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City University of New York PH.D. 1983

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GENETIC AND PHYSICAL ANALYSIS OF THE IMMUNITY REGION OF *Bacillus subtilis* PHAGE ø105

by

DORIS F. CULLY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

GENETIC AND PHYSICAL ANALYSIS OF THE IMMUNITY REGION OF

Bacillus subtilis PHAGE ø105

by

DORIS F. CULLY

Advisor: Anthony J. Garro, Ph.D.

This thesis has examined the genes and gene products involved in the regulation of lysogeny in the temperate *Bacillus subtilis* phage ø105. Little is known about lysogeny in gram-positive bacteria, although lysogeny of gram-negative bacteria by phages such as lambda and P22 has been well studied.

The ø105 repressor gene responsible for the maintenance of repression during lysogeny has been identified and isolated. This gene was first isolated by cloning several restriction fragments from the phage genomic DNA into *B.subtilis* cloning vectors. These hybrid plasmids were then tested for the expression of a protein which could render *B.subtilis* cells immune to superinfection by ø105. The proteins expressed by the hybrid plasmids were also examined in *B.subtilis* minicells and *E.coli* maxicells using SDS-polyacrylamide gel electrophoresis.

To examine the molecular organiz ation of the genes coding for the proteins being expressed, the phage DNA fragment responsible for the immune phenotype was sequenced. The molecular weights of the proteins predicted from the DNA sequences roughly corresponded with those expressed in the *B.subtilis* and *E.coli* cells. This sequence data made it possible to subclone portions of the immunity region to identify the smallest fragment which could confer ø105 immunity in *B.subtilis*.

The smallest cloned DNA fragment which renders cells immune was a 740 bp HindIII-PvuII fragment. The DNA sequence of this region showed three open reading frames with predicted protein molecular weights of 16.5K, 7.3K, and 5.5K. The coding sequence for the 16.5K protein was found on one strand, and the coding region for the 7.3K and 5.5K proteins was found on the complementary strand. These coding regions overlap each other and therefore could not be separated using subcloning strategies to determine which protein was the ø105 repressor.

The immunity regions of two ø105 clear-plaque deletion mutants which are defective in the maintenance of repression were examined by DNA restriction and sequence analysis. These analyses indicated that a complete defect in lysogeny is produced by any deletion of DNA coding for the 16.5K, 7.3K, and 5.5K proteins. It is therefore likely that at least one of these proteins is the protein required for maintenance of repression during lysogeny.

Since the 16.5K coding sequence is found on the complement of the 7.3K and 5.5K coding sequences it was possible to determine which DNA strand codes for the ø105 repressor by examining the RNA produced in an established ø105 lysogen. The ø105 lysogenic RNA, which by analogy to other temperate phages codes for the repressor for maintenance of lysogeny, was mapped by hybridizing single-stranded DNA isolated from the 740 bp HindIII-PvuII fragment to RNA isolated from a ø105 lysogen. The DNA in the RNA-DNA hybrid was extended with reverse transcriptase using the RNA as a template and the resulting product examined by gel electrophoresis. These results showed that the RNA made during ø105 lysogeny is transcribed from the DNA strand coding for the 16.5K protein. The size of the extended DNA predicts that the 5'-end of the RNA was initiated in the region immediately preceding the coding sequence for the 16.5K protein. This region contains RNA polymerase recognition sequences which are found in most bacterial promoters as well as an eight base sequence complementary to the 3'-end of 16S rRNA of B.subtilis. Thus, this region appears to be functioning as the promoter for the maintenance of ø105 lysogeny.

In conclusion, I have identified the ø105 repressor and promoter required for the maintenance of lysogeny in Bacillus subtilis. Other ø105 proteins were shown to be nonessential for lytic phage growth and are likely to be involved in the regulation of phage lysogenic replication.

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INTRODUCTION

The bacteriophages are a diverse group of viruses which have been used throughout the history of molecular biology as models for studying the regulation of prokaryote gene expression. The study of lysogeny in temperate phages in particular has contributed to our understanding of the complex balance of regulatory mechanisms involved in phage development. The most extensively studied temperate phages have been isolated from the gram-negative bacteria Escherichia coli and Salmonella typhimurium. What has been learned about these phages has contributed to our knowledge of transcription and translation in gram-negative bacteria and prokaryotes in general. Gene expression in the gram-positive bacteria and their temperate phages has not been as well characterized and it appears, in fact, that the gram-positive and gram-negative cells may have subtle differences in the mechanisms by which they regulate transcription and translation. While E.coli is able to express genes from both gram-negative and gram-positive species, heterologous gene expression in B.subtilis is limited almost exclusively to genes from other gram-positive bacteria (Cohen et al., 1973; Ehrlich, 1978; Kreft et al., 1978). This limited range of gene expression in B.subtilis may be due to host range limitations in transcription, translation, or post-translational processing of genetic information.

