USAFA-TR-91-3

BACTERIOPHAGE T4D GENE 42 MUTANTS EXHIBIT A DEFECTIVE GENETIC EXCLUSION PHENOTYPE

LIEUTENANT COLONEL JOHN W. OBRINGER

DEPARTMENT OF BIOLOGY
USAF ACADEMY, COLORADO 80840

FEBRUARY 1991

DEAN OF THE FACULTY
UNITED STATES AIR FORCE ACADEMY
COLORADO 80840

USAFA-TR-91-3

Technical Review by Lt Col David McCloy
United States Air Force Academy Clinic Administration
USAFA Academy, Colorado 80840

Technical Review by Major R. Patrick McCreary
Department of Biology
USAF Academy, Colorado 80840

Editorial Review by Lt Col Donald C. Anderson
Department of English
USAF Academy, Colorado 80840

This research report entitled "Bacteriophage T4D Gene [illegible] Mutants Exhibit a Defective Genetic Exclusion Phenotype" [illegible] presented as a competent treatment of the subject, worthy [illegible] publication. The United States Air Force Academy vouches for th[illegible] quality of the research, without necessarily endorsing th[illegible] opinions and conclusions of the author.

This report has been cleared for open publication and publ[illegible] release by the appropriate Office of Information in accordance with AFM 190-1, AFR 12-30, and AFR 80-3. This report may hav[illegible] unlimited distribution.

Robert K. Morrow Jr.
ROBERT K. MORROW JR., Lt Col, USAF
Director of Research

6 MAR 91
Dated

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 1991	Final

4. TITLE AND SUBTITLE
Bacteriophage T4D Gene 42 Mutants Exhibit a Defective Genetic Exclusion Phenotype

5. FUNDING NUMBERS

6. AUTHOR(S)

John W. Obringer

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Department of Biology, USAF Academy, CO
Department of Microbiology and Immunology
University of AZ, Tucson, AZ

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a DISTRIBUTION / AVAILABILITY STATEMENT

Unlimited Distribution

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Genetic exclusion in phage T4 is the prime responsibility of the imm and sp genes. The map region containing imm does not allow sufficient coding space to encode for proteins the size reported for the imm gp. After assaying 30 mutants of the genes adjacent to imm, I found 7 in gene 42 that were defective in the imm phenotype. Upon reverting one of these, amNG411(42), the mutant most defective exclusion, for its gene 42 phenotype the exclusion phenotype also reverted toward wildtype. When assayed in UGA suppressor hosts, imm+ phage showed a decreased exclusion ability indicating that an opal codon is involved in production of the functional imm gp. I concluded that imm and gene 42 overlap in an out-of-phase orientation with the involvement of an opal readthrough. This overlap has implications in the genetic regulation of this region.

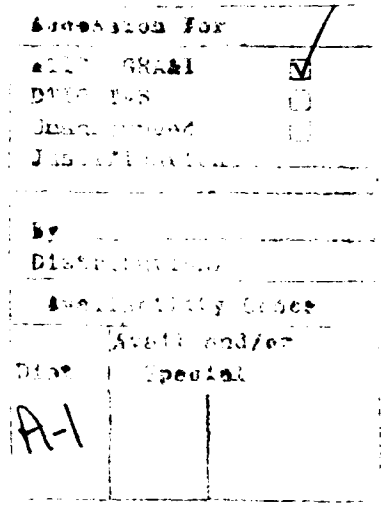
14. SUBJECT TERMS	15. NUMBER OF PAGES
Bacteriophage T4, Genetic Exclusion	50
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

BACTERIOPHAGE T4D GENE 42 MUTANTS EXHIBIT A DEFECTIVE GENETIC EXCLUSION PHENOTYPE

BY

JOHN W. OBRINGER, Lt Col, Ph. D., USAF



February 1991

Department of Biology

U. S. Air Force Academy, CO 80840

ABSTRACT

Genetic exclusion in phage T4 is the prime responsibility of the imm and sp genes. The map region containing imm does not allow sufficient coding space to encode for proteins the size reported for the imm gp. After assaying 30 mutants of the genes adjacent to imm, I found 7 in gene 42 that were defective in the imm phenotype. Upon reverting one of these, amNG411(42), the mutant most defective exclusion, for its gene 42 phenotype the exclusion phenotype also reverted toward wildtype. When assayed in UGA suppressor hosts, imm+ phage showed a decreased exclusion ability indicating that an opal codon is involved in production of the functional imm gp. I concluded that imm and gene 42 overlap in an out-of-phase orientation with the involvement of an opal readthrough. This overlap has implications in the genetic regulation of this region.

LIST OF FIGURES

FIGURE		PAGE
1.	Figure 1: Morphology of Phage T4D	6
2.	Figure 2: Phage T4 Genetic Map	11
3.	Table 1: Bacterial and Phage Strains	15
4.	Table 2: Genetic Exclusion by Phage in Suppressor Hosts	26
5.	Figure 3: Gene 42 Genetic Map	28
6.	Table 3: Immunity of Gene 42 and Gene 43 Amber Mutants	29
7.	Table 4: Immunity of amNG411 Revertants	31
8.	Figure 4: Immunity of Gene 42 & 43 Mutants	36
9.	Figure 5: Exclusion by amNG411 Revertants	38 & 38a
10.	Figure 6: Immunity of T4 Mutants in Suppressor Strains	41

TABLE OF CONTENTS

SECTION	PAGE
Introduction	5
Materials and Methods	14
Results and Discussion	24
Conclusions	35
Bibliography	44

INTRODUCTION

Bacteriophage T4 is a large bacterial virus of Escherichia coli containing a single linear double-stranded DNA molecule of about 166 x 10^3 base pairs in length. Its tightly organized genome encodes close to 200 genes which choreograph a complex, well regulated developmental process (reviewed in Guttman and Kutter, 1983). Bacteriophage T4, henceforth referred to as phage T4 or T4D+, consists of a virion approximately 215nm in total length and 80nm in width at the head (Fig. 1). The capsid or head is an icosahedron made primarily of a protein layer attached to the connector vertex of the head. A neck structure containing a whiskered collar joins to the tail. The tail is 100nm long and composed of 20 different species of protein and is the smallest contractile organ known to man. T4 consists of an outer sheath surrounding an inner tube through which the phage's DNA passes during the infection process. The distal portion of the tail is attached to a complex baseplate fitted with long and short tail fibers. The tail fibers are essential for infection by providing the primary host range determinants and effecting the adsorption process (Fig. 1).

Phage T4 is morphologically classified as a urophage. It can be further classified as a member of the T-even-like phages along with its cousins T2 and T6 (Guttman and Kutter, 1983;see also Birge, 1981, for an overview). The T-even-like phages include numerous types; many of them sharing serological as well as morphological characteristics (Schwartz, 1980; Schwartz et al., 1980). They also share a high degree of DNA homology (Kim and Davidson, 1974). Of all of the T-even-like phages, phage T4

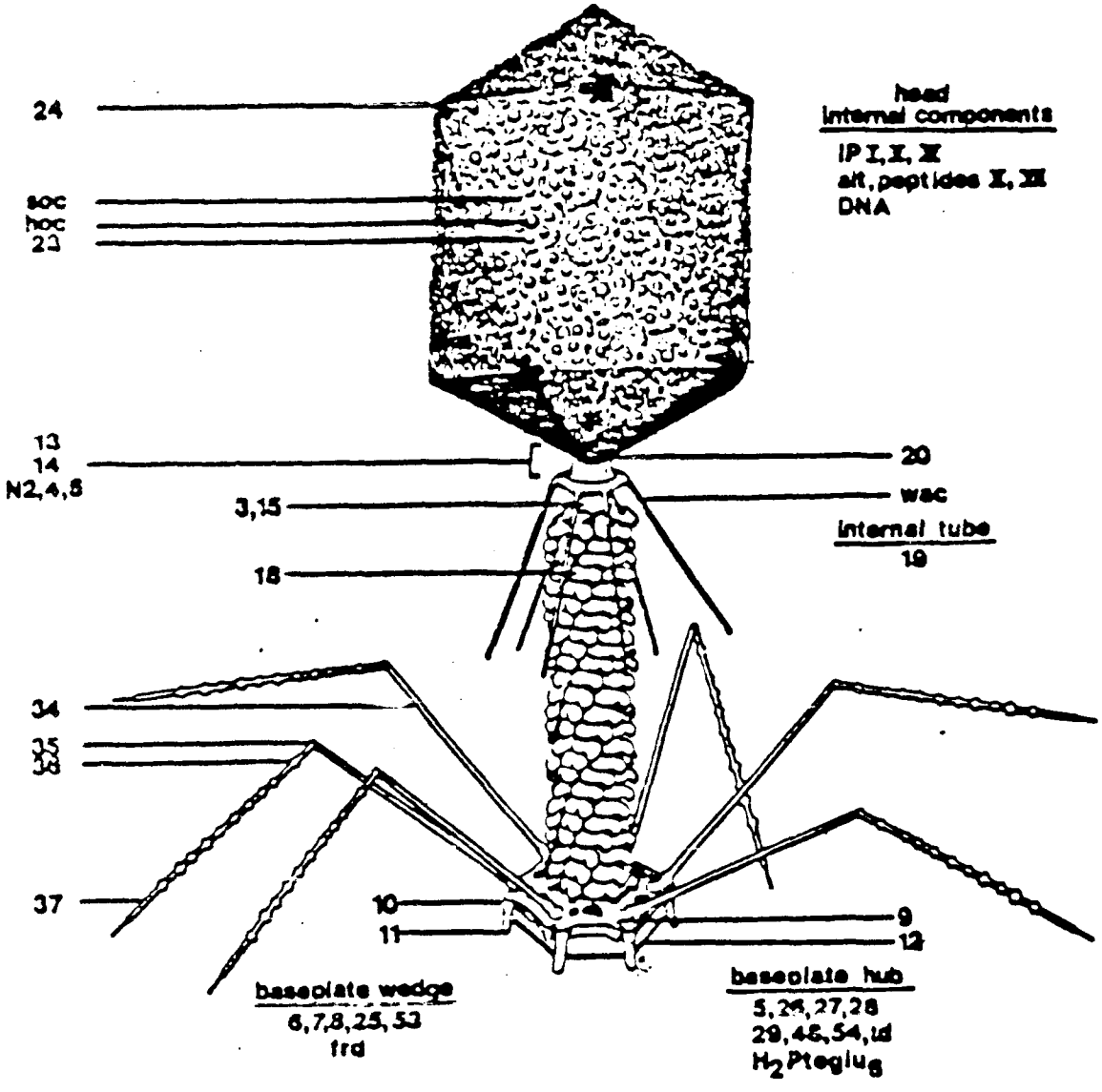


Figure 1: Structure of Bacteriophage T4D. The numbers and other designations represent gene products from the phage T4D genome that comprise the structure of the phage. (Taken from Kutter and Ruger, 1985).

has been the experimental workhorse. The past 50 years of experimentation have resulted in T4 being the most well understood phage genetic system available for studying basic molecular phenomena. One of these molecular phenomenon is genetic exclusion.

