

The functions of the phage T4 immunity and spackle genes in genetic exclusion

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Summary

Genetic exclusion is the ability of a primary infecting phage to prevent a secondary infecting phage from contributing its genetic information to the progeny. The molecular mechanism of the phenomenon is not well understood. The two genes in phage T4 mainly responsible for genetic exclusion are the immunity (*imm*) gene and the spackle (*sp*) gene. Evidence is presented that the *imm* gp enables the host exonuclease V to degrade superinfecting phage DNA. This appears to be accomplished by the *imm* gp altering gp 2/64, the presumed pilot protein, which protects the 5' end(s) of the phage DNA. Exonuclease III is also involved in genetic exclusion but its action does not appear to depend upon the *imm* or *sp* gene products. Gp *sp* appears to interfere with the lysozyme activity of gp 5, a component of the central base plug, postulated to aid in tail tube penetration during the injection process. A molecular model of genetic exclusion is proposed. Genes *imm* and *sp* are part of a cluster of genes which also includes 42, beta-glucosyltransferase, and *uvsX*. The genes of this cluster encode proteins apparently adapted for competition and defence at the DNA level. These genes may encode fundamental adaptive strategies found throughout nature.

1. Introduction

Genetic exclusion in phage T4 is the ability of a primary infecting phage to prevent secondary infecting phage from contributing genetic information to the progeny. The exclusion phenomenon in T-even phages was first reported by Dulbecco (1952) and has been studied by several investigators since then (e.g. Visconti, 1953; Fielding & Lunt, 1970; Anderson *et al.* 1971; Sauri & Earhart, 1971; Vallee *et al.* 1972; Okamoto, 1973; Yutsudo & Okamoto, 1973). All of the T-even-like phages have been shown to express some degree of exclusion (Dulbecco, 1952; Anderson & Eigner, 1971). In phage T4, genetic exclusion approaches 100% efficiency. The two phage T4 genes shown to be primarily responsible for exclusion are immunity (*imm*) and spackle (*sp*) (Mufti, 1972; Childs, 1970, 1973; Cornett, 1974; Vallee & deLapeyrière, 1975). However, the mechanism has not been well defined.

The *imm* gene was named for its ability to provide 'immunity' to superinfecting phage T4 and to the disruptive effects of superinfecting T4 ghosts. It is expressed as an immediate early function (Dulbecco,

1952; Peterson *et al.* 1972; O'Farrell & Gold, 1973; Yutsudo & Okamoto, 1973). The *imm* gene product (gp) acts in a stoichiometric rather than in a catalytic manner (Vallee & Cornett, 1973) and accounts for approximately 50% of the exclusion phenotype at 37 °C, 4 min post infection (Dulbecco, 1952; French *et al.* 1952; Sauri & Earhart, 1971; Cornett, 1974; Vallee & deLapeyrière, 1975). The gp *imm* is postulated to act at the cell wall or membrane (Vallee & Cornett, 1973; Yutsudo & Okamoto, 1973), but its exact mechanism of action remains unknown.

The first spackle (*sp*) mutant was isolated by Emrich (1968) as a suppressor of an *e* mutant (lysozyme defective) in phage T4. This *sp* mutant had reduced resistance to lysis from without, suggesting that gp *sp* is a phage-directed component of the bacterial cell wall. Subsequently it was determined that *sp* is an immediate early gene and that it accounts for approximately 20% of the wild-type exclusion phenotype (Cornett, 1974; Vallee & deLapeyrière, 1975; Peterson *et al.* 1972). Evidence was also presented by Kao & McClain (1980*a, b*) that gp *sp* is a lysozyme inhibitor working against gp 5, a component of the baseplate central plug (Kikuchi & King, 1975) that has lysozyme activity (Nakagawa *et al.* 1985). Recently the *sp* gene has been shown to be the

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same gene as gene 40 (Obring *et al.* manuscript submitted). Throughout this paper the reference to sp will be to the sp phenotype of the gene *sp/40*.

2. Materials and methods

(i) Media

Bacteria were grown in Hershey's Broth (HB) or on enriched Hershey's agar plates. These were prepared as described by Steinberg & Edgar (1962). When antibiotic media were required, the appropriate amount of antibiotic, salts or stock solution, was added at the concentrations recommended in Maniatis *et al.* (1982). M9 adsorption salts solution (Clowes & Hayes, 1968) was used in diluting phage and for promoting adsorption of phage to host cells. Soft top agar (enriched Hershey's plate media with half the agar added) was used at a temperature of 45 °C where it is liquid. To titre phage, indicator cells and phage were mixed in the soft agar and then plated.

(ii) Bacteria, phage and plasmid strains

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

(iii) Cultures

Bacterial and phage cultures were prepared and handled as described previously (Bernstein, 1987).