This thesis is concerned with the temperate phage ø105 which lysogenizes the gram-positive bacterium B.subtilis. This study of the ø105 genes involved in lysogeny will hopefully contribute to our understanding of the mechanisms involved in gene expression in gram-positive cells. To aid in understanding the experimental rationale of this thesis, a brief outline of the phenomenon of lysogeny as it is understood in the prototype temperate coliphage lambda is presented in the following section.

I. CLASSIFICATION OF BACTERIOPHAGE

The assortment of viruses which infect bacteria vary widely in size, shape, and the type of nucleic acid (DNA or RNA) which they contain. Based on these criteria bacteriophages have been classified into 18 groups which include three types of tailed phages, cubic phages with single-stranded DNA or RNA and filamentous phages (Bradley, 1967). Because of this complex classification, bacteriophages have been more generally grouped on the bases of their interaction with host bacteria as virulent, temperate, or persistent. Infection by virulent phage is characterized by the ultimate lysis of the infected host. Persistent phage do not lyse the infected bacterium but continuously replicate and release phage. Cells infected with temperate phage can show two alternative responses. Some infected cells are killed by the temperate phage in a manner analogous to virulent phage infection, while other cells survive the infection, and give rise to lysogenic clones. The temperate phage DNA persists in these lysogens in a repressed state, either in the form of a plasmid, or as a prophage covalently integrated into the host chromosome. Unlike cells infected with a persistent phage, lysogenized cells do not continuously produce phage but are capable of dividing many times without shedding phage particles. This lysogenic state although fairly stable is reversible, and spontaneous prophage induction, which results in lytic replication occurs at a rate of about 10^{-4} per bacterial generation.

II. TEMPERATE BACTERIOPHAGE

The repressed lysogenic state was described experimentally in the 1950s by Lwoff and Gutman who showed, in a *B.megaterium* phage system, that lysogenized cells still possessed the capacity to produce phage (Lwoff and Gutman, 1950). Occasionally the lysogenic state

would break down spontaneously, resulting in cell lysis and phage production. Jacob and Wollman (Jacob and Wollman, 1953), working with an E.coli lysogen, showed that lysogenized cells were immune to infection by the phage used to lysogenize the cells. It was shown that the infecting phage could adsorb and inject its nucleic acid into the lysogenized cells (Lwoff, 1953), but the injected DNA did not replicate (Jacob and Wollman, 1953). The immune state could be abolished, however, by treating the cells with various DNA damaging agents, which ultimately resulted in cell lysis and the release of viable phage particles (Lwoff et al., 1950). Thus, lysogenic cells carry phage DNA for many generations as a dormant prophage that renders the cell immune to infection by related homoimmune phage. Temperate phage which were able to infect each other's lysogens were considered heteroimmune.

To explain the phenomenon of immunity in lysogenic bacteria, Jacob and Monod (Jacob and Monod, 1961) proposed that prophages produce a trans-acting repressor molecule which inhibits the expression of phage genes necessary for replication and cell lysis. The proposed repressor molecule would be responsible both for the maintenance of lysogeny and for the immunity towards superinfecting phages (Kaiser and Jacob, 1957), and homoimmune phages would possess functionally interchangeable repressor molecules. The inactivation of the repressor through environmental stimuli, which damage DNA, would result in the expression of the repressed genes required for lytic development. The ability to induce lytic replication, in essentially 100% of the lysogenized cells in a culture, through the use of DNA damaging drugs, irradiation, or the direct inactivation of thermolabile repressors has contributed to the use of temperate phages as model systems for studying gene regulation.

