. Ligations were performed at 15°C for 18 hours in T4 DNA ligase buffer using 2 units of T4 DNA ligase per $\mu$g of DNA (Gibco-BRL, Burlington, Canada). DNA was subjected to electrophoresis in 0.75% agarose gels, unless otherwise indicated, in TAE buffer [40 mM Tris-acetate (pH 8.0), 1 mM EDTA]. Size-selected DNA fragments were purified using the Geneclean II Kit (Bio 101, Mississauga, Canada). One microgram of pFUSLUX, pAG32 or pAG34 was routinely used for transformations.

### 3.3.4 Transformation

An overnight culture of strains LF20102 or LF20150 was diluted 20-fold into fresh LB media and grown to an $A_{550}$ of 0.4. The cells were then subjected to centrifugation at 3000 x g in an IEC Model Centra-8R Centrifuge (International Equipment Centrifuge, Needham Hts., USA) at 4°C for 8 minutes. The cell pellets were resuspended in 0.6 volumes of 2 mM $MgSO_4$, 2 mM HEPES-NaOH (pH 7.5) and incubated on ice for 20 minutes. The cells were then collected by centrifugation and resuspended in 0.6 volumes of 50 mM $CaCl_2$, 2 mM HEPES-NaOH (pH 7.5). After 25 minutes on ice, the cells were again collected by centrifugation and resuspended in 0.1 volumes of 50 mM $CaCl_2$, 2 mM HEPES-NaOH (pH 7.5). One microgram of DNA, in a total volume of 200 $\mu$l of 50 mM $CaCl_2$, 2 mM HEPES-NaOH (pH 7.5), was mixed with 200 $\mu$l of competent cells and incubated on ice for 25 minutes. The

mixture was incubated at 37°C for 3 minutes, and then 1.5 ml of fresh LB broth was added. After 1.5 hours of incubation at 37°C, the mixture was plated onto LB agar plates containing the appropriate antibiotics. After incubation at 37°C for 24 hours, the petri dishes were placed at room temperature.

### 3.3.5 Plasmid Constructions

Plasmid pAG32 was constructed by linearizing pFUSLUX (Guzzo and DuBow, 1991) with *Sal* I and subsequent ligation with the 1.4 kb *kan* DNA fragment purified from pUC71K (Vieira and Messing, 1982) after hydrolysis with *Sal* I. The ligation mixture was transformed into *E. coli* strain JM105 and selected on LB plates containing Tc and Kn. Plasmid pAG34 was obtained by hydrolysis of pFUSLUX with *Sst*I, purifying the 19.5 kb fragment [thus removing 300 bp downstream of *luxB* (Swartzman *et al.*, 1990)], and ligation with the 1.5 kb *bla* gene purified from plasmid pJoA (Harel *et al.*, 1990), also digested with *Sst*I. The ligation mixture was transformed into *E. coli* JM105 and selected on LB plates containing Tc and Ap. Plasmid pAG42 was constructed by linearizing pTF421 (Fitzwater *et al.*, 1984) with *Pst*I, followed by ligation with the 1.4 kb *kan* gene purified from pUC71K (Vieira and Messing, 1982) after hydrolysis with *Pst*I. The ligation mixture was transformed into *E. coli* strain DH1 and selected on LB plates containing Kn.

### 3.3.6 Analysis of Colony Plasmid Content

Each colony was picked with a toothpick, dispersed in 10 $\mu$l of LB broth, and then the toothpick used to streak LB plates containing the appropriate antibiotics. To determine the plasmid content of the original colony dispersed in 10 $\mu$l of LB broth, 10 $\mu$l of 100 mM Tris-HCl (pH 7.5), 4 mM EDTA, 2% (w/v) sodium dodecyl sulfate, 0.8 M sucrose and 0.2 mg/ml bromophenol blue was then added to the LB

broth. After incubation at room temperature for 10 minutes, the cell debris was removed by centrifugation at 15000 x g for 15 minutes at 4°C, and the supernatant fluid, containing the cellular nucleic acids, was subjected to electrophoresis in a 0.5% agarose gel. The DNA was bidirectionally transferred to two Hybond-N nylon membranes (Amersham, Oakville, Canada) by the method of Smith and Summers (1980). One filter was hybridized to a digoxigenin-dUTP-labeled 3.2 kb *Bam*HI *luxAB* DNA fragment isolated from pFUSLUX (Guzzo and DuBow, 1991), and the other was hybridized to a digoxigenin-dUTP-labeled pTF421 fragment (Fitzwater *et al.*, 1984) linearized with *Sal* I, according to the DIG DNA Labeling and Detection Kit protocol (Boehringer Mannheim, Laval, Canada).

### 3.3.7 Analysis of Chromosomal IS*1*, IS*2* and IS*5* Content

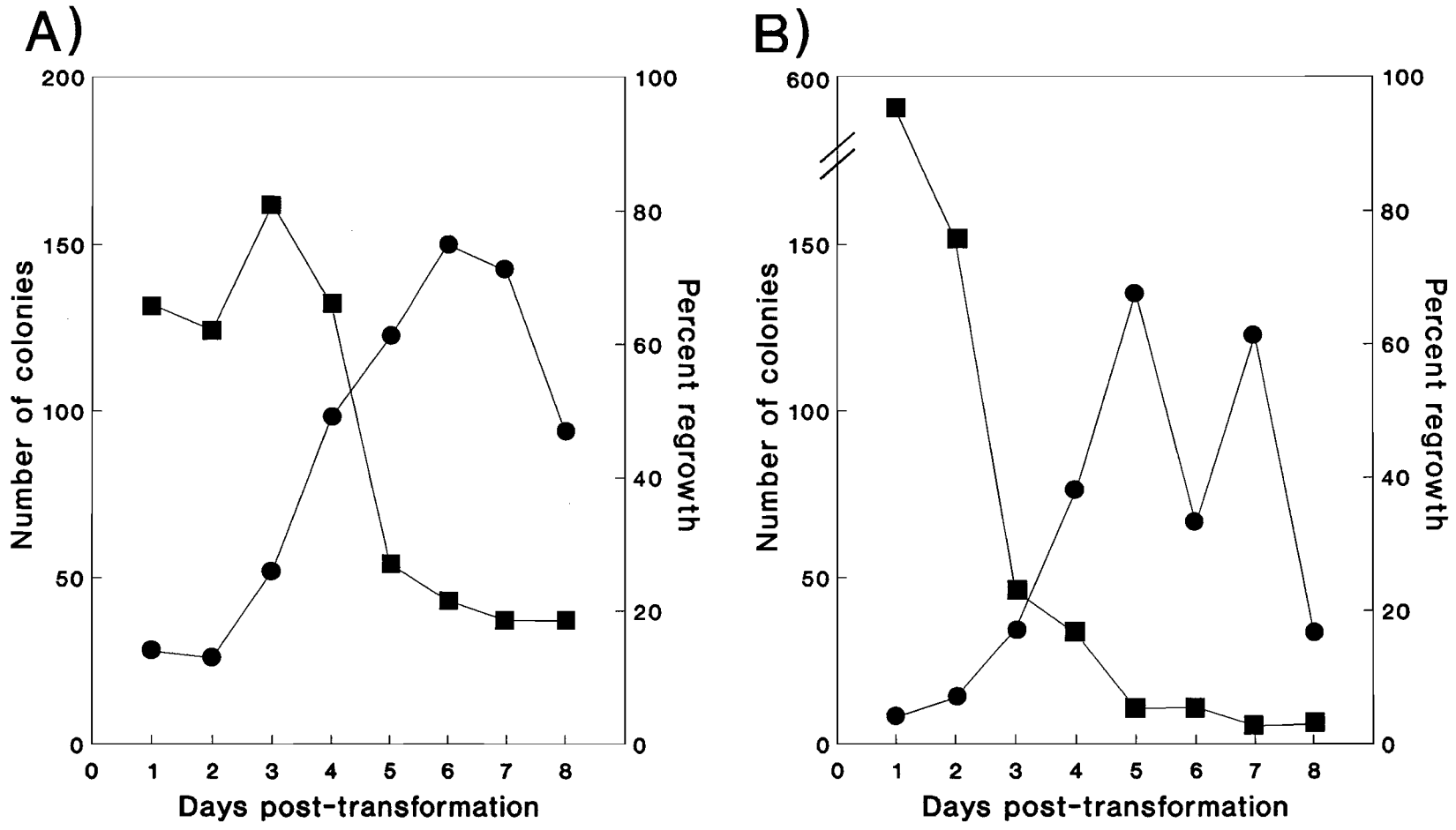
Ten micrograms of total cellular DNA (from clones isolated at various days post-transformation of pFUSLUX into strain LF20102, or of strain DH1 alone) were isolated (Guzzo and DuBow, 1991) and hydrolyzed with *Bam*HI or *Hin*dIII. After electrophoresis through 0.75% agarose gels, the DNA was denatured and transferred to a Hybond-N nylon membrane (Smith and Summers, 1980) and hybridized with 0.1 $\mu$g of each of the digoxigenin-dUTP-labeled IS element probes. The IS*1* probe was obtained after purifying a 1.6 kb DNA fragment from plasmid pBRG36 (Biel *et al.*, 1984) hydrolyzed with *Pvu*II plus *Eco*RI. The IS*2* probe was isolated as a 0.72 kb DNA fragment from plasmid pBRK10 (Dykhuizen *et al.*, 1985) hydrolyzed with *Hpa*I plus *Hin*dIII. The IS*5* probe was obtained by isolating a 0.94 kb DNA fragment after digesting plasmid pLX2 (Green *et al.*, 1984) with *Bgl* II plus *Eco*RI.

## 3.4 RESULTS

### 3.4.1 A Plasmid-Based System to Acquire Single-Copy Tn*5* Chromosomal Insertions

We have previously described a system that permits the isolation of single-copy, promoterless *luxAB* genes in the *E. coli* chromosome (Guzzo and DuBow, 1991). These clones were created by the use of a plasmid, pFUSLUX, which contains the *luxAB* genes and a $Tc^R$ selectable marker embedded within a modified Tn*5* element consisting of 23 bp of IS*50*L and a complete copy of IS*50*R (Johnson and Reznikoff, 1983). Plasmid pFUSLUX replicates intracellularly, via its ColE1 replicon, to approximately 15 copies per cell (Staudenbauer, 1978). In order to inhibit pFUSLUX replication after transformation, and thus select for *luxAB* element transpositions into the chromosome, pFUSLUX was introduced into an *E. coli* strain harboring an $Ap^R$ p15A-based plasmid, pTF421, which inhibits the replication of ColE1 plasmids by constitutively expressing ColE1's RNA1 from the *trp* promoter (Fitzwater *et al.*, 1984). After transformation of pFUSLUX into a *recA*$^-$ *E. coli* strain harboring pTF421 (strain LF20102) and selection on LB plates containing Tc and Ap, colonies were obtained that contained the Tn*5*-*luxAB* element transposed into the chromosome at a single location (Guzzo and DuBow, 1991). However, these single insertion clones only began to appear 3 days post-transformation. Moreover, colonies appeared that, upon subsequent restreaking on LB plates containing Ap plus Tc, did not grow. We found that the number of new colonies appearing each day increased in numbers up to 3-4 days post-transformation, and then slowly decreased (Figure 1A). The percent regrowth of the total collection of colonies, after restreaking on LB plates containing Ap plus Tc, increased over time post-transformation, and reached a peak at day 6 post-transformation. We wanted to investigate why colonies appeared that would not grow after restreaking, and the

Figure 1. Analysis of the transformants obtained using strain LF20102. Depicted are the number of colonies (■) and percent regrowth (●) after transformation of A) pFUSLUX into LF20102, selecting on Ap plus Tc; B) pAG32 into LF20102, selecting on Ap plus Kn. Refer to Materials and Methods for experimental details.


A)
B)
Number of colonies
Percent regrowth
Days post-transformation
0
50
100
150
200
600
20
40
60
80
1
2
3
4
5
6
7
8

reason for the time-dependent increase in the number of stable $Ap^R$ plus $Tc^R$ transformants.

### 3.4.2 Colonies Form That Do Not Regrow

Since Tc is bacteriostatic, the *E. coli* cells containing only pTF421 would not be killed after the transformation and plating procedure. The $Ap^R$ gene (*bla*), encoding a TEM-type $\beta$-lactamase, is located in the periplasm (Sykes and Matthew, 1976), and can cause a zone of Ap degradation around the cells (Sambrook *et al.*, 1989). Hence, it is possible that *E. coli* cells, after transformation and plating, can continue to divide (and form colonies), as long as the cells retained the $Tc^R$ determinant from plasmid pFUSLUX. To test this hypothesis, a pFUSLUX derivative, designated pAG32, was constructed that contained the *kan* gene. When pAG32 was transformed into strain LF20102, the total number of colonies appearing post-transformation was now found to decrease, rather than increase, over time (Figure 1B). However, the percent growth after restreaking on plates containing Kn plus Tc was again found to increase, until a maximum was reached at day 5.

The appearance of colonies that do not regrow upon restreaking could be due to the formation of mixed colonies on the original transformation plates, such that some cells in the colony contain pFUSLUX, while others contain pTF421. To test whether the cells in the colony were mixed, colonies appearing post-transformation were streaked on LB agar plates containing Ap, or Tc, or Ap plus Tc. Colonies that did not regrow on LB plates containing Tc plus Ap were found to regrow on plates containing either Ap or Tc. To determine the plasmid content of the colonies, eight colonies per day were analyzed, up until 7 days post-transformation of pFUSLUX into strain LF20102. It was found that free pFUSLUX plasmid could be detected in all of the colonies up until day 3, and that none of the examined colonies could grow upon restreaking on LB plates containing Ap plus Tc (Table 2). By day 4,

**Table 2: Plasmid Analysis of Colonies Post-Transformation**

| | Plasmid Detected | | |
|---|---|---|---|
| Days Post-transformation | pTF421 | pFUSLUX | Regrowth[a] |
| 1 | 0 | 8 | 0 |
| 2 | 7 | 8 | 0 |
| 3 | 8 | 8 | 0 |
| 4 | 8 | 4 | 5 |
| 5 | 8 | 3 | 6 |
| 6 | 8 | 4 | 7 |
| 7 | 8 | 0 | 3 |

a: Regrowth is scored as the ability of a colony to regrow after streaking on LB plates containing Tc plus Ap.

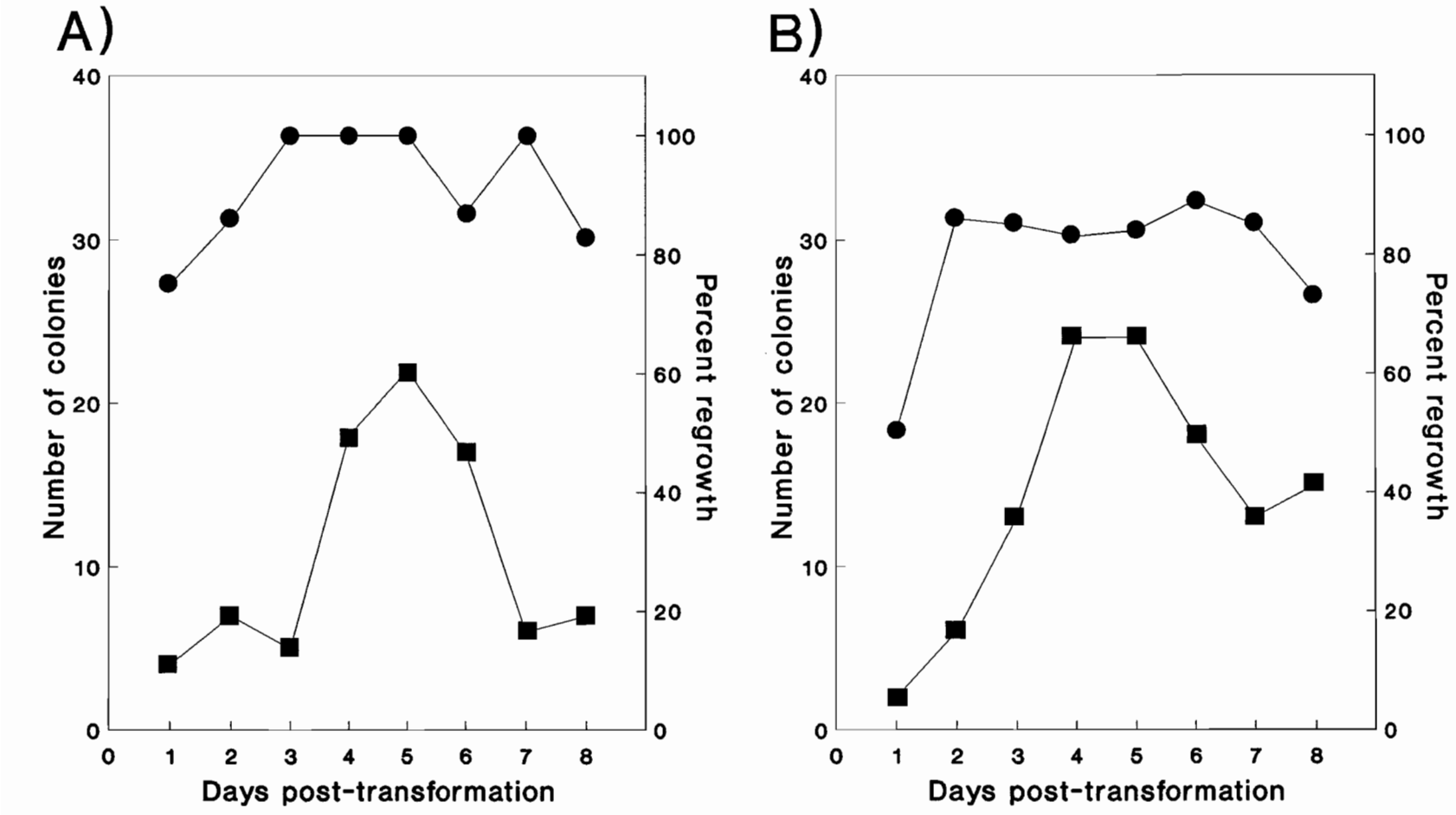
colonies that grew upon restreaking were obtained, and these were found to contain plasmid pTF421 (Table 2). Moreover, these $Ap^R$ and $Tc^R$ clones displayed hybridization of *luxAB* in the chromosomal DNA, corresponding to transposition of the Tn*5* element (Guzzo and DuBow, 1991). The plasmid pTF421 was not detected in the colonies which arose on the first day post-transformation, yet was detected in the colonies that arose after further incubation (2 to 7 days post-transformation). This suggests that it is the loss of pTF421, rather than transposition, that is the rate limiting step for colony formation in the first few days post-transformation, while Tn*5* transposition occurs only after further incubation.

To confirm whether Ap degradation around the pTF421-containing cells could account for the appearance of colonies that did not grow upon restreaking, the gene encoding $Kn^R$ was cloned into pTF421, as this antibiotic is not degraded around the cells. The *kan* gene was cloned into pTF421, simultaneously disrupting the *bla* gene, yielding the plasmid pAG42. As well, the *bla* gene was cloned into the unique *Sal* I site of the Tn*5* element of pFUSLUX, yielding plasmid pAG34, which is both $Ap^R$ and $Tc^R$. When pAG34 was transformed into strain LF20150 (*E. coli* DH1 containing pAG42), and selected either on LB plates containing Kn plus Tc (Figure 2A), or LB plates containing Kn plus Cb, another $\beta$-lactam antibiotic (Figure 2B), the vast majority of colonies that appeared at all times post-transformation could now grow upon restreaking onto double antibiotic-containing plates.

### 3.4.3 Other Gene Rearrangements in Stable, Tn*5* Chromosomal Insertion Clones

When pAG34 was transformed into LF20150, the number of stable Tn*5* chromosomal insertion colonies was found to increase over time (Figure 2A and B). The ability to detect increased frequencies of Mu and IS*103* excision after several days of incubation on petri dishes, under selective conditions, has been previously

Figure 2. Analysis of the transformants obtained using strain LF20150. Depicted are the number of colonies (■) and percent regrowth (●) after transformation of A) pAG34 into LF20150, selecting on Kn plus Tc; B) pAG34 into LF20150, selecting on Kn plus Cb. Refer to Materials and Methods for experimental details.


A)
B)
Number of colonies
Percent regrowth
Days post-transformation
0
10
20
30
40
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8

observed (Hall, 1988; Shapiro, 1984; see Discussion). Furthermore, *E. coli* clones containing transposition of an IS element into the *spc* operon were reported to contain one to three unselected IS*1* elements transposed to new chromosomal loci (Read and Jaskunas, 1980). Since some common cellular physiological mechanism/factor which affects transposition could be responsible for the transposition of Tn*5* over time, the movement of other transposable elements in the clones that had acquired a chromosomal Tn*5* element was investigated. The distribution of IS elements was examined by Southern blot analysis. The results are summarized in Table 3. An event involving an IS element was defined as the loss, or alteration of migration, of an IS element band in the Southern blots hybridized with the various IS elements. No new copies of an IS element were observed. However, it was found that 11% of the Tn*5-luxAB* containing clones had also undergone an IS*1* event, 6% had a detectable IS*2* event and 3% had a detectable IS*5* event (Table 3). These clones all arose from independent colonies on different petri dishes, and thus are unlikely to represent reisolation of siblings after transformation. It is interesting to note that 0.9% of the clones had also accumulated conditional lethal mutations. It was found that 0.5% were temperature sensitive, 0.2% were cold sensitive and 0.2% were anaerobic sensitive for growth (not shown).

## 3.5 DISCUSSION

During the study of transposition of a Tn*5-luxAB* element from a multicopy plasmid whose replication was inhibited *in trans*, it was found that new colonies, which had acquired a chromosomal Tn*5* insertion, appeared for over a week post-transformation, and that the total number of colonies increased over time (Figure 2A and B). The appearance of colonies over time, as observed here, under selective conditions is a hallmark of what are called adaptive mutations (Foster, 1993). Using

**Table 3: Frequency of IS and/or Chromosomal Events[a]**

| Element | Frequency of Events(%) |
|---|---|
| IS*1* | 11 |
| IS*2* | 6 |
| IS*5* | 3 |
| Cumulative | 20 |

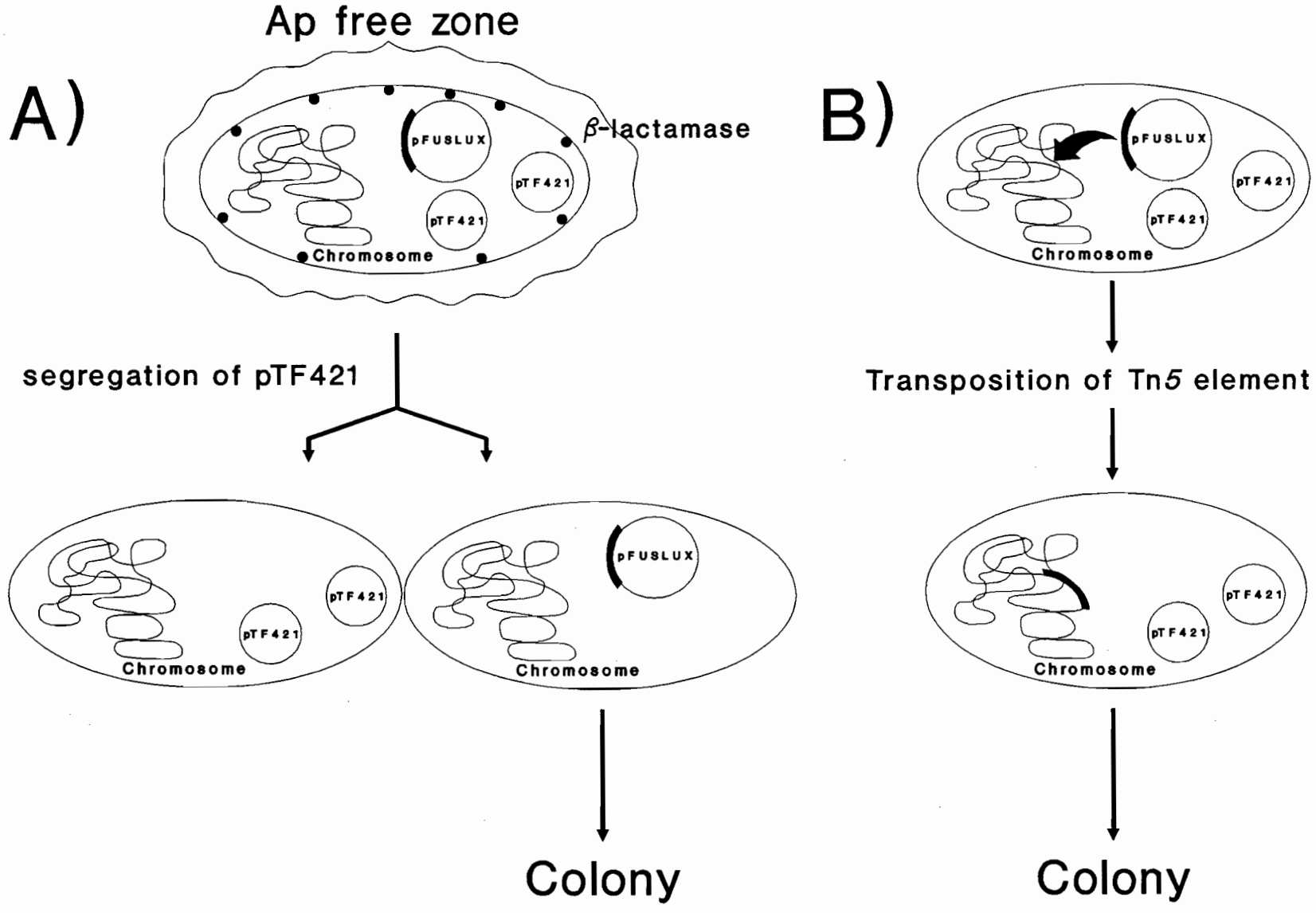
a: An event is defined as a change in the pattern of IS elements after hydrolysis of total cellular DNA, gel electrophoresis, Southern blotting and hybridization with the respective IS element.