(iv) Methods

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 progeny. The quantitative expression of the exclusion phenotype, as determined by this assay, is the 'immunity value'. This is a measure of immunity to superinfection. The immunity value (IV) was determined as follows: A primary infection of a Su^- host was carried out using

Table 1. *E. coli*, Plasmids and Phage Strains Used

Strains	Genotype or comments	Source/reference
<i>E. coli</i>		
S/6	Su^-	This lab
CR63	Su^+ (SuI, serine)	This lab
KP360	<i>recBC, sbcB</i>	K. Peterson
DE828	594 (<i>recD1014</i>)	D. Ennis
594	Su^- (parent of DE828)	K. Peterson
CES201	<i>recBC, sbcB</i>	CGSC
JC5519	Su^+ , <i>recBC</i>	CGSC
JC7623	Su^+ <i>recBC, sbcB</i>	CGSC
DH5	Su^+ 2, <i>lacZdel</i> , r^-	M. Moran
KL16	Su^- (parent of BW9101)	B. Wein
BW9101	<i>xth</i> (exo III ⁻)	B. Wein
Plasmids		
pBSK101	amp, tet	Fujisawa <i>et al.</i> , 1985
pUC18	amp, expression vector	BRL
pJO11	amp, T4 sp clone	This study
Phage T4		
T4D ⁺	wild type	This lab
amE142, imm ⁻	(39 amber, imm)	This lab
amNG372	(55 amber)	This lab
amNG205	(42 amber)	This lab
amNG205 sp ⁻	(42 amber, sp)	This lab
amNG205 imm ⁻ , sp ⁻	(42 amber, imm, sp)	This lab
5tsl	(5 ts)	Kao & McClain, 1980
amN51	(2 amber)	This lab
Other Phages		
T2	wild type	W. Wood
T6	wild type	W. Wood

CGSC is the *E. coli* Genetic Stock Center, Yale University, School of Medicine. W. Wood is at the Univ. of Colorado, Boulder. J. Wiberg is at the Univ. of Rochester, Medical Center. B. Wein is at Johns Hopkins University, School of Medicine. D. Ennis, M. Moran and K. Peterson are at the Univ. of Arizona. BRL is Bethesda Research Laboratories. The information in parentheses under the genotype column indicates the phage genes bearing the mutational defects and type of defects, if known.

Table 2. Genetic exclusion by Phage T4 *imm*[±], *sp*[±] strains in various *E. coli* hosts (endonuclease *I*[±] and exonuclease *V*[±])

Phage T4 mutant	<i>E. coli</i> hosts				
	594 exoV ⁺ endoI ⁺	DE828 (594, <i>recD</i>) exoV ⁻ endoI ⁺	JC5519 (<i>recBC</i>) exoV ⁻ endoI ⁺	KP360 (<i>recBC</i> ; <i>sbcB</i>) exoV ⁻ endoI ⁻	CES201 (<i>recBC</i> ; <i>sbcB</i>) exoV ⁻ endoI ⁻
NG372(55) <i>imm</i> ⁺ <i>sp</i> ⁺	0.06 ± 0.01	0.15 ± 0.00	0.25	0.23 ± 0.02	0.16
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁺	0.11 ± 0.01	0.20 ± 0.01	0.26	0.13 ± 0.01	0.24 ± 0.01
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁺	0.35 ± 0.01	0.34 ± 0.00	0.44	0.31 ± 0.04	0.30 ± 0.05
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁻	0.24 ± 0.02	0.40 ± 0.02	0.31	0.32 ± 0.03	0.36 ± 0.07
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁻	0.86 ± 0.02	0.89 ± 0.03	0.54	0.66 ± 0.05	0.54 ± 0.01

The above numbers represent the average Immunity Value for each phage strain plus or minus the standard error. NG205 was used as the superinfecting phage when NG372 was the primary infecting phage and vice versa. A minimum of three trials was done to determine the values having standard errors. One experiment was done in host *E. coli* CES201 with primary phage NG372 and in the series of experiments done in *E. coli* JC5519. Although *E. coli* JC5519 is Su 2 it did not suppress the mutations in phages NG205 and NG372 used in these experiments. Standard errors shown as 0.00 were less than 0.005.

the phage strain being assayed for its exclusion phenotype. A conditional lethal amber mutation was present in this strain. After a delay, superinfection was carried out with a second conditional lethal mutant defective in a different gene from the first. The titre of infective centres was then measured under restrictive growth conditions. This titre was then divided by the titre of infective centres measured from a simultaneous infection of the same two phages to give the IV value. This is indicated by the equation:

$$IV = \frac{\text{titre of infective centres after delayed superinfection}}{\text{titre of infective centres after simultaneous infection}}$$

Because complementation is required for phage growth, only cells that contain both mutant phage genomes will give rise to an infective centre. Those cells containing only one of the mutant genomes will not. As will be seen below (e.g. Table 2), *imm*⁺ *sp*⁺ phage have an IV of 0.06–0.11 at 37 °C. This indicates that the success of delayed infections is much lower than of simultaneous ones. An *imm* *sp* mutant has an IV of 0.86 at 37 °C (Table 2) indicating that in this case delayed infections are more successful. The protocol for this standard exclusion assay is described in detail in Obringer *et al.* (manuscript submitted).