A) THE BACTERIOPHAGE LAMBDA

The prototype temperate phage used in the early studies of lysogeny was the E.coli phage lambda. Lambda is a member of a major family of temperate coliphages that are referred to as lambdoid phages. These large, icosahedral, tailed phage have as their genetic material double-stranded DNA which possess complementary cohesive termini. The lambdoid phages can undergo genetic recombination with each other and their prophages are inducible by ultraviolet irradiation. The regulation of lysogeny of these coliphages appear to be similar to other phages of the enterobacter family in that they require the presence of a repressor for the maintenance of lysogeny and the sequential expression of early genes for the establishment of lysogeny.

One of the early approaches used to study lysogeny in lambda was to analyze lambda transcription during the shift from lytic to lysogenic regulation. During lytic growth lambda produces mRNAs which code for factors necessary for DNA replication and recombination and phage structural proteins (Sly et al., 1965). The lambda lysogen, however, synthesizes only a single mRNA which codes for a protein involved in the maintenance of lysogeny and superinfection immunity (Szybalski, 1969; Taylor et al., 1967). This latter transcript corresponds to approximately 4-5% of the 48,500 base-pair (bp) lambda genome, and is transcribed leftward from the l strand of the DNA (Szybalski, 1969; Taylor et al., 1967). Derepression of prophage replication is associated with the synthesis of other mRNAs, in particular with two transcripts which code for the positive control factors N and Q (Taylor et al., 1967; Kumar et al., 1969). These factors permit the expression of the delayed early and late genes necessary for lytic replication.

The identification of the genes involved in lambda lysogeny was greatly aided by the isolation and characterization of lambda mutants defective for this mode of replication. Lysogenization-defective lambda mutants produce plaques which are clearer than the turbid pla-

ques formed by the wild-type phage. These lambda mutants can be placed into three complementation groups: CI, which produces clear plaques and, CII and CIII, which produce hazy plaques less turbid than those of wild type phage (Kaiser, 1957). The study of these mutants helped to identify three separate lambda genes that cooperate in the establishment of immunity. The product of the integrase or int gene, which specifically controls lambda prophage integration, is also required for the establishment of lysogeny. This gene was not identified in the early analysis of lambda plaque mutants since the int mutants still produce turbid plaques. Of the three genes, CI, CII, and CIII, only the CI gene product is required for the maintenance of lysogeny.

The mechanisms by which the lambda proteins act to enable the formation of a lambda prophage has become a classic model for gene regulation in prokaryotes. The CII and CIII proteins which are produced early in a lambda infection promote lysogenization by enhancing the transcription of the CI and int genes. Purified CII protein has been shown to activate transcription in vitro from the early promoters (PE and PI) for the CI and int genes, respectively (Shimatake and Rosenberg, 1981). The CII protein is very labile and is stabilized by the CIII protein (Hoyt et al., 1982). In addition, the host E.coli himA and himD gene products are believed to enhance the production of the CII protein and thus play a part in the fate of infected cells (Hoyt et al., 1982).

The CI gene product, which is the lambda repressor protein, was isolated by Ptashne (Ptashne, 1967) who demonstrated that it binds in vitro to wild type DNA (Ptashne and Hopkins, 1968). Steinberg and Ptashne (Steinberg and Ptashne, 1971) have shown that this binding blocks in vitro transcription of the early lambda transcripts required for lytic replication. Repressor molecules bind to the left and right operators (OL and OR), thus preventing the interaction of RNA polymerase with the two promoters PL and PR (Ptashne and Hopkins, 1968). These operator and promoter sites are located in the lambda genome on

either side of the region coding for the lambda repressor. Located next to the PR promoter is the promoter PM which is responsible for the transcription of CI and thus for the maintenance of repression. Repressor binding to the OR operator also acts to both stimulate and inhibit transcription from the PM promoter. The control of transcription from the PL, PR and PM sites are dependent on the concentration of repressor in the lysogen (see below).