Genetic exclusion in phage T4 is the ability of a primarily infecting phage to prevent a secondarily infecting phage from contributing its genetic information to the progeny. Primarily infecting or primary phage is/are the first phage(s) to infect a cell; whereas, the secondarily infecting phage(s) or secondary phage(s) are those that infect after the primary phage(s) have begun its infectious cycle. Two or more genetically distinct T4 phage may contribute to the progeny if the infection is carried out simultaneously. Also if the infections are conducted sequentially in the presence of a host cell metabolic inhibitor, such as KCN, at sub-lethal concentrations both types of infecting phage will contribute to the progeny after the inhibitor is diluted out to innocuous levels (Cohen, 1949). However once the infectious cycle has begun, addition of secondarily infecting (or superinfecting) phage prompts the infected cell to respond in a number of characteristic ways, some of which lead to genetic exclusion.

Genetic exclusion or superinfection exclusion, first noted by Delbruck and Luria (1942) as infection "interference" between various coliphages, was shown by Delbruck and Bailey (1946) to be the inability of superinfecting phage to contribute their genetic information to the progeny. The exclusion phenomenon in T-even phages was first described by Dulbecco in

1952 and has been of interest to several investigators since then (Visconti, 1953; Fielding and Lunt, 1970; Anderson, Williamson and Eigner, 1971; Sauri and Earhart, 1971; Vallee, Cornett and Bernstein, 1972; Okamoto, 1973; Yutsudo and Okamoto, 1973). Despite the interest in the phenomenon among investigators, there has been virtually no discussion of the adaptive benefit it has for the phage until recently (Obringer, 1988, 1987; Abedon, 1990).

The exclusion by primary phages of superinfecting phage genomes can be classified ecologically as competition-either interspecific (between species) or intraspecific (among individuals of the same species) (reviewed in Smith, 1966). Each type of competitive exclusion presumably bestows an advantage to the primary infecting phage. Interspecific exclusion allows the primary phage (predator) to sequester the resources of the host cell (prey) for the exclusive production of its own genome and incidentally that of its species. With respect to intraspecific competition, exclusion may also act as an adaptive mechanism for promoting individual fitness since the protected resource is used for the production of the primary infecting phage's own unique genome. In other words it is beneficial for the individual phage to be selfish or practice genetic exclusion.

The imm gene was named for its ability to provide "immunity" to superinfecting phage T4 (which leads to genetic exclusion) and the disruptive effects, such as cell lysis, of superinfecting T4 ghosts (phage with their DNA removed). Imm falls into a class of phage T4 genes known as the immediate early

genes because they are transcribed by the host unmodified RNA polymerase and their expression begins immediately after infection (Dulbecco, 1952; Peterson, Cohen and Ennis, 1972; O'Farrell and Gold, 1973; Yutsudo and Okamoto, 1973). Expression of the imm gene is blocked by drugs that inhibit the E. coli gyrase implying that the imm gene is under host gyrase control (Sinden and Pettijohn, 1982). Expression of the imm protein (gp imm) requires de novo protein synthesis, its level of expression is independent of the multiplicity of infection and the wildtype protein acts in a stoichiometric rather than in a catalytic manner (Vallee and Cornett, 1973). Although the synthesis of imm increases linearly for up to 10 minutes post infection (pi), it bestows nearly complete immunity by 3 minutes pi at 37 °C. The imm gene mutants have been shown by various investigators to be approximately 50% defective in the exclusion phenotype at 37 °C, 4 minutes pi (Dulbecco, 1952; French et al., 1952; Sauri and Earhart, 1971; Cornett, 1974; Vallee and de Lapeyriere, 1975; Obringer, 1988). By comparing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein gels of wildtype versus imm- phage, Yutsudo (1979) attributed two proteins to the imm gene (77 kilodaltons [kDa] and 45 kDa), while O'Farrell and Gold (1973) in a similiar manner assigned weights of 40 kDa and 28 kDa to the imm gene product(s). It is not clear why in both cases two proteins, rather than one, are altered in the imm- mutant and why the values from the two labs disagree. O'Farrell and Gold's molecular weight determinations were corroborated by Sinden and Pettijohn (1982). Recent DNA sequence data predicts gp imm to be approximately 83 or 73 amino

acid residues in size and lipophilic in nature (Lu and Henning, 1989). The imm protein is postulated to act at the cell wall or membrane (Vallee and Cornett, 1973; Yutsudo and Okamoto, 1973; Lu and Henning, 1989), because superinfecting phage have a substantially reduced efficiency of DNA injection (Bayer, 1968; Cornett 1974). Most of the DNA that is injected by superinfecting phage is subsequently hydrolyzed by host endonuclease I found in the periplasmic space (French et al., 1951; Lesley et al., 1951; Hershey et al. 1954; Fielding and Lunt, 1970; Anderson and Eigner, 1971; Anderson et al., 1971; Yutsudo and Okamoto, 1973). The mechanism of action of gp imm is currently thought to involve the the inactivation or alteration of the incoming chromosome's pilot protein (Obringer, 1988). The interaction of gp imm with the pilot protein presumably exposes the superinfecting phage's double stranded DNA to destruction by the host's DNA degrading enzymes, such as exonuclease V.

Although the immunity function had been known since the late 1940's the responsible gene was not discovered until the early 1970's when two laboratories independently found and mapped a mutant defective in imm. Mufti (1972) was the first to report a mutant that was defective in the immunity function. Following up this finding in the same lab, Vallee and Cornett isolated and mapped a non-amber imm- mutant to a position between genes 42 (dCMP-hydroxymethylase) and 43 (DNA polymerase) using standard genetic recombinational mapping techniques (Vallee and Cornett, 1972; Cornett and Vallee, 1973). Independently, Childs (1973) using incomplete phage genomes confirmed the map position [Fig. 2,

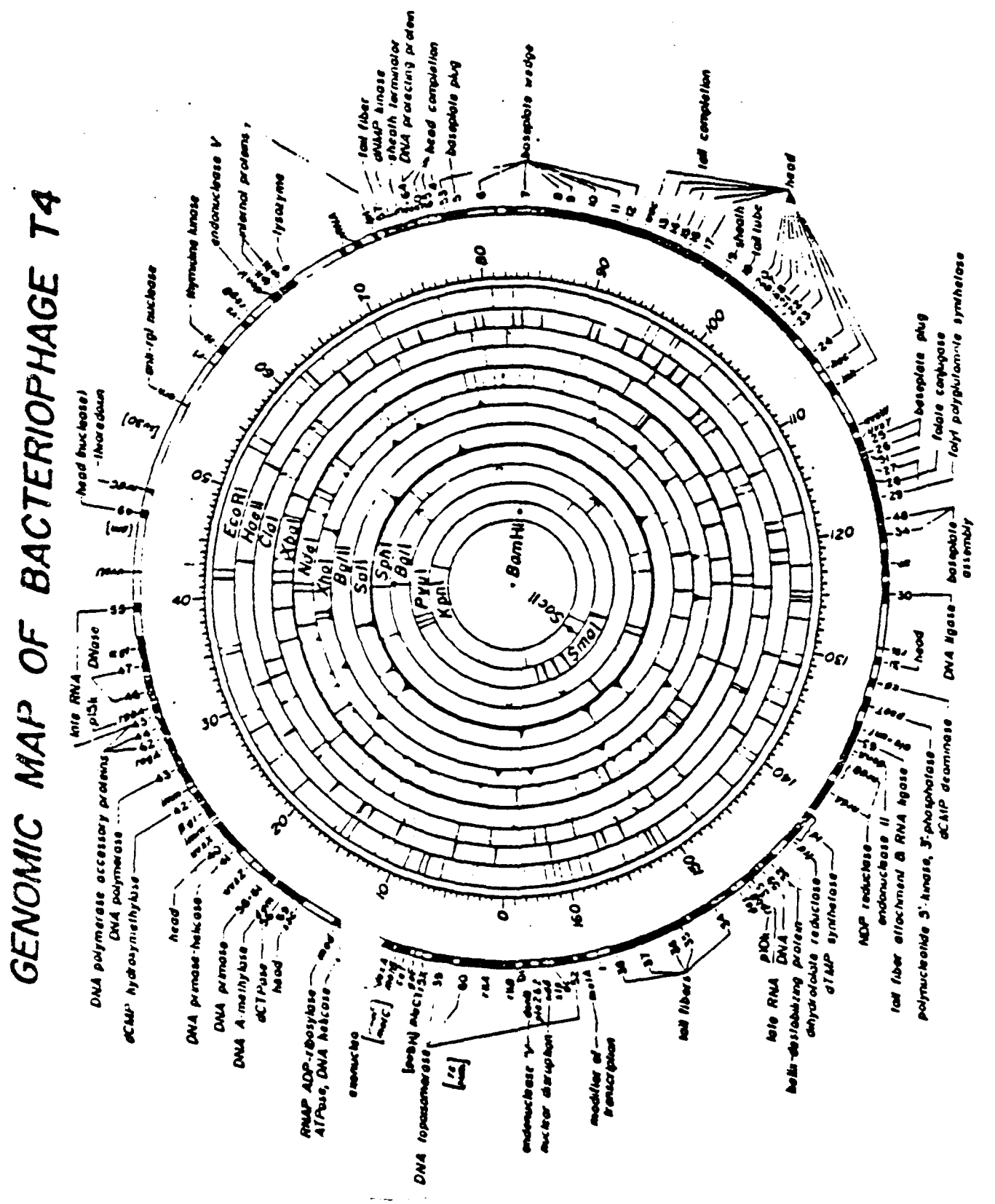


Fig 2: Genomic Map of Bacteriophage T4(taken from Kutter and Ruger, 1985).

map position about 26.5 kilobase (kbp) from the border of the rIIA-rIIB genes]. Cornett (1974) determined that his mutant and that of Childs' were isoallelic. Supporting the above map postion with molecular evidence, Jiang, Na and Xu (1984) and Lu et al. (1982) reported that a clone of the imm gene also contained a considerable portion of gene 42.

More recent information concerning the gene 43-imm-gene 42 region supports the previous work, but also poses a new dilemma. The 1985 "Bacteriophage Genomic Map" (Kutter and Ruger) places the carboxy end of gene 43 at approximately the 26.71 kb position and the amino terminal of gene 42 at approximately 26.2 kb. An immediate early promoter with the direction of transcription toward gene 42 has been mapped to the 26.3 kb region (Gram et al., 1984) (Fig. 2). All of this taken together leaves about 0.5 kb between genes 42 and 43 to accommodate imm. Thus there is only room for at most a 18.5 kDa protein, which is only 24 to 66 percent of the information needed (the percentage depends on which of the reported sizes of the imm protein[s] is correct).

Although the imm gene seems to account for the majority of the exclusion phenotype it is not the only gene involved in the process of genetic exclusion. Gene sp, located in approximately the same area of the genome is also responsible for aiding in genetic exclusion. Gene sp, acting plieotropically, has recently been shown to be the same gene as gene 40 which is also involved in head morphogenesis (Obringer, McCreary and Bernstein, 1988), and has been renamed, sp=40 (Kutter, 1990).