Efficiency of plating. The host cells were grown to approximately 5 × 10⁸/ml in HB plus antibiotic as required, pelleted by centrifugation and resuspended to the original titre in fresh HB. Approximately 1 × 10⁸ cells plus phage were mixed in 3 ml of soft top agar and then plated directly onto enriched HB agar plates. The plates to be incubated at 37 or 43 °C were prewarmed to 37 °C prior to plating. The plates were then incubated at the experimental temperature until an adequate indicator lawn was formed. The

plaques were tabulated as the mean number per plate ± the standard error for each temperature category.

Construction of expression vector plasmid pJO11. Plasmid pJO11 is a recombinant plasmid of the expression vector pUC18 with a 367 bp insert of T4 DNA from the region 22.014 to 21.647 kbp on the T4 restriction map (Kutter & Ruger, 1985). This fragment expresses gene *sp/40*. Plasmid pJO11 was constructed by removing this fragment from plasmid pBSK101 by double digestion with HindIII and BglII and inserting it into pUC18 at the HindIII and BamHI sites in the correct transcriptional orientation. A detailed description of its construction is presented elsewhere (Obringer *et al.* manuscript submitted).

3. Results

(i) The effect of exonuclease V in genetic exclusion

Some inferential evidence has been obtained concerning the action of *gp imm* in exclusion (Vallee & Cornett, 1973; Vallee & deLapeyrière, 1975; Yutsudo & Okamoto, 1973), but the molecular mechanism is still unclear. The available evidence indicates that *gp imm* acts at the level of the membrane, either altering a phage adsorption site or interfering with secondary phage DNA ejection or uptake. However, the possibility that *gp imm* may be involved in a cytoplasm-based exclusion response has not been considered in the literature.

Exonuclease V (*exo V*) is a cytoplasmic enzyme produced by the *E. coli recBCD* genes. It has several functions but its ability to degrade DNA from the 5' end is regarded as its most biologically significant activity (reviewed in Telander, Muskavitch & Linn, 1981; Amundsen *et al.* 1986). Phage T4 amber mutants

defective in genes 2 and 64 when grown in a restrictive host form progeny which can adsorb normally to the host cell and kill it, but cannot produce a productive infection (Granboulan *et al.* 1968; Silverstein & Goldberg, 1976*a*). The *E. coli* *exo V* was shown to degrade phage T4 glucosylated 5-hydroxymethylcytosine containing DNA (HMC-DNA) in these infections. However, gene 2 and 64 mutants do form plaques on *exo V* defective hosts. Genes 2 and 64 are now thought to be one gene (rather than two) which acts to protect the phage DNA from exonucleolytic degradation (Goldberg, 1983).

One possible strategy by which primary phage might exclude superinfecting phage DNA is to render it susceptible to host exonucleases. A simple and economical way to accomplish this would be to inactivate the protective proteins of the superinfecting DNA. To pursue this idea, I first addressed the question of whether *exo V* is involved in the genetic exclusion process at all.

Therefore the standard exclusion assay was performed on several phage strains. Two were *imm*⁺ *sp*⁺ (one carried on amber mutation in gene 55; the other in gene 42). I also used an *imm* mutant (*imm2*), a *sp*⁻ mutant and an *imm2*, *sp*⁻ double mutant. All of the *imm* and/or *sp*⁻ mutants had the same amber mutation in gene 42 as the *imm*⁺ strain, thus providing a consistent genetic background for comparison purposes. The assays were performed in strains of *E. coli* that were either wild-type or defective for *exo V*. *E. coli* 594 is wild-type for *exo V* and is the parent of DE828. DE828 carries a *recD* mutation and has no detectable *exo V* activity (Amundsen *et al.* 1986). *E. coli* JC5519 is another *exo V* defective strain having a mutation in the *recBC* locus. Strains KP360 and CES201 are defective in both *exo V* and endonuclease I (endo I). As can be seen from Table 2, when the primary infecting phage is *imm*⁺ the IVs in the *exo V*⁻ hosts are consistently higher than in the *exo V*⁺ host. The average increase is 2.4-fold for the two *imm*⁺ *sp*⁺ strains, and 1.4 for the *imm*⁺ *sp*⁻ strain. The most reliable comparison is between the values obtained in *E. coli* 594 and the isogenic DE828 strain. Collectively these results show that when the primary phage is *imm*⁺ there is decreased exclusion (i.e. increased IV) of the secondary phage in the *exo V* deficient strains. This demonstrates that *exo V* is involved in the exclusion process. This result is also consistent with the hypothesis that gp *imm* alters the secondary infecting phage's incoming DNA to expose it to *exo V* degradation. On the other hand the data on *imm*⁻ *sp*⁺ in Table 2 suggest that the gp *sp* has no significant involvement in *exo V* mediated exclusion.