Mutations in the repressor binding sites OR and OL can render lambda insensitive to the action of the CI protein, producing virulent strains which can grow on and destroy both lysogenic and nonlysogenic cells (Ptashne and Hopkins, 1968). These virulent lambda mutants have been useful in defining the nature of the interaction between repressor molecules and DNA. Three repressor-binding sites (1,2, and 3), each 17 bp long, are found in the OR and OL sites. These 17 bp long sequences are separated from each other by spacers five to seven bp long. The DNA sequences of the two operator regions, OR and OL, are nearly identical (Maniatis et al., 1975; Ptashne et al., 1976). while each of the three 17 bp binding sites within OR and OL exhibit slightly different DNA sequences and show two-fold rotational symmetry. DNA-protein binding studies with lambda virulent mutants have shown that the three 17 bp binding sites have different affinities for repressor (Johnson et al., 1979). The OR1 site has the highest affinity for the CI protein and is the site of preferential binding at low CI concentrations. Binding at this site inhibits transcription from the PR promoter, and thus of the lambda early genes whose products are required for lytic growth. Conversely, binding of CI protein at OR1 stimulates transcription from PM, the promoter from which the CI gene is transcribed during the maintenance of repression. At high CI concentrations all three OR sites are occupied, includng OR3, the lowest affinity site. Binding at OR3 turns off transcription from PM, thus synthesis of the CI repressor may be considered to be autoregulated (Johnson et al., 1979). Low CI concentrations stimulate CI synthesis while high CI concentrations inhibit CI synthesis.

Transcription of the CI gene from PM, the promoter involved in maintenance of repression, is inhibited by the action of the cro gene which codes for the so-called lambda "antirepressor" (Johnson et al., 1978). This protein inhibits CI synthesis by binding to the same three OR sites to which the repressor binds. The cro protein is required for lambda lytic development by directly inhibiting the continued synthesis of the repressor. The cro protein also binds to the OL operator and thus regulates the synthesis of the delayed early (N) and late genes (Q) required for lytic development. The cro gene is located immediately to the right of the PM PR OR site on the lambda genome and is transcribed from the PR promoter. Thus, the synthesis of the "antirepressor" cro is also under the control of the repressor. The region which contains the genes CI and cro and the operator OL and OR and promoters PL, PM, and PR, is responsible for controlling the immune state of a lambda lysogen and is thus called the lambda "immunity region".

During the establishment of lysogeny, the circularized lambda genome integrates into the host chromosome via a site-specific recombination event (Campbell, 1962). These circular intermediates are formed through the joining of the single-stranded 5'-end complementary termini on the lambda DNA molecule. The region of the phage genome which attaches and recombines with the host chromosome is called the attachment (att) site and is located near the middle of the phage DNA, proximal to the integrase (int) gene. Thus, the genetic map of the lambda prophage is circularly permuted with respect to the genetic map of the vegetatively replicating phage. Lambda integration usually occurs at one or two preferred sites on the host chromosome. This mechanism by which lambda inserts its DNA into the host chromosome is very different than that of another E.coli temperate phage, $Mu^{-}1$, which integrates at a large and apparently random number of sites in the E.coli chromosome through an attachment (att) site located near the end of the phage linear DNA molecule (Couturier, (1976).

B) THE BACTERIOPHAGE ø105

The bacteriophage ø105 infects and lysogenizes the transformable strains of the gram-positive bacterium B.subtilis 168. ø105 was originally isolated in 1965 by Reilly while studying bacteriophage present in soil bacteria (B.E. Reilly, Ph.D. Dissertation, Western Reserve University, Cleveland, Ohio, 1965). The ø105 particle has an icosahedral head and a long flexible non-contractile tail which lacks a sheath (Boice et al., 1969). The genome of ø105 consists of double-stranded DNA with a M_r of approximately 26,2(+0.3) x 10^6 daltons, or 40,000 bp as determined by both electron microscopy and alkaline gradient sedimentation velocity studies (Birdsell et al., 1969). ø105 DNA, as extracted from the phage particle, sediments as a non-covalently closed circular molecule (Scher et al., 1977), and circular structures are also seen by electron microscopy (Birdsell et al., 1969). These circles are unstable and are converted to double-stranded linear molecules by exposure to formamide or dimethyl formamide (Chow et al., 1972). The two lines of evidence which suggest that circularization occurs via the presence of complementary single-stranded ends in a manner analogous to lambda, namely: 1. the circles can be covalently closed by DNA ligase (Scher et al., 1977) and 2. the circle to linear conversion is reversible (Birdsell et al., 1969. Compared to lambda the ø105 circles dissociate more readily, i.e. at lower formamide concentrations, but also reassociate more readily (Chow et al., 1972). From these observations it has been suggested that the ø105 single-stranded termini are either shorter than those of lambda which has 13 unpaired bases at its ends, or are lower in guanine and cytosine (G + C) content than the lambda ends which are 83.3% G + C (Chow et al., 1972). The G + C content of the entire ø105 DNA molecule is approximately 43.5% (Birdsell et al., 1969).

