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GENETIC AND PHYSIOLOGICAL STUDIES OF *BACILLUS SUBTILIS* BACTERIOPHAGE ø105 MUTANTS WITH ALTERED LYSOGENIZATION CHARACTERISTICS

by

MING-FAN LAW

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Chairman of Examining Committee

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Abstract

GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS SUBTILIS BACTERIOPHAGE ø105 MUTANTS WITH ALTERED LYSOGENIZATION CHARACTERISTICS

by

Ming-Fan Law

Adviser: Professor Anthony J. Garro

Mutants of the Bacillus bacteriophage ø105 and SPO2 with altered lysogenization and induction phenotypes were isolated and characterized to investigate the effect of lysogeny on DNA mediated transformation, and to identify and map the ø105 genes which function in the establishment or maintenance of the prophage state.

The low transformation efficiency of B. subtilis lysogenic for phages ø105 or SPO2 is shown to result from the induction of lytic phage replication during the development of the physiological state of competence for transformation. Lysogenic competent cells have a higher rate of spontaneous prophage induction than noncompetent cells. Lysogens formed by non-inducible mutants of ø105 and SPO2 exhibited higher transformation levels than those formed by wild-type phage. These results suggest that the physiological state of competence in B. subtilis promotes prophage derepression leading to cell death and the loss of

potential transformants.

Six complementation groups, mutations in which decreased the ability of ø105 to lysogenize infected cells were identified and their map order determined. These genes are tightly linked and appear to be clustered between genes D and E on the ø105 map. Unequivocal mapping of this block of genes affecting the ability of ø105 to lysogenize has been hampered by the anomalous recombination frequencies observed in crosses involving these genes and outside markers.

In infections with the wild-type phage up to 4 hours may be required to produce a stably lysogenized cell. During this time the replication of the infecting phage appears to be repressed but the cell is still sensitive to superinfection. The infected cells, which will ultimately give rise to lysogenic clones, segregate uninfected daughter cells during cell divisions which occur at early times after infection.

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CHAPTER I

INTRODUCTION

Depending on the response they elicit upon infecting bacteria, bacteriophages may be broadly divided into three categories, the virulent phages, the persistent phages, and the temperate phages. The virulent phages display an extreme form of parasitism in which the infected bacteria lyse at the end of the phage growth cycle releasing the newly formed phage particles. This mode of replication results in cell death and is referred to as lytic replication. In contrast to the virulent phages, both the persistent phages and the temperate phages are capable of a more subtle form of parasitism. Persistent phages replicate in and are released continuously from growing and dividing carrier bacteria without markedly harming the bacteria. These carrier bacteria are somewhat unstable but can be subcultured and will continue to release phage particles (Marvin and Hohn, 1969). Temperate phages, on the other hand, replicate either lytically or lysogenically. In the latter mode of replication, the infected bacteria survive and give rise to lysogenic progeny (Lwoff, 1953). The phage DNAs persist in a repressed state in the lysogenic bacteria for many cell generations. The persistence of phage DNAs in the host cells produces the phenomenon of lysogeny, which in turn has provocative implications

for many biological problems. First of all, the switch between the vegetative and lysogenic states by temperate bacteriophage provides a useful system for studies of differentiation. Secondly, it throws light on the origin of viruses and the role of the prophage in the evolution of bacteria. Moreover, lysogeny is useful as a prokaryotic model for the provirus hypothesis (Temin, 1971) and the oncogene hypothesis (Huebner and Todaro, 1969). An understanding of phage-host interaction, and the mechanism and processes involved in the regulation of lysogeny may provide the basis for a more complete understanding of viral oncogenesis.

In a lysogenic bacterium, the phage genome can be present either in the form of a plasmid, or as an integrated prophage which is covalently linked to the bacterial DNA. The state of the phage genome is a general characteristic of the particular infecting phage. The Escherichia coli phage P1 appears to lysogenize as a plasmid whereas others such as E. coli phage lambda and Mu-1, Salmonella typhimurium phage P22 and Bacillus subtilis phages ø105 and SP02, lysogenize as a prophage in the chromosome. The functions of the phage genes required for the vegetative replication of the phage are repressed by a phage-coded repressor. Repression can be reversed by a variety of circumstances. Derepression occurs spontaneously with a frequency of approximately 10^{-4} to 10^{-6} per bacterial

generation and can be artificially induced by agents such as UV and x-ray irradiation, thymine starvation, and treatment with alkylating agents such as mitomycin C (MC). Under these conditions which interfere with host DNA replication, the repressed prophage switchs from lysogenic to vegetative replication and more phage particles are produced.

Lysogenic bacteria acquire unique characteristics which are not present in the non-lysogenic strains. Since the latent phage genome can be activated to replicate, they transfer to their offsprings the ability to produce bacteriophage. Lysogenic bacteria are immune to superinfection by bacteriophages homoimmune to the residing prophage because of the presence of the repressor in the cell cytoplasm. One consequence of the formation of immune cells is that the plaques produced by the temperate bacteriophages are usually turbid. As will be discussed in a latter section the conversion from a turbid to clear plaque phenotype can be used to identify lysogenization-defective mutants. In addition to the production of repressor, certain prophages can endow the lysogenized cells with new phenotypes by the continued expression of some phage genes. This is the phenomenon of lysogenic conversion. Examples are the alteration of somatic O antigens in Salmonella typhimurium (Robbins and Wright, 1971), the formation of diphtheria toxin by Corynebacterium

diphtheriae (Barksdale, 1971; Pappenheimer, 1973), and the production of the competence factors for transformation in Bacillus stearothermophilus (Streip and Young, 1971; Yasbin et al., 1975) and in Staphylococcus aureus (Sjöström et al., 1973; Rudin et al., 1974).

Studies of the regulation of lysogeny to a large extent have been limited to the enteric bacteriophages, most notably the E. coli phages lambda and Mu and the Salmonella typhimurium phage P22. In the case of lambda and P22, multiple phage genes whose functions are required for the establishment and maintenance of the prophage state, have been identified. These two phages also resemble each other in having a preferred integration site on the bacterial genome. Their phage attachment (att) sites, through which integration with the host chromosome occurs is situated towards the middle portion of the phage DNAs. Integration in both cases occurs via circular integrative intermediates and site-specific recombination as described in the Campbell model (1962). The third bacteriophage, the mutator phage Mu, differs from lambda and P22 in having its att site located at the ends of the phage genome. It integrates into a large if not random number of sites on the E. coli chromosome (Couturier, 1976).

ø105 is a temperate phage which infects the transformable strain of Bacillus subtilis 168. The phage particle has an icosahedral head and a flexible,

non-contractile tail (Birdsell et al., 1969). The phage DNA is double-stranded, with a molecular weight of 26.2 ($\pm$0.3) x 10^6 daltons (Birdsell et al., 1969; Chow et al., 1972). The DNA consist of a unique, linear nucleotide sequence and is not circularly permuted (Armentrout and Rutberg, 1970; Armentrout et al., 1971; Chow et al., 1972). ø105 forms an integrated prophage and only a single integration site has been observed, between the bacterial markers phe-1 and ilv-A1 (Rutberg, 1969). The ø105 prophage can be induced to replicate vegetatively by mitomycin C or uv-irradiation (Birdsell et al., 1969). There is evidence from both genetic and physical studies that the genetic markers and nucleotide sequences of vegetative and prophage ø105 DNA are colinear, and not circularly permuted (Armentrout and Rutberg, 1970; Chow and Davidson, 1973). The linear phage DNA has cohesive ends and is capable of reversible cyclization (Birdsell et al., 1969; Chow et al., 1972). In fact, as isolated from the phage particles, ø105 DNA consists primarily of circular molecules formed through the association of complementary nucleotide sequences at the ends of the DNA (Scher et al., in press).

Our interest in ø105 has been stimulated by evidence which indicated that the mechanisms of ø105 prophage excision and possibly integration differ in several respects from the mechanisms of prophage integration and excision

exhibited by lambda and P22. For example, the ø105 att site appears to involve the ends of phage DNA (Armentrout and Rutberg,1970; Chow and Davidson, 1973; Shapiro et al., 1974). Also, following induction, the derepressed ø105 prophage is not excised from the bacterial chromosome prior to replication but rather replicates as a complex of phage and bacterial DNA (Armentrout and Rutberg, 1971; Rutberg, 1973). In these respects, ø105 resembles the mutator phage, Mu, but as previously noted, ø105, unlike Mu, integrates perferentially at a single site on its host chromosome.