Additionally, there was no consistent effect on exclusion of an additional endo I mutation in the *exo V*⁻ strains (strains KP360 and CES201 in Table 2). That is, there was no additional increase in IVs in the doubly defective hosts. This result is consistent with the observations of several investigators that endo I is

not involved in genetic exclusion although it catalyses breakdown of superinfecting DNA in the periplasmic space (Fielding & Lunt, 1970; Anderson & Eigner, 1971; Anderson *et al.* 1971; Silverstein & Goldberg, 1976*b*).

(ii) *The effect of a mutated gene 2/64 (pilot protein) on genetic exclusion in an *exo V*⁻ host*

One can speculate that the gp *imm* alters or removes a protective protein from superinfecting phage DNA thereby exposing it to *exo V* degradation. Such a hypothetical protective protein is referred to as a pilot protein (Goldberg, 1983). It follows from this speculation that the level of exclusion seen in an *exo V*⁻ host superinfected with phage DNA having a defective pilot protein would approximate the level of exclusion seen in the same host superinfected with protected DNA. In other words the presence or absence of a functional pilot protein would be expected to make no difference in the level of exclusion in an *exo V*⁻ host.

Goldberg (1983) maintains that gene 2/64 is the T4 pilot protein. Silverstein & Goldberg (1976*a*) developed a method to grow gene 2 amber mutants in *Su*⁻ hosts yielding phage (2.*Su*⁻) that will only form plaques on *exo V*⁻ hosts. By contrast, DNA from 2.*Su*⁻ phage is rapidly degraded in *exo V*⁺ *E. coli*. Reasoning that 2.*Su*⁻ phage would be excluded at the same rate as phage possessing an intact pilot protein in an *exo V*⁻ host, the standard exclusion assay was performed using 2.*Su*⁻ as the secondary infecting phage. By comparing the results in Table 3 with those in Table 2 for the same host/phage combinations, it can be seen that the IVs are essentially the same, supporting the above prediction.

(iii) *Exonuclease III involvement in genetic exclusion*

E. coli also produces a nuclease that catalyses the 3' to 5' stepwise removal of mononucleotides from double-stranded DNA having an exposed 3'-OH group (Weiss, 1976). The enzyme, a product of the *xth* gene, as been classified as exonuclease III (*exo III*).

Table 3. Genetic exclusion of 2.*Su*⁻ superinfecting phage DNA by phage T4 *imm*[±], *sp*[±] strains in *E. coli* DE828 (*exo V*⁻)

Phage T4 mutant	<i>E. coli</i> host DE828 (594, <i>recD</i>) <i>exo V</i> ⁻
NG372(55) <i>imm</i> ⁺ <i>sp</i> ⁺	0.18 ± 0.05
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁺	0.20 ± 0.03
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁺	0.41 ± 0.04
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁻	0.45 ± 0.05
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁻	0.85 ± 0.05

The above numbers represent the average Immunity Value from a minimum of three trials for each phage strain plus or minus the standard error.

Table 4. Genetic exclusion by phage T4 *imm*[±], *sp*[±] strains in various *E. coli* hosts (exonuclease III[±])

Phage T4 mutant	<i>E. coli</i> hosts	
	KL16 (<i>xth</i> ⁺)	BW9101 (<i>xth1</i>)
NG372(42) <i>imm</i> ⁺ <i>sp</i> ⁺	0.03 ± 0.01	0.10 ± 0.00
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁺	0.06 ± 0.00	0.10 ± 0.01
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁺	0.32 ± 0.01	0.47 ± 0.01
NG205(42) <i>imm</i> ⁺ <i>sp</i> ⁻	0.20 ± 0.00	0.25 ± 0.00
NG205(42) <i>imm</i> ⁻ <i>sp</i> ⁻	0.75 ± 0.03	0.83 ± 0.08

The above numbers are the average Immunity Values for each phage strain plus or minus the standard error. The above values were obtained from a minimum of three trials. Standard errors shown as 0.00 were less than 0.005.

Table 5. Plaque forming ability of phage T4 (2.Su⁻) on eight *E. coli* strains with wild-type and defective exonucleases III and V, endonuclease I and amber suppressors at 37 °C

<i>E. coli</i> strain	Endo I	Exo		Su	T4 strain	
		III	V		Wild type	2.Su ⁻
S/6	+	+	+	-	P	0
CR63	+	+	+	+	P	0
594	+	+	+	-	P	0
DE828	+	+	-	-	P	P
JC7623	-	+	-	+	P	P
CES201	-	+	-	-	P	P
KL16	+	+	+	-	P	0
BW9101	+	-	+	-	P	0

+ indicates the presence of the specified endonuclease, exonuclease or suppressor in the *E. coli* strain shown to the left, - indicates absence of same. In the columns under T4 strains, P indicates plaque formation on the *E. coli* strain indicated, 0 indicates no plaques formed.