As previously mentioned, gene 42 is located in the

neighborhood of sp=40, and is next door to gene 1mm. Bacteriophage T4 gene 42 (reviewed by Mathews and Allen, 1983), an essential gene, produces an enzymatic protein called deoxycytidylate monophosphate hydroxymethylase (dCMP-HMase). This enzyme, discovered in 1957 by Flaks and Cohen, catalyzes the conversion of deoxycytidylate monophosphate to 5-hydroxymethyldeoxycytidine by the addition of a hydroxymethyl group to the 5 carbon postion of the N-base. Gene 42 mutants completely lack DNA unless there are accompanying phage mutations which do not degrade unmodified DNA. Gp 42 has been shown by genetic evidence to participate in the phage replication apparatus suggesting direct or indirect interaction and kinetic coupling with the DNA polymerase. The obvious advantage of hydroxymethylated DNA in the phage genome is that it is protected while phage DNA-degrading enzymes hydrolyze host DNA. Another advantage is that the usual arsenal of restriction endonucleases used by the host to degrade foreign DNA are rendered useless against phage DNA because of the hydroxymethylation of cytosine.

The infection process of phage T4 essentially allows the phage to inject its genome into a suitable E. coli host cell permiting a cycle of phage growth. Infection by phage T4 has been extensively studied (reviewed by Goldberg, 1983). If all goes well during host cell takeover and T4 gene expression the various components of the progeny phage are constructed, the original DNA is multiply replicated, and then all of the components are assembled in a neatly organized particle. After

about 20-30 minutes at 37 degrees Celsius and the production of about 100 new phage particles, a phage encoded lysozyme lyses the membranes of the host cell, and the progeny phage are released into the environment to begin the cycle anew. The proposed role of the imm gene in this cycle is to establish a territory, the E. coli cell, whose resources are available for the exclusive use of the primary infecting phage in progeny production since secondary infecting phage, who would share the resource if allowed to infect, are excluded from infection by gps imm and sp (Obringer, 1988). This function alone is sufficient to account for the evolutionary selection of the imm gene (Abedon, 1990, personal communication).

MATERIALS AND METHODS

Media

Bacteria were grown in Hershey's broth (HB) or on enriched Hershey's agar plates. These were prepared as prescribed by Steinberg and Edgar (1962). When antibiotic medium was required the appropriate amount of antibiotic, salts or stock solution, was added to the concentrations recommended in Maniatus, Fritsch and Sambrook (1982). M9 adsorption salts solution was used in diluting phage and for promoting adsorption of phage to host cells in various assays. To prepare this solution the following were added per liter of distilled deionized water: 5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, and the pH adjusted to 6.8 to 7.0. All media were autoclaved, cooled to 55 C to add non-autoclavable supplements, and then allowed to cool further to room temperature before use. Soft top agar (enriched Hershey's

plate media with 1/2 the agar added) was used at a temperature of 45°C where it is liquid. Indicator cells and phage were mixed in the soft agar before plating to titer phage.

Bacteria and Phage Strains

The strains used, their relevant characteristics and the source or a reference are listed in Table 1.

Table 1: Bacteria and Phage Strains Used

Bacteria Strain	Genotype or comments(e)	Source/ reference
E. coli		
S/6	su-	Berstein Lab (a)
CR63	Su I, serine	Berstein Lab
K803	su+	Berstein Lab
594	su-(parent of DE828)	K. Peterson (a)
G240	su-	CGSC (b)
G240R4	Su 9, UGA, trp	CGSC
U11R1d	Su 5, UAA-UAG, lysine	CGSC
H12R7a	Su 4, UAA-UAG, tyrosine	CGSC
DH5	Su 2, lacZdel, r-	M. Moran (a)
T4 Phage		
T4D+	wildtype	Berstein Lab
am21x3	(42 amber)	J. Wiberg (c)
am269x3	"	"
amC87	"	"
amNG93	"	"
amE142, imm-	(39 amber, imm)	Berstein Lab
amNG372	(55 amber)	"
amNG205	(42 amber)	"
amNG205 sp-	(42 amber, sp)	"
amNG205 imm-, sp-	(42 amber, imm, sp)	"
amN93	(11 amber)	"
amE498	(42 amber)	B. Wood (d)
amN122	"	"
amNG411	"	"
amE117	"	"
amE774	"	"
amNG55x5	"	"
amNG554	(42 amber)	B. Wood

amCT36	(42 amber)	B. Wood
amE645	"	"
amNG394	"	"
amNG352	"	"
amNG385	"	"
4304	(43 amber)	Berstein Lab
4315	"	"
4306	"	"
4312	"	"
4335	"	"
4316	"	"
B22	"	"
4305	"	"
4322	"	"
E192	"	"
4314	"	"
4309	"	"
4302	"	"
R1 - R10	revertants of amNG411	This study
X655	(rIIB opal)	B. Wood
5ts1	(5 ts)	Kao and McClain, 1980
imm2	(imm)	Berstein Lab

(a) The Berstein Lab, K. Peterson and M. Moran are at the Univ. of Arizona.

(b) CGSC is the E. coli Genetic Stock Center, Yale University, School of Medicine.

(c) J. Wiberg is at the Univ. of Rochester, Medical Center.

(d) B. Wood is at the Univ. of Colorado, Boulder.

(e) The parenthesis under the genotype column in the phage strains indicates the genes bearing the defect and type of defect, if known.

Bacterial cultures

Bacterial cultures were stored for long term use in agar slants and stabs or by freezing at -70 °C in a mixture of 50% glycerol and 50% overnight culture. Overnight cultures were grown in HB at 37 °C from slant inocula weekly or as required. Experimental host bacteria were prepared by diluting overnight cultures 100-fold into the appropriate medium and incubating them

at the experimental temperature with aeration. The bacterial densities were determined at frequent intervals using a Petroff-Hauser counting chamber. The bacteria were generally grown to 1-2 x 10^8 /ml and diluted to the desired concentration. Centrifugation of cells was generally conducted at 2,000 x g RCF. Plating indicators were prepared by growing the host cells to about 4 x 10^8 /ml, concentrating 25-fold by centrifugation, and resuspending in fresh HB.

Titration of cells and phage plaque forming units

All titering of cell colony forming units (cfu) was carried out on enriched Hershey's agar plates with top agar overlays. Phage titers were determined after dilution by plaque formation on agar plates by the agar overlay method of Adams (1959). An aliquot of the phage-containing dilution was dispensed into soft top agar along with approximately 0.2 ml of plating indicator, and then spread evenly over the surface of an agar plates by gently tilting the plate in a circular manner. Then the soft agar was allowed to harden on a level surface, plates were incubated for a minimum of 12 hours at the appropriate temperature followed the plaque tabulation.

Preparation of phage

Phage stocks were prepared by either the plate or bottle lysate method. In the plate lysate procedure 1 x 10^5 phage and 2 x 10^9 bacteria were added to 2.5 ml of soft top agar plus 3 ml of HB, and then poured over the surface of a Hershey's plates. After overnight incubation at 30 C several drops of chloroform were added to each plate plus 5 ml of M9 salts solution. Two hours were allowed for the cells to lyse. Then the phage

containing fluid was decanted and cellular debris removed by centrifugation at 10,000 x g for 10 minutes. This procedure normally yielded phage titers around 5×10^{10}. If higher titer phage stocks were desired, the phage suspensions were sedimented by centrifugation at 23,000 x g for 2 hours. All centrifugation was conducted at 4^{o} C. Then the pellet was resuspended in 1-3 ml of M9 salts solution. This usually resulted in a 10-50 fold increase in titer.

Bottle lysates were prepared by inoculating a 250 ml bottle of HB with 1×10^{4} to 10^{6} phage and 1×10^{8} cfu of host cells. The mixture was incubated overnight at 30^{o} C with aeration. Following incubation several drops of chloroform were added, the mixture stirred vigorously, and let stand for 2 hours. The bacterial debris was removed by a low speed centrifugation as above.

Verification of suppressor host strains

Verification of the presence of a nonsense mutation suppressor in a host strain was accomplished by titering a phage mutant with a known type of nonsense mutation (i.e. amber, opal, ochre). The type of mutant used corresponded to the type of suppressor being tested. A significant increase in the efficiency of plating by nonsense mutants of a particular type on the presumed suppressor strain, as compared to the control strain (known non-suppressor), was taken as evidence for the presence of the corresponding suppressor in the host strain. For example, I used a known opal mutant (X655) in the T4 rIIB gene to verify that E. coli G240R4 and CAJ64 possessed opal suppressors before

using them in further experiments.

Standard genetic exclusion assay

The standard exclusion assay measures a primary infecting phage's ability to prevent superinfecting phage from contributing their genetic markers to the progeny. The quantitative expression of the exclusion phenotype as determined by this assay is referred to as the "immunity value." This is a measure of immunity to superinfection or genetic exclusion. The immunity value (IV) was determined as follows: The titer of infective centers was measured when a delayed superinfection (D) by a phage T4 amber mutant was carried out after a primary infection by the amber mutant being assayed for its exclusion phenotype. This titer was then divided by the titer of infective centers measured from a simultaneous infection (S) of the same two phage as indicated by the equation:

$$IV = \frac{D \text{ (titer of infective centers after delayed superinfection)}}{S \text{ (titer of infective centers after simultaneous infection)}}$$

Wildtype imm+ phage have an IV of 0.08 at 37 C since the success of delayed infections is much lower than of simultaneous ones. Conversely, the standard imm-2 mutant has an IV of 0.47 at 37 C indicating that in this case delayed infections are more successful.

The procedure for measuring the IV began by growing the host cells to 1-2 X 10^8 /ml at the experimental temperature (ET), and concentrating the host cells by centrifugation at the ET if possible. The hosts were resuspending in prewarmed M9 salts

solution (Clowes and Hayes, 1968) to approximately 5 X 10^8 /ml and starved for a minimum of 30 min. For the delayed superinfection (D), at t = 0, 0.1 ml of amber phage #1 (moi < 10^{-3}) was added to 1.5 ml of the su- bacteria and the mixture incubated at the ET. [Very low mois (10^{-3} - 10^{-4}) were used to ensure that only single infections occurred, thereby avoiding dosage artifacts.] After 4 min. to allow adsorption, an equal volume of prewarmed double concentration Hershey's broth was added to provide nutrients to permit expression of the exclusion genes. Because complementation or cross-reactivation is required for phage growth, only cells that contain phage #1 and phage #2 will give rise to an infective center. Those cells containing only one of the mutants will not. At t = 8 min amber mutant #2 (having a mutation in a different gene than that of amber phage #1) was added and allowed to adsorb for 4 min before titering on E. coli S/6 (su-). The simultaneous superinfection (S) was carried out in a similar manner except that both amber phages were added at t = 0 min and titered at t = 10 min. A gene 39 double mutant (amber E142, imm-2) was used as an imm- control while a gene 55 amber mutant (NG372, imm+) provided an imm- control.

Reversion of amNG411

Revertants of amNG411(gene 42) were obtained by plating this mutant at high titer on a non-suppressing host. The plaques that arose resulted from am+ revertant phage. The reversion rate of amNG411(42) was approximately 2 x 10^{-6}, so about 1 x 10^7 phage were plated on E. coli S/6 (su-) to yield a sufficient number of plaques to test. Ten independent revertant plaques were selected at random, chorded and grown to high titer stocks for further

use.