Whether an infecting phage replicates lytically or lysogenically is known, for lambda and P22, to be influenced by the interaction of both phage and bacterial gene products. Studies of lambda and P22 have shown that lysogenization-defective phage mutants generally can be classified into two functional groups, one defective in the establishment of lysogeny and the other in its maintenance. For example, in the case of lambda, there are four genes, cI, cII, cIII, and int, required for establishment and only one, cI, required for maintenance of the prophage state. The cI gene product is the lambda repressor which represses transcription of the genes essential for lambda replication; cII and cIII functions are required for efficient expression of the cI gene early in infection, and int function is needed for prophage integration

(Gingery and Echols, 1967; Gottesman and Yarmolinsky, 1968; Echols and Green, 1971). Similarly, in P22, there is a cluster of immunity genes consisting of genes c1, c2 and c3 which are primarily responsible for the establishment and maintenance of lysogeny. Functional c2 gene product is required continuously for the maintenance of the prophage state. Genetic studies indicate that the c2 gene of P22 codes for a repressor, the behavior of which is similar to that of the lambda repressor (Levine, 1972). However, the regulation of lysogeny in P22 is more complicated than lambda in that there is a second immunity region consisting of genes ant and mnt; ant codes for an antirepressor which antagonizes the c2-mediated repression of lytic replication, and mnt is a regulatory gene which negatively controls the antirepressor operon (Levine, 1972; Levine et al., 1975). The integration of P22 into the bacterial chromosome is mediated by a diffusible product produced by gene L of P22 (Smith and Levine, 1967). In the case of ø105, although a number of lysogenization-defective mutants had been described (Rutberg, 1969; Armentrout and Rutberg, 1971), the genes involved in the regulation of lysogeny had not been systematically studied.

B. subtilis is a transformable bacterial species. Although all the transformable strains carry an inducible-defective prophage, PBSX (Haas and Yoshikawa, 1969; Garro and Marmur, 1970), without any apparent effect on their

transformability, the presence of a ø105 or SPO2 prophage was known to greatly reduce transformation efficiences: The transformation efficiency of these lysogens is approximately 1% that of non-lysogens (Peterson and Rutberg, 1969; Yasbin and Young, 1972; Yasbin et al., 1973). It had been suggested that the reduced transformation efficiency of lysogens was either a form of lysogenic conversion in which the continued expression of some prophage gene(s) interfered with this mode of genetic exchange, or that the effect was related to cell death caused by induction of lytic phage replication during the development of competence (Garro, 1973; Yasbin et al., 1973).

The purposes of the present investigation were twofold: (1) to study the relationship between lysogeny and transformation in order to determine the cause for the impaired transformability of B. subtilis lysogens, and (2) to enumerate and map the phage genes which function in the establishment or maintenance of lysogeny, in the hope of identifying potential regulatory sites on the phage genome.

CHAPTER II

MATERIALS AND METHODS

1. Bacteria and Phage Strains

The bacteria strains employed are listed in Table 1. All strains are *Bacillus subtilis* 168 derivatives. BD99 was used as the host for determination of lysogenization frequencies for the clear plague mutants. This strain and its lysogenic derivatives were also used as the recipients for all transformation experiments. Phage stocks were prepared on strain SR135. All phage crosses were performed in strain MB228 which carries the *su*$^{+3}$ suppressor. This strain also was used to assay PFU by ø105 *sus* mutants. The *su*$^{-}$ strain 44AO was used to assay PFU by all *sus*$^{+}$ phages. GB75 is non-inducible for the defective phage PBSX which is caused by all *B. subtilis* 168 strains. Lysogens of this strain carrying a ø105 prophage were induced to prepare large quantity of wild type ø105.

The wild type strain of ø105, the conditional lethal mutants and the two lysogenization-defective mutants, ø105*cts*23 and ø105*c*4, were kindly provided by L. Rutberg. Wild type SPO2 was the generous gift of J. Marmur.

2. Chemicals and Enzymes

Mitomycin C (MC) was purchased from Sigma Chemical Co., N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) from Aldrich

TABLE 1

BACILLUS SUBTILIS STRAINS EMPLOYED

Strain	Relevant properties[a]	Source
BD99	*his*A-1, *trp*C-2, *thr*-5	D. Dubnau
BR95	*phe*-1, *ilv*A-1, *try*C-2	L. Rutberg
SR135	*spo*A-9, (*trp*-7), *su*$^{+3}$	L. Rutberg
44AO	*trp*C-2, *thy*, *str*^{r}	K. Bott
MB228	*leu*-8, (*met*B, *thr*-5), *su*$^{+3}$	J. Marmur
GB75	*xin*, *met*C, *pyr*A	A. Garro

[a]Properties: *spo*A-9, asporogenous; *su*$^{+}$, supresses the auxotrophic phenotypes in bracket; *str*^{r}, resistant to 5 mg of streptomycin sulfate per ml; *xin*, noninducible for PBSX.

Chemical Co., polyethylene glycol 6000 (PEG) from Fisher Scientific Co., and optical grade CsCl for density gradients from Harshaw Chemical Co. Renografin-76 (methylglucamine N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate) is supplied as a 76% solution by E. R. Squibb and Sons. Deoxyribonuclease 1, ribonuclease A and ribonuclease T_1, were from Worthington Biochemical Corp.

3. Media and Growth of Phage and Bacteria

A. Media

Min-CH medium (Rutberg, 1969) contained Spizizen's salts (Anagnostopoulos and Spizizen, 1961), 0.5% glucose, 25 µg/ml each of aspartic acid and glutamic acid, 0.05% casamino acids and 10^{-5}M $MnCl_2$. Required amino acids were added to a final concentration of 50 µg/ml. Veal yeast (VY) broth contained 2.5% veal infusion (Difco) and 0.5% yeast extract. Colony-forming units (CFU) were assayed on plates of either tryptose blood agar base (TBAB, Difco) or tryptone broth solidified with 1.5% agar. *His*$^+$ transformants of BD99 were scored on Min-CH agar (2% agar) plates supplemented with tryptophan and threonine.

The overlay plates used to assay for PFU contained tryptone broth supplemented with 10^{-2}M $MgSO_4$ and 0.2% maltose. The concentrations of the bottom and the top agar in these overlay plates were 1.5% and 0.6%, respectively.

B. Growth and Fractionation of Competent Cells

Bacteria were grown to competence and transformed using the two-step growth procedures of Anagnostopoules and Spizizen (1961) as modified by Rudner and Remeza (1973). Transforming DNA was prepared by the Marmur method (1961).

In some experiments, after development of competence, competent and non-competent cell populations were separated by Renografin density gradient centrifugation (Hadden and Nester, 1968). Linear Renografin gradients (35% to 52%) were employed. In these gradients the Renografin solution, as supplied by the manufacturer, is taken as 100%. Approximately 5×10^7 bacteria of a competent culture were layered on the preformed gradients in cellulose nitrate tubes (Spinco SW27 tube, Beckman Instruments). Following centrifugation for 30 min at 20,000 rev/min in a SW27 rotor, the cells separated into two fractions which were removed by puncturing the tube with a hypodermic syringe in the region of each cell band.

C. Preparation of ø105 and anti-ø105 antiserum

Bacteriophage ø105 was prepared by induction of *B. subtilis* GB75(ø105). Mitomycin C was added at a concentration of 0.4 μg/ml to cultures grown to a density of 5×10^8 CFU/ml in VY broth (Garro, 1973). After 120 min incubation at 37C a drop of $CHCl_3$ was added to complete lysis. The lysate was incubated for 30 min at 37C with

1 μg/ml of both ribonuclease and deoxyribonuclease. NaCl was added to 0.5M and cell debris removed by centrifugation for 10 min at 7,000 rev/min, in a Sorvall SS-34 or GSA rotor. The phage lysate was concentrated by polyethylene glycol precipitation (Yamamoto et al., 1970) and purified by banding in a CsCl step gradient prepared in phage diluent which contained 0.01M Tris pH 7.4, 5×10^{-3}M $MgCl_2$, 2×10^{-5}M $MnCl_2$ and 0.01 mg/ml gelatin. The gradient layers consisted of 3 ml 1.65 g/cm^3 CsCl, 4 ml 1.43 g/cm^3 CsCl and 5 ml of the concentrated phage resuspended in phage diluent. The layered gradient was centrifuged for 16 h at 33,000 rev/min and 10C in a Spinco SW41 rotor. The purified phage was dialysed overnight against 2,000 volumes of phage diluent.