It seemed reasonable to suppose that this enzyme also might be involved in the exclusion of super-infecting phage T4 DNA. The results (Table 4) of the standard exclusion assay performed in an *xth* mutant and its isogenic parental strain show consistently decreased exclusion ability (increased IVs) of the primary infecting phage in the *exo* III defective mutant. Therefore, *exo* III is involved in the exclusion process, but its effect does not appear to be linked to the presence or absence of the *imm* or *sp* gene products.

(iv) Plaque forming ability of 2.Su⁻ phage on various *E. coli* hosts

As seen in previous experiments *exo* III and *exo* V are involved in the exclusion process while *endo* I is not. 2.Su⁻ phage are unable to form plaques on *exo* V⁺ hosts, presumably because the presence of a defective pilot protein (gp 2/64) allows degradation of the 2.Su⁻ DNA by *exo* V. The pilot protein therefore presumably protects the 5' end(s) of the infecting T4 chromosome. Since *exo* V is involved in exclusion and

exo V hosts will support the growth of 2.Su⁻ phage, then by analogy an *exo* III defective host (*xth* mutant) may also support the growth of 2.Su⁻ phage.

The data in Table 5 show that 2.Su⁻ phage form plaques on *exo* V⁻ hosts, in agreement with the report of Silverstein & Goldberg (1976a), but not on an *exo* III defective host. This implies that the pilot protein defect renders the 2.Su⁻ phage DNA sensitive to *exo* V activity specifically. The results with the *endo*I *exo*III⁺ *exo*V⁻ hosts imply that *exo* III acting alone is unable to degrade 2.Su⁻ phage DNA. The results with the *endo*I⁺ *exo*III⁻ *exo*V⁺ host imply that *exo*III is not required for degrading 2.Su⁻ phage DNA when the other host enzymes are present. The results also indicate that the presence of an additional *sbcB* mutation does not suppress the effect of the *exo* V⁻ mutation in its ability to allow growth of 2.Su⁻ phage (Table 5, hosts JC7623 and CES201). Additionally, the presence of an amber suppressor in the host has no effect on its ability to allow 2.Su⁻ phage to form plaques (Table 5, hosts CR63 and JC7623). This supports the idea that the 2.Su⁻ defect is a structural one (i.e. a pilot protein) rather than a defective

Table 6. Genetic exclusion of phage T4 (T45tsl) by primarily infecting phage T4 imm[±], sp[±] strains in *E. coli* host S/6

Primary infecting phage	Superinfecting phage	
	5 ⁺	T45tsl
NG372(55) imm ⁺ sp ⁺	0.08 ± 0.01	0.36 ± 0.07
NG205(42) imm ⁺ sp ⁺	0.04 ± 0.01	0.22 ± 0.03
NG205(42) imm ⁺ sp ⁻	0.26 ± 0.03	0.31 ± 0.10
NG205(42) imm ⁻ sp ⁺	0.47 ± 0.03	0.64 ± 0.05
NG205(42) imm ⁻ sp ⁻	0.70 ± 0.06	0.73 ± 0.04

The above numbers represent the average Immunity Value determined by the standard exclusion assay from a minimum of three trials at 37 °C for each phage strain plus or minus the standard error. Infective centres were titred on *E. coli* S/6 at 43 °C, the restrictive condition for phage T45tsl and the amber mutants.

product resulting from expression of the infecting parental genome.

(v) Genetic exclusion of a T4 mutant (T45tsl) defective in gene 5

The mechanism of gp sp action in genetic exclusion is not known, but some insight may be gained from the role of gp sp in resisting lysis from without and lysis from within. Gene product 5, a component of the central base plug, acts as a lysozyme that can cause host cell lysis at a sufficiently high phage multiplicity of infection (Kao & McClain, 1980a; Nakagawa *et al.* 1985). Gp sp has been shown genetically to interact with gp 5, and presumably to inactivate its lysozyme function (Kao & McClain, 1980b). It has been proposed that gp 5 aids penetration of the tail tube during infection by the enzymic digestion of the peptidoglycan layer of the host cell wall (Kao & McClain, 1980b). This suggests that the contribution that gp sp makes to genetic exclusion results from inhibition of superinfecting phage injection. This then leads to degradation of the superinfecting DNA in the periplasmic space as previously described. To investigate this idea I obtained a unique gene 5 mutant of phage T4 (T45tsl), isolated by Kao & McClain (1980b), that has a temperature sensitive defect which prevents interaction with gp sp.