*lacZam* mutations, Cairns *et al.* (1988) showed that a number of Lac$^+$ revertants arose after several days when the cells were plated on lactose minimal media, and did not occur when the selective agent was not present. Shapiro (1984) showed that in a strain that had the *araB* and *lacZY* genes separated by a defective Mu phage, fusion of *araB* to *lacZ* [to produce Lac(Ara) $^+$ colonies] occurred 5 to 6 days after plating on lactose-arabinose minimal media, and rose to a maximum level after 20 days. The frequency of gene fusion formation after plating was 1 X $10^{-8}$ per cell, but before plating could not be detected at a measured frequency of 3 X $10^{-11}$ per cell. Cairns *et al.* (1988) showed that fusion formation occurred only when both lactose and arabinose were present. Starvation alone did not cause Lac$^+$ reversion or Lac(Ara) $^+$ fusion formation. The presence of the selective agents was also required. Furthermore, strains that were Lac(Ara) $^-$ due to point mutations in *araC* or *lacZ* did not show a delay in the appearance of Lac(Ara) $^+$ colonies. Hence the formation of a gene fusion is a late event (Cairns *et al.*, 1988), and is dependent upon Mu transposition functions (Shapiro, 1984). Hall (1988) studied mutations in the *bgl* operon that required two events to allow utilization of the sugar substrate salicin; a point mutation in *bglR* (*blgR*$^o$ to *bglR*$^+$) and excision of an IS*103* in *blgF*, which occurred at independent rates of 4 X $10^{-8}$ and $< 2$ X $10^{-12}$ per cell division, respectively. Two to three weeks after plating on MacConkey salicin plates, double revertants appeared at a frequency of $10^{-8}$ per cell. Furthermore, the first, and rate-limiting, event was shown to be the excision of IS*103* in 1 to 10% of 8 to 12 day old colonies from which *blgR*$^+$ revertants developed. The frequency of IS transpositions to a P1 prophage was also found to be higher in the stationary phase of growth (Arber *et al.*, 1981; Iida *et al.*, 1983). Since it is not known if the cells in the system described here are growing after transformation, or what the growth rate is, it is not possible to determine whether there is an increase in the transposition frequency or rate over time.

However, it appears that the plasmids transformed into the cells are able to persist for several days, during which time the number of cells containing a chromosomal copy of Tn*5* increases. Furthermore, 20% of Tn*5-luxAB* containing clones had a change in the chromosomal arrangement of IS*1*, IS*2* or IS*5* elements (Table 3).

It should be noted that in the system employed here the cells are not starved, but instead their growth is restricted due to the presence of antibiotics in the growth media. The events described here can be divided into an early phase, where colony formation appears to be predominantly due to segregation and loss of pTF421 (Figure 3A), and a late phase where the predominant event allowing colony formation is chromosomal insertion of Tn*5* (Figure 3B). Colonies that did not grow upon restreaking after transforming the $Tc^R$ plasmid pFUSLUX into strain LF20102 (*E. coli* DH1 containing the $Ap^R$, RNA1-overproducing plasmid pTF421) were examined and found to contain predominantly pFUSLUX. If pTF421 is poorly partitioned, and ultimately lost, from a cell that contains pFUSLUX, a colony could form due to prior Ap degradation around the pTF421-containing cells (Sambrook *et al.*, 1989). This would release pFUSLUX replication from RNA1 inhibition, allowing this cell to grow and divide in an area devoid of active Ap (Figure 3A). Hence, we can hypothesize that the cells may grow slowly on the petri dishes post-transformation until either pTF421 is lost from the cell, or transposition occurs, resulting in growth and colony formation. Lenski and Mittler (1993) argued that adaptive mutations may appear under selective conditions due to slow growth of the mutant on the selective media, which would allow replication-dependent mutations to occur. When a mutation occurs that allows optimal growth of the mutant, a colony will form. Slow growth can occur if the mutation is leaky, by cross-feeding, or by growth-supporting contaminant substrates in the selective media. We also propose that the cells are growing at a low rate until either pTF421 segregation (Figure 3A) or Tn*5* transposi-

Figure 3. The events that give rise to a colony after transformation. After transformation of pFUSLUX into *E. coli* DH1 containing pTF421 and selection for $Tc^R$ plus $Ap^R$, two events may give rise to a colony: A) Cell division leading to pTF421 segregation. Since Ap is degraded around the pTF421-containing cells, an Ap free zone is formed allowing the pFUSLUX-containing cells to grow and form a colony; B) If Tn*5*-*luxAB* ($Tc^R$) transposes to the chromosome with the concomitant loss of pFUSLUX, cells containing pTF421 can divide and form a colony. Plasmid pFUSLUX contains the *luxAB* and *tet* genes, flanked by Tn*5*, on a ColE1-based replicon.


Ap free zone
A)
pFUSLUX
β-lactamase
pTF421
pTF421
Chromosome
segregation of pTF421
pTF421
pTF421
Chromosome
pFUSLUX
Chromosome
Colony
B)
pFUSLUX
pTF421
pTF421
Chromosome
Transposition of Tn5 element
pTF421
pTF421
Chromosome
Colony

tion (Figure 3B) occurs, that then allows the cells to grow exponentially and form a colony. It is possible that cells post-transformation share similar properties to cells under starvation stress, and may be in a similar physiological state due to some other underlying factor such as a low metabolic rate. Genetic controls such as DNA repair pathways have been explored (Foster, 1993). However, it is also possible that there is a protein(s) that is expressed under these conditions that regulates both transposition frequency, plasmid partitioning and mutation rates. The Clp protease has been implicated in affecting Mu-mediated rearrangements (Mhammedi-Alaoui *et al.*, 1994; Shapiro, 1993) and in the degradation of proteins expressed during carbon starvation (Damerau and St. John, 1993). The signal for increased synthesis of $\sigma^S$, a stationary-specific sigma factor, was shown to be homoserine lactone, a metabolite synthesized from intermediates in threonine biosynthesis (Huisman and Kolter, 1994), and is another potential candidate for regulating cellular processes during slow growth.

It is surprising that the resident plasmid, pTF421, is lost at such a seemingly high frequency. Plasmid stability is proportional to copy number, partitioning, oligomer formation, plasmid load and host-killing systems (Nordström and Austin, 1989; Summers, 1991). Models for the loss of multicopy, nonconjugative plasmids, such as pTF421, are based upon a random distribution. Hence, the probability of forming a plasmid-free daughter is a function of the copy number at cell division. Those cells with fewer pTF421 plasmids will form a colony early. Thus, the more pTF421 replicates in the cells that contain pFUSLUX, the longer it will take to lose pTF421 from the cells and the longer the time before a colony appears post-transformation, accounting for their appearance over several days. Recent evidence for pBR322-derived plasmids (also using a ColE1 replicon) suggests that there is a high variance within a population of cells for the production of plasmid-free daugh-

ter cells, and that a subpopulation of cells exists which can give rise to plasmid-free cells at a high rate (Tolker-Nielsen and Boe, 1994).

These studies have demonstrated the transposable element-mediated acquisition of antibiotic resistance determinants in cells after prolonged periods of incubation on petri dishes. Bacteria spend only a fraction of their existence in exponential phase due to the limited availability of nutrients (Tormo *et al.*, 1990). The *in situ* generation time for *E. coli* in the intestine was estimated to be 12 hours (Roszak and Colwell, 1987). Slow growth, as demonstrated here, may favor gene acquisition, transposition, and plasmid partitioning and loss. These results may also have serious implications for human health since transposition from plasmids is often accompanied by antibiotic resistance gene acquisition. The reemergence of antibiotic resistant strains of bacteria is still a public health threat (Davies, 1994). Further insights into how antibiotic resistance is acquired and disseminated via transposons and plasmids should provide interesting information on microbial ecology and physiology. Concomitant changes in gene structure, as observed here with IS elements, also demonstrates how evolution need not be a gradual process. In maize, stress conditions can cause major gene rearrangements, such as chromosome translocations, breaks and fusions, and can also activate the movement of transposable elements (McClintock, 1984). Transposition of multiple mobile elements within the same *Drosophila melanogaster* germ cells were found and termed 'transposition bursts' (Georgiev, 1984). These major gene rearrangements may also explain the evolution of new plant and animal species, as well as augment a cell's arsenal to survive prolonged environmental stress (McClintock, 1984).

# CHAPTER 4

## Transcription of the *Escherichia coli fliC* Gene is Regulated by Metal Ions

## PREFACE

In Chapter 2, a library of 3000 single-copy *Escherichia coli* chromosomal *luxAB* gene fusions was created. By screening the library of *luxAB* gene fusions in the absence and presence of 1 and 10 $\mu$g/ml of aluminum at pH 5.5, two clones were found that displayed increased luminescence with increasing aluminum concentrations. The characterization of one of the clones is described in Chapter 4.

## 4.1 ABSTRACT

A library of *luxAB* gene fusions in the *Escherichia coli* genome were used to screen for clones displaying transcriptional changes in the presence of aluminum. One clone was found that contained a luciferase gene fusion in which transcription was increased in the presence of aluminum, and subsequently shown to be induced by copper, iron and nickel. Cloning of the metal-regulated gene, hybridization to the ordered phage λ bank of the *E. coli* chromosome, and sequencing of DNA adjacent to the *luxAB* fusion revealed that the insertion occurred within the *fliC* (*hag*) gene of *E. coli*. This gene encodes flagellin, the filament subunit of the bacterial motility organ, and is under the control of several regulatory cascades. These results suggest that environmental metals may play a role in regulation of the motility potential of *E. coli*, and that this bioluminescent gene fusion clone (or derivatives thereof) may be used to prepare a biosensor for the rapid detection of metal contamination in water samples.

## 4.2 INTRODUCTION

Toxic metals can have deleterious effects by blocking essential functional groups, displacing essential metal ions, or modifying the active conformation of biological molecules (Collins and Stotzky, 1989). Bacterial cells have evolved mechanisms to deal with elevated levels of these toxic environmental metals. The plasmid-borne heavy metal resistance operons found in many species of bacteria are a good example of this (Hennecke, 1990; Silver and Misra, 1988).

Aluminum is a non-essential metal generally present in natural waters at concentrations ranging from approximately $< 0.03\ \mu g/ml$ to $> 0.67\ \mu g/ml$ (Birchall *et al.*, 1989). Aluminum becomes more soluble with increasing acidity and can often be the most toxic element in acidic waters (Cronan and Schofield, 1979; Johnson

and Wood, 1990). Several mechanisms have been postulated to explain aluminum toxicity (Birchall *et al.*, 1989; Schindler, 1988), including its potential to bind to DNA in *Rhizobium* spp. (Johnson and Wood, 1990). Genetically-programmed responses to elevated levels of aluminum in bacteria have not yet been defined. We chose to search for genes in the *Escherichia coli* chromosome that may play a role in cellular responses to elevated levels of aluminum in the environment. The rationale behind our approach was that genes (and their encoded products) that play a role in responding to elevated levels of aluminum could be induced (or repressed) at the level of transcription in the presence of the metal. In order to detect these transcriptional changes, a library of approximately 3000 *E. coli* clones was created, each of which contained a random, single, chromosomally-located insertion of a promoterless *luxAB* transcriptional reporter gene cloned within a truncated Tn*5* element (Guzzo and DuBow, 1991). The gene product of the *Vibrio harveyi luxAB* genes, luciferase, is a mixed function oxidase composed of an $\alpha$ and $\beta$ subunit that catalyzes oxidation of an aldehyde in a reaction that results in the emission of light (Ziegler and Baldwin, 1981). The amount of light produced by *E. coli* cells carrying the *luxAB* genes downstream from promoters is proportional to the amount of transcription of *luxAB* (Engebrecht *et al.*, 1985).

By monitoring changes in light emission of clones from the *E. coli luxAB* library in the absence and presence of aluminum, we identified a clone with a gene fusion whose transcription is induced in the presence of aluminum. We present evidence that transcription of this gene is also affected by other metals, and we identify this gene as *fliC* (formerly called *hag*), which encodes flagellin, the structural protein of the flagellar filament (Macnab, 1987a).

## 4.3 MATERIALS AND METHODS

### 4.3.1 Bacterial Strains

*E. coli* strain DH1 [F⁻ *recA1 endA1 gyrA96 thi hsdR17* ($r_k^-$, $m_k^+$) *supE44 relA1*] (Hanahan, 1983) was used to prepare the library of *luxAB* transcription fusion clones (Guzzo and DuBow, 1991). Strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd5* ($r_k^-$, $m_k^-$) (F' *proAB lacI*$^q$*ZΔM15*)] (Gough and Murray, 1983) was used for subcloning the chromosomal DNA from gene fusions originally present in strain DH1. Strain NM621 (*hsdR mcrA mcrB recD1009 supE44*) (Whittaker *et al.*, 1988) was used for propagation of the λ phage *E. coli* chromosomal library prepared by Kohara *et al.* (1987).

### 4.3.2 Media

Strains were routinely grown at 37°C in LB broth or on LB plates containing 1.5% agar (Miller, 1972), and supplemented with antibiotics when indicated. Tc was used at a final concentration of 10 μg/ml in broth and 20 μg/ml in agar. Ap was used at a final concentration of 40 μg/ml.

### 4.3.3 DNA Manipulations

Plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis *et al.*, 1982). All restriction enzyme digestions and ligations were performed as previously described (Tolias and DuBow, 1987). Transformations were performed (Mandel and Higa, 1970) and incubated at 37°C. Isolation of total cellular DNA was performed as previously described (Guzzo and DuBow, 1991).

### 4.3.4 Construction of the *luxAB* Gene Fusion Library

Construction of the *luxAB* gene fusion library has been described elsewhere (Guzzo and DuBow, 1991). Briefly, the ColE1-based plasmid pRZ341-21 (Johnson and Reznikoff, 1983), containing a *tet* gene plus the *luxAB* genes within a left end-truncated Tn*5* element, was constructed and designated pFUSLUX. The plasmid pFUSLUX was introduced into an *E. coli* strain DH1 that contained an $Ap^R$ plasmid, pTF421, which inhibits the replication of ColE1 plasmids via overproduction of RNA1 (Fitzwater *et al.*, 1984). Transformation on media containing Tc plus Ap selects for cells that contain the *luxAB* genes inserted into the chromosome at a single location. A library of approximately 3000 such clones was created.

### 4.3.5 Construction of Plasmid pAG5

Plasmid pAG5 was constructed by ligating 3.0 $\mu$g of *Bam*HI-cleaved total cellular DNA isolated from strain LF20111 to 1.0 $\mu$g of *Bam*HI-cleaved pUC119 (Vieira and Messing, 1987). The ligation mixture was transformed into strain NM522 (Gough and Murray, 1983) and transformants were selected on Tc-containing LB plates (Figure 2).

### 4.3.6 Mapping Using the Kohara Library

The Kohara *et al.* (1987) ordered $\lambda$ library of the *E. coli* genome was propagated on *E. coli* strain NM621 and plated as described elsewhere (Whittaker *et al.*, 1988). Transfer to nitrocellulose filters (Xymotech Biosystems, Town of Mount Royal, Canada) was performed following the plaque lift procedure for DNA (Maniatis *et al.*, 1982). The probe was prepared by isolating 1.0 $\mu$g of a 2.3 kb *Hin*d III fragment from plasmid pAG5 and then labeled and hybridized using the DIG DNA Labeling and Detection Kit (Boehringer-Mannheim, Laval, Canada)

according to the manufacturer's instructions.

### 4.3.7 Southern Blotting and Hybridization of Probe

Ten micrograms of total cellular DNA was used per restriction endonuclease hydrolysis and then subjected to electrophoresis through a 0.75% agarose gel. Transfer of total cellular DNA to Hybond-N (Amersham Ltd., Oakville, Canada) by the bidirectional method and hybridization were performed as described elsewhere (Smith and Summers, 1980). A sample (0.1 $\mu$g) of a 2.3 kb *Hin*dIII fragment was isolated from plasmid pAG5 and labeled by using the random-primed labeling method (Feinberg and Vogelstein, 1983; 1984) with [$\alpha$-$^{32}$P]dGTP (3000 Ci/mmol; ICN Biomedicals, Mississauga, Canada).

### 4.3.8 DNA Sequencing

Single-stranded phagemid DNA preparation from plasmid pAG5 was performed as described elsewhere (Vieira and Messing, 1987). Dideoxy sequencing reactions using [$\alpha$-$^{35}$S]ATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada) were performed with a Sequenase kit (U.S. Biochemical Corp., Cleveland, USA) according to the manufacturer's protocol. To determine the sequence at the junction between IS*50*R and the chromosomal DNA in pAG5, an oligonucleotide ($^{5'}$AAGGTTCCGTTCAGGAC$^{3'}$) corresponding to base pairs 1497 to 1513 of IS*50*R (Auerswald *et al.*, 1981) was synthesized (Sheldon Biotechnology Centre, Montreal, Canada) and used as a primer. To determine the sequence at the *Bam*HI site in the chromosomal DNA, the *Bam*HI fragment of plasmid pAG5 (containing the *tet* gene, IS*50*R, and adjacent chromosomal DNA sequences) was cloned into the *Bam*HI site in the opposite orientation in the same vector (pUC119), and the -40 primer provided in the Sequenase kit was used.

4.3.9 Assay for Luciferase Induced Light Emission

This technique is taken from Miyamoto *et al.* (1985). Briefly, 1-$cm^2$ patches of cells were grown overnight on an LB agar petri dish, placed upside down, and exposed to KODAK XAR-5 film at 23°C after addition of 50 $\mu$l of dodecyl aldehyde (Aldrich, Milwaukee, USA) to the cover of the petri dish. The X-Ray films were developed after various times.

## 4.4 RESULTS

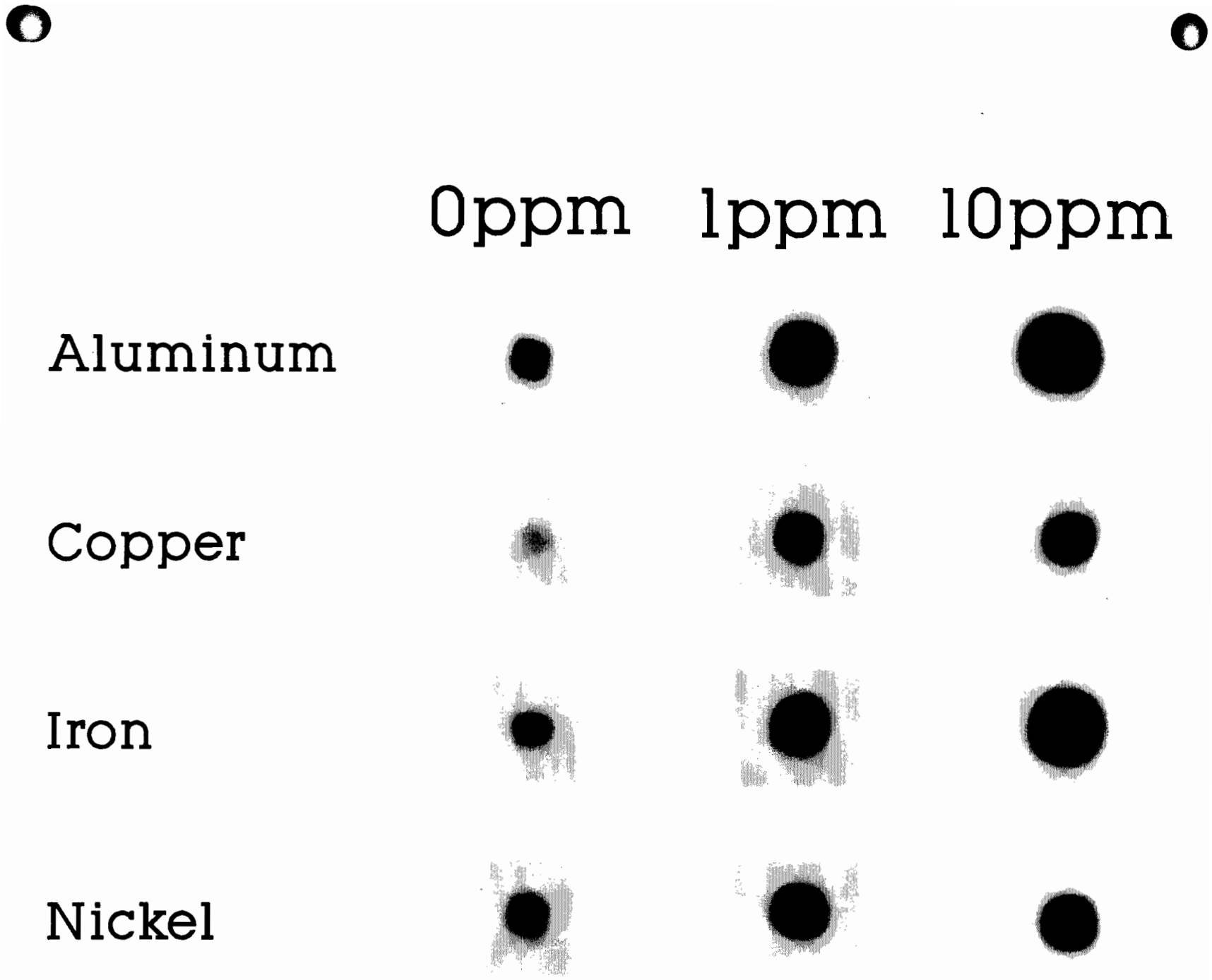
### 4.4.1 Identification of a Gene Whose Transcription is Increased in the Presence of Aluminum

Aluminum toxicity increases with decreasing pH due to its increased solubility at lower pHs; the toxic effects occur maximally between pH 5.0 and 6.0 (Wood, 1988). The light emission of each individual clone of the *E. coli* luciferase gene fusion library was monitored in the absence of aluminum and in the presence of aluminum at 1 and 10 $\mu$g/ml (1 ppm and 10 ppm) at a pH of 5.5; this level of aluminum is above background levels (approximately $< 0.03$ $\mu$g/ml to $> 0.67$ $\mu$g/ml) found in natural waters (Birchall *et al.*, 1989). One clone, designated LF20111, was identified by its increasing gene expression (light emission) with increasing concentrations of aluminum (Figure 1). Strain LF20111 was screened in the presence of other metals to determine the specificity of the transcriptional response as measured by light emission. Transcription was also found to increase in the presence of copper, iron, and nickel at 1 and 10 $\mu$g/ml at pH 5.5 (Figure 1).

### 4.4.2 Cloning and Mapping of the Metal-Responsive Gene

It was determined, by Southern blotting analysis, that the Tn*5-luxAB* element was present in the chromosome at a single location (data not shown). Thus, cloning

Figure 1. Light emission of 1-$cm^2$ patches of strain LF20111 grown in the presence of the indicated quantities of added aluminum, copper, iron and nickel (as $AlCl_3$, $CuSO_4$, $FeCl_3$ and $NiSO_4$) at pH 5.5. See Materials and Methods for experimental details.


0ppm
1ppm
10ppm
Aluminum
Copper
Iron
Nickel

of the right end of the gene fusion could be accomplished in one step by using the *tet* gene in the Tn*5-luxAB* element as a selectable marker. Cloning was performed by cleaving total cellular DNA from strain LF20111 with *Bam*HI, and ligating the DNA to plasmid pUC119 (Vieira and Messing, 1987) hydrolyzed with the same enzyme (Figure 2). The ligation mixture was transformed into strain NM522 (Gough and Murray, 1983) and selection for $Tc^R$ was performed. Because there are no *Bam*HI sites in *tet* and IS*50*R, the only plasmids that survive this selection should contain *tet*, IS*50*R and the chromosomal sequences between IS*50*R and the adjacent, chromosomal *Bam*HI site (Figure 2). The plasmid clone designated pAG5 was obtained by this procedure.