Recombinational mapping of phage mutants

The map position of the T4 mutant amN122(42) is not reported in the literature. I mapped this mutation using the two-factor cross method described by Holmes (1975). The crosses to other previously mapped gene 42 amber mutants were performed in E. coli CR63(su+). To determine the map position of amN122, it was crossed with amNG385(42) and amE498(42). The map distance from amN122 to amNG385 was 0.1 map units, and to amE498 it was 0.6 map units. This allowed positioning of amN122 at approximately the middle of gene 42. The sum total of the two intervals is 0.7 which agrees exactly with the distance Holmes (1975) found between the two end markers of amNG385 and amE498.

Non-standard exclusion assay

This exclusion assay was employed when the phage being assayed did not carry a conditional lethal mutation, which would be lethal under the assay conditions. I used it to assay the amNG411 revertant phages, since the amber mutation that was formerly in gene 42 was reverted to a non-lethal condition. The assay began by inoculating 1×10^9 E. coli S/6 (su-) with the primary infecting phage at an moi of about 0.1. The primary infecting phage is the phage being assayed for its exclusion phenotype. The host cells were previously starved for a minimum of 30 minutes in prewarmed M9 salts solution. After 4 minutes of adsorption (t = 4) an equal volume of double concentration HB, prewarmed to 37 °C, was added to allow expression of the exclusion genes. At t = 8 minutes, amNG205 (gene 42 amber mutant) was

used to secondarily infect at an moi of 8 to 10. At t = 12 an aliquot of the culture was sampled for free (unadsorbed) phage and for infective centers to assess the adsorption of the phage to the host cells and their ability to produce productive infections. In order to prevent progeny phage released after a single cycle of infection from colliding with and adsorbing to cells prior to the lysis of the whole population, the superinfected cells were diluted (Ellis and Delbruck, 1939) into 37 C HB within 20 minutes pi. The infected cells were then allowed to incubate for 1 hour. Chloroform was then added to artificially lyse the cells that had not already done so. The progeny phage were plated on E.coli S/6(su-) and on E. coli CR63 (su+). The fraction of the superinfecting phage able to contribute their genetic markers to the progeny was calculated from the plaques formed on each host. The E. coli S/6 strain titers only the non-amber (am+) phage while the E. coli CR63 strain titers all of the phage in the culture. The plaques formed on E. coli CR63 represent the progeny of both the primary and secondary phage. But the plaques formed on E. coli S/6 represent only the progeny of the primary infecting phage because the secondary phage carries an amber mutation and cannot grow on a su- host like E. coli S/6. The titers on the different hosts were adjusted for the efficiency of plating (eop) of T4D+ (wildtype) on that host for each experiment to allow the results to be directly comparable.

The degree of genetic exclusion was calculated as follows: The eop adjusted titer on E. coli CR63 minus the eop adjusted titer on E. coli S/6 was divided by the eop adjusted titer on E.

coli S/6. This number expresses the fraction of superinfecting phage that were able to participate in progeny production. This measures the primarily infecting phage's ability to exclude the secondarily infecting phage. The number is actually the ratio of progeny phage with the secondary infecting phage genotype (2^o) to the number of progeny phage with the primary infecting phage genotype (1^o) or $2^o/1^o$. Imm+ primary infecting phage yield a $2^o/1^o$ ratio of about 1, while imm- primary phages give a ratio about 4-5 times higher. This is expected since many more secondary infections can take place in a cell primarily infected with an imm- phage than an imm+ phage.

A variation of this protocol was used when assaying the exclusion phenotype of a primary am, rather am+, infecting phage. In this case the host cell used was E. coli CR63 (su+) which allows growth of the am mutant as well as the secondary infecting am+ phage. The calculations were adjusted to yield the ratio of 2^o to 1^o infecting phage as before. This variation in protocol was used in assaying the exclusion phenotype of amNG411 to enable direct comparison of this am parent to its am+ revertants. This method provides a convenient measure of the success of a secondary infection without the primary phage having to carry a conditional lethal mutation in an unrelated essential gene as is required in the standard exclusion assay. Despite the apparent simplicity of this assay, it is not the preferred method because it is more difficult to perform and lacks the precision and accuracy of the standard exclusion assay.

RESULTS AND DISCUSSION

The imm Gene

Characterization of the imm-2 mutant

The imm gene mutant isolated by Vallee and Cornett (1973) was designated as imm-2 and it proved to be allelic with Childs' (1973) mutant, designated as imm 1. In my work imm-2 was used as the standard imm- mutant. The imm-2 mutation is not suppressible in amber suppressor containing hosts, so imm-2 is not an amber mutation (Vallee and Cornett, 1973). [The three nonsense or termination codon mutation designations are: (i) amber having a UAG sequence on the messenger RNA (mRNA). (ii) Opal - UGA. (iii) ochre or umber - UAA (Birge, 1981).]

To facilitate future investigation of the imm gene's involvement in genetic exclusion, I attempted to further characterize the imm-2 mutation. Using the standard genetic exclusion assay designed to quantitate the degree of exclusion of the primarily infecting phage, the imm-2 mutant was assayed in various E. coli hosts and at a range of temperatures. Briefly, the assay yields a value, henceforth referred to as the immunity value (IV), that reflects the amount of successful infection by the secondarily infecting phage. Therefore low IV, indicates a low rate of successful superinfection, i.e. a high level of exclusion. A high IV, indicates a successful superinfection, i.e. a defective exclusion phenotype. For example a phage that is wildtype for exclusion characteristically yields an IV of 0.08 at 37 C in E. coli S/6, whereas imm-2 yields an IV of 0.47 due to the increased success of superinfection. The IVs can vary slightly depending upon the host.

To ascertain whether or not the imm-2 mutant had an opal or ochre defect, I assayed its ability to genetically exclude superinfecting phage in various E. coli nonsense suppressor strains. If imm-2 is suppressed by the suppressor carrying strain the resultant IV would be expected to move from that of the imm- phage to that of the imm+ phage. As can be deduced from the results presented in Table 2, the imm-2 mutation present in the strain amE142(gene 39), imm- is not an opal (UGA) suppressed by the insertion of tryptophan, nor is it an ochre/amber mutant (UAA/UAG) suppressed by the insertion of lysine or tyrosine. In no case did the imm mutant exhibit a wildtype phenotype. Since the mutation in the imm-2 mutation was not suppressed it probably is not a nonsense mutation, at least one corrected by the insertion of the above amino acids. In my succeeding experiments with imm-2 I took this conclusion into account.

The last column in Table 2 shows data for amNG411(gene 42) which has a defective exclusion phenotype and is used here as an imm- control. I will return to these results after reviewing the data in Table 3.

The exclusion phenotype of gene 42 and 43 amber mutants

As mentioned in the introduction, there is an inconsistency between the phage T4 restriction map and the reported imm gene product (gp) molecular weignts. When the reported sizes for the imm gp are compared with the coding region available between genes 42 and 43, one finds that there are insuffient base pairs to code for the entire imm gp. Lamm, et al. (1987) suggest a smaller imm gp size deduced from the sequence data and cloned

expression, but whether or not these data accurately reflect the case during the phage life cycle is an open question. A conservative explanation is that the imm gene overlaps an adjacent gene. Two lines of evidence suggest that this is the case. First, Childs (1973) using partial T4 genomes as the basis for mapping only obtained the imm+ phenotype when a gene 42 marker was also present in the phage. Second, Lu (1982) reported screening 5000 clones from a phage T4 genomic library and finding at least a portion of gene 42 on every one containing the imm gene. Furthermore, a strong early promoter has been mapped to the reputed imm gene region (26.3 kbp) providing a likely candidate for the imm gene's regulatory element. If an overlap exists, a nonsense mutation of a neighboring gene

Table 2: Genetic Exclusion by Three Phage Strains in Various E. coli Nonsense Codon Suppressor Hosts.

E. coli Strain	Phage T4 Strain		
	amNG372 (gene 55, imm+)	amE142 (gene 39), imm-2	amNG411 (gene 42)*
U11R1d (Su 5, lysine- UAA-UAG)	.11±.01	.39±.02	.34±.01
H12R7a (Su C, tyrosine- UAA-UAG)	.12±.03	.57±.01	.39±.01
CAJ64 (Su 9, tryptophan- UGA)	.20±.01	.29±.02	.58±.06
G240R4 (Su 9, tryptophan- UGA)	.12±.02	.52±.04	.43±.05

The above numbers represent the average Immunity Value obtained by the standard genetic exclusion assay at 37oC for each phage strain plus or minus the standard error. *Phage amNG411(gene 42) exhibits a defective exclusion phenotype.

located in the shared region may also appear as a mutation in the imm gene. Therefore, I assayed amber (UAG) mutants defective in genes 42 and 43 (the neighbors bordering imm) for their effect on the imm phenotype. If the genes overlap in the same reading frame, a nonsense mutation in one gene will also be a nonsense mutation in the other. On the other hand if the overlaps are out-of-phase then a nonsense mutation in one gene (either gene 42 or 43) may introduce a missense mutation in the other (imm). In this case, the different amber mutations in the overlap region would be expected to have different effects on the imm phenotype. Some of the resultant missense mutations in the imm gene might be silent, and some might be temperature or cold sensitive. Others might cause a mild perturbation in the imm gp structure with a small functional loss, while others may result in a totally defective protein and a corresponding imm- phenotype.

Because of the expected variation, I screened 17 amber mutations in gene 42 and 13 in gene 43, which mapped along nearly the entire length of each gene (Fig. 3), at various temperatures. As a positive control an imm+ phage with an amber mutation in an unrelated gene 55 was used, and as a negative control an imm- mutant with a second amber mutation in gene 39 was used. The second amber mutation is required for the experimental protocol but has been shown to have no affect on the immunity phenotype (Vallee and Cornett, 1973). The protocol for the standard genetic exclusion assay is described in Materials and Methods. The results in Table 3 show that seven amber mutants, all in gene 42, are significantly defective in imm function. One (amNG411) was defective at all five temperatures, while amE498

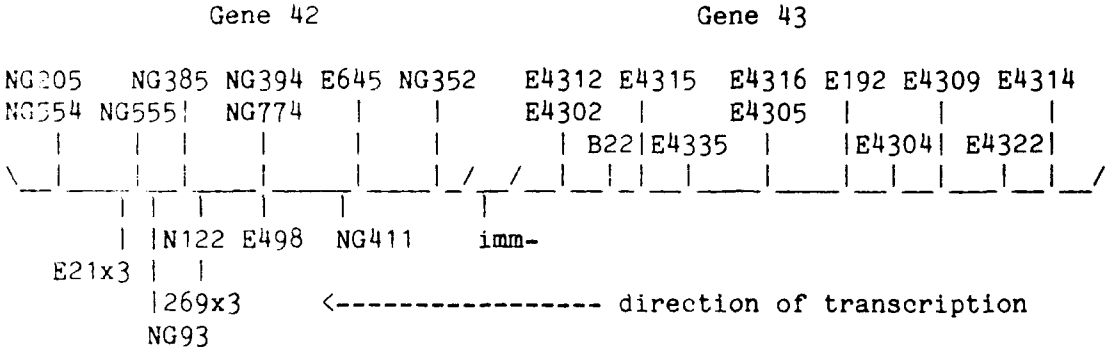


Fig. 3: A genetic map of the amber mutation sites in genes 42 and 43 (plus the imm gene). All mutants that were assayed for their immunity phenotype are indicated according to the site of their defect. The map positions of the gene 43 mutants were taken from Reha-Krantz and Bessman (1981) and Allen, Albrechet, and Drake (1970). The map positions of most of the gene 42 mutant sites were taken from Holmes (1975) with the exception of amN122(gene 42), which I mapped using the two-factor cross method described by Holmes. The positions of E21x3 and 269x3 that were kindly provided by J. S. Wiberg (personal communication, 1986). The mutant designations appearing above the horizontal line displayed an imm+ phenotype, while those below exhibited some degree of defective immunity. Two other gene 42 mutants, CT36, E117 which were not mapped also showed an imm+ phenotype, while phage C87, also unmapped, showed a slightly defective imm phenotype.

