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Characterization of gene expression of the left end regulatory region of the *Pseudomonas* transposable bacteriophage D3112

By

Kirsty Anne Salmon

Department of Microbiology and Immunology
McGill University, Montreal
August, 1999

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree Doctor of Philosophy

This thesis is dedicated to the memory of my Dad, Robert C. Salmon, who passed away suddenly December 30, 1998. You were not only a great father but also a wonderful teacher and supporter of everything I did (even hockey!).

Thanks Dad.

Abstract

Bacteriophage D3112 is a Mu-like transposable bacteriophage of *Pseudomonas aeruginosa*. D3112 has a double-stranded DNA genome of approximately 38kbp with a genetic organization similar to that of coliphages Mu and D108. Genetic mapping and DNA sequence analysis has identified the left-end of the phage genome as encoding the transposase enzyme *(A)* and the lysogenic *(c)* repressor. As genetic control of the lytic-lysogenic switch in D3112 has not been elucidated, characterization of the expression and activity in this region was undertaken. The *c* open reading frame (ORF), located at the left-most end of the phage genome, has four possible GTG initiation codons. Site-directed mutagenesis, along with an *in vivo* immunity assay, were used to determine that the second GTG is used as the translation-initiation codon. Northern blotting analysis, transcriptional *lacZ* fusions and primer extension analyses were used to define the promoter, P*c*, to bp972-940 from the left-end of the phage genome. Overexpression, purification and activity analysis of the D3112 c repressor demonstrated that it binds to a 261bp *Pvu*II fragment localized directly upstream of the *c* repressor ORF. We have located a previously unidentified Ner homologue in D3112. Using cloned fragments containing the D3112 promoter P*c* fused to a *lacZ* reporter gene, β-galactosidase expression was blocked when the fragment was lengthened from bp1099 to bp1439. A previously unidentified ORF was located from bp1033-1384 that demonstrated significant homology to the Ner proteins of coliphages Mu and D108. The *ner* promoter, P*ner*, has been located to bp957-992 using β-galactosidase assays and primer extension

analyses, and Northern blotting demonstrated that D3112 *ner* is part of a 2.2kb mRNA transcript. As the *ner* gene begins at bp1033 and the transposase gene begins at bp2539, this transcript is not large enough to encode for the *A* or *B* genes, as is seen in the early transcripts of coliphages Mu and D108. The *ner* promoter (P*ner*) was shown to be under the control of the D3112 repressor. When D3112 Ner was overexpressed and purified, this protein bound to the 261bp *Pvu*II fragment from the D3112 intergenic region. The D3112 early promoter P*e* was located, by β-galactosidase assays, directly upstream of the *A* gene to a 507bp fragment (bp2234-2741). These results suggest that the D3112 lytic-lysogenic switch uses Mu and D108-like functions but that their roles may be quite different. A model for the lytic-lysogenic switch in D3112 is presented.

Résumé

Le bactériophage D3112 de *Pseudomonas aeruginosa* est un phage transposable semblable au coliphage Mu. Le phage D3112 possède un génome d'ADN à double brin d'environ 38kpb et son organisation génétique ressemble à celle des coliphages Mu et D108. La cartographie génétique et l'analyse de la séquence de l'ADN ont identifié et situé les séquences codantes de l'enzyme transposase *(A)* et du répresseur lysogénique *(c)* à l'extrémité gauche du génome du phage. Le contrôle génétique du passage lytique-lysogénique n'ayant pas été élucidé chez le phage D3112, l'étude de l'expression et de l'activité dans cette région a été entreprise. Le cadre de lecture ouvert du gène *c*, situé à l'extrême gauche du génome du phage, présente quatre codons d'initiation GTG possibles. Il a été démontré, par mutagénèse dirigée et par un test immunitaire *in vivo*, que le deuxième GTG sert de codon d'initiation lors de la traduction. Une analyse par transfert de Northern, des fusions transcriptionnelles *lacZ* et des extensions d'amorces ont été utilisées pour définir la position du promoteur, P*c*, aux paires de bases 972-940 à partir de l'extrémité gauche du génome du phage. La surexpression, la purification et l'analyse de l'activité du répresseur c du phage D3112 ont démontré qu'il se lie un fragment *Pvu*II de 261 paire de bases situé directement en amont du cadre de lecture ouvert du répresseur c. Nous avons localisé chez le phage D3112 un homologue de Ner n'ayant jamais été identifié auparavant. En utilisant des fragments clonés contenant le promoteur P*c* du phage D3112 fusionnés gène rapporteur *lacZ*, la β-galactosidase n'a pas été exprimée lorsque le fragment a été étendu des paires de bases 1099 à 1439. Un cadre

de lecture ouvert non identifié précédemment a été localisé aux paires de bases 1033-1384. Il présente une homologie significative avec les protéines Ner des coliphages Mu et D108. Le promoteur de *ner*, P*ner*, a été localisé aux paires de bases 957-992 par des tests de β-galactosidase et des extensions d'amorces. L'analyse par transfert de Northern a démontré que le *ner* du phage D3112 fait partie d'un transcrit d'ARNm essager de 2.2kb. Le gène *ner* débutant à la paire de bases 1033 et le gène transposase à la 2539^{me}, ce produit n'est pas assez grand pour encoder les gènes *A* ou *B*, comme le démontrent les transcrits précoces des coliphages Mu et D108. Il a été démontré que le promoteur de *ner* (P*ner*) est sous le contrôle du répresseur du phage D3112. Lorsque le Ner du phage D3112 a été surexprimé et purifié, cette protéine s'est liée au fragment *Pvu*II de 261 paires de bases de la région intergénique du phage D3112. Le promoteur précoce P*e* du phage D3112 a été localisé, par des tests de β-galactosidase, à un fragment de 507 paires de bases (pb 2234-2741) directement en amont du gène *A*. Ces résultats suggèrent que le passage lytique-lysogénique du phage D3112 met en jeu des fonctions semblables à celles des phages Mu et D108, mais que leurs rôles peuvent être bien différents. Un modèle du passage lytique-lysogénique chez le phage D3112 est présenté.

Acknowledgments

I would first like to thank my supervisor Dr. Michael S. DuBow. When I first came to Mike's lab I knew almost nothing about bacteriophages or *Pseudomonas*. I now leave McGill not only with this experience, but with a completely different perspective on how science can be approached. Thank you for allowing me experimental independence, yet challenging me to question my results. I have learned a lot.

I would like to express an extreme gratitude to "YODA" (a.k.a. Bruce W. Ritchings now found at UCSD) for his e-mail responses to my, "DOTEWN's", endless technical questions: May the force be with you! To Caro (Diorio), you've been a great friend, moral supporter and sounding board. Good luck in everything you do. To my comp study partners Djenann St.-Dic and Julie Guzzo—we made it!! I would also like to thank Julie for introducing me to hockey...2 mugs...2 years: can't beat that...P.S. I'm a wall!! Thank you also to Djenann, Manuelle Rongy-Mimouni, and M.-C. Ouimet for the french translation of my Abstract. I couldn't have done it without you!!

To all the others who have helped me these past years. Dr. Peter Ulycznyj for countless discussions on the ins and outs of working with *Pseudomonas aeruginosa* and D3112. A big Thank you to Dr. Greg Marczynski for scientific discussions and a different point of view. Thanks also to Doris Fortin and Ian Siboo for scientific discussions, and to Felix Sieder and David Alexander for their endless help with my computer woes.

I would also like to express my sincere gratitude to Orit Freedman, one of my honour's students, who kept me on my toes with her challenging questions. Thank you not only for your work in the lab (which became part of Chapter 2), but your wonderful sense of being. I wish you happiness in life.

To the rest of you: D3112, Mayla, Madani, Nicha, Karen, Rania, Dr. Brian Mee, Gaëlle, Nicola, Anne and all the past 'DuBowians'—thanks for the camaraderie! Thanks also goes to the office staff: Jeanie, Carol Anne, Jennifer, Wayne, and Jayne. Thanks for all of your help and smiling faces. To my hockey teams, BOSS and the Mother Puckers, you made my time at McGill very enjoyable.

Finally, I would like to thank my family for their support and to David Dahan—I couldn't ask for a better partner in life. Not only are you a terrific scientist, but you are also a wonderful person.

Financial support was provided by Fellowships from the Natural Sciences and Engineering Council of Canada; the Faculty of Medicine, McGill University; and the Department of Microbiology and Immunology (F.C. Harrison Fellowship).

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 4, inclusive) in the form of original papers. A provision in the guidelines concerning thesis preparation reads as follows:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g. appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is

made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilites of all the authors of the co-authored papers.

With regard to these conditions, I have included as Chapters of this thesis, three original papers which are all being submitted for publication. Chapters 2 through 4, inclusive, each contain an Abstract, Introduction, Materials and Methods, Results, and Discussion section. Chapters 3 and 4 contain prefaces that serve as the connecting text to bridge the manuscripts. A Literature Review (Chapter 1) and a Summary (Chapter 5) 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.

Manuscripts and Authorship

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

1. Salmon, K.A., O. Freedman, B.W. Ritchings, and M.S. DuBow. 1999. Characterization of the lysogenic repressor *(c)* gene of the *Pseudomonas aeruginosa* transposable bacteriophage D3112. To be submitted to Virology.

2. Salmon, K.A., and M.S. DuBow. 1999. Identification and characterization of a phage Mu/D108 Ner homologue in the *Pseudomonas aeruginosa* transposable bacteriophage D3112. To be submitted to the Journal of Bacteriology.

3. Salmon, K.A., and M.S. DuBow. 1999. Identification of the early promoter, P*e*, of the *Pseudomonas aeruginosa* Mu-like transposable bacteriophage D3112. To be submitted to FEMS Micro. Letters.

As has been attested by the supervisor (Dr. M.S. DuBow), I was responsible for all the research described in Chapters 2 to 4 inclusively, with the following exception:

1. In Chapter 2, Orit Freedman and I contributed equally to the cloning and sequencing of the wild type D3112 repressor gene and to the hydroxylamine mutagenesis study involving the D3112 repressor. B.W. Ritchings served as a technical consultant to the study.

Contributions to Original Knowledge

1. Using site-directed mutagenesis, along with an *in vivo* immunity assay, I provided evidence for the start codon for the D3112 repressor.
2. Along with Ms. Orit Freedman (an honour's student), we identified that there is a single amino acid mutation between the wild type D3112 repressor and the temperature sensitive mutant (G6D).
3. Along with Ms. Orit Freedman, we used secondary structure predictions and chemical mutagenesis to demonstrate residues crucial to the function of the D3112 repressor, including a prediction of the helix-turn-helix DNA-binding motif in the N-terminus of the protein.
4. Using Northern blotting analysis, I demonstrated the monocistronic transcription of D3112 *c*.
5. I identified the promoter, P*c*, of D3112 using both *lacZ* transcriptional fusions and primer extension analysis.
6. I have overexpressed and purified D3112 cts-6HIS and Δcts-6His.
7. Using band retardation assays, I have demonstrated that D3112cts-6HIS binds specifically to a 261bp *Pvu*II fragment located directly upstream of the *c* gene.
8. Using β-galactosidase assays of the P*c* promoter, I have located a previously unidentified Mu/D108 *ner* homologue in D3112.

9. Using β-galactosidase assays, I have demonstrated that D3112 Ner inhibits transcription from P*c in vivo*.

10. I have overexpressed and purified D3112 Ner.

11. Using band retardation analyses, I have demonstrated that D3112 Ner binds specifically to a 261bp *Pvu*II fragment located within the intergenic region (between the D3112 *c* and *ner* genes).

12. I have identified a novel promoter, P*ner*, using both *lacZ* transcriptional fusions and primer extension analyses.

13. Using β-galactosidase assays, I have demonstrated that P*ner* is repressed by D3112 c.

14. By Northern blotting analysis, I have demonstrated that the *A* and *B* genes of D3112 are not transcribed with D3112 *ner*.

15. By Northern blotting analysis, I have demonstrated that the *cip* locus is transcribed only during the lytic cycle with D3112 *ner* from P*ner*.

16. Using β-galactosidase assays, I have identified the early promoter, P*e*, directly upstream of the *A* gene.

17. By Northern blotting analysis, I have demonstrated that the *A* and *B* genes of D3112 are transcribed together as a single transcript from P*e*.

18. I have developed a model for the lytic-lysogenic switch in D3112.

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List of Abbreviations

A	transposase of phage Mu or D108 or *A* gene product of phage D3112
Ap	ampicillin
bp	base pair(s)
CT DNA	calf thymus DNA
dNTP	deoxyribonucleoside triphosphate
ds	double stranded
DTT	dithiothreitol
HA	hydroxylamine
His6	6 histidine tag
IHF	integration host factor
IPTG	isopropyl-β-D-thiogalactopyranoside
kb(p)	kilobase(s), kilobase pair(s)
kDa	kilodalton(s)
Km	kanamycin
min	minutes
MW	molecular weight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming units
pip	piperacillin
PMSF	phenyl-methylsulfonyl fluoride
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
Spc	spectinomycin
ss	single stranded
Str	streptomycin
Tc	tetracycline
Tn	transposon
ts	temperature sensitive
U	units

Chapter 1

Introduction

Temperate Phages: Evolution of the Lytic-Lysogenic Switch

"Anything that is produced by evolution is bound to be a bit of a mess."

Sydney Brenner
(cited in Casjens *et al.*, 1992)

1.1 Evolution of Bacteriophages

Over 4500 different bacteriophages (or phages) have been described in the scientific literature (Ackermann and DuBow, 1987; Coetzee, 1987), and there have been many attempts to understand the evolution of viruses in general (Koonin, 1992; Gibbs *et al.*, 1993; Morse, 1993; Gibbs, 1994). Nearly 20 years ago, however, virologists realized that certain principals regarding the evolution of animal viruses may not apply to bacteriophages (Casjens *et al.*, 1992; Brüssow *et al.*, 1998). For example, phylogenetic trees based on morphology and/or nucleic acid homology are not easily constructed for phages (Campbell, 1994a; Brüssow *et al.*, 1998; Ackermann, 1999; Pecenkova and Paces, 1999), and no clear linear descent based on the gradual accumulation of point mutations has been established (Desiere *et al.*, 1998). Application of traditional methods for reconstructing the mechanisms of phage origin and evolution is complicated by several obstacles. First, phages do not have typical markers of evolution such as rRNA, and second, their genomes are characterized by high plasticity and undergo extensive lateral gene transfer.

One hypothesis for the origin of phages postulates that phages developed from cell components (i.e. from 'selfish' DNA) (Hartl *et al.*, 1984). Further evolution likely involved the acquisition of genes from plasmids, transposons, host chromosomes, or later from other phages. Within certain groups, conservation of gene order suggests descent from a common phage ancestor (Franklin, 1985; Eppler *et al.*, 1991; Hatfull *et al.*, 1994; Ford *et al.*, 1998a; Lucchini *et al.*, 1998; Hendrix *et al.*, 1999), and occasionally considerable similarity in genome organization is observed between "taxonomically" distant phages. For example, the genome organization of transposable coliphages Mu and D108 is very similar to that of the *Pseudomonas* phage D3112 (reviewed in DuBow, 1994), even though their hosts diverged over 140 million years ago (Ochman and Lawrence, 1996).

How a putative common phage ancestor evolved towards today's phages has also been debated. The construction of viable hybrids between phages λ and P22 supports a theory of modular evolution, which proposes that phages have evolved through the exchange of interchangeable genetic elements (or modules) that each carry out a particular function (Botstein and Herskowitz, 1974; Susskind and Botstein, 1978, Botstein, 1980). As modules are exchanged by recombination among phages belonging to the same interbreeding population, both highly similar and widely different phages can be produced. This theory has also been useful in our understanding of the evolution of many other phages such as T4 (Monod *et al.*, 1997) and has subsequently been accepted as a standard hypothesis for the evolution of all bacteriophages.

The mechanism by which modules are exchanged between phages is largely unknown (Casjens *et al.*, 1992; Monod *et al.*, 1997). It has been speculated that modular

exchanges might be mediated by homologous recombination using either highly conserved motifs within coding sequences or short regions of homology in regulatory elements (Casjens *et al.*, 1992). Site-specific recombination could also be involved in shuffling these modules, as might illegitimate recombination, which may result in the joining of two nonhomologous DNAs in a manner that does not disturb normal function.

Whether temperate phages evolved from virulent ones or vice versa is also a matter of debate. For example, studies of temperate and lytic *Streptococcus thermophilus* phages ϕSfi19 and ϕSfi21, DT1 and ϕO1205, and ϕSfi11 and ϕO1205 have shown a close genetic relationship, and similar findings were also found between *Lactococcus* phages ϕ31 and ϕr1t (Brüssow *et al.*, 1994b; Brüssow and Bruttin, 1995; Bruttin *et al.*, 1997b; Desiere *et al.*, 1998; Lucchini *et al.*, 1998; Walker *et al.*, 1998). Whether this phage immunity module, which is responsible for the lytic-lysogenic switch in these temperate phages, was recently acquired is unknown. It seems unlikely that the first phages would have adopted a system of lytic infection which would have resulted in the development of host cell lysis. Instead, it has been suggested that the first phages were pseudolysogens (Letarov, 1998) where replication takes place in a cell that continues to divide, but that release of phage particles either doesn't occur or happens without host cell lysis. This condition is believed to exist in natural environments, and has been observed under laboratory conditions using *Pseudomonas aeruginosa* and phages F116 and UT1, in which the starved host cell coexists with its viral genome in an unstable relationship for extended periods (Ripp and Miller, 1998). Examples of this life cycle can also be found in the filamentous phages (e.g. f1, fd, M13 of *E. coli* and VSK and CTXϕ of *Vibrio*

cholerae) which are continually extruded from their host without lysis (Model and Russel, 1988; Kar *et al.*, 1996; Neidhart *et al.*, 1996; Waldor *et al.*, 1997), and in some unusual archaephages (e.g. SIRV and TTVI) where viral particles are infective but unable to conduct a true lytic cycle under the majority of conditions studied (Reiter *et al.*, 1988; Zillig *et al.*, 1988). The addition of a lysogeny module (or repressor gene) to this ancestral phage would have permitted suppression of virus production under adverse conditions, and would have resulted in true lysogeny. Moreover, if this ancestral phage had been plasmid-based, the acquisition of a recombination system could have lead to the phage recombining into the host chromosome, as seen in contemporary prophages during lysogeny.

How could a lysogeny module evolve? The modular theory of bacteriophage evolution has been used extensively to show the possible evolution of entire phage genomes, but not of the individual modules themselves. The question of how these immunity modules or regions have developed or evolved is a complicated one: phages are believed to have evolved through a series of point mutations, intragenomic rearrangements, and recombination events (Casjens *et al.*, 1992; Ackermann *et al.*, 1995). This review will summarize the essential features of the lytic and lysogenic pathways which are common to a variety of temperate phages, and attempt to chart a possible evolutionary pathway of development of the genetic switch governing these life cycles. Beginning with the simplest scenario through more complex regulatory regimes, this review will use examples of phages from the Gram-negative bacteria, the Gram-positive bacteria, and the archae to offer a possible mechanism by which the lytic-lysogenic decision may have evolved.

1.2 Divergent Life Styles: The Lytic and Lysogenic Life Cycles

When a temperate phage infects a bacterium, there are typically two developmental pathways open to the phage: the lytic cycle, which culminates in the production of new progeny virus particles along with lysis of the cell; and the lysogenic cycle, in which the host cell survives with the lytic capacity of the virus turned off. The mechanisms by which temperate phages lysogenize and/or lyse their hosts have been the subject of extensive investigation over the last half a century. The choice between these two pathways and the eventual establishment of one of them depends on a complex network of negative and positive controls.

If the lytic cycle is initiated, the repressor protein is turned off and the early viral genes are expressed, leading to the production of progeny virus. This process varies among the different phages, however, following infection (or release from lysogeny) transcription, translation, genome replication and encapsidation, particle assembly and finally release of progeny phage occurs. Generally, the phage (whether temperate or virulent) accomplishes all of this by the sequential expression of viral genes such that the proteins for replication and recombination are synthesized first, and the proteins involved in head and tail structures and cell lysis are made at a later time. Therefore, one can define an early and a late stage of lytic development. The temperate enterobacterial phages Mu (Howe, 1987a, 1987b; Harshey, 1988), P1 (Yarmolinsky and Sternberg, 1988), P2 and P4 (Bertani and Six, 1988; Lindqvist *et al.*, 1993; Ziegelin and Lanka, 1995), P22 (Susskind and Botstein, 1978; Poteete, 1988), and λ (Enquist and Skalka,

1973; Furth and Wickner, 1983; Campbell, 1994b) have all had their lytic cycles well documented.

If the lysogenic cycle is chosen, three fundamentally different problems must be solved: establishment, maintenance and future induction. The establishment of lysogeny in an infected cell generally requires two functionally separate events: repression of the genes required for the lytic cycle as well as disguising of the viral DNA within the host cell. The placement of the phage genome varies and it is either integrated into the host's chromosome (such as with λ, Mu and most other temperate phages) or it is maintained extrachromosomally in plasmid form that replicates in synchrony with the host (such as with P1 [Ikeda and Tomizawa, 1968] and the *P. aeruginosa* general transducing phage F116 [Miller *et al.*, 1977]). In the case of integrated prophages, the establishment stage of the lysogenic pathway requires coordination between the expression of the genes required for integration and repression of the genes required for the lytic cycle. It is interesting to note that integration and plasmid maintenance lifestyles are also seen among the archaephages. In particular, *Halobacterium* ϕH has a plasmid stage, while ϕCh1 of *Natronobacterium* integrates into the host genome (Schnabel., 1984; Witte *et al.*, 1997). Since both lifestyles are seen in two different Kingdoms of life, it is not yet possible to say which lifestyle developed first (Ackermann, 1999). Interestingly, integrated prophages are more frequent than plasmid prophages, and many plasmid prophages can integrate their genomes within host DNA. For example, phage P1 can integrate within host DNA (Yarmolinsky and Sternberg, 1988) although the plasmid-mode of replication is more frequent. Alternatively, coliphage λ integrates at a specific

location and will only replicate as a plasmid, *λdv*, if some early genes (such as *N*) are eliminated (Enquist and Skalka, 1973; Kleckner and Signer, 1977).

The maintenance of lysogeny is inherently simpler than establishment. The repression of viral genes must be maintained and this is accomplished through the action of the c or cI repressor protein (Table 1.1). The repressor gene is normally referred to as the *c* or *cI* gene because mutations within this gene cause the phage's normally turbid phage plaques (due to growth of lysogenic bacteria) to become *c*lear. During lysogeny, the repressor protein generally acts to cause complete turnoff of all genes required for lytic functions. Since most temperate phages employ a single phage-encoded protein to activate the later stages of lytic development (e.g. protein C of phage Mu, or Ogr of phage P22), maintenance of repression requires a turnoff of only those genes active during the earliest stages of the lytic cycle.

Once established, the lysogenic state is quite stable. However, many temperate phages have evolved the option to undergo induction through environmental signals such as UV light, mitomycin C, or thymine deprivation. This ability for induction, often a result of induction of SOS functions, including the RecA protein (reviewed in Altschuler *et al.*, 1993; Ogawa and Ogawa, 1990), also enables the prophage to avert destruction when the host chromosome, of which it is usually a part, loses the capacity to replicate. How certain temperate phages evolved UV-inducibility is not understood, however, it was likely a recent acquisition. Induction from the lysogenic state requires two functionally separate events: release of repression and, in the case of integrated prophages, excision of the viral DNA from the host DNA. The lytic development that

Table 1.1. List of characterized temperate phages, their hosts and their putative or known repressor proteins.

Host	Phage	Repressors*	Reference or Review
Eubacteria			
Bacillus	ϕ105	c_{105}, Orf3	Van Kaer *et al.*, 1987
	SPβ	c/d	Zahler, 1982; McLaughlin *et al.*, 1986
Escherichia	D108	c, Ner	DuBow, 1994
	434	cI, Cro	Ptashne, 1992
	HK022	c1, Cro	Oberto *et al.*, 1989
	λ	cI, Cro	Ptashne, 1992
	Mu	c, Ner	DuBow, 1994
	186	cI, Apl	Dodd and Egan, 1996; Shearwin and Egan, 1996
	P1	c1/c4, Coi	Heinrich *et al.*, 1995a, 1995b
	P2	c, Cox	Egan and Dodd, 1994
	P4	*cI*†	Campbell, 1996
	P7	c1/c4, Coi	Heinrich *et al.*, 1995a, 1995b
	ϕ80	cI, gp30	Rybchin, 1984
Haemophilus	HP1	cI, Cox	Esposito *et al.*, 1996
Lactobacillus	ϕadh	Rad, Tec	Engel *et al.*, 1998
	ϕg1e	Cpg, Cng	Kodaira *et al.*, 1997
Lactococcus	BK5-T	cI, (Orf63)	Boyce *et al.*, 1995a, 1995b
	r1t	Rro, Tec	Nauta *et al.*, 1996
	TP901-1	gp4, gp5	Madson and Hammer, 1998
Mycobacterium	L1	cI	Chaudhuri *et al.*, 1993
	L5	gp71	Hatfull and Sarkis, 1993
Myxococcus	Mx8	Imm	Salmi *et al.*, 1998
Pseudomonas	D3	cI, Cro	Farinha *et al.*, 1994
	D3112	c, Ner	Autexier *et al.*, 1991 Salmon and DuBow, submitted (Chapter 3)
	ϕCTX	unknown	Nakayama *et al.*, 1999
Salmonella	P22	c2, Cro, Ant	Poteete, 1994

Table 1.1. Con't.

Host	Phage	Repressors	Reference or Review
Staphylococcus	ϕPVL	Orf31, Orf34	Kaneko *et al.*, 1998
Streptococcus	ϕO1205	gp4	Stanley *et al.*, 1997
	ϕSfi21	cI, Cro, Ant	Bruttin *et al.*, 1997a
	ϕTP-J34	cI, Cro, Ant	Neve *et al.*, 1998
Streptomyces	ϕC31	c_{54}/c_{42}, Orf12	Smith and Owen, 1991 Wilson *et al.*, 1995
Vibrio	K139	cI, Cox	Nesper *et al.*, 1999
Archaea			
Halobacterium	ϕH	rep/T_{ant}‡	Ken and Hackett, 1991; Stolt and Zillig, 1992
Natronobacterium	ϕCh1	unknown	Witte *et al.*, 1997

*Listed in order for lysogenic then lytic repressor proteins, followed by accessory repressors (e.g. antirepressors), where known.

†The *cI* product in P4 is not a protein repressor but rather a heterogeneous collection of short RNA transcripts of cI DNA.

‡T_{ant} of ϕH is an antisense RNA that also contributes to immunity by this phage.

ensues is identical to that found after infection, except (in some cases) for the additional requirement for excision of the viral DNA. Not all temperate phages have the capacity for induction by specific agents, or the correct conditions for prophage induction have not, as yet, been determined. These include phages such as P2 (Bertani, 1968) and Mu (Howe and Bade, 1975; Kupelian and DuBow, 1986; DuBow and Shinder, 1983).

The choice between the lytic cycle and the lysogenic cycle, as well as whether to maintain lysogeny or undergo induction is complicated, yet is a crucial step in the phage life cycle. High lysogeny rates and low induction rates are presumably advantageous in environments where there are few susceptible cells. If there is a preponderance of susceptible cells to infect, however, low lysogeny rates and higher induction rates may be favoured. Some factors that influence the decision are known. For example, for coliphage λ, infection of a host by only one phage particle (i.e. low multiplicity of infection, m.o.i.) or a high rate of bacterial growth favours the lytic cycle; on the other hand, infection of a single host cell by many phages (i.e. high m.o.i.), or poor growth conditions result in a higher efficiency of lysogenization (Kourilsky, 1973). In P1, the probability of lysogeny greatly increases if the host cells are grown at low temperatures (Bertani and Nice, 1954), as seen in coliphage λ (Gabig *et al.*, 1998). How these factors directly affect the choice between the lytic and lysogenic life cycles is generally unknown. Thus lysogeny and induction rates may depend upon a number of factors including the concentration of resources, the density of susceptible cells and the way these factors vary over time (Mittler, 1996).

1.3 Temperate Phages

Temperate phages have been identified that infect most of the major groups of Eubacteria and Archaea (Table 1.1). These phages are found wherever their bacterial hosts exist, although only a small number have been investigated in detail (Bergh *et al.*, 1989; Ackermann, 1996). As a benefit, lysogeny is thought to confer its greatest advantage by offering protection from infection by homoimmune temperate phage and some lytic phages (Stewart and Levin, 1984). For example, *Salmonella* ε-prophages (e.g. ε-15) are able to alter the antigenic specificity of the host lipopolysaccharide and therefore confer resistance to infection by other phages (Uetake *et al.* 1958). In the case of *Bacillus subtilis*, the *mpi* gene in the SPβ prophage renders cells immune to infection by the unrelated virulent phage ϕ1*m* (Rettenmeier *et al.*, 1979). These effects are similar to that observed by the *rexAB* genes of coliphage λ, which protect lysogens of this phage from T4rII mutants (Engelberg-Kulka *et al.*, 1998; reviewed in Court and Oppenheim, 1983; Snyder and Kaufman, 1994; Parma *et al.*, 1992), and the *sieA, sieB* and *a1* genes of the *Salmonella* phage P22. In P22 lysogens, SieA interferes with DNA injection by P22 and related phages (Susskind *et al.*, 1974a; Taneja and Chakravorty, 1978; Hofer *et al.*, 1995); SieB causes the lytic cycle of other *Salmonella* phages (not P22) to abort at an early stage (Susskind *et al.*, 1974b); and A1 alters the structure of the lysogen's O-antigen thereby interfering with adsorption by P22 and related phages (Yamamoto, 1978).

In addition to providing protection to their host bacteria, some prophages produce toxins that are harmful or lethal to humans (e.g. diphtheria toxin by phage β of *Corynebacterium diphtheria*) and therefore contribute directly to the pathogenesis (and

thus the spread) of the bacteria they inhabit. Other prophages indirectly affect bacterial pathogenesis by altering their hosts. For example, the *bor* gene of coliphage λ is expressed during lysogeny and promotes bacterial resistance to serum complement killing (Barondess and Beckwith, 1990, 1995).

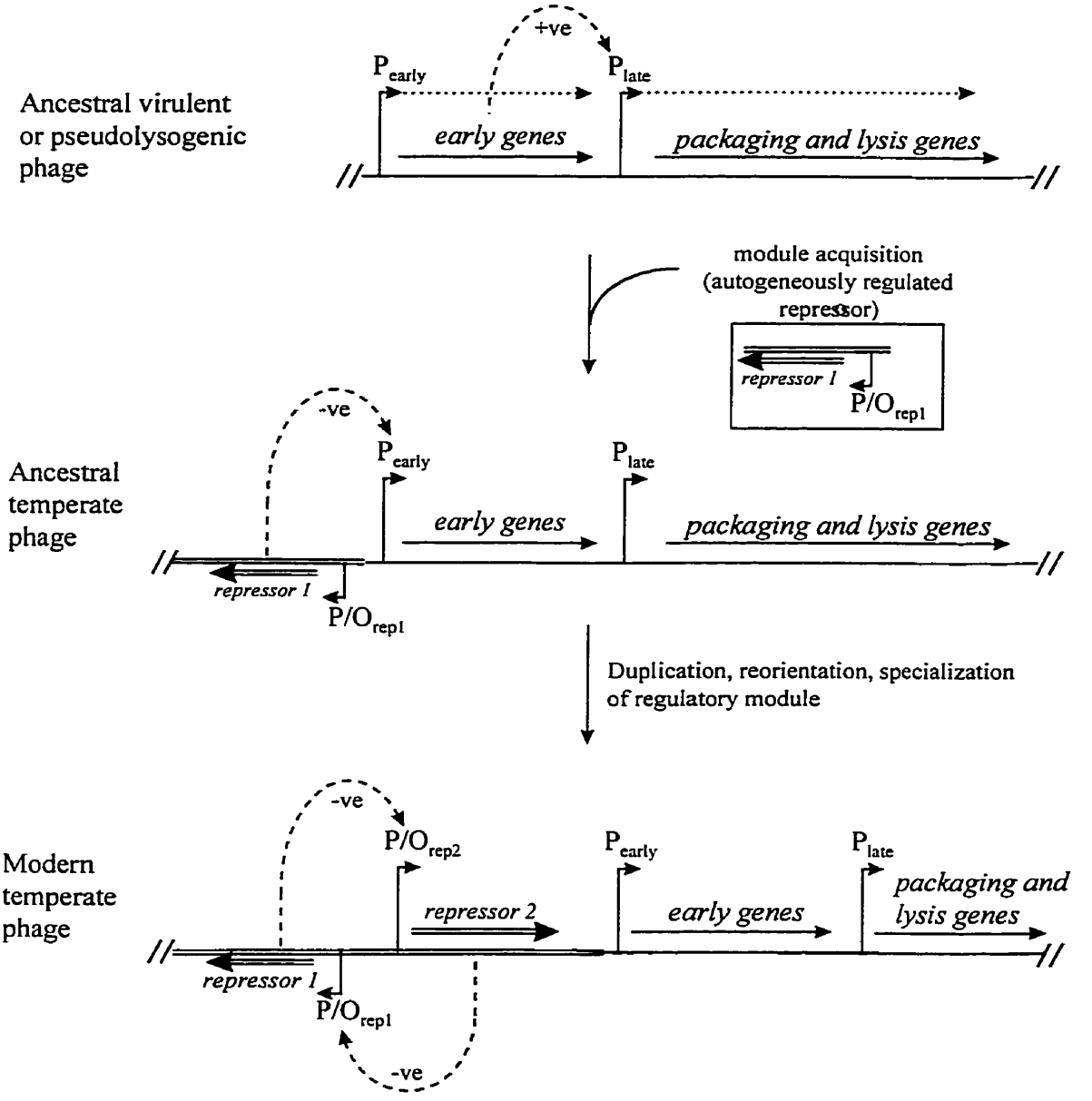
1.4 Immunity Regions and the Genetic Switch

In all temperate phages, the lysogenic state is determined by the activity of the phage immunity or regulatory region, in addition to various host factors such as integration host factor (IHF), H-NS, HflA and HflB. Temperate phages usually contain one (e.g. λ and its relatives, Mu) or two (e.g. P22) immunity regions which encode a repressor and an antirepressor, respectively. P1, and its close relative P7, are exceptional in that they contain a tertiary immunity region, *imm*T, in addition to these functions.