Plasmid pAG5 was digested with *Hin*dIII, and a fragment containing 1.1 kb of IS*50*R and approximately 1.2 kb of adjacent chromosomal DNA was isolated (Figure 2). This fragment was labeled and hybridized to the ordered phage λ bank of the *E. coli* chromosome (Kohara *et al.*, 1987) and was found to hybridize to phages 341 and 342 (Figure 3A). Thus, the junction between IS*50*R and the chromosomal DNA was contained in the area of the bacterial genome common to both phages 341 and 342.

The exact size of the chromosomal *Bam*HI fragment in which the Tn*5-luxAB* element had inserted was determined by digesting the cellular DNA of the parent *E. coli* strain DH1, in which the fusions were prepared, with *Bam*HI and hybridizing to the *Hin*dIII probe (Figure 3C). The band was determined to be 1.8 kb in size. In strain LF20111, in which the *Tn5-luxAB* element is inserted in the chromosome, the band increases in size to approximately 8.5 kb. Another band, approximately 5 kb in size, appears that is due to cross-hybridization of the probe to the ColE1 RNA1-overproducing plasmid pTF421 (data not shown).

By combining the information obtained from mapping and analysis via Southern

Figure 2. Schematic diagram for the cloning of the metal-responsive gene. Restriction enzyme sites are abbreviated as follows: *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). See Materials and Methods for experimental details.

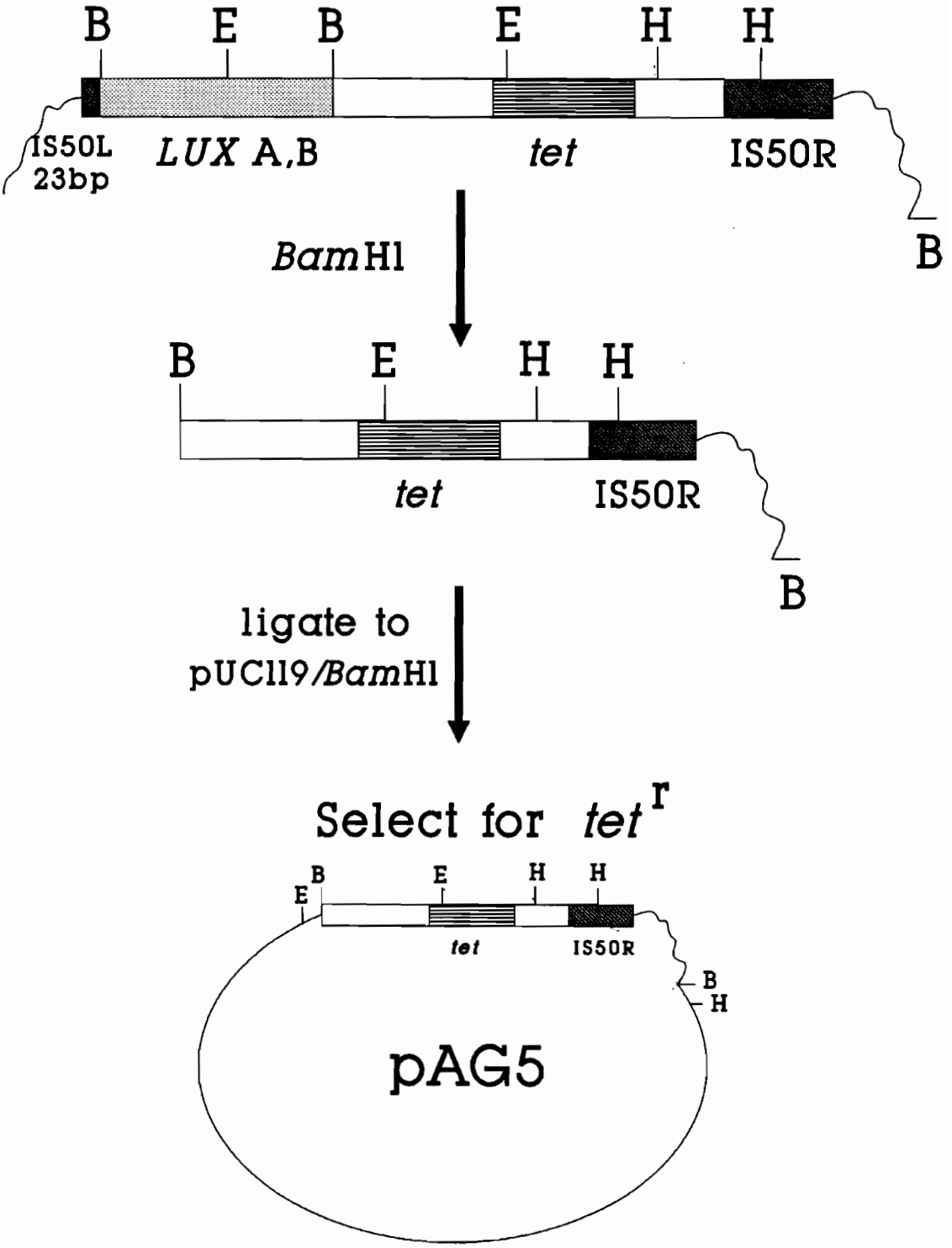
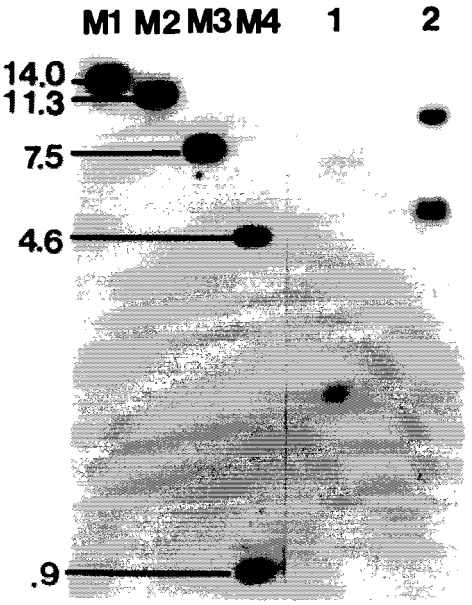

B
E
B
E
H
H
IS50L
23bp
LUX A,B
tet
IS50R
B
BamHI
B
E
H
H
tet
IS50R
B
ligate to
pUC119/BamHI
Select for tet r
B
E
E
H
H
tet
IS50R
B
H
pAG5

Figure 3. Strategy for the determination of the genomic location of the metal-responsive gene. A) Hybridization to the ordered phage λ *E. coli* gene bank (Kohara *et al.*, 1987). Shown is the hybridization to plaques containing DNA from phages 341 and 342. Plaques containing DNA from phages 340 and 343 are also shown. B) Transcription map from the *fliC* promoter/operator through the *luxAB* genes in relation to the published *Bam*HI restriction map corresponding to phages 341 and 342 (Kohara *et al.*, 1987). Sequencing localized the site of the Tn*5-luxAB* insertion to between bp 97 and 98 with respect to the start of the *fliC* coding sequence (Kuwajima *et al.*, 1986). C) Southern blot analysis of *Bam*HI-cleaved DNA of the parent strain DH1 (lane 1) and strain LF20111 (lane 2). Sizes in kb of marker DNAs (lanes M1 - M4) are indicated at the left.

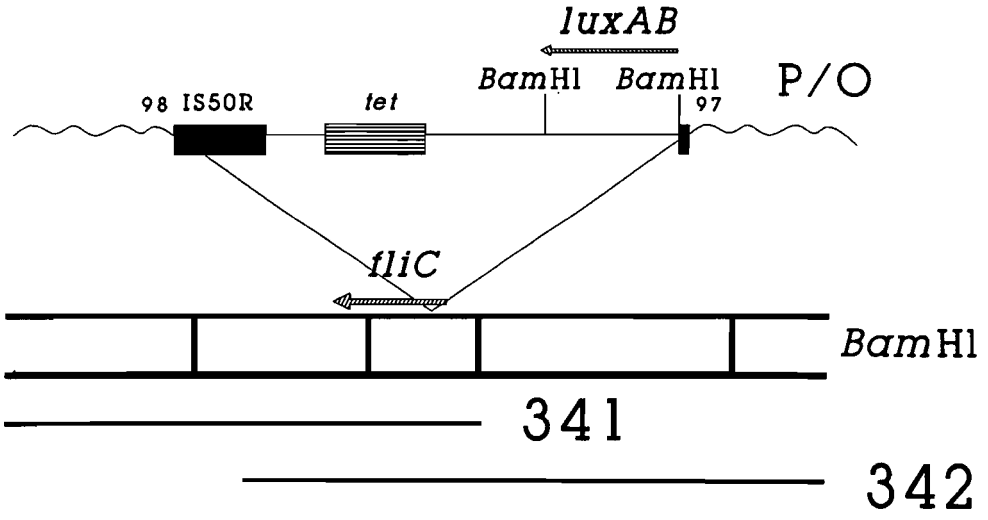
A


340
341
342
343

C


M1 M2 M3 M4
1
2
14.0
11.3
7.5
4.6
.9

B


luxAB
BamHI
BamHI
P/O
98 IS50R
tet
97
fliC
BamHI
341
342

blotting, it was determined that the Tn*5-luxAB* element had inserted approximately 1.2 kb from the first *Bam*HI site common to both phages in a right-to-left orientation on the Kohara *et al.* (1987) restriction map of the *E. coli* genome at approximately 42.4 minutes, in the vicinity of *fliC* (Bachmann, 1990).

### 4.4.3 Sequencing and Identification of the Metal-Responsive Gene

To precisely identify the metal-responsive gene, the junction between IS*50*R and the site of Tn*5-luxAB* insertion was sequenced from the plasmid pAG5. The sequence obtained was analyzed by a computer homology search against the GenBank data bank; the *fliC* gene, encoding flagellin, was identified. The site of insertion is 97 bp downstream from the start of the *fliC* coding sequence (Kuwajima *et al.*, 1986) (Figure 3B) and in the correct orientation for the *luxAB* genes to be under the control of the *fliC* promoter region. DNA sequencing was also performed at the other *Bam*HI junction to rule out the possibility of chromosomal rearrangements that may have occurred at the site of insertion. This was found to be the *Bam*HI site situated at bp 1224 of *fliC*, in good agreement with the distances obtained from gel electrophoresis of pAG5 restriction fragments and the Southern blot of strains DH1 and LF20111 (Figure 3C).

## 4.5 DISCUSSION

Aluminum is the most abundant metal in the Earth's crust (Johnson and Wood, 1990). It has been noted that aluminum displays increasing toxicity to living organisms as the pH is lowered below 6.0, presumably due to its increasing solubility (Birchall *et al.*, 1989; Cronan and Schofield, 1979; Cronan *et al.*, 1986; Johnson and Wood, 1990; Wood, 1988). To identify any genetically programmed responses that play a role in coping with the stress of elevated levels of environmental aluminum,

*luxAB* gene fusions in the *E. coli* chromosome were screened at pH 5.5 in the absence and presence of aluminum. A clone was found that had the *luxAB* genes fused to a gene whose transcription was increased in the presence of aluminum, as well as copper, nickel and iron, at pH 5.5 (Figure 1). Zinc was also found to induce transcription, whereas lithium has little, if any, effect (data not shown). Cloning, mapping and sequencing revealed that the *luxAB* genes were inserted 97 bp downstream from the start codon of the *fliC* coding sequence (Figure 3).

The *fliC* gene encodes flagellin, which is the single protein that makes up the filament of the *E. coli* flagellum (Macnab, 1987a). Gene fusions of *fliC-lacZ* have been previously studied to determine the effects on transcription by other proteins involved in the assembly and regulation of the flagellum (Komeda, 1982; Komeda and Iino, 1979). It was found that *fliC* transcription is controlled by many steps in a complex, regulatory cascade mechanism. Any defect in a step in the regulatory cascade resulted in decreased expression of *fliC* and thus reduced synthesis of the flagellar filament (Komeda, 1982; Komeda and Iino, 1979; Macnab, 1987a). Assembly of the flagellum is costly to the cell: the growth disadvantage of synthesis relative to cells not synthesizing a flagellum was estimated to be about 2%, as measured on the basis of growth rate (Macnab, 1987a). However, the flagellum can also provide a growth advantage, since it is part of the machinery that enables *E. coli* to migrate toward nutrients and away from toxicants (Macnab, 1987b).

We have found that *fliC* transcription can be induced by the presence of low concentrations of metal ions. It is still not clear at which step, if any, in the regulatory cascade the metal ions mediate the increase in *fliC* transcription. Stimulation of *fliC* gene expression in the presence of aluminum occurred at pH 5.5 but very little occurred at pH 7.0 (data not shown). This response may be biologically relevant, since the cell should be expending energy only in the presence of aluminum at doses

that would harm the cell and only at concentrations that are above the natural background levels in the environment. At pH 7.0, the toxicity of aluminum is reduced due to its decreased solubility, possibly explaining the lack of *fliC* induction at pH 7.0. If the *fliC* gene product were important to cell survival in the presence of heavy metals, the Tn*5-luxAB* insertion in the gene would disrupt it, and thus strain LF20111 would be more sensitive than the wild-type strain DH1 to elevated concentrations of aluminum. No significant differences in MIC were found when strains LF20111 and DH1 were plated on LB media containing aluminum at pH 5.5 or at pH 7.0 (data not shown).

Two transcriptional regulatory proteins have been shown to require iron (Fur) or divalent metal ions (Fnr) for activity. Both the Fur and Fnr proteins have a consensus binding sequence upstream of the start of transcription (Hennecke, 1990). Neither consensus binding site was found upstream of the start site of *fliC* transcription.

Heavy metals have complex chemistries, and their concentrations in aquatic environments depend upon many factors including pH, $E°$, and the presence of organic ligands (Cronan *et al.*, 1986; Wood, 1988). Also, metal concentrations are not always constant. For example, in spring, melting snow can produce a strong acid and aluminum pulse (Schindler, 1988). The ability to quickly and easily measure the bioavailability of metal ions above background levels in the environment would be useful for early detection of metal ion contamination in aqueous samples. Strain LF20111 showed increased light emission in the presence of several metal ions. However, the *fliC*::Tn*5-luxAB* bacterial clone described here may prove useful as the starting material for engineering a bioluminescent biosensor to measure elevated amounts of only heavy, toxic metal ions in aquatic environments with current *in vivo* and *in vitro* genetic manipulations. The advantages of using a microorganism for

detection are that the amount of metal measured is equivalent to the amount available to living organisms and the rapidity and ease of the bioluminescence assay (Bulich, 1984). Moreover, since the reagent (strain LF20111) is living, unlimited quantities can be inexpensively prepared.

# CHAPTER 5

## A *luxAB* Transcriptional Fusion to the Cryptic *celF* Gene of *Escherichia coli* Displays Increased Luminescence in the Presence of Nickel

## PREFACE

In Chapter 2, a library of 3000 single-copy *Escherichia coli* chromosomal *luxAB* gene fusions was created. By screening the library of *luxAB* gene fusions in the absence and presence of 1 and 10 $\mu$g/ml of nickel, one clone was found that displayed increased luminescence with increasing concentrations of nickel. Chapter 5 describes the characterization of this clone.

## 5.1 ABSTRACT

From a library of 3000 *Escherichia coli* clones, each containing a single, chromosomally-located *luxAB* transcriptional gene fusion, one clone was found in which luminescence increased in the presence of 1 to 50 parts per million of $NiSO_4$. A molecular analysis revealed that the insertion occurred within the *celF* gene of *E. coli*. This gene encodes the phospho-β-glucosidase involved in cleavage of the sugars cellobiose, salicin and arbutin. Cloning and sequencing of DNA downstream of the *celF* gene revealed three open reading frames (potentially encoding polypeptides of 9.9-, 14.1- and 28.5-kDa) that could be coexpressed with the *celF* gene and which may underlie the observed induction of the *celF* gene by nickel. A polypeptide of 26-kDa was produced when this region was placed under the control of the $P_{tac}$ promoter. Moreover, this region was found to be directly adjacent to, and transcribed in the opposite orientation from, the *katE* gene of *E. coli*.

## 5.2 INTRODUCTION

Nickel is a metal that is toxic at elevated concentrations and is carcinogenic to humans (Costa, 1991). Nickel compounds yield negative results for bacterial mutagenesis using *Salmonella typhimurium* and *Escherichia coli* tester strains, but can test positive in mammalian mutagenicity assays (Costa, 1991; Sunderman Jr., 1989). Although toxic at high doses, nickel is an essential trace element required for bacterial ureases, hydrogenases, methylcoenzyme M reductases, and CO dehydrogenases (Hausinger, 1987; Wu *et al.*, 1991).

We are interested in studying the regulation of chromosomal gene expression by metals (Guzzo *et al.*, 1991), including nickel. To our knowledge, only one bacterial gene, the hydrogenase of *Bradyrhizobium japonicum*, has been proven to be induced in the presence of nickel. The regulatory sequences required for nickel-

induced gene expression were delineated to bp -168 to -118 upstream of the transcriptional start site (Kim *et al.*, 1991). In an effort to identify other chromosomal genes and physiological functions which are regulated by nickel, a library of *E. coli* clones that contains the promoterless *luxAB* reporter genes inserted at different, single loci (Guzzo and DuBow, 1991) was screened to find genes that change their expression (i.e. light emission) in the presence of nickel. Screening of this library, in the absence and presence of nickel, allowed the identification of a single clone that contains the *luxAB* genes within a gene whose expression is elevated in the presence of nickel. We report the identification of this gene as *celF*, the distal-most cistron of an, at mesophilic temperatures, cryptic (Droffner and Yamamoto, 1992) operon of *E. coli*, involved in the metabolism of cellobiose, salicin and arbutin (Parker and Hall, 1990a). Moreover, we found three ORFs, adjacent to the *celF* gene, which may be coexpressed with the *celF* gene, and which are adjacent to, and in the opposite orientation from, the *katE* gene (von Ossowski *et al.*, 1991).

## 5.3 MATERIALS AND METHODS

### 5.3.1 Bacterial Strains and Media

*E. coli* strain DH1 [$F^-$ *recA endA1 gyrA96 thi hsdR17* ($r_k^-$, $m_k^+$) *supE44 relA1*] (Hanahan, 1983) was used to prepare the library of *luxAB* transcription fusion clones (Guzzo and DuBow, 1991). Strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd-5* ($r_k^-$, $m_k^-$) (F' *proAB lacI*$^q$*ZΔM15*)] (Gough and Murray 1983) was used for subcloning the chromosomal DNA from gene fusions originally present in strain DH1. Strain LF20143 is strain DH1 with a Tn*5-luxAB* element inserted in the *xyl* operon (Guzzo and DuBow, 1991). Strains were routinely grown at 37°C in LB broth (pH 7.2) or on LB plates containing 1.5% agar, supplemented with Tc (final concentration of 10 μg/ml in broth and 20 μg/ml in agar). Nickel sulfate (11.15 mg/ml),

copper sulfate (2.76 mg/ml), cobalt chloride (10.09 mg/ml) or cellobiose (5% w/v) stock solutions, prepared in sterile deionized water, were added to achieve the indicated final concentrations in culture media.

### 5.3.2 Construction of Plasmids pAG27, pAG28, pAG29 and pAG30

Plasmid pAG27 was constructed by ligating 4.0 μg of *Bam*HI-cleaved total cellular DNA, isolated from strain LF20112 (the *luxAB* gene fusion strain inducible by nickel, see Results) to 1.0 μg of *Bam*HI-cleaved pUC119 (Vieira and Messing, 1987) and was isolated after selecting transformants on Tc-containing media (Guzzo *et al.*, 1991). The 2.75 kb *Bam*HI-*Hin*dIII fragment from pAG27 (0.2 μg) was ligated to 0.1 μg each of pUC118 and pUC119 (Vieira and Messing, 1987), also cleaved with *Bam*HI plus *Hin*dIII, and transformed into strain NM522. The resultant plasmids were designated pAG28 and pAG29, respectively. The 3' recessed ends of 1.0 μg of the 2.75 kb *Bam*HI-*Hin*dIII fragment from plasmid pAG27 were filled-in and ligated to 0.5 μg of plasmid pKK223-3 (Brosius and Holy, 1984) cleaved at the *Sma*I site, and transformed into strain NM522, yielding plasmid pAG30.

### 5.3.3 DNA Sequencing and Mapping with the Kohara Library

Labeling of the cloned chromosomal DNA and mapping using the λ library of the *E. coli* genome (Kohara *et al.*, 1987) was performed as previously described (Guzzo *et al.*, 1991). The probe was prepared by isolating 1.0 μg of a 2.75 kb *Bam*HI-*Hin*dIII DNA fragment from plasmid pAG27. Determination of the sequence at the junction between IS*50*R and the adjacent chromosomal DNA cloned into plasmid pAG27 was performed as previously described (Guzzo *et al.*, 1991).

### 5.3.4 Sequencing of the Genomic Junction Between *celF* and *katE*

Single-stranded phagemid DNA was prepared (Vieira and Messing, 1987) from plasmids pAG28 and pAG29. Dideoxy sequencing reactions with [$\alpha$-$^{35}$S]dATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada) were performed with a Sequenase kit (U.S. Biochemical Corp., Cleveland, USA) according to the manufacturer's protocol. To determine the sequence at the 3' end of the *cel* operon, an oligonucleotide ($^{5'}$TCTGGCCATAGGGATGC$^{3'}$), corresponding to bp 4924 to 4940 (Parker and Hall, 1990a), was synthesized (Sheldon Biotechnology Centre, Montreal, Canada) and used as a primer in the sequencing reaction with plasmid pAG28. Sequencing at the 3' end of the *katE* gene was performed using an oligonucleotide ($^{5'}$ATGTCAACACATCCAGC$^{3'}$), corresponding to bp 3400 to 3416 (von Ossowski *et al.*, 1991), as a primer with plasmid pAG29.

### 5.3.5 Visualization of Plasmid-Encoded Polypeptides

Polypeptides expressed from the 3' end of the *cel* operon were labeled *in vivo* with $^{35}$S-methionine using the chloramphenicol release procedure (Neidhardt *et al.*, 1980). Radiolabeled ([$^{35}$S]-methionine; 1000 Ci/mmol; Amersham Ltd., Oakville, Ontario) gene products were visualized by autoradiography after electrophoresis on a 22.5% sodium dodecyl sulfate-polyacrylamide gel (Giulian *et al.*, 1985) lacking glycerol.

### 5.3.6 Assay for Luciferase-Induced Light Emission

Light emission of clones grown in petri dishes was performed as previously described (Guzzo and DuBow, 1991). Quantitation of light emission was performed by growing strain LF20112 or strain LF20143 for 18 to 24 hours on LB plates containing $NiSO_4$, $CoCl_2$, $CuSO_4$ or cellobiose. The cells were resuspended in LB broth

containing $NiSO_4$, $CoCl_2$, $CuSO_4$ or cellobiose. One milliliter samples were introduced into a Tropix Optocomp I Luminometer (MGM Instruments, Hamden, USA) and the total RLU was determined for a 10 second interval after injection of 100 $\mu$l of dodecyl aldehyde (Aldrich, Milwaukee, USA) (diluted 1:100 in LB broth). The number of photons emitted (per second) is derived by multiplying the RLU by 10 and dividing by the calibration factor (set to 1.0). The $A_{600}$ of the culture was also recorded.