TABLE 3: Immunity of gene 42 and gene 43 amber mutants

Gene(s)	Amber Mutant	Immunity values obtained at the temperatures indicated				
		25°C	30°C	32°C	37°C	42°C
39, *imm*-2	E142	.67±.05	.54±.03	.52±.03	.47±.03	.35±.03
55, *imm*+	NG372	.21±.01	.13±.01	.11±.01	.08±.01	.06±.01
42	NG411	.50±.03	.43±.02	.29±.01	.46±.08	.28±.05
	E498	.44±.02	.33±.05	.08±.00	.08±.00	.04±.01
	N122	.30±.02	.21±.01	.09±.01	.05±.01	.05±.01
	NG93		.21±.03		.15±.01	
	E21x3		.21±.02		.17±.03	
	C87		.19±.04		.10±.01	
	269		.18±.03		.07±.01	
	NG205		.14±.03		.04±.01	.08±.02
	E117		.13±.02		.05±.01	.06±.02
	E774		.12±.01		.06±.01	.07±.02
	NG555	.22±.01	.11±.01		.12±.02	.05±.01
	NG554		.11±.00		.06±.01	.07±.01
	CT36	.23±.02	.11±.02		.18±.03	.04±.00
	E645	.24±.00	.10±.03		.11±.02	.04±.00
	NG394	.22±.02	.10±.01		.09±.02	.06±.00
	NG352		.07±.00		.05±.00	.05±.01
43	4304		.15±.01		.09±.00	.10±.01
	4315		.15±.03		.04±.01	.05±.00
	4306		.13±.02		.04±.00	.04±.01
	4312		.12±.07		.09±.01	.05±.00
	4335		.11±.01		.05±.00	.06±.01
	4316		.10±.02		.05±.01	.04±.00
	B22		.10±.01		.05±.01	.04±.01
	4305		.10±.01		.10±.02	.07±.01
	4322		.09±.01		.06±.02	.05±.00
	E192		.09±.03		.05±.00	.05±.00
	4314		.09±.01		.03±.00	.05±.00
	4309		.07±.01		.04±.01	.05±.01
	4302		.01±.01		.04±.01	.04±.00

29

and amN122 were measurably defective at 25°C and 30°C, but not at 32°C and above. Two others (amNG93 and amE21x3) were defective to an intermediate degree with no observed temperature sensitivity. Finally, amC87 and am269x3 are significantly defective in imm function only at 30°C. Fig. 4 provides a graphic representation of the IVs of three exclusion defective gene 42 mutants compared to the imm+ and imm-2 controls. The figure shows the consistent imm- phenotype of amNG411(42) at all temperatures and the cold sensitive phenotype of E498(42) and amN122(42). Amber mutations which do not exhibit a defective imm phenotype are interspersed among those that do (Fig. 3). Therefore genes 42 and imm probably do not overlap in the same reading frame, but rather overlap in an out-of-phase orientation. Assuming the recombinational map of gene 42 represents the actual linear order and approximate spacing of the sites of the mutations, the imm gene shares approximately 50% of gene 42's base pairs.

Additionally, by comparing the row of IVs in Table 3 for phage E142(39), imm-2 to IVs for the imm+ control (NG372), one can see that imm-2 is not a temperature sensitive or cold sensitive mutant.

The exclusion phenotype of amNG411 revertants

One possible explanation for the strongly defective exclusion phenotype of amNG411(gene 42) is that it is a double mutant; that is, it may have an amber mutation in gene 42 and a second mutation in a non-overlapping imm gene. To investigate this possibility I selected 10 independent spontaneous revertants of amNG411 based on growth of the phage on a host lacking an

amber suppressor. I then assayed these am+ revertants for their exclusion phenotype. As controls, the amNG411 parent as well as imm+ and imm-2 phage were tested for their exclusion phenotype. The technique I employed was developed by S Abedon (personal communication, 1986, see Material and Methods). Its advantage for measuring the exclusion phenotype of the revertants is that it does not require an unrelated conditional lethal mutation in the phages being assayed. This non-standard exclusion assay measures the exclusion phenotype of the primarily infecting phage as the ratio of the secondary infecting phage's genotype to the primary's in the progeny. Under the assay condition used, the ratio found when the primary phage was imm+ was 0.65 at 37 C

Table 4: Genetic Exclusion by amNG411 (gene42) Revertants at 37 C

Phage strain	2 /1 ±SE
imm+ (T4D+)	0.65±0.16
R1	1.36±0.04
R2	1.07±0.15
R3	0.90±0.22
R4	1.60±0.36
R5	1.08±0.25
R6	0.99±0.13
R7	0.88±0.06
R8	1.07±0.10
R9	1.26±0.38
R10	1.07±0.35
imm-2	3.65±1.35
amNG411 (parent)	3.96±0.24

The above values are measures the exclusion phenotype of the designated phage using the non-standard genetic exclusion assay. The assays of the amNG411 revertants (designated R 1 - 10) were performed in E. coli S/6(su-) at 37oC. The amNG411 parent was assayed in E. coli CR63(su+) because the gene 42 amber mutation would intefere with the progeny production in a Su- host. The imm+ and imm-2 controls were tested on both E. coli S/6 and CR63. The values of amNG411 determined on E. coli CR63 were normalized to the E. coli S/6 scale for the purposes of direct comparison.

(Table 4), while the value for primary imm-2 phage was 3.65. This greater ratio reflects an increase in the proportion of the genotype of the secondary phage in the progeny when the imm gene is defective. (Note that amNG411 is also exclusion defective in this assay yielding a ratio of 3.96.) The $^{o}2\,/\,^{o}1$ ratios of the amNG411 revertants are intermediate, but more closely resemble the $^{o}2\,/\,^{o}1$ ratio of imm+ than of imm-2 or the amNG411 parent (Table 4 and Fig. 5). These results demonstrate conclusively that when the amber mutation in amNG411 is reverted for the gene 42 conditional lethal phenotype, the imm- character also reverts to a more nearly imm+ phenotype. These are exactly the results predicted if amNG411 does not contain a second mutation, and the amber mutation in gene 42 is the same mutation that exhibits a defective exclusion phenotype. If amNG411 was actually a double mutant, the chance of all 10 revertants having spontaneously reverted at both sites is approximately 1×10^{-22}. These results not only indicate that amNG411 does not have a second site mutation in the imm gene (or any other exclusion related gene), but also provide further evidence that genes 42 and imm overlap.

The effect of opal suppression on exclusion

Preliminary DNA sequence information (H. Gram, personal communication, 1985) shows a long open reading frame (ORF) extending from a promoter in the putative imm region into structural gene 42 in an out-of-phase orientation. If it were not for a single UGA (opal) termination codon 256 base pairs into the imm gene ORF and prior to the gene 42 promoter region, I would consider this conclusive evidence of the hypothesized overlap.

At first glance the presence of the termination codon, if the sequence data is correct, would negate the proprosed overlap, but there is considerable precedent in the literature for read-through of termination codons (Garen, 1968; Philipson et al., 1978; Geller and Rich, 1980; Birge, 1981), specifically opals (Garen, 1968; Weiner and Weber, 1971; Yates et al., 1977; Nasmyth and Tatchell, 1980), especially in phage (Weiner and Weber, 1973; Yates et al., 1977). As a matter of fact an opal termination codon read-through hypothesis would nicely account for the here-to-fore inexplicable observations by two independent laboratories (O'Farrell and Gold, 1973; Yutsudo, 1979) that imm is responsible for two proteins. This could be accounted for if there were a less than 100% efficient read-through of the opal codon, as seen in the phage lambda O gene (Yates et al., 1977). Although the reported molecular weights differ considerably between the two laboratiories, the ratio of the large imm protein to the small protein are comparable: 40/28 = 1.4 (O'Farrell and Gold, 1973); 77/45 = 1.7 (Yutsudo, 1979).

To address the subject of an opal (UGA) nonsense codon in the sequence of imm prior to gene 42, I designed an experiment based on the following logic. If the opal termination codon is read-through to produce a functional imm gp then growing imm+ phage T4 in an E. coli opal suppressing host may result in a measurable change in the imm phenotype. The two opal suppressing strains used insert the amino acid tryptophan (trp) at the site of the opal termination codon. In one case the su- parent was available as a control, in the other I could not

obtain the isogenic su- parent. There are three possible outcomes of preferentially inserting trp in place of the opal codon: (i) trp is the amino acid inserted by the "normal" read-through mechanism so that no phenotypic change will result, (ii) trp is not the usual amino acid at this position, so that insertion of trp improves the function of the imm gp, resulting in a qualitative increase in imm function which may or may not be measurable; (iii) trp is not the usual amino acid at this position, so that its insertion causes some degree of impaired function which can be measured.

Using E. coli G240R4 (Su 9, trp), an opal suppressor strain, I found a small but definite increase in IV (decrease in immunity) for each of 7 mutants previously determined to be imm+, compared to the immunity value obtained by the same phage in the suppressor minus parent strain E. coli (G240) (Fig. 6). In E. coli CAJ64, the other UGA suppressor strain, the immunity values were 2-3 fold greater than the values obtained in the su- hosts of E. coli G240 or E. coli S/6 implying an even greater loss of immunity (Fig. 6). E. coli S/6 was the su- minus host used in the gene 42 and 43 mutant screening experiments (Table 5). In general these results show a decrease in exclusion of imm+ phage in the presence of an opal suppressor. They can be explained by reasoning that the trp inserting opal suppressor competes with the normal read-through mechanism to yield at least some imm gp which is defective due to the trp substitution. These results indicate that an opal termination codon is involved in the full phenotypic expression of the imm gene, and taken in light of the sequence data they further support the hypothesis

that the imm gene overlaps with gene 42.

Thus far I have obtained strong genetic evidence for the existence of an out-of-phase overlap between the imm gene and gene 42 with probable involvement of an opal termination codon.