The results in Table 6 show that when sp⁺ phage were used for the primary infection the IVs increased considerably when superinfection was with T45tsl phage compared with 5⁺ phage. The IVs from the T45tsl superinfections approximate the IVs of cells primarily infected with sp⁻ phage and superinfected with gene 5⁺ phage. Also the IVs obtained with cells primarily infected with sp⁺ phage are not significantly different from the values of the sp⁻ infected cells when both are superinfected with T45tsl. Collectively, these observations suggest that because the lysozyme specified by T45tsl is not susceptible to inactivation by gp sp, the superinfection by T45tsl is not blocked by gp

sp. This results in an increased IV when T45tsl are used to superinfect cells primarily infected with sp⁺ phage. The increased IV is the same as that obtained with cells primarily infected with sp⁻ phage. In wild-type phage infections, gp sp apparently inhibits gp 5, the lysozyme of the phage's baseplate central plug, thus interfering with its ability to aid in tail tube penetration. This contributes to the exclusion of the secondarily infecting phage's DNA from the progeny.

(vi) Efficiency of plating of phages T4D⁺ and T45tsl on induced DH5:pJO11

To examine further gp sp action in an environment isolated from the effects of the remainder to the phage genome, efficiency of plating (eop) experiments were conducted comparing T4D⁺ and T45tsl on two *E. coli* hosts: *E. coli* DH5 containing the plasmid pUC18 (non-recombinant) and on DH5 containing plasmid pJO11 (a recombinant of pUC18 with an inserted T4 DNA fragment having the expressible sp gene). The experiments were conducted at temperatures ranging from 22 to 43 °C. As can be seen in Fig. 1, T4D⁺ is efficiently excluded by DH5:pJO11, especially at the lower temperatures. This alone shows that gp sp is capable of excluding phage and supports the body of data implicating gp sp in genetic exclusion.

As expected, T45tsl (Fig. 1) is excluded considerably less efficiently by DH5:pJO11 than T4D⁺. This confirms the ability of T45tsl phage to penetrate the sp barrier, and supports the findings of the genetic exclusion assays in which the host cells were primarily infected by sp⁺ phage using T45tsl as the secondarily infecting phage (Table 6).

(vii) Efficiency of plating of phages T2 and T6 on induced DH5:pJO11

Since phages T2 and T6 are closely related to T4, it is of interest to examine the ability of T4 gp sp to exclude T2 and T6. T4 has been shown by several

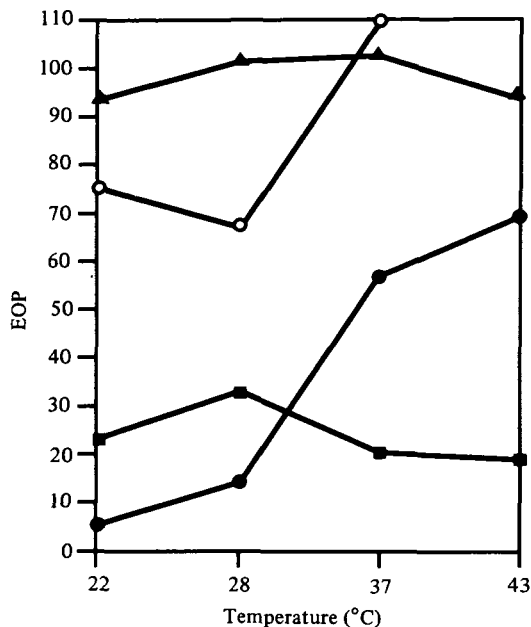


Fig. 1. Efficiency of plating (EOP) of phages T4D⁺ (●), T45tsl (○), T2 (■) and T6 (▲) on a gp sp/40 expressing clone. Each of the values represented above were obtained by dividing the number of plaques from an equal aliquot of phage titred on DH5:pJO11 (sp/40⁺) × 100 by the number of plaques formed on DH5:pUC18. This yields the percentage of plaques formed on the sp containing clone.

investigators using different techniques (reviewed in Birge, 1982) to be more closely related to T2 than T6, so one would expect interspecific exclusion by T4 gp sp to be more effective against T2 than T6. The phage T4 gp sp producing clone was tested for its exclusion ability of the T-even phages at temperatures ranging from 22 to 43 °C under inducing conditions. The eop of T2, T4 and T6 on DH5:pJO11 are shown in Fig. 1. As an example of the results obtained, at 28 °C the sp clone (DH5:pJO11) excludes 86% of T4, 67% of T2 and 0% of T6 compared to the ability of the same phages to titre on the same *E. coli* host containing the non-recombinant parental plasmid (DH5:pUC18). These results support the expectation that T2 should be excluded more effectively than T6.