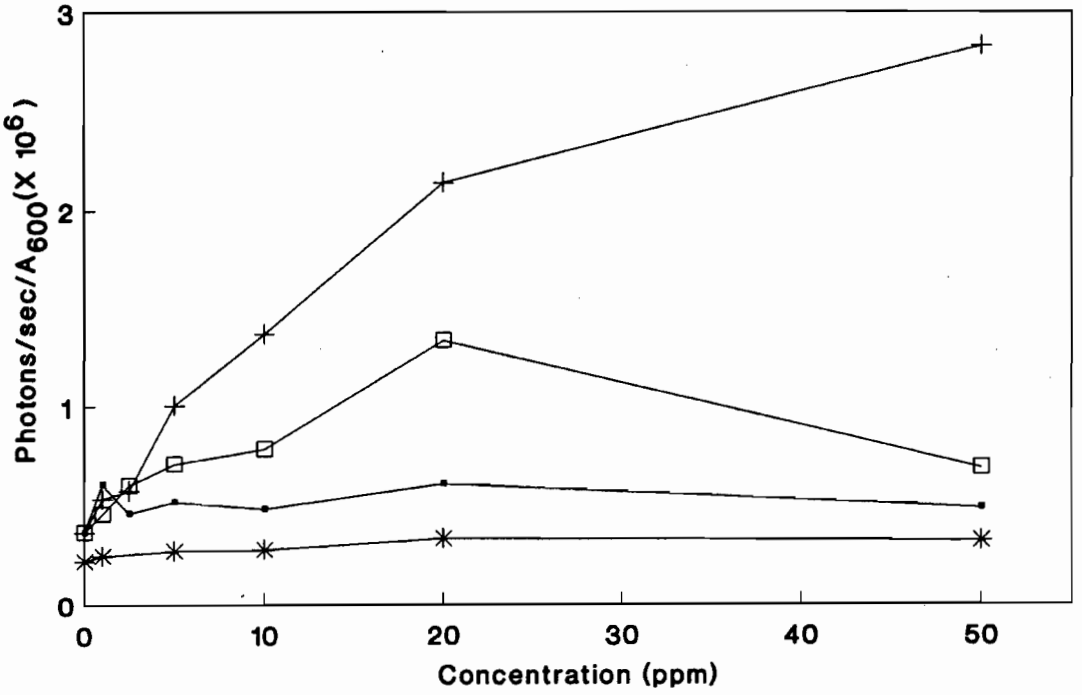
## 5.4 RESULTS

### 5.4.1 Identification of a Clone that Contains the *luxAB* Genes in a Gene Transcribed in the Presence of Nickel

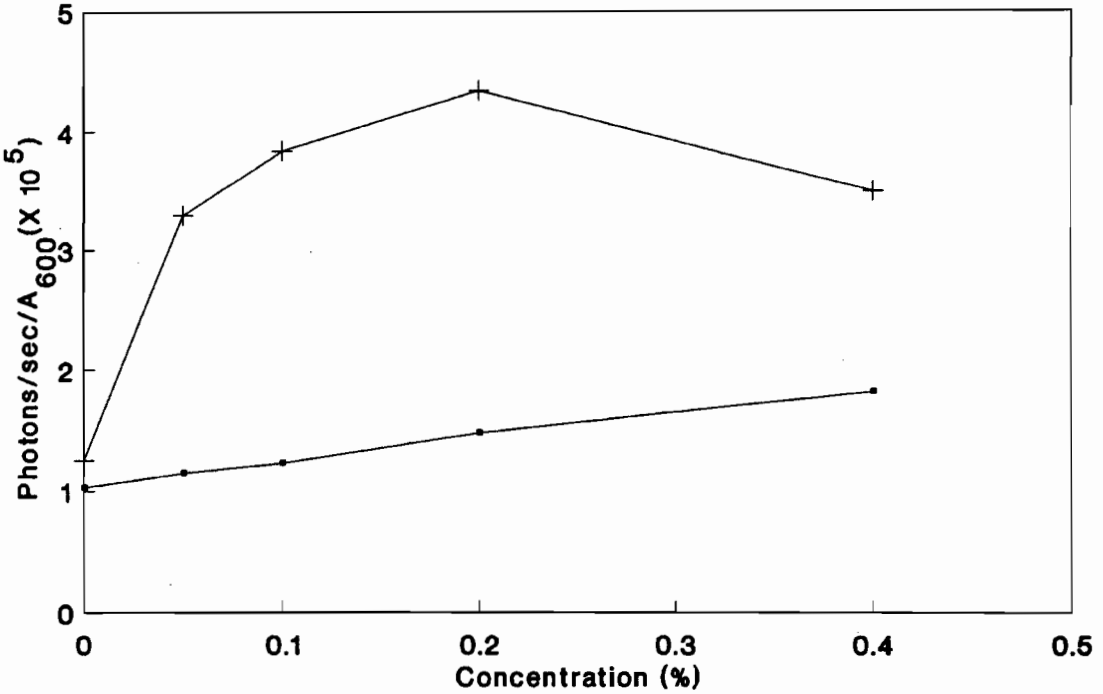
A library of 3000 *E. coli* clones has previously been constructed that contained the promoterless *luxAB* genes from *Vibrio harveyi* flanked by a left end-truncated Tn*5* element, transposed into single, random chromosomal locations (Guzzo and DuBow, 1991). The library was screened in the absence and presence of 1 and 10 ppm $NiSO_4$. One gene fusion clone was found that increased its expression in a dose-dependent manner, as measured by an increase in luminescence, with increasing concentrations of 1, 2.5, 5, 10, 20 and 50 ppm of $NiSO_4$ (Figure 1A). This clone was designated strain LF20112. Maximal induction occurred at 50 ppm of $NiSO_4$, a level 7.7-fold over that recorded without the addition of $NiSO_4$. The addition of increasing concentrations of cobalt, an element with similar chemical properties to nickel, yielded a 3.0-fold increase in luminescence at 20 ppm, which decreased at 50 ppm, possibly due to cytotoxic effects (Webb, 1970) (Figure 1A). Moreover, no significant increase in luminescence was observed in the presence of increasing concentrations of $CuSO_4$ (Figure 1A) or other metals such as $MgSO_4$, $FeCl_3$, or $ZnCl_2$ (data not shown). To determine whether these results were due to non-specif-

Figure 1A. Luminescence of strain LF20112 in the presence of increasing concentrations of $CuSO_4$ ( ▪ ), $NiSO_4$ (+) or $CoCl_2$ (□) and of strain LF20143 in the presence of increasing concentrations of $NiSO_4$ (✳). B) Luminescence of strain LF20112 in the presence of increasing concentrations of cellobiose in the absence ( ▪ ) and presence (+) of 20 ppm $NiSO_4$.

A


Photons/sec/A600(X 10^6)
3
2
1
0
0
10
20
30
40
50
Concentration (ppm)

B


Photons/sec/A600(X 10^5)
5
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
Concentration (%)

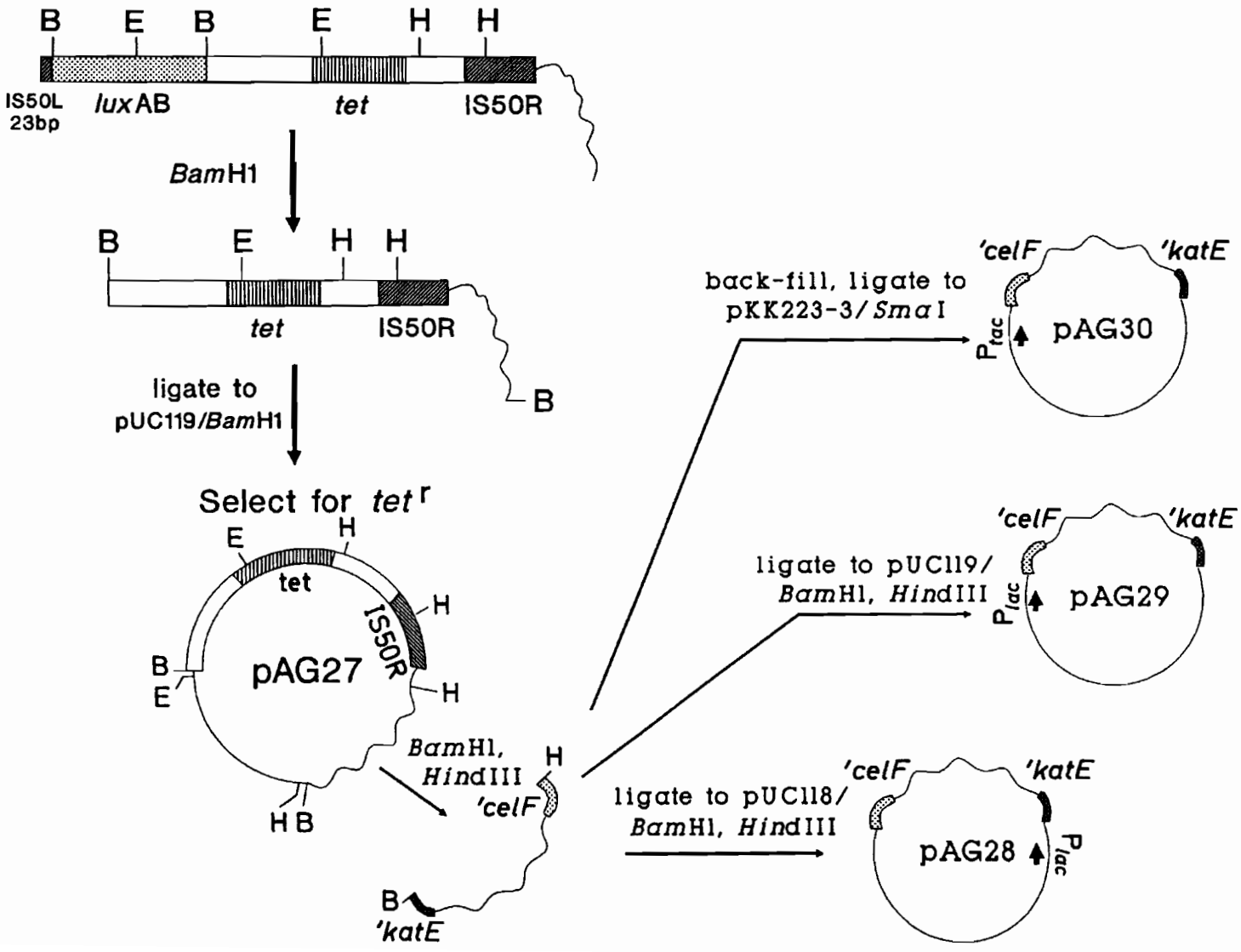
ic effects of $NiSO_4$ on luciferase expression or activity, strain LF20143, which contains the *luxAB* genes in the *xyl* operon (Guzzo and DuBow, 1991), was assayed in the presence of increasing concentrations of $NiSO_4$. No induction of luminescence was observed (Figure 1A). Moreover, P1-mediated transduction of the *luxAB* gene fusion into *E. coli* W3110, a *gyrA*$^+$ strain, still displayed a similar increase in luminescence with increasing concentrations of nickel (data not shown).

5.4.2 Determination of the Identity of the Gene that is Transcriptionally Induced in the Presence of Nickel

Southern blotting analysis (Guzzo *et al.*, 1991) of strain LF20112 revealed that the Tn*5-luxAB* element was present in single copy (data not shown). The right end of the gene fusion was cloned in one step using the *tet* gene in the Tn*5-luxAB* element as a selectable marker (Guzzo *et al.*, 1991), resulting in plasmid pAG27 (Figure 2). Plasmid pAG27 was digested with *Bam*HI plus *Hin*dIII, and a 2.75 kb DNA fragment spanning the cloned chromosomal DNA was isolated (Figure 2). This DNA fragment was labeled and hybridized to the ordered phage λ bank of the *E. coli* chromosome (Kohara *et al.*, 1987) and found to hybridize to both phages 325 and 326 (data not shown). Using the restriction map generated from plasmid pAG27, the site of the Tn*5-luxAB* insertion was estimated to be at approximately 38.0 min and transcribed in a right-to-left orientation on the Kohara *et al.* (1987) restriction map of the *E. coli* genome.

The junction of the Tn*5-luxAB* insertion in the chromosomal DNA of plasmid pAG27 was sequenced and found to be 3205 basepairs downstream of the *cel* operon transcriptional start site, and within the *celF* gene (Figure 3B) (Parker and Hall, 1990a). The orientation of the Tn*5-luxAB* insertion is colinear with *celF* gene transcription. To determine whether the *luxAB* fusion in *celF* is induced by cello-

Figure 2. Schematic diagram for the cloning of the nickel-responsive gene. The *Bam*HI-*Hin*dIII fragment from plasmid pAG27 was isolated and cloned into plasmids pAG28 and pAG29 for sequencing the intercistronic region between *celF* and *katE*, and into plasmid pAG30 for visualization of polypeptides downstream of *celF*. Restriction enzyme sites are abbreviated as follows: *Bam*HI (B), *Eco*RI (E) and *Hin*dIII (H).


B E B E H H
IS50L 23bp
luxAB
tet
IS50R
BamH1
B E H H
tet
IS50R
B
ligate to pUC119/BamH1
Select for tet r
E H
tet
IS50R
H
B
E
pAG27
H
H B
BamHI, HindIII
H
'celF
B
'katE
back-fill, ligate to pKK223-3/SmaI
'celF
'katE
Ptac
pAG30
ligate to pUC119/ BamHI, HindIII
'celF
'katE
Ptac
pAG29
ligate to pUC118/ BamHI, HindIII
'celF
'katE
pAG28
Ptac

biose, increasing concentrations of cellobiose were added to strain LF20112, and found to cause an approximately 2-fold increase in *celF* expression (Figure 1B), a result consistent with the 3-fold induction (measured by enzyme assays) of *celF* levels (Parker and Hall, 1990b) under similar conditions. When nickel is added at 20 ppm, increasing cellobiose concentrations could cause up to a 4.5-fold increase in luminescence (Figure 1B), suggesting a synergistic effect of these two compounds on *celF* expression, or the presence of a nickel-activated promoter upstream of the *celF* gene.

### 5.4.3 Identification of ORFs Downstream of the *celF* Gene

The *celF* gene had previously been proposed to be the most distal gene in the *cel* operon, however no transcription termination signals were found (Parker and Hall, 1990a). In order to determine whether there were other genes, downstream of *celF*, whose expression (and thus, function) may also be induced by nickel, the DNA sequences downstream of the *celF* gene were cloned and sequenced. The results of the DNA sequence analysis (Figure 3A) revealed the presence of three ORFs. Moreover, downstream of these ORFs are two unique and inverted PUs (Gilson *et al.*, 1984; Stern *et al.*, 1984), followed by the *katE* gene of *E. coli* (von Ossowski *et al.*, 1991), which is transcribed in the opposite direction from the *cel* operon and adjacent downstream ORFs (Figure 3B).

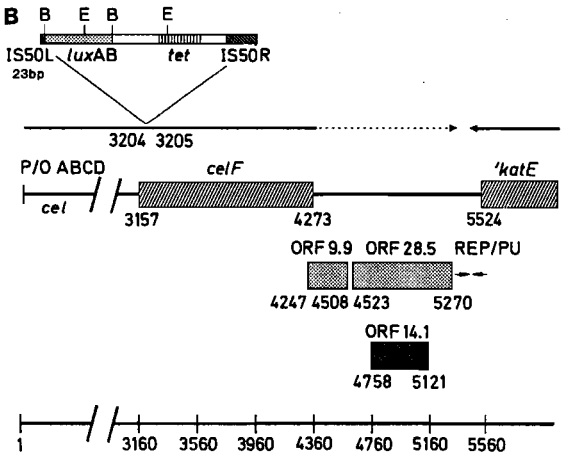
Codon usage of these three ORFs suggests that they would be weakly expressed (Sharp and Li, 1986) in *E. coli*. However, when sequences comprising the 3' end of the *celF* gene, plus the adjacent ORFs, were placed under the control of the $P_{tac}$ promoter [plasmid pAG30 (Figure 2)] and subjected to the chloramphenicol release procedure (Neidhardt *et al.*, 1980), expression of only a 26-kDa polypeptide was observed (Figure 3C). The size of this polypeptide is consistent with the expres-

Figure 3. DNA sequence of, and potential polypeptides encoded by, the intercistronic region between *celF* and *katE*. A) The new DNA sequences reported here are enclosed within parentheses and have been assigned accession number X66725 by the EMBL Data Library. The last 11 amino acids of CelF are depicted in reading frame 1. Starting within the carboxy terminus of CelF, in reading frame 2, is ORF 9.9, which is immediately followed by ORF 28.5. Embedded in ORF 28.5, in reading frame 3, is ORF 14.1. The last 11 amino acids of KatE are shown on the opposite strand. Dashed lines denote the PU elements, whose direction is indicated by an arrow. B) Physical map of the *cel-katE* intergenic region. The Tn*5-luxAB* element was determined to be located in the *celF* gene, 3204 bp downstream of the start, and in the same direction, of *cel* transcription. DNA sequence numbering shown at the bottom begins with bp 1 as the start of *cel* transcription [which is 238 bp downstream from the *cel* operon sequence as reported by Hall and Parker (1990a)]. Depicted are the genes coding for *celF*, *katE* and ORFs 9.9, 28.5 and 14.1. Two conserved (REP)/PU elements (Gilson *et al.* 1984; Stern *et al.* 1984) are found between ORF 28.5 and *katE*. C) Polypeptides expressed from the intercistronic region between *celF* and *katE*. $^{35}$S-labeling of plasmid pAG30 encoded gene products was performed as described in Materials and Methods. Shown are the polypeptides expressed from strain NM522 without any plasmid (Lane 1); containing plasmid pKK223-3 after 20 (Lane 2), 40 (Lane 3) and 60 (Lane 4) minutes of $^{35}$S-methionine addition; and containing plasmid pAG30 after 20 (Lane 5), 40 (Lane 6) and 60 (Lane 7) minutes of $^{35}$S-methionine addition. Size markers, in kDa, are indicated at the left. The arrow indicates the 26-kDa polypeptide expressed from plasmid pAG30.

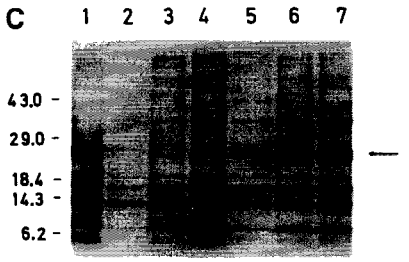
A

```
GGGCGCGATG GCGCGACGCC ACATCCGCGC AATTAGGCAT TTCGATGATA AAGTAATGGG GCTGTTCACA CCATTAAAGG CTTCAAGATT GCTGCCAGTA  100
 G  R  N  G  A  T  P  H  P  R  N  * end [CelF]
       M  A  R  R  H  I  R  A  I  R  H  F  D  D  L  V  M  G  L  F  T  P  L  L  A  S  R  L  L  P  V
       start [ORF 9.9]

ACGCCGCAAC TTAACGGAGA ATTGAACGAT ATGTTACTGG CGCTAAACCT TAGTCCGTTG GTGCATTCCG ATCGCGATGC TGAGCTGCTG GCACGCGAGA  200
 T  P  Q  L  N  G  E  L  N  D  M  L  L  A  L  N  L  S  P  L  V  H  S  D  R  D  A  E  L  L  A  R  G

TGATTCTGGC GCACGAGAAA TGGCTGCCAA ACTTTGCCGA CTGCATCGCA GAGCTTAAAA AAGCACATTA ACCGAGGCTG ATTATGGAAC GCTTACTGCT  300
M  I  L  A  H  E  K  W  L  P  N  F  A  D  C  I  A  E  L  K  K  A  H  * end [ORF 9.9]  M  K  R  L  L  L
                                                                                          start [ORF 28.5]

TGTTAATGCC GATGATTTTG GCTTAAGTCA AAGGACAGAA CTACGGCATT ATCGAGGCCT GTCGCAATGG GATTGTCACT GTCGACGACG TCACTGTGAA  400
  V  N  A  D  D  F  G  L  S  Q  R  T  E  L  R  H  Y  R  G  L  S  G  W  D  C  H  C  R  R  R  H  C  E

TGGCAGGCTA TTGACCATGC GGTGCATTTG AGTTGTGATG AACCAATTCT GGCCATAGGG ATGCACTTTG TCCTTATTAT GGGTAAGCCA CTGACAGCTA  500
 W  Q  A  I  D  H  A  V  H  L  S  C  D  E  P  I  L  A  I  G  M  H  F  V  L  I  M  G  K  P  L  T  A

TGCCGGGGTT AACCCGCGAT GGTGTGCTGG GAAAATGGAT CTGGCAGTTG GCAGAAGAAG ATGCTTTACC GCTGGAAGAA ATTACTCAGG AGCTTGTCAG  600
M  P  G  L  T  R  D  G  V  L  G  K  W  I  W  Q  L  A  E  E  D  A  L  P  L  E  E  I  T  Q  E  L  V  S
                   M  V  C  W  E  N  G  S  G  S  W  Q  K  K  M  L  Y  R  W  K  K  L  L  R  S  L  S
                   start [ORF 14.1]

TCAGTATTTG CGTTTCATTG AGCTATTTGG ACGCAAACCT ACGCATCTTG ATAGCCATCA TCATGTGCAT ATGTTCCCGC AGATTTTCCC GATTGTTGCC  700
 Q  Y  L  R  F  I  E  L  F  G  R  K  P  T  H  L  D  S  H  H  H  V  H  M  F  P  Q  I  F  P  I  V  A
V  S  I  C  V  S  L  S  Y  L  D  A  N  L  R  I  L  I  A  I  I  M  C  I  C  S  R  R  F  S  R  L  L  P

AGGTTTGCGG CAGAGCAGGG CATTGCGTTG CGGGCGGATC GTCAGATGGC GTTTGATTTG CCGGTCAACC TGCGCACTAC CCAGGGATTC AGCAGTGCAT  800
 R  F  A  A  E  Q  G  I  A  L  R  A  D  R  Q  M  A  F  D  L  P  V  N  L  R  T  T  Q  G  F  S  S  A
 G  L  R  Q  S  R  A  L  R  C  G  R  I  V  R  W  R  L  I  C  R  S  T  C  A  L  P  R  D  S  A  V  H

TCTACGGTGA AGAGATCAGT GAGTCGCTGT TCCTGCAAGT GCTGGATGAC GCAGGCCATC GGGGTGATCG TTCGCTGGAG GTGATGTGTC ATCCGGCGTT  900
 F  Y  G  E  E  I  S  V  E  S  L  F  L  Q  V  L  D  D  A  G  H  R  G  D  R  V  S  L  E  V  M  C  H  P  A  F
 S  T  V  K  R  S  V  S  R  C  S  C  K  C  W  M  T  Q  A  I  G  V  I  V  R  W  R  * end [ORF 14.1]

TATCGATAAT ACTATCCGTC AGAGTGCTTA CTGTTTCCCA CGCTTAACGG AGCTGGATGT GTTGACATCA GCGTCGTTGA AAGGTGCTAT CGCTCAGCGT 1000
  I  D  N  T  I  R  Q  S  A  Y  C  F  P  R  L  T  E  L  D  V  L  T  S  A  S  L  K  G  A  I  A  Q  R

GGTTATCGGT TGGGGAGTTA TCGGGATGTG TAAGTGAGGA TGTTTGCCAG ATCGGCGTGA ACCTTGCATC CGGCGATTAA TTCCTACAAA GGCGGCGTAA 1100
 G  Y  R  L  G  S  Y  R  D  V  * end [ORF 28.5]

                                                                                                        <-
CTGCCGCCGC TTGAGACTGC TGACAAACGC AAAACTGCCT GATGGCTTCG CTTATCAGGC CTACGTGTTT CCTGCAATAT ATTGAATTTG CACAGTTTTG 1200
                                                                                  end [KatE] * A  P  I  K
                                                                                             AGTCC GTCCTTAAAA
TAGGCCGGAT AAGGCGTTCA CGCGCATCCG GCATAAACAA AGCGCACTAT GTAAATCATT GAGGCGGCGC AATTGCGCGC TCCCATCAGG CAGGAATTTT 1300
```

B




C



sion of ORF 28.5, which is preceeded by a Shine-Dalgarno sequence with 4/6 nucleotide homology (AGGCTG) to the consensus.

## 5.5 DISCUSSION

We have found that a fusion of the *luxAB* genes to the *celF* gene of *E. coli* conferred nickel-mediated induction of *luxAB* gene expression. Sequencing of the *cel* operon revealed five potential coding sequences in the order *celABCDF*, with *celF* believed to be the distal-most cistron (Parker and Hall, 1990a). The *celF* gene encodes a phospho-$\beta$-glucosidase that cleaves the phosphorylated $\beta$-glucoside sugars. This operon is cryptic in wild-type *E. coli* at mesophilic temperatures, yet can be moderately expressed at elevated (48°C - 54°C) temperatures (Droffner and Yamamoto, 1992). We have found that expression of the distal portion (*celF*) of this operon is also induced in the presence of nickel. RNA blotting analysis, using strain LF20112, showed that there is an approximate 4-fold increase in *luxAB*-specific transcripts in the presence of 10 ppm $NiSO_4$ (data not shown). This correlates well with the 3.7-fold induction of luminescence measured in the presence of 10 ppm $NiSO_4$ (Figure 1). The relationship of the gene products of *cel* (and the ORFs) to nickel metabolism is not currently known, though *E. coli* strain LF20112, containing the *luxAB* insertion in *celF*, did not display an increased sensitivity to nickel when compared to the parent strain DH1 (data not shown).