CONCLUSIONS

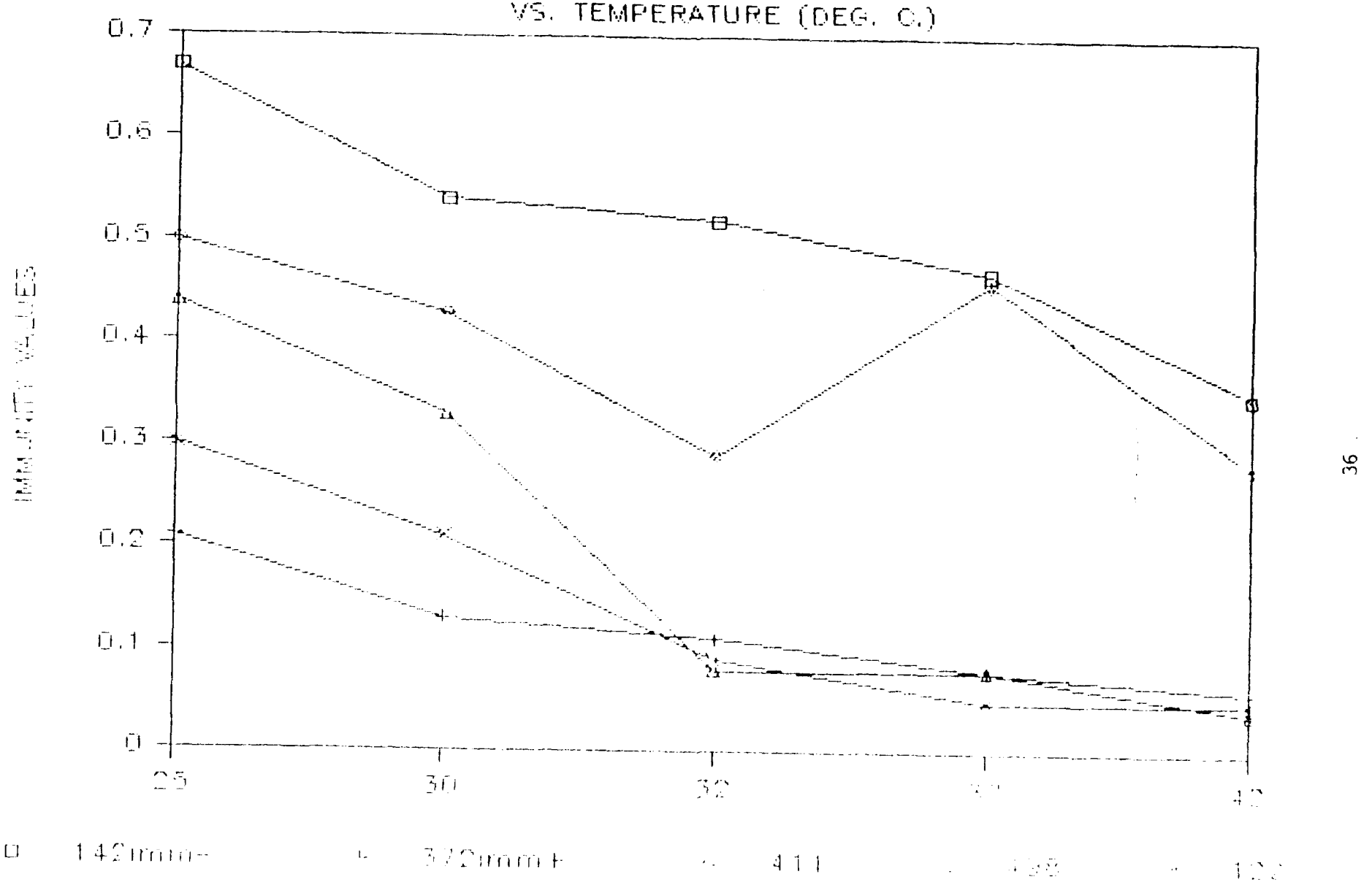
Characterization of the imm-2 mutant phenotype

To aid in the investigation of genetic exclusion, the phenotype of the standard imm- mutant, imm-2, was further characterized. The protein molecular weight findings (Yutsudo, 1979; O'Farrell and Gold, 1973) suggest that imm-2 might be a nonsense mutant since protein bands were missing on SDS-PAGE gels of the protein products of imm-2. However, Vallee and Cornett (1973) had determined that imm-2 was not an amber mutation. As the results in Table 2 show, I found that imm-2 is also not a opal nonsense mutant suppressed by the insertion of tryptophan, nor is it an amber or ochre nonsense mutation suppressed by the insertion of lysine or tyrosine. These experiments do not entirely rule out the possibility that imm-2 is a nonsense mutant, since it may require suppression by an amino acid not inserted in the above hosts.

As indicated in Table 3 and the corresponding lines in Fig. 4 a comparison of the imm mutant's Immunity Values (IVs) with those of the imm+ wildtype over a range of temperatures from 25 ^{o}C to 43 ^{o}C shows that imm-2 never attains the wildtype IV. This indicates it is neither cold nor temperature sensitive.

Based on the above results imm-2 is not obviously suppressible, not temperature sensitive and not cold sensitive.

Fig. 4: Vs OF GENE 42 & 43 MUTANTS



As a result, all subsequent experiments using imm-2 were designed to take these characteristics into account.

The imm gene shares an out-of-phase overlap with gene 42

I have obtained genetic data which provides strong support for the hypothesis that the imm gene overlaps substantially with gene 42. Much of the discussion concerning the overlap between the imm gene and gene 42 has been presented previously with the data, so only a brief review of the findings is presented here. I assayed 30 amber mutants defective in genes 42 and 43 (the two neighbors bounding the imm gene) and found 7 mutants in gene 42 that displayed a significantly defective exclusion phenotype (Table 3, Fig. 4). Six of the seven map to the amino terminal one-half of gene 42. (The seventh mutation site has not been mapped.) Since other amber mutants upstream of the most distal exclusion defective gene 42 mutant show a wildtype exclusion phenotype, I concluded that the imm gene and gene 42 substantially overlap in an out-of-phase orientation (Fig. 3).

amNG411 is not a double mutant

Phage amNG411(42) showed a very strong exclusion defective phenotype. To counter the argument that it may contain two mutations (one in gene 42 and the other in the imm or sp gene), I determined the exclusion phenotype of amNG411 phage reverted for the conditional lethal gene 42 phenotype. All of the revertants more closely resembled wildtype in their exclusion than either the amNG411 parent or the imm-2 control (Table 4, Fig. 5). This indicates that amNG411 is not a double mutant, and that gene 42 is somehow involved in the exclusion process.

One might be tempted to postulate that gene 42 has a dual

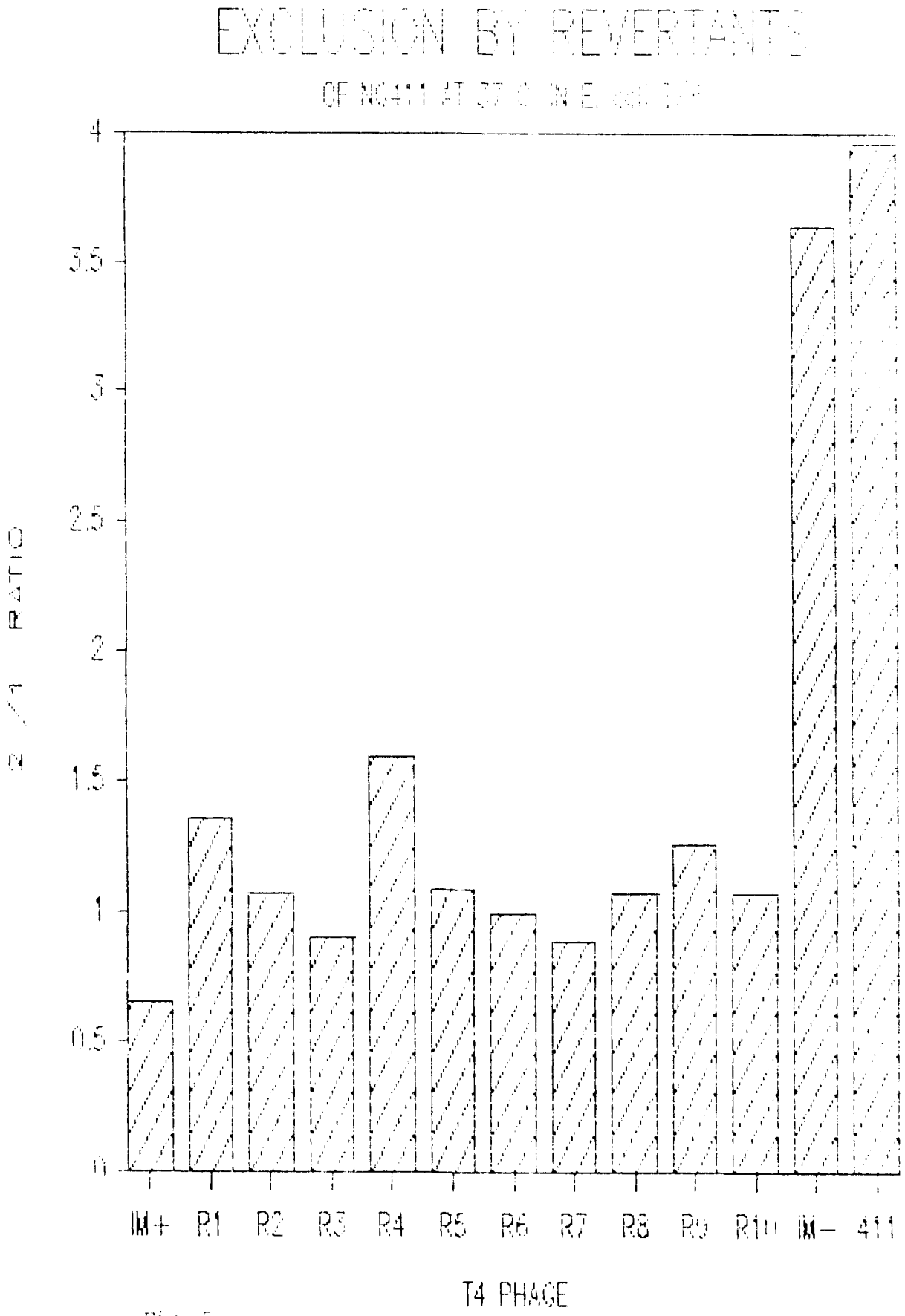

EXCLUSION BY REVERTANTS
T4 PHAGE
M+
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
M-
411

Fig. 5

Fig. 5: The measure of exclusion in this assay is the ratio of the primary phage to secondary phage genotype among the progeny. This ratio, $\frac{2}{1}$ ratio, defines the exclusion phenotype of each phage as determined by the non-standard exclusion assay. IM+ represents the imm+ control (T4D+), IM- the imm- control (imm-2), 411 the amNG411 parent of the revertants, and R1 - R10 the independent revertants of amNG411. See the footnote to Table 4, and Materials and Methods for further explanation.

function, one of which is to promote genetic exclusion. There are two lines of evidence that argue against this idea. First, if that were true then one would expect all amber mutants whose defect is upstream of the most distal exclusion defective gene 42 mutant to also be defective in the exclusion phenotype, but this is not the case (Fig. 3). Second, when amNG411 was assayed for its exclusion phenotype in a suppressor host one would reasonably expect the exclusion phenotype to be suppressed also. However this is not the case (Fig. 5).