1.4.1 The Simple Switch

If we postulate that the first temperate phage evolved from an ancestral pseudolysogenic phage, the first step may have been the addition of a simple control segment to the phage genome (Figure 1.1). In this simple temperate phage, a repressor (acting alone) would function in the complete switching off of the lytic cycle and thus establishment of true lysogeny. Modern temperate phages are able to alternate lytic infection with lysogenization, whereas this type of presumptive ancestor could only lysogenize its host. Viral particles would only be formed when environmental conditions led to inactivation

Figure 1.1. A model for the evolution of the lytic-lysogenic switch. In this model, a putative ancestral virulent or pseudolysogenic phage may have acquired a single repressor gene (or control segment) in its genome. Expression of this repressor protein would have led to the inhibition of the expression of the lytic genes, thereby maintaining the lysogenic state, and giving rise to an ancestral temperate phage. Lytic gene expression, therefore, would have only occurred due to inactivation of the repressor protein. Over the course of evolution, gene duplication of the control segment may have taken place. This duplicated control segment may have also inverted and then specialized, giving rise to the divergent repressor system seen in many modern temperate phages. P/O refers to the promoter/operator of the putative repressor gene(s); the terms +ve and -ve refer to positive and negative regulation of the corresponding promoter.


Ancestral virulent or pseudolysogenic phage
+ve
P early
P late
early genes
packaging and lysis genes
module acquisition (autogeneously regulated repressor)
repressor 1
P/O repl
Ancestral temperate phage
-ve
P early
P late
early genes
packaging and lysis genes
repressor 1
P/O repl
Duplication, reorientation, specialization of regulatory module
Modern temperate phage
-ve
P/O rep2
P early
P late
repressor 2
early genes
packaging and lysis genes
repressor 1
P/O repl
-ve

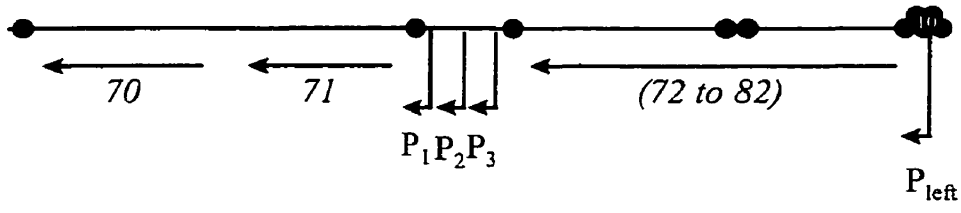
of the repressor protein and lysogen induction. To date, only mycobacteriophage L5 fits this simple model. Mycobacteriophage L5 was originally isolated in 1960 from a lysogenic strain of *Mycobacterium smegmatis* (Doke, 1960). L5 forms stable lysogens in *M. smegmatis,* but requires special conditions (i.e. high Ca^{2+} conditions) in order to infect *M. tuberculosis* or bacille Camlette-Guérin (BCG) (reviewed in Snapper *et al.*, 1988; Hatfull, 1994; Fullner and Hatfull, 1997). *M. smegmatis* L5 lysogens contain a single L5 prophage integrated into a chromosomal *attB* site (Lee *et al.*, 1991) and are immune to superinfection.

The genome of L5 was completely sequenced in 1993 (Hatfull and Sarkis, 1993), and the immunity region proposed (Figure 1.2). Interestingly, the immunity region appears to be quite simple in that all of the early lytic genes are transcribed leftwards from a single promoter, P_{left}, including the putative lysogenic repressor transcribed from gene *71*.

Gene *71* of L5 produces a protein of 183 amino acids (gp71) which contains a putative H-T-H DNA-binding motif in the N-terminus, and appears to have an analogous function to the *c1* repressor gene of coliphage λ (Donnelly-Wu *et al.*, 1993). Phages with mutations in gene *71* that have a temperature-sensitive clear-plaque phenotype are also thermoinducible, illustrating that gp71 is required for maintenance of lysogeny. Gene *71* is also able to confer immunity to superinfection, not only to L5 but also to the related virulent phage D29. Further analysis of a number of L5 mutants have also suggested that gene *71* is likely the only gene required for lysogeny (Donnelly-Wu *et al.*, 1993).

Figure 1.2. Genetic organization of the L5 immunity region (not to scale). The positions of the L5 genes are shown as arrows. Potential gp71 repressor binding sites are shown by ●, including the 10 sites surrounding P_{left} (adapted from Brown *et al.*, 1997).

L5



There are three promoters (P_1, P_2, and P_3) located immediately upstream of gene *71*, all of which are active in L5 lysogeny. However, the early lytic promoter, P_{left}, may be responsible for initiating all early transcription (Nesbit *et al.*, 1995). Although L5 appears quite similar to our ancestral temperate phage model, its repressor, gp71, binds to at least 24 asymmetric DNA-binding sites throughout the L5 genome, called 'stopoperator' sites (Brown *et al.*, 1997). Binding of gp71 to these sites is believed to play a critical role in inhibiting downstream gene expression, from P_{left} as well as other lytic promoters, thereby ensuring that the prophage remains transcriptionally silent.

A model has been proposed for the regulation of gene *71* synthesis and control of the lytic-lysogenic decision (Nesbit *et al.*, 1995). When L5 infects an *M. smegmatis* cell, transcription from P_{left}, P_1, P_2, and P_3 result in the immediate synthesis of gp71. The newly synthesized gp71 is then subject to degradation or modification by a host-encoded enzyme(s) which may be inhibited by a putative L5 protein encoded by a gene in the *72→82* region (Donnelly-Wu *et al.*, 1993; Sarkis *et al.*, 1995). Stabilization of gp71 protein thereby allows for an increase in the concentration of gp71, which then binds to P_{left} and represses further transcription. Repression at P_{left} would therefore contribute directly to the formation of the lysogenic state. To maintain lysogeny, the $P_1 \rightarrow P_3$ promoters would continue expression of gp71, which would then continue to repress P_{left} as well as the other lytic promoters located throughout the phage genome.

Another temperate mycobacteriophage, L1, has also been described, however, only a putative cI-like repressor has so far been observed in the immunity region (Chaudhuri *et al.*, 1993).

1.4.2 The Simple Switch Revisited

From this primordial one-repressor immunity region, a different type of immunity region may have arisen through duplication of the control segment, followed by inversion and specialization (Figure 1.1). The only phages studied thus far that show any resemblance to this stage of temperate phage development are members of the P2-family, and the transposable phages Mu and D108. In these phages, two different repressors act at nearby binding sites to turn off the expression of the other. For the transposable phages to have arisen, a transposon would have also had to insert within (or flank) this primordial temperate phage (likely related to Tn*3*; see Kamp, 1987) and given rise to the precursor of the transposable phage family.

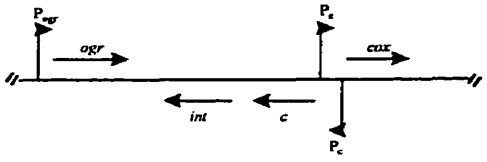
1.4.2.1 P2-family

Coliphage P2 was first isolated by Bertani in 1951. Numerous phages serologically related to P2 have been described and are widespread in nature, however, only phages P2 and 186 have been extensively studied (reviewed in Bertani and Six, 1988; Egan and Dodd, 1994). Although 186 is a close relative of the non-inducible P2 family (Bertani, 1968), phage 186, like coliphage λ, is UV-inducible (Woods and Egan, 1974), and as will be shown later, appears to have a more complex genetic switch.

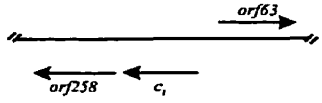
The immunity region of P2 is shown in Figure 1.3. The lytic-lysogenic switch is made up of both a transcriptional switch (which determines whether transcription will lead to lytic or lysogenic growth), and a recombinational switch (which determines whether the phage chromosome exists integrated into the chromosome or excised from it)

Figure 1.3. Immunity regions of the P2-family of phages (not to scale). Shown are the maps of coliphage P2 (adapted from Egan and Dodd, 1994); *Bacillus subtilis* phage ϕ105 (adapted from Van Kaer *et al.*, 1989); *Haemophilus influenzae* phage HP1 (adapted from Esposito *et al.*, 1997); *Lactobacillus* phages ϕadh (adapted from Engel *et al.*, 1998), ϕg1e (adapted from Kodaira *et al.*, 1997), and A2 (adapted from García *et al.*, 1999); *Lactococcus* phages BK5-T (adapted from Boyce *et al.*, 1995a; 1995b), r1t (adapted from van Sinderen *et al.*, 1996), and TP901-1 (adapted from Madsen and Hammer, 1998); *Streptococcus thermophilus* phages ϕO1205 (adapted from Stanley *et al.*, 1997); ϕSfi21 (adapted from Bruttin *et al.*, 1997b), and ϕTP-J34 (adapted from Neve *et al.*, 1998); as well as the *Vibrio cholerae* phage K139 (adapted from Nesper *et al.*, 1999).

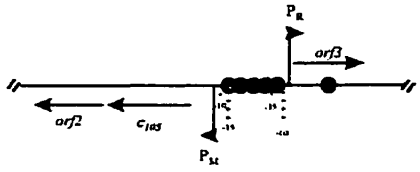
P2



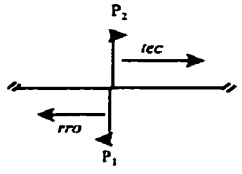
BK5-T



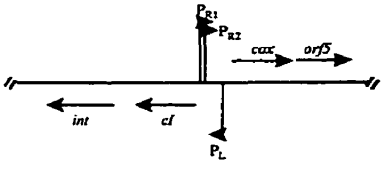
ϕ105



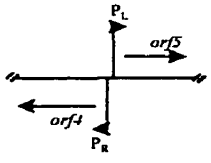
r1t



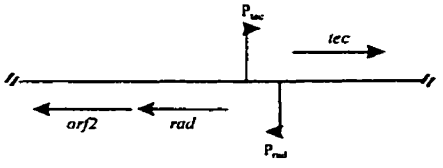
HP1



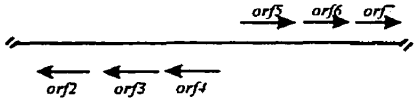
TP901-1



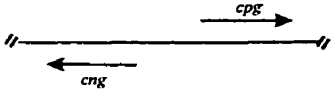
ϕadh



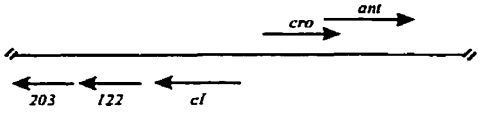
ϕO1205



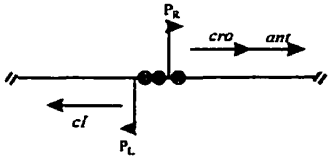
ϕg1e



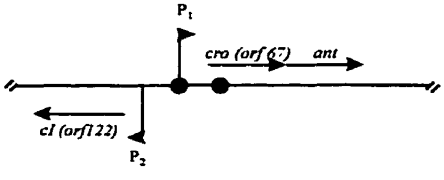
ϕSfi21



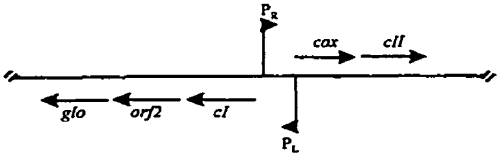
A2



ϕTP-J34



K139



(reviewed in Egan and Dodd, 1994). The transcriptional switch (Geisselsoder *et al.*, 1973) is comprised of *C*-P_e-P_c-*cox*. P_e is the promoter for lytic transcription and is repressed, during lysogeny, by the C repressor (Geisselsoder *et al.*, 1973). During the lytic cycle, the promoter P_c is repressed by Cox.

Whether infection by phage P2 results in lysogenization of the host or lytic growth of the phage depends upon a competition between transcription from the repressor promoter P_C, and the early promoter, P_e. Transcription from these promoters is mutually exclusive, since the P_C repressor Cox is formed from the P_e transcript and the P_e repressor C from the P_C transcript, and the two transcripts overlap by about 30 nucleotides. For the establishment of lysogeny, both C and *E. coli* IHF are required; in IHF mutants, transcription from P_C is inhibited (Saha *et al.*, 1990).

Of the *Bacillus* temperate phages, ϕ105 is the best characterized and its immunity region resembles that of P2, except that the promoters (P_M and P_R) are not face-to-face (Figure 1.3). In ϕ105, early gene expression is controlled, at least in part, by specific interaction of a repressor (c_{105}; Dhaese *et al.*, 1985a, 1985b; Cully and Garro, 1985) with six 14bp sequences (O_{R1}→O_{R6}; Van Kaer *et al.*, 1987, 1989) that are organized as direct repeats and together (except O_{R3}) constitute the ϕ105 (rightward) operator. Binding of the c_{105} repressor to these sites stimulates transcription from P_M (the promoter for its own gene), while at the same time repressing P_R (the early lytic promoter).

The ϕ105 repressor-operator system presents some interesting and unusual aspects. Two of the operators (O_{R1} and O_{R2}) are located close together in the non-transcribed region between the divergent P_M and P_R promoters, but do not overlap with

the -35 or -10 RNA polymerase recognition sites. A third operator, O_{R3}, is located within the coding region of the first gene transcribed from P_R. O_{R4} overlaps with the -35 of P_M (Van Kaer *et al.*, 1989), and therefore, the organization of O_{R4}, O_{R1} and O_{R2} appears to be similar to the O_{R1}, O_{R2}, O_{R3} operators of coliphage λ (see Section 1.4.3) (Ptashne *et al.*, 1992). Although not yet fully characterized, a comparison with λ and its operators (see Section 1.4.3) would suggest that occupation of O_{R1} (and to a lesser extent O_{R2}) by c_{105} would induce favourable interactions with RNA polymerase (Meyer and Ptashne, 1980; Hochschild *et al.*, 1983), and thus stimulate P_M activity. The resulting increase in c_{105} concentration might then lead to binding at O_{R4} which would then block further P_M transcription by the RNA polymerase leading to autoregulation by the repressor.

Repression of transcription at P_R is likely a result of c_{105} binding to the O_{R5}, O_{R6} and O_{R3} sites. Repressor binding at O_{R6} competes with binding of RNA polymerase to P_R, thus negatively regulating this promoter (Van Kaer *et al.*, 1989). Any residual transcription would be further blocked by the repressor occupying the O_{R3} site.

Whether or not *orf3* plays a role in the control of lysogeny is unclear. The *orf3* gene encodes a putative protein of 89 amino acids and may be the functional equivalent of P2 Cox. A fragment containing *orf3* and introduced into *B. subtilis* on a multi-copy plasmid was able to confer immunity to ϕ105 superinfection and an HTH motif was identified within the N-terminus of the ORF (Van Kaer *et al.*, 1987). Further studies are required in order to elucidate the role of *orf3* in the lytic-lysogenic switch of ϕ105, including binding studies to determine whether or not this putative protein is able to turn off expression of c_{105} by binding to operator sites O_{R4} and possibly O_{R1}.

The *Haemophilus influenzae* temperate phage HP1 is related to the P2 family of phages (Esposito *et al.,* 1996), and was the first phage identified for this host (Harm and Rupert, 1963). The complete nucleotide sequence of HP1 was completed in 1996 (Esposito *et al.,* 1996) and the genetic switch defined (Esposito *et al.,* 1997) (Figure 1.3). During the lytic cycle, the HP1 Cox protein represses transcription from the P_L transcript. However, during lysogeny, the HP1 cI protein represses lytic transcription from the P_R promoters. Operators have also been defined for both Cox and cI within the early promoter region. In addition, the Orf5 protein shows similarity to the 186 cII protein. However, it is unclear what role this protein might play in the lytic-lysogenic decision of HP1.

Among the *Lactobacillus* phages, only three temperate phages have been well characterized (ϕadh, ϕg1e, and A2), and all show a similarity in their immunity regions to P2 (Kodaira *et al.,* 1997; Engel *et al.,* 1998; Garcia *et al.,* 1997; 1999) (Figure 1.3): the immunity regions all appear to consist of two divergent repressors which (likely) act at nearby operator sites to turn off the expression of the other.

The immunity region of ϕadh was determined in 1998 (Engel *et al.*, 1998) (Figure 1.3). An ORF, referred to as *rad*, was found to code for a protein which was able to repress transcription from the two divergent facing promoters, P_{tec} and P_{rad}. An interesting observation, however, was that overexpression of *orf2* with *rad* was able to alleviate the repressing effect of Rad on P_{rad} (Engel *et al.,* 1998). It is unclear what role *orf2* plays in the lytic-lysogenic decision. The protein predicted to be encoded by the *tec* (*t*echnically *e*quivalent to *c*ro) gene shows no homology or similarity with any of the Cro-like proteins

of other phages and its function is not known at this time. However, this gene likely encodes a protein required for the lytic cycle.

The complete sequence of *Lactobacillus* phage ϕg1e was determined in 1997 (Kodaira *et al.*, 1997). Although, at this time, only sequence data is available, it appears that ϕg1e uses a simple switch, like P2, to control its lytic-lysogenic decision. The immunity region (Figure 1.3) consists of two divergent repressor-like genes, *cng* and *cpg*, although their actual functions have not yet been determined (Kodaira *et al.*, 1997). The N-terminal domain of Cpg resembles that of the λ repressor family, and contains a potential H-T-H motif. The N-terminal domain of Cng resembles that of Cpg (38% identity) and also contains a potential H-T-H motif. In addition, Cng resembles the λ Cro repressor with 33% overall identity. The location of the putative P_{cng} and P_{cpg} promoters will be important in our understanding of this lytic-lysogenic decision in this phage.

The immunity region of the *Lactobacillus* phage A2 also resembles that of P2 (Figure 1.3) (Ladero *et al.*, 1998; García *et al.*, 1999). Characterization of the *cI* gene demonstrated that: (i) it encodes a 225 amino acid protein with DNA-binding and RecA cleavage motifs; (ii) it is expressed in lysogenic cultures; and (iii) it confers superinfection immunity to its host (Ladero *et al.*, 1998). Adjacent, but divergently transcribed, an ORF homologous to λ Cro was also identified. Purification of the cI protein demonstrated that it interacts specifically with three operators ($O_1 \rightarrow O_3$) located upstream of P_L. When cI bound to O_{R1} and O_{R2}, it was found to inhibit transcription at P_R, and to increase transcription at P_L (García *et al.*, 1999). Binding at O_{R3} results in

repression of P_L, and thus suggests that A2 cI autoregulates itself. The role of the Cro and Ant homologues have not yet been determined.

Of the lactococcal temperate phages, r1t, BK5-T, TP901-1, and Tuc2009 have had their immunity regions studied in some detail (van de Guchte *et al.*, 1994; Boyce *et al.*, 1995a, 1995b; van Sinderen *et al.*, 1996; Madsen and Hammer, 1998) (Figure 1.3) with phage r1t being completely sequenced (van Sinderen *et al.*, 1996). Although the genetic switch has not been characterized in any of these phages, the r1t immunity regions contains two divergently oriented genes, *rro* encoding the phage repressor, and *tec*. Band retardation assays demonstrated that Rro binds to the intergenic region and that, *in vivo*, Rro represses transcription from the lytic promoter P_2 (Nauta *et al.*, 1996). It therefore appears that the r1t strategy to achieve lysogeny is similar to that used by coliphage P2. It still needs to be determined if the *tec* gene actually directs the phage into the lytic cycle and/or if it is the functional equivalent of Cro.

Similar genes encoding repressors have been identified in the other phages (*orf4* in TP901-1, *orf279* in BK5-T, and *cI* in Tuc2009). However, the individual repressors show very little homology with each other except for 73% overall identity between *orf279* of BK5-T and the putative repressor gene of Tuc2009 (Boyce *et al.*, 1995a; van de Guchte *et al.*, 1994), and a small region of similarity between TP901-1 *orf4* and r1t *rro* (Madsen and Hammer, 1998). A *cro* homologue has not yet been identified in BK5-T, however, several ORFs (including *orf63*) may fulfill this function.

There are many temperate phages that infect *Streptococcus thermophilus*, a Gram-positive lactic acid bacterium used as a starter in industrial milk fermentation (Mercenier,

1990; Carminati and Giraffa, 1992; Fayard *et al.*, 1993; reviewed in Brüssow *et al.*, 1998). The immunity regions of ϕSfi21, ϕTP-J34 and ϕO1205 are shown in Figure 1.3.

The lytic-lysogenic switch of ϕSfi21 appears to be similar to that of coliphage P2, consisting of two repressors (cI and Cro) divergently transcribed. The putative repressor protein of ϕSfi21, cI, shows very weak homology with the cI-like putative repressor of the lactococcal phage Tuc2009, and appears to have a H-T-H motif and a RecA cleavage site (Bruttin *et al.*, 1997a). A Cro-like protein (Orf75, Cro) has also been identified whose N-terminus shows homology to that of the 434 cI repressor. In addition, one ORF (*orf287, ant)*, showed significant homology to the antirepressor of phage P1, and may be involved in the lytic-lysogenic switch. If this is the case, the genetic switch of ϕSfi21, may be more complex than that of the P2-family, however, the role of Ant has not yet been determined. Orf203 may be involved in a phage resistance phenotype such as seen with the *rex* genes of coliphage λ.

The sequence of the immunity region of the *S. thermophilus* phage ϕTP-J34 has also been determined (Neve *et al.*, 1998), and shows similar gene organization to the immunity regions of the P2-family. Two divergent promoters, P_2 and P_1, appear to be responsible for the transcription of two repressor-like genes, *orf122* and *orf67*, although unlike P2, these two promoters are back-to-back. Orf122 (cI) showed significant similarity to a number of phage repressor proteins such as the *Salmonella* P22 c2 repressor, the *E. coli* 434 cI repressor, the *B. subtilis* phage PBSX repressor and the putative repressors from the *Lactobacillus* phages ϕg1e and Tuc2009. A second ORF, Orf67, was named Cro due to its homology with the cI and Cro repressors of coliphages

434 and ϕ80 and the *Lactobacillus* phage BK5-T, as well as its orientation with respect to Orf121 (cI). The intergenic region between the ϕTP-J34 *cI* and *cro* ORFs contains the two divergent putative promoter elements, as well as two putative operator sites: one overlapping the P_I promoter; and the other upstream of the putative antirepressor.

Phage ϕO1205 was completely sequenced in 1997 (Stanley *et al,* 1997). Only one gene, *orf4*, expected to be part of the immunity region has been identified. Orf4 exhibits limited similarity at the N-terminus of the coliphage HK022 cI repressor. Further characterization of the other ORFs in this region *(orf2, orf3, orf5→7)* may shed light on the lytic-lysogenic switch in this *Streptococcus* phage.

Recently, a temperate phage from *Vibrio cholerae*, K139, has been described whose immunity region resembles that of the P2-family (Reidl and Mekalanos, 1995; Nesper *et al.,* 1999) (Figure 1.3). Phage K139 contains divergent lytic and lysogenic operons that appear to be transcribed from face-to-face promoters P_L and P_R. In the transcriptional direction of the putative lytic promoter (P_R), homologues of the P2 *cox*, phage 186 *apl*, and phage 186 *cII* genes are encoded (see Section 1.4.3). There is also some amino acid similarity of the K139 Cox to that of the *H. influenzae* HP1 Cox. It is not yet known what role the *cII* ORF plays in the lytic-lysogenic decision.

During the lysogenic state, the *cI, orf2, glo*, and *int* genes are likely initiated from the P_L promoter. It has not yet been shown, however, if the *cI* gene encodes the lysogenic repressor. The *orf2* gene shows no significant homology to other proteins, however, Glo is believed to participate in phage exclusion.

The immunity regions of members of the P2-family therefore appear to represent a somewhat simple genetic switch. In each case (or will likely be shown), the lytic-lysogenic decision appears to be the result of expression from two divergent promoters of two repressor-like proteins that turns off the expression of the other. These promoters can be face-to-face (as seen in phages such as P2) or back-to-back (as seen in phages such as ϕ105). It is not known if this difference in promoter placement is significant in terms of evolution, however, the transposable phages also use this simple-type switch to control their lytic-lysogenic decision, and their divergent promoters are face-to-face.

1.4.2.2 Transposable Phages

To date, only three transposable phages have been isolated from *E. coli*: phages Mu (Taylor, 1963), D108 (Mise, 1971) and B278 (Fusté *et al.*, 1980). The best studied are Mu and D108 (reviewed in DuBow, 1994; Pato, 1989), and heteroduplex analysis has shown the two phages to be 90% homologous at the DNA level (Gill *et al.*, 1981). Most of the non-homologous regions between these two phages lie within the left-end immunity region (Mizuuchi *et al.*, 1986; Tolias and DuBow, 1985; Toussaint *et al.*, 1983), resulting in a lack of immunity to superinfection by the other phage (Hull *et al.*, 1978). Variation within the immunity region likely provides a selective advantage (Campbell and Botstein, 1983) and is easily achieved; only a few base pair changes are required to evolve a new repressor-operator combination (Wharton and Ptashne, 1985).

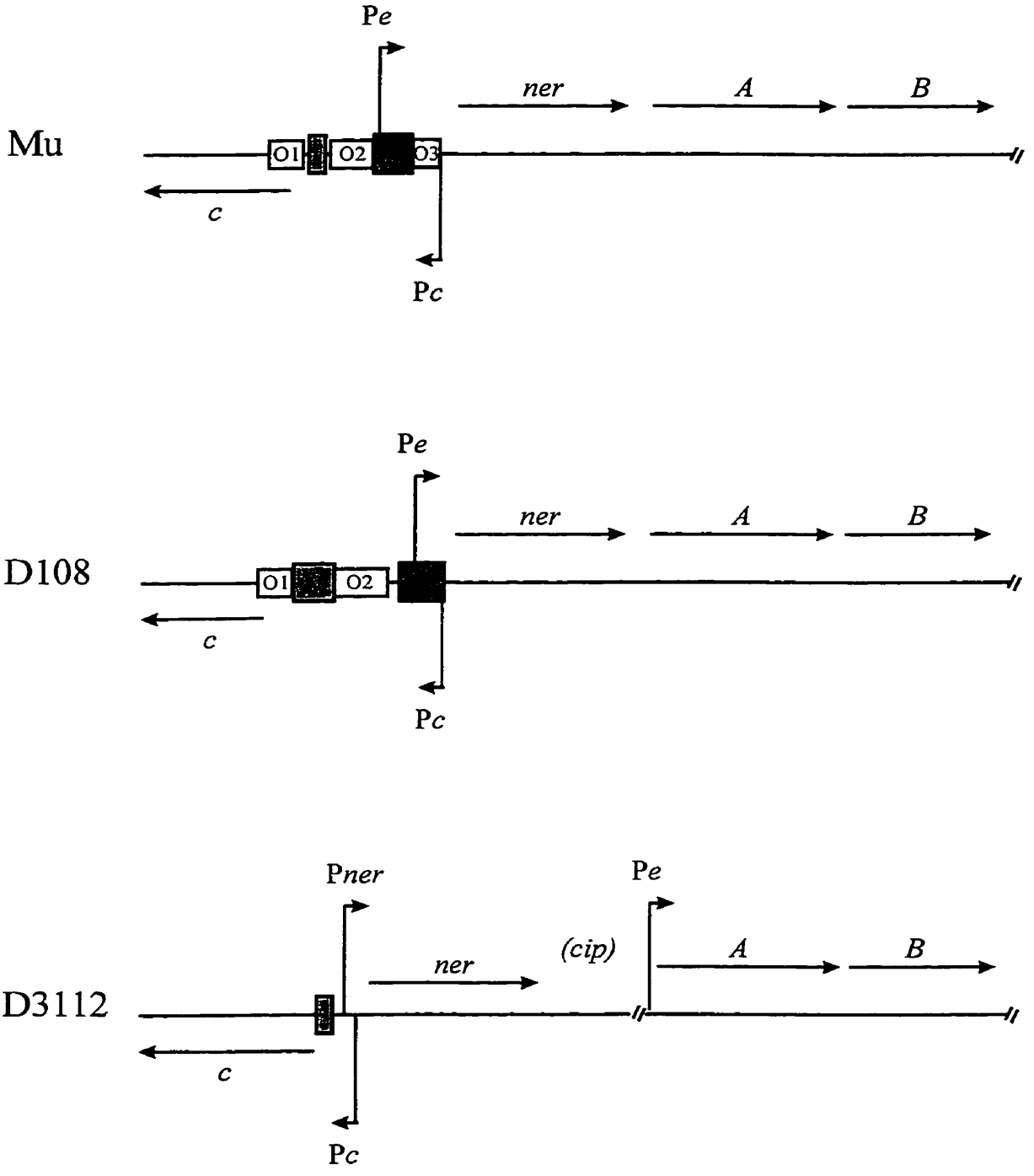
In contrast to *E. coli*, over 60 different transposable phages have been isolated from *Pseudomonas aeruginosa* (Akhverdyan *et al.*, 1984, 1985; Yanenko *et al.*, 1979). These phages have been divided into two groups according to their best studied members:

B3 and D3112. Based on DNA homology, the B3 group has been further subdivided into three subgroups: B3, PM681 and PM57 (Krylov *et al.*, 1986). The *Pseudomonas* transposable phages share many properties with coliphages Mu and D108 (Krylov *et al.*, 1980a, 1980b; Bogush *et al.*, 1981). They possess linear double stranded DNA genomes of about 37kbp, with approximately 2.5kbp of host DNA sequence at the right extremity of the phage genome. In addition, the left end of the B3 group (Krylov *et al.*, 1980b) and of D3112 (Autexier *et al.*, 1991) contain host DNA (50-150bp in B3, 30-33bp in D3112), as in Mu and D108 (54-150bp; George and Bukhari, 1981; Gill *et al.*, 1981).

Phage D3112, the subject of this thesis, is the best studied of the transposable *Pseudomonas* phages. Although there is no detectable DNA homology between D3112 and Mu (Rehmat and Shapiro, 1983), these phages share a similar life cycle, except that D3112 shows a smaller burst size of 10-50 phage particles per cell (Krylov *et al.*, 1980b). Like Mu, D3112 generates a 5bp duplication upon insertion that starts and ends with the sequence 5'-TG-3' (Darzins and Casadaban, 1989a; Autexier *et al.*, 1991). However, while Mu is not inducible by UV light (Howe and Bade, 1975), mitomycin C (DuBow and Shinder, 1983), or γ-rays (Kupelian and DuBow, 1986), the *Pseudomonas* phages are readily inducible by both UV light and mitomycin C (Krylov *et al.*, 1980b, 1982; Plotnikova *et al.*, 1982).