One other bacterial gene that has been shown to be induced in the presence of nickel is the gene that encodes the hydrogenase of *B. japonicum* (Kim *et al.*, 1991). No homologies were found between the sequences responsible for the nickel-mediated induction of the hydrogenase gene, located between positions -168 to -118 upstream of the start of transcription, and the *cel* operon (data not shown). Thus, induction of *cel* expression in the presence of nickel may occur via a different

mechanism from that occurring in the presence of cellobiose or at elevated temperatures, and from that in *B. japonicum.*

Cloning and sequencing of the DNA downstream of the *cel* operon revealed the presence of three ORFs prior to the appearance of potential transcription termination signals (see below). Immediately adjacent to these sequences is the *katE* gene (von Ossowski *et al.*, 1991), transcribed in the opposite direction to that of the *cel* operon. Thus, our DNA sequencing links these two operons onto a single contiguous DNA sequence (Figure 3A). We found that a protein of 26-kDa could be expressed in *E. coli* from the region downstream of *celF* (Figure 3C), and may represent the expression of ORF 28.5. None of these three ORFs contain extensive amino acid homologies to any known proteins present in the data banks. There are many examples of metalloregulatory proteins (Hennecke, 1990). Although a nickel-binding motif has not been elucidated, it probably occurs via coordination with two to three cysteines/histidines properly oriented in a spatial fashion that would not be evident from the primary structure (Higaki *et al.*, 1992). It is not yet known whether nickel-induced gene expression begins at the *cel* promoter or initiates from a promoter within the *cel* operon, located upstream of the *celF* gene. The presence of internal, and differentially expressed, promoters within a single operon has been observed previously, for example, in the *aroB*-URF74.3-*dam* operon of *E. coli* (Jonczyk *et al.*, 1989).

Two PU elements located immediately adjacent to the translation termination codon of ORF 28.5 were found (Figure 3A). The DNA sequences flanking PU elements in *E. coli* are conserved and can be classified into different groups, termed BIMEs (bacterial interspersed mosaic elements), which may perform different functions (Gilson *et al.*, 1991), including transcription termination. As no transcription termination signals were detected downstream of the *cel* operon (Parker and

Hall, 1990a), termination of transcription of the *cel* operon (plus the ORFs), and that of *katE* may be occurring in this conserved, PU-containing, DNA segment.

The observed induction of the *celF* gene in the presence of nickel is not due to a generalized effect of nickel on transcription, since only one of the 3000 *luxAB* gene fusion clones was found to be affected by nickel. It is possible that the low level of nickel-mediated induction of *celF* expression reflects this operon's cryptic state, as the extent of induction is comparable to that seen in the wild-type cryptic *cel* operon by cellobiose (Parker and Hall, 1990b). Retention of the *cel* operon, plus adjacent ORFs, in a cryptic state may be analogous to that observed for genes within cryptic λ prophages (Bouché *et al.*, 1990). Induction of expression of *celF*, plus the adjacent ORFs, by nickel may have once played a functional role, which may have since become dispensable. Sensitive reporter genes, such as *luxAB* (Guzzo and DuBow, 1991), may thus prove useful to identify and dissect other cryptic bacterial genes and help elucidate these evolutionary processes.

# CHAPTER 6

## Identification, sequencing and characterization of an aluminum-inducible *Escherichia coli* gene

## PREFACE

The library of 3000 *Escherichia coli luxAB* gene fusions described in Chapter 2 was used to find two clones that displayed increased luminescence with increasing concentrations of aluminum. In Chapter 4 it was shown that one of these clones was induced by other metal ions, and that the *luxAB* genes were located 97 bp downstream of the *fliC* translational start site. Chapter 6 describes the characterization of the second aluminum-inducible clone.

## 6.1 ABSTRACT

Aluminum, although considered for many years to be biologically inert, is now known to be toxic. Due to pollution and acid rain, the pH of natural waters is decreasing, resulting in the bioavailability of aluminum and toxicity in the environment. Organisms frequently change the expression of certain genes after exposure to toxic metals in order to respond to metal-induced effects on cell physiology. In order to find genes whose expression is inducible by aluminum, a library of *luxAB* chromosomal gene fusions was constructed in *Escherichia coli*, and cellular luminescence was assayed in the absence and presence of aluminum. One clone was found that showed increased luminescence in the presence of aluminum, and subsequently by iron, but not nickel, copper, magnesium or zinc. Cloning of the junction of *luxAB* insertion and hybridization to the λ library of the *E. coli* genome, revealed that the aluminum-inducible gene mapped to 2 378 450 bp. A northern blotting analysis revealed that a transcript of 800 nucleotides was induced in the presence of aluminum or iron, with aluminum being a more potent inducer at pH 5.5 than at pH 7.0. An S1 nuclease analysis determined that transcription started at two sites corresponding to 82 and 102 bp upstream of the gene's translational start site. DNA sequencing of 1286 bp surrounding the site of *luxAB* insertion and a search for potential open reading frames showed that either a 22.2-kDa or 10.6-kDa (designated ORF 22.2 or ORF 10.6, respectively) polypeptide could be expressed from this region. Cloning of the DNA coding region and analysis of its expression *in vivo* revealed that ORF 22.2 is expressed. The gene coding for ORF 22.2 was hence designated *ais* (aluminum and iron stimulated). A protein homology search indicated that ORF 22.2 is highly homologous to the AfrS protein, whose function is unknown but is potentially involved in AF/R1 pilus expression and/or assembly.

## 6.2 INTRODUCTION

Aluminum is the third most abundant crustal element (Martin, 1988). Aluminum is not thought to participate in any biological processes (Ganrot, 1986), although it has been observed to activate the guanine nucleotide binding regulatory component of adenylate cyclase (Sternweis and Gilman, 1982). Aluminum was considered to be biologically inert, but its toxic effects are now being realized (Ganrot, 1986). In humans, aluminum is believed to contribute to a variety of neurological and skeletal pathologies (MacDonald and Martin, 1988), including senile dementias and Alzheimer's disease. It has been shown that aluminum ions promote aggregation of high density $\beta$-amyloid plaques in the brain tissue of Alzheimer's patients (Mantyh *et al.*, 1993). Other studies have shown that micromolar quantities of aluminum can inhibit the voltage dependent anion-selective channels in *Neurospora crassa* mitochondria (Dill *et al.*, 1987), and can activate a metal-ion activated non-specific $Ca^{2+}$-driven channel in mouse N1E-115 cells (Oortgiesen *et al.*, 1990). Aluminum was also shown to bind to DNA in *Rhizobium* spp. (Johnson and Wood, 1990).

Aluminum is more toxic at acidic pH, presumably due to its increased solubility (Birchall *et al.*, 1989; Cronan and Schofield, 1979; Cronan *et al.*, 1986; Wood, 1988). At a pH $< 5$, aluminum is present in its soluble form as $Al(H_2O)_6^{3+}$, abbreviated as $Al^{3+}$ (MacDonald and Martin, 1988). However, due to the high charge and small size of aluminum (Ganrot, 1986), as pH increases, $Al(H_2O)^{3+}$ deprotonates resulting in the formation of different hydroxy complexes, eventually yielding the insoluble form $Al(OH)_3$ at neutral pH (MacDonald and Martin, 1988; Martin, 1988). The solubility of aluminum is increased by ligands that complex $Al^{3+}$, preventing the formation of $Al(OH)_3$, but organically bound aluminum is nontoxic compared to monomeric aluminum species (Driscoll *et al.*, 1980). Furthermore,

time-dependent dimers and polynuclear complexes are formed as the pH is raised above 5.0 (MacDonald and Martin, 1988). Polynuclear aluminum species were shown to inhibit root formation in wheat and soybean at a lower concentration than $Al^{3+}$ (Parker *et al.*, 1989).

In an effort to find aluminum-mediated effects on cell physiology, and the cell's response to them, we searched for genetically-programmed responses to aluminum exposure in *Escherichia coli.* A library of 3000 *E. coli* clones, each containing the promoterless *Vibrio harveyi luxAB* genes in single, random chromosomal loci, was previously created (Guzzo and DuBow, 1991). Changes in transcription, as measured by a change in luminescence, were monitored in the absence and presence of aluminum at pH 5.5 after overnight growth on petri dishes. One of the two clones that was isolated, strain LF20111, was previously shown to contain the *luxAB* genes inserted 97 bp downstream of the *E. coli fliC* translational start site. In addition to aluminum, luminescence of this clone was also inducible by copper, iron and nickel on LB agar medium (Guzzo *et al.*, 1991). Here, we present the characterization of a second *E. coli* gene whose expression is induced (as measured by luciferase and northern blotting to the cloned gene) by aluminum. Moreover, this previously uncharacterized gene, mapped to 2 378 450 bp on the *E. coli* genetic map, is also shown to be induced by iron, but by no other metal. Induction was observed to occur within 10 minutes after aluminum addition.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Bacterial Strains and Media

*E. coli* strain DH1 [$F^-$ *recA1 endA1 gyrA96 thi hsdR17* ($r_k^-$, $m_k^+$) *supE44 relA1*] (Hanahan, 1983) was used to prepare the library of *luxAB* transcription fusion clones (Guzzo and DuBow, 1991). Strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd-5* ($r_k^-$,

$m_k^+$) (F' *proAB lacI*$^q$*ZΔM15*)] (Gough and Murray, 1983) was used to transform all DNA ligation reactions, except for plasmids pAG51 and pAG53, which were isolated after transforming strain DH1. *E. coli* strains were propagated at 37°C, unless otherwise stated, in LB broth or on LB plates containing 1.5% agar (Miller, 1992) and supplemented with antibiotics when indicated. Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada), 40 μg/ml; Cm (Sigma, St. Louis, USA), 50 μg/ml; Tc (Boehringer Mannheim, Laval, Canada), 10 μg/ml in broth, 20 μg/ml in agar. Aluminum chloride (22.38 mg/ml) (Anachemia, Montreal, Canada), ferric chloride (2.91 mg/ml) (BDH, Montreal, Canada) and nickel sulfate (11.15 mg/ml) (Anachemia, Montreal, Canada) stock solutions, prepared in sterile deionized water, were added to achieve the indicated final concentrations. The pH of LB was adjusted to 5.5 with 12 M HCl and to 7.0 with 2 N NaOH. Noble Agar (Difco Laboratories, Detroit, USA) was used instead of Bacto agar (Difco Laboratories, Detroit, USA) for luminescence assays. M9 broth is M9 salts (Miller,1992) supplemented with 1.5% (w/v) casamino acids, 0.4% (w/v) glucose, 40 μg/ml L-tryptophan, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$ and 10 μg/ml (w/v) thiamine.

### 6.3.2 DNA Manipulations

All restriction endonuclease hydrolyses were performed in 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 75 mM NaCl, 6 mM 2-mercaptoethanol and 0.25 mg/ml bovine serum albumin at 37°C for 2 hours using 3 units of enzyme per μg DNA, except for *Sma*I which was performed according to the manufacturer's protocol (Gibco-BRL, Burlington, Canada). Partial *Bam*HI hydrolysis was performed by treating 5 μg of pFUSLUX with 1 unit of enzyme for 10 minutes at 37°C. Calf intestinal alkaline phosphatase reactions (Du Pont Canada Inc., Mississauga, Canada)

were performed using 1 μl of the enzyme [diluted 50-fold in 10 mM Tris-HCl (pH 10.0)] per μg of DNA for 30 minutes at 37°C in a final volume of 30 μl of 10 mM Tris-HCl (pH 10.0). Back-filling 3' recessed ends of DNA fragments was performed as previously described (Guzzo and DuBow, 1991). Ligations were performed at 15°C for 18 hours following the manufacturer's protocol (Gibco-BRL, Burlington, Canada). DNA was subjected to electrophoresis in 0.75% agarose gels (Sambrook *et al.*, 1989). Size-selected DNA fragments were purified using the Geneclean II Kit (Bio 101, Mississauga, Canada). DNA transformations were performed as previously described (Mandel and Higa, 1970).

6.3.3 Construction of Plasmids

Plasmid pAl-1 was constructed by ligating *Bam*HI-cleaved chromosomal DNA (Guzzo and DuBow, 1991) from strain LF20110 to *Bam*HI-cleaved pUC119 (Vieira and Messing, 1987), transforming the ligation mixture into *E. coli* NM522, and selecting for $Tc^R$ clones. Plasmid pAG27 was cloned in the same manner as pAl-1, but the total cellular DNA was hydrolyzed with *Hin*dIII. The *Hin*dIII fragment from pAG17, containing *luxAB*, *tet* and chromosomal DNA upstream of the Tn*5-luxAB* insertion, was cloned into plasmid pSU19 (Bartolomé *et al.*, 1991), resulting in plasmid pAG51. Plasmid pAG3 was obtained by performing a partial *Bam*HI digestion on plasmid pFUSLUX (Guzzo and DuBow, 1991), religating, and screening for clones that had lost the *Bam*HI site situated between *luxB* and the *tet* gene. Plasmid pAG52 was constructed by ligating the *Bam*HI-*Hin*dIII fragment from pAG3, containing *luxAB* and the *tet* gene, to plasmid pSU19 cleaved with the same enzymes. The 700 bp *Bam*HI-*Hin*dIII fragment from pAG17 was isolated, back-filled and recleaved with *Alu*I. A 370 bp fragment was then isolated using the "Crush and Soak" procedure (Sambrook *et al.*, 1989) and ligated to pAG52 cleaved with

*Sma*I and treated with calf intestinal alkaline phosphatase. A plasmid that showed aluminum-inducible luminescence was isolated and named pAG53. Phage 379 (Kohara *et al.*, 1987) was hydrolyzed with *Eco*RI, and the fragment spanning 2 378 150 to 2 388 150 bp on the *E. coli* map (Rudd, 1992) was ligated into *Eco*RI-cleaved pUC119 and designated pAG11. Phage 379 was also cleaved with *Sal* I and the fragment spanning 2 369 550 bp to a site at approximately 2 379 050 bp (Rudd, 1992) on the *E. coli* map (160 bp upstream of bp 1 in Figure 2) was cloned into *Sal* I-hydrolyzed pUC119, resulting in plasmid pAG12. Plasmid pAG43 was constructed by isolating a 5.0 kb *Hin*dIII-*Bam*HI fragment from pAG12 and ligating to plasmid pUC119 cleaved with the same enzymes. Plasmid pAG46, used for the chloramphenicol release procedure, was constructed by isolating a 5.0 kb *Hin*dIII-*Bam*HI fragment from plasmid pAG12, back-filling and ligating to plasmid pKK223-3 (Brosius and Holy, 1984) cleaved with *Sma*I and treated with calf intestinal alkaline phosphatase. The $P_{tac}$ promoter directs synthesis of RNA starting at the *Hin*dIII end towards the *Bam*HI site.

### 6.3.4 Luminescence Assay

An overnight culture of strain LF20110, grown in LB broth at 32°C, was diluted 100-fold into fresh LB broth at pH 5.5 or pH 7.0 and grown for 1 hour. The culture was then separated into different flasks. A 5 ml aliquot was taken out for the 0 time reading. One flask contained no exogenous metal, whereas the appropriate volume of aluminum or ferric chloride solution was added to each of the other flasks to reach a final concentration of 1, 5, 10 and 20 $\mu$g/ml aluminum or iron. Samples were then removed at various intervals and luminescence measured as previously described (Guzzo *et al.*, 1992).

### 6.3.5 DNA Sequencing and Mapping with the Kohara Library

Labeling of cloned chromosomal DNA and mapping using the λ library of the *E. coli* genome (Kohara *et al.*, 1987) was performed as previously described (Guzzo *et al.*, 1991). The probe was prepared by isolating 1.0 μg of a 4.0 kb *Bam*HI-*Eco*RI DNA fragment from plasmid pAl-1. For sequencing, single-stranded phagemid DNA was prepared from pUC119- or pUC118-based plasmids as described elsewhere (Vieira and Messing, 1987). Dideoxy sequencing reactions, with [α-$^{35}$S]dATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada), were performed with a Sequenase kit using the universal primer (U.S. Biochemical Corp., Cleveland, USA). Determination of the sequence at the junction between IS*50*R and the adjacent chromosomal DNA, cloned into plasmid pAl-1, was performed as previously described (Guzzo *et al.*, 1991).

### 6.3.6 Northern Analysis

An overnight culture of strain DH1 was diluted 100-fold into LB broth adjusted to either pH 5.5 or 7.0, and grown at 32°C. When the culture reached an $A_{550}$ of 0.2, they were separated into different flasks. Aluminum chloride, ferric chloride or nickel sulfate were added to the flasks to achieve a final concentration of 10 μg/ml. There was also one flask that had no metal added. After the cells had grown for 80 minutes, RNA was extracted and purified using the RNaid II Kit (Bio 101, Mississauga, Canada). Five micrograms of RNA was subjected to electrophoresis through a 1% denaturing agarose gel according to Sambrook *et al.* (1989), except that the RNA was prestained by the addition of 100 μg/ml ethidium bromide to the loading buffer, and the running buffer contained 7.4% (v/v) formaldehyde, 40 mM MOPS-NaOH (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After electrophoresis, the gel was soaked in 20XSSC [3 M NaCl, 0.3 M sodium citrate (pH 7.0)]

for 20 minutes and then transferred to a Hybond-N membrane (Amersham Ltd., Oakville, Canada) for 16 hours. The filter was baked in a vacuum oven at 80°C for 2 hours, and then hybridized to a $^{32}$P-labeled probe according to Shackelford and Varmus (1987). The probe was a 700 bp *Bam*HI-*Hin*dIII fragment isolated from plasmid pAG17, labeled with [$\alpha$-$^{32}$P]dGTP (3000 Ci/mmol, ICN Biomedicals, Mississauga, Canada) according to the procedure of Feinberg and Vogelstein (1983; 1984).

6.3.7 S1 Nuclease Analysis

An oligonucleotide $^{5'}$GGTCGCAACGTTCAGCATGACGAA $^{3'}$, corresponding to the complement of bp 598 to 575 (Figure 3A) was synthesized (Sheldon Biotechnology Centre, Montreal, Canada), labeled at its 5' end with [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol; Amersham Ltd., Oakville, Canada), and extended using single-stranded DNA isolated from pAG43 as a template (Green and Struhl, 1989). The double-stranded radioactive products were subsequently hydrolyzed with *Pst*I (Figure 3 bp 39), subjected to electrophoresis through an alkaline agarose gel, and the single-stranded S1 nuclease probe was purified (Green and Struhl, 1989). The probe ($10^5$ cpm) was hybridized to 10 $\mu$g of RNA isolated from strain DH1 grown in the presence of 10 $\mu$g of aluminum at pH 7.0 (see section 6.3.6) in aqueous hybridization buffer in a total volume of 15 $\mu$l at 55°C (Green and Struhl, 1989). After 12 hours, 135 $\mu$l of S1 nuclease mix, containing 250 units of S1 nuclease (Pharmacia Biotech, Montreal, Canada), was added and the mixture incubated at 37°C for 60 minutes. The reaction was subsequently stopped, precipitated and resuspended in 5 $\mu$l of 0.1 M NaOH. An equal volume of formamide loading dye was added and half of the reaction was subjected to electrophoresis on a 5% denaturing polyacrylamide gel (Green and Struhl, 1989). A dideoxy sequencing reaction was also performed

using the synthesized oligonucleotide and single-stranded plasmid pAG43 as a template (see section 6.3.5).

### 6.3.8 Visualization of Plasmid-Encoded Polypeptides

Polypeptides expressed from plasmids pKK223-3 (Brosius and Holy, 1984) and pAG46 were labeled *in vivo* with [$^{35}$S]methionine using a modified chloramphenicol release procedure (Neidhardt *et al.*, 1980). Briefly, overnight cultures of strain NM522 containing plasmid pKK223-3 (Brosius and Holy, 1984) or pAG46 were diluted 12.5-fold in fresh M9 broth. When an $A_{550}$ of 0.5 was reached, Cm (final concentration 68 $\mu$g/ml) was added. After 16 hours, the cells were pelleted by centrifugation at 3000 x g for 8 minutes and washed with 2 volumes of M9 salts solution. The cells were pelleted again and resuspended in 0.1 volume of M9 salts solution. Cells (100 $\mu$l) were mixed with 100 $\mu$l of labeling mix [200 $\mu$Ci/ml [$^{35}$S]methionine (1500 Ci/mmol; Amersham Ltd., Oakville, Canada), 2 mM IPTG, 15.75% methionine assay medium (5.25 g dissolved in 37.5 ml $H_2O$, boiled 2 minutes, and filter sterilized; Difco Laboratories, Detroit, USA)]. Aliquots of 50 $\mu$l were removed at 0, 20, 40 and 60 minutes, to which 1 $\mu$l of L-methionine (1 mg/ml) was added. The aliquots were subjected to centrifugation at 15000 x g for 10 minutes, the cell pellets resuspended in 100 $\mu$l of Giulian sample buffer (Giulian *et al.*, 1985), and then boiled for 5 minutes. The supernatant fluid was collected after a 15 minute centrifugation. The gene products were visualized by autoradiography after electrophoresis on a 22.5% SDS-polyacrylamide gel lacking glycerol (Giulian *et al.*, 1985).

## 6.4 RESULTS

### 6.4.1 Isolation of an Aluminum-Inducible *luxAB* Gene Fusion Clone

A library of 3000 *E. coli* clones, each containing a single chromosomal copy of the *V. harveyi luxAB* genes in random positions, was previously constructed (Guzzo and DuBow, 1991). The library was screened in the absence and presence of 1 and 10 μg/ml of aluminum on LB plates adjusted to pH 5.5. One of two clones found to display increased luminescence with increasing concentrations of aluminum (Guzzo *et al.*, 1991) was isolated and designated strain LF20110.

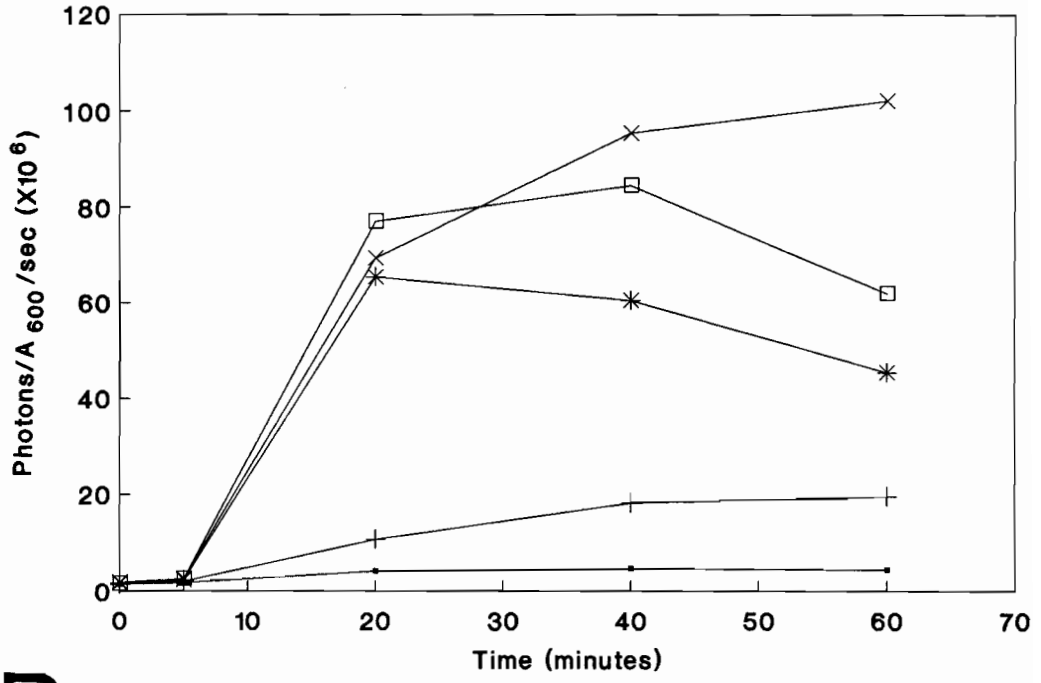
Luminescence of strain LF20110 was quantitated over a 60 minute interval in the absence and presence of 1, 5, 10 and 20 μg/ml aluminum in LB broth adjusted to pH 5.5 (Figure 1A) or pH 7.0 (Figure 1B). The rate of increase in light production in the presence of aluminum was faster at pH 5.5 than 7.0. At pH 5.5, luminescence reached a maximum by 20 minutes, whereas at pH 7.0 the maximum was attained at approximately 40 minutes post-aluminum exposure. Furthermore, 1 μg/ml of aluminum induced a detectable elevation of luminescence at pH 5.5, but not at pH 7.0. Luminescence was also found to be induced in the presence of iron (as ferric chloride) (Figure 2). The expression of luminescence in the presence of iron at pH 5.5 (Figure 2A) is similar to that displayed in the presence of aluminum at pH 5.5. The final level of luminescence, after 60 minutes of exposure to ferric chloride at pH 7.0 (Figure 2B), is lower than aluminum at pH 7.0, but the pattern of induction is similar. The luminescence of strain LF20110 is not inducible by nickel sulfate, copper sulfate, zinc chloride or magnesium sulfate (data not shown).