4. Discussion

(i) The molecular mechanism of genetic exclusion

(1) *Host exonuclease involvement in T4 genetic exclusion.* The results obtained show that primary infecting phages recruit at least two host exonucleases, exo III and exo V, to exclude superinfecting phage genomes. The data in Table 2 indicate that the role of exo V in exclusion depends on the gp imm. That is, exo V does not participate in the exclusion process unless the primary infecting phage carries a functional imm gene. One interpretation of the results in Table 2 is that gp imm interacts with the superinfecting DNA

and exposes the 5' end as a substrate for host exo V degradation.

Goldberg (1983) has proposed that T4 DNA is protected by the product of gene 2/64, the hypothetical pilot protein. The results in Table 3 show that a functional gp 2/64 is not required for protection of superinfecting phage DNA in an exo V⁻ host. This supports the idea that gp imm inhibits the protective capacity of the pilot protein. If the superinfecting T4 chromosome is unprotected it presumably becomes a substrate for exo V.

In addition to having nuclease activity, exo V has been shown to bind to double strand (ds) DNA ends, and then to move rapidly along the DNA, unwinding it. This produces single-strand loops which the enzyme cuts to release single stranded fragments (reviewed in Telander-Muskavitch & Linn, 1981). These fragments then become a substrate for additional host DNA degrading enzymes. Primary phage apparently protect their own DNA from exo V during replication and recombination by producing a protein (T4 rec inhibitor) that inhibits exo V at 10 min post-infection (Behme *et al.* 1976).

As originally postulated by Kornberg (1974), the function of the pilot protein of the phage nucleoid is to provide specificity and possibly structural help in transferring the phage DNA from the virion into the host cell cytoplasm and to aid in initiation of DNA replication. If it is true that the T4 pilot protein, gp 2/64, aids in replication, then the interaction of gp imm with the pilot protein of superinfecting phage DNA may also interfere with its replication.

The exo III participation in T4 genetic exclusion shown in Table 4 does not depend upon the presence of gp imm or gp sp. Possibly its activity is regulated by another phage product. Alternatively, exo III may function in a more general fashion by degrading any unprotected DNA in the cytosol. In any case, some phage function presumably makes the superinfecting ds-DNA susceptible to exo III.

(2) *The action of gp sp.* The data presented in Table 6 indicate that gp sp acts in genetic exclusion by inhibiting the lysozyme function of a phage tail central base plug component, gp 5. This then prevents successful superinfection. In order to accomplish this, gp sp presumably either acts at the bacterial cell wall or at a site exterior to the murein layer. The basis for this assumption is that gp sp must either alter the recognition site of gp 5 action, or directly interact with gp 5 before it reacts with its peptidoglycan substrate in the murein layer, or both. Cornett (1974), observed a reduced efficiency of DNA injection by superinfecting phage into cells primarily infected with sp⁻ phage compared to sp phage. This observation suggests that the sp gp affects a phage recognition site in the cell envelope. Since DNA injection is triggered by irreversible phage binding to the cell surface it would seem that the gp sp does alter an adsorption site. However Cornett's observation seems the reverse of

what one might expect. If gp sp is acting to exclude superinfecting phage, one would expect cells infected with sp⁺ phage, not sp⁻ phage, to cause a reduced efficiency of secondary phage injection. I suggest the following explanation for this dilemma, based on the notion that an effective defence can counter an offence only if the opponent can be engaged. Together the above findings suggest that gp sp increases the efficacy of exclusion by molecularly highlighting the gp sp containing adsorption sites. Presumably, this would decoy the phage to adsorb preferentially at these altered sites. Irreversible binding would occur, immediately triggering injection. The injection would proceed normally until the tail tube approaches the murein layer where the appropriate counter measure (gp sp's anti-lysozyme activity) is stationed to inactivate gp 5, thereby preventing further tail tube penetration. The DNA might then be extruded into the periplasmic space and degraded by periplasmic enzymes. So despite the fact that gp sp increases the efficiency of injection, the net effect is to decrease the chance of a successful superinfection.

(ii) *A molecular model of T4 exclusion by gps imm and sp*

Among the earliest genes expressed after infection are *imm* and *sp*. As proposed by Vallee & Cornett (1972, 1973) gp *imm* probably occupies a position at the adhesion sites (Bayer, 1968) where the cell wall and cytoplasmic membrane are contiguous. These adhesion sites have been shown to be the adsorption and injection sites for phage T4, and several other phages, particularly T2 (Bayer, 1970). The gp *sp* may join gp *imm* at or near these sites. The *sp* protein presumably further modifies the outer membrane to highlight molecularly the adsorption site. As a secondary phage approaches, it is attracted to the modified site, adsorbs and attempts to inject. But due to the anti-lysozyme activity of the gp *sp* on the incoming gp 5 the injection is blocked in some but not all superinfections. The blockage occurs because of the inability of the tail tube to penetrate the cell wall when the lysozyme is inactivated. The DNA extruded from the successful injections then encounters the *imm* protein. The *imm* gp probably interacts with the superinfecting chromosome's terminal pilot protein. This interaction has a number of consequences. First, the ds-DNA ends are exposed to exo V degradation and probably unwinding. The unwound single strands are cut into single stranded fragments that are further degraded by host enzymes such as exo III. Also, the interaction of gp *imm* with the pilot protein may inhibit initiation of replication of the superinfecting chromosome.