In the coliphages, the early genes, including *ner*, *A*, *B*, and the repressor *(c)* gene, are located in the leftmost 5kbp of the genome and encode functions involved in the regulation of integration, transcription and DNA transposition (reviewed in DuBow, 1994) (Figure 1.4). The gene organization of D3112 is similar to Mu and D108, with the genes involved in the regulation of transcription and transposition located at the left-end

Figure 1.4. Maps of the Mu, D108 and D3112 left end immunity regions (not to scale). The arrows represent the genes and their direction of transcription. The binding sites for the repressor (□), Ner (■), and IHF (■) proteins are shown in addition to the relative positions and direction of transcription for the P*e* and P*c* promoters (adapted from DuBow, 1994).


Mu
Pe
ner
A
B
O1
O2
O3
c
Pc
D108
Pe
ner
A
B
O1
O2
c
Pc
D3112
Pner
Pe
ner
(cip)
A
B
c
Pc

(Bidnenko *et al.*, 1989; Autexier *et al.*, 1991; Ulycznyj *et al.*, 1995). In D3112, a function called *cip* (*c*ontrol of *i*nteraction of *p*hages), has been located in the immunity region (Gerasimov *et al.*, 1985). Although referred to as the *ner*-like homologue, the *cip* function has not yet been fully characterized. It has been suggested that *cip* may confer B39 immunity to D3112 polylysogens (i.e. a host cell lysogenized by more than one D3112 prophage; Gerasimov *et al.*, 1985; Bidnenko *et al.*, 1996), however, conclusive evidence for this remains to be established. The *cip* region may have been either a recent addition to D3112 or was lost (or moved to another location) from Mu and D108 during evolution.

In the coliphages, transcription of the repressor proceeds from right to left from the promoter P*c* (Figure 1.4) (van Meeteren *et al.*, 1980; Mizuuchi *et al.*, 1986; Levin and DuBow, 1987). When expressed during lysogenic development, repressor (c) binds to each of its operator sites (three in Mu and two in D108) blocking transcription of the early genes, which are transcribed as a single polycistronic mRNA from left to right from the early promoter, P*e* (Bade, 1972; Wijffelman *et al.*, 1974a, 1974b). The repressor proteins of the two coliphages are nonhomologous and are incapable of binding to each other's operator sites or conferring immunity to superinfection by the other phage (reviewed in DuBow, 1994).

Lytic development is dependent on expression of the *ner* (*n*egative *e*arly *r*egulation) gene (Figure 1.4), which is the first gene transcribed from P*e*. In the early stages of lytic development, Ner inhibits repressor gene transcription from P*c* (van Leerdam *et al.*, 1982). In late stages of lytic development, Ner accumulates and inhibits its own transcription from P*e* (Wijffelman *et al.*, 1974a, 1974b). This genetic switch

results in both the immediate inhibition of repressor transcription and delayed negative regulation of early gene expression later in the lytic cycle (van Meeteren and van de Putte, 1980; van de Putte *et al.*, 1981). Late negative regulation of early gene expression is crucial in the controlled expression of the transposition functions (encoded by the *A* and *B* genes) late in lytic development (Pato and Reich, 1984; Baker *et al.*, 1991). The *A* gene encodes the transposase protein and *B* the transposase-associated protein, both of which participate in the integration of infecting phage DNA and its subsequent replicative transposition to other sites in the host chromosome (reviewed in Charconas *et al.*, 1996; Craigie, 1996; Lavoie and Charconas, 1996; Stellwagen and Craig, 1998). The Mu A protein is not catalytically turned over, and is used stoichiometrically in the transposition reaction (Pato and Reich, 1984), yet Mu A cannot be overexpressed since high concentrations lead to aberrant transposition products (Baker *et al.*, 1991). Thus, tight regulation of *A* gene expression is critical during lytic growth.

E. coli IHF is also required for lytic growth in Mu and D108 (Krause and Higgins, 1986). The P*e* promoter of Mu is specifically inhibited by the binding of H-NS to the promoter region (van Ulsen *et al.*, 1996). Binding by IHF at its consensus site (Goosen and van de Putte, 1984; Krause and Higgins, 1986; Van Rijn *et al.*, 1988) (Figure 1.4) interferes with the binding of H-NS to P*e* (van Ulsen *et al.*, 1996), and allows for the stoichiometric production of transposase required for transposition (Pato and Reich, 1982, 1984).

The transposable coliphage switch is therefore relatively simple: it consists of two antagonistic repressors vying to turn the other off. Binding by one of the two (c or Ner) occludes the transcription of the other. The lytic lysogenic switch in D3112 has not yet

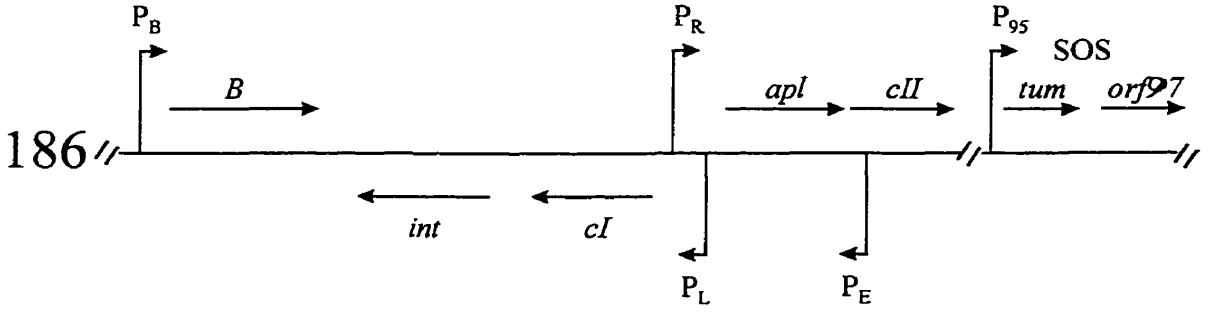
been fully characterized. With the recent identification of a *ner* homologue in this phage (Salmon and DuBow, submitted [Chapter 3]), the presence of the *cip* function, and the location of the *A* and *B* genes further upstream and expressed from their own promoter (Salmon and DuBow, submitted [Chapter 4]), it is likely that the switch in this *Pseudomonas* transposable phage will be different from that seen in the transposable coliphages.

Reports of transposable phages from bacterial species other than *E. coli* and *P. aeruginosa* have been rare. Nonetheless, phage VcA1, from *Vibrio cholera*, has been shown to be a Mu-like transposable phage (Johnson and Romig, 1981). Its genome is approximately 37kbp in length (like Mu and D108), however, very little is known of its genetics or mechanism of transposition. Like Mu and D108 (Taylor, 1963), phage VcA1 appears to randomly insert within the *V. cholera* chromosome (Johnson and Romig, 1981).

Potential Mu-like phages have also been reported in both *Haemophilus influenzae* and *Shigella dysenteriae*. In 1995, following the sequencing of the *H. influenzae* Rd genome, a cryptic Mu-like phage was identified in the chromosome (Fleischmann *et al.*, 1995). Homologous ORFs to the transposase *(A)*, transposase-associated *(B)*, and *ner* genes have been identified. In *Shigella dysenteriae* the site-specific recombinase *pinD* was proposed to be derived from a Mu-like prophage due to high sequence homology with Mu *gin* (Tominaga, 1997). Adjacent to *pinD*, an ORF homologous to *mom* of Mu was also identified (Tominaga, 1997).

From these "simple"-type switches, more complicated switches may have arisen. For example, from P2, phage 186 (Figure 1.5) may have evolved due to the insertion of a

Figure 1.5. Immunity region of coliphage 186 (adapted from Neufing *et al.*, 1996; and Shearwin *et al.*, 1998) (not to scale).


P_B
P_R
P_95
SOS
B
apl
cII
tum
orf97
186
int
cI
P_L
P_E

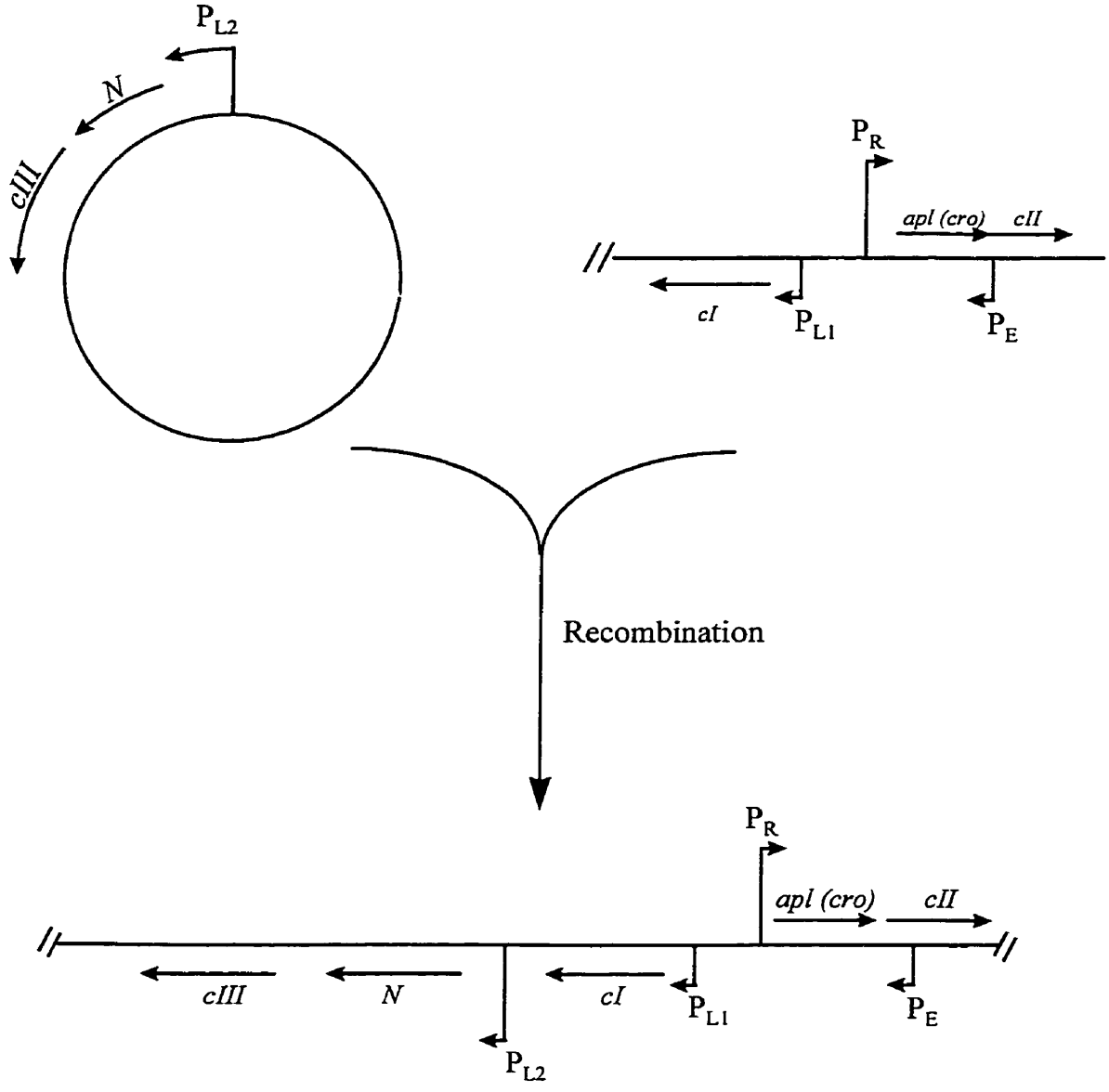
cII gene, in addition to the requirement for a third promoter, P_E, to allow for adequate lysogenic repressor production. Further evolution from the 186-like immunity region may have lead to more complex λ-like switches comprised of five different genes *(cIII, N, cI, cro, cII)*. If we accept the modular theory of evolution, the module from a 186-like phage may have either legitimately or illegitimately recombined with a plasmid or phage which possessed a module consisting of a gene for antitermination (an *N*- or *nun*-like gene), in addition to a protease-protection gene *(cIII)* (Figure 1.6). Recombination of this genetic switch within this plasmid, and further specialization, could have resulted in an ancestor of the lambdoid family of phages.

1.4.3 The Intermediate Switch

As observed in the simple switch of coliphage P2, only the *C* gene is required for the establishment of lysogeny. However, in coliphage 186, a close relative of P2, both the *cI* and the *cII* genes are required (Kalionis *et al.*, 1986a, 1986b; Lamont *et al.*, 1993). The 186 *cII* gene, likely a more recent addition to the phage genome, is not required for maintenance of lysogeny (Lamont *et al.*, 1993), and its transcription is repressed by cI during this cycle (Dodd *et al.*, 1990). Like P2, the lytic-lysogenic decision in 186 occurs at the level of transcription from two promoters: P_R, the lytic promoter which is active during vegetative growth, and P_L, the lysogenic promoter (Figure 1.5).

During lysogeny, transcription from P_L yields the cI protein, which represses P_R. During the lytic cycle, transcription from P_R yields the Apl protein which, analogous to P2 Cox, represses P_L (Dodd et al. 1990). In phage 186, P_R is much stronger than P_L

Figure 1.6 A model for the evolution of the "intermediate" λ-type genetic switch. In this model, a 186-like genetic switch module may have inserted within a plasmid (or phage) which possessed a second module consisting of a promoter, an antitermination-type *N* gene, and a protease degradation protection gene *(cIII)*. The recombination of the two modules may have given rise to the λ-family of phages.


P_L2
N
cIII
P_R
apl (cro)
cII
cI
P_L1
P_E
Recombination
P_R
apl (cro)
cII
cIII
N
cI
P_L1
P_E
P_L2

(Dodd *et al.*, 1990). In order to activate transcription from P_L, the cII protein must first activate the P_E promoter which spans the *apl-cII* intergenic region, upstream of P_L (Neufing *et al.*, 1996). This P_E transcript extends into the lysogenic operon past P_R, leading to the production of cI. The cI protein, in turn, represses P_R and relieves the inhibition of P_L by P_R. The activity of P_L is then sufficient to maintain production of cI. In addition to its repression of P_R, cI also directly represses transcription of the late control gene, *B*, from the P_B promoter (Dibbens *et al.*, 1992).

As an added module, an antirepressor, Tum, is required for induction of the 186 prophage. The SOS operon of 186 (Figure 1.5), contains two genes (*tum* and *orf97*) transcribed from the P_{95} promoter, which is under host LexA control (Lamont *et al.*, 1989; Lewis *et al.*, 1994; Brumby *et al.*, 1996). Exposure of a 186 lysogen to a DNA-damaging agent leads to the activation of RecA, which in turn yields cleaved LexA, resulting in both the host's SOS response as well as the relief of the LexA-mediated repression of P_{95}. The Tum protein, now produced, inactivates the cI repressor thereby relieving repression of the lytic promoters (Shearwin *et al.*, 1998). It is not known how Tum inactivates cI.

1.4.3.1 The Lambdoid Phages

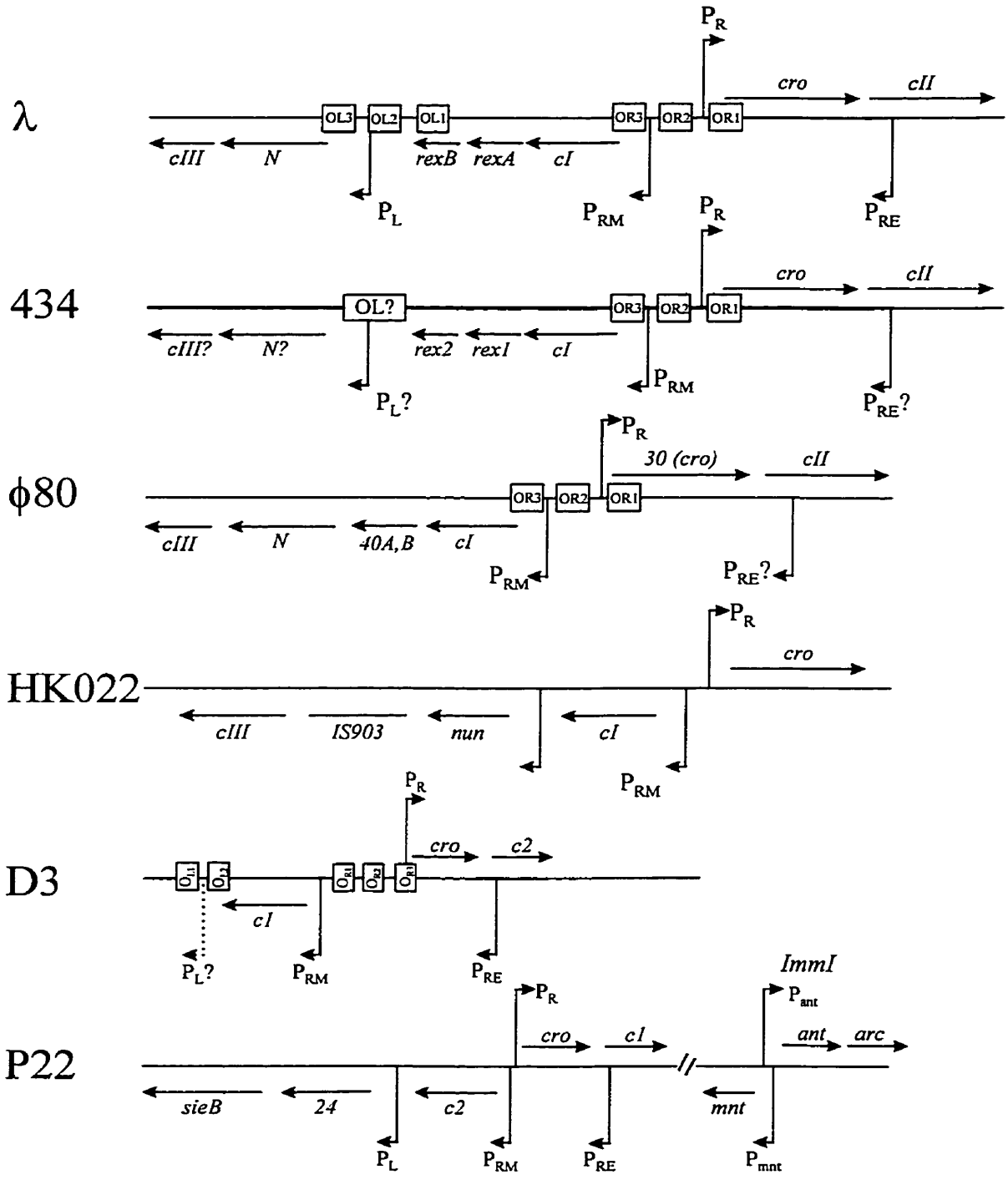
Relatives of coliphage λ have been isolated from various sources (Bezdek and Amati, 1967; Kitamura and Mise, 1970; Hershey and Dove, 1971; Krylov and Tsygankov, 1976; Dhillon *et al.*, 1980). However, phage λ itself was naturally isolated only once (Casjens *et al.*, 1992). Most λ-like phages grow on *E. coli*, but a few (such as

P22, L, and LP-7) infect *Salmonella typhimurium* or, in the case of D3, *P. aeruginosa* (Miller *et al.*, 1974).

All of the lambdoid-type phages have a single immunity region. Three genes, normally identified as *cI, cII,* and *cIII*, are involved in the lytic-lysogenic switch (Figure 1.7). All of these phages also have an *N*-like gene (although not yet shown for D3), and a *cro*-like gene. The *cro*-like gene resembles the *cox* gene of P2, and the *ner* gene of the transposable coliphages, and is involved in the commitment to the lytic cycle. The *N* gene, encodes an antiterminator that enables RNA polymerase to transcribe through regions of DNA that would otherwise cause the mRNA to terminate by binding at a specific sequence called *Nut* (*N utilization*) (reviewed in Ptashne, 1992).

The establishment of stable lysogeny for phage λ requires both repression of lytic functions as well as the insertion of the viral DNA into the host chromosome (reviewed in Ptashne, 1992). The primary regulatory element for the establishment of repression is the product of the *cII* gene (reviewed in Ho and Rosenberg, 1988; Obuchowski *et al.*, 1997). The *cII* gene is transcribed from P_R with *cro,* following antitermination by the *N* gene product (reviewed in Ptashne, 1992) (Figure 1.7). The cII protein activates transcription from three promoters: P_{RE}, P_I and P_{aQ} (reviewed in Ho and Rosenberg, 1988). The P_{RE} promoter controls the expression of the repressor *(cI)*, which inhibits transcription of the lytic functions, while the P_I promoter controls the expression of integrase *(int)*, which catalyzes the recombination of the viral DNA into the host genome (reviewed in Herskowitz and Hagen, 1980; Wulff and Rosenberg, 1983). The third promoter, P_{aQ} , is thought to direct the synthesis of an antisense RNA, which reduces the expression of the

Figure 1.7. Immunity regions of the λ-like family of phages (not to scale). Shown are the maps of coliphages λ (adapted from Ptashne, 1992), 434, HK022 (adapted from Cam *et al.,* 1991), and ϕ80 (adapted from Rybchin, 1984); the *P. aeruginosa* phage D3 (adapted from Farinha *et al.,* 1994); and the *S. typhimurium* phage P22 (adapted from Poteete, 1994). The arrows represent the genes and their direction of transcription. The operator binding sites (□) are shown in addition to the relative positions and direction of transcription for the various promoters.


λ
P_R
cro
cII
OL3
OL2
OL1
OR3
OR2
OR1
cIII
N
rexB
rexA
cI
P_L
P_RM
P_RE
434
P_R
cro
cII
OL?
OR3
OR2
OR1
cIII?
N?
rex2
rex1
cI
P_RM
P_L?
P_RE?
ϕ80
P_R
30 (cro)
cII
OR3
OR2
OR1
cIII
N
40A,B
cI
P_RM
P_RE?
HK022
P_R
cro
cIII
IS903
nun
cI
P_RM
D3
P_R
cro
c2
cI
P_L?
P_RM
P_RE
ImmI
P22
P_R
P_ant
cro
cI
ant
arc
//
sieB
24
c2
mnt
P_L
P_RM
P_RE
P_mnt

λ late gene transcription antiterminator, gpQ (Ho and Rosenberg, 1985; Hoopes and McClure, 1985).

The level of cII protein in an infected cell is proposed to be the crucial determinant in the lytic-lysogenic decision of phage λ (Herskowitz and Hagen, 1980). This level, however, depends on the balance between the rate of synthesis and degradation of cII (reviewed in Ptashne, 1992). Cells in which cII is rapidly degraded do not produce repressor, increase in Q and Cro protein levels, and grow lytically. The cII protein is susceptible to cleavage by the HflA and HflB proteases and requires the *cIII* gene product to protect it from proteolysis (Echols, 1986; Cheng *et al.*, 1988; Ho and Rosenberg, 1988; Herman *et al.*, 1993, 1997). Alternatively, in lysogens where cII is highly active, transcription of *cI,* and *int* proceeds at a high rate, integration of the prophage occurs, and lysogeny is established.

The maintenance of lysogeny in phage λ is the simplest stage of lysogenic development. It involves only the maintenance of viral repression through the action of a single phage protein: the product of the *cI* gene (reviewed in Ptashne, 1992; Cambell, 1996). Once initially expressed, the cI repressor begins to stimulate its own transcription from a cI-dependent promoter called P_{RM}. The cI repressor also negatively controls genes required for the lytic cycle at P_R and P_L by binding to two tripartite operators (O_L and O_R) (Figure 1.7). Each operator consists of three oligonucleotide sites (e.g. O_L consists of O_{L1}, O_{L2}, and O_{L3}) separated by short spacers. Binding by cI at the rightward operator (O_{R1}) negatively controls transcription of the lytic cycle genes from P_R, yet positively controls transcription of *cI* from P_{RM} (by binding at O_{R2}). Rightward transcription is repressed by

the cooperative binding of repressor to sites O_{R1} and O_{R2}. Activation of P_{RM} by repressor bound at O_{R2} requires specific contacts between the RNA polymerase and the repressor protein itself (Guarente *et al.*, 1982; Hochschild *et al.*, 1983). Binding of cI to the leftward operator (O_L) represses transcription of the *N* gene, which is required for lytic development. Through continued production of the cI protein, prophage repression is therefore maintained and repression of superinfecting λ phage gene expression is also accomplished. Thus a λ lysogen is immune to further infection by λ phage.

Progression to the lytic cycle requires the action of Cro. If the level of cII in a newly infected cell is low, or if a breakdown in repressor function in a lysogen occurs, transcription of *cro* from P_R occurs. Cro inhibits the synthesis of cI, thus antagonizing the establishment (or re-establishment) of lysogeny. For progression to the lytic cycle, Cro binds to operators O_L and O_R. Cro bound to O_{R3} (for which it has the highest affinity) prevents transcription of *cI* from P_{RM}. At high Cro concentrations, O_{R2} and O_{R1} are filled and rightward transcription from P_R shuts off as well. Once transcription of *cI* has been shut down and early transcription established from P_R, late transcription can begin (reviewed in Ptashne, 1992). The result is the production of new progeny phage.

For λ, 434, ϕ80, HK022 and P22, the general structures of the operators, the patterns of positive and negative control and the types of cooperativity shown by both the repressor and Cro are similar in all five phages despite protein and DNA sequence differences (Levine, 1972; Susskind and Botstein, 1978; Poteete, 1988; Poteete, 1994) (Figure 1.7). These common features serve the same vital role for each of the five

phages: they ensure stable lysogeny and allow efficient switching to lytic growth upon induction.

For coliphage 434, the immunity region differs slightly from that of λ in several respects: (i) the repressor and Cro proteins show only slight amino acid sequence similarity; (ii) the 434 right operators are shorter than the right operators of λ and show no sequence similarity (14bp vs. 17bp, Lauer *et al.*, 1981; Wharton *et al.*, 1984; Bushman and Ptashne, 1986, 1988); and (iii) the relationship of the three operator sites to the promoters they control differs for each phage (Bushman, 1993).

Footprinting experiments *in vitro* have established that the repressor and Cro proteins of 434, like those of λ, each bind to the three O_R sites with opposite orders of affinity: repressor binds most tightly to O_{R1}, whereas Cro binds most tightly to O_{R3} (Wharton *et al.*, 1984). However, in 434 Cro primarily represses transcription from P_R, by binding to O_{R1} (Wharton *et al.*, 1984; Bushman, 1993), whereas binding of λ Cro to O_{R1} and O_{R2} contribute equally to repression (Meyer and Ptashne, 1980). This effect is similar to that seen in phages P22 and ϕ80 (Ogawa *et al.*, 1988a, 1988b; Poteete *et al.*, 1986). In addition, 434 *cI*, unlike λ *cI* and HKO22 *cI*, has a recognizable Shine-Dalgarno ribosome-binding site (RBS) upstream of the start codon when expressed from P_{RM} (Bushman, 1993; Resch *et al.*, 1995; Cam *et al.*, 1991). Although the λ *cI* message from P_{RM} begins with the AUG itself (Resch *et al.*, 1995; Cam *et al.*, 1991), it does have a downstream sequence that is required for efficient translation (Balakin *et al.*, 1992; Shean and Gottesman, 1992).

HK022 is a temperate coliphage related to λ, which excludes superinfecting λ. The HK022 *nun* gene is responsible for this effect and encodes a transcription termination factor that prevents transcription of genes that lie promoter distal to λ *Nut* sites (Robert *et al.*, 1987; Robledo *et al.*, 1990) (Figure 1.7). Although the *nun* gene is in a position that corresponds precisely with that of λ *N*, Nun is not required to complete either the lytic or lysogenic life cycle (Oberto *et al.*, 1989). In addition, HK022 *nun* is expressed in the lysogen (Robert *et al.*, 1987) from P_M (Cam *et al.*, 1991). Therefore, although regulation of phage HK022 seems similar to its lambdoid relatives, this phage's early gene expression has unique features.

The genetic organization of the φ80 immunity region is largely similar to that of λ, (Ogawa *et al.*, 1988a, 1988b) (Figure 1.7). However, differences between these two phages include (i) the presence of a ρ-independent terminator of transcription immediately after the φ80 *N* gene; and (ii) the lack of a t_{R1}-type terminator as seen in λ (Ogawa *et al.*, 1988b). The other major difference is the uniqueness of the structure and function of the operators. For example, binding of φ80 cI at O_{R1} is not as tight as binding at O_{R2} or O_{R3}, and φ80 gp30 (Cro-like protein) binds to O_{R1} as tightly as it does to O_{R3}. These differences in binding affinity may reflect evolutionary maturation. It is probable that the first tripartite operators arose due to simple duplications. As phages evolved, the operators specialized such that the DNA-binding proteins bound to them with different affinities.

The *P. aeruginosa* phage D3 is a UV-inducible phage that will likely turn out to be a member of the lambdoid family (Farinha *et al.*, 1994). The immunity region of D3

resembles that of coliphages P2 and λ (Figure 1.7). The early promoters, P_R and P_{RM} have been located and sequenced, and the *c1* and *cro* genes identified (Farinha *et al.*, 1994). Three 14bp near-palindromic sequences located between the *c1* and *cro* genes (Farinha *et al.*, 1994) bound purified D3 c1 repressor (Farinha and Kropinski, 1997). In addition, two other potential 14bp operators, downstream of the *c1* gene, may indicate the position of the putative P_L promoter. The presence of only two operator sequences at P_L is different from that seen in coliphage λ, and may represent an evolutionary divergence of these phages. Although the genetic switch has not yet been completely characterized, the D3 c1 protein is able to provide immunity to superinfection by D3 and λ *in vivo* (Miller and Kokjohn, 1987). Further characterization of the D3 immunity region, including the role of the Cro protein, and localization of the putative *N*, *cII*, and *cIII* genes, will be useful in the understanding D3's placement in the evolution of the λ family of phages.

For the *S. typhimurium* phage P22, the immunity region resembles that of coliphage λ, however, a new module, the *immI* region, has likely recently been acquired (Figure 1.7). Immediately following infection, three small mRNAs are produced by three different promoters: P_L, P_R, and P_{ant} (reviewed in Poteete, 1988, 1994). The P_L transcript encodes the gene *24* protein which, like *N* of λ, causes antitermination at the ends of the short P_L and P_R transcripts. The P_R transcript encodes the Cro protein, which binds to operators at P_L and P_R to turn down the amount of transcription of these two promoters (Poteete *et al.*, 1986). This repression of the P_L and P_R transcription is necessary for entry into the lytic pathway. If not, production of the c1 protein channels the phage into the lysogenic cycle.

The *c1* gene is transcribed as a result of antitermination by the gene *24* protein. The regulation of c1 protein synthesis and stability is complex and only partially understood (reviewed in Poteete, 1988; 1994). When present in sufficient concentrations, c1 protein stimulates transcription from P_{RE} (Figure 1.7). The c2 protein (equivalent to cI of λ), encoded by the P_{RE} transcript, is then synthesized and acts by binding to operators overlapping P_L and P_R, where it represses transcription. In addition to this repression, c2 also turns on its own transcription from the otherwise silent promoter P_{RM}.

The P*ant* promoter is responsible for the transcription of both *ant* (the antirepressor) and *arc*. Although its mechanism is not yet understood (Susskind and Botstein, 1975; De Anda, 1985), the antirepressor appears to induce lytic growth of any P22-related prophages that may be present in the infected cell (Susskind, 1980; Susskind and Youderian, 1982; Youderian *et al.*, 1982). Transcription of *ant* is repressed mainly by Mnt. The *mnt* gene is located in the *immI* region of phage P22, and its gene product acts as a second transcriptional repressor that is required for maintenance of the lysogenic state (reviewed in Poteete, 1984). Mnt binds to an operator site which overlaps the P*ant* promoter and the -35 region of P*mnt* (Vershon *et al.*, 1987b). During the lysogenic life cycle, binding of Mnt to this site represses transcription from P*ant* and enhances its own transcription from P*mnt* (Sauer *et al.*, 1983; Vershon *et al.*, 1985; 1987a). The Arc protein binds to an operator (O_{arc}) between the P*mnt* and P*ant* promoters, and is required to inhibit overproduction of Ant during the lytic cycle, which is lethal to the cell (Susskind, 1980). In addition to the regulation of P*ant* by Mnt and Arc, a third repressor, *sar*, is also involved. The *sar* gene product is an antisense RNA, produced from P*sar*,

that is partly responsible for the negative regulation of antirepressor synthesis (Liao *et al.*, 1987; Wu *et al.*, 1987).