### 6.4.2 Cloning and Sequencing of the Aluminum-Inducible Gene

A Southern blot was performed, and confirmed that the Tn*5-luxAB* element was present at a single chromosomal location in strain LF20110 (data not shown).

Figure 1. Measurement of luminescence emitted by strain LF20110 grown in the presence of aluminum at pH 5.5 (A) and pH 7.0 (B). Aluminum was added to a final concentration of: 0 (▪), 1 (+), 5 (✳), 10 (□) and 20 (×) μg/ml.

A


120
100
80
60
40
20
0
Photons/A 600/sec (X10 6)
0
10
20
30
40
50
60
70
Time (minutes)

B

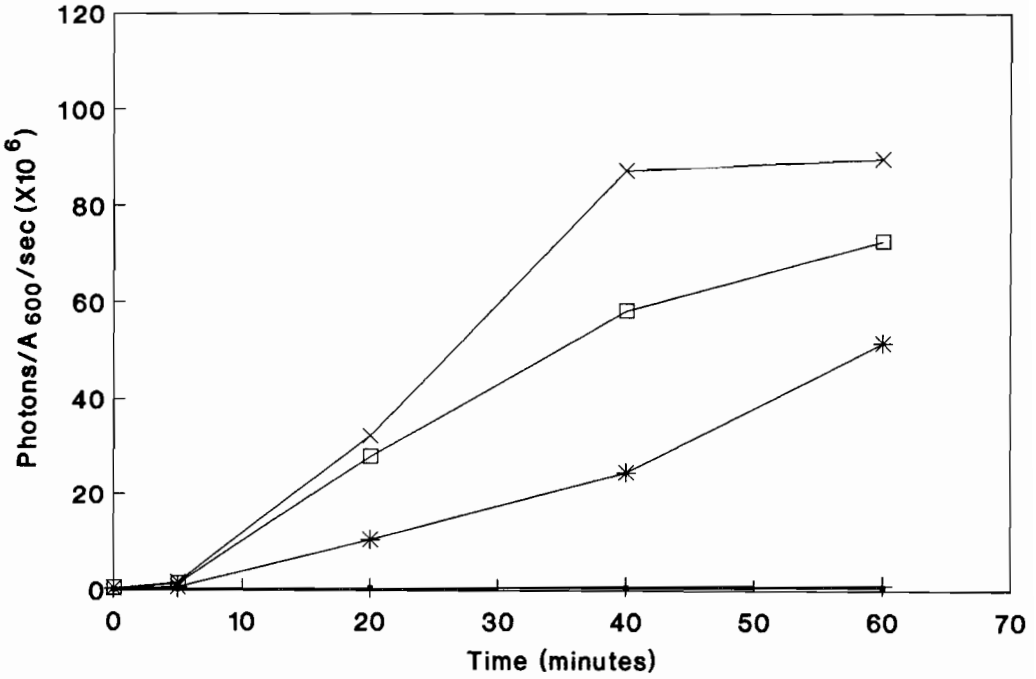
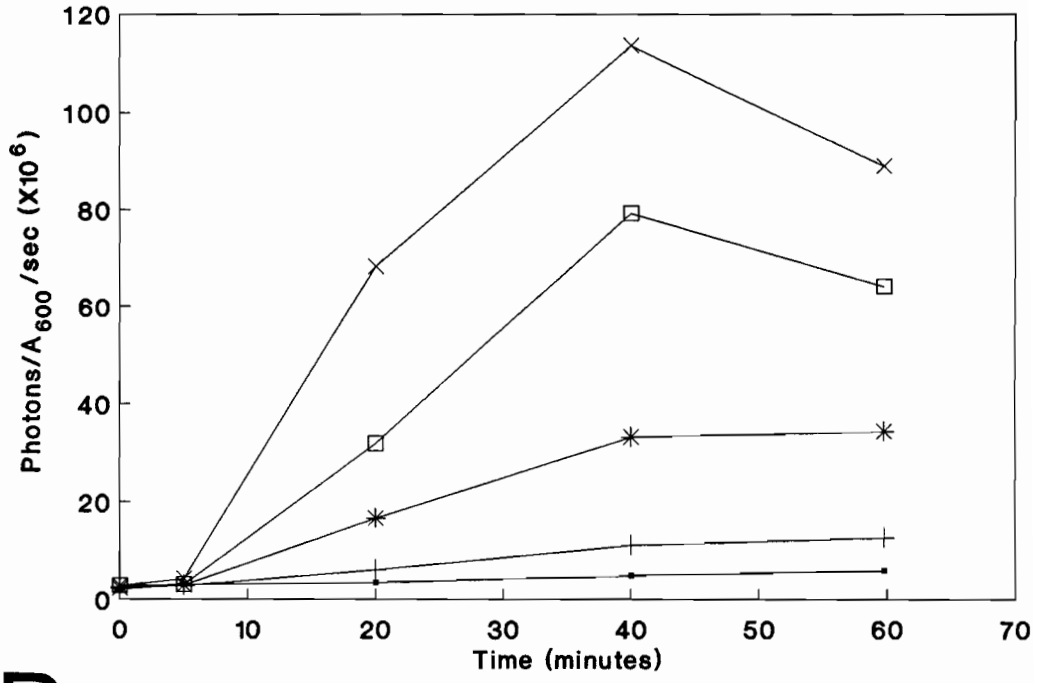
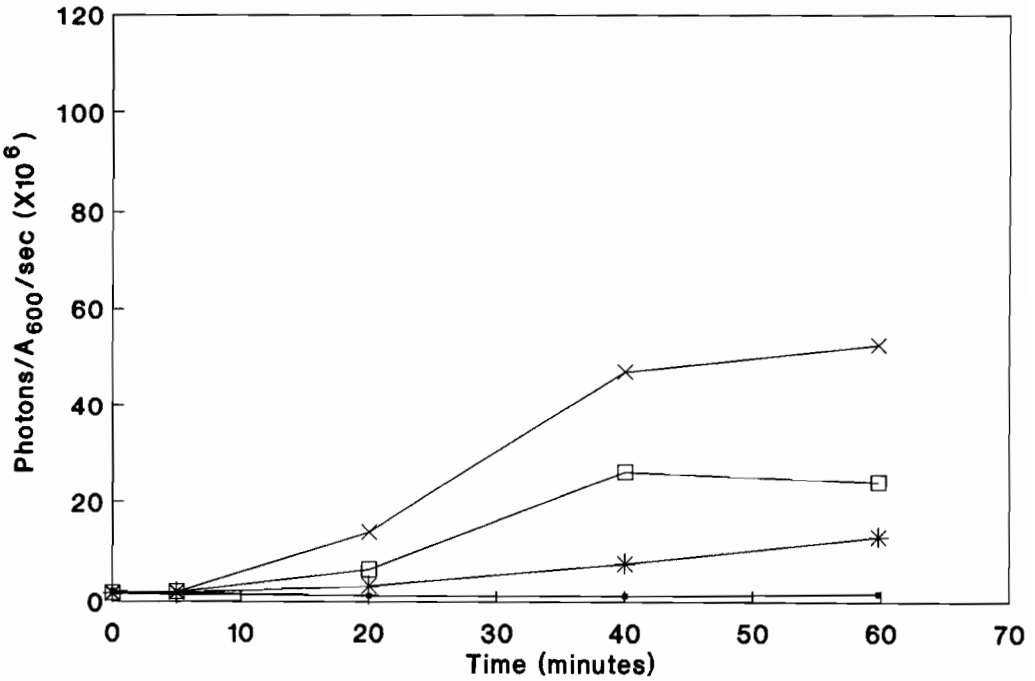

120
100
80
60
40
20
0
Photons/A 600/sec (X10 6)
0
10
20
30
40
50
60
70
Time (minutes)

Figure 2. Measurement of luminescence emitted by strain LF20110 grown in the presence of iron at pH 5.5 (A) and pH 7.0 (B). Iron was added to a final concentration of: 0 ( ▪ ), 1 (+), 5 (✳), 10 (□) and 20 (×) μg/ml.

A


120
100
80
60
40
20
0
Photons/A600/sec (X10^6)
0
10
20
30
40
50
60
70
Time (minutes)

B

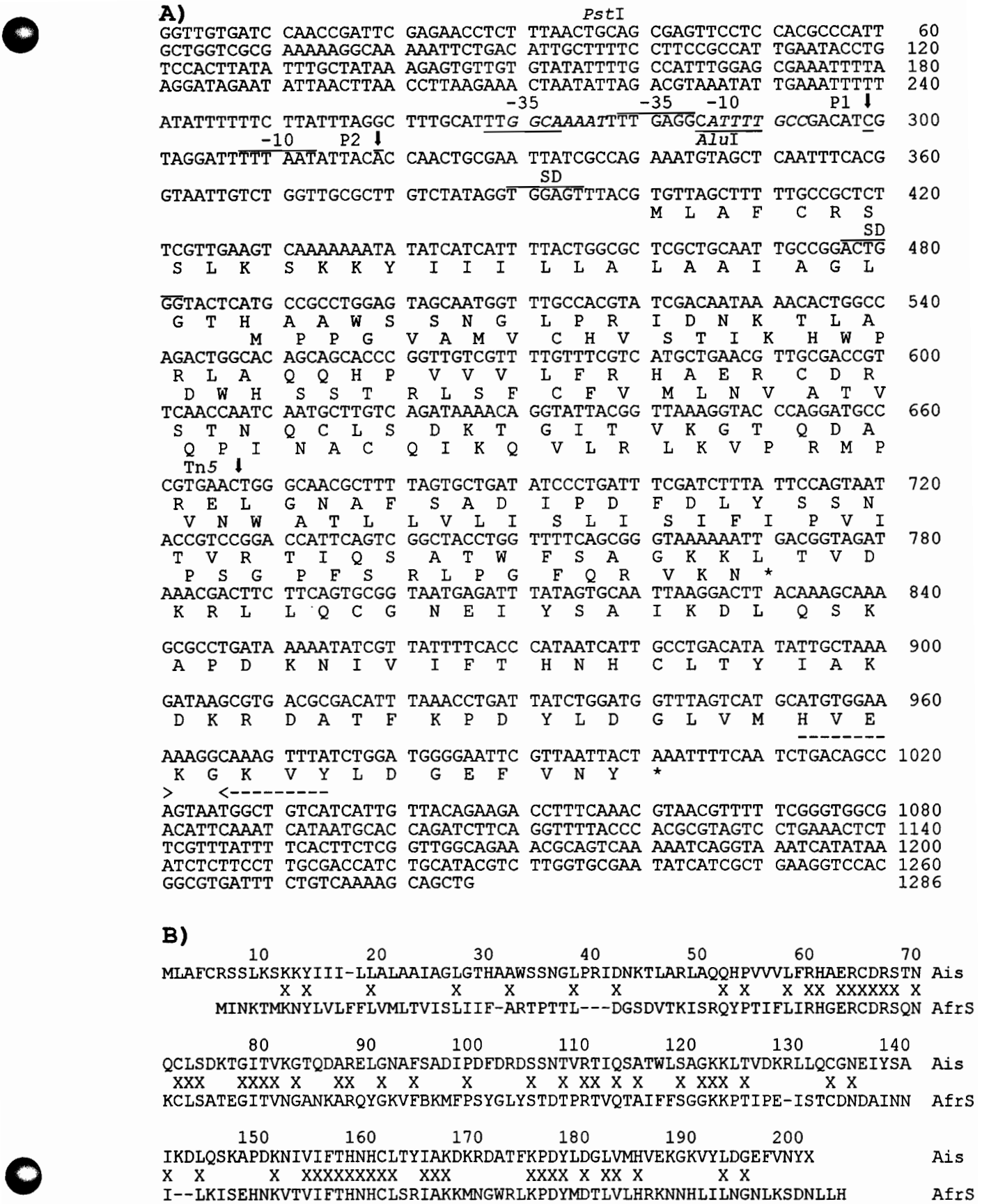

120
100
80
60
40
20
0
Photons/A600/sec (X10^6)
0
10
20
30
40
50
60
70
Time (minutes)

The right end of the gene fusion was cloned in one step using the *tet* gene in the Tn*5-luxAB* element as a selectable marker (Guzzo and DuBow, 1991; 1994a). The enzyme *Bam*HI cleaves to the left of the *tet* gene. Hence, digestion of total chromosomal DNA with *Bam*HI, ligation to *Bam*HI-cleaved pUC119 (Vieira and Messing, 1987), and selection of transformants on LB containing Tc selects for plasmids that contain the *tet* gene, IS*50*R and chromosomal DNA downstream of the site of Tn*5-luxAB* insertion. The resulting plasmid, pAl-1, was shown to contain approximately 4.3 kb of chromosomal DNA, with an *Eco*RI site located 4.0 kb away from the chromosomal *Bam*HI site. The site of insertion was sequenced using a primer complementary to IS*50*R as previously described (Guzzo *et al.*, 1991). Comparison of the sequence with the bacterial sequences in the GenBank database revealed that this region had not been previously sequenced. The 4.0 kb *Bam*HI-*Eco*RI fragment was labeled and found to hybridize to phage 379 of the λ library of the *E. coli* chromosome (Kohara *et al.*, 1987). Comparison of the restriction map of the cloned DNA fragment to that of the *E. coli* chromosome revealed that the Tn*5-luxAB* element had inserted at approximately 2 378 450 bp on the *E. coli* map (Rudd, 1992) and is transcribed in a right-to-left orientation. A total of 1286 bp, spanning the site of insertion, was sequenced and found to reveal two ORFs of 200 and 94 amino acids starting with a GTG and an ATG codon, respectively (Figure 3A).

### 6.4.3 Analysis of Transcription

To determine the transcription of this region, the left end of the gene fusion was cloned into pUC119 using *Hin*dIII and selection for $Tc^R$, resulting in plasmid pAG17. When the Tn*5-luxAB* element inserts into the chromosome, a *Bam*HI site is placed 23 bp downstream of the site of insertion. Hydrolysis of pAG17 with *Hin*dIII and *Bam*HI produced an approximately 700 bp fragment. The fragment was labeled

Figure 3. DNA sequence around the site of Tn*5-luxAB* insertion and homology of ORF 22.2/Ais to AfrS. A) Shown are the amino acid sequences in one-letter code of ORF 22.2 in the first reading frame and of ORF 10.6 in the second reading frame. Potential Shine-Dalgarno (SD) sequences for each ORF are overlined. The two start sites of transcription, P1 and P2, are indicated by vertical arrows. The putative $\sigma^{70}$ "-10"and "-35"sequences are underlined for P1 and overlined for P2. An inverted repeat embedded in the $\sigma^{70}$ "-10"and "-35"sites is italicized. The site of Tn*5-luxAB* insertion was determined to be between bp 667 and 668 and is denoted by a vertical arrow. An inverted repeat downstream of ORF 22.2, which is a potential terminator, is indicated by horizontal arrows. Relevant restriction sites are shown. B) Homology of ORF 22.2/Ais to AfrS. The amino acid sequences in one-letter code are aligned to give maximum homology. Identical residues are indicated by an X. The numbering corresponds to the amino acid sequence of ORF 22.2/Ais. The sequence for AfrS was obtained from the Genbank database accession number L08467.

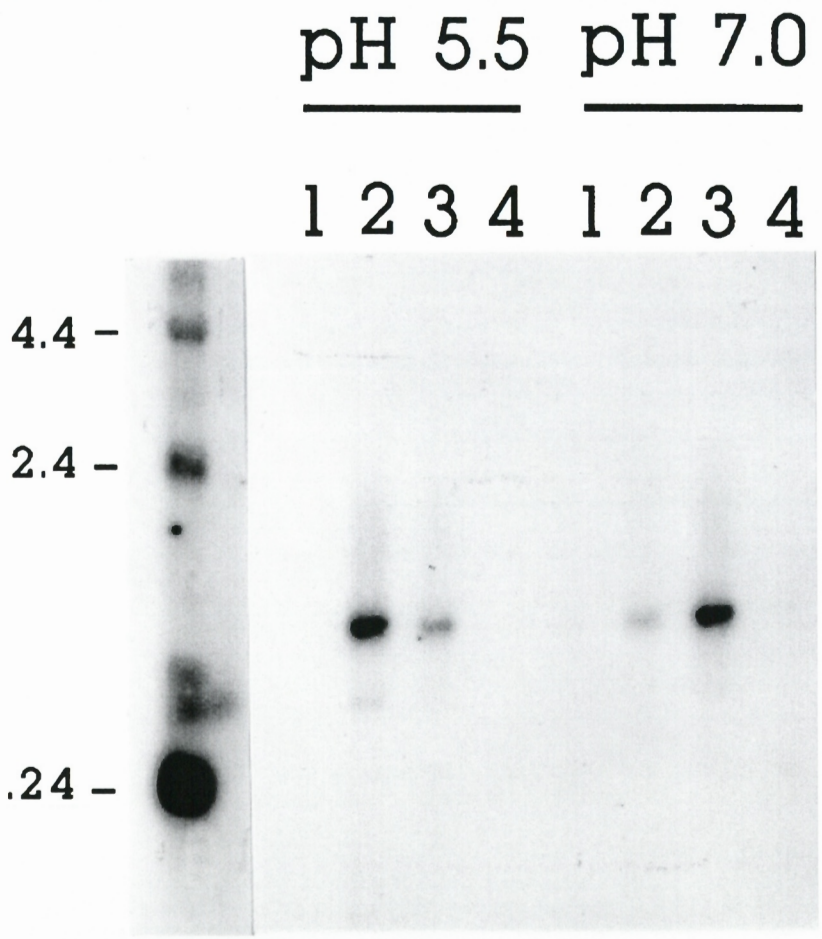

A)
PstI
-35
-35
-10
P1
-10
P2
AluI
SD
SD
Tn5
B)
Ais
AfrS
Ais
AfrS
Ais
AfrS

and hybridized to RNA isolated from *E. coli* DH1 (no *luxAB* insertion) grown in the absence and presence of 10 μg/ml aluminum chloride, ferric chloride and nickel sulfate either at pH 5.5 or at pH 7.0 (Figure 4A). A mRNA approximately 800 nucleotides in length was produced. The transcript was more abundant at pH 5.5 than pH 7.0 in aluminum exposed cells, but was present in higher amounts from cells exposed to iron at pH 7.0 than at pH 5.5. The level of mRNA from cells exposed to nickel sulfate is comparable to unexposed cells. These results also show that increased luminescence is due to an increase in *luxAB* transcription (Forsberg *et al.*, 1994).

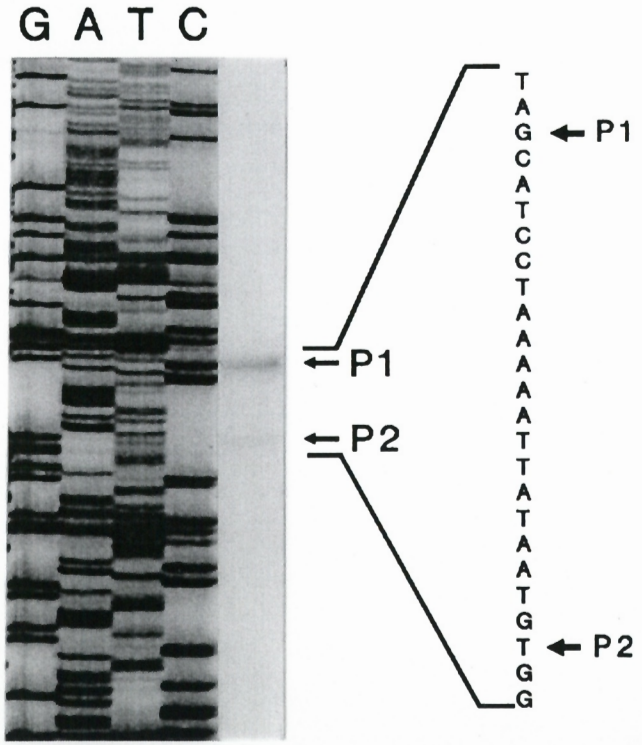
An S1 nuclease analysis was performed on RNA from cells exposed to aluminum (Figure 4B). Two protected fragments were seen migrating with nucleotides 299 and 319, localizing the transcriptional start site to a C and an A residue, respectively (Figure 3A). The promoter starting at bp 299, which produced a more abundant amount of protected fragment, was called P1, while the promoter starting at bp 319 was designated P2. If the transcript is 800 nucleotides long, then a transcriptional stop site should be located near bp 1119. The plasmid pAG17, which contains the *tet* and *luxAB* genes, 23 bp of IS*50L* and *E. coli* DNA sequences from the site of Tn*5-luxAB* insertion to the upstream *Hin*dIII site (Figure 3, 28 bp upstream of bp 1 to bp 677) cloned into the high copy number vector pUC119 (Vieira and Messing, 1987), did not show increased luminescence in the presence of aluminum. When the *Hin*dIII fragment from pAG17 was cloned into the intermediate copy number plasmid pSU19 and transformed into *E. coli* DH1, aluminum was now able to induce *luxAB* transcription. It was also found that plasmid pAG53, containing a *Hin*dIII-*Alu*I fragment, allowed aluminum induction of gene transcription (not shown), localizing the aluminum responsive promoter regulatory signals to be between 28 bp upstream of bp 1 to bp 348 in Figure 3.

Figure 4. Northern blot and S1 nuclease analysis of the aluminum-inducible transcript. A) Northern blot of RNA isolated from cells grown in the absence (lanes 1) or presence of 10 $\mu$g/ml aluminum chloride (lanes 2), ferric chloride (lanes 3) or nickel sulfate (lanes 4) at pH 5.5 or pH 7.0. The RNA was probed using a 700 bp *Hin*dIII-*Bam*HI DNA fragment from pAG17 (refer to Materials and Methods for details). Sizes in kb of an RNA ladder marker (Gibco-BRL, Burlington, Canada) are indicated at the left. B) S1 nuclease of the transcript induced in the presence of aluminum. The DNA sequence, generated using the oligonucleotide for the S1 nuclease reaction, is shown. The two protected fragments, labeled P1 and P2, are indicated by arrows.

A


pH 5.5
pH 7.0
1 2 3 4 1 2 3 4
4.4 –
2.4 –
.24 –

B


G A T C
P1
P2
TAGCATCCTAAAAATTATAATGTGG
P1
P2

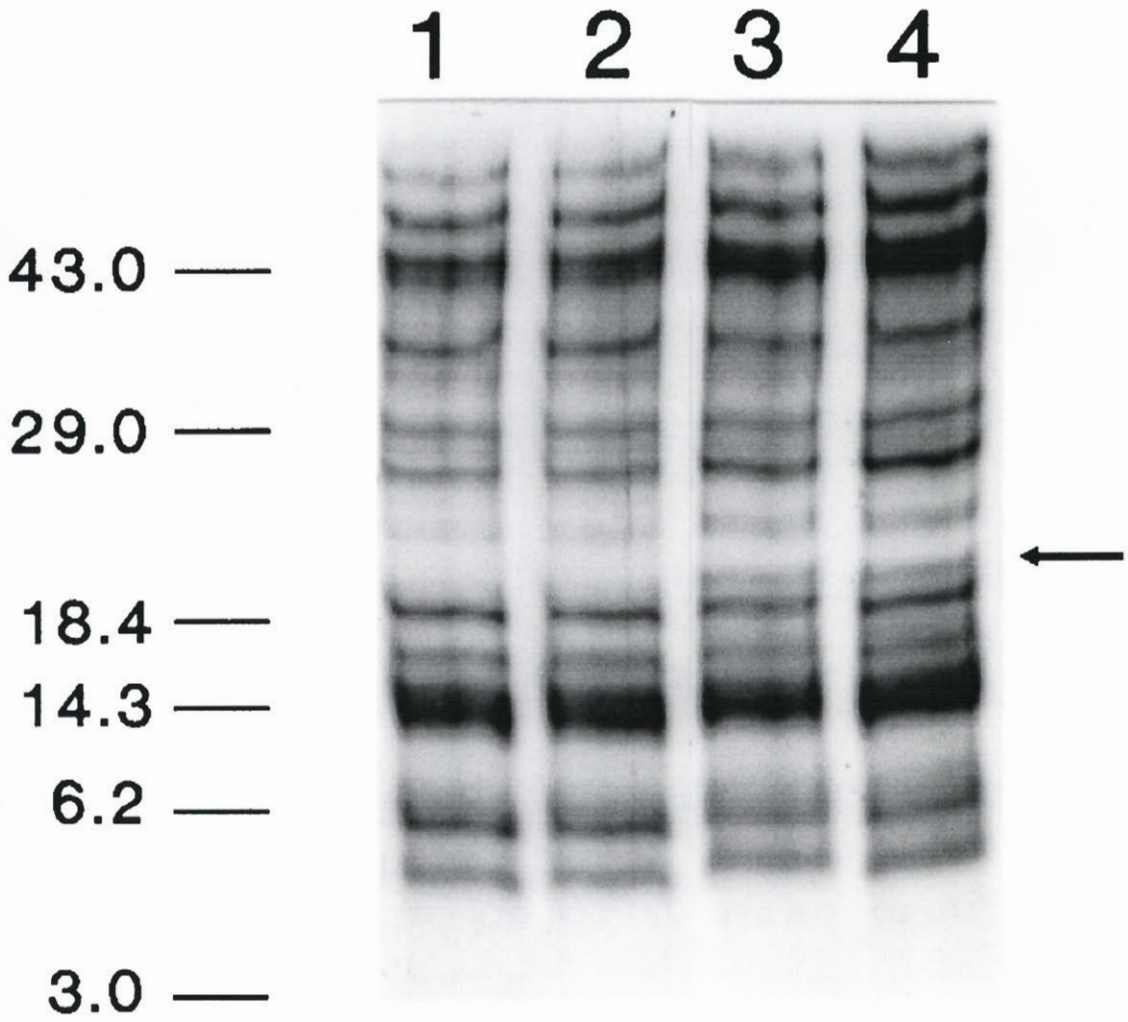
### 6.4.4 Expression of ORFs

Translation of the DNA sequence upstream of bp 319, using ATG as a start codon, revealed a potential ORF with a predicted molecular weight of 10.6-kDa spanning bp 488 to 769 (Figure 3A). Another potential ORF, coding for a polypeptide of 8.8-kDa, was also predicted from bp 1025 to bp 1249. However, this would place the coding segment downstream of the predicted transcriptonal stop site. Thus, it is unlikely that ORF 8.8 is made from the aluminum-inducible transcript. Translation of the DNA sequence using GTG, an alternative start codon, revealed that an ORF with a predicted molecular weight of 22.2-kDa could be coded from the region spanning bp 400 to 999. Shine-Dalgarno sequences are present upstream of both ORF 10.6 and ORF 22.2. When the expression of [$^{35}$S]methionine-labeled products was monitored *in vivo* using a DNA fragment containing a *Hin*dIII (28 bp upstream of bp 1, Figure 3A) to a *Bam*HI site (5 kb downstream of bp 1 in Figure 3A) expressed from the $P_{tac}$ promoter, a polypeptide migrating at approximately 22-kDa was seen (Figure 5), consistent with the expression of ORF 22.2.