ø105 forms an integrative prophage which is inducible with ultraviolet irradiation and mitomycin C (Birdsell et al., 1969). Only a single integration site which is located between the bacterial mark-

ers phe-1 and ilv-A1 has been identified (Peterson and Rutberg, 1969). Genetic and physical studies show that the vegetative and prophage ø105 DNA maps are colinear and not circularly permuted (Armentrout and Rutberg, 1970; Chow and Davidson, 1973). The structure of the integrative form of ø105 DNA, whether it is a circular or linear molecule, has not as yet been determined. There are two possible explanations for the colinearity of the vegetative and prophage maps: first, the integrative intermediate may be a linear molecule with each end participating in the integration event; and second, the integrative intermediate may be a circular molecule with the att site situated at or close to the end joint. In this regard ø105 resembles coliphage Mu-1 whose att site also involves the ends of the phage genome. Mu-1, however, differs from ø105 in that Mu-1 prophage integrates at numerous sites on the E.coli genome in a manner analogous to insertion sequences (IS) and Tn elements (Bukhari et al., 1977).

On the basis of its size the ø105 genome should have a coding capacity for approximately 25 to 30 proteins. However, only 11 essential genes have been identified by studies with conditional lethal mutants and these map in a unique linear sequence (Armentrout and Rutberg, 1970). The order of these genes as they appear in the ø105 prophage are ilvC/LKJHGFEDCBA/phe-1, where ilvC and phe-1 are the B.subtilis markers found adjacent to the ø105 prophage. The products of these ø105 essential genes and their functions have not been characterized, with the exception of the gene K product which has been shown to be essential for phage DNA replication (Armentrout and Rutberg, 1970). Under nonpermissive conditions the conditional lethal mutants ø105Ksus7 and Kts31 fail to replicate their DNA and thus, were unable to produce infectious DNA in a transfection assay. The product of the J gene may also be involved in ø105 DNA replication since ø105Jsus11 mutants grown in nonpermissive cells produce DNA which is less infectious than wild type DNA (J.I. Flock, Ph.D. Dissertation, Karolinska institutet, Stockholm, Sweden, 1978).

Reeve has examined the ø105 proteins which are synthesized in ø105 infected B.subtilis minicells and has identified 23 phage-specific polypeptides (Reeve, 1977). The molecular weights of these polypeptides, as estimated from SDS-polyacrylamide gel electrophoresis, range from 7,000 to 138,000. Six of these 23 polypeptides were classified as early gene products on the basis of the ability of their genes to be transcribed by unmodified host RNA polymerase. In these experiments B.subtilis minicells were infected with ø105 in the presence of chloramphenicol which, while allowing transcription, blocked translation of phage RNAs into products which might modify the host polymerase. The chloramphenicol block was removed and the RNAs which had been synthesized were allowed to be translated in the presence of rifampicin. The early ø105 proteins have estimated molecular weights of 102,000; 29,500; 22,000; 20,300; 18,000 and 8,500. The infection of minicells derived from a ø105 lysogen results in the production of only 10 phage-specific polypeptides. The molecular weights of the more predominant of these polypeptides are 102,000; 41,000; 29,500; 22,000; 18,000, and 7,000. Some of these ø105 proteins produced in immune minicells appear to be similar in size to the early ø105 proteins. The ø105 phage proteins observed in these studies have not been further characterized.

The genetic map of ø105 has been correlated with the restriction endonuclease EcoRl cleavage map of ø105 DNA by marker rescue experiments using genetically mapped conditionally lethal phage mutations (see Fig.1) (Scher et al., 1978). Cleavage of ø105 DNA with the endonuclease EcoRl generates 10 fragments, one of which, namely A, consists of the terminal fragments C and D associated noncovalently, through their "sticky" ends (Scher et al., 1977). The size in base pairs of the EcoRl generated fragments, as determined by Scher et al., are as follows: A 16,600; B 10,800; C 8,800; D 7,900; E 5,300; F 3,000; G 900; H 600, and (I,J) 300 (Scher et al., 1977).