Anti-ø105 antiserum was prepared by immunizing rabbits with CsCl purified ø105 (32 mg/ml) suspended in complete Freund's adjuvant (Freund, 1951).

4. Isolation of Phage Mutants

A. Mutagenesis

Bacteria expotentially growing in Min-CH medium were infected with wild-type phage at a multiplicity of 0.5. After allowing 15 min for adsorption, MNNG was added to a final concentration of 200 μg/ml. Incubation was continued for 30 min at 37C at which point the cultures were diluted 1:1,000 with VY broth and 1.0 ml aliquots distributed to

separate culture tubes. After another 90 min of incubation, a drop of $CHCl_3$ was added to each tube, and the lysates were screened for mutants as described below.

B. Induction-defective (ind$^-$) Phage Mutants

Mutants that would form lysogens which had low rates of spontaneous induction were sought among those mutants resistant to MC induction. These were detected by overlaying the mutagenized lysates with 44AO on tryptone broth agar plates containing 0.05 μg of MC per ml in the case of ø105 and 0.04 μg/ml for SPO2. Under these conditions the MC ind$^-$ mutants form turbid plaques, whereas those of wild-type phage are clear (Horiuchi and Inokuchi, 1967). The turbid plaques were picked to 1 ml of VY and the phage was purified by replating. Phage stocks were prepared by infecting cultures of SR135 and overlaying the infected cells on MB plates with 0.2% top agar. The confluently lysed lawns were harvested with 10 ml of VY and sterile-filtered (HAWP 025, 0.45μ m pore size; Millipore Corp.).

BD99 lysogens of the mutants were isolated from overlay plates by stabbing the center of turbid plaques and streaking for single colony-forming units on TBAB plates.

C. Lysogenization-defective ø105 Mutants

Phage mutants which are impaired in their ability to lysogenize produce plaques which are clear or less turbid

than those of the wild-type phage. Spontaneously derived and MNNG-induced mutants were isolated. The spontaneous mutants were obtained from broth cultures of cells lysogenized by one of the induction-defective ø105 mutants, ø105*ind*-1. Culture of this *ind*$^-$ lysogen contained several thousandfold fewer spontaneously released phage than culture of wild-type lysogens. Thus, it was possible to detect, among the released phage, rarely occurring mutants which have lost the capacity to maintain the lysogenic state. In fact, approximately 50% of the phage found in the culture supernatants of the *ind*$^-$ lysogens are clear plaque mutants.

The procedures employed in the isolation of clear plaque mutants by MNNG mutagenesis were the same as described for the isolation of *ind*$^-$ mutants. The mutagenized phage lysates in this case, however, were screened for clear plaque formers. Only a single clear plaque mutant was picked from each mutagenized lysate to avoid the isolation of siblings. The mutants were purified by re-plaquing and stocks prepared by harvesting confluently-lysed lawns from overlay plates. All stocks were sterile-filtered before use. In general, approximately 0.5% of the phage which survived MNNG treatment produced clear plaques.

5. Complementation Analysis

In order to determine the number of genes involved in ø105 lysogenization, the clear plaque ø105 mutants were tested for their ability to complement each other, with respect to the production of lysogenized cells, using a procedure similar to one described by Kaiser (1957) for clear plaque lambda mutants. In this test an aliquot of exponentially growing cells, about 5×10^7 CFU, is first infected at a multiplicity of 5 or more with one mutant, and the infected cells overlayed on a ø105 assay plate. At this multiplicity of infection, the infected cells would form a confluently-lysed lawn upon overnight incubation. Prior to incubation, however, the cells were superinfected by spotting on the overlay, within 10 min of its hardening, 10 µl samples containing 5×10^6 PFU of the other test phages. If complementation occurred, the lysogens formed grew and produced colonies within the area of the spotted lysate.

6. Phage Crosses

Exponentially growing MB228$\underline{su}^+$ cells from a VY broth culture were infected with a mixture of two mutants at a multiplicity of 5 for each parental type. After a 15 min incubation at 37C to allow for adsorption, the unadsorbed phage were removed by pelleting the cells at 3,000 x g for 10 min at 4C and resuspending them in warm

VY which contained anti-ø105 antiserum at a K=2.5. After 10 min further incubation the culture was diluted 10^{-4} with VY and incubated for 120 min. A drop of $CHCl_3$ then was added to complete lysis. For crosses involving temperature-sensitive (ts) mutants all incubations were carried out at 32C. The lysates were assayed for total phage (PFU on su^+ at 32C), ts^+sus^+ phage (PFU on su^- at 40C), sus^+ phage (PFU on su^- at 32C), ts^+ phage (PFU on su^+ at 40C) and also scored for turbid and clear plaque phenotypes. Percent recombination is expressed as 200% x No. of recombinants/total phage.

7. Measurement of Spontaneous Induction Rates

The spontaneous induction rates of ø105 and SPO2 lysogens were calculated as the ratio of the number of spontaneously released PFU to the number of CFU in the cultures. Estimates of the spontaneous induction rate of the bacteria lysogenized by the ø105ind^- and SPO2ind^- mutants were made from the number of PFU in the sterile-filtered samples of cultures at both the pre-competent and the competent stages of development.

For the Renografin-fractionated cell populations, cells from each band were washed on a membrane filter (Millipore Corp.) with VY medium and then resuspended in fresh VY broth. They were then incubated at 37C and samples were removed at different time intervals and

assayed for PFU in the supernatant.

8. Measurement of Lysogenization Frequencies

The frequencies at which wild-type and mutant ø105 lysogenized infected cells were assayed by scoring the infected cells both for immunity to superinfection and for the spontaneous release of phage. The cells were infected with the test phage using the procedure described for the phage crosses. Immediately upon resuspension in the anti-ø105 antiserum-containing VY broth the culture was assayed for the following: 1. for infective centers, by overlaying with a lawn of sensitive cells; 2. for total surviving CFU, by plating on nutrient agar spread with anti-ø105 antiserum; 3. for immune CFU, by plating on nutrient agar spread with 10^8 PFU of ø105csi-17. After 17 h incubation at 37C the colonies which grew on the antiserum-containing medium were tested for their ability to release phage by stabbing them into lawns of sensitive cells and for immunity by streaking on ø105csi-17-seeded plates. Lysogenization frequencies were calculated as the ratio of the immune cells/ml to the infective cneters/ml.

CHAPTER 3

RELATIONSHIP BETWEEN LYSOGENY, SPONTANEOUS INDUCTION, AND TRANSFORMATION EFFICIENCIES IN BACILLUS SUBTILIS

Strains of Bacillus subtilis 168 lysogenic for ø105 and/or SPO2 transform with bacterial DNA at a much lower efficiency than non-lysogenic strains (Peterson and Rutberg, 1969; Yasbin and Young, 1972; and Yasbin et al., 1973). Five possible explanations had been suggested for this decrease in transformation efficiency in lysogenic cultures (Yasbin et al., 1973): (i) restriction of unmodified DNA, (ii) deficiencies in recombination enzymes needed in transformation, (iii) alteration of cell surface resulting in reduced uptake of transforming DNA, (iv) preferential degradation of bacterial genes by nucleases, and (v) preferential lysis of competent cells by their prophage. The first four explanations which were postulated to be possible consequences of lysogenic conversion seemed to be unlikely on the basis of the following observations: (i) DNA isolated from bacteria lysogenic for ø105 and/or SPO2 was no more efficient in transforming the lysogenic strains than DNA isolated from non-lysogenic bacteria (Yasbin et al., 1973), (ii) the presence of prophage ø105 and/or SPO2 did not interfere with transfection by DNA from phage ø29 or SPO1, nor did they affect PBS1- or SP10-

mediated transduction (Peterson and Rutberg, 1969; Yasbin and Young, 1972; and Yasbin et al., 1973), (iii) at early stages (first 60 min) of competence, lysogenic cultures of B. subtilis were capable of binding irreversibly as much bacterial DNA as the non-lysogenic cultures. At the same stage, transformation efficiency of the lysogenic cultures was severely reduced when compared with that of the non-lysogenic cultures (Yasbin et al., 1973).

Positive evidence in support of any particular explanation, on the other hand, had been lacking. Garro (1973) reported that cells lysogenized by a clear plaque ø105 mutant, ø105c4, exhibited low rates of spontaneous induction and high transformation efficiency. Although this suggested a relationship between spontaneous prophage induction and the loss of transformed cells, the procedures used to isolate these lysogens may have selected for cellular mutants which suppressed the expression of some prophage genes. We therefore sought to determine if there was in fact a general relationship between spontaneous prophage induction and low transformation levels. For this purpose, the susceptibility of competent cells to spontaneous phage induction was examined. In addition, phage mutants with altered induction characteristics were isolated and their effect on transformation was studied.