## 6.5 DISCUSSION

We have isolated a gene inducible by both aluminum and iron, as measured by an increase in luminescence from a gene fusion and by northern blotting. We have designated this gene as *ais* (aluminum and iron stimulated). Aluminum-mediated induction of transcription, measured by a northern blot, is more pronounced at pH 5.5 than at pH 7.0, while the situation is reversed for iron. Induction of transcription, as measured by luminescence, occurs at lower exogenous aluminum and iron concentrations at pH 5.5. Thus, it is possible that the solubility of aluminum, namely $Al^{3+}$, plays a role in the induction of gene expression. The study of aluminum toxicity to *E. coli* suggests that the toxic species is $Al^{3+}$ (Guida *et al.*,

Figure 5. Polypeptides expressed from the region of the genome inducible by aluminum. Shown are the $^{35}$S-labeled polypeptides expressed *in vivo* from strain NM522 containing plasmid pKK223-3 after 40 (lane 1) and 60 minutes (lane 2) post-[$^{35}$S]methionine addition; and containing plasmid pAG46 after 40 (lane 3) and 60 minutes (lane 4) post-[$^{35}$S]methionine addition. The arrow indicates the 22-kDa polypeptide expressed from plasmid pAG46. Sizes of marker polypeptides (in kDa) are shown at the left.


1
2
3
4
43.0
29.0
18.4
14.3
6.2
3.0

1991). However, transcription could be induced by an aluminum hydroxy complex, the formation of which is pH dependent (MacDonald and Martin, 1988). This is possible since iron, which displays similar complexing properties and pH solubility relationships to aluminum (Brown and Schwartz, 1992), also induces luminescence in LF20110. The ionic radius of $Al^{3+}$ most closely resembles that of $Fe^{3+}$ (Ganrot, 1986; Martin, 1986). Thus $Al^{3+}$ can bind to the same sites as $Fe^{3+}$ to form complexes with siderophores, transferrin and citrate (Fatemi *et al.*, 1991, Martin, 1986). Guida *et al.* (1991) showed that iron deficiency increased the toxicity of aluminum to *E. coli.*

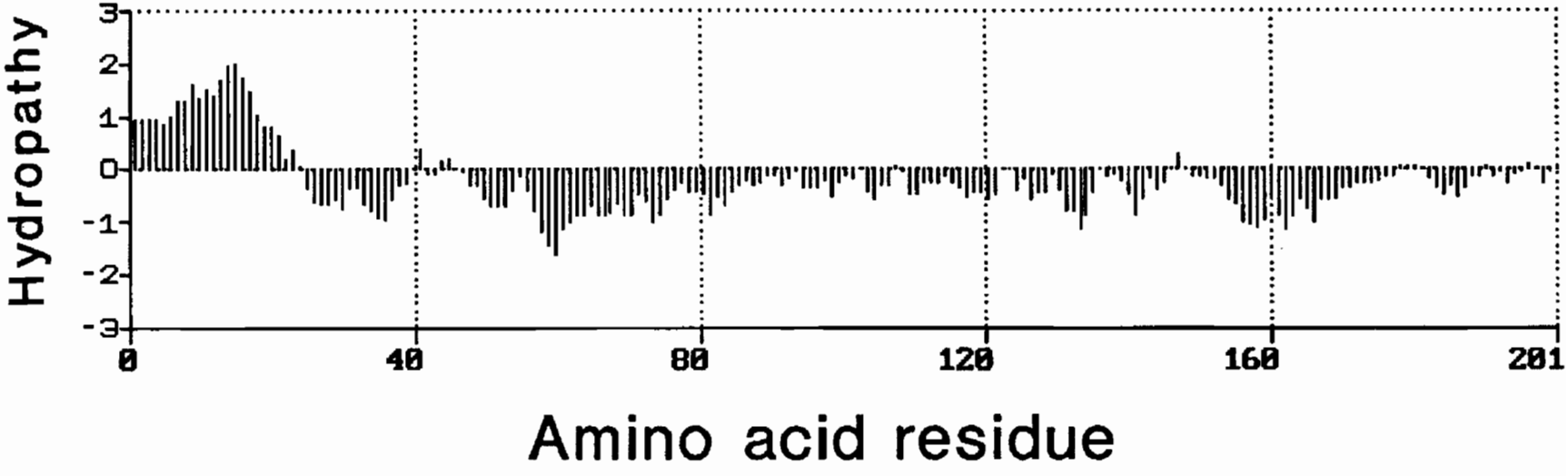
The level of induction of *ais* expression, as measured using luminescence (Figures 1 and 2), does not reflect the levels measured using northern blots (Figure 4A). In northern blotting, the measured amount of RNA after aluminum-exposure is much higher at pH 5.5 than at pH 7.0, whereas for iron the level is much higher at pH 7.0. Using luminometry, the final level of luminescence is higher at pH 5.5 than at pH 7.0 after cellular exposure to iron, whereas the levels are similar for aluminum at both pHs. However, a strict quantitative interpretation of transcription using luciferase as a measure of gene expression can be problematic in some circumstances (Forsberg *et al.*, 1994; Guzzo *et al.*, 1992).

The Tn*5-luxAB* element was shown to be inserted in a previously uncharacterized gene. The DNA sequence spanning the insertion site was determined, and an S1 nuclease analysis showed that transcription could start at two positions, termed P1 and P2, corresponding to bp 299 and 319, respectively (Figure 3A). Potential $\sigma^{70}$ "-10" and "-35" recognition sites for these promoters are indicated in Figure 3A. The occurrence of two closely spaced transcriptional start sites has been found, for example, for the *E. coli lac* operon (Malan and McClure, 1984) and the *Vibrio cholerae viuB* gene (Butterton and Calderwood, 1994). Analysis of the poten-

tial ORFs downstream of bp 319 revealed that there were two possible candidates: ORF 10.6 with an ATG start codon and ORF 22.2 with a GTG start codon. When this DNA region was expressed *in vivo* from the $P_{tac}$ promoter, a polypeptide migrating at approximately 22-kDa was seen (Figure 5). A DNA sequence homologous to the region of the *E. coli* genome, containing the DNA sequence which codes for ORF 22.2, was also found (by Southern blotting) in *Shigella sonnei*, but not in other *Enterobacteriaceae* species (data not shown). An inverted repeat, suggestive of a transcription terminator, was found starting 10 bp downstream from the ORF 22.2/Ais translational stop site (Figure 3A).

The function of the protein that is induced in the presence of aluminum and iron is unknown. A hydropathy plot of ORF 22.2/Ais revealed that it contains a large hydrophobic region at the amino terminus and is then predominantly hydropholic over the rest of the protein (Figure 6), which could indicate a membrane spanning domain. Furthermore, a potential signal sequence is present, consisting of a basic N-terminal region (amino acids 1 to 14), a hydrophobic core (amino acids 15 to 25) and a net negatively charged C-terminal region. Two putative consensus sites were found that would direct cleavage either between amino acids 22 and 23 or 25 and 26, indicating that this could be a membrane bound or periplasmic protein (Pugsley, 1993; Ray *et al.*, 1986). Comparison of the protein sequence against all other known sequences revealed that it has strong homology to AfrS (Genbank accession number L08467) (Figure 3B). The function of AfrS is unknown, but it is part of a plasmid-located operon coding for proteins involved in the synthesis of the AF/R1 pilus (Wolf and Boedeker, 1990). AF/R1 pili are located on the surface of *E. coli* RDEC-1 and promote attachment of the bacteria to rabbit intestinal brush borders. It is interesting to note that exposure of *Pseudomonas fluorescens* ATCC 13525 to aluminum caused an increase in secretion of a gelatinous, lipid-rich residue

Figure 6. Hydropathy plot for ORF 22.2/Ais. The plot was generated using the hydrophobicity scales of Kyte and Doolittle (1982) with a window of 19 amino acids. An index $>1$ indicates hydrophobicity, whereas an index of $<1$ corresponds to hydrophilicity.


Hydropathy
3
2
1
0
-1
-2
-3
0
40
80
120
160
201
Amino acid residue

which bound aluminum, decreasing its bioavailability (Appanna *et al.*, 1994). It will be interesting to determine whether ORF 22.2/Ais is involved in aluminum metabolism or detoxification. However, strain LF20110, containing the Tn*5-luxAB* insertion within the coding sequence of ORF 22.2/Ais and hence disrupting it, did not display increased sensitivity to aluminum when compared to wild type strain DH1 (data not shown).

It was shown that a *Hin*dIII-*Alu*I DNA fragment (Figure 3, from 28 bp upstream of bp 1 to bp 348) was able to confer aluminum inducibility of *luxAB* on the intermediate copy number plasmid pSU19. Inducibility was not seen when pUC119, a high copy number vector, was used, which suggests a limiting cellular factor for aluminum inducibility (Gill and Ptashne, 1988). The DNA binding proteins Fur (Ferric Uptake Regulator) and Fnr (Fumarate Nitrate Reductase) regulate gene transcription through iron availability or deficiency (Hennecke, 1990). The closest sequence found to an iron binding protein consensus sequence (Kammler *et al.*, 1993) was 9 out of 19 bp to the Fur box (Figure 3A bp 298 to 316) or 7 out of 16 bp to the Fnr consensus (Figure 3A bp 233 to 253 or bp 273 to 293). However, transduction of the *ais*::*luxAB* gene fusion into an *E. coli fnr*$^-$ strain did not abolish aluminum- or iron-mediated induction of luminescence (not shown). Fur represses transcription at high $Fe^{2+}$ concentrations and is responsive to other divalent cations (Hennecke, 1990), whereas induction of *ais* transcription occurs at high iron concentrations and is not responsive to other divalent cations. An 8 bp inverted repeat, separated by a 7 bp spacer ($^{5'}$GGCAAAAT(N)$_7$ATTTTGCC $^{3'}$), was found to overlap with the putative $\sigma^{70}$ "-10"and "-35"sequences of P1 and the $\sigma^{70}$ "-35"site of P2 (Figure 3A). Hence, a limiting cellular protein could bind to this sequence to activate transcription in the presence of aluminum or iron.

Future experiments to determine the minimal DNA sequences required, and

identification of factor(s) responsible, for aluminum-mediated transcriptional induction, and the role of ORF 22.2/Ais in aluminum and iron metabolism are currently underway.

# CHAPTER 7

## Metallothionein-II and Ferritin H mRNA Levels are Increased in Arsenite Exposed HeLa Cells

## PREFACE

Chapters 4 to 6 describe the identification and characterization of three *Escherchia coli* genes transcriptionally induced in the presence of the metals aluminum or nickel. Chapter 7 describes a search for genes that produce mRNAs which are more abundant after exposure to arsenite using HeLa cells in culture.

## 7.1 ABSTRACT

Arsenite is extremely toxic and, though non-mutagenic, is a carcinogen. To determine the effects of arsenite on changes in cell physiology, we searched for genes in HeLa cells whose mRNAs are more abundant after cellular exposure to arsenite. A cDNA subtraction was performed between cDNA synthesized from RNA extracted from HeLa cells grown in the absence and presence of 5 $\mu$M sodium arsenite. Isolation and sequencing of three clones that showed a higher hybridization signal to RNA from arsenite exposed cells, versus unexposed cells, revealed that two of the cDNAs coded for human ferritin H chain and the other coded for metallothionein-II. These results suggest the possibility that arsenite exposure may lead to increased levels of oxygen radicals, which augmented metallothionein and ferritin can act to detoxify.

## 7.2 INTRODUCTION

Arsenic is extremely toxic and has no known nutritional value. Epidemiological studies have linked arsenic exposure to cancer in humans (Smith *et al.*, 1992). Although arsenite is non-mutagenic using bacterial and mammalian tester strains (Jacobson-Kram and Montalbano, 1985), it potentiates the mutagenicity of UV and alkylating agents (Li and Rossman, 1989). In addition, arsenite exposure induces sister chromatid exchanges and chromatid aberrations in human peripheral lymphocytes and fibroblasts (Jacobson-Kram and Montalbano, 1985; Jha *et al.*, 1992), as well as augments X-Ray- and UV-induced chromosomal damage (Jha *et al.*, 1992). Arsenite ion interacts with the sulfhydryl groups of amino acids, thereby inhibiting thiol-dependent enzymes (Dong and Luo, 1993; Jha *et al.*, 1992). It has also been suggested that the arsenite ion may inhibit DNA repair via inactivation of DNA ligase (Jha *et al.*, 1992; Li and Rossman, 1989), consistent with its non-mutagenic but

genotoxic properties. Arsenite ion exposure was found to lead to DNA-protein cross-links and DNA strand breaks (Dong and Luo, 1993), suggesting additional mechanisms of DNA damage and carcinogenicity for this metalloid.

Eukaryotic cellular defense systems to toxic metal exposure include the modulation of gene expression. Arsenite ion exposure induces the synthesis of some heat shock proteins (Taketani *et al.*, 1989). Heme oxygenase was also shown to be induced by arsenite exposure in human cells (Keyse and Tyrrell, 1989; Taketani *et al.*, 1989). Heme oxygenase catalyzes the oxidative degradation of protoheme to biliverdin and other products, which are effective peroxide radical scavengers. Hence, it was suggested that heme oxygenase acts to protect against oxidative damage (Keyse and Tyrrell, 1989), caused directly or indirectly by arsenite. When rats were fed arsenite, hepatic metallothionein was augmented (Albores *et al.*, 1992). In rats and mice, DNA damage in the lung caused by dimethylarsinic acid, a major metabolite of inorganic arsenicals, was due to production of dimethylarsenic peroxyl radical and other active oxygen species (Yamanaka *et al.*, 1991). In addition, arsenite stimulates the synthesis of a 31-kDa nuclear membrane protein in an SV40-transformed Balb/c cell line (Disa *et al.*, 1993).

In order to identify genes in HeLa cells whose expression is induced, or mRNA stability augmented, in the presence of sodium arsenite, a subtraction "library" was created between cDNA made from cells exposed to 5 $\mu$M sodium arsenite versus unexposed cells. Described here is the method used to subtract, clone, and screen the cDNAs, and the identification of two genes, whose mRNAs are more abundant after a 24 hour exposure to arsenite, as metallothionein-II and the ferritin H chain.

## 7.3 MATERIALS AND METHODS

### 7.3.1 Cell Culture

HeLa cells were grown on plates in DMEM (pH 7.0) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Burlington, Canada), 2.5 $\mu$g/ml amphotericin B (Fungizone, Squibb, Montreal, Canada), 10 units/ml penicillin and 100 $\mu$g/ml streptomycin (Pen-Strep, Gibco-BRL, Burlington, Canada) at 37°C with a 5% $CO_2$ atmosphere until a cell density of $6.7X10^5$ cells/ml was reached. The cells were then transferred to DMEM (pH adjusted to 5.5 or 7.0 with 12 N HCl) or to DMEM (pH 7.0) containing 5 $\mu$M sodium arsenite. After 24 hours, the cells were trypsinized and pelleted.

### 7.3.2 DNA Manipulations

Hydrolysis with *Eco*RI, and calf intestinal alkaline phosphatase treatment, were performed as previously described (Tolias and DuBow, 1987). Labeling of DNA with [$\alpha$-$^{32}$P]dNTPs (3000 Ci/mmol; ICN Biomedicals, Mississauga, Canada) was accomplished with the random prime method using 100 ng of cDNA (Sambrook *et al.*, 1989).

### 7.3.3 RNA Isolation, cDNA Synthesis and Subtraction

RNA was isolated using the RNaid II Kit (Bio 101, Mississauga, Canada). The subtraction method was performed according to Schraml *et al.* (1993), except for the modifications described here. The cDNA from unexposed HeLa cells was synthesized directly on oligo(dT)$_{25}$ Dynabeads (Dynal Inc., Lake Success, USA) according to Schraml *et al.* (1993), except that the cDNA synthesis buffer was modified to contain 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 20 mM DTT. From 2.16 mg of total cellular RNA, it was estimated that 8.6

μg of single-stranded driver cDNA, bound to the Dynabeads, was synthesized (Sambrook *et al.*, 1989). Double-stranded tracer cDNA, from RNA extracted from arsenite-exposed cells, was synthesized using a Time Saver cDNA synthesis kit with the addition of *Eco*RI/*Not* I linkers (Pharmacia Biotech, Montreal, Canada). In a 500 μl screw-cap polypropylene tube, 0.5 μg of denatured, double-stranded tracer cDNA was mixed with 8.6 μg of driver Dynabead-bound cDNA (driver:tracer ratio of 34:1) in hybridization buffer [0.75 M NaCl, 25 mM HEPES (pH to 7.5 with 2 N NaOH), 5 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate] at 68°C for 72 hours. The supernatant fluid, containing annealed non-subtracted cDNA (enriched in double-stranded augmented cDNAs from exposed cells), was magnetically separated from the Dynabead-bound cDNA and precipitated using 60 mM ammonium acetate, 20 μg glycogen and 70% (v/v) ethanol. After incubation at -20°C for 16 hours, the DNA was pelleted by centrifugation at 15000 x g for 30 minutes at 4°C and resuspended in 10 μl of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

7.3.4 Cloning of Subtracted cDNA

Five μl of the subtracted cDNA was ligated to 50 ng of pUC119 (Vieira and Messing, 1987), previously hydrolyzed with *Eco*RI and treated with calf intestinal alkaline phosphatase, at 15°C for 15 hours (Sambrook *et al.*, 1989). An aliquot of the ligation reaction was electroporated into 40 μl of electrocompetent *Escherichia coli* strain DH5αF'(Raleigh *et al.*, 1989) according to the manufacturer's protocol (Bio-Rad Laboratories, Mississauga, Canada) using the Bio-Rad Gene Pulser apparatus set at 25 μFarads, 2.5 kV and 200 ohms, and then plated on LB agar (Sambrook *et al.*, 1989) containing 40 μg/ml Ap, 1 mM IPTG and 40 μg/ml Xgal.

### 7.3.5 Screening Procedure

Total cellular DNA was isolated from 1-$cm^2$ patches of white clones using the rapid cleared lysate procedure (DuBow and Lalumière, 1994) and subjected to electrophoresis through 0.75% agarose gels. The DNA in the gels was then denatured and bidirectionally transferred to Hybond-N membranes (Amersham Ltd., Oakville, Canada) (Smith and Summers, 1980). One filter was hybridized (Chaconas *et al.*, 1980) with [$\alpha$-$^{32}$P]-labeled cDNA synthesized from RNA isolated from unexposed cells. The other filter was hybridized (Chaconas *et al.*, 1980) to [$\alpha$-$^{32}$P]-labeled cDNA synthesized from RNA isolated from sodium arsenite exposed cells.

### 7.3.6 Northern Blot and Slot Blot Analysis

For northern blotting, 5 $\mu$g of RNA was subjected to electrophoresis through 1% denaturing agarose gels (Sambrook *et al.*, 1989), except that the RNA was prestained by the addition of 100 $\mu$g/ml ethidium bromide to the loading buffer, and the running buffer contained 7.4% (v/v) formaldehyde, 40 mM MOPS-NaOH (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After electrophoresis, the gel was soaked in 20XSSC [3 M NaCl, 0.3 M sodium citrate (pH 7.0)] for 20 minutes and the RNA transferred to a Hybond-N membrane for 16 hours (Sambrook *et al.*, 1989). RNA was treated (Sambrook *et al.*, 1989) and applied through a Bio-Rad Bio-dot apparatus onto a Hybond-N membrane. The filters were hybridized (Shackleford and Varmus, 1987) to [$\alpha$-$^{32}$P]-labeled probes. The probes were isolated after hydrolysis of pUC119 plasmids containing cloned cDNAs with *Eco*RI and gel purifying the cDNA fragment (Sambrook *et al.*, 1989). The actin probe was isolated as a 1.15 kb *Pst*I DNA fragment from plasmid 91 (Minty *et al.*, 1981). The probes were removed from the filters by washing twice in 0.5 N NaOH at 37°C.

### 7.3.7 DNA Sequencing

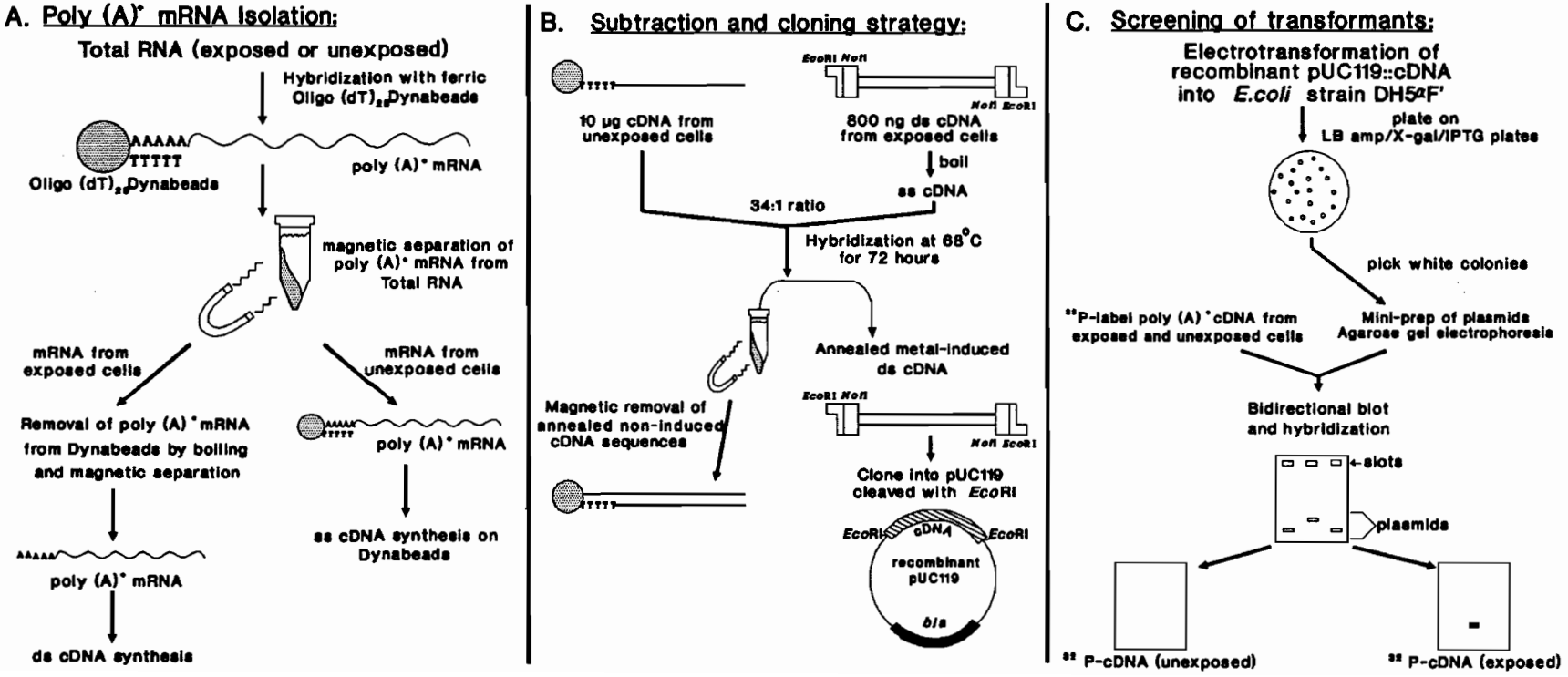
For DNA sequencing, single-stranded phagemid DNA was prepared from the pUC119-based plasmids (Vieira and Messing, 1987). Dideoxy sequencing reactions with [$\alpha$-$^{35}$S]dATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada) were performed with a Sequenase kit using the universal primer (U.S. Biochemical Corp., Cleveland, USA).