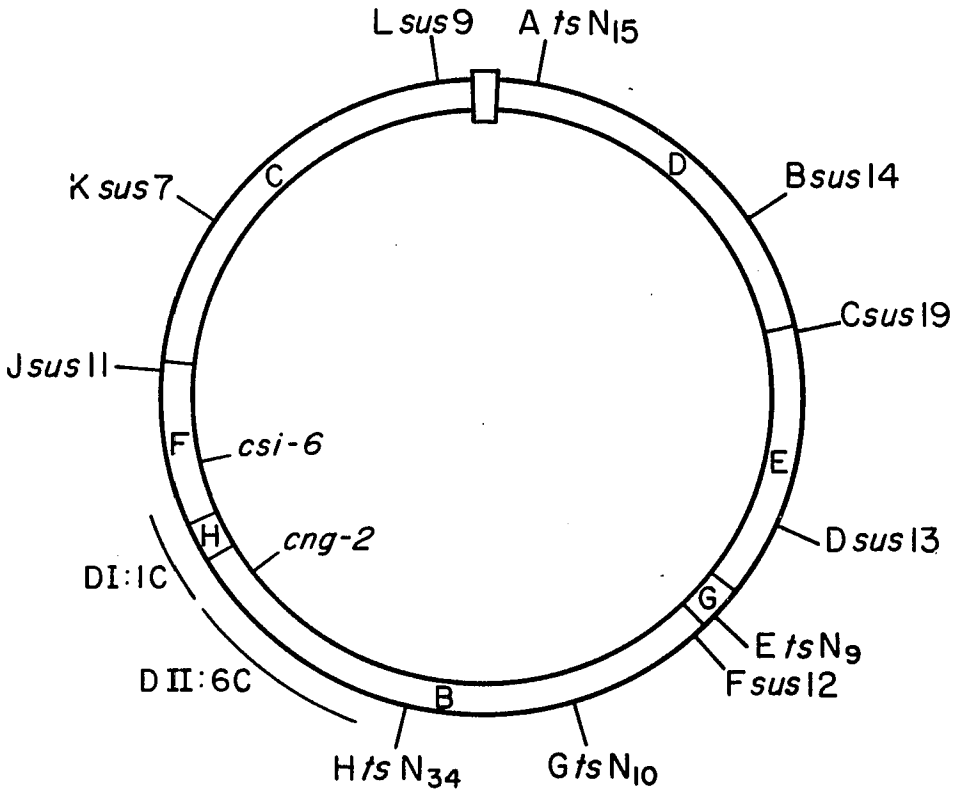
Lysogenization-defective ø105 mutants produce either clear or hazy plaques which are less turbid than those of wild type phage. It

FIGURE 1

Correlated genetic and EcoRl cleavage map of ø105 DNA.

The sizes of the EcoRl fragments are proportional to their molecular weights. The localization of the genetic markers to specific EcoRl fragments is based on the data of Scher et al. (Scher et al., 1978). The distances between markers are based on the recombination frequencies obtained by Armentrout and Rutberg (Armentrout and Rutberg, 1970). The positions of the deletions DI:1C and DII:6C are based on the data of Flock (Flock, 1977). Figure was taken from Scher et al. (Scher et al., 1978).

Figure 1


L sus 9
A ts N15
B sus 14
C sus 19
D sus 13
E ts N9
F sus 12
G ts N10
H ts N34
DII:6C
DI:1C
cng-2
csi-6
J sus 11
K sus 7
A
B
C
D
E
F
G
H

is generally believed that temperate phage with a clear-plaque phenotype are defective in the maintenance of phage repression while mutants with a hazy-plaque phenotype are thought to be defective in the establishment of lysogeny (Belfort and Wulff, 1974). Heteroduplex analysis of ø105 DI:1C with two other ø105 clear-plaque deletion mutants, DI:2C and DI:4C, showed that the DI:1C deletion overlaps the deletions present in these two mutants (Flock, 1977). The deletion present in ø105 DII:6C does not have any overlap with the DI:1C deletion and is indistinguishable, by heteroduplex analysis, from the deletions found in two hazy plaquing mutants ø105 DI:1t and DI:29t (Flock, 1977). Flock, who isolated these deletion mutants, has proposed that the ø105 DII:6C mutant actually carries an additional point mutation which is responsible for its clear-plaque phenotype (Flock, 1977). Preliminary data supporting the presence of a point mutation in ø105 DII:6C which maps in the vicinity of the clear-plaque mutations has recently been obtained in the laborartory of A.Garro (personal communication). Thus, the deletions present in ø105 DII:6C, DI:1t, and DI:29t, together with those of DI:1C, DI:2C, and DI:4C define a region of the ø105 genome which, while not essential for replication, is involved in lysogen formation.