1. Results

A. Spontaneous Prophage Induction in Competent and Noncompetent Cells

If the physiological state of competence results in prophage derepression, one would expect cultures enriched for competent cells to produce higher levels of phage than cultures of noncompetent cells. BD99(ø105) was grown to competence and fractionated into two populations of cells by Renografin density gradient centrifugation. When incubated in VY broth, the less dense population which contained the competent cells (Table 2) released more phage than the denser banding noncompetent cells (Fig. 1).

B. Induction Characteristics of ø105 and SPO2 *ind*$^-$ Mutants

Approximately 30% of the ø105 and SPO2 isolates which formed turbid plaques on the overlay plates containing MC produced lysogens which had reduced spontaneous induction rates when grown in VY broth. They were designated as *ind*$^-$ mutants. Three ø105*ind*$^-$ and three SPO2*ind*$^-$ mutants were selected for further study. Table 3 shows the number of spontaneously released PFU/ml from cultures of *B. subtilis* lysogenized with the selected ø105*ind*$^-$ and SPO2*ind*$^-$ mutants. For the ø105 mutants, ø105*ind*-1 and *ind*-2 exhibited spontaneous prophage induction rates that were about 10^{-3} that of wild-type ø105,

TABLE 2

TRANSFORMATION EFFICIENCIES OF RENOGRAFIN FRACTIONATED CELLS

Fractionated Cells[a]	His^+ transformants/10^8 cells[b]
Unfractionated	4.9×10^3
Top band cells	1.6×10^4
Bottom band cells	< 100

[a]Top and bottom refer to the positions of the cells in the Renografin gradient, the top band cells being less dense than the bottom band cells.

[b]Transforming DNA was added to a final concentration of 2 μg/ml.

Figure 1

Release of spontaneously induced phage from competent and noncompetent cell populations. A 5-ml amount of a competent culture of BD99(ø105) was layered onto a 32-ml continuous Renografin gradient (35 to 52%) and centrifuged for 30 min at 20,000 rpm in an SW27 rotor. The cells separated into two bands which were removed by puncturing the tube with a hypodermic syringe in the region of each band. The less dense band, which contained all the competent cells, and the denser banding cells were each washed on a membrane filter (Millipore Corp.) with 25 ml of VY medium and then resuspended in 3 ml of the same medium and incubated at 37C. At intervals, samples were removed and assayed for plaque-forming units on overlay plates containing 3 mg of streptomycin sulfate per ml. The results are expressed relative to the number of colony-forming units in each sample at 0 min. Symbols: competent cells (●); noncompetent cells (o).

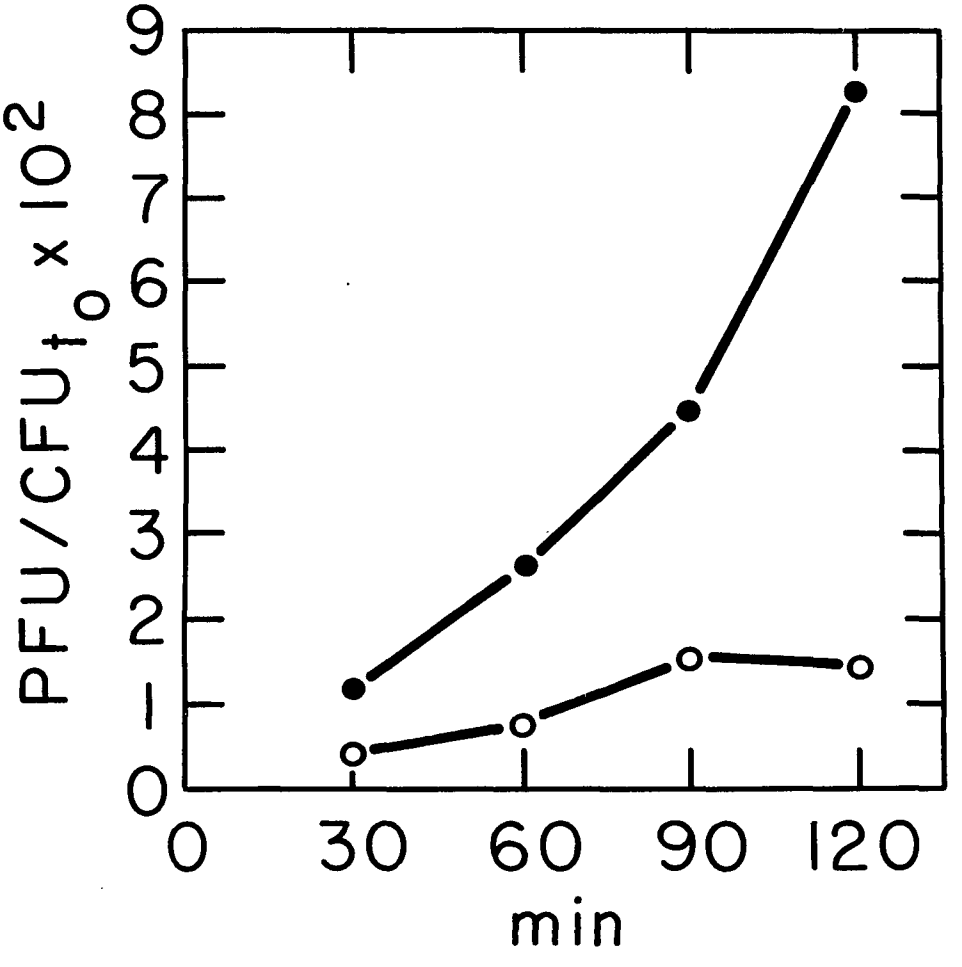
PFU/CFU$_{t_o}$ x 10^2
0
1
2
3
4
5
6
7
8
9
0
30
60
90
120
min

TABLE 3

THE NUMBER OF SPONTANEOUSLY RELEASED PFU/ml IN THE SUPERNATANTS OF CULTURES OF *B. SUBTILIS* LYSOGENS IN VY BROTH[a]

Strains	PFU/ml
BD99(ø105)	5.6×10^5
BD99(ø105*ind*-1)	<100
BD99(ø105*ind*-2)	<100
BD99(ø105*ind*-3)	2.4×10^4
BR95(SPO2)	2.9×10^4
BR95(SPO2*ind*-1)	<100
BR95(SPO2*ind*-2)	<100
BR95(SPO2*ind*-3)	<100

[a]The number of spontaneously released PFU/ml in the supernatant were determined, after incubation for 120 min, for cultures which had been pre-washed to remove free phage in the supernatant at 0 min.

whereas that of the third one, ø105*ind*-3 was approximately 10^{-1} that of wild-type prophage. All three of the SPO2 mutants selected for study formed lysogens which had spontaneous induction rates of 10^{-2} that of wild-type SPO2 prophage.

The pattern of spontaneous induction for representative mutants then was determined during growth to competence. In the two-step competence regimen used, cells are grown to early stationary phase in a relatively rich medium and then are diluted 10-fold into a step-down medium, reaching maximal competence after another 90 min of incubation. Phage levels present immediately prior to the step-down (precompetent) and at the end of the 90 min step-down incubation (competent) are given in Table 4. When compared with their respective wild-type lysogens, the mutants produced reduced levels of spontaneously induced phage at both precompetent and competent stages of growth. These levels are especially lowered for the ø105*ind*-1 and SPO2*ind*-1 lysogens, which also do not exhibit the large increase in the ratio of plaque-forming units to colony-forming units seen with the wild-type and ø105*ind*-3 lysogens during the step-down incubation.