If one assumes that the amber mutations in gene 42 are reverse polarity mutations one might attempt to explain these effects on imm gene function. Precedence for this comes from the classic work of Bauerle and Margolin (1966). They described a system in the S. typhimurium tryptophan pathway where mutations in a downstream gene had a severe, reverse polar affect on the function of an upstream gene. Mutations in the downstream gene closer to the operator-proximal end had a more severe inhibitory effect on expression of the upstream gene. They further demonstrated that the two gene products in question function in a unique multifunctional enzyme complex. They explained their results by reasoning that as the mutations became more operator-proximal the downstream polypeptide was progressively shorter and therefore less likely to interact positively in the functional complex. This mimicked a defective phenotype in the upstream gene, since its product could only function in the complexed form. An explanation such as this does not seem applicable to the gene imm/42 relationship. The results in Table 3 show that the

most operator-proximal mutation in gene 42 (amNG352) assayed as wildtype for exclusion. Also as mentioned above, when amNG411 is assayed for exclusion in suppressing conditions it remains defective for exclusion, even though the gp has hydroxymethylase function (Fig. 5). Additionally, I know of no work indicating that gp imm and gp 42 function in a complex.

I conclude that the best explanation of the gene 42 mutant exclusion results and the amNG411 revertant results is that genes imm and 42 significantly overlap in an out-of-phase orientation. This would represent the first case of an extensive gene overlap in phage T4. This has significant evolutionary, ecological and gene regulation implications as discussed below. [Some short overlaps have been found such as the 4 bp overlap involving a termination and start codon described by Chu et al. (1984)]

An opal codon appears to be involved in exclusion

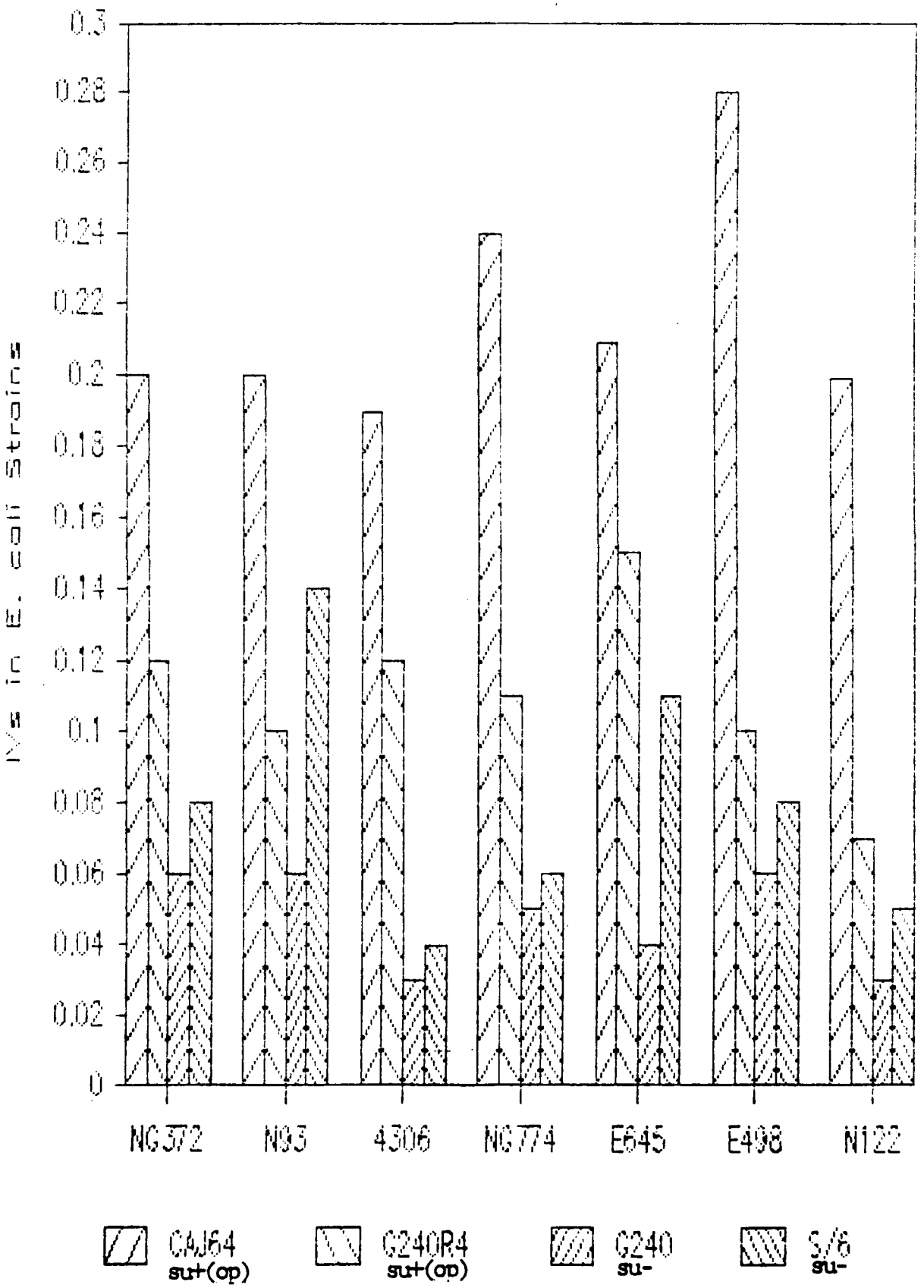
Preliminary sequence analysis of the imm gene region (Gram, personal communication, 1985), indicates one opal codon in an ORF that extends from the imm gene region well into the structural portion of gene 42. Based on the results of the exclusion assays done in opal(UGA) suppressing hosts (Fig. 6), I conclude that the opal codon in the imm gp is normally read-through as some amino acid, but that the preferential insertion of trp at this site reduces imm function. These results provide further evidence supporting the gene imm/42 overlap hypothesis. They also imply that the larger imm protein seen in SDS-PAGE gels is the gp responsible for the exclusion function.

A Multiple Overlap is Postulated

To explain the occurence of the two imm proteins that have

Fig. 6 : Immunity Values of T4 Mutants

in Various su+/− Hosts



been reported by two independent labs (as reviewed previously), I further postulate that the opal codon on the mRNA transcript of the region is normally read-through with less than 100% efficiency. Assuming that gene 42 and imm overlap and that the imm gene actually encodes for two proteins, then a multiple overlapping gene situation would exist within that region. Yutsudo (1979) refers to the sequence that codes for the larger imm protein as gene imm a and the one that codes for the smaller protein as gene imm b. Using this terminology, there would be an out-of-phase overlap between imm a and gene 42 plus an additional in-phase overlap between imm a and imm b. The entirety of imm b would share the first 256 bp with imm a and presumably be terminated within imm a during translation by the embedded opal termination codon. It is commonly held that in-phase overlaps (reviewed in Normark et al, 1983) are a way to produce a pair of proteins that either (i) interact with the same target with their common region while performing different functions via their unique domains or (ii) interact directly to produce a functional complex (van de Hondel, Konings and Shoenmakers, 1975; Smith and Parkinson, 1980). Although no function has been assigned to the imm b gene product, a theoretical argument can be made for its utility based on the continued existence of its opal termination codon. Simply, a genetic element as powerful as a termination codon would not be selectively maintained in the phage genome in the middle of a gene (i.e. imm a) were it not an advantage. The selective advantage is most likely due to the function of the protein that the nonsense codon terminates so the region of DNA responsible for the product does constitute a gene

(i.e. imm b). There is an example in yeast that sets a precedent for the hypothesized imm a/imm b two protein situation in T4. It seems that the MATa gene cassette that determines mating type in yeast also produces two proteins from the same section of DNA. And the longer protein is produced by the read-through of an embedded opal codon less than 100% of the time (reviewed in Lewin, 1987). The larger protein participates in mating functions, while the smaller protein's function is unknown.

Significance of the Gene imm/42 Gene Overlap

From a gene regulation perspective overlapping genes are of interest because according to Normark et al. (1983), they can have "important regulatory implications both at the level of expression and at the level of protein-protein interaction." Interestingly enough, several lines of evidence show gp 42 to function in a kinetically coupled dNTP-synthesizing complex thought to be attached to the host membrane that works in close association with the gene 43 containing replication complex (reviewed in Mathews and Allen, 1983). Although I know of no evidence directly connecting the imm gp to this complex, there is considerable evidence, reviewed previously, indicating that imm also functions at the membrane.

Normark's group (1983) also suggests that overlapping genes may provide a tool to study the evolution of coding and control sequences. A case has been made for the natural selection of overlapping genes to code for a particular protein quality such as membrane binding. Studies of overlapping genes in phage MS2 (Beremand and Blumenthal, 1979) and in ØX174 (Barrel, Air and

Hutchison, 1976) have shown that a +1 shift in reading frame results in the encoding of a very hydrophobic (possibly membrane binding) protein. Interestingly, the sequence data for imm predicts a large hydrophobic amino terminal region (Gram, 1985, personal communication).

Ecological Aspects of the Gene imm/42 Overlap

The possible regulatory connection between imm and gene 42 takes on additional significance when one considers the ecological aspects of their functions. The imm gene by providing immunity to superinfection is, in effect, establishing a territory for the first infecting phage. The infected cell becomes the exclusive resource of the primary infecting phage for use in self propagation. Immunity may thus be an adaptation to intraspecific competition. Gene 42 (dCMP-hydroxymethylase) alters phage DNA in a way that protects progeny phage from the host (prey) restriction enzymes. Thus gp 42 is an adaptation in a form of interspecific competition.

Another way of looking at the association of the imm gene and gene 42 is that the overlap is part of a regulatory device to prevent expression of gene 42 (essential for phage DNA replication) until after the imm gene is transcribed. This would postpone DNA replication until competing genomes have been excluded by the action of the imm gp thus ensuring that only the initial phage's own DNA is replicated.

BIBLIOGRAPHY

Abedon, S. (1990). Evolution of Territoriality in Bacteriophage T4. (Manuscript in Preparation)

Adams, M. H. (1959). Bacteriophages Interscience Publishers, Inc., New York.

Allen, E. F., I. Albrechet and J. W. Drake (1970). Properties of Bacteriophage T4 Mutants Defective in DNA Polymerase. Genetics 65:187-200.

Anderson, C. W. and J. Eigner (1971). Breakdown and Exclusion of Superinfecting T-even Bacteriophage in Escherichia coli. J. Virol. 8:869-886.

Anderson, C. W., J. R. Williamson and J. Eigner (1971). Localization of Parental Deoxyribonucleic Acid from Superinfecting T4 Bacteriophage in Escherichia coli. J. Virol. 8:887-893.

Barrell, B. G. , G. M. Air and C. A. Hutchison (1976). Overlapping Genes in Bacteriophage ØX174. Nature 264:34-41.

Bauerle, R. and P. Margolin (1966). A Multifunctional Enzyme Complex in the Pathway of S. typhimurium: Conparison of Polarity and Pseudopolarity Mutations. Cold Spring Harbor Symposia of Quantitative Biology 31:203-213.

Bayer, M. E. (1968). Adsorption of Bacteriophages to Adhesions Between Wall and Membrane of Escherichia coli. Virology 2(4):346-356.

Beremand, M. N. and T. Blumenthal (1979). Overlapping Genes in RNA Phage: A New Protein Implicated in Lysis. Cell 18:257-266.

Birge, E. A. (1981). Bacterial and Bacteriophage Genetics Springer-Verlag, New York.

Childs, J. D. (1973). Superinfection Exclusion by Incomplete Genomes of Bacteriophage T4. J. Virol. 11:1-8.