(iii) *Ecological significance of the genes in the region from imm to sp*

Despite the interest in genetic exclusion among investigators, there has been virtually no discussion of the adaptive benefit it has for the phage. The exclusion by primary phages of superinfecting phage genomes by *imm* and *sp* can be classified ecologically as competition-intraspecific when acting against individuals of the same species or interspecific when acting against organisms of a different species (reviewed by Smith, 1966). Competition is the endeavour of two organisms of the same or different species to gain their share of the same limited resource (Milne, 1961). Adaptations that promote success in either type of competition presumably are advantageous to the phage. It is known that interspecific mixed phage infections result in lowered fitness among progeny (Mahmood & Lund, 1972). Therefore genetic exclusion, by reducing interspecific mixed infections, increases progeny fitness. With respect to intraspecific competition, exclusion may act as an adaptive mechanism for promoting individual fitness since these functions protect the host cell as a resource for the production of the primary infecting phage's own unique genome. Other genes in the same genomic region as *imm* and *sp* function to protect the phages own DNA from the nucleases of the host and lysogenic phage. Together these genes allow the primary phage to take over the host cell and sequester it as a resource for the phage's own self-propagation.

Genes *imm*, 42, beta-glucosyltransferase, *uvsX* and *sp* are clustered in the phage genetic map. Since clustering of genes with related functions is common in the phage T4 genome, it is reasonable to consider whether these particular genes have related functions. The argument is made below and summarized in Table 7 that these genes share the characteristic of being involved in phage competition/defence at the DNA level.

The *imm* gene, by subjecting superinfecting phage DNA to nuclease degradation, is, in effect, establishing a territory (the host cell) for the first infecting phage. The infected cell then becomes the exclusive resource of the primary infecting phage for use in self-propagation.

The next gene in a counterclockwise direction is gene 42 (dCMP-hydroxymethylase). Gp 42 alters phage DNA in a way that protects progeny phage from host restriction enzymes and also protects the phage DNA from degradation by the phage enzymes endonuclease II (gp den A) and endonuclease IV (gp den B) that degrade C-DNA to scavenge nucleotides for phage growth.

The next gene, proceeding in the same direction, is the gene for beta-glucosyltransferase. This enzyme glucosylates phage hydroxymethyldeoxycytosine containing DNA. Together, genes 42 and beta-glucosyltransferase protect the phage DNA during takeover

Table 7. A tabulation of five linked T4 genes with their enzymic and competition/defence functions

Gene	Product/function	Competition/defence function
imm	Unknown/inactivation of pilot protein of superinfecting phage DNA	Exclude competing DNA of same species
42	dCMP hydroxymethylase/leads to substitution of HMC for C in T4 DNA	Protect own DNA from host nucleases and from phage nucleases endo II and endo IV
β -gt	β -glucosyl transferase/glucosylates HMC residues in T4 DNA	Protect own DNA from nucleases of host and phage P1 lysogen
uvsX	rec-A analogue/required for recombinational repair	Repair own DNA
sp	= gp40/inactivates the baseplate gp5 lysozyme, blocks superinfecting phage penetration of peptidoglycan layer	Exclude competing DNA of same or similar species

of the *E. coli* host by rendering the host nucleases ineffective against the phage DNA. Glucosylation of the phage T4 DNA also protects against restriction by other lysogenic phage, such as phage P1 (reviewed in Revel, 1983).

Further counterclockwise is the *uvsX* gene. This gene encodes a protein similar in function to the *E. coli* *recA* protein (Fujisawa, 1985). It has a key role in recombinational repair of damaged phage DNA (see Bernstein & Wallace, 1983, for review).

Next in the sequence is the *sp* gene. The location of this gene was recently determined (Obringer *et al.*, manuscript submitted) as being in the region between 21·647 and 22·014 kbp on the T4 restriction map (Kutter & Ruger, 1985). As shown here, *sp* excludes superinfecting phage T4 DNA through its anti-lysozyme function. It also excludes phage T2 (Fig. 1). Therefore, *sp* gp acts in both intra- and interspecific competition.

In phage T4 it appears that the strategies of intra- and interspecific competition and defence at the level of the DNA molecule are not only conceptually linked, but their genetic determinants are physically linked and possibly co-regulated on the same section of DNA. Taken together this group of genes may represent a coadaptive gene cluster. A coadaptive gene cluster is a group of tightly linked genes whose products function cooperatively in determining the fitness of the organism (Stahl & Murray, 1966). Although often considered of secondary importance, the genes of this cluster may encode fundamental adaptive strategies which are universal in nature.

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