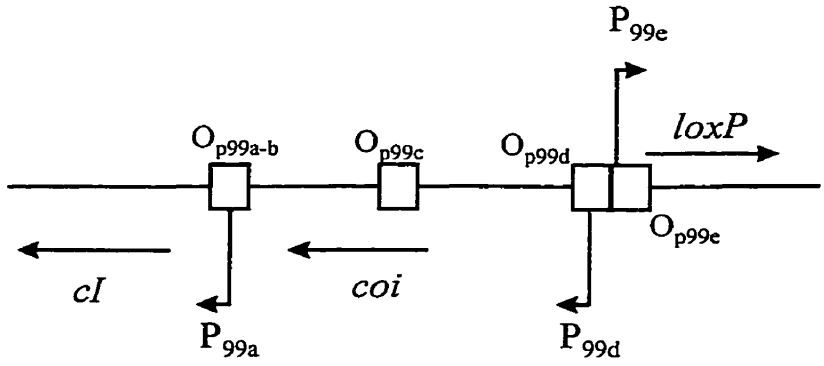
1.4.4 The Complex Switch

From its humblest beginnings, the primordial genetic switch has been subjected to many changes. As described, the "simple" genetic switch may have acquired more genes, giving rise to a switch of "intermediate" complexity, and which may have subsequently evolved into one possessing two immunity modules, like P22. More complex regulation consequently occurred when a phage possessing two immunity modules, may have acquired a third immunity module, as seen in coliphage P1 (Figure 1.8).

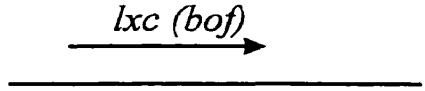
Phage P1, like P2, was originally isolated in 1951 by Bertani from a lysogenic *E. coli* strain and is also able to lysogenize *Shigella dysenteriae.* Initially, P1 went unstudied until 1955, when Lennox discovered P1-mediated generalized transduction of chromosomal markers between strains of *E. coli* and *S. dysenteriae.* The temperate phages P1 (Bertani, 1951; Ikeda and Tomizawa, 1968), P7 (originally designated ϕamp; Smith, 1972) and D6 (Mise and Suzuki, 1970) exist as plasmids in the lysogenic state, in contrast to the transposable and lambdoid phages which are integrated as prophages into the host chromosome. The P1 plasmid prophage genome comprises about 90kb, is maintained at about one copy per cell nucleoid, and is almost identical to P7 (Ikeda and Tomizawa, 1968). Like other temperate phages, P1 prophage is repressed for production of lytic phage functions and a P1 lysogen exhibits immunity to superinfection by other P1 phages (reviewed in Yarmolinsky and Sternberg, 1988). However, among the temperate phages, P1 employs the most complex regulatory apparatus for the establishment and

Figure 1.8. Immunity region of coliphage P1 (not to scale). Shown are the *immC*, *immI*, and *immT* modules. The immunity region of P7 is genetically identical. The arrows represent the genes and their direction of transcription. The operator binding sites (□) are shown in addition to the relative positions and direction of transcription for the various promoters (adapted from Heinrich *et al.*, 1995b).

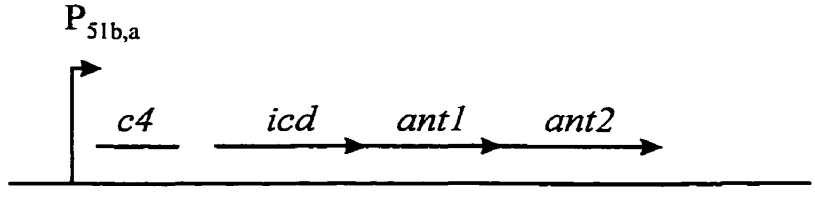
ImmC



ImmT



ImmI



maintenance of lysogeny. Regulation of lysogeny by P1 requires the expression of genes located within three discrete regions of the phage genome: the tripartite immunity system composed of the *imm*C, *imm*I and *imm*T regions.

The *imm*C regions of P1 and P7 contain a cluster of operators and the genes for *c1* and the c1 inactivator *coi*. The c1 proteins of P1 and P7 repress their respective phage's lytic functions and are responsible for maintaining the prophage in the lysogenic state. The c1 repressor of P1 is functionally identical to the c1 repressor of P7, and the two can be interchanged, however, P1 and P7 are heteroimmune indicating that the c1 repressor is not responsible for immune specificity (Chesney and Scott, 1975; Wandersman and Yarmolinsky, 1977).

Synthesis of c1 is produced from the promoter P_{99a} and is autoregulated via the bipartite operator Op99a·b (reviewed in Yarmolinsky and Sternberg, 1988). The P1 c1 repressor binds to at least 17 operators which are distributed widely over the P1 genome (Citron *et al.*, 1989; Heinzel *et al.*, 1989; Velleman, 1987; Lehnherr *et al.*, 1992). Upstream of P_{99a} there are three operators in addition to *coi*. The *coi* gene encodes a highly negatively charged protein of 69 amino acids (Heinzel *et al.*, 1990; Schaefer and Hays, 1990), and exerts its inactivating function by binding to the c1 repressors of P1 and P7 non-covalently (Heinzel *et al.*, 1992). The existence of a protein which antagonizes the action of the c1 repressor suggests that Coi participates in the decision process for the lytic or lysogenic pathway.

The *immI* region of P1 and P7 represent a single operon which is under the dual control of the c1 and c4 repressors (Heisig *et al.*, 1989). The c1 repressor controls transcription from promoter P_{51a} via the operator O_{p51}, and therefore P_{51a} is shut off in a

lysogen. Expression from P_{51b}, however, results in the expression of the *c4, icd,* and *ant1* and *ant2* genes. The *c4* gene is an antisense RNA that negatively regulates *icd, ant1* and *ant2* synthesis (Citron and Schuster, 1990; Citron and Schuster, 1992). The *ant1* and *ant2* genes code for two antirepressors (Heilmann *et al.,* 1980; Heisig *et al.,* 1989), and the *icd* gene codes for a protein that interferes with cell division (Riedel *et al.,* 1993). During lysogeny, c4 RNA directly represses translation of the translationally coupled *icd* and *ant1* genes by binding to two short sequences surrounding the RBS of the *icd* gene (Citron and Schuster, 1990; Reidel *et al.,* 1993; Heinrich *et al.,* 1994). A second RBS is found in front of *ant2*.

P1 and P7 are heteroimmune because of differences in their *c4* genes (reviewed in Yarmolinsky and Sternberg, 1988). Sequence comparison of the two *c4* genes revealed two bp substitutions that alter specificity of the *c4* antisense RNA (Citron and Schuster, 1990). As regulation by the *immI* region is effectively controlled by c4 antisense RNA, the involvement of c1 is unclear. How antirepressor synthesis is ever accomplished is only recently becoming understood.

In a P1 or P7 lysogen, translation of *ant* mRNA is blocked because c4 occludes the corresponding RBS. As a consequence, transcription of *ant* is prematurely terminated via a Rho-dependent terminator and no *ant*-specific RNA is found in the lysogen (Biere *et al.,* 1992). However, when bacteria are infected by P1 or P7, *ant* mRNA is found in the bacteria population.

In vivo, P1 and P7 *c4* RNAs are processed by *E. coli* RnaseP (Hartmann *et al.,* 1995). The decision for synthesis of either *c4* or *ant* mRNA is determined by the outcome of a race between *c4* RNA processing from the primary transcript by RnaseP and

other, unknown enzymes, and the ongoing transcription which leads to the completion of *ant* mRNA synthesis. If the processing is not accomplished in this time, Ant is synthesized to force lytic growth of the phage.

The regulatory function of the *imm*T region is encoded by the *lxc* (or *bof*) gene (reviewed in Heinrich *et al.*, 1995b). It has been shown that Lxc is a small protein of 82 amino acids, and that it decreases cI repressor synthesis *in vivo* (Schaefer and Hays, 1991; Velleman *et al.*, 1992). In the presence of Lxc, c1 is able to cause increased repression at operator sites by forming a c1-Lxc-operator ternary complex, including the operator site surrounding P_{99a} (Velleman *et al.*, 1992). In addition to this ability to increase repression during lysogeny, Lxc is also able to prevent the inactivating action of Coi on the c1 repressor.

1.4.5 Undefined Switches

Although the genetic switch behind the lytic-lysogenic decision has been well characterized for some temperate phages, there are many phages for which the switch has not yet been defined. A large number of these phages have been either partially or completely sequenced, yet, the proteins, promoters, and operators have not been characterized. This section gives a brief summary of phages (by host species) whose immunity regions are just beginning to be defined.

1.4.5.1 *Bacillus* Phages

Many *Bacillus* strains are lysogenic for one or more prophages. However, many of these prophages are defective and cannot produce infectious virions (reviewed in

Zahler, 1988). For example, the *B. subtilis* strain 168 contains both prophage SPβ (Zahler, 1982) as well as the defective phage PBSX (Seaman *et al.*, 1964). Studies on the non-defective temperate phages from *Bacillus* show they have life cycles similar to coliphage λ, and site-specifically integrate within the bacterial chromosome. No transposon-like phages with multiple insertion sites (as with coliphage Mu) have been found, and only a single P1-like phage, ϕ20, from *B. anthracis* has been reported (Inal and Karunakaran, 1996).

Phage SPβ (Warner *et al.*, 1977) is a specialized transducing phage (Zahler, 1982). Although the immunity region of this phage has not yet been completely defined, control of the lytic-lysogenic switch was shown to be controlled by its *d* gene (McLaughlin *et al.*, 1986). The *d* gene produces a protein of 22kDa, which was shown to confer immunity to superinfecting phages to its host *in trans*. In addition to controlling immunity, the *d* gene also appears to be involved in the establishment of lysogeny, as a mutation in *d* (called *d2*) caused the SPβ phage to grow lytically and to form clear plaques on sensitive bacteria. Interestingly, however, a cloned *d* gene could not complement this *d2* mutation, suggesting that the *d* gene product may act to block the repressor function or that it is involved in the regulation of another essential step in the establishment of lysogeny. Clearly, regulation of the lytic-lysogenic switch in SPβ is complex and the *d* gene product may have more than one regulatory role in controlling phage immunity and lysogenization.

1.4.5.2 *Streptomyces* phages

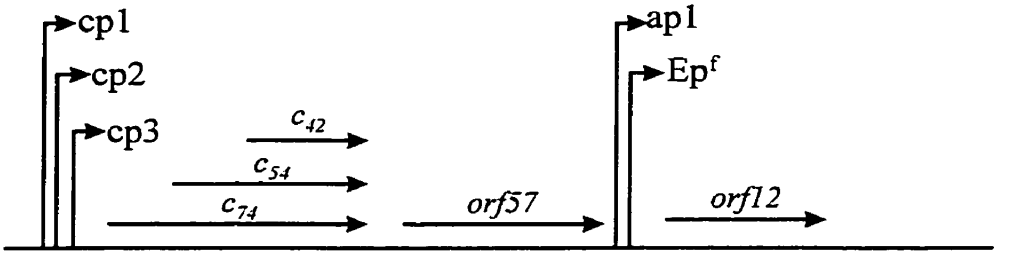
The genus *Streptomyces* consists of Gram-positive bacteria that develop morphologically complex mycelial colonies and produce a wide variety of commercially important secondary metabolites (Chater, 1989). Among the temperate phages of *Streptomyces*, ϕC31 is the most extensively studied (Chater, 1986), and its immunity region resembles that of a simple switch (Figure 1.9). However, unlike the single repressor seen in the simple switch, the ϕC31 *c* gene produces three N-terminally different, C-terminally identical proteins of 74, 54 and 42kDa (Sinclair and Bibb, 1989; Smith and Owen, 1991). Transcription studies of the *c* gene demonstrated that the promoter, *c*p1, has the potential to express all three repressor proteins, whereas the internal promoter, *c*p2, is only capable of expressing the 54 and 42kDa proteins (Sinclair and Bibb, 1989; Smith and Owen, 1991) (Figure 1.9). A third promoter, *c*p3, was also identified. However, activity at this putative promoter was weak and suggests that *c*p3 may require activation by a phage-encoded regulator (Smith and Owen, 1991).

A model has been proposed for the functions of the repressor proteins and the lytic-lysogenic decision (Smith and Owen, 1991). In this model, the 54kDa and 42kDa proteins are the true repressor proteins and the 72kDa protein functions as the antirepressor. In this manner, the genetic switch may be determined by differential expression of the three *c*-encoded proteins.

The lytic cycle of ϕC31 is believed to be initiated by deactivation of the repressor proteins (perhaps by c_{74}), along with the expression of immediate-early genes (Suárez *et al.*, 1992), one of which likely encodes the activator of the early gene promoters (Ingham

Figure 1.9. Immunity region of *S. coelicolor* ϕC31 (not to scale). The arrows represent the genes and their direction of transcription. The relative positions and direction of transcription for the various promoters is also shown (adapted from Hartley *et al.*, 1994 and Wilson *et al.*, 1995).

φC31



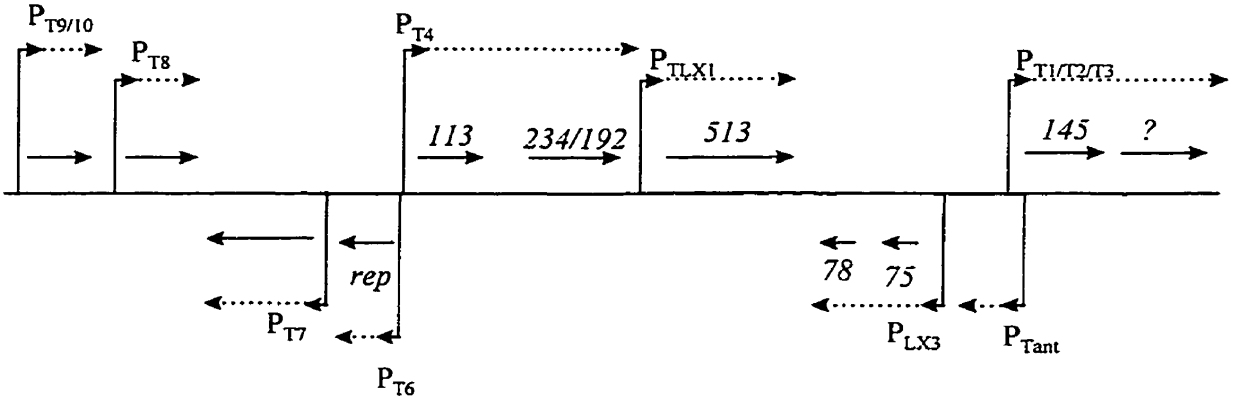
et al., 1993). Moreover, in a departure from typical temperate phage promoters, the early gene promoters of ϕC31 are likely controlled by a σ^{54}-like RNA polymerase, although RNA polymerase containing σ^{54} has not yet been identified in streptomycetes (Ingham *et al.*, 1993).

1.4.5.3 Phages of the *Archaebacteria*

The archaephage ϕH of *Halobacterium salinarium* is the most extensively studied archaephage (reviewed in Reiter *et al.*, 1987; Zillig *et al.*, 1988, 1996). Phage ϕH does not integrate into the host DNA, but is present as a plasmid in the lysogen. The sequence of the immunity region (referred to as the central L region) has been elucidated (Gropp *et al.*, 1992) (Figure 1.10). Part of the immunity conferred by the region has been shown to be due to a repressor gene, *rep*, the product of which specifically shuts off the synthesis of the major early lytic transcript *T4* (Ken and Hackett, 1991; Stolt and Zillig, 1992). Specifically, Rep acts at the transcriptional level by blocking the promoter for *T4* (Stolt and Zillig, 1994). The promoter for the *rep* gene itself is positioned back-to-back to the promoter for T4, in a manner similar to that of the P2 simple switch. Transcription of *rep* does not occur during the lytic cycle, and appears to be simply due to transcription from the stronger promoter for *T4*. A second contribution to immunity comes from an antisense-RNA transcript, T_{ant}, which, by pairing with the first part of the early lytic transcript *T1*, mediates specific endonucleolytic cleavage at the ends of the RNA duplex thus formed (Stolt and Zillig, 1993a). In addition, the *T9* transcript, though on its own

Figure 1.10. The immunity region of *Halobacterium salinarium* ϕH (not to scale). The solid arrows represent the genes and their direction of transcription, and the dashed arrows represent the major transcripts that have been detected (adapted from Gropp *et al.*, 1992 and Stolt and Zillig, 1993b).

ϕH1


$P_{T9/10}$
P_{T8}
P_{T4}
P_{TLX1}
$P_{T1/T2/T3}$
113
234/192
513
145
?
rep
78
75
P_{T7}
P_{T6}
P_{LX3}
P_{Tant}

has no influence on phage growth or immunity, has a co-operative effect on the ϕH repressor to promote lysogeny (Stolt and Zillig, 1993b).

The archaephage ϕCh1 was first isolated from *Natronobacterium magadii* in 1997 (Witte *et al.*, 1997). This phage is unusual in that its dsDNA genome is packaged together with host-encoded RNA into the mature phage particle. Although ϕCh1 has been shown to be a temperate phage with a chromosomally integrated prophage, the components of its genetic switch has not yet been characterized.

1.4.6 Complete Divergence from the Theme

In most known temperate bacteriophages, prophage immunity is determined by a repressor protein which inhibits expression of replication functions and of other genes involved in the lytic cycle. The *E. coli* satellite phage P4 deviates from this model in several respects. First, P4 prophage immunity does not prevent transcription initiation of the operon encoding the replication functions (α operon). Rather it promotes the premature termination of transcription that initiates at P_{LE}, the constitutive promoter of the α operon (Dehò *et al.*, 1988; Ghisotti *et al.*, 1992). Second, the immunity factor is not a protein, but a small, 69nt RNA (CI RNA) that exhibits complementarity with the nontranslated leader region of the transcripts it regulates (Dehò *et al.*, 1992; Ghisotti *et al.*, 1992).

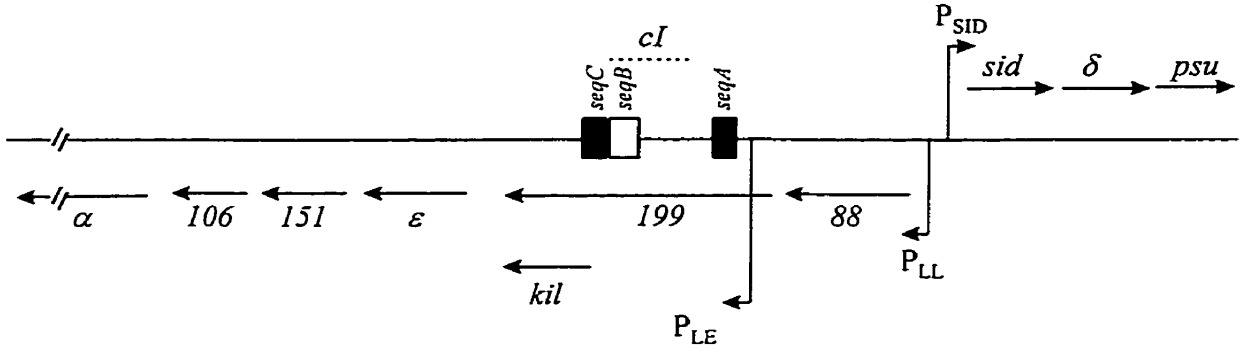
Phage P4 was isolated in 1963 by Six, and was originally classified as a defective bacteriophage because it depends on a lysogenic or coinfecting helper phage, such as coliphage P2, in order to undergo lytic development (Six and Klug, 1973). P2 acts as a

helper by providing gene products used by P4 for cellular lysis and for the assembly of its virion (Six, 1975). Despite this dependent relationship, the genome of P4 is unique: the P4 genome shows no organizational or sequence similarity to P2 or 186 (Figure 1.11). When P4 infects an *E. coli* host that has previously been lysogenized by a P2 or P2-like helper prophage, it may enter either the lysogenic or lytic pathway (Six, 1975, Six and Klug, 1973). In the absence of the helper phage, infection of *E. coli* by P4 may lead to either the immune-integrated condition, analogous to the typical lysogenic state, or to the establishment of a multicopy plasmid mode of maintenance (reviewed in Bertani and Six, 1988; Lindqvist *et al.*, 1993; Egan and Dodd, 1994).

Early after infection, the P_{LE} promoter produces a transcript that extends beyond the replicase gene α, however transcription from P_{LE} is intrinsically termination prone, and transcripts of different lengths are produced. Later in infection, as well as during the lysogenic state, the production of P_{LE} transcripts longer than 0.3 to 0.4kb is prevented by premature transcription termination (Dehò *et al.*, 1988; Dehò *et al.*, 1992) at a rho-dependent terminator located within the first transcribed gene of the left operon (Briani *et al.*, 1996). This premature termination is dependent on an RNA factor (CI RNA), encoded by the untranslated leader region of the α operon itself (*cI* locus), and which is generated by the processing of the primary transcript (Dehò *et al.*, 1992; Ghisotti *et al.*, 1992). A host protein, polynucleotide phosphorylase (encoded by the *pnp* gene), is also required for the establishment of P4 immunity, though, its mechanism of action is not yet understood (Piazza *et al.*, 1996).

Figure 1.11. Immunity region of coliphage P4 (not to scale). The solid arrows represent genes and their direction of transcription. The dashed line represents the location of the *cI* locus, and the boxes represent the sequences with complementarity (*seqA* and *seqC* [■] to *seqB* [□]) within this area. Also shown is the location of the promoters and their direction of transcription (adapted from Sabattini *et al.,* 1995).

P4



A region in the CI RNA *(seqB)* exhibits high complementarity with *seqA* and *seqC*, two sequences downstream of P_{LE} that are both necessary to establish immunity upon infection (Sabbattini *et al.*, 1995). Thus, in P4, both immunity and replication functions are coded for by the same operon. Since transcription of the distal portion of the replication operon is decreased soon after infection, expression of the α operon in the plasmid condition (and in the lytic cycle) occurs by the activation of another promoter, P_{LL}. This positively regulated promoter, located 400nt upstream of P_{LE} (Dehò *et al.*, 1988), is insensitive to transcription termination by CI RNA (Polo *et al.*, 1996).

The genetic switch underlying the choice between the integrative and the plasmid life cycles is complex and beyond the scope of this literature review. Briefly, in the plasmid state, promoters P_{SID} and P_{LL} are active together with P_{LE}. Promoters P_{SID} and P_{LL} require the P4 δ protein for activity (Dale *et al.*,1986; Dehò *et al.*, 1988). Since the δ protein is transcribed from P_{SID}, the plasmid transcriptional state is maintained by a positive feedback loop. It is not known how this state is established. It has been hypothesized that a low level of δ expression may occur during the uncommitted phase of P4 replication and that this could prime P_{SID} and P_{LL}, or that some other P4 function, encoded in the left operon and expressed from either P_{LE} or P_{LL}, may help activate P_{SID} (reviewed in Lindqvist *et al.*, 1993).

In the plasmid state, P_{LE} is still active. Short transcripts characteristic of the lysogenic condition, are abundantly produced and only a small amount of full-length transcripts are detected (Dehò *et al.*, 1988). Thus, the immune and the plasmid modes are not mutually exclusive. Rather, the plasmid mode of transcription is superimposed on the

immunity regulation. In addition, transcription from P_{LL} during the plasmid state is not subject to the strong transcription termination mechanism caused by P4 immunity and operating on the RNA starting at P_{LE} (Dehò *et al.,* 1992). This may be due to translation of *orf88* and *orf199* and/or to differences in the secondary structure of the RNA transcribed from P_{LL}, which may prevent the interaction between CI RNA and the SeqA/SeqC targets.

The P4 lytic cycle requires efficient exploitation of the helper genetic information to produce progeny virions (for a review see Lindqvist *et al.,* 1993; Egan and Dodd, 1994). This is accomplished both by utilizing the P2-encoded regulatory mechanisms normally acting on the helper, and by carrying out new P4-encoded functions that bypass or enhance P2 control systems.

1.5 Conclusions

The debate surrounding the theory of evolution is polarized around two models: the punctuated equilibrium hypothesis and the gradual view. The theory of punctuated equilibrium states that the evolution of organisms is marked by long periods of very little evolution interrupted by periods of relatively rapid evolutionary change (reviewed in Moller and Pomiankowski, 1993; Coyne and Charlesworth, 1996; Mlot, 1996). In contrast, the gradual view states that the rate of evolution is gradual, albeit with variable rate (reviewed in Moller and Pomiankowski, 1993). Current theories on the evolution of phages favour the punctuated evolution model with incidents of large genome reshuffling.

Phages seem to have evolved through insertions, deletions, horizontal transfer of genes between phage and host, and between phages when they coinfect the same host.

Within the phage itself, the various modules, believed to be the main focus of phage evolution, have also, themselves, been subject to evolution. This Chapter has focused on the immunity region and suggested that this module has also been subject to evolutionary development. Small deletions, insertions, mutations and DNA rearrangements have likely played key roles in the evolution of this module. Comparison of the defined and putative genetic switches from a phylogenetically diverse group of phages suggests that although many share a common bi-repressor-type switch, many different evolutionary solutions are possible in its construction. Changes in the proteins involved in the genetic switch are also likely to have had very large effects on many regulatory circuits, whereas changes in the binding sites and changes in the spacing between these sites are likely to have been specific to a particular locus (Liu and Little, 1998). Analyzing the genetic switch of more temperate phages in detail should enhance our understanding of the evolution of this important module.

1.6 Rational and Objectives of the Thesis

Bacteriophage D3112 of *P. aeruginosa* is extremely similar to coliphages Mu and D108 in genetic structure and its proteins appear to be analogous in function. However, information for this phage has been mainly limited to genetic and sequencing data, and

only one factor involved in the lytic-lysogenic decision, the D3112 repressor, has been genetically mapped. Thus, further characterization of the D3112 immunity region was initiated.

Chapter 2 presents the characterization of the expression and activity of the D3112 c repressor. The correct start codon, as well as important residues in repressor functioning were identified, in addition to the localization of the repressor promoter, P*c*. The D3112 repressor was purified and shown to bind to a region directly upstream of the *c* ORF.

In Chapter 3, I describe the identification of a previously unmapped Ner homologue in the D3112 genome. D3112 *ner* was located directly upstream of the D3112 *c* ORF, in a position analogous to that of Mu and D108 Ner. D3112 *ner* is not transcribed in conjunction with the transposase genes (*A* and *B*) as seen in the two coliphages, instead, D3112 *ner* is transcribed from a novel promoter, called P*ner*. Purification of D3112 Ner demonstrated that it binds to a fragment located in the intergenic region (between *c* and *ner*). In Chapter 4, the location of a promoter directly upstream of the D3112 *A* gene, called P*e*, is described. A model for the lytic-lysogenic switch is presented.

Finally, Chapter 5 contains a summary of the data. The significance of the data is discussed, and suggestions are made for future areas of research.

Chapter 2

Characterization of the lysogenic repressor *(c)* gene of the *Pseudomonas aeruginosa* transposable bacteriophage D3112

2.1 Abstract

Bacteriophage D3112 is a Mu-like temperate transposable phage of *Pseudomonas aeruginosa*. Genetic mapping and DNA sequence analysis has identified the left-end of the phage genome as encoding the transposase enzyme *(A)* and the temperate repressor *(c)*. As genetic control of the lytic-lysogenic switch in D3112 has not been elucidated, characterization of the expression and activity of the c repressor was undertaken. The *c* open reading frame (ORF), located at the left-most end of the phage genome and transcribed from right to left, has four possible GTG initiation codons. Each of the four GTG codons was modified using site-directed mutagenesis to the non-f-methionine, but still valine-encoding codon, GTA. Plasmids containing either the wild type repressor ORF or the ORFs containing mutated GTG codons into *P. aeruginosa* resulted in the loss of immunity to superinfection by D3112 lysates only when the second GTG was mutated. Northern blotting analysis demonstrated that the D3112 *c* repressor is transcribed as a 900nt mRNA. The promoter region was defined by transcriptional *lacZ* fusions and primer extension analyses to bp972-940 from the left-end of the phage genome. When the D3112 c repressor was overexpressed and purified as a fusion protein with a C-terminal 6-Histidine extension (cts15-His6) this protein bound to a 261bp *Pvu*II fragment localized directly upstream of the *c* repressor ORF. The results of this study indicate that although D3112 c shows higher homology to the λ family of repressors, that it appears more functionally analogous to that of coliphages Mu and D108.

2.2 Introduction

Bacteria of the genus *Pseudomonas* are infected by more than 60 distinct temperate, transposable bacteriophages, including phage D3112 (Akverdyan *et al.*, 1984; DuBow 1994). Such transposable bacteriophages are rare in the Enterobacteriaciae. The two known transposable coliphages, Mu and D108, have been well studied and are able to function as both transposons and as viruses (reviewed in Pato, 1989; DuBow, 1994). During their lytic cycle, the infecting phage DNA first integrates conservatively (via DNA transposition) into the bacterial chromosome at random locations. During lytic growth, these prophages amplify their genomes through replicative transposition to new locations within the host chromosome. In the lysogenic state, the prophage lytic functions are repressed by the action of a repressor (the *c* gene product).

Bacteriophage D3112 has a 38 kilobase pair (kbp) linear double stranded DNA genome and possesses a genetic organization similar to that of the transposable coliphages Mu and D108 (Krylov *et al.*, 1980a, 1980b; Yanenko *et al.*, 1983, 1988; DuBow, 1994). Genetic mapping, using deletion mutants of an RP4::D3112 plasmid (Krylov *et al.*, 1982; Yanenko *et al.*, 1983, 1988; Gerasimov *et al.*, 1985), was used to localize the lytic-lysogenic regulatory region to the leftmost end of the phage genome. Bacteriophage D3112, like the two coliphages, is also capable of acting as an insertional mutagen in Pseudomonads, and lysogens of the phage have insertions in many different locations (Plotnikova *et al.*, 1983; Rhemat and Shapiro, 1983).

Transposable phages have proven to be enormously useful in the study of gene structure and expression in their hosts. Mini-D3112 transposons which lack the genes essential for phage growth but which retain the terminal sequences required *in cis* for transposition, have been developed for use in *Pseudomonas* strains (Darzins and Casadaban, 1989a, 1989b). An understanding of the lytic-lysogenic switch and regulation of phage-mediated DNA transposition would be beneficial for both the future development of D3112 as a genetic tool and our understanding of gene regulation in *P. aeruginosa* and its bacteriophages.

The leftmost 5.5 kbp of the D3112 genome have been cloned and sequenced (Autexier *et al.*, 1991; Ulycznyj *et al.*, 1995). Several open reading frames (ORFs) have been identified, and their locations suggest strong functional similarity with the leftmost ends of Mu and D108 (Yanenko *et al.*, 1983, 1988; DuBow, 1994). The putative lysogenic repressor, encoded by the *c* gene, is transcribed from right to left (as in Mu and D108) and a cloned fragment containing the *c* ORF (bp1174-0) conferred immunity to D3112 superinfection *in vivo* (Autexier *et al.*, 1991). The proteins required for replicative transposition by D3112 (expressed from the *A* and *B* genes) have been identified and characterized (Ulycznyj *et al.*, 1995), while the *cip* (*c*ontrol of *i*nteraction of *p*hages) function has been mapped to the right of the *c* gene (Gerasimov *et al.*, 1985; Bidnenko *et al.*, 1996). Although located in a position analogous to *ner*, the *cip* function may be more analogous to Rex of λ (reviewed in Court and Oppenheim, 1983; Snyder and Kaufman, 1994); as its role has been described as preventing infection by another transposable phage, B39, in lysogens containing multiple D3112 prophages (Gerasimov *et al.*, 1985; Bidnenko *et al.*, 1996).

In order to understand the genetic control of the lytic-lysogenic switch in D3112, the expression and activity of the *c* repressor gene must be determined. This gene has been mapped to the leftmost end of the phage genome and shown to consist of a single large ORF transcribed from right to left, as in Mu and D108 (Autexier *et al.*, 1991). In this study, we demonstrate the use of an internal GTG as the start codon, in addition to identifying amino acids important in the proper functioning of the protein. Localization of the promoter, P*c*, was also accomplished. Finally, purification of the D3112 repressor helped demonstrate that it binds to a region directly upstream of the D3112 *c* ORF.

2.3 Materials and Methods

2.3.1 Bacterial strains, phages, plasmids and culture conditions.

The bacterial strains, bacteriophages and plasmids used in this study are listed in Table 2.1. All cultures were grown at 37°C in Luria Bertani broth and agar (Sambrook *et al.*, 1989) except for PAS429 and strains containing plasmids expressing the temperature-sensitive c repressor (cts), which were grown at 32°C. When necessary, antibiotics were added at the following concentrations: for *P. aeruginosa*, pipercillin (Pip; 100μg/ml); streptomycin (Str, 300μg/ml); tetracycline (Tc; 200μg/ml); for *E. coli*, ampicillin (Ap; 50μg/ml), kanamycin (Km; 50μg/ml); Str (100ug/ml); Tc (10μg/ml).