Chu, F. K., G. F. Maley, F. Maley and M. Belfort (1984). Intervening Sequence in the Thymidylate Synthase Gene of Bacteriophage T4. PNAS(USA) 81:3049-3053.

Clowes, R. C. and W. Hayes (1968). Experiments in Microbial Genetics John Wiley and Sons, Inc., New York.

Cohen, J. D. (1949). Growth Requirements of Bacterial Virus. Bact. Rev. 13:1-24.

Cornett, J. B. (1974). Spackle and Immunity Functions of Bacteriophage T4. J. Virol. 13:312-321.

Cornett, J. B. and M. Vallee (1973). The Map Position of the Immunity (imm) Gene of Bacteriophage T4. Virology 51:506-508.

Delbruck, M. and W. T. Bailey (1946). Induced Mutations in Bacterial Viruses. Cold Spring Harbor Symp. of Quant. Biol. 11:33-37.

Delbruck, M. and S. E. Luria (1942). Interference Between Bacterial Viruses. I. Interference Between Two Bacterial Viruses Acting Upon the Same Host, and the Mechanism of Virus Growth. Arch. Biochem. 1:11-141.

Dulbecco, R. (1952). Mutual Exclusion Between Related Phages. J. Bacteriol. 63:209-217.

Ellis, E. L. and M. Delbruck (1939). The Growth of Bacteriophage. J. Gen. Genet. 22:365-385.

Emrich, J. (1968). Lysis of T4-Infected Bacteria in the Absence of Lysozyme. Virology 35:158-165.

Flaks, J. G. and S. S. Cohen (1957). The Enzymatic Synthesis of 5-Hydroxymethyldeoxycytidylic Acid. Biochem. Biophys. Acta 25:667-669.

Fielding, P. E. and M. R. Lunt (1970). The Relationship Between Breakdown of Superinfecting Virus Deoxyribonucleic Acid and Temporal Exclusion Induced by T4 and T5 Bacteriophages. J. Gen. Virol. 6:333-342.

French, R. C., S. M. Lesley, A. F. Graham and C. E. van Rooyen (1952). Studies on the Relatioship Between Virus and Host Cell. III. The Breakdown of T2r+ Bacteriophage upon Infection of its Host. Can J. Med. Sci. 29:108-143.

Garen, A. (1968). Sense and Nonsense in the Genetic Code. Science 160:149-159.

Geller, A. I. and A. Rich (1980). A UGA Termination Suppressor tRNA (Trp) Active in Rabbit Reticulocytes. Nature 283:41-52.

Goldberg, E. (1983). Recognition, Attachment and Injection. in Bacteriophage T4. C. K. Matthews. E. M. Kutter, G. Mosig and P. B. Berget (eds.), American Society for Microbiology, Washington D.C., pp.32-39.

Guttman, B. and E. Kutter (1983). Overview. in Bacteriophage T4. C. K. Matthews, E. M. Kutter, G. Mosig and P. B. Berget (eds.), American Society for Microbiology, Washington D.C., pp.8-10.

Gram, H., H. Liebig, A. Hack, E. Niggeman and W. Ruger (1984). A Physical Map of Bacteriophage T4 Including the Postions of the Strong Promoters and Terminators Recognized In Vitro. Mol. Gen. Genet. 194:232-240.

Hershey, A. D., A. Garen, D. K. Fraser and J. D. Hudis (1954). Growth and Inheritance in Bacteriophage. Carnegie Inst. Wash. Year B. 53:210-225.

Hendrix, R. W., J. W. Roberts, F. W. Stahl and R. A. Weisenberg

(eds.) (1983). Lambda II Cold Spring Harbor Laboratory, pp. 437-438.

Holmes, G. E. (1975). Intragenic Complementation by Gene 42 Mutations of Bacteriophage T4. J. Virol. 16(4):1085-1089.

Jiang, N., S. Na and X. Xu (1984). Preliminary Study on the Superinfection Immunity of Cloned Bacteriophage T4 imm Gene. Yichuan 6(2):3-5.

Kim, J. and N. Davidson (1974). Electron Microscope Heteroduplex Study of Sequence Relations of T2, T4 and T6 Bacteriophage DNAs. Virology 57:93-111.

Kutter, E., et al (1990). Genetics Maps, Cold Spring Harbor Laboratory, Cold Spring Harbor MA.

Kutter, E. and W. Ruger (1985). Bacteriophage Genomic Map, August, 1985. Abstracts of the 1985 Evergreen International Bacteriophage T4 Meeting, Evergreen State College, Olympia WA.

Lamm, N., J. Tomaschewski and W. Ruger (1987). Nucleotide Sequence of the Deoxytidylate Hydromethylase Gene of Bacteriophage T4 (g42) and the Homology of its Gene with Thymidylate Synthase of E. coli. Nucleic Acids Res. 15(9):39920

Lesley, S. M., R. C. French, A. F. Graham and C. E. van Rooyen (1951). Studies on the Relationship Between Virus and Host Cell. III. The Breakdown of T2r+ Bacteriophage upon Infection of its Host. Can. J. Med. Sci. 29:108-143.

Lewin, B. (1987). Genes III John Wiley and Sons, New York. p. 664.

Lu, M., U. Henning (1989). The Immunity (imm) Gene of Escherichia coli Bacteriophage T4. J. of Virology 63(8):3472-3478.

Lu, S., Y. Chen, X. Xu and P. Ji (1982). Identification and Analysis of Clones Carrying Bacteriophage T4 Genes 42 and 43 DNA Fragments. ACTA GENET SIN 9(3): 165-171.

Maniatus, T., E. F. Fritsch and J. Sambrook (1982). Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory.

Mathews, C. K. and J. R. Allen (1983). DNA Precursor Biosynthesis. in Bacteriophage T4 C. K. Matthews. E. M. Kutter, G. Mosig and P. B. Berget (eds.), American Society for Microbiology, Washington D.C., pp. 59-70.

Mufti, S. (1972). A Bacteriophage Mutant Defective in Protection

Against Superinfecting Phage. J. Gen. Virol. 17:119-123.

Nasmyth, K. and K. Tatchell (1980). The Structure of Transposable Yeast Mating Type Loci. Cell 19:753-766.

Normark, S., S. Bergstrom, T. Edlund, T. Grundstrom, B. Jaurin, F. P. Lindberg and O. Olsson (1983). Overlapping Genes. Ann. Rev. Genet. 17:499-525.

Obringer, J. (1987). Genetic Exclusion in Bacteriophage T4. Doctoral Dissertation, Univ. of Arizona.

Obringer, J. (1988). The functions of the Phage T4 Immunity and Spackle Genes in Genetic Exclusion. Genet. Res. 52:81-90.

Obringer, J., R.P. McCreary and H. Bernstein (1988). Bacteriopnage T4 Genes sp and 40 Apparently Are the Same J. of Virol. 62(8):3043-3045.

O'Farrell, P. Z. and L. M. Gold (1973). The Identification of Prereplicative Bacteriophage T4 Proteins. J. Biol. Chem. 248:5499-5501.

Okamoto, K. (1973). Role of T4 Phage-directed Protein in the Estabishmnent of Resistance to T4 Ghosts. Virology 56:595-603.

Peterson, R. F., P. S. Cohen and H. L. Ennis (1972). Properties of Phage T4 Messenger RNA Synthesized in the Absence of Protein Synthesis. Virology 48:201-206.

Philipson, L., P. Anderson, U. Olshevsky, R. Weinberg and D. Baltimore (1978). Translation of MuLV and MSV RNAs in Nuclease Treated Reticulocyte Extracts: Enhancement of the gag-pol Polypeptide with Yeast Suppressor tRNA. Cell 13:189-197.

Rena-Krantz, L. J. and M. J. Bessman (1981). Studies on the Biochemical Basis of Mutation VI: Selection and Characterization of a New Bacteriophage T4 Mutator DNA Polymerase. J. Mol. Biol. 145:677-695

Sauri, C. J. and C. F. Earhart (1971). Superinfection in Bacteriophage T4: Inverse Relationship Between Genetic Exclusion and Membrane Association of Deoxyribonucleic Acid of Secondary Bacteriophage. J. Virol. 8:856-859.

Schwartz, M. (1980). Interactions of Phages with their Receptor Proteins. in Virus Receptors (Receptors and Recognition), Series B, volume 7, L. L. Randall and L. Philipson (eds.), Chapman and Hall, London, pp. 60-94.

Schwartz, H. I. Riede, I. Sonntagh and U. Henning (1980). Degrees of Relatedness of T-even Type E. coli Phages Using Different or the Same Receptors and Topology of Serologically Cross-reacting Sites. EMBO J. 2:375-390.

Sinden, R. R. and D. E. Pettijohn (1982). Torsional Tension in fntracellular Bacteriophage T4 DNA: Evidence that a Linear DNA Complex can be Supercioled In Vivo. J. Mol. Biol. 162:659-677.

Smith, R. A., and J. S. Parkinson (1980). Overlapping Genes of the cheA Locus of Escherichia coli. PNAS(USA) 77:5370-5374.

Smith, R. L. (1966) Ecology and Field Biology Harper and Row, New York, pp. 424-484.

Steinberg, C. M. and R. S. Edgar (1962). A Critical Test of a Current Theory of Recombination in Bacteriophage. Genetics 47:187-208.

van de Hondel, C. A., R. N. Konings, and J. G. Shoenmakers (1975). Regulation of Gene Activity in Bacteriophage M13 DNA: Coupling Transcription and Translation of Purified Genes. Virology 67:487-497.

Vallee, M. and J. B. Cornett (1972). A New Gene of Bacteriophage T4 Determining Immunity Against Superinfecting Ghosts and Phage in T4-infected Eschricnia coli. Virology 48:777-784.

Vallee, M., J. B. Cornett, and H. Bernstein (1972). The Action of Bacteriophage T4 Ghosts on Escherichia coli and the Immunity to this Action Developed in Cells Preinfected with T4. Virology 48:766-776.

Vallee, M. and J. B. Cornett (1973). The Immunity Reaction of Bacteriophage T4: a Noncatalytic Reaction. Virology 53:441-447.

Vallee, M. and de Lapeyriere (1975). The Role of the Genes imm and s in the Development of Immunity Against T4 Ghosts and Exclusion of Superinfecting Phage in Escherichia coli Infected with T4. Virology 67:219-233.

Visconti, N. (1953). Resistance to Lysis from Without in Bacteria Infected with T2 Bacteriophage. J. Bactiol. 66:247-253

Weiner, A. M. and Weber K. (1971). Natural Read-through at the UGA Termination signal of QB Coat Protein Cistron. Nature 234:206-209.

Yates, J. L., W. R. Gette, M. E. Furth, and M. Nomura (1977). Effects of Ribosomal Mutations in the Read-through of a Chain Termination Signal: Studies on the Synthesis of Bacteriophage Lambda O Gene Protein In Vitro. PNAS (USA) 74: 689-693.

Yutsudo, M. (1979). Regulation of imm Gene Expression in Bacteriophage T4-infected Cells. J. Gen. Virol. 45:351-359.

Yutsudo, M. and K. Okamoto (1973). Immediate-Early Expression of the Gene Causing Superinction Breakdown in Bacteriophage T4B. J. Virol. 12:1628-1630