C. Transformation Efficiencies

If the poor transformability of ø105 and SPO2 lysogens

TABLE 4

SPONTANEOUS INDUCTION OF ø105 AND SPO2 LYSOGENS

Strain	CFU[a]/ml	PFU/ml	PFU/CFU
Precompetent cultures			
BD99(ø105)	1.5×10^{9}	5.7×10^{5}	3.8×10^{-4}
BD99(ø105*ind*-1)	2.6×10^{9}	2.7×10^{2}	1.0×10^{-7}
BD99(ø105*ind*-3)	3.2×10^{9}	3.8×10^{4}	1.2×10^{-5}
BD99(SPO2)	1.5×10^{9}	3.2×10^{4}	2.1×10^{-5}
BD99(SPO2*ind*-1)	1.8×10^{9}	50	2.8×10^{-8}
Competent cultures			
BD99(ø105)	3.4×10^{8}	2.0×10^{7}	6.0×10^{-2}
BD99(ø105*ind*-1)	4.8×10^{8}	1.3×10^{2}	2.7×10^{-7}
BD99(ø105*ind*-3)	3.2×10^{8}	9.4×10^{5}	2.9×10^{-3}
BD99(SPO2)	2.7×10^{8}	1.0×10^{7}	3.7×10^{-2}
BD99(SPO2*ind*-1)	2.1×10^{8}	50	2.4×10^{-7}

[a]CFU, Colony-forming units; PFU, plaque-forming units.

is a consequence of prophage induction, this effect of lysogeny should be modified by the *ind*⁻ mutations. The transformation efficiencies of the lysogens, which are presented in Table 5, support this notion. Lysogens of the ø105 and SPO2 mutants that had low spontaneous induction rates were as transformable as the nonlysogenic strains, and, although the presence of the ø105*ind*-3 prophage reduced transformation efficiencies, the reduction was less than that produced by wild-type ø105. The relative transformation efficiency of BD99(ø105*ind*-3) is consistent with a spontaneous induction rate intermediate to that of ø105*ind*-1 and wild-type ø105 lysogens.

2. Discussion

The results presented indicate that the poor transformability of ø105 and SPO2 lysogens is a consequence of prophage induction in the competent cell population. Phage mutations conferring resistance to induction counteract this effect of lysogeny, and their capacity to do so appears to vary with the extent to which they lower the rate of spontaneous induction. The gene products modified by the ø105*ind* and SPO2*ind* mutants have not been identified; however, mutation in phage lambda producing an *ind*⁻ phenotype all have been localized in the gene coding for the lambda repressor (Jacob and Campbell, 1959; Eshima et al., 1972).

TABLE 5

TRANSFORMATION EFFICIENCIES OF LYSOGENIC AND NONLYSOGENIC STRAINS

Recipient strain	His^+ transformants/10^8 cells[a] Expt 1	Expt 2	Relative Transformation Frequency[b]
BD99	917,000	1,259,000	1.00
BD99(ø105)	9,900	12,700	0.01
BD99(ø105*ind*-1)	941,000	1,007,000	0.90
BD99(ø105*ind*-2)	770,455		0.71
BD99(ø105*ind*-3)	43,000	59,000	0.05
BD99(SPO2)	6,000	14,000	0.009
BD99(SPO2*ind*-1)	1,192,000	1,242,000	1.12
BD99(SPO2*ind*-2)	1,176,000		1.08
BD99(SPO2*ind*-3)	981,000		0.90

[a]Recipient strains were transformed at a DNA concentration of 10 µg/ml.

[b]Relative transformation frequency is the average frequency of transformation for each sample divided by the average frequency of transformation for BD99.

In contrast to transformation, transfection does not require continued cell viability and, although lysogenization by ø105 decreases transfection by ø1 DNA (Peterson and Rutberg, 1969), essentially normal levels of transfection by ø29 and SPO1 DNAs have been reported for ø105 and SPO2 lysogens (Yasbin and Young, 1972; Yasbin et al., 1973). Inhibition of transfection, by induction of a resident prophage, probably would not be apparent unless the replication cycle of the transfecting phage was either subject to prophage-mediated interference (Rettenmier and Hemphill, 1973; Rettenmier and Hemphill, 1974) or could not be completed prior to cell lysis. Prophage-induced cell lysis is the most likely explanation for the observation of Yasbin et al. (1973) that the normal period during which competent cells may be transfected is shortened in ø105 and SPO2 lysogens.

Preferential lysis by prophage induction of the competent cell population in a competent culture of lysogens also explains the following observations: (i) the effect of lysogeny on transformation efficiencies is additive i.e. B. subtilis strains lysogenic for both ø105 and SPO2 are more severely impaired in their ability to be transformed than either of the single lysogens (Yasbin et al., 1973), and (ii) after the first 60 min of competence during which lysogens are capable of binding DNA at wild-type level, there is a rapid decrease in the binding of DNA

(Yasbin et al., 1973). Strains of B. subtilis doubly lysogenic for ø105 and SPO2 are expected to have greater probability of being lysed during the stages of competence than single lysogens because both prophages in the double lysogens could lyse the cells, thus resulting in a further decrease in the number of transformable bacteria. The rapid decrease of irreversible DNA binding by lysogens at late stages of competence is due to lysis of competent cells instead of a deficiency in DNA uptake. Preferential lysis of transformed lysogens should result in the release of transforming DNA from competent lysogens. In fact, Yasbin et al. (1975) showed that there was a loss, over a two hour period at late stages of competence, of 95% of the transforming DNA which had been taken up by the competent lysogens.

The physiological changes associated with the development of competence apparently interfere not only with the maintenance of lysogeny but also with the establishment of the prophage state. Stewart and Pagel (1973) have reported that in SPO2-infected competent cells, the choice between lysogeny and lysis is shifted to the latter mode of replication. One possible explanation for these effects of competence on prophage establishment and maintenance is the depressed rate of DNA synthesis in competent cells (McCarthy and Nester, 1967; Dooley et al., 1971). All of the common treatments used to initiate prophage

induction such as MC and ultraviolet, and X-irradiation also inhibit DNA replication.

One additional comment on the relationship between lysogeny and transformation is warranted. All of the transformable strains of B. subtilis which have been examined have been shown to carry an inducible defective phage called PBSX or PBSH (Subbaiah et al., 1965; Haas and Yoshikawa, 1969; and Garro and Marmur, 1970). Using an assay which monitors the killing activity of PBSX for B. subtilis W23 (Garro et al., 1970), we have been unable to detect an increased production of PBSX particles in competent cultures. This may reflect the fact that even in the presence of MC, PBSX is only poorly inducible from late-exponential phase cells (Haas and Yoshikawa, 1969). Recently, it has also been found that essentially all transformable strains of Bacillus subtilis 168 are lysogenic for an infectious bacteriophage SPβ (Warner et al., 1977). This phage, in contrast to ø105 and SPO2, is not readily inducible. Even under inducing conditions, such as treatment of cultures with mitomycin C or nitrosoguanidine, less than 1% of the cells in the cultures release infectious SPβ particles (Warner et al., 1977). It also appears that growth to competence does not cause the induction of SPβ since cells cured of the phage do not exhibit an increased transformation efficiency (Zahler and Hemphill, personal communication).

CHAPTER 4

GENETIC ANALYSIS OF LYSOGENIZATION-DEFECTIVE MUTANTS OF THE TEMPERATE *BACILLUS* BACTERIOPHAGE ø105

Using conditional-lethal mutants, Armentrout and Rutberg (1970) had identified 11 essential genes in ø105, and a recombination map of temperature-sensitive (*ts*) and suppressable (*sus*) mutants of ø105 had been established by two- and three-factor crosses (Fig. 2). Considering the molecular weight of the double stranded ø105 DNA, the ø105 genome carries enough information for approximately 25 genes. Genes non-essential for phage replication, such as those involved in the regulation of lysogeny had not been mapped. Three ø105 mutants with altered lysogenization properties had been described. Two of these, ø105*cts*23 and ø105*ind*, were thought to be mutated in genes involved in the repression of phage replication. The *cts*23 mutant forms a thermally inducible prophage (Armentrout and Rutberg, 1971) whereas the prophage formed by the *ind* mutant exhibits a spontaneous induction rate which is 10^{-3} that of the wild-type phage and is also non-inducible by mitomycin C (see previous chapter). Another mutant, ø105*c*4, is defective for lysogen formation, but retains the ability to lysogenize some infected cells. The lysogens formed were non-inducible by mitomycin C but were induced when grown to competence and exposed

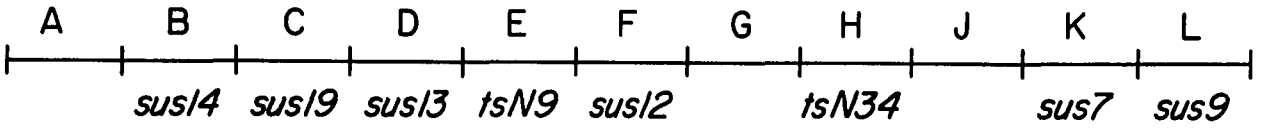

Figure 2

Genetic map of ø105. The map and the location of the sus and ts mutants are from Armentrout and Rutberg (1970).

to B. subtilis 168 DNA (Garro, 1973a; Garro, 1973b).