2.3.2 Enzymes and chemicals

All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Superscript II Reverse Transcriptase were purchased from either Amersham-Pharmacia (Baie d'Urfe,

Table 2.1. Bacterial strains, phages and plasmids used in this study.

Strain/Phage/ Plasmid	Relevant characteristics	Reference/Origin
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type, prototroph	Holloway, 1969
PAS429	PAO1 *ami*::D3112*cts*15	J. Shapiro
Escherichia coli		
BL21(DE3)	F⁻ *ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm* (DE3)	Studier and Moffatt, 1986
DH5α	*supE44 ΔU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
Phages		
D3112 *cts*15	*cts*, temperature-sensitive repressor (38kb)	J. Shapiro
MK1307	ssDNA helper phage	Vieira and Messing, 1987
Plasmids		
pBR322	Ap^R, Tc^R, cloning vector	Bolivar *et al.*, 1977
pUC119	Ap^R;cloning vector	Vieira and Messing, 1987
pET29-b	Km^R; C-terminal 6XHIS expression vector	Novagen, Inc.
pDN19lacΩ	Str^R/Spc^R, Tc^R; Broad host range, promoterless *lacZ* vector	Totten and Lory, 1990
pTJS140	Ap^R; rep$_{RK2}$ *ori*T$_{RK2}$ rep$_{pMB1}$ *lac'IPOZ*	Darzins and Casadaban, 1989a
pB28$_L$	left-end (backfilled-*Hin*dIII) fragment of D3112 *cts*15 into the *Sma*I-*Hin*dIII site of pUC119	Autexier *et al.*, 1991
pSWL12	*Nru*I-*Eco*RI fragment from pB28$_L$ into the *Sma*I-*Eco*RI site of pTJS140	Autexier *et al.*, 1991
pOF4	left-end (backfilled-*Hin*dIII) fragment of D3112c$^+$ into the *Sma*I-*Hin*dIII site of pUC119	This study
pOF6	*Nru*I-*Eco*RI fragment from pOF4 into the *Sma*I-*Eco*RI site of pTJS140	This study
pKAS210	D3112 *cts*15 in pET29b	This study
pKAS211	D3112 *cts*15 (from 3rd GTG) in pET29b	This study
pKAS220	bp1174-bp366 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS224	bp1172-bp518 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS228	bp1172-bp772 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS230	bp838-bp1099 *Pvu*II fragment of D3112*cts* cloned into the *Sma*I site of pUC119	This study

Table 2.1. Con't.

Strain/Phage/ Plasmid	Relevant characteristics	Reference/Origin
pKAS305	*Nru*I-*Eco*RI fragment from $pB28_L$ (1^{st} GTG to GTA) into the *Sma*I-*Eco*RI site of pTJS140	This study
pKAS306	*Nru*I-*Eco*RI fragment from $pB28_L$ (2^{nd} GTG to GTA) into the *Sma*I-*Eco*RI site of pTJS140	This study
pKAS307	*Nru*I-*Eco*RI fragment from $pB28_L$ (3^{rd} GTG to GTA) into the *Sma*I-*Eco*RI site of pTJS140	This study
pKAS308	*Nru*I-*Eco*RI fragment from $pB28_L$ (4^{th} GTG to GTA) into the *Sma*I-*Eco*RI site of pTJS140	This study
pOF7	pSWL12/L22P	This study
pOF8	pSWL12/L36P	This study
pOF14	pSWL12/L37F	This study

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Spc^R, spectinomycin resistance; Str^R, streptomycin resistance; Tc^R, tetracycline resistance

QC) or GIBCO-BRL (Burlington, ON). *Taq* DNA polymerase was purchased from Promega (Madison, WI). The Sequenase Version 2.0 Kit was purchased from Amersham-Pharmacia, and the Isotherm Sequencing Kit was purchased from Epicenter Technologies Inc. (Madison, WI). All other chemicals were purchased from either Fisher (Nepean, ON) or Sigma (St. Louis, MO).

2.3.3 Bacteriophage lysates and in vivo immunity assay.

Bacteriophage lysates were prepared using strain PAS429 grown at 32°C until an A_{550}= 0.6, shifted to 42°C and incubated until the A_{550}< 0.1. The phage lysate was titered on TCMG plates (Schumm *et al.*, 1980) as previously described (Ljundquist and Bukhari, 1977). The *in vivo* immunity assay was performed as previously described (Levin and DuBow, 1989).

2.3.4 Site-directed mutagenesis.

Site-specific mutagenesis was performed with the U.S.E. Mutagenesis Kit (Pharmacia Biotech) using the *ScaI*/*MluI* U.S.E. selection primer (Pharmacia Biotech) for the initial screening procedure, according to the manufacturer's instructions. Primers KS15 (5'GTCTGATTTTACGATAAAAAAGCCG3'), KS16 (5'GCAGCCTAGCTACC TCCGCG3'), KS17 (5'CCAATCCAGACTTACACGACCAGCC3') and KS18 (5'GGCTTCGCAGCTACCTCTTCCCC3') were used to specifically mutagenize the first, second, third and fourth GTG codons to GTA codons (underlined) in the *c* repressor gene. The primers were purchased from GIBCO-BRL.

2.3.5 PCR amplification.

PCR reactions were performed in a DNA Thermal Cycler (MJ Research, Inc.). For the PCR amplification of the various *c* ORFs, the reactions were performed in 50μl volumes. Each reaction mixture contained 50ng of DNA template, 2U of *Taq* DNA polymerase, 4mM $MgCl_2$, 0.1mM deoxynucleoside triphosphate mix, and 0.2μM primers (see below). Thirty cycles were performed for each reaction. Each cycle consisted of incubations for 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C. The primers used for the PCR reactions were purchased from GIBCO-BRL. Restriction enzyme sites were added at the 5' ends of the primers (shown below in boldface) to facilitate subsequent cloning of the PCR products. Additional nucleotides were added 5' to the restriction enzyme sites to ensure efficient cleavage. The following primers were used in the PCR reactions: primers KS6 (5'-CCCC**CATATG**GCTAGGCTGC-3') and KS25 (5'-CCCC**CTCGAG**AACCATCCAGCGGC-3') were used for the PCR amplification of the *c* ORF from the second GTG start codon; and KS12 (5'-CCCCCC**CATATG**AGTCGGA TTGGC-3') and KS25 were used for the PCR amplification of the *c* ORF from the third GTG. In each case, one of the primers (KS6 or KS12) modifies the original GTG start codon such that there is an ATG within the PCR product.

2.3.6 Hydroxylamine (HA) mutagenesis of pSWL12.

CsCl-purified pSWL12 plasmid DNA was mutagenized using hydroxylamine (HA) as previously described (Miller, 1972) for 24hr at 37°C. Following mutagenesis,

the DNA was dialyzed in 2L T.E. buffer (Sambrook *et al.*, 1989) at 4°C, for 24hrs with 2 changes of T.E. buffer and then ethanol precipitated as previously described (Miller, 1972).

2.3.7 DNA sequencing.

Single stranded DNA required for sequencing reactions was obtained by superinfection with phage M13K07 (Vieira and Messing, 1987) or by denaturing dsDNA as described (Lim and Pene, 1988). DNA sequencing was performed using either the Sequenase version 2.0 Kit or the Isotherm Sequencing Kit according to the manufacturer's instructions. Sequencing reactions were subjected to electrophoresis on either 5% polyacrylamide or 5% Long Ranger (JT Baker, Phillipsburg, NJ) sequencing gels as previously described (Maxam and Gilbert, 1980), and visualized by autoradiography after exposure to Kodak XAR-5 film for 24-72 hrs at -70°C under Dupont Cronex intensifying screens.

2.3.8 Plasmid constructions.

Plasmids used for the *in vivo* immunity assay were constructed in the following manner: Plasmid $pB28_L$, containing the left-most 1.8kbp of the D3112*c*ts15 genome in pUC119 (Autexier *et al.*, 1991), was used as the target plasmid for the site-specific mutagenesis. In four separate reactions, each of the four potential GTG start codons was replaced with the non-f-methionine codon GTA, generating plasmids pKAS301, pKAS302, pKAS303 and pKAS304. The plasmids were sequenced to confirm that no

other mutations had been incorporated into the mutagenesis product. The plasmids were then hydrolyzed with *Nru*I/*Eco*RI and the resulting fragments were subcloned into the *Sma*I/*Eco*RI sites of the broad host range plasmid pTJS140, producing plasmids pKAS305, pKAS306, pKAS307 and pKAS308 (Table 2.1).

Cloning of the left-end of D3112 c^+ phage DNA for sequencing and functional studies was accomplished by first ensuring that the left-end of the D3112 c^+ was blunt with T4 DNA polymerase (Sambrook *et al.*, 1989). The genome was then hydrolyzed with *Hin*dIII and a 1.8kbp DNA fragment was isolated from a 0.7% agarose gel using Gene Clean (Bio 101, Vista, CA) according to the manufacturer's instructions. This fragment was then ligated, using T4 DNA ligase, to the expression vector pUC119 previously hydrolyzed in its polylinker with *Sma*I and *Hin*dIII, resulting in the plasmid pOF4. Cloning of the D3112 c^+ repressor gene into a broad host-range vector was accomplished by hydrolyzing pOF4 with *Nru*I and *Eco*RI and isolating a 1.1kbp fragment (as described above). This fragment was then ligated, using T4 DNA ligase, to vector pTJS140 that had been previously cleaved in its polylinker at the *Sma*I and *Eco*RI sites generating plasmid pOF6.

From the mutagenesis experiments, HA-treated pSWL12 plasmids (see above) that lost the ability to provide immunity to superinfection *in vivo* were digested with *Pst*I and *Eco*RI, and a 1.1kbp fragment was isolated (as described above). This fragment was then ligated to a non-mutagenized pTJS140 vector previously digested with *Pst*I and *Eco*RI. The resultant plasmids (named pOF7, pOF8, pOF9, pOF10, pOF12, pOF14 and pOF15) were again screened for loss of immunity function. For these mutant plasmids, the *Pst*I/*Eco*RI fragment was also ligated into the vector pUC119, previously cleaved in

its polylinker with *PstI*/*Eco*RI, for sequencing (pOF107, pOF108, pOF109, pOF110, pOF112, pOF114 and pOF115).

Plasmids for the promoter determination via β-galactosidase promoter-probe fusions were constructed in the following manner: various fragments from the D3112*cts*15 genome (see Figure 2.1) were made blunt using T4 DNA polymerase (if required), subcloned into the *Sma*I site of pUC119 and the orientation determined by restriction enzyme hydrolyses. Plasmids containing the proper orientation were hydrolyzed with *Eco*RI and *Bam*HI and the resulting fragments isolated and subcloned into the *Eco*RI/*Bam*HI sites of pDN19lacΩ (Totten and Lory, 1990). The resulting plasmids are listed in Table 2.1.

Plasmids for the overexpression of D3112 cts15, using either the second or third GTG as the start codon, were constructed in the following manner: the PCR products from each ORF were hydrolyzed with *Nde*I/*Xho*I and ligated to the *Nde*I/*Xho*I site of pET29-b. Once constructed, the plasmids were sequenced using the T7 promoter primer and the T7 terminator primer (Novagen, Inc., Madison, WI) to ensure there were no alterations arising from the PCR reactions. The resulting plasmids were called pKAS210 and pKAS211, respectively (Table 2.1).

2.3.9 Transformations and Electroporations.

Transformations of *E. coli* were performed using a $RbCl_2$ method (Hanahan, 1983). Electroporation of *P. aeruginosa* strains was performed according to the method of Farinha and Kropinski (1990) using 15% glycerol/1mM MOPS as the buffer. The

DNA used for the transformations and electroporations was prepared using either an alkaline-lysis method (Sambrook *et al.*, 1989) or Qiagen quick-spin columns (Qiagen, Mississauga, ON).

2.3.10 β-galactosidase assays.

Expression of the *lacZ* gene, under the control of the putative P*c* promoter region, was measured using β-galactosidase assays as described by Miller (1972).

2.3.11 Northern blot hybridization.

Total cellular RNA was prepared from *P. aeruginosa* strains PAO1, PAS429 (grown at 32°C) and PAS429^{IND} (PAS429 grown at 32°C to A_{550}=0.7 and then induced for 8 min at 42°C) by an adaptation of the CsCl purification procedure (Glisin *et al.*, 1974), as described by Deretic *et al.* (1987). RNA, 10μg from each strain, was subjected to electrophoresis on a 1.2% agarose/formaldehyde gel (Ausubel *et al.*, 1989), transferred and fixed to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as described previously (Cai and DuBow, 1996). The 0.4 to 9.5kb RNA ladder (GIBCO-BRL) was subjected to electrophoresis in parallel with the above samples and visualized by ethidium bromide staining prior to transfer to the nitrocellulose membrane. A 0.6kb *Nde*I/*Xho*I fragment from pKAS210, containing the entire D3112 *c* gene, was used to detect D3112 *c* mRNA (Table 2.1). The DNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, 1Ci=37Gbq, Amersham-Pharmacia) using the random priming method (Sambrook *et al.*, 1989) and random hexanucleotide primers (GIBCO-BRL).

Hybridization was carried out as previously described (Mahmoudi and Lin, 1989). Unhybridized probe was removed by washing the filters twice at 68°C in 2X SSC, 1% SDS. The blots were air dried and exposed to a Molecular Dynamics Storage Phosphor Screen and visualized using a PhosphorImager SF and Image Quant Software Program (Molecular Dynamics, Sunnyvale, CA).

2.3.12 Transcriptional start site determination.

The transcriptional start site of the *c* gene was determined by primer extension. Primer KS21 (5'-GCGTCGAAGTCGGAACAGC-3'), which complements the noncoding strand near the 5' end of the *c* ORF, was end-labeled with [γ-^{32}P]ATP (5000Ci/mmol, Amersham-Pharmacia) using T4 polynucleotide kinase according to the manufacturer's instructions (GIBCO-BRL). The labeled primer was purified using the Nucleotide Removal Kit (Qiagen Inc., Mississauga, ON) and the resulting labeled primer was used for the reverse transcription (RT) reactions. The total RNA template for the primer extension reaction was prepared as described above, and was pre-treated with DNAseI (Boehringer Manneheim) prior to the RT reaction. Superscript II reverse transcriptase was used for the RT reactions under the conditions recommended by the manufacturer (GIBCO-BRL). The DNA template for the sequencing reaction was $pB28_L$ and the primer was KS21. The products of the sequencing and RT reactions were subjected to electrophoresis on a 5% polyacrylamide sequencing gel and visualized using autoradiography as described above.

2.3.13 Overexpression and purification of D3112 cts15.

E. coli BL21(DE3) cells were transformed with plasmids pKAS210, pKAS211, or the vector control plasmid pET29-b. Bacterial cultures were grown to an A_{550}= 0.4 and expression of T7 RNA polymerase, required to transcribe the T7 promoter in the plasmids, was induced in the host strain by the addition of isopropylthio-galactopyranoside (IPTG) to a final concentration of 1mM (Studier and Moffatt, 1986). The cultures were grown for an additional three hours and harvested by centrifugation for 20min at 4000 ⏃ *g*, at 4°C. Purification of the two proteins were performed under native conditions using Ni-NTA agarose (Qiagen Inc) according to the manufacturer's instructions with the following modifications: the lysis buffer contained 5mM imidazole and 1mM phenyl-methylsulfonyl fluoride (PMSF); two different wash buffers were used containing 1mM PMSF and either 20mM or 50mM imidazole, respectively; the elution buffer contained 250mM imidazole and 1mM PMSF. Samples of the crude extracts, washes and elutions were subjected to electrophoresis on a 20% SDS-PAGE gel (Laemmli, 1970)

2.3.14 Operator binding assay.

Binding of the D3112 repressor to DNA was assayed using the gel electrophoretic shift method (Fried and Crothers, 1981; Garner and Revzin, 1981). The buffer used in the binding reactions contained 20mM Tris-HCl, pH7.5, 1mM EDTA, 10mM 2-mercaptoethanol, 50mM NaCl, 100μg/ml bovine serum albumin and specified amounts of sonicated calf thymus DNA. Protein-DNA binding reactions were performed and then

subjected to electrophoresis on 5% polyacrylamide gels as described in Kukolj and DuBow (1991).

2.4 Results

2.4.1 Site-specific mutagenesis to determine the c repressor start codon

The original sequencing of the D3112 *c* gene revealed no ATG start codon. Instead, four GTG codons were found, each of which were in the same reading frame (Autexier *et al.*, 1991). In order to determine which of the four GTG codons is used as the start codon for translation of the c repressor, site-specific mutagenesis was performed. Plasmid $pB28_L$, containing the left-most 1.8kbp of the D3112*c*ts15 genome (Autexier *et al.*, 1991), was used as the target for mutagenesis. Each of the four putative GTG codons was mutagenized to a GTA codon which also encodes a valine but is no longer able to function as a start codon (Neidhart *et al.*, 1990). From these mutagenized plasmids, the *Nru*I/*Eco*RI fragment (bp1174-1 of the D3112 *c*ts15 genome) was subcloned into the broad host-range vector pTJS140 and electroporated into *P. aeruginosa* PAO1 cells. Lawns of each of these strains were prepared and tested for their ability to confer immunity to D3112 superinfection (see Materials and Methods) and the results are shown in Table 2.2. Strain PAO1 shows a high level of susceptibility to superinfection *in vivo*. Strain PAO1/pTJS140 also displays a high susceptibility to D3112 superinfection, indicating that the vector alone confers little significant immunity or resistance to D3112

Table 2.2. Immunity to D3112 superinfection conferred by cloned D3112 left-end DNA-containing plasmids in PAO1.

	Titer of Phage Lysate (pfu/ml)									
Plasmid	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}
32°C										
None	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
pTJS140	–	–	–	+	++	++	+++	+++	+++	+++
pSWL12	–	–	–	–	–	–	–	–	–	–
pKAS305	–	–	–	–	–	–	–	–	–	–
pKAS306	–	–	–	++	++	+++	+++	+++	+++	+++
pKAS307	–	–	–	–	–	–	–	–	–	–
pKAS308	–	–	–	–	–	–	–	–	–	–
pOF6	–	–	–	–	–	–	–	–	–	–
42°C										
pSWL12	–	–	–	–	+	++	+++	+++	+++	+++
pOF6	–	–	–	–	–	–	–	–	–	–

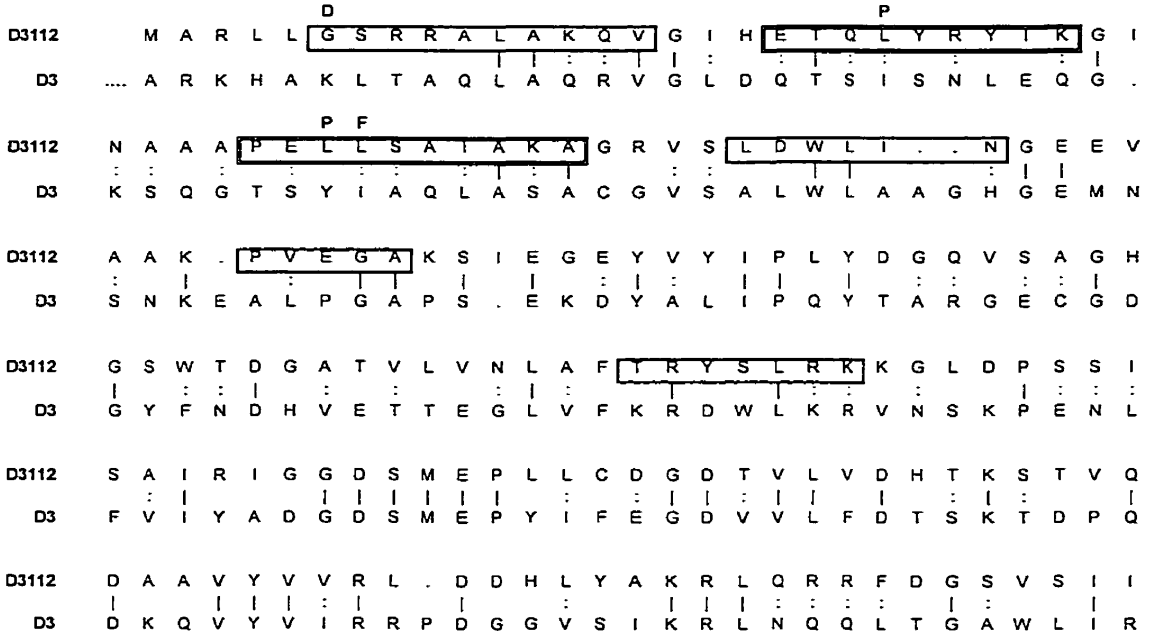
–=no plaque, + = slight clearing, ++ = turbid region of lysis, +++ = complete lysis

superinfection. As a positive control, strain PAO1/pSWL12 (pTJS140 containing the original left-end fragment and expressing the temperature-sensitive repressor cts15) shows a high level of immunity to D3112 superinfection at all phage dilutions tested at 32°C (Autexier *et al.*, 1991). Of the mutagenized plasmids, only pKAS306 displays reduced immunity to D3112 superinfection. Immunity is at a level comparable to that of the vector alone, indicating that the second GTG is most probably used as the translational start codon.

2.4.2 Cloning and sequencing of the wild type repressor ORF

Since only the temperature-sensitive (ts) repressor had been previously sequenced (Autexier *et al.*, 1991), we sought to determine the sequence of the wild type D3112 c repressor protein. To accomplish this, the left-end *Hin*dIII fragment from the D3112 c^+ phage genome was cloned into the vector pUC119 generating plasmid pOF4. DNA sequencing revealed a single TA→CG mutation at bp 780 (Autexier *et al.*, 1991). This single bp mutation results in a change from a glycine in the wild type repressor at amino acid position 6 to an aspartic acid in the ts mutant (Figure 2.1). In addition, plasmid pOF6, containing the wild type repressor ORF, was able to confer immunity to D3112 superinfection at 42°C as well as 32°C, unlike the ts repressor ORF, from plasmid pSWL12, which functions only at 32°C (Autexier *et al.*, 1991) (Table 2.2).

Figure 2.1. Comparison of the amino acid sequences of the lysogenic repressor proteins of *Pseudomonas* phages D3112 and D3. Straight lines (|) indicate identity between amino acids, colons (:) indicate similarity between amino acids. Boxed amino acids indicate predicted helices of D3112 (see text) including the predicted helix-turn-helix motifs of both proteins (double lines). Shown above the D3112 sequence is the location of the *c*ts mutation (D6G) and the three repressor mutants L22P, L36P, L37F.


D3112 M A R L L G S R R A L A K Q V G I H E T Q L Y R Y I K G I
D3 .... A R K H A K L T A Q L A Q R V G L D Q T S I S N L E Q G .
D3112 N A A A P E L L S A I A K A G R V S L D W L I . . N G E E V
D3 K S Q G T S Y I A Q L A S A C G V S A L W L A A G H G E M N
D3112 A A K . P V E G A K S I E G E Y V Y I P L Y D G Q V S A G H
D3 S N K E A L P G A P S . E K D Y A L I P Q Y T A R G E C G D
D3112 G S W T D G A T V L V N L A F T R Y S L R K K G L D P S S I
D3 G Y F N D H V E T T E G L V F K R D W L K R V N S K P E N L
D3112 S A I R I G G D S M E P L L C D G D T V L V D H T K S T V Q
D3 F V I Y A D G D S M E P Y I F E G D V V L F D T S K T D P Q
D3112 D A A V Y V V R L . D D H L Y A K R L Q R R F D G S V S I I
D3 D K Q V Y V I R R P D G G V S I K R L N Q Q L T G A W L I R

2.4.3 Isolation of Repressor mutants unable to confer immunity

To define important motifs of the c repressor protein, plasmid pSWL12, which is able to confer immunity (Autexier *et al.*, 1991), was mutagenized with hydroxylamine (HA). The chemical mutagen HA is able to induce transition mutations within DNA of either GC→AT or AT→GC (Freese *et al.*, 1961a, 1961b; Tessman *et al.*, 1967). These mutagenized plasmids were electroporated into PAO1 cells and screened for the loss of the ability to confer immunity to D3112 superinfection using an *in vivo* immunity assay (Levin and DuBow, 1989; see Material and Methods). From the primary screening, 11 plasmids with reduced ability to confer immunity to D3112 superinfection were isolated (data not shown). The *PstI/Eco*RI fragment from the 11 mutagenized plasmids were cloned into pTJS140 and again screened to confirm that this loss in immunity was associated with a mutation within the *c* ORF and not the vector itself. After this second round of screening, seven of the original plasmids were still unable to confer immunity to superinfection *in vivo* (Table 2.3). These seven plasmids were subsequently subcloned into pUC119 for sequencing. Of the seven plasmids, five showed a mutation of an AT→GC at position 732, which resulted in the change Leu22Pro (e.g. pOF7, Table 2.3). One mutant (pOF8) contained the mutation AT→GC at position 690, which resulted in the change Leu36Pro. The other mutant contained a change of GC→AT at position 688, which resulted in Leu37Phe (pOF14).

Table 2.3. Identification of mutations within plasmids (and in the protein ORF) that conferred immunity following a second screening.

Plasmid	Mutation*	
pOF7	AT→GC	L22P
pOF8	AT→GC	L36P
pOF14	GC→AT	L37F

*mutations are indicated as bp mutation followed by mutation within the coding region of the protein.

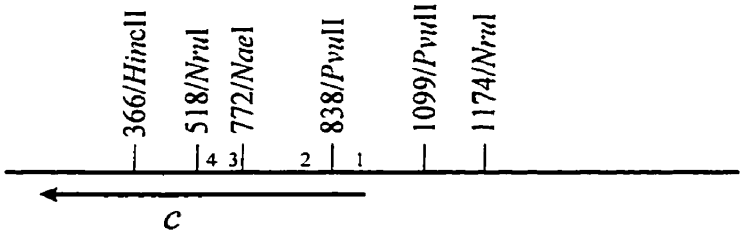
2.4.4 Analysis of Pc, the D3112 c repressor promoter

The promoter for the *c* ORF (P*c*) was located using β-galactosidase assays. Fragments spanning a site from inside the *c* ORF to upstream of this ORF were subcloned into the *lacZ* transcriptional fusion broad host range plasmid pDN19lacΩ (Totten and Lory, 1990) (Figure 2.2). When expressed in *P. aeruginosa* wild type strain PAO1, plasmid pKAS220 (containing an *Nru*I-*Hin*cII fragment from bp1174-366) produced 1206 Miller units of β-galactosidase. Further deletions of this fragment in pKAS224 (*Nru*I-*Nar*I), pKAS228 (*Nru*I-*Nae*I) and pKAS232 (*Pvu*II-*Pvu*II) displayed statistically insignificant increases in the amount of promoter activity (Figure 2.2). From these results, the smallest fragment to show promoter activity resided within a *Pvu*II fragment spanning bp1099-838 (pKAS232).

To determine the size of the *c* transcript, total cellular RNA was isolated from PAO1, PAS429 and PAS429^{IND} (PAS429 induced; see Materials and Methods) cells, subjected to electrophoresis on a 1.2% agarose/formaldehyde gel, and transferred to a nitrocellulose membrane. The *c* gene (bp770-170) was used as the probe. Following hybridization, a 900nt transcript was detected only from RNA isolated from PAS429 cells (Figure 2.3).

The position of the P*c* promoter and the transcriptional start site were mapped using primer extension (Figure 2.4). The transcriptional start site was found to be located at position 940, centered 154bp upstream of the translation initiation codon (second GTG).

Figure 2.2. Analysis of deletion constructs for P*c* promoter activity. The insert in each plasmid, cloned just 5' of the promoterless β-galactosidase gene in pDN19lacΩ is shown along with levels of β-galactosidase activity in *P. aeruginosa* strain PAO1. The results represent the average of a minimum of three separate experiments, and Standard Deviations are shown. The arrow reflects the direction of transcription through the promoterless *lacZ* gene. The top of the Figure shows a restriction enzyme map of the very left-end of the D3112 genome with the location of the *c* repressor ORF and positions of the four putative GTG start codons (1→4).



Plasmid		β-Galactosidase Activity (Miller Units)
pDN19lacΩ		45 ± 3
pKAS220	←	1206 ± 110
pKAS224	←	1251 ± 59
pKAS228	←	1262 ± 70
pKAS232	←	1275 ± 77

Figure 2.3. Northern blot analysis of total cellular RNA from *P. aeruginosa* strains PAO1, PAS429 and PAS429^{IND} using the D3112 *c* gene as a probe. On the left of the figure is the 0.4 to 9.5kb RNA ladder. The arrow points to a 0.9kb mRNA that hybridized to the probe.

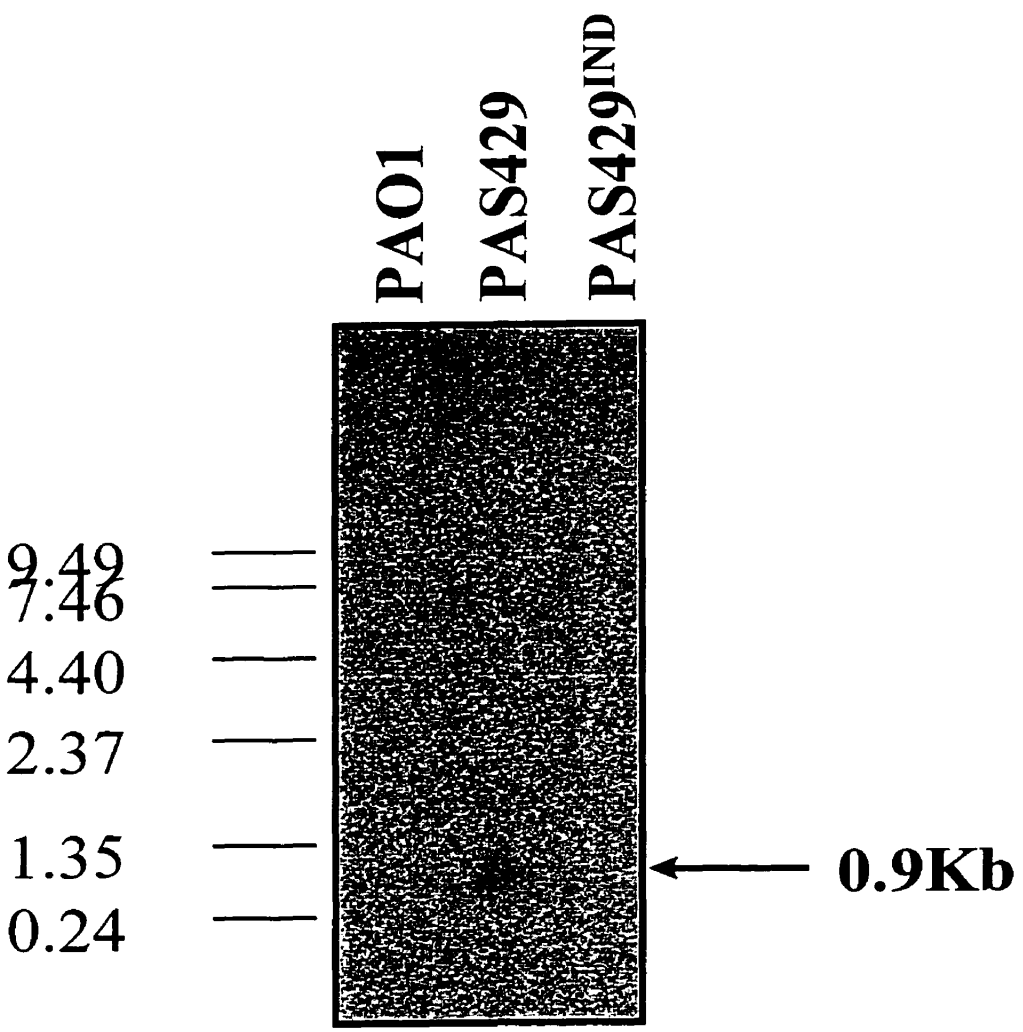
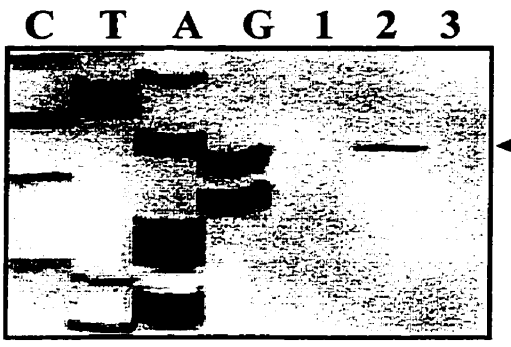

PAO1
PAS429
PAS429IND
9.49
7.46
4.40
2.37
1.35
0.24
0.9Kb

Figure 2.4. (A) Transcriptional start site of D3112 *c* mRNA. Lanes: 1, PAO1; 2, PAS429; 3, PAS429^{IND}; C, T, A, and G, nucleotide-specific sequencing reactions. The arrow points to the extended product on the sequence corresponding to the start site. (B) Sequence of the P*c* promoter region (numbered from the left-end of the D3112 genome). Boxed nucleotides indicate the putative –10 and –35 regions as well as the +1 transcription start site.