## 7.4 RESULTS

### 7.4.1 cDNA Subtraction, Cloning and Screening

In an effort to find genes whose expression, or mRNA stability, is augmented by cellular exposure to sodium arsenite, a cDNA subtraction was performed between cDNAs synthesized from 5 $\mu$M arsenite exposed versus unexposed cells. The cDNA from the unexposed cells was synthesized directly on ferric Dynabeads (Figure 1A) with the subsequent separation of non-subtracted, double-stranded cDNA using a magnet, thus allowing the direct cloning of the remaining non-subtracted, double-stranded cDNAs (Figure 1B) (Schraml *et al.*, 1993). By comparing the transformation efficiency of cloned cDNAs ligated before subtraction to that after subtraction, it was estimated that an approximate 21-fold enrichment was achieved (not shown). To screen for cloned cDNAs that encoded augmented mRNAs after a 24 hour exposure to 5 $\mu$M arsenite, 234 non-subtracted cDNAs, cloned into pUC119 (Vieira and Messing, 1987), were hybridized to total radiolabeled cDNA synthesized from unexposed versus exposed cells (Figure 1C). Three plasmids that showed a difference in hybridization between the two probes, designated pAG60, pAG61, and pAG62, were further analyzed.

Figure 1. Outline of the method for (A) poly(A) $^{+}$ mRNA isolation, (B) subtraction and cloning and (C) screening of transformants. For details, see Materials and Methods.


A. Poly (A)+ mRNA Isolation:
Total RNA (exposed or unexposed)
Hybridization with ferric Oligo (dT)25 Dynabeads
AAAAA
TTTTT
Oligo (dT)25 Dynabeads
poly (A)+ mRNA
magnetic separation of poly (A)+ mRNA from Total RNA
mRNA from exposed cells
mRNA from unexposed cells
Removal of poly (A)+ mRNA from Dynabeads by boiling and magnetic separation
poly (A)+ mRNA
poly (A)+ mRNA
ds cDNA synthesis
ss cDNA synthesis on Dynabeads
B. Subtraction and cloning strategy:
EcoRI NotI
TTTT
10 µg cDNA from unexposed cells
800 ng ds cDNA from exposed cells
NotI EcoRI
boil
ss cDNA
34:1 ratio
Hybridization at 68°C for 72 hours
Annealed metal-induced ds cDNA
Magnetic removal of annealed non-induced cDNA sequences
EcoRI NotI
NotI EcoRI
Clone into pUC119 cleaved with EcoRI
EcoRI
cDNA
EcoRI
recombinant pUC119
bla
C. Screening of transformants:
Electrotransformation of recombinant pUC119::cDNA into E.coli strain DH5αF'
plate on LB amp/X-gal/IPTG plates
pick white colonies
32P-label poly (A)+ cDNA from exposed and unexposed cells
Mini-prep of plasmids Agarose gel electrophoresis
Bidirectional blot and hybridization
slots
plasmids
32 P-cDNA (unexposed)
32 P-cDNA (exposed)

### 7.4.2 RNA Analysis

To quantify the increase in mRNA abundance after arsenite exposure, the individual cDNAs were isolated, purified, radiolabeled, and used to probe northern and dot blots of total RNA from exposed and unexposed cells (Figure 2). In addition, the blots were stripped of radioactivity and hybridized to an actin probe, used as an internal control. RNA was extracted from unexposed cells grown at pH 5.5 and pH 7.0 to rule out possible pH effects. Densitometric scanning of the blots (Tolias and DuBow, 1987) revealed that plasmids pAG60 and pAG61 contained a cDNA that hybridized to an approximately 0.8-0.9 kb mRNA whose abundance is augmented at least 2-fold in arsenite-exposed cells (Figure 2A), while plasmid pAG62 hybridized to a 0.3-0.4 kb mRNA whose abundance is augmented approximately 6-fold upon arsenite exposure (Figure 2B).

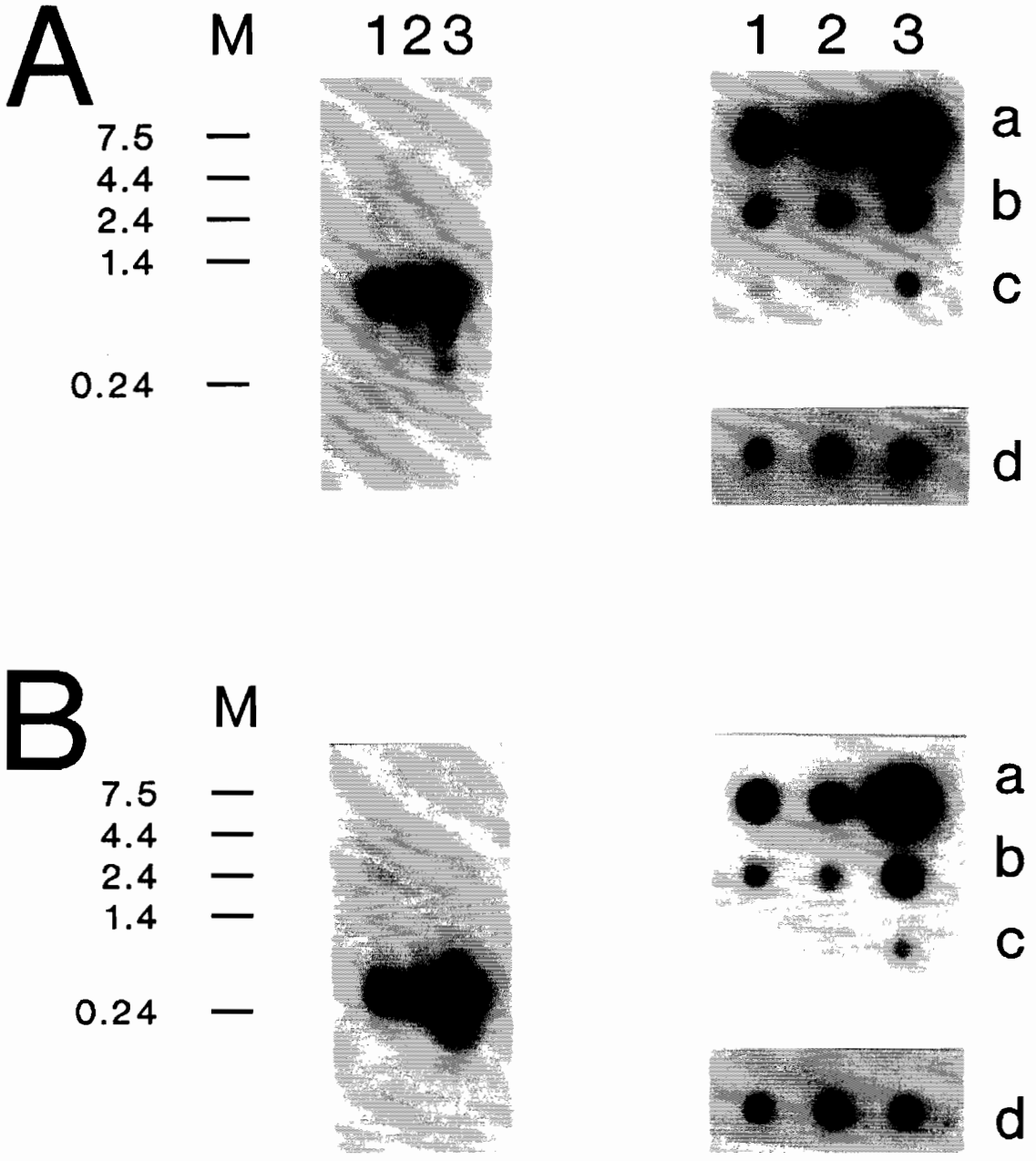
### 7.4.3 Identification of the cDNAs

The three cDNAs were sequenced, and it was found that plasmids pAG60 and pAG61 contained an identical DNA sequence. When the two different cDNA sequences were compared against the DNA sequences present in the GenBank database, plasmids pAG60 and pAG61 were shown to contain the cDNA encoding the human ferritin H chain (Boyd *et al.*, 1985), while plasmid pAG62 contained a cDNA coding for human metallothionein-II (Karin and Richards, 1982).

## 7.5 DISCUSSION

In order to find genes induced by the toxic arsenite ion, a cDNA subtraction was performed between cDNA from unexposed HeLa cells versus those prepared after a 24 hour exposure to 5 $\mu$M sodium arsenite. After cloning and analysis of three arsenite-induced cDNAs, we found that human metallothionein-II and ferritin

Figure 2. Northern and mRNA dot blots of cloned cDNAs. The cDNAs from plasmids pAG60 (A) or pAG62 (B) were isolated, [$\alpha$-$^{32}$P]-labeled and hybridized to 5 $\mu$g of total cellular RNA that was subjected to electrophoresis through formaldehyde agarose denaturing gels (left panel), or hybridized to 2.5 (a), 0.25 (b) or 0.025 $\mu$g (c) of total cellular RNA on a nylon membrane dot blot (right panel). Also shown is the hybridization of [$\alpha$-$^{32}$P]-labeled actin to 2.5 $\mu$g (d) of total cellular RNA on a nylon membrane dot blot. The RNA was isolated from HeLa cells grown at pH 5.5 (lane/slot 1), pH 7.0 (lane/slot 2) or at pH 7.0 plus 5 $\mu$M arsenite (lane/slot 3). Sizes in kb of an RNA ladder (Gibco-BRL, Burlington, Canada) marker are indicated in lanes M.


A
M
123
1 2 3
7.5
4.4
2.4
1.4
0.24
a
b
c
d
B
M
7.5
4.4
2.4
1.4
0.24
a
b
c
d

H mRNA levels are elevated approximately 6- and 2-fold, respectively, in HeLa cells exposed for 24 hours to 5 $\mu$M arsenite *in vitro* (Figure 2). We do not, as yet, know if this increase is due to an increase in transcription or mRNA stability. Metallothionein exists in two distinct isoforms, designated MT-I and MT-II, that bind and sequester heavy metals, thus lowering their availability and toxicity (Hamer, 1986). Ferritin is an iron storage protein made up of 24 subunits of H and L chains in varying ratios (Boyd *et al.*, 1985). Arsenite ingestion was found to induce rat hepatic metallothionein *in vivo* (Albores *et al.*, 1992). Hepatic MT-I mRNA was increased 1.6-fold, while MT-II mRNA was induced 35-fold at 24 hours post-arsenite treatment *in vivo* (Albores *et al.*, 1992). However, the high level of apparent MT-II mRNA induction was found to be mainly due to its low initial hepatic levels.

Metallothionein is also believed to play a role in protection against oxygen radicals (Sato and Bremner, 1993). Increased production of ferritin in cultured endothelial cells was found to increase the cells' resistance to oxidant-mediated damage (Balla *et al.*, 1992). Hence, it is possible that metallothionein-II and ferritin H mRNA levels were increased by a common signal, such as oxidative-mediated damage. Arsenite exposure may augment oxidative damage by directly participating in the formation of oxygen radicals (Yamanaka *et al.*, 1991). Alternatively, it has been suggested that an arsenite-mediated reduction or modification in the intracellular antioxidant glutathione may be the signal, a result consistent with the observed augmentation of heme oxygenase levels in arsenite-exposed cells (Keyse and Tyrrell, 1989). Moreover, increased levels of glutathione correlated with arsenite resistance in a chinese hamster ovary cell line (Lo *et al.*, 1992). Although the mechanism(s) and signal(s) for arsenite-induced increases in metallothionein and ferritin remain to be elucidated, these results add credence to the hypothesis that arsenite toxicity may also be manifested through oxygen radical generation (or stability).

# CHAPTER 8

## Summary, Conclusions and Future Prospects

Paracelsus (1493-1541) stated: "All substances are poisons; there is none that is not a poison. The right dose differentiates a poison and a remedy" (Hodgson, 1987). Some metals are essential for life (e.g. Co, Cu, Mo, Mn, Ni, Se, Zn) because they provide essential cofactors for metalloproteins and enzymes, while others are not (e.g. As, Al, Cd) (Hughes and Pool, 1989). However, at elevated concentrations, metals can act in a deleterious manner by blocking essential functional groups, displacing essential metal ions, or modifying the active conformation of biological molecules (Collins and Stotzky, 1989). Microorganisms can survive a wide range of changes in their environment (including elevated concentrations of toxic compounds) by altering gene expression (and thus their physiology). To identify chromosomal genes, the expression of which changes in the presence of a metal, it was necessary to rapidly quantify gene expression. The luciferase-encoding *Vibrio harveyi* *luxAB* genes were chosen, as they catalyze a reaction that oxidizes an aldehyde and $FMNH_2$ to emit light as an end product (Meighen, 1991). The amount of *luxAB* expressed is thus proportional to the amount of cellular luminescence. Insertion of the *luxAB* genes (minus their own promoter) within a gene places *luxAB* production under the control of the regulatory region of the bacterial gene (Chapter 1). Since the location (or the existence) of the metal-regulated gene(s) in the *E. coli* chromosome is unknown, it was necessary to monitor the expression of every gene, of which there are approximately 3000 (Kohara, 1989).

The ColE1-based plasmid pFUSLUX was constructed to contain the *luxAB* genes, plus a $tet^R$ gene (which acts as a selectable marker), within the ends of an enfeebled Tn*5* transposon, composed of a truncated IS*50*L and a complete IS*50*R (Johnson and Reznikoff, 1983). To create a library of 3000 *E. coli* clones, each containing the *luxAB* genes inserted at single and random chromosomal loci, plasmid pFUSLUX was transformed into *E. coli* strain DH1 containing a p15A-based,

$Ap^R$ plasmid which overproduces RNA1, an inhibitor of ColE1 replication. After selection for $Tc^R$ plus $Ap^R$ clones, colonies appeared which contained a single-copy insertion of *luxAB* in the *E. coli* chromosome (Chapter 2). However, it was also found that colonies containing Tn*5* transposed into the chromosome increased in number 3 to 6 days post-transformation, 20% of which also displayed changes in IS*1*, IS*2* or IS*5* element distribution (Chapter 3). Furthermore, colonies that would not grow upon restreaking were found to decrease in percentage in the same time period. These colonies arose due to the apparent loss of pTF421 from cells containing pFUSLUX, concomitant with apparent Ap degradation around the pTF421-containing cells. It is thus possible that *E. coli* grown for extended periods of time under adverse conditions (such as those shown in Chapter 3) display increased frequencies of so-called "adaptive mutations" and DNA rearrangements. This could be due to some common underlying physiological factor that is expressed under these conditions. For example, the Clp protease has been implicated in affecting Mu-mediated rearrangements (Mhammedi-Aloui *et al.*, 1994; Shapiro, 1993) and in the degradation of proteins expressed during carbon starvation (Damerau and St. John, 1993). It should be possible to test the affects of selected chromosomal mutations (such as *clp*) on Tn*5* transposition over several days in the system described here. Any mutation affecting this process could alter the frequency of Tn*5*-*luxAB* insertions.

The level of luminescence of each of the 3000 *E. coli* clones was monitored in the absence and presence of 1 and 10 $\mu$g/ml of aluminum at pH 5.5, and of nickel at pH 7.0. Aluminum is the most abundant crustal metal (MacDonald and Martin, 1988), yet has no biological function (Hughes and Pool, 1989). However, aluminum exposure can cause diverse effects (Ganrot, 1986; Massey and Taylor, 1988; Sigel and Sigel, 1988) such as damage to the gill epithilia of fish (Bichall *et al.*, 1989), in

addition to being responsible for a multitude of disorders in human hemodialysis patients (MacDonald and Martin, 1988). Nickel is an essential trace element for bacterial ureases, hydrogenases, methylcoenzyme M reductases, and CO-dehydrogenases (Hausinger, 1987). Although nickel compounds yield negative results using bacterial mutagenesis tests, they can test positive in mammalian mutagenicity assays (Costa, 1991; Sunderman Jr., 1989). Nickel has adverse growth and differentiation effects on human cells *in vitro* (Lechner *et al.*, 1984) and is carcinogenic (Costa, 1991). Two clones were found that displayed increased luminescence in the presence of aluminum (strains LF20110 and LF20111) and one clone that increased in the presence of nickel (strain LF20112).

The luminescence of strain LF20111 also increased in the presence of copper, nickel and iron on LB agar plates. Cloning and hybridization to an ordered phage λ library of the *E. coli* genome, and sequencing of part of the *luxAB* gene fusion of strain LF20111, revealed that the *luxAB* genes were inserted 97 bp downstream of the *E. coli fliC* translational start site (Chapter 4). The *fliC* gene encodes the flagellin protein that constitutes the major portion of the bacterial motility organ. The transcription of *fliC* is regulated in a complex, cascade mechanism. Primary regulation in the cascade is by cAMP (MacNab, 1987a). Aluminum-mediated induction of the *fliC::luxAB* gene fusion was abolished in an *E. coli cya*$^-$ strain (not shown), suggesting that regulation occurs at some step in this cascade. Interestingly, induction of *fliC* transcription did not occur in the presence of metal ions when *E. coli* was grown in liquid media, but did occur when grown on LB agar plates. Bacteria growing on solid media behave differently than in liquid media. Biofilms form on solid surfaces due to the excretion of an exopolysaccharide matrix that mediates aggregation and adsorption (Costerton *et al.*, 1994). The biofilm matrix may have some function in the cell's ability to cope with metal ions, either by

affecting metal bioavailability or transport, which may account for *fliC* transcriptional induction on solid media. When exposed to aluminum, *Pseudomonas fluorescens* increases secretion of a gelatinous, lipid-rich residue which binds aluminum and decreases its bioavailability (Appanna *et al.*, 1994)

Strain LF20112, inducible by nickel and cobalt, was shown to contain the *luxAB* genes inserted 3204 base pairs downstream of the *cel* operon transcriptional start site, in the *celF* gene (Chapter 5). This operon is cryptic in wild type *E. coli*, and its polypeptides are involved in the catabolism of β-glucosides (Parker and Hall, 1990a). The relationship of nickel to these gene products was not understood. However, DNA sequencing revealed the presence of a gene that encodes an expressible 28-kDa polypeptide located immediately downstream of *celF*. Nickel binds to two to three cysteines/histidines oriented in a proper spatial fashion (Higaki *et al.*, 1992). Although it is impossible to predict the nickel-binding motif, close to the amino-proximal end of ORF 28.5 is the amino acid sequence CHC---HC (amino acids 31 to 38) which could potentially bind nickel. It would be interesting to determine whether ORF 28.5 binds nickel by transferring ORF 28.5 to a nitrocellulose membrane and allowing it to bind to radioactive nickel, $^{63}Ni$.

Strain LF20110, inducible by aluminum and iron, contains the *luxAB* genes in a previously uncharacterized gene, designated *ais* (aluminum and iron stimulated), that was cloned and sequenced (Chapter 6). An mRNA of 800 nucleotides in length was produced in the presence of aluminum and iron, and two closely spaced transcriptional start sites were localized. Furthermore, preliminary promoter resection delineated the aluminum-mediated transcriptional signals to 370 bp. It is now possible to determine the minimal aluminum/iron-responsive sequence by performing exonuclease digestion on the 370 bp fragment and screening for aluminum/iron-mediated induction. Once that is done, *in vitro* directed mutagenesis can be per-

formed to determine the specific DNA sequences responsible for metal-mediated regulation of gene expression. Furthermore, any proteins binding to the aluminum/iron-mediated transcriptional sequences can be identified and purified by fractionating and testing DNA binding using gel retardation assays. Alternatively, random genomic clones can be screened for their effect on *ais* expression using *luxAB* as a marker. The two iron binding transcriptional factors, Fur and Fnr, bind to $Fe^{2+}$, and repress or activate transcription under anaerobic growth conditions (Hennecke, 1990). Induction by Fnr does not appear likely. Due to the similar complexing and pH solubility characteristics of $Al^{3+}$ to $Fe^{3+}$ (Brown and Schwartz, 1992), it seems more likely that transcription of the *ais* gene is induced by $Fe^{3+}$, not $Fe^{2+}$. This could be tested by pretreating the growth medium with an $Fe^{3+}$ chelating agent such as ferrichrome and determining whether transcriptional induction is abolished. However, a mutant would have to be created in the bacterial receptor for ferrichrome uptake, FhuA, to prevent its uptake into the cell (Moeck *et al.*, 1994). If induction is by $Fe^{3+}$, then it would be interesting to determine whether mutations in any of the *E. coli* ferric uptake systems (Klebba *et al.*, 1993) abolishes transcriptional induction of the *ais* gene by aluminum, since bacterial transporters for aluminum are unknown.

A polypeptide of 22-kDa was expressed *in vivo* from the aluminum-inducible transcript (Chapter 6). A potential signal sequence was found in a hydrophobic amino-terminal segment. By amino acid sequencing the mature polypeptide, it will be possible to determine whether ORF 22.2/Ais is cleaved. Furthermore, fractionation studies should be performed to determine where in the cell this protein is located. The involvement of ORF 22.2/Ais in aluminum detoxification is not yet known, though insertion of Tn*5-luxAB* into the *ais* DNA coding sequence does not affect viability of *E. coli* exposed to high aluminum concentrations. However, this

does not rule out the possibility that ORF 22.2/Ais is involved in aluminum metabolism. The amino acid sequence of ORF 22.2/Ais was found to be homologous to the protein AfrS. Although the function of AfrS is unknown, it is transcribed as part of an operon which codes for AF/R1 pili, responsible for adherence of *E. coli* RDEC-1 to rabbit intestinal brush borders (Wolf and Boedeker, 1990).

The method of gene fusions was successfully employed to find genes in *E. coli* induced by the metals aluminum and nickel. The knowledge gained can provide an understanding of how microorganisms respond to toxic metal exposure. We were also interested in finding genes in human cells inducible by toxic metals to determine other modes of metal detoxification and metabolism. This was done *in vitro* using the HeLa cell line. The metal ion arsenite was used since it is known to be toxic, but the mechanisms are not clear.

Arsenite is extremely toxic. Although non-mutagenic, it is a carcinogen. A cDNA subtraction was performed between cDNA synthesized from HeLa cells grown in the absence and presence of 5 $\mu$M arsenite for 24 hours. Cloning and sequencing of three cDNAs that showed a higher hybridization signal to RNA from HeLa cells in the presence, versus the absence, of arsenite showed that two of the cDNAs coded for human ferritin H and the other coded for metallothionein-II (Chapter 7). The mRNA for heme oxygenase was previously shown to be induced by arsenite (Keyse and Tyrrell, 1989; Taketani *et al.*, 1989). The common property of all these proteins are their ability to aid in cellular defense to oxidative damage. This oxidative damage could occur by the metabolic transformation of arsenite to methylated derivatives, which can then directly participate in oxidative damage, or indirectly by binding to the thiol-rich antioxidant glutathione. It would be interesting to determine whether arsenite-mediated induction of metallothionein-II, ferritin H and heme oxygenase is abolished by pretreating the cells with glutathione (Chang *et*

*al.*, 1991). We also do not as yet know whether a 24 hour exposure to, or a dose of 5 $\mu$M, arsenite is optimal for RNA augmentation. Northern and dot blots should be performed on RNA extracted from HeLa cells at different times after exposure to arsenite at different concentrations. Furthermore, whether augmentation is due to increased transcription or mRNA stability should also be explored.

Described in this thesis are molecular approaches that can be used to identify genetically programmed responses to toxic metal exposure. The library of 3000 *E. coli luxAB* gene fusions can be screened at any time with other metals. Since gene fusions are versatile and can be created in virtually any prokaryote, this approach can be extended to many other bacteria. The uninduced library of cDNA bound to oligo(dT)$_{25}$ can be used to subtract cDNA synthesized from cells exposed to other metals. This approach can also be adapted to other eukaryotic organisms. The information extracted from using these approaches can provide insight into the functions of cellular targets, signal transduction pathways, and the mechanisms of sensitivity to metal exposure.

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