In an attempt to study the ø105 genes involved in the regulation of lysogeny, a series of clear plaque mutants were isolated and their genetics analysed. A tightly linked cluster of 5-6 such genes has been identified. The immunity region, defined by this cluster, has been tentatively located towards the middle of the phage genome between genes D and E. As described below the mapping studies have been complicated by what appears to be an unusually high frequency of recombinant production in crosses of immunity region mutants with outside markers.

1. Results

A. Complementation Groups

A total of 33 clear plaque mutants, including the previously noted ø105cts23 and ø105c4, were selected randomly for study. With respect to the new mutants, 24 were isolated following MNNG mutagenesis and 7 were of spontaneous origin from the ind-1 lysogen. The MNNG-induced mutants exhibited a variety of plaque phenotypes ranging from hazy to very clear plaques.

Six complementation groups were identified. Their numbering, cI -cVI, corresponds to the map order which was determined for the immunity region genes from the genetic crosses described below. The distribution of mutants among these groups and the average lysogenization

frequencies of representative mutants are listed in Table 6. A majority of the mutants appeared to belong to a single complementation group, number V. This group also includes: the temperature-conditional clear plaque mutant ø105cts23; ø105c4; all the MNNG-induced mutants which form large clear plaques and, with one exception, all of the spontaneously derived mutants. However, as will be discussed below, some of the mutants in this group may belong to group VI.

In general mutants classified in the same group fail to complement each other whereas all mutants within a group complement any mutant of another group for the production of lysogens. There is one exception to this, namely the group VI mutant csi-6 which complements csi-1 of group V but not the other members of this group. The complementation between csi-6 and csi-1 is also unusual in that it is unidirectional. Complementation between these mutants was observed only when csi-6 infected cells were superinfected with csi-1, but not when csi-1 infected cells were superinfected with csi-6.

B. Lysogenization and Development of Immunity to Superinfection

The lysogenization frequencies observed correlated in a general way with the plaque phenotypes of the mutants. Mutants in groups V and VI produce large clear plaques

TABLE 6

COMPLEMENTATION GROUPS AND LYSOGENIZATION FREQUENCIES OF CLEAR-PLAQUE MUTANTS OF ø105

Group	Number of Mutants	Representative Mutants	Lysogenization Frequencies[a]
cI	1	cng-2	1×10^{-4}
cII	5	cng-24	1×10^{-3}
cIII	2	cng-14	1×10^{-3}
cIV	3	cng-10	1×10^{-4}
cV	21	csi-17	5×10^{-6}
		csi-1	4×10^{-6}
cVI	1	csi-6	2×10^{-5}

[a]The cells were infected at a multiplicity of 5, as described under Methods. The values given are the ratio of immune to infected cells assayed 35 min post infection. Measured in this way, the lysogenization frequency of the wild-type phage is 6×10^{-3}.

and lysogenize less frequently than mutants in the other groups whose plaques, though less turbid than the wild-type, are still hazy. These lysogenization frequencies were calculated, as described under Methods, as the ratio of immune to infected cells at a time approximately 35 min post infection. In these experiments, however, it was evident that the number of immune CFU were only a small fraction of the total CFU which would survive infection if unchallenged by a superinfecting phage. Also, the lysogenization frequency of 6×10^{-3} observed for the wild-type phage was significantly lower than the frequency of $10\text{-}30 \times 10^{-2}$ which had previously been reported (Birdsell et al., 1969). It seemed likely that these differences were related to the time when the infected cells were tested for immunity since the higher values were obtained by allowing the infected cells to form colonies on nutrient agar before challenge.

In order to examine the relationship between survival after infection and immunity in more detail a time-course study was conducted. The results of a typical experiment are shown in Fig. 3. At early times after infection about 10-30% of the CFU exposed to the wild-type phage are capable of forming colonies when plated on nutrient agar; however, less than 10% of these surviving CFU are immune to superinfection. The number of surviving CFU and immune CFU are observed to increase with time but at different

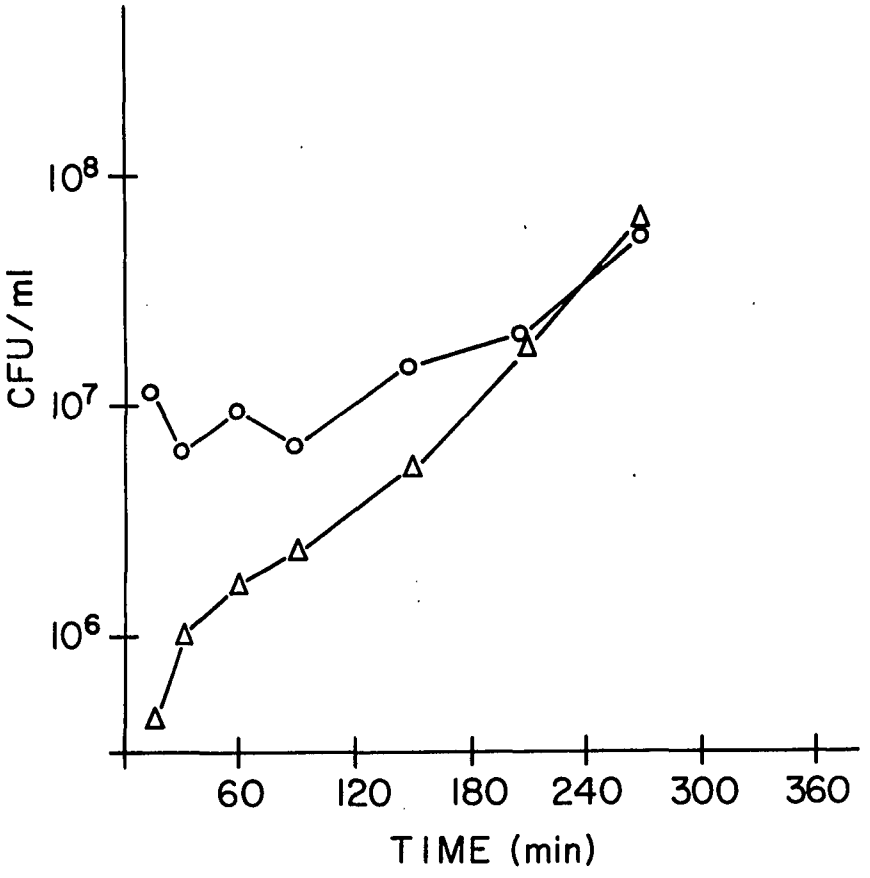

Figure 3

Development of immunity to superinfection. An exponentially growing culture, 1.3 x 10^8 CFU/ml, in VY broth was infected with wild-type ø105 at a multiplicity of 5. After allowing 15 min at 37C for adsorption, free phage were neutralized by the addition of anti-ø105 antiserum. Samples were removed at the times indicated and assayed for total CFU (o) on nutrient agar plates and immune CFU (Δ) on plates seeded with the clear plaque mutant ø105csi-17.

rates. Complete immunity among the surviving CFU is not achieved until approximately 4 h post infection. This long lag between infection and the development of immunity is not an artifact related to the use of the csi-17 mutant as the superinfecting phage since similar results were obtained when the infected cells were challenged by superinfection with the wild-type phage.

The fact that infected cells which ultimately give rise to stable lysogens continue to divide before immunity is established suggested that some of the daughter cells produced might not inherit the prophage. This was shown to be the case in the following experiment. Colonies produced by plating infected cells in the presence of anti-ø105 antiserum were picked, dispersed in broth and then tested for total CFU and immune CFU. The results presented in Table 7 indicate that the colonies which contain lysogenized cells are actually heterogeneous in this regard.

C. Map of the Immunity Region Genes

Initial studies of the distribution of the immunity region genes indicated that they were tightly clustered. The recombination frequencies observed in two-factor crosses between the group V mutant, csi-17, and the representative mutants of groups I, II, IV and VI were 1.6, 0.2, 0.1 and 0.6 per cent respectively. This low level

TABLE 7

THE OCCURRENCE OF IMMUNE AND NON-IMMUNE CELLS IN COLONIES DERIVED FROM CELLS WHICH HAD SURVIVED INFECTION WITH WILD-TYPE ø105[a]

Colony No.	Total CFU[b]	Immune CFU[c]	Per Cent Immune
1.	5.8×10^6	1.6×10^6	27.6
2.	3.4×10^7	1.0×10^5	0.9
3.	7.9×10^5	1.2×10^5	15.2
4.	1.0×10^7	4.0×10^6	40.0
5.	1.8×10^7	1.1×10^7	61.1
6.	2.3×10^7	2.3×10^7	100.0
7.	1.4×10^7	1.3×10^7	92.8
8.	1.2×10^7	N.D.[d]	----
9.	1.5×10^7	N.D.	----
10.	6.9×10^7	N.D.	----

[a]Cells were infected at a multiplicity of 5-10 and 35 min later plated on nutrient agar in the presence of anti-ø105 antisera.