A.



3′
T
T
C
A
A*
G
G
C
G
G
5′

B.

+1

5′-AACATAACACATCTGATTGGCTGATCAAGACGCAACAGTAAAGTTCCG-3′
TTGTATTGTGTAGACTAACCGACTAGTTCTGCCTTGTCATTTCAAGGC

972 940

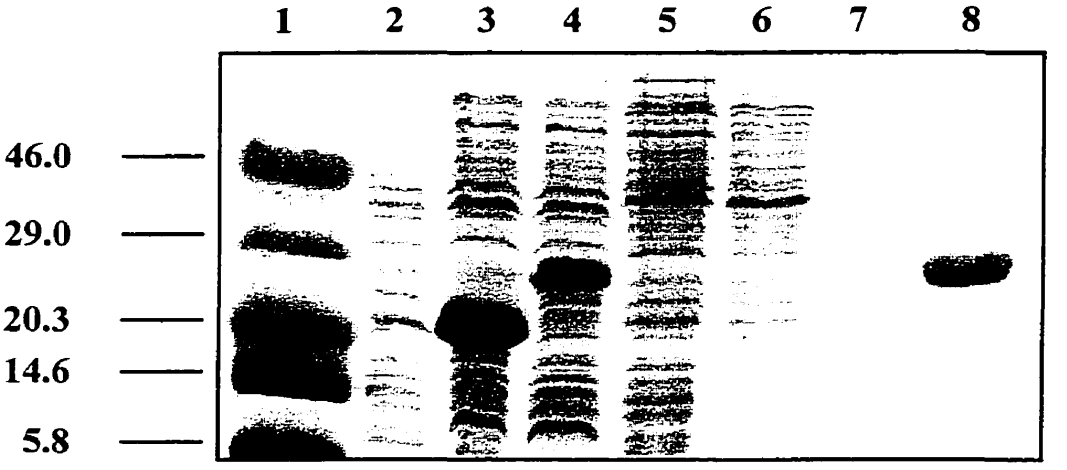
2.4.5 Overexpression and purification of the D3112 cts repressor protein

To facilitate further analysis of the D3112 repressor, two plasmids containing the *c* ORF, commencing with either the second or third GTG and ending with the last codon of the ORF, were constructed (see Materials and Methods). The cloning procedure (two different PCR products cloned into plasmid pET29-b) created fusions of six histidine (His6) codons, in frame with the *c* ORFs, at their 3' ends. The resulting plasmids, pKAS210 and pKAS211, were used to transform *E. coli* BL21(DE3) cells, which contain the T7 RNA polymerase gene inserted into the chromosome (Studier and Moffatt, 1986) and induced as outlined in the Materials and Methods. The uninduced and induced whole cell extracts expressing the D3112 *c*ts15 fusions (cts15-His6 from the second GTG and Δcts15-His6 from the third GTG) were analyzed on a 20% SDS polyacrylamide gel (PAGE). A band, representing the cts15-His6 repressor, migrated at an apparent molecular weight of 28.4kDa (Figure 2.5, lane 4) as compared with the molecular weight markers (lane 1), unlike induced extracts containing the vector alone (lane 2). A band, representing the Δcts15-His6, migrated at an apparent molecular weight of 19kDa (lane 3). Both the cts15-His6 (lane 7) and the Δcts15-His6 (not shown) proteins were purified using Ni-NTA agarose as described in the Material and Methods.

2.4.6 Preliminary characterization of cts-His6 and Δcts15-His6

A band retardation assay (Fried and Crothers, 1981; Garner and Revzin, 1981; Kukolj and DuBow, 1991) was performed to characterize the DNA binding properties of the D3112 repressor. In this assay, a non-specific ^{32}P-labeled *Hin*fI fragment from

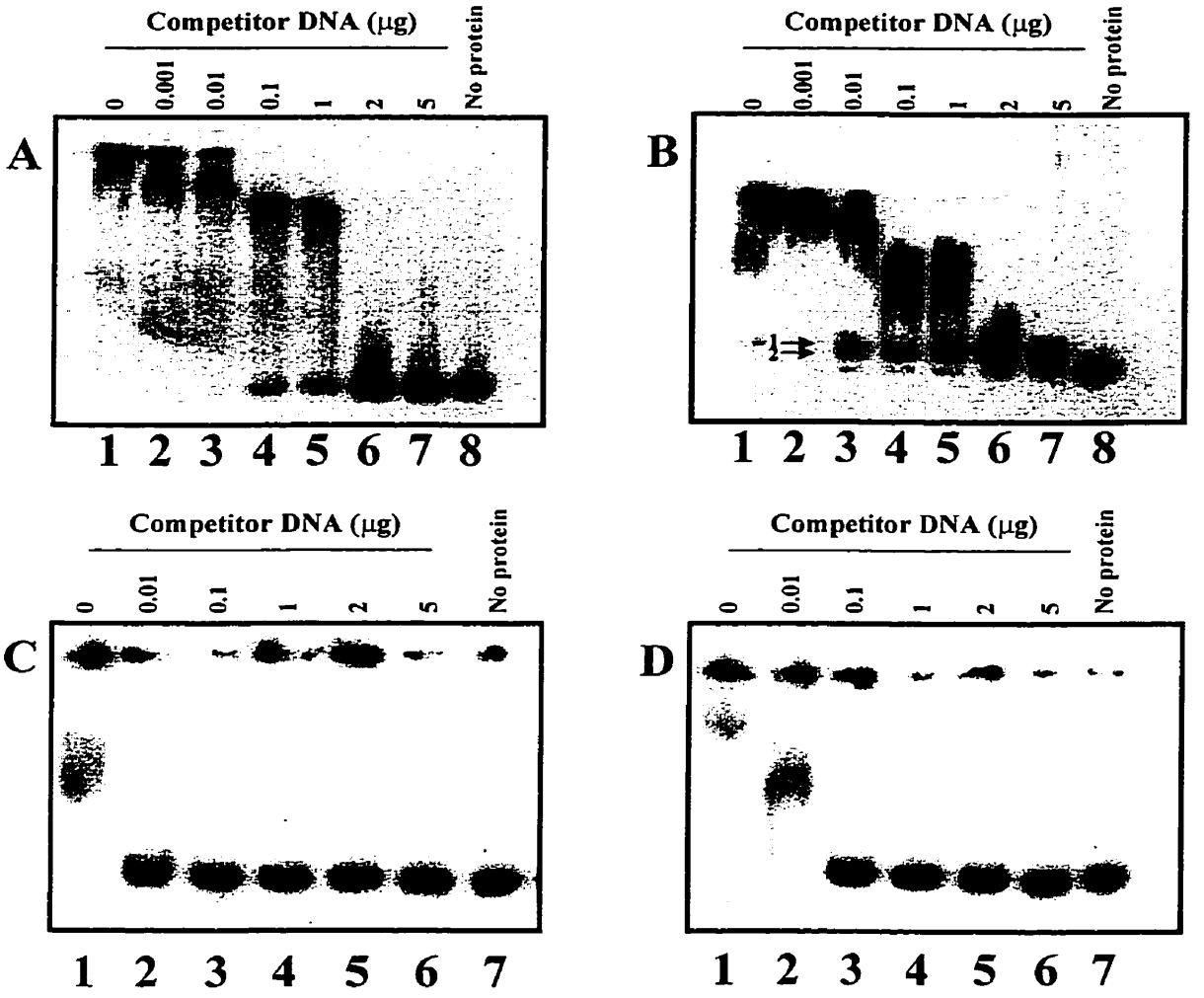
Figure 2.5. SDS-PAGE analysis of D3112 c repressors. Lane 1, prestained molecular weight markers; lane 2, crude extract of BL21(DE3)/pET29-b induced for 4hrs at 1mM IPTG; lane 3, crude extract of BL21(DE3)/pKAS211 induced for 4 hrs at 1mM IPTG; lane 4, crude extract of BL21(DE3)/pKAS210 induced for 4hrs at 1mM IPTG; lanes 5-8 are some of the purification steps of cts15-His6. Lane 5; flow-through; lane 6, 20mM Imidazole wash, lane 7, 80mM Imidazole wash; lane 8, elution at 250mM imidazole.


1
2
3
4
5
6
7
8
46.0
29.0
20.3
14.6
5.8

pBR322, or a ^{32}P-labeled *Eco*RI-*Hin*dIII fragment from pKAS230, containing the region flanking the P*c* promoter (herein referred to as the specific fragment), was incubated with 500ng of purified cts15-His6. The cts15-His6 protein readily bound to both of these fragments (Figure 2.6A, lane 1 and Figure 2.6B, lane 1, respectively). Upon addition of non-labeled competitive substrate (2μg sonicated calf thymus DNA), binding of cts15-His6 to the non-specific pBR322 fragment was abolished (Figure 6A, lane 6) whereas the binding of the specific fragment was not completely abolished even after the addition of 5μg competitor DNA (Figure 2.6B, lane 7). The electrophoretic migration of cts15-His6 with the specific DNA fragment displayed two fragments whose migration was inhibited (Figure 2.6B, lanes 3-7).

Using the same specific and non-specific ^{32}P-labeled fragments, binding of 500ng of purified Δcts15-His6 protein was also studied. As illustrated in Figure 2.6C (lane 1) and 2.6D (lane 1), Δcts15-His6 was able to bind to both the non-specific and specific fragments. However, binding by Δcts15-His6 to the non-specific fragment was abolished upon the addition of 0.01μg non-labeled competitor DNA (Figure 2.6C, lane 2). Binding to the specific fragment was abolished upon the addition of 0.1μg competitor DNA (Figure 2.6D, lane 3).

Figure 2.6. Band retardation assays with cts15-His6 and Δcts15-His6. (A) Band retardation assay of cts15-His6 with a 154bp *Hin*fI non-specific fragment from pBR322. Lanes 1-7: 5000cpm radiolabeled fragment with 500ng purified cts15-His6 incubated with increasing amounts of sonicated calf thymus DNA (competitor DNA). Lane 8 is 5000cpm radiolabeled fragment alone. (B) Band retardation assay of cts15-His6 with a 261bp *Pvu*II fragment from the D3112 left-end. Lanes 1-7: 5000cpm radiolabeled fragment with 500ng purified cts15-His6 incubated with increasing amounts competitor DNA. Lane 8 is 5000cpm labeled fragment alone. The arrows in (B) indicate the two fragments whose migration was inhibited upon incubation with cts15-His6. (C and D) Band retardation assays as in (A) and (B) except that 500ng of purified Δcts15-His6 was used.


A
Competitor DNA (μg)
0
0.001
0.01
0.1
1
2
5
No protein
1 2 3 4 5 6 7 8
B
Competitor DNA (μg)
0
0.001
0.01
0.1
1
2
5
No protein
1 2 3 4 5 6 7 8
C
Competitor DNA (μg)
0
0.01
0.1
1
2
5
No protein
1 2 3 4 5 6 7
D
Competitor DNA (μg)
0
0.01
0.1
1
2
5
No protein
1 2 3 4 5 6 7

2.5 Discussion

This paper describes the initial characterization of the lysogenic c repressor from the *Pseudomonas* transposable phage D3112. Previous inspection of the D3112 left-end sequence (Autexier *et al.*, 1991) revealed the existence of a single large ORF commencing with a GTG codon. Three internal in-frame GTG codons, which could also be used as the start codon for c repressor translation, were also identified. Through site-specific mutagenesis, each of the four GTG codons was converted to a GTA codon. This preserved the incorporation of a valine such that internal codons would be unaffected, however, the new GTA codon would be unable to act as a start codon for translation of the protein (Neidhardt *et al.*, 1990). D3112 c repressor activity *in vivo* was detected by subcloning the mutagenized *c* ORFs into the broad host range vector pTJS140 (Darzins and Casadaban, 1989a) and transformed into *P. aeruginosa* PAO1 cells. Lawns of these transformed cells were then tested with various dilutions of D3112 phage lysate and incubated overnight to allow infection to occur (*in vivo* immunity assay). As reported previously, plasmid pSWL12 containing the leftmost 1.8kbp of D3112 DNA (Autexier *et al.*, 1991) conferred immunity to D3112 superinfection at all dilutions of the D3112 phage lysate tested, as did plasmids pKAS305, 307 and 308 (Table 2.2). In contrast, plasmid pKAS306 was unable to confer immunity to D3112 superinfection, indicating that the second GTG is the probable start codon for the lysogenic c repressor.

Sequencing of the wild type D3112 c^+ gene and comparison with the D3112 *c*ts15 mutant (Autexier *et al.*, 1991) revealed a single AT→GC base pair mutation that

results in an amino acid change from a glycine, in the wild type repressor at position 6, to an aspartic acid, in the temperature-sensitive repressor (Figure 2.1; putative α-helix 1). Interestingly, the change of a glycine to an aspartic acid leading to a temperature-sensitive protein function is found in at least five other proteins: the Mu c repressor (Vogel *et al.*, 1991), phage T4 lysozyme (Gray and Mathews, 1987), *E. coli* ribosomal protein L24 (Nishi *et al.*, 1987), phage P22 tail spike endorhamnosidase (Yu and King, 1984) and the λ cI repressor (Nelson *et al.*, 1983; Groisman *et al.*, 1984). Although these conserved temperature-sensitive changes do not occur within currently identifiable protein motifs, in each of these proteins the change does occur before a putative α-helix region. In our case, as is seen with Mu c repressor (Vogel *et al.*, 1991), this mutation occurs in the first putative α-helix in the N-terminus of the D3112 c repressor (Figure 2.1).

The protein with the highest homology to D3112 c is the cI repressor from the λ-like *P. aeruginosa* bacteriophage D3 (Farinha *et al.*, 1994) (Figure 2.1). The two proteins share 64.3% similarity (including 33.6% identity) and the majority of the similarity appears to lie within the C-termini of these proteins. This may be significant, as Farinha *et al.* (1994) have predicted that the C-terminus of the D3 cI repressor contains the dimerization domain whereas the putative helix-turn-helix (H-T-H) motif is located within the N-terminal region. It is thus possible that the C-terminal region of the D3112 repressor could also contain the dimerization domain, while the N-terminus contains the DNA binding domain. This result is supported by the fact that putative α-helices were identified only in the N-terminus of the protein (Figure 2.1) using a secondary structure prediction algorithm (McClelland and Rumelhart, 1988; Kneller *et al.*, 1990).

In order to identify residues of the D3112 c repressor protein required for proper functioning, we employed a random mutagenesis protocol. Mutagenesis of the complete repressor coding region, present in plasmid pSWL12 (Autexier *et al.*, 1991), was performed with the chemical mutagen hydroxylamine, which causes predominantly GC→AT (and sometimes AT→GC) transitions, mainly as a result of its reaction with cytosine (Freese *et al.*, 1961a, 1961b; Tessman *et al.*, 1967). Out of 100 originally selected mutated plasmids, only seven showed significant reduction in function following a second screening. All of these mutations were found to be within the N-terminal region of the protein and within a putative H-T-H motif (Figure 2.1, Table 2.3). Of the seven D3112 repressor mutants sequenced in this study, five were found to have the same mutation of Leu22Pro in the N-terminus of the protein. From computer structure predictions, this mutation appears to lie in the first α-helix of our predicted H-T-H motif (Figure 2.2). Leucine is an aliphatic, hydrophobic amino acid, which makes it an ideal residue for the inside of proteins and regions of α-helices that do not have contact with the aqueous surroundings (Branden and Tooze, 1991). In contrast, proline is an imino acid and a known α-helix-disrupter with a tendency to introduce sharp kinks in protein secondary structure. A mutation of a leucine to a proline in this α-helix would change a functional α-helix to a dysfunctional motif which could no longer fold correctly or bind DNA. Of the two other mutations detected using this protocol (Leu36Pro and Leu37Phe), both appear to be located in the third putative α-helix which is the second α-helix of the putative H-T-H motif. In other DNA-binding proteins, such as λ cI, this second α-helix of the H-T-H motif is the so-called DNA-recognition α-helix (Pabo and

Lewis, 1982). Therefore, if this second α-helix is, in fact, the c repressor DNA recognition α-helix, disruption would clearly abolish specific DNA binding by the repressor protein and result in the loss of activity of the protein. The Leu36Pro mutation would likely disrupt this third α-helix. However, the Leu37Phe change is not as obviously disruptive to protein structure, as both are aliphatic hydrophobic amino acids. One difference between the two, however, is that while leucine is a branched chain amino acid, phenylalanine contains a bulky aromatic ring. This difference may somehow destabilize protein function enough to abrogate function.

Further understanding of the D3112 *c* repressor ORF was accomplished by defining the promoter, P*c*. Fragments spanning the upstream regions of the *c* ORF were subcloned into the broad host range promoterless *lacZ* vector pDN19lacΩ (Totten and Lory, 1990) and used to transform the wild type *P. aeruginosa* strain PAO1. From the β-galactosidase assays (Figure 2.2), the fragment spanning bp1099-838 (a *Pvu*II fragment, pKAS232) is the smallest fragment conferring β-galactosidase activity. This indicates that the promoter, P*c*, resides within these 261bp.

Determination of the transcriptional start site of the *c* gene using a primer extension analysis was used to precisely map the position of P*c* (Figure 2.4). The transcriptional start site was located 154bp upstream from the second GTG at position 940 and was centered 8bp downstream of a putative σ^{70} promoter sequence [-10 (AACAGT)/-35 (CTGATT)] (Hawley and McClure, 1983) identified with the Neural Network Promoter Prediction algorithm (Reese, 1994; Reese and Eeckman, 1995; Reese *et al.*, 1996). In addition, the *c* repressor gene transcribed as a 900nt mRNA (Figure 2.3)

indicating that D3112 *c* is monocistronic, as seen in the two coliphages Mu and D108 (Krause *et al.*, 1983; Levin and DuBow, 1989).

To overexpress and purify the D3112 repressor, the *c* gene, beginning with the second and third GTG codons, were amplified by PCR and cloned into the pET29-b expression vector. The purified D3112 cts15-His6 repressor protein (Figure 2.5) bound specifically to a fragment located directly upstream of the *c* ORF (Figure 2.6B) but the Δcts15-His6 protein (expressed from the third GTG) did not (Figure 2.6D). Neither the cts15-His6 nor the Δcts15-His6 purified proteins bound with high affinity to a pBR322 *Hin*fI fragment (Figure 2.6A, and C). D3112 cts15-His6 bound to the specific fragment produces two distinct bands upon binding (Figure 2.6B, lanes 3-7). The formation of these two bands suggests that there may be two operators located within this fragment to which repressor binds, a result also observed with the c repressor from the transposable coliphage D108 (Kukolj and DuBow, 1991). As both Mu and D108 repressors have been shown to bind specifically to operators located in positions analogous to this fragment, this data suggests that putative operators for D3112 repressor exist in this region.

Binding of cts15-His6, but not the Δcts15-His6 protein, to the specific fragment also supports the second GTG as the start codon. Our prediction of the H-T-H motif beginning at amino acid 19 and ending at amino acid 43 would therefore not be expressed as part of the third GTG codon-initiated protein (Δcts15-His6) as the location of the third GTG is at position 661 (amino acid 46 of the second GTG codon initiated protein). As Δcts15-His6 failed to demonstrate specific binding activity with a fragment found directly

upstream of the *c* ORF, this suggests that the DNA-binding domain is lacking from this protein.

The D3112 c repressor region has been shown to have an organization similar to that seen in the two coliphages Mu and D108. However, the c protein exhibits higher homology to the λ-related family of cI repressor proteins. The location of the promoter, P*c*, in a position analogous to that seen in Mu and D108, and specific binding to this region, also suggests an evolutionary relatedness to the two coliphages. Moreover, in Mu and D108, binding of the repressor to its operators is positioned to ensure the obstruction of transcription from the early promoter P*e*, and subsequent repression of transcription of the *ner* gene during lysogeny. D3112 has not been shown to possess a *ner* homologue in the analogous position (Bidnenko *et al.*, 1996; Krylov *et al.*, 1980a, 1980b; Yanenko *et al.*, 1983, 1988). The localization of the other components of early gene regulation will be important in the understanding of D3112 c repressor function in the lytic-lysogenic switch of this *Pseudomonas* transposable bacteriophage.

Chapter 3

Identification and characterization of a phage Mu/D108 Ner homologue in the *Pseudomonas aeruginosa* transposable bacteriophage D3112

Preface to Chapter 3

In the previous Chapter, characterization of the D3112 repressor and the regulation of the repressor *c* gene were undertaken. The results of this study indicated that D3112 is organizationally similar to the coliphages Mu and D108 with respect to location and transcription of the repressor gene. Unlike the two coliphages, however, D3112 has not been shown to possess an equivalent of the *ner* gene, which is required for lytic development in Mu and D108. This Chapter presents the identification and characterization of a *ner* homologue in D3112 and evidence that regulation of the lytic-lysogenic switch in D3112 is likely different to that found in the two coliphages.

3.1 Abstract

The *ner* gene of transposable phages Mu and D108 is the first gene of the early (transposase) operons, where it functions to turn down repressor gene expression during lytic growth and transposase gene expression later in the lytic cycle. Homologues of Ner have been found in the genomes of eubacteria and humans. We have identified a Ner homologue in the *Pseudomonas aeruginosa* Mu-like transposable bacteriophage D3112. Using cloned fragments containing the D3112 promoter, P*c*, fused to a *lacZ* reporter gene, β-galactosidase expression was blocked when this fragment was lengthened from bp1099 to bp1439. A previously unidentified open reading frame (ORF) was located from bp1033-1384 that demonstrated 61% similarity and 35% identity to the Ner homologue in *Neisseria meningitidis* and 60% similarity and 40% identity to the Ner-like protein (Nlp) of *Escherichia coli.* In addition, the D3112 Ner protein shares 54% similarity (33% identity) to the Ner protein of coliphage D108 and 57% similarity (33% identity) to the Ner protein of coliphage Mu. The *ner* promoter has been located to bp957-992 using β-galactosidase assays and primer extension analyses, and Northern blotting demonstrated that D3112 *ner* is part of a 2.2kb mRNA transcript. As the *ner* gene begins at bp1033 and the transposase *A* gene begins at bp2539, this transcript is not large enough to encode for the *A* and *B* genes, as is seen in the early transcripts of coliphages Mu and D108. Between the D3112 *ner* gene and *A*, there are three putative ORFs not present in the Mu or D108 early regions. The *ner* promoter was shown to be under the control of the D3112 repressor, as demonstrated using *in vivo* β-galactosidase

assays. When D3112 Ner was overexpressed and purified, it bound to a 261bp *Pvu*II fragment from the D3112 intergenic region. Our results suggest that D3112 regulation uses Mu and D108-like functions, but that their roles may be different.

3.2 Introduction

Bacteriophage D3112 is a temperate, transposable bacteriophage that infects the medically and environmentally important bacterium *Pseudomonas aeruginosa.* Its genetic organization was shown to be similar, but not homologous, to that of coliphages Mu and D108 (Krylov *et al.*, 1980a, 1980b; Yanenko *et al.*, 1983, 1988; Akhverdyan *et al.*, 1985; reviewed in DuBow, 1994). Mu and D108 are temperate bacteriophages of *Escherichia coli* that propagate their DNA by undergoing up to 100 cycles of DNA transposition per hour during the lytic cycle (reviewed in Pato, 1989; DuBow, 1994). In the two coliphages, the choice between lytic and lysogenic development is regulated by several host functions and two phage-encoded gene products, the repressor (c) and the product of the *ner* gene, both of which act at the level of transcription (van de Putte *et al.*, 1980). During the lysogenic cycle, repressor *(c)* is transcribed from right to left from its own promoter, P*c* (van Meeteren *et al.*, 1980; Kukolj and DuBow, 1991). Repressor binds to each of its three (Mu) or two (D108) operators and blocks transcription from the early promoter, P*e* (Krause and Higgins, 1986; Kukolj and DuBow, 1991). During lytic development, *ner* is the first gene expressed from P*e* (Krause *et al.*, 1983; Marrs and Howe, 1990), along with the transposase *(A)* and *B* genes. Ner turns off production of the repressor gene *c* by binding to its operator site, which blocks expression from P*c* (Barlach and Shumann, 1983; Kukolj *et al.*, 1989; Tolias and DuBow, 1986; Wijffelmann *et al.*, 1974). By binding to this operator, Ner is also capable of turning down expression of the early operon to allow a level of early gene expression sufficient to amplify, via DNA

transposition, the viral genome. The *E. coli* histone-like DNA-binding protein H-NS also appears to specifically inhibit P*e* transcription (van Ulsen *et al.*, 1996). Binding of Integration Host Factor (IHF) to its site upstream of P*e* results in the direct stimulation of P*e* (Giphart-Gassler *et al.*, 1979; Goosen and van de Putte, 1984; Krause and Higgins, 1986; Levin and DuBow, 1989; Kukolj and DuBow, 1992). The binding by IHF at its site also interferes with the formation of P*e*-H-NS DNA-protein complexes, thereby alleviating H-NS-mediated repression at P*e* (van Ulsen *et al.*, 1996).

Although much work has been done to understand the genetics behind D3112 infection of *P. aeruginosa*, the mechanism underlying the lytic/lysogenic decision is not well understood. For example, previous genetic mapping (Gerasimov *et al.*, 1985, Yanenko *et al.*, 1983, 1988) and nucleotide and protein homology searches (Autexier *et al.*, 1991) have not located any gene resembling *ner* in the early region. In its place, *cip*, or *c*ontrol of *i*nteraction of *p*hages (Gerasmiov *et al.*, 1985; Bidnenko *et al.*, 1996) has been located. Although referred to as the *ner*-like homologue in D3112 (Bidnenko *et al.*, 1996), the proposed *cip* function appears to be more analogous to Rex of λ (reviewed in Court and Oppenheim, 1983; Synder and Kaufaman, 1994) than to Ner of Mu and D108, as the *cip* function has been described as conferring immunity to phage B39 infection in D3112 polylysogens (Gerasimov *et al.*, 1985; Bidnenko *et al.*, 1996).

As D3112 shows the potential to be developed into a very powerful genetic tool in *P. aeruginosa* much like phage Mu in *E. coli* (Groisman *et al.*, 1984; Groisman and Casadaban, 1986; Groisman and Casadaban, 1987a, 1987b), it becomes important to understand the molecular controls governing its lytic/lysogenic switch. In this paper, we describe the identification, characterization, and cloning of a *ner* homologue in D3112,

located between the repressor *c* gene and the region known as *cip*, and the regulation of its expression.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids and growth conditions.

All bacterial strains and plasmids used in this work are described in Table 3.1. Cells were grown at 37°C in Luria Bertani medium (Sambrook *et al.*, 1989) for both *P. aeruginosa* and *E. coli*, except for PAS429, which was grown at 32°C. Plasmids were transformed into *E. coli* using the rubidium chloride method (Hanahan, 1983) and into *P. aeruginosa* using electroporation with 15% glycerol/1mM MOPS as the buffer (Farinha and Kropinski, 1990). The DNA used for the transformations and electroporations was prepared using either an alkaline-lysis method (Sambrook *et al.*, 1989) or Qiagen quick-spin columns (Qiagen, Mississauga, ON). Where required for plasmid maintenance, antibiotics were added at the following concentrations: for *E. coli*, ampicillin (Ap) at 50μg/ml, kanamycin (Km) at 50μg/ml, streptomycin (Str) at 100μg/ml, and tetracycline (Tc) at 10μg/ml; for *P. aeruginosa*, pipercillin (Pip), 100μg/ml, Str at 300μg/ml, and Tc at 200μg/ml.

Table 3.1. Bacterial strains, phages and plasmids used in this study.

Strain/Phage/ Plasmid	Relevant characteristics	Reference/Origin
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type, prototroph	Holloway, 1969
PAS429	PAO1 *ami*::D3112 *cts*15	J. Shapiro
Escherichia coli		
BL21(DE3)	F⁻ *ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm* (DE3)	Studier and Moffatt, 1986
DH5α	*supE44 ΔU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
Plasmids		
pBR322	Ap^R, Tc^R, cloning vector	Bolivar *et al.*, 1977
pCR2.1	T-cloning vector	Invitrogen
pUC119	Ap^R;cloning vector	Vieira and Messing, 1987
pET29-b	Km^R; C-terminal 6XHIS expression vector	Novagen, Inc.
pDN19lacΩ	Str^R/Spc^R, Tc^R; Broad host range, promoterless *lacZ* vector	Totten and Lory, 1990
$pB28_L$	left-end (backfilled-*Hin*dIII) fragment of D3112 *cts*15 into the *Sma*I-*Hin*dIII site of pUC119	Autexier *et al.*, 1991
pSWL12	*Nru*I-*Eco*RI fragment from $pB28_L$ into the *Sma*I-*Eco*RI site of pTJS140	Autexier *et al.*, 1991
pKAS220	bp 1174-bp 366 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS221	bp366-bp1174 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS225	bp518-bp1174 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS229	bp772-bp1172 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
PKAS230	bp838-bp1099 *Pvu*II fragment of D3112*cts* cloned into the *Sma*I site of pUC119	This study
pKAS232	bp838-bp1099 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS244	bp1818-bp366 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS249	bp1-bp1103 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study

Table 3.1. Con't.

Strain/Phage/ Plasmid	Relevant characteristics	Reference/Origin
pKAS252	bp1439-bp772 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS314	D3112 *ner* (PCR product) in pCR2.1	This study
pKAS315	D3112 *ner* (from pKAS314) in pET29-b	This study

Ap^R, ampicillin resistance; K^{mR}, kanamycin resistance; Spc^R, spectinomycin resistance; Str^R, streptomycin resistance; Tc^R, tetracycline resistance

3.3.2 Enzymes and chemicals.

All restriction enzymes, T4 DNA ligase, T4 DNA Polymerase (backfilling reactions), and Superscript II Reverse Transcriptase, were purchased from either GIBCO-BRL Inc. (Burlington, ON) or Amersham-Pharmacia (Baie d'Urfe, QC). The Isotherm Sequencing Kit was purchased from Epicenter Technologies Inc. (Madison, WI). All other chemicals were purchased from either Fisher (Nepean, ON) or Sigma (St. Louis, MO).

3.3.3 PCR amplification.

PCR reactions were performed in a DNA Thermal Cycler (MJ Research, Inc.). For the PCR amplification of the *ner* ORF, reactions were performed in 50µl volumes containing *Taq* DNA polymerase. Each reaction mixture contained a final concentration of 50ng of DNA template, 2U of *Taq* DNA polymerase, 4mM $MgCl_2$, 0.1mM deoxynucleoside triphosphate mix, and 0.2µM primers (see below). Thirty cycles were performed for each reaction. Each cycle consisted of incubations for 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C. The primers used for the PCR reactions were purchased from GIBCO-BRL. Restriction sites were added at the ends of the primers (shown below in boldface) to facilitate subsequent cloning of the PCR products. Additional nucleotides were added 5' to the restriction sites to ensure efficient cleavage. The following primers were used in the PCR reactions. KS23 (5'-CCCC**CTCGAG**AGCCCTC GCTTCGTC-3') which places a unique *Xho*I site at the 3' end of the *ner* gene just before the natural

stop codon; and KS24 (5'-CCCCC**CATATG**ATTGAGATGAACATAGC-3') which places a unique *Nde*I site at the 5' end of D3112 *ner*.

3.3.4 DNA sequencing.

Single stranded DNA required for sequencing reactions was obtained by denaturing dsDNA as described (Lim and Pene, 1988). DNA sequencing was performed using the Isotherm Sequencing Kit (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions, and the products subjected to electrophoresis on either 5% polyacrylamide or 5% Long Ranger (JT Baker, Phillipsburg, NJ) sequencing gels as previously described (Maxam and Gilbert, 1980). The results were visualized by autoradiography using Kodak XAR-5 film for 24-72hrs at -70°C under Dupont Cronex intensifying screens.