[b]CFU from dispersed colonies plated on nutrient agar.

[c]CFU from dispersed colonies plated on nutrient agar spread with 10^8 PFU ø105csi-17.

[d]N.D. = None detected at the highest concentration of cells assayed.

of recombination, approximately 2% across the entire immunity region, can be contrasted with the recombination frequencies of the mapped conditional-lethal mutations which range from 1.0% between genes B and C to 6-13% between genes C and D (Armentrout and Rutberg, 1970). The vegetative map of ø105 shown in Fig. 2 also indicates the loci of the conditional lethal mutations which were used in other crosses described below.

In order to determine the recombination frequencies in the cI - cVI interval it was desirable to reduce the background of parental phage which came through the crosses. This was done by constructing a set of sus-clear double mutants. The mutation carried by each representative clear-plaque mutant was coupled by recombination with sus14 and sus9. We had established by two-factor crosses that the immunity region was situated between these markers which map in the end-proximal genes B and L respectively. In these two-factor crosses the average recombination frequency between sus14 and sus9 was 24.8% whereas the recombination frequencies of the clear-plaque mutations with these markers was in the range of 7-17.5% (Table 8). The constructed sus-clear double mutants were crossed in su^+ cells and the relative frequency of wild-type phage produced was assayed. The rationale used to interpret these crosses is illustrated in Fig. 4. The results of the crosses which are presented in Table 9, indicate the

TABLE 8

TWO-FACTOR RECOMBINATION BETWEEN *sus* AND CLEAR PLAQUE MUTANTS OF ø105[a]

	*sus*14	*sus*9
cI *cng*-2	12.02	n.d.[b]
cII *cng*-24	17.43	7.37
cIII *cng*-14	11.65	13.15
cIV *cng*-10	9.92	14.88
cV *c*4	12.95	11.85
csi-1	n.d.	16.37
csi-17	9.00	n.d.
cVI *csi*-6	n.d.	n.d.
*sus*9	24.80	---

[a]The crosses were performed as described in Methods. The values presented are percent recombination.

[b]not done.

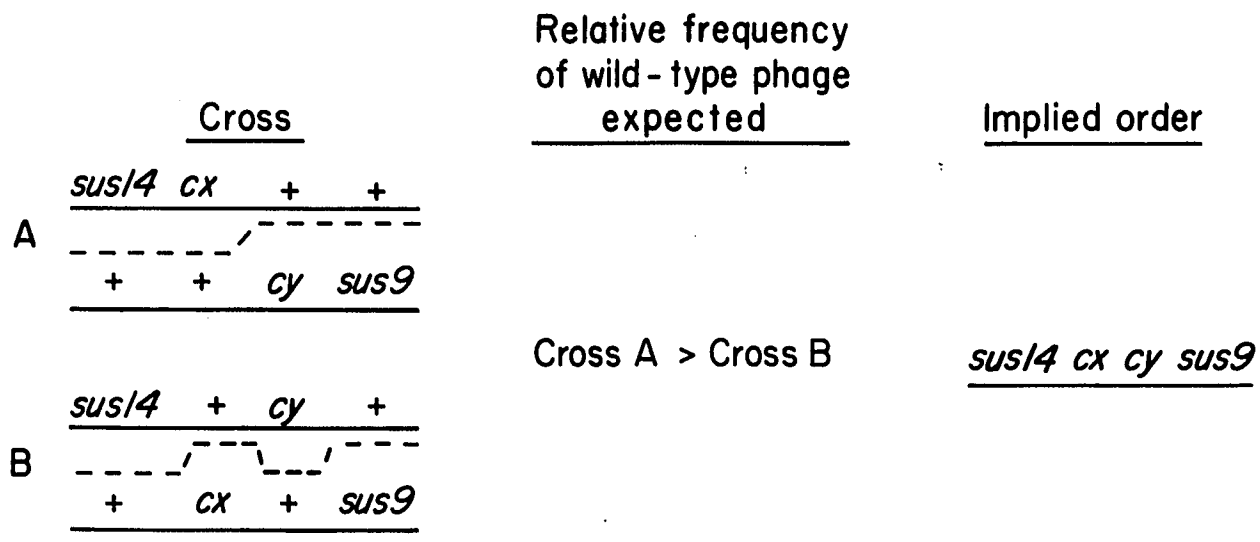

Figure 4

Rationale for the interpretation of the four-factor crosses between the sus-clear double mutants. The relative order of the clear plaque marker in each set of crosses was determined, as illustrated, from the ratios of turbid PFU on su^- cells to the total PFU on su^+ cells.

TABLE 9

THE PER CENT OF WILD-TYPE PHAGE PRODUCED IN FOUR-FACTOR CROSSES OF *sus*-CLEAR DOUBLE MUTANTS[a]

sus9-cy	sus14-cx: cI cng-2	cII cng-24	cIII cng-14	cIV cng10	cV csi-1	cV csi-17	cVI csi-6
cI cng-2	---	0.053	0.045	0.040	0.075	0.081	0.080
cII cng-24	0.369	---	<0.001	0.001	0.026	0.014	0.080
cIII cng-14	0.145	0.007	---	<0.001	0.017	0.010	0.011
cIV cng-10	0.240	0.006	0.003	---	0.006	0.005	0.010
cV csi-1	0.269	0.048	0.066	0.012	---	0.016	0.031
csi-17	0.234	0.076	0.029	0.030	0.019	---	0.006
cVI csi-6	0.272	0.055	0.014	0.077	0.168	0.019	---

[a]The crosses were performed as described in Methods. The values given are the per cent turbid plaques on *su*⁻ cells.

following map order: cng-2, cng-24, cng-14, cng-10, (csi-1, csi-17), csi-6.

D. Location of the Immunity Region Genes

Attempts to position the immunity region genes on the ø105 map by three-factor crosses were unsuccessful. These crosses involved clear-plaque mutations in cI and cVI and sus and ts mutations in genes D, E, F and H. The rationale behind these crosses, the results of which are presented in Table 10, was as follows. If the clear plaque mutation was situated between a pair of sus and ts mutations then a cross between susx cy + x + + tsz should produce approximately equal numbers of turbid and clear plaquing progeny when plated on an su^- host at 40C. The same results should be obtained when the clear-plaque mutation is coupled with the same ts mutation and crossed with the same sus, i.e., susx + + x + cy tsz. If on the other hand the clear plaque mutation lay outside the pair of sus and ts mutations grossly unequal numbers of clear and turbid plaques were expected because of the requirement for a multiple recombinational event to produce one or the other class of phage. Applying this rationale to the results of the three-factor crosses, however, would lead to the conclusion that the immunity region maps in three different loci, namely between genes F and H, genes E and F and genes D and E.

TABLE 10

THREE-FACTOR CROSSES INVOLVING THE cI AND cVI CLEAR-PLAQUE MUTANTS

Crosses[a]	Per cent clear plaques	
Set A, genes: F, H, cI, cVI	su^+ at 32C	su^- at 40C
1. sus12, cng-2 x tsN34	62.5	48.5 (38.7)[b]
2. tsN34, cng-2 x sus12	49.9	58.9 (59.0)
3. sus12, csi-6 x tsN34	55.6	41.2 (37.1)
4. tsN34, csi-6 x sus12	43.4	51.5 (59.4)
Set B, genes: E, F, cI, cVI		
1. tsN9, cng-2 x sus12	78.1	68.2 (43.7)
2. sus12, cng-2 x tsN9	61.3	66.7 (54.4)
3. tsN9, csi-6 x sus12	73.5	51.7 (35.2)
4. sus12, csi-6 x tsN9	50.0	47.7
Set C, genes: D, E, cI, cVI		
1. tsN9, cng-2 x sus13	85.5	64.0 (37.4)
2. tsN9, csi-6 x sus13	86.7	62.4 (36.0)

[a]Crosses were performed as described in Methods. Lysates were plated on both su^+ lawns at 32C and su^- lawns at 40C and scored for turbid and clear plaques.

[b]The numbers in parentheses are normalized with respect to the per cent clear plaques observed under the permissive conditions. The corrected values were calculated in each case as:

$$50\% \times \frac{\%\text{ clear plaques on } su^- \text{ at } 40C}{\%\text{ clear plaques on } su^+ \text{ at } 32C}$$

Since the ø105 genome is known, from physical studies, to consist of a unique nucleotide sequence (Armentrout et al., 1971; Chow et al., 1972) the immunity region genes must occupy a unique position on the map. A series of two-factor crosses involving the same clear and conditional-lethal mutations was therefore conducted in an attempt to identify this locus. The recombination frequencies observed between the clear and conditional-lethal mutations (Table 11), although higher than expected for mid-chromosomal markers were internally self-consistent. That is, the cI mutation always mapped closer than the cVI mutation to genes C and D whereas the converse was true with respect to genes E, F and H. One interpretation of these results which also takes into account the tight linkage between the clear-plaque mutations is that the entire immunity region is located between genes D and E.

E. Evidence for High-Frequency Double Recombinations

It would be possible to explain the anomalous recombination frequencies observed between the clear plaque mutations and outside markers as well as the unusual results of the three-factor crosses if the immunity region underwent a high frequency of double recombinational events with adjacent genes. Two crosses were performed to examine this possibility as follows. We defined interval I as the interval between gene D and the immunity region and II as

TABLE 11

PER CENT RECOMBINATION IN TWO-FACTOR CROSSES BETWEEN CONDITIONAL-LETHAL AND CLEAR-PLAQUE MUTANTS OF ø105

Gene		C	D	E	F	H
	Mutant	sus19	sus13	tsN9	sus12	tsN34
D	sus13	12.9	---			
E	tsN9	14.4	2.5	---		
F	sus12	14.1	2.9	0.5	---	
H	tsN34	13.2	14.7	11.8	5.9	---
cI	cng-2	8.3	12.6	19.4	11.9	20.8
cVI	csi-6	15.6	20.4	13.8	4.4	8.5

the interval between gene E and the immunity region and then assayed for progeny from crosses involving genes D, E, cI and cVI for phage which resulted from: (i) recombination in interval I but not II; (ii) recombination in interval II but not I and (iii) recombinations in intervals I and II. The results of these crosses are presented in Table 12. It can be seen that while the recombination frequency in interval I (RFI) is less than 1% as is RFII, the frequency of double recombinants, RFI and II, is significantly higher. In other words, the number of double recombinants, instead of being some fraction of the singles, exceeded them by a factor of 10 to 20. Furthermore if the double recombinants are not taken into account, as would be the case in a two-factor cross between sus13 and tsN9, the sum of RFI and RFII approximates the observed recombination frequency between sus13 and tsN9.

2. Discussion

The results of the present study indicate that there are at least five and most likely six genes involved in the establishment and maintenance of lysogeny by ø105. Identification of cVI as a separate complementation group from cV was based on the ability of the csi-6 mutant to complement the group V mutant csi-1. The possibility that this may represent intragenic complementation cannot be excluded at present. However, in addition to its ability

TABLE 12

ANALYSIS OF SINGLE AND DOUBLE RECOMBINANTS PRODUCED IN CROSSES OF + c tsN9 x sus13 + + PHAGE

	I II + cng-2 tsN9 x sus13 + +	I II + csi-6 tsN9 x sus13 + +
Total progeny	1.2×10^6	1.5×10^6
+ + +	1.5×10^3	2.8×10^3
RFI[a]	0.3%	0.4%
+ c +	2.7×10^3	4.6×10^3
RFII	0.5%	0.6%
+ + tsN9	2.9×10^4	4.6×10^4
sul3 c +	4.5×10^4	6.7×10^4
RFI and II	6.2%	7.5%

[a]RFI, RFII and RFI and II are recombination frequencies in the respective intervals as described in the text.

to complement csi-1, two lines of evidence indicate that the csi-6 mutant might indeed represent another complementation group. Firstly, in the four-factor crosses, csi-6 was mapped to the right of csi-1 and csi-17 (Table 9). Secondly, the lysogenization frequency of csi-6 is different from that of group V mutants (Table 6). The failure of csi-6 to complement most of the group V mutants may reflect the existence of double mutations in some of the group V mutants. A majority (15/21) of the group V mutants were MNNG-induced. Because of the potential of MNNG to produce multiple, closely linked mutations (Adelberg et al., 1965; Oeschger and Berlyn, 1974), it is possible that some of the MNNG-induced group V mutants are actually double mutants which carry mutations in both genes V and VI. An alternative explanation for the inability of csi-6 mutant to complement most of the group V mutants may be that the cV and cVI gene products interact to form a functional product. Inactivation of either one may disrupt the function of the joint molecule. Thus, some of the mutants classified in group V may actually belong to group VI. Along these lines, the cts-23 mutant, which forms a thermally inducible prophage, and the c4 mutant, which forms stable, immune lysogens, had been assumed, on the basis of their different phenotypes, to carry different mutations inactivating separate gene products (Garro, 1973a). Nevertheless, they were identified

by complementation analysis to be members of the same complementation group, group V.

The physiological function of the genes identified has not been determined. The range in lysogenization frequencies observed for the mutants clearly indicates that some, particularly cV and cVI, are more critical to lysogen formation than the others. The procedure used to measure lysogenization frequencies would probably not differentiate between repression-defective and integration-defective mutants. Thus the results presented in Table 6 should not be interpreted as indicating that all the mutants form stable lysogens which carry an integrated prophage. The presence of immune cells capable of liberating infectious phage could result from the formation of a "pseudolysogenic" carrier state (Hemphill and Whiteley, 1975) which has been reported for other Bacillus phages (Takahashi, 1964; Bott and Strauss, 1965). Unequivocal demonstration of an integrated prophage will require mapping studies showing, in each case, linkage between bacterial and phage genes. This has been done for the group V mutant, ø105c4, which appears to integrate at the normal prophage insertion site (Garro, 1973a).

The time course study revealed that even in the case of wild-type ø105 the formation of a stably integrated prophage may not occur until several hours post infection. During this time the lytic functions of the infecting

phage must be repressed since the infected cells survive and can divide to give rise to noninfected cells. The form of repression evident at early times after infection is unusual in that it appears to be cis-dominant. That is, it does not extend to superinfecting phage. This may simply reflect low levels of a repressor which at higher concentrations renders stable lysogens immune to superinfection. Alternatively, it may indicate that the early stages of lysogen formation are under the control of a separate repressor which does not act on superinfecting phage. Echols et al. (1976) have speculated that regulatory proteins with preferential cis-activity, such as the λQ-gene product, might be the result of either a limited binding specificity or metabolic instability of such proteins.

We are tentatively suggesting that the entire immunity region is located between genes D and E on the ø105 map. This suggestion is based on the tight linkage between the immunity region genes and the recombination frequencies observed in the two-factor crosses reported in Table 11. If we assume, as indicated by their tight linkage, that the immunity region genes are contiguous and not separated by any of the known essential ø105 genes, then the only locus which fits the two-factor cross data is one between D and E. However, because of the anomalous recombination frequencies observed in crosses of the clear-plaque

mutations with outside markers, unequivocal mapping of this region will probably require a physical rather than a genetic approach. The unusual behavior of ø105 clear-plaque markers in genetic crosses had been noted previously by Armentrout and Rutberg (1970). In fact the inclusion of clear-plaque markers in the first mapping study of ø105 (Rutberg, 1969) led to the erroneous conclusion that the phage had a circularly permuted genome.

The molecular basis for these anomalous recombination frequencies is not known. It should be noted that the clear-plaque phenotypes of the mutants employed in the crosses is not supressible. They also produce equivalent numbers of plaques on $\underline{su}^+$ and $\underline{su}^-$ hosts. Thus the hosts employed for the crosses or to assay the recombinants should not have affected the observed recombination frequencies. The possibility that the mutations responsible for the clear-plaque phenotypes also had a direct effect on phage recombination is also unlikely. The presence of the clear-plaque mutations in the phages used in three-factor crosses did not affect recombination frequencies between other markers in the same phage. It would appear from the results of the crosses presented in Table 12 that the immunity region genes participate in an unusually high frequency of double-recombinational events with adjacent markers. If this interpretation of these crosses is correct it suggests that the immunity region may be transferred from chromosome to chromosome as a block of genes in a manner analagous to the transposible genetic elements recently described in a number of plasmids and phages (Cohen and Kopecko, 1976).

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