3.3.5 Plasmid constructions.

Plasmids for the promoter-probe β-galactosidase assays were constructed in the following manner. Fragments from the D3112*c*ts15 genome (Figure 3.1) were made blunt (if required) and subcloned into the *Sma*I site of pUC119 and the orientation was determined by restriction enzyme digests. The plasmid containing the proper orientation was digested with *Eco*RI and *Bam*HI and the resulting fragment subcloned into the *Eco*RI/*Bam*HI site of the *lacZ*-based promoter probe plasmid pDN19lacΩ (Totten and Lory, 1990). The resulting plasmids are listed in Table 3.1.

Plasmid pKAS315, containing the D3112 *ner* gene, was constructed as follows: the *ner* gene was first amplified using PCR and primers KS23 and KS24 and ligated into the T-vector pCR2.1 (Invitrogen, San Diego, CA) to generate pKAS314. Plasmid pKAS314 was sequenced to ensure there were no mutations using the M13 Reverse Primer and the M13 Forward (-40) Primer (Epicentre Technologies). Plasmid pKAS314 was digested with *Nde*I and *Xho*I and the *ner* fragment was cloned into the *NdeI/XhoI* sites of the expression vector pET29-b (Novagen, Madison, WI) to generate pKAS315. This plasmid expresses D3112 *ner* which produces D3112 Ner with a 6-His tag at the C-terminus of the protein (D3112Ner-His6).

3.3.6 β-Galactosidase assays.

Expression of the *lacZ* gene, under the control of the putative *ner* promoter region, was measured by β-galactosidase assays as described by Miller (1972).

3.3.7 Northern blot hybridization.

Total cellular RNA was prepared from *P. aeruginosa* strains PAO1, PAS429 (grown at 32°C) and PAS429^{IND} (PAS429 grown at 32°C to $A_{550} = 0.7$ and then induced for 8 min at 42°C) by an adaptation of the CsCl purification procedure (Glisin *et al.*, 1974) as described by Deretic *et al.* (1987). Ten μg of RNA from each strain was subjected to electrophoresis on a 1.2% agarose/ formaldehyde gel (Ausebel *et al.*, 1989) and transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as previously described (Cai and DuBow, 1996). The 0.4 to 9.5kb RNA ladder (GIBCO-

BRL) was subjected to electrophoresis in parallel with the above samples and visualized by ethidium bromide staining prior to transfer to the nitrocellulose membrane. The probe used to detect D3112 *ner* mRNA was a 0.6kb *NdeI/XhoI* fragment from pKAS314 containing the entire D3112 *ner* gene (Table 3.1). The DNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, 1Ci=37Gbq, Amersham-Pharmacia) using the random priming method (Sambrook *et al.*, 1989) and random hexanucleotide primers (GIBCO-BRL). Hybridization was carried out as previously described (Mahmoudi and Lin, 1989). Unhybridized probes were removed by washing the filters twice at 68°C in 2X SSC, 1% SDS. The blots were air dried and exposed to a Molecular Dynamics Storage Phosphor Screen and visualized using a PhosphorImager SF and Image Quant Software Program (Molecular Dynamics, Sunnyvale, CA).

3.3.8 Transcriptional start site determination.

To establish the transcriptional start site of the *ner* transcript, primer KS22 (5'-GCTGGTACTTGATCCACTCCC-3'), complementary to the noncoding strand near the 5' end of the *ner* ORF, was end labeled with [γ-^{32}P]ATP (5000Ci/mmol; Amersham-Pharmacia) using polynucleotide kinase as instructed by the manufacturer (GIBCO-BRL). The labeled primer was purified using the Nucleotide Removal Kit (Qiagen Inc.) and the resulting labeled primer was used for the reverse transcription (RT) reactions. The total RNA template for the primer extension reactions were prepared as described above, and pre-treated with DNAseI prior to the RT reaction. Superscript II reverse transcriptase was used for the RT reactions as recommended by the manufacturer

(GIBCO-BRL). The DNA template for the sequencing reaction was $pB28_L$ and the primer was KS22. The products of the sequencing and RT reactions were subjected to electrophoresis on a standard 5% polyacrylamide sequencing gel and visualized using autoradiography as described above.

3.3.9 Protein overexpression and purification.

Plasmid pKAS315 was transformed into the *E. coli* strain BL21(DE3). A 5ml aliquot of an overnight of this strain was diluted into 200ml of LB medium and grown to OD_{550}=0.4, at which point isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 1mM. The IPTG was required to induce production of the T7 RNA polymerase from the host strain required to express the D3112 *ner* gene from the T7 promoter in pKAS315 (Studier and Moffatt, 1986). These cells were left to grow for an additional 4 hours. Purification of the D3112 Ner protein was performed under native conditions using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions with the following modifications: The lysis buffer contained 10mM imidazole and 1mM phenylmethylsulfonyl fluoride (PMSF); three wash buffers were used containing 20mM, 50mM or 80mM imidazole as well as 1mM PMSF; the elution buffer contained 250mM imidazole and 1mM PMSF. Samples of the crude lysate, washes and elutions were subjected to electrophoresis on a 20% SDS-PAGE gel as described (Laemmli, 1970).

3.3.10 Operator binding assay.

Specific binding of the D3112 Ner protein was detected using the gel electrophoretic shift method (Fried and Crothers, 1981; Garner and Revzin, 1981). The

buffer used in the binding reactions contained 20mM Tris-HCl, pH7.5, 1mM EDTA, 10mM 2-mercaptoethanol, 50mM NaCl, 100μg/ml bovine serum albumin and specified amounts of sonicated calf thymus DNA. Protein-DNA binding reactions were performed and subjected to electrophoresis through 5% polyacrylamide gels as described in Kukolj and DuBow (1991).

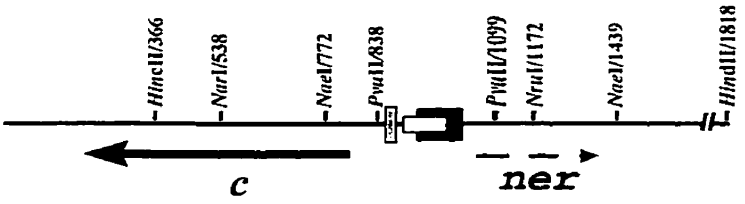
3.4 Results

3.4.1 Identification of a D3112 Ner homologue

We have previously characterized the lysogenic repressor *(c)* gene of D3112 (Chapter 2) in which we discovered the location of the P*c* promoter by cloning fragments of the D3112 genome into the *lacZ* transcriptional fusion vector pDN19lacΩ (Totten and Lory, 1990). When expressed in the wild type *P. aeruginosa* strain PAO1, plasmid pKAS220 (containing an *Nru*I-*Hin*cII fragment from the D3112 left-end regulatory region) produced approximately 1206 Miller Units of β-galactosidase activity (Figure 3.1). Further lengthening of this fragment to a *Hin*dIII site (bp1818; pKAS244, Figure 3.1) revealed a marked reduction of activity at P*c* (75 Miller Units). This effect was also found using plasmid pKAS252, which contains an *Nae*I fragment (72 Miller Units; bp1439-772).

These results suggest that there may be a repressor-like protein encoded within this region (bp772-1439) and thus a search for ORFs within this area was undertaken.

Figure 3.1. Analysis of constructs for promoter activity. The insert in each plasmid, cloned just 5' of the promoterless *lacZ* gene in pDN19lacΩ is shown along with levels of β-galactosidase activity in *P. aeruginosa* strain PAO1. The results represent the average of a minimum of three separate experiments, and Standard Deviations are shown. The arrow reflects the direction of transcription through the promoterless *lacZ* gene. The top of the Figure shows a restriction enzyme map of the very left-end of the D3112 genome with the location of the *c* repressor ORF (black arrow), the location of P*c* (□), the location of the D3112 *ner* ORF (hatched arrow), the location of the P*ner* promoter (■), and the location of the IHF consensus site (◘).



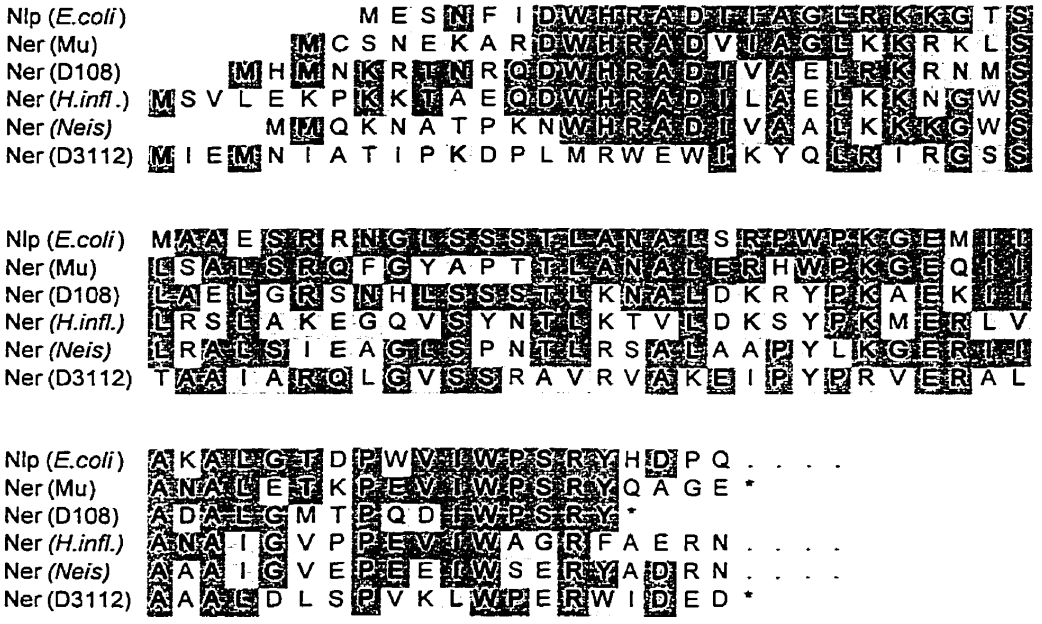
Plasmid		Activity
pKAS220	←	1206 ± 110
pKAS221	→	11,175 ± 206
pKAS225	→	11,306 ± 111
pKAS229	→	11,310 ± 136
pKAS233	→	11,310 ± 127
pKAS244	←//	75 ± 22
pKAS252	←	72 ± 27

One potential ORF, beginning with an ATG codon, was located at bp1033-1386 which could encode a protein of 117 amino acids. Subsequent translation and BLAST searches (Altschul *et al.*, 1997) indicated that this ORF shares 61% similarity and 35% identity with the Ner homologue of *Neisseria meningitidis* (Tinsley and Nassif, 1996) and 60% similarity and 40% identity with the Ner-like protein (Nlp) protein of *E. coli* (Choi *et al.*, 1989) over a 55 amino acid region. In addition, this ORF shares 54% similarity (33% identity) to the Ner protein of coliphage D108 (Tolias and DuBow, 1985) over a 65 amino acid region, and shares 57% similarity (33% identity) to the Ner protein of coliphage Mu (Priess *et al.*, 1982) over a 53 amino acid region. Figure 3.2 shows the homology of the D3112 Ner amino acid sequence with sequences of other Ner homologues found using the Gapped BLAST search program (Altschul *et al.*, 1997).

3.4.2 Analysis of the promoter, P*ner*, of the D3112 *ner* gene

The promoter for the *ner* ORF was located by cloning fragments from the D3112 genome into the *lacZ* transcriptional fusion broad host range plasmid pDN19lacΩ (Totten and Lory, 1990) (Figure 3.1). When expressed in the *P. aeruginosa* wild type strain PAO1, plasmid pKAS221 (containing a *Hin*cII-*Nru*I fragment) produced 11,175 Miller Units of β-galactosidase activity which is approximately ten-fold the activity seen at P*c* (pKAS220). Subsequent shortening of this fragment in pKAS225 (*Nar*I-*Nru*I) and pKAS229 (*Nae*I-*Nru*I) displayed only slight increases in the amount of promoter activity. From these results, the smallest fragment still able to produce promoter activity resided within a *Pvu*II fragment spanning bp838-1099 (pKAS233).

Figure 3.2. Comparison of the amino acid sequences of the Ner homologues to that of D3112 Ner (Altschul *et al.*, 1997). Shown are the amino acid sequences of the Nlp protein of *E. coli* (Choi *et al.*, 1989), coliphage Mu Ner (Preiss *et al.*, 1982), coliphage D108 Ner (Mizuuchi *et al.*, 1986), a Ner-like protein from *Haemophilus influenzae* Rd (Fleischmann *et al.*, 1995), and the Ner-like protein of *Neisseria meningitidis* (Tinsley and Nassif, 1996). Identical amino acids are shown in dark shading, similar amino acids are shown in light shading. The * indicates the stop codon.


Nlp (E.coli)
Ner (Mu)
Ner (D108)
Ner (H.infl.)
Ner (Neis)
Ner (D3112)

To determine the size of the *ner* transcript, total RNA was isolated from *P. aeruginosa* strains PAO1, PAS429 and PAS429^{IND} (PAS429 incubated at 43°C for 8min). Northern blotting analysis, using the D3112 *ner* gene as a probe, revealed a 2.2kb mRNA transcript only with RNA isolated 8min post-induction of a lysogen (PAS429^{IND}) (Figure 3.3). To more precisely map the position of the P*ner* promoter, we determined the transcriptional start site using a primer extension analysis (Figure 3.4). The transcriptional start site was found to be located at bp992, centered 41bp upstream of the Ner AUG translation initiation codon. A putative –10 (TATGTT) and-35 (TTGATC) was located just upstream from the +1 site (Hawley and McClure, 1983).

As the *ner* promoter in coliphages Mu and D108 (P*e*) is under the control of their respective repressors, we wished to determine if P*ner* is under the control of the D3112 repressor. Plasmid pKAS249, containing bp 1-1103 (1-*Fsp*I) of the D3112 left-end cloned into the promoterless *lacZ* broad host range plasmid pDN19lacΩ, was constructed. This plasmid contains the entire D3112 *c*ts repressor gene, under the control of P*c*, in addition to a portion of the D3112 *ner* gene (bp 1033-1103) fused to the promoterless *lacZ* gene under the control of P*ner*. This plasmid was then electroporated into the *P. aeruginosa* wild type strain PAO1 and assayed for β-galactosidase activity at 32°C, 37°C and 42°C (Table 3.2). When the cells were grown at 32°C, 110 Miller Units of β-galactosidase activity was observed. However, when these cells were grown at either 37°C or 42°C, the amount of β-galactosidase activity was significantly increased to over 10,000 Miller Units of activity. These results suggest that the D3112 repressor is able to regulate D3112 *ner* expression from the P*ner* promoter.

Figure 3.3. Northern blot analysis of total cellular RNA from *P. aeruginosa* strains PAO1, PAS429 and PAS429^{IND} using the D3112 *ner* gene as a probe. On the left of the figure is the 0.4 to 9.5kb RNA ladder, as described in the Materials and Methods. The arrow points to a 2.2kb mRNA transcript that hybridized to the probe.

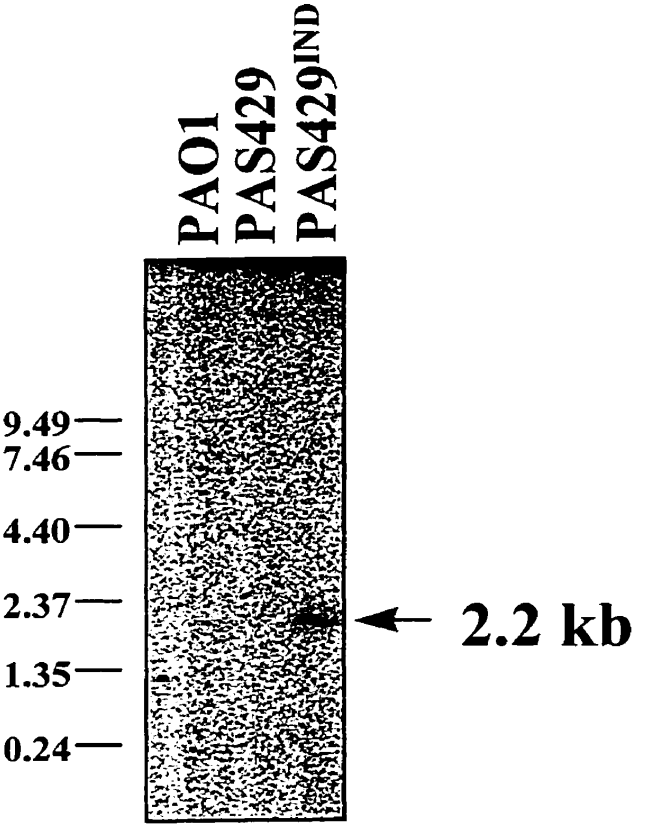
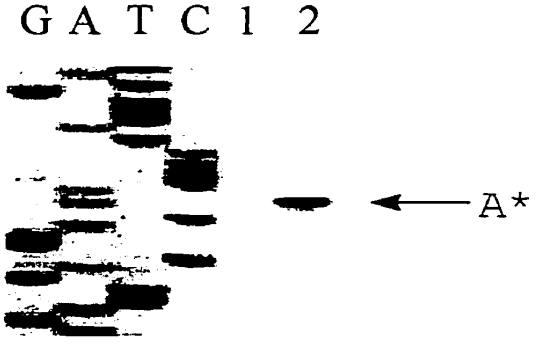

PAO1
PAS429
PAS429IND
9.49
7.46
4.40
2.37
1.35
0.24
2.2 kb

Figure 3.4. (A) Transcriptional start site of D3112 *ner* mRNA. Lanes 1, PA429; 2, PAS429^{IND}; G, A, T, and C, nucleotide-specific sequencing reactions. The arrow points to the extended product on the sequence corresponding to the start site. (B) Sequence of the P*ner* promoter region (numbered from the left-end of the D3112 genome). Boxed nucleotides indicate the putative -10 and -35 regions as well as the +1 transcription start site.

A.



B.

```
                -35                      -10          +1
5′-TTCCGTCTTGATCAGCCAATCAGATGTGTTATGTTTATCCCCAACAG-3′
3′-AAGGCAGAACTAGTCGGTTAGTCTACACAATACAAATAGGGGTTGTC-5′
```

Table 3.2. β-galactosidase activity (in Miller Units) of PAO1 cells containing plasmid pDN19*lac*Ω or pKAS249 at different temperatures. The results represent the average of a minimum of three separate experiments, and Standard Deviations are shown.

Temperature	pDN19*lac*Ω	pKAS249
32°C	45 ± 12	110 ± 21
37°C	75 ± 23	10,011 ± 193
42°C	51 ± 16	10,027 ± 201

3.4.3 Overexpression and purification of D3112 Ner

Overexpression and purification of D3112 Ner was performed. Cloning of the D3112 *ner* gene into the expression vector pET29-b (pKAS315) created a six histidine (His-6) fusion in frame with the 3' end of the *ner* ORF. Plasmid pKAS315 was transformed into *E. coli* BL21(DE3) cells, which contain the T7 RNA polymerase gene inserted into the chromosome under the control of the *lacUV5* promoter (Studier and Moffatt, 1986) and induced by the addition of 1mM IPTG (final concentration). The induced whole cell extract, expressing the D3112 Ner fusion (D3112Ner-His6) was analyzed on a 20% SDS PAGE gel. Overexpression (Figure 3.5, lane 2), and subsequent purification of D3112Ner-His6 using Ni-NTA agarose (lane 8) demonstrated that D3112Ner-His6 has an apparent molecular weight of 14.6kDa.

3.4.4 Characterization of the DNA-binding activity of D3112Ner-His6

For characterization of the DNA-binding activity of the D3112 Ner protein to its presumed binding site, an operator binding assay (Fried and Crothers, 1981; Garner and Revzin, 1981; Kukolj and DuBow, 1991) was performed. A 154bp ^{32}P-labeled DNA fragment from plasmid pBR322 was used as the nonspecific DNA substrate. In addition, a 261bp *Eco*RI-*Hin*dIII ^{32}P-labeled DNA fragment from the D3112 intergenic region (pKAS230) was also used (herein referred to as the specific fragment). This specific DNA fragment contains the intergenic region between the repressor and *ner* coding regions (the presumed Ner binding region).

In this assay, 200ng of purified D3112Ner-His6 was incubated with either the ^{32}P-labeled non-specific fragment (Figure 3.6A, lane 2), or with the ^{32}P-labeled specific

Figure 3.5. SDS-PAGE analysis of D3112 Ner. Lane 1, prestained molecular weight markers; lane 2, crude extract of BL21(DE3)/pKAS315 induced for 4hrs at 1mM IPTG; lanes 3-7 are some of the purification steps of D3112Ner-His6. Lane 3; flow-through; lane 4, 20mM Imidazole wash; lane 5, 50mM Imidazole wash; lane 6, 80mM Imidazole wash; lane 7, elution at 250mM Imidazole. The arrow points to the D3112 Ner band.

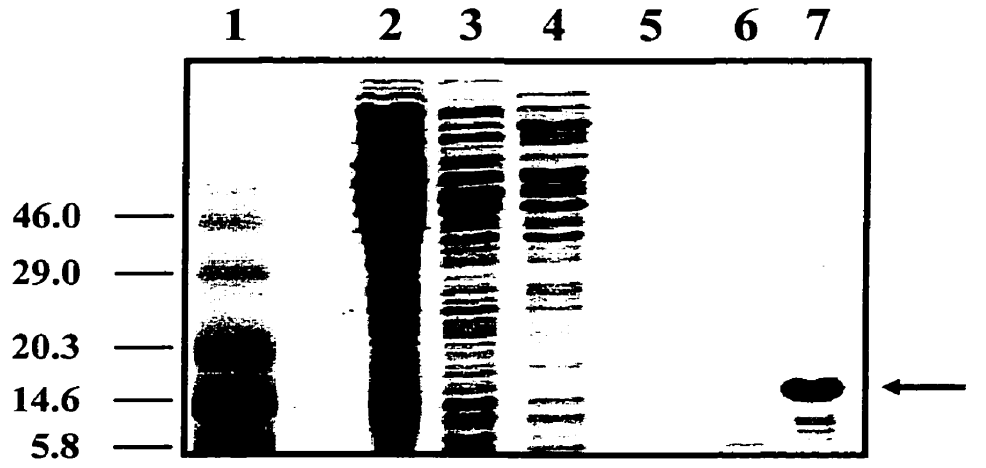
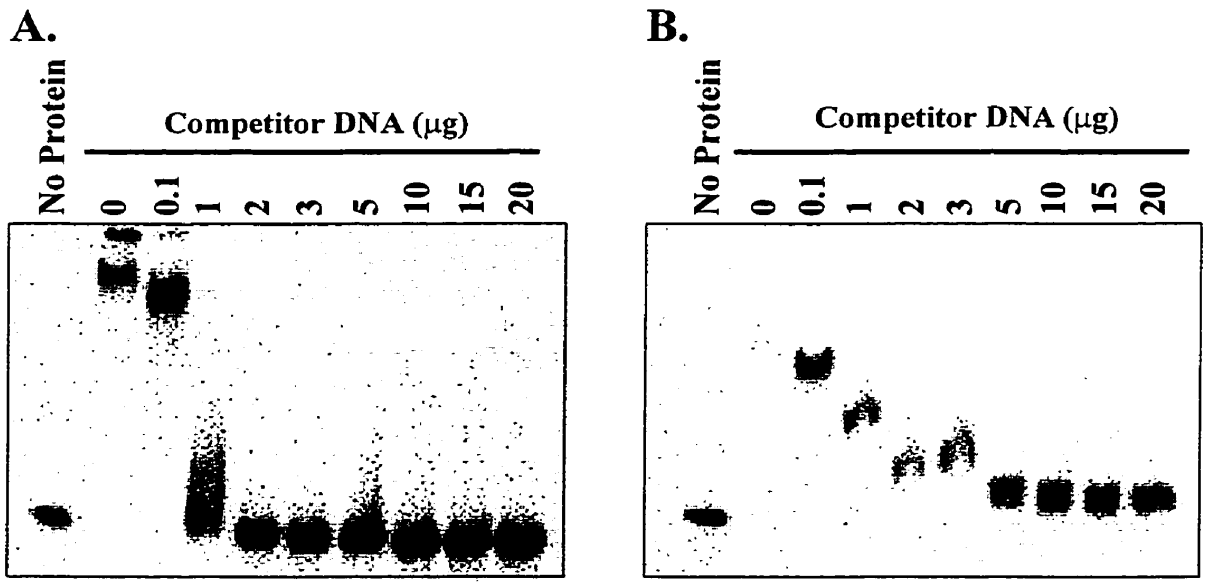

1
2
3
4
5
6
7
46.0
29.0
20.3
14.6
5.8

Figure 3.6. Band retardation assays with D3112Ner-His6 (see Materials and Methods) with either a 154bp *Hin*fI non-specific fragment from pBR322 (A), or a 261bp *Pvu*II fragment from the D3112 intergenic region (B). The first lane in each gel represents 5000cpm radiolabeled fragment alone. The remaining lanes represent 5000cpm radiolabeled fragment incubated with 200ng of purified D3112Ner-His6 and varying amounts of competitor DNA (sonicated calf thymus DNA) as indicated.


A.
No Protein
Competitor DNA (μg)
0
0.1
1
2
3
5
10
15
20
B.
No Protein
Competitor DNA (μg)
0
0.1
1
2
3
5
10
15
20

fragment (Figure 3.6B, lane 2). The D3112Ner-His6 protein readily bound to each of these fragments. Upon the addition of non-labeled competitive substrate (sonicated calf thymus DNA), however, it was observed that the binding by D3112Ner-His6 to the non-specific pBR322 fragment was abolished upon the addition of 1μg competitor DNA (Figure 3.6A, lane 5). However, binding of the specific fragment was still not completely abolished even after the addition of 20μg of competitor DNA (Figure 3.6B, lane 10).

3.5 Discussion

We have identified and cloned a previously unidentified Ner homologue of the *P. aeruginosa* transposable phage D3112. During characterization of the repressor promoter, P*c*, we discovered a fragment (bp366-1818; pKAS244) that, although containing P*c*, showed a lack of promoter expression (Figure 3.1). Subsequent shortening of this fragment to bp772-1439 (pKAS229) and ORF searches identified a 351bp gene (bp1033-1384), which is transcribed from left to right. BLAST searching (Altschul *et al.*, 1997) determined that this ORF shared significant similarity and identity with the Ner homologue of *N. meningitidis* (Tinsley and Nassif, 1996), the Ner-like protein (Nlp) of *E. coli* (Choi *et al.*, 1989), the Ner protein of coliphage D108 (Tolias and DuBow, 1985), and the Ner protein of coliphage Mu (Priess *et al.*, 1982) (Figure 3.2). The location of this ORF just to the right of the *c* ORF in the phage genetic map is similar to the location of *ner* in the two well-studied coliphages Mu and D108, and has now been named D3112

ner. In addition, the loss of activity at Pc when D3112 *ner* is present, suggests a role for D3112 Ner as a regulatory protein involved in initiating lytic development by repressing the expression of the D3112 repressor. This is similar to the role of the Mu and 108 Ner proteins (Goosen and van de Putte, 1984, 1986; Tolias and DuBow, 1986).

With the identification of the D3112 *ner* ORF, we wished to locate the promoter for this gene. Using β-galactosidase assays (Figure 3.1) and primer extension analysis (Figure 3.3), the *ner* promoter has been located to bp957-992. As we have previously located Pc to bp972-940, this indicates that, although divergent, the transcripts from these two promoters do not overlap as seen in Mu and D108 (Krause *et al.*, 1983; Goosen *et al.*, 1984; Levin and DuBow, 1989). However, the RNA polymerase binding sites (the -10/ -35 of each promoter) are overlapping. As the strength of the *ner* promoter is also approximately ten times that seen at the lysogenic promoter, Pc, and this may suggest that, upon infection, transcription from the *ner* promoter is favoured, leading to the lytic cycle. This is supported by the fact that only 5-10% of D3112-infected log phase cells go on to form lysogens (reviewed in DuBow, 1994).

Northern blotting (Figure 3.4) demonstrated that *ner* is transcribed as part of a 2.2kb mRNA. This transcript is large enough to code for not only the D3112 *ner* gene but also the three putative *cip* ORFs located directly downstream of the *ner* ORF (Autexier *et al.*, 1991). However, this transcript is not large enough to code for the transposase *(A)* or *B* genes, as is seen in coliphage Mu (Krause *et al.*, 1983). This suggests that the early promoter, required to transcribe *A* and *B* for the lytic cycle, is located further downstream, and most likely just preceding the *A* gene itself. In a recent paper, Bidnenko *et al.* (1996) described promoter activity in a fragment cloned upstream

from the *ner* ORF. Due to the fact that this upstream promoter activity is likely attributable to an early promoter, and the fact that a *ner*-containing transcript is too short to also encode the *A* and *B* gene products, we have named the D3112 *ner* promoter, P*ner*.

The *cip* region is proposed to encode six putative ORFs (Autexier *et al.*, 1991). However, none of the putative ORFs show homology to other proteins (or DNA), although the *cip* region has been suggested to confer immunity to B39 infection in polylysogens (Gerasimov *et al.*, 1995; Bidnenko *et al.*, 1996). Our results demonstrate that the *ner-cip* mRNA is extensively transcribed at a detectable level only during the lytic cycle and not during lysogeny. Thus, the role of *cip* in D3112 remains unknown.

In the two coliphages, P*e* (responsible for the transcription of the *ner*, *A*, and *B* genes) is under the control of the repressor (c). Therefore, it would be important to know if P*ner* is also under the control of the D3112 repressor (c). Plasmid pKAS249 was constructed which contained bp1-1103 of the D3112 left-end. This fragment contains the entire D3112 *c*ts gene, under the control of P*c*, as well as a portion of the *ner* gene fused to a promoterless *lacZ* reporter gene under the control of P*ner* (Figure 3.1). In this construct, the temperature-sensitive repressor protein (cts15) should be fully functional at 32°C. However, when the temperature is shifted to either 37°C or 42°C, the repressor is inactive and therefore not likely to be able to repress early gene expression (Autexier *et al.*, 1991). When pKAS249 was electroporated into PAO1 and grown at 32°C, only 110 Miller Units of β-galactosidase activity was seen (Table 3.2). A shift to either 37°C or 42°C, where the repressor is no longer functional, demonstrated significant increase in activity at P*ner*. A previous study (Chapter 2) also demonstrated that purified D3112

repressor bound to a fragment from the D3112 intergenic region. Together, these results strongly suggest that P*ner* is negatively regulated during the lysogenic cycle by the binding of D3112 repressor.

Preliminary *in vivo* experiments suggest that D3112 Ner is able to repress transcription from P*c* (compare pKAS220 with pKAS244 and pKAS252, Figure 3.1) as seen in coliphages Mu and D108 (van Meeteren and van de Putte, 1980; van Leerdam *et al.*, 1982; Tolias and DuBow, 1985; Goosen and van de Putte, 1986). To begin characterization of this activity, D3112 Ner was overexpressed and purified (Figure 3.5). D3112Ner-His6 has an apparent molecular weight of 14.6kDa. This is significantly larger than the Ner proteins of Mu and D108 which are only 8kDa. However, there are large blocks of conserved amino acids between the three proteins, indicating that although the Ner proteins have diverged, that they have maintained a significant degree of structural homology.

Purified D3112Ner-His6 bound specifically to a fragment from the D3112 intergenic region (Figure 3.6B). The location of this fragment, within the intergenic region, suggests that D3112 Ner functions to repress transcription at P*c* by binding to an operator in this region. This also suggests that the genetic switch in D3112 may be similar to that seen in the two coliphages, except in the control of transposase *(A)* gene expression. In the coliphages, early lytic development requires the continual synthesis of low levels of transposase (Pato and Reich, 1984), which is tightly regulated by Ner protein binding at P*e* (Goosen and van de Putte, 1986). This study demonstrates that D3112 Ner is able to function in the negative regulation of the lysogenic repressor. However, its role in the regulation of transposase expression is not yet understood. The

results of this study, therefore suggest, that the genetic switch in D3112 is likely more complicated than that seen in Mu and D108.

Chapter 4

Identification of the early promoter, P*e*, of the *Pseudomonas aeruginosa* Mu-like bacteriophage D3112

Preface to Chapter 4

In Chapter 2, we provided evidence for a Mu/D108 Ner homologue in D3112. These studies also demonstrated the location of a promoter, P*ner*, just upstream of the *ner* gene in D3112. Although similar to the position of P*e* in Mu and D108, the transcript from P*ner* was not large enough to encode for either the transposase *(A)* or *B* genes. This suggested that D3112 early promoter, P*e*, required to transcribe the transposase genes required for the lytic cycle, would be further downtream (i.e. toward the right-end of the phage genome). This Chapter provides evidence for the location of the D3112 P*e* directly upstream of the *A* gene and which produces a transcript large enough to encode for both the *A* and *B* genes. Furthermore, the results of the three studies presented in this thesis has led to the development of a model for the lytic-lysogenic switch in D3112 which is presented in this Chapter.

4.1 Abstract

The early promoter, Pe, of the *Pseudomonas aeruginosa* Mu-like transposable bacteriophage D3112 has been identified. This promoter maps to a 507bp *TthIII*-*Bal*I fragment from the D3112 genome. Northern blot analysis indicates that the transposase *(A)* gene is transcribed as part of a transcript of around 4kb after prophage induction, which suggests that the early promoter we identified is responsible for transcription of the transposase *(A)* and *B* genes of D3112. A model for the lytic-lysogenic switch in D3112 is proposed.

4.2 Introduction

The temperate transposable coliphages Mu and D108 use converging promoters of their early and lysogenic repressor operons (termed P*e* and P*c*, respectively) to regulate their lytic and lysogenic pathways (reviewed in DuBow, 1994; Pato, 1989). This convergent promoter organization results in a 35bp overlap in Mu and a 21bp overlap in D108, of the 5' ends of their respective transcripts (Krause *et al.*, 1983; Goosen *et al.*, 1984; Levin and DuBow, 1989). The decision between lytic and lysogenic development is controlled by two phage-encoded proteins: the c repressor (transcribed from P*c*) and the Ner protein (transcribed from P*e*). During the lysogenic state, the repressor (c) binds to the promoter-operator region of the early operon, thus blocking transcription from P*e* (reviewed in DuBow, 1994). During lytic growth, the early genes are expressed from P*e*. This results in the production of proteins from the *ner* gene and the *A* (transposase) and *B* genes, in addition to the genes of the semi-essential early region located downstream of the *B* gene. The Ner protein binds to its operator and acts as a repressor to turn off expression of the repressor operon and commit the phage to the lytic cycle. By binding this operator, Ner protein is also capable of turning down expression of the early operon to allow a level of early gene expression sufficient to amplify, via DNA transposition, the viral genome. Studies have also shown that the *E. coli* histone-like DNA-binding protein H-NS also appears to specifically inhibit P*e*. Integration Host Factor (IHF), binding to its site upstream of P*e*, counteracts the H-NS-mediated repression of this promoter (van Ulsen *et al.*, 1996).

Transposable *Pseudomonas* phage D3112 bears striking similarities, though not homologies, to transposable coliphages Mu and D108. Phage D3112 also encodes a repressor protein in its left end, transcribed from right to left on the phage genome as in Mu and D108, which can act to confer immunity to D3112 superinfection *in vivo* (Autexier *et al.*, 1991). In the previous Chapter, a Ner homologue was been identified in D3112 in a position analogous to the *ner* genes of the two coliphages. Expression of *ner in vivo* demonstrated that it represses expression of the *c* gene from P*c*, and that repressor regulates expression of *ner* from the *ner* promoter, P*ner*.

An important difference between the two coliphages and D3112 is the fact that the transposase gene of D3112 does not begin until bp2539 from the left end (Ulycznyj *et al.*, 1995) as compared with bp1328 for Mu (Priess *et al.*, 1987) and bp1290 for D108 (Mizuuchi *et al.*, 1986). A function, called *cip* (*c*ontrol of *i*nteraction of *p*hage) has been genetically mapped in D3112 between the *ner* homologue gene and the transposase gene (Gerasimov *et al.*, 1985; Yanenko *et al.*, 1983; 1988). This region, originally postulated to be the functional equivalent to the *ner* gene product of the coliphages, is believed to inhibit the lytic growth of heteroimmune related phage B39 and to function in the lysogen (Gerasimov *et al.*, 1985; Bidnenko *et al.*, 1996). In the previous chapter, we showed that this region is only expressed from P*ner* during lytic growth.

Promoter activity has been identified within this *cip* region (bp1818-3523; Bidnenko *et al.*, 1996). However, results from studies with D3112 *ner* expression indicated that the *cip* region is transcribed with *ner* from P*ner* (Chapter 3). In this paper we report on the discovery of promoter activity directly upstream of the transposase gene *(A)* and discuss the probability that this promoter activity is that of the early promoter, P*e*.

4.3 Materials and Methods

4.3.1 Bacterial strains, phages, plasmids and culture conditions.

The bacterial strains, bacteriophages and plasmids used in this study are listed in Table 4.1. All cultures were grown at 37°C in Luria Bertani broth and agar (Sambrook *et al.*, 1989) except for PAS429 which was grown at 32°C. When necessary, antibiotics were added at the following concentrations: for *P. aeruginosa*, streptomycin (Str, 300μg/ml); tetracycline (Tc; 200μg/ml); for *E. coli*, ampicillin (Ap; 50μg/ml); Str (100ug/ml); Tc (10μg/ml).

4.3.2 Enzymes and chemicals.

All restriction enzymes, T4 DNA ligase, and T4 DNA polymerase, were purchased from either Amersham-Pharmacia (Baie d'Urfe, QC) or GIBCO-BRL (Burlington, ON). All other chemicals were purchased from either Fisher (Nepean, ON) or Sigma (St. Louis, MO).

4.3.3 Plasmid constructions.

Plasmids for the promoter determination via β-galactosidase promoter-probe fusions were constructed in the following manner: various fragments from the D3112*c*ts15 genome (see Figure 4.1) were made blunt using T4 DNA polymerase (if required), subcloned into the *Sma*I site of pUC119 and the orientation determined by restriction enzyme hydrolyses. Plasmids containing the proper orientation were

Table 4.1. Bacterial strains, phages and plasmids used in this study.

Strain/Phage/ Plasmid	Relevant characteristics	Reference/Origin
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type, prototroph	Holloway, 1969
PAS429	PAO1 *ami*::D3112 *c*ts15	J. Shapiro
Escherichia coli		
DH5α	*supE44 ΔU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
Plasmids		
pUC119	Ap^R;cloning vector	Vieira and Messing, 1987
pDN19lacΩ	Str^R/Spc^R, Tc^R; Broad host range, promoterless *lacZ* vector	Totten and Lory, 1990
pKAS206	D3112 *A* gene in pKK223-3	Ulycznyj *et al.*, 1995
pKAS273	bp1818-bp2741fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS277	bp2030-bp2741 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pKAS280	bp2234-bp2741 fragment of D3112*cts* cloned into the *Eco*RI/*Bam*HI site of pDN19lacΩ	This study
pSH8	*Hin*dIII (1.8kbp)-*Sal*I (11.8kbp) of D3112*cts* cloned into the *Hin*dIII/*Sal*I site of pUC119	Autexier, 1991

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Spc^R, spectinomycin resistance; Str^R, streptomycin resistance; Tc^R, tetracycline resistance

hydrolyzed with *Eco*RI and *Bam*HI and the resulting fragments isolated and subcloned into the *Eco*RI/*Bam*HI sites of pDN19lacΩ (Totten and Lory, 1990). The resulting plasmids are listed in Table 4.1.

4.3.4 Transformations and Electroporations.

Transformations of *E. coli* were performed using a $RbCl_2$ method (Hanahan, 1983). Electroporation of *P. aeruginosa* strains was performed according to the method of Farinha and Kropinski (1990) using 15% glycerol/1mM MOPS as the buffer. The DNA used for the transformations and electroporations was prepared using either an alkaline-lysis method (Sambrook *et al.*, 1989) or Qiagen quick-spin columns (Qiagen, Mississauga, ON).

4.3.5 β-galactosidase assays.

Expression of the *lacZ* gene, under the control of the putative P*c* promoter region, was measured using β-galactosidase assays as described by Miller (1972).

4.3.6 Northern blot hybridization.

Total cellular RNA was prepared from *P. aeruginosa* strains PAO1, PAS429 (grown at 32°C) and PAS429^{IND} (PAS429 grown at 32°C to A_{550}=0.7 and then induced for 8 min at 42°C) by an adaptation of the CsCl purification procedure (Glisin *et al.*, 1974), as described by Deretic *et al.* (1987). RNA, 10μg from each strain, was subjected to electrophoresis through a 1.2% agarose/formaldehyde gel (Ausubel *et al.*, 1989),

transferred and fixed to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as described previously (Cai and DuBow, 1996). The 0.4 to 9.5kb RNA ladder (GIBCO-BRL) was subjected to electrophoresis in parallel with the above samples and visualized by ethidium bromide staining prior to transfer to the nitrocellulose membrane. A 2.1kbp *Eco*RI-*Hin*dIII fragment from pKAS206, containing the entire D3112 *A* gene, was used to detect D3112 *A* mRNA. A 276bp *Alu*I fragment from pSH8, containing the *cip3* putative ORF, was used to detect D3112 *cip* mRNA. The DNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, 1Ci=37Gbq, Amersham-Pharmacia) using the random priming method (Sambrook *et al.*, 1989) and random hexanucleotide primers (GIBCO-BRL). Hybridization was carried out as previously described (Mahmoudi and Lin, 1989). Unhybridized probe was removed by washing the filters twice at 68°C in 2X SSC, 1% SDS. The blots were air dried and exposed to a Molecular Dynamics Storage Phosphor Screen and visualized using a PhosphorImager SF and Image Quant Software Program (Molecular Dynamics, Sunnyvale, CA).

4.4 Results

4.4.1 Identification of a fragment containing the transposase *(A)* promoter

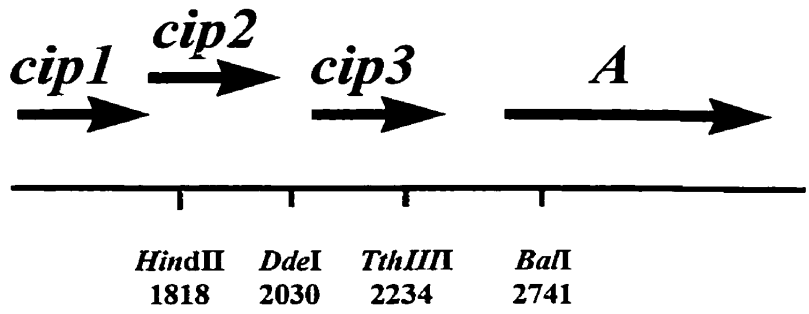
The location of the promoter for the D3112 *A* gene was identified by cloning fragments from the D3112 genome into the *lacZ* transcriptional fusion broad host range vector pDN19*lac*Ω (Totten and Lory, 1990) (Figure 4.1). When expressed in the *P.*

aeruginosa wild type strain PAO1, plasmid pKAS273 (containing a *Hin*dIII-*Bal*I fragment) produced 2350 Miller Units of β-galactosidase activity. Similar to the *Eco*RI site of the fragment from the study by Bidnenko *et al.* (1996), the *Bal*I site is within the coding sequence of the transposase *(A)* gene. Subsequent shortening of this fragment in pKAS277 (*Dde*I-*Bal*I fragment) and pKAS280 (*TthIII*-*Bal*I fragment) displayed only slight differences in the amount of promoter activity, even when no complete *cip* ORF was present. These results suggest that the 507bp *TthIII*-*Bal*I fragment most likely contains the transposase *(A)* promoter.

4.4.2 Determination of the size of the transcript from the transposase promoter

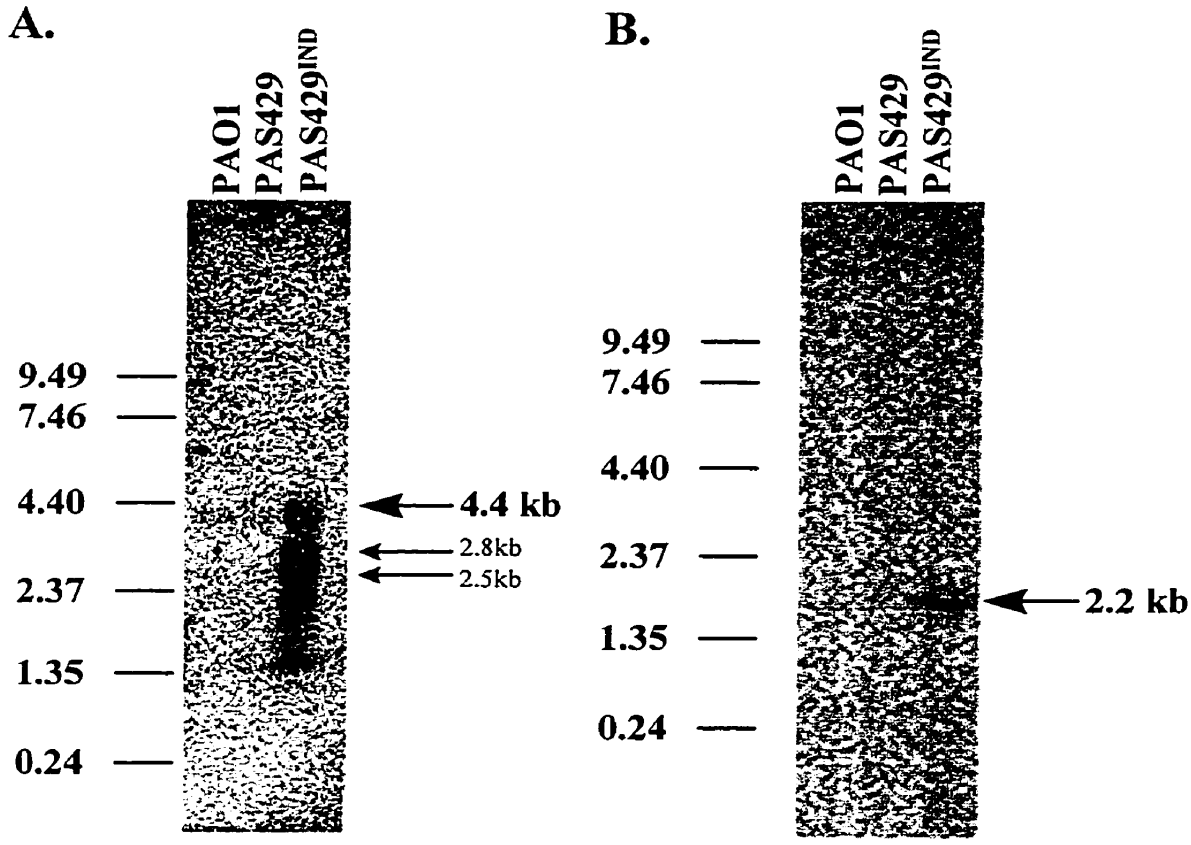
To determine the size of the transposase transcript, total RNA was isolated from *P. aeruginosa* strains PAO1, PAS429 and PAS429^{IND} (PAS429 incubated at 42°C for 8 min following growth to A_{550}=0.7). Hybridization of the Northern blots using the D3112 *A* or *cip3* genes as probes yields two different results. When the transposase *(A)* gene was used as the probe, a smeared band beginning around 4.4kb was detected only in RNA isolated at 8min post-induction of phage lytic growth (PAS429^{IND}; Figure 4.2A), in addition to two bands at 2.8kb and 2.5kb. When the third putative *cip* ORF (*cip3*; Autexier *et al.*, 1991) was used as a probe, a 2.2kb transcript was observed only in RNA isolated at 8min post-induction of phage lytic growth (PAS429^{IND}; Figure 4.2B).

Figure 4.1. Analysis of deletion constructs for P*e* promoter activity. The insert in each plasmid, cloned just 5' of the promoterless *lacZ* gene in pDN19lacΩ, is shown along with levels of β-galactosidase activity in *P. aeruginosa* strain PAO1. The results represent the average of a minimum of three separate experiments and the Standard Deviations (S.D.) are shown. The arrow reflects the direction of transcription through the promoterless *lacZ* gene. The top of the Figure shows a restriction enzyme map of the region numbered from the very left-end of the phage genome (not to scale). The *cip1*, *cip2*, and *cip3* genes represent the 3 *cip* ORFs located on the upper strand of the D3112 genome (Autexier *et al.*, 1991).



Plasmid		Activity +/- S.D.
pDN19lacΩ		45 ± 3
pKAS273	⟶	2350 ± 97
pKAS277	⟶	2401 ± 156
pKAS225	→	2513 ± 212

Figure 4.2. Northern blot analysis of total cellular RNA from *P. aeruginosa* strains PAO1, PAS429, and PAS429^{IND}. (A) Northern blot hybridized with the D3112 transposase *A* gene; (B) An identical Northern blot prepared as in (A), hybridized with the D3112 *cip3* gene.


A.
PAO1
PAS429
PAS429IND
9.49
7.46
4.40
2.37
1.35
0.24
4.4 kb
2.8kb
2.5kb
B.
PAO1
PAS429
PAS429IND
9.49
7.46
4.40
2.37
1.35
0.24
2.2 kb

4.5 Discussion

We have identified promoter activity directly upstream of the gene encoding the D3112 transposase *(A)*, to a 507bp *TthIII*-*Bal*I fragment. Previous studies (Bidnenko *et al.*, 1996) had located promoter activity in this region, but had mapped it only to a 1.6kbp fragment which covered part of the *cip* region as well as the transposase gene.

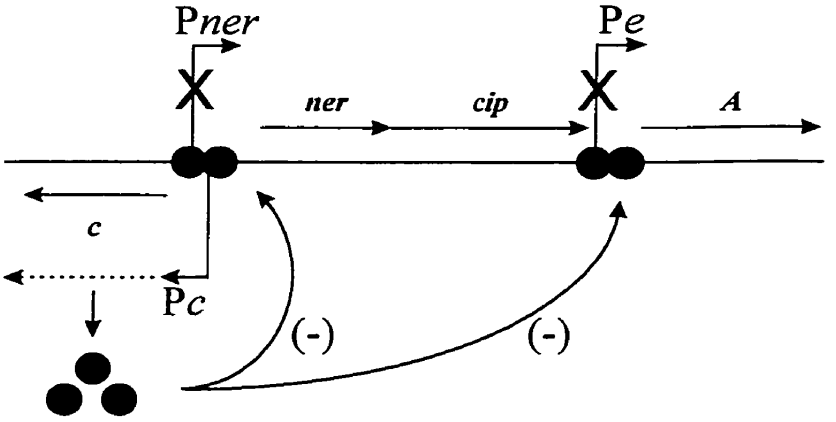
A Northern blotting analysis was performed to further refine the early operon transcript. When total RNA from *P. aeruginosa* strains PAO1, and PAS429, plus PAS429^{IND} (8min post-induction of a D3112 lysogen) were probed with the D3112 *A* gene, a smear of RNA, beginning at 4.4kb was observed, in addition to the observation of two bands occurring at 2.8kb and 2.5kb (Figure 4.2A). These results may indicate that the 4.4kb mRNA is quickly processed and/or degraded, resulting in the appearance of 2.8kb and 2.5kb bands. Alternatively, a larger RNA is produced from P*e*, as is observed with phage Mu (Marrs and Howe, 1990), which is unstable or rapidly processed (as observed in phage T7 early mRNA; Yamada *et al.*, 1975) to yield the bands detected in the Northern blots.

When the same total RNA was probed with a fragment equal to the *cip3* ORF, a single 2.2kb transcript was observed (Figure 4.2B). These results imply that the *cip* region, which has been shown to be transcribed with the D3112 *ner* gene (Chapter 3), is not transcribed with the transposase gene. Due to the size of the transcript observed upon hybridization of the *A* gene, the results suggest that the D3112 *A* and *B* genes are transcribed from their own promoter located directly upstream of the *A* gene (P*e*).

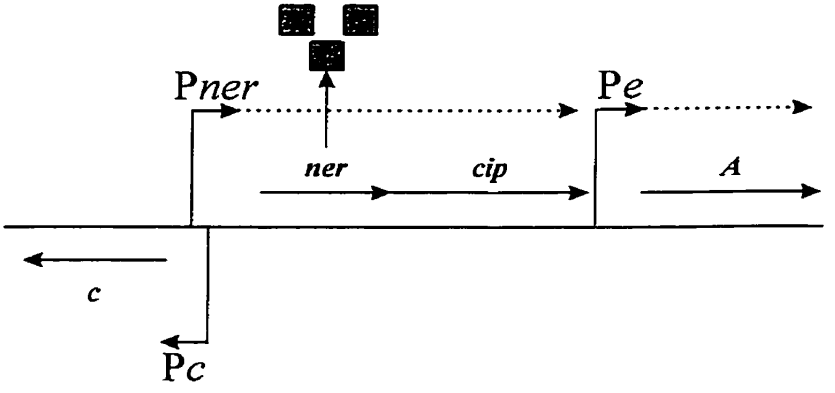
A model for the lytic-lysogenic switch in D3112 can be inferred and is depicted in Figure 4.3. During the lysogenic cycle, the repressor *(c)* gene is transcribed from P*c*. Binding by the repressor to one or more operators located within the intergenic region would inhibit the transcription of the *ner* gene from P*ner*. During the lytic cycle (or release from lysogeny), the *ner* gene is transcribed from P*ner*. Binding by the Ner protein to its operator site within the intergenic region would inhibit transcription from P*c*, thereby inhibiting the production of repressor. This first part of this model would therefore appear to be similar to that of the coliphages Mu and D108 (reviewed in DuBow, 1994). The question of control of expression from the early promoter, P*e*, remains problematic. During the lysogenic cycle, the repressor (c) protein should bind to operator sites surrounding the P*ner* and P*e* promoters and inhibit their transcription. Therefore, no transposase would be produced during lysogeny. During the lytic cycle, the repressor protein would cease to inhibit transcription, thereby allowing transposase protein to be expressed. Following expression of *ner* from P*ner*, the Ner protein would then accumulate in sufficient amounts to bind operator site(s) located near P*ner* and P*e* to inhibit excessive levels of transposase from being produced. In Mu, the control of transposase expression late in early gene expression is crucial (Pato and Reich, 1984; Baker *et al.*, 1991), as high transposase concentrations lead to aberrant transposition products (Baker *et al.*, 1991).

Figure 4.3. A model for the lytic-lysogenic switch in D3112. (A) Model for the molecular events leading to lysogeny (see text for details). (B) Model for the molecular events during the early lytic cycle (see text for details). (C) Model for the molecular events late in the lytic cycle (see text for details).

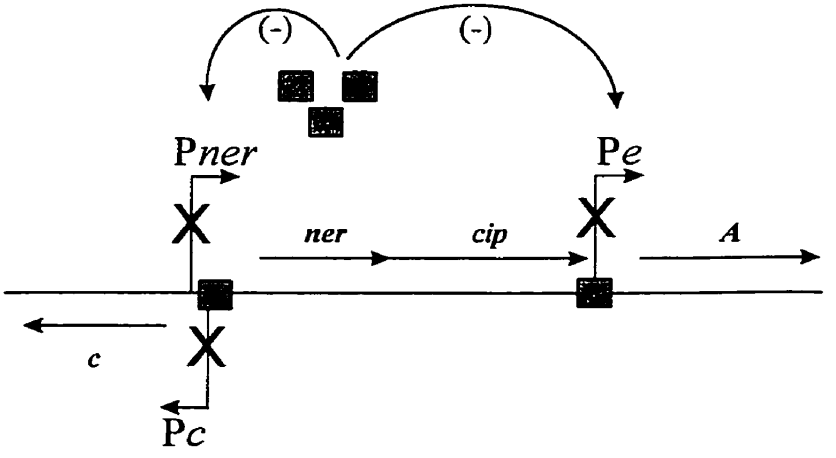
A. Lysogeny



B. Lytic Cycle; Early



C. Lytic Cycle; Late



Chapter 5

Summary and Future Prospects

When a temperate phage infects a bacterial cell, it has the ability to propagate itself in one of two essentially different ways. Either it can multiply and kill its host cell in the lytic cycle, or it can establish a quasi permanent symbiotic association resulting in a lysogenic bacterium. The mechanism governing the genetic switch between the lytic and lysogenic responses in temperate phages is complex and has changed greatly over the course of evolution.

The accumulation of sequence and biochemical data detailing the factors involved in the genetic switch of a variety of temperate phages has provided for possibilities to examine its evolution (Chapter 1). With more and more temperate phages being sequenced, emphasis on the molecular characterization of their lytic-lysogenic switches is required in order to further develop these ideas.

The genetic switch of the transposable *Pseudomonas aeruginosa* phage D3112 was originally believed to be similar to that of coliphages Mu and D108, due to its genome organization. Previous sequencing of the D3112 left-end (Autexier *et al.*, 1991) had revealed a single large ORF, transcribed from right to left, and commencing with a GTG codon. Three internal, in-frame GTG codons were also identified as possible start codons for the D3112 c repressor translation. Using site-directed mutagenesis, each of the four GTG codons was converted to a GTA codon. This 'silent' mutagenesis therefore preserved the incorporation of a valine such that the internal codons would be unaffected, however, the new GTA codon would be unable to act as a start codon for translation of the protein (Neidhart *et al.*, 1990). Once mutagenized, the ORFs containing the mutated codons were subcloned into a broad host range vector and transformed into *P. aeruginosa* PAO1 cells and tested for their ability to confer immunity to superinfection *in vivo* (Table

2.2). The results of this study indicated that the second, internal GTG, is used as the start codon.

Sequencing of the wild type c^+ gene and comparison with the previously sequenced *c*ts15 mutant (Autexier *et al.*, 1991) revealed a single AT→GC bp mutation that results in an amino acid change from a glycine, in the wild type repressor at position 6, to an aspartic acid, in the temperature-sensitive repressor (Figure 2.1). This mutation occurs within the first putative α-helix of this protein, and not within the putative H-T-H motif. Modeling of the D3112 repressor to other phage repressors may help to localize the context of G6 within the repressor structure.

Further random mutations were made to the D3112 repressor protein in order to identify residues important in its function. Using chemical mutagenesis, three residues, all within the putative H-T-H motif, were identified as being important in proper protein functioning, as determined by *in vivo* immunity assays (Table 2.3). When this putative H-T-H motif was deleted from the repressor protein (3GTG-His6; Figure 2.6D), DNA-binding was abrogated.

We have localized the P*c* promoter to a 261bp *Pvu*II fragment from the D3112 left-end (bp1099-838; Figure 2.2) and through primer extension analysis, we have precisely mapped the transcriptional start site of this monocistronic mRNA (Figure 2.3) to bp940 (Figure 2.4). In Mu, IHF, in conjunction with the c repressor, promotes the formation of a stable nucleoprotein structure with the early operator *in vitro* and allows the establishment and maintenance of lysogeny (Alazard *et al.*, 1992; Gama *et al.*, 1992; Vogel *et al.*, 1991; Betermier *et al.*, 1995). As there is a consensus IHF site immediately

downstream of P*c*, the β-galactosidase assays shown in Figure 2.1 should be repeated in a PAO1 *himA* deletion strain (Dan Wozniak, personal communication) to determine if IHF has an effect at this promoter. An understanding of the role of IHF at P*c*, if any, is crucial to our understanding of the lytic-lysogenic switch in D3112.

DNA binding assays of purified D3112 repressor (cts15-His6; Figure 2.5) showed binding of a 261bp *Pvu*II fragment from the D3112 left-end. This fragment displayed two distinct bands upon binding by cts15-His6 (Figure 2.6B). As this suggested the presence of two or more operators within this fragment, footprinting analysis of this fragment should determine the number of operators and the affinity of D3112 repressor for these sites, as well as the putative c repressor consensus DNA binding sequence.

In Chapter 3, we report the discovery of a Mu/D108 Ner homologue in D3112 which shares 54% similarity (33% identity) to the Ner protein of coliphage D108 over a 65 amino acid region, and shares 57% similarity (33% identity) to the Mu Ner protein over a 53 amino acid region (Figure 3.2). The D3112 *ner* promoter, P*ner*, was located to bp957-992 (Figure 3.3). Northern blotting analysis (Figure 3.4) demonstrated that *ner* is transcribed as part of a polycistronic 2.2kb mRNA transcript that codes for *ner* as well as three putative *cip* ORFs (Autexier *et al.*, 1991). *In vitro* transcription-translation assays, as well as other *in vivo* assays of this cloned region, need to be performed to determine which of these *cip* ORFs (if any) are actually translated. Also unclear is the function of the *cip* region and its potential proteins which have no homologues in the protein databanks. Bidnenko *et al.* (1996) and Gerasimov *et al.* (1985) have suggested that *cip* functions to confer resistance of D3112 polylysogens to B39 infection (another transposable *Pseudomonas* phage). However, we demonstrated that the *ner-cip* mRNA is

extensively transcribed at a detectable level only during the lytic cycle and not during lysogeny. Construction of a D3112Δ*cip* phage may provide some insight into the role of *cip*.

β-galactosidase assays indicated that (i) D3112 Ner represses P*c* activity (Figure 3.1) and (ii) that D3112 repressor inhibits P*ner* activity (Table 3.2). This is reminiscent of the activity seen at P*c* and P*e* of coliphages Mu and D108. DNA-binding studies using purified D3112 Ner showed that this protein bound specifically to a 261bp *Pvu*II fragment from the D3112 intergenic region (Figure 3.6B). DNA footprinting will help define the D3112 Ner operator site within this fragment. In addition, it would be interesting to determine the effect of IHF and H-NS on Ner binding to its operator. In coliphages Mu and D108, binding of IHF relieves H-NS mediated repression at P*e* thereby allowing an optimal amount of transcription from this promoter (van Ulsen *et al.*, 1996; Kukolj and DuBow, 1992). Also in Mu development, IHF plays an important role in the lytic cycle as the phage does not plaque on *himA* or *himD* mutant strains (Miller and Friedman, 1977). Plaque assays of D3112 on a *himA* mutant of PAO1 would confirm if this is also the case in D3112. Repetition of the previous work on D3112 should be performed in a *himA* mutant and will extend our understanding of the control of the lytic cycle in this *Pseudomonas* phage.

In coliphages Mu and D108, the *ner* gene is transcribed from P*e* as part of a polycistronic mRNA that includes the transposase *A* and *B* genes. However, in Chapter 3, we showed that D3112 *ner* is not transcribed in conjunction with the transposase genes and therefore its novel promoter was called P*ner*. In Chapter 4, we locate the D3112 P*e*

promoter to a 507bp fragment (bp2234-2741) downstream of the transposase *A* gene (Figure 4.1). We showed, by Northern blot analysis, that the D3112 *A* and *B* genes are encoded by a 4.4kb bicistronic mRNA (Figure 4.2) from P*e*.

We have proposed a testable model for the genetic switch in D3112 (Figure 4.3). During lysogeny, the D3112 repressor *(c)* is transcribed from P*c*. D3112 c binds to its operator(s) and effectively shuts off transcription from P*ner* and likely P*e* (see below). Alternatively, during the lytic cycle, *ner* is transcribed from P*ner*. D3112 Ner binds to its operator which shuts off transcription of repressor from P*c*. By alleviating repressor transcription, the transposase will eventually be transcribed from P*e* following inactivation and release of repressor bound to this promoter region. We have not yet identified factors regulating transcription from P*e*, however, this promoter is not active during lysogeny (Figure 4.2). An experiment where P*e*, fused to a reporter gene such as *lacZ*, is cotransformed with a plasmid expressing either the D3112 repressor or D3112 Ner would demonstrate whether or not either of these proteins is involved in the regulation of this promoter. Binding of Ner at P*e*, or another as yet unidentified factor, may be required to repress transcription at this promoter in order to allow for only stoichiometric amounts of transposase to be produced during the lytic cycle, as seen in Mu and D108 (Pato and Reich, 1984). Characterization of the regulation of P*e* during both the lytic and lysogenic cycles is crucial in our understanding of this phage.

This thesis has described a preliminary analysis of the lytic-lysogenic switch of D3112. Further characterization should lead to a fuller understanding of the molecular events of the genetic switch of this phage. In terms of evolution of the lytic-lysogenic switch seen in the transposable phages, D3112 may represent a novel form of this

module. It may become apparent that the *cip* function represents another level of regulation of the lytic-lysogenic switch (i.e. such as the *mnt/ant* system of P22) that has been inserted recently. Alternatively, the presence of the *cip* function within D3112 may depict an earlier form of the transposable phages. The presence of a promoter upstream of the *A* and *B* genes suggests that this whole region may have been inserted within the phage genome as a module (likely from a transposon). The coliphages may have lost the *cip* function recently, leading to the use of a single promoter for both the *ner* gene and the *A* and *B* genes. Further characterization of other *Pseudomonas* transposable phages may give us insight regarding this quandary.

A thorough understanding of phage evolution will require the study of phages from a wide variety of hosts. Fortunately, the new sciences of genomics and bioinformatics offers hope for a new generation of well-characterized phages. A complete understanding of phage biology is integral to our understanding of a world dominated by bacteria.

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