Other clear and hazy plaquing ø105 mutants have been isolated and preliminarily grouped into three to four cistrons by complementation tests (M.F.Law, Ph.D. Dissertation, CUNY, NY, NY, 1977). One of these complementation groups, namely CI, contains all but one of the clear-plaque mutants which have been isolated. An example of a ø105 CI mutant is the ø105csi-6 which carries a mutation that has been localized on the ø105 EcoRl fragment F (Fig.1) (Scher et al., 1978). ø105csi-6 was isolated by a procedure devised for isolating lambda CI repressor defective mutants (Blattner et al., 1974). Cells lysogenized by a non-inducible prophage, ø105ind-1, were grown in broth and the culture supernatants, which contain very few spontaneously liberated phage (Garro and Law, 1974) were screened for the occurrence of spontaneous clear-plaque mutants. Such mutants, in the case of lambda, generally escape the block imposed by the Ind^- mutation by

loosing the capacity to produce a gene product necessary for maintenance of repression. The only clear-plaque mutant isolated to date which does not fall into the CI complementation group is ø105cng-2 which carries a mutation that has been localized on the ø105 EcoRl fragment B (Fig.1) (Scher et al., 1978). The low frequency with which the cng-2-like-mutation occurs suggests that the area mutated is a promoter or operator region involved in the regulation of ø105 lysogeny. The two remaining complementation groups, namely CII and CIII, produce hazy plaques which are less turbid than wild type. The CII and CIII complementation mutants map between the csi-6 and the cng-2 markers (see Fig.1) and may correlate with the region deleted in the ø105 hazy-plaque deletion mutants DI:1t and DI:29t (M.F.Law, Ph.D. Dissertation, CUNY, NY,NY, 1977). The hazy-plaque mutants which represent the CII and CIII complementation groups are probably defective in the establishment of a ø105 lysogen. The mutants in these complementation groups and the clear and hazy-plaque deletion mutants all map in the non-essential region of the ø105 genome which will be referred to as the immunity region. Although only a relatively small number of ø105 clear and hazy-plaque mutants have been mapped to date, none have been found to map outside this immunity region (M.F. Law, Ph.D. Dissertation, CUNY, NY,NY, 1977).

III. PROKARYOTIC TRANSCRIPTION AND TRANSLATION INITIATION SITES

Transcription and translation of prokaryotic genes involve a complex array of cellular components. Some of these components, namely the DNA dependent RNA polymerases and 16S ribosomal RNAs, in addition to their polymerization functions also play direct roles in the control of RNA and protein synthesis through their ability to recognize and associate with their respective templates. The mechanisms by which these components recognize and interact with DNA or RNA has been studied in both E.coli and B.subtilis.

To initiate transcription, RNA polymerase recognizes and binds to specific sites on DNA which have been termed promoter sites. Analysis of promoter sites in both E.coli and B.subtilis has revealed 2 regions of conserved sequence which generally are located 35 and 10 bases upstream of the mRNA start point. The -10 region, or Pribnow sequence, is generally homologous to the six base sequence TATAAT (Pribnow, 1975). Approximately twenty-five bases upstream from the Pribnow sequence is the -35 region which usually conforms with the six-base sequence TTGACA where the first three bases TTG are invariant (Rosenberg and Court, 1979). The function of these regions as promoters has been confirmed by in vitro DNA binding studies with purified DNA dependent RNA polymerases from E.coli and B.subtilis. Mutations in the conserved -35 and -10 sequences have been shown to alter the ability of RNA polymerase to recognize and interact with these promoter sites (Rosenberg and Court, 1979).

Although the -35 and -10 regions of E.coli and B.subtilis are similar in sequence (Moran et al., 1982), transcriptional differences do exist. For instance, RNA polymerase isolated from B.subtilis does not efficiently transcribe E.coli DNA in a purified in vitro system (Murray and Rabinowitz, 1982). However, E.coli can efficiently express in vivo drug resistance genes derived from gram-positive bacteria (Cohen et al., 1973; Ehrlich, 1978). Thus, there is a transcriptional barrier which impedes transcription of E.coli DNA by B.subtilis RNA polymerase which can not be accounted for by differences found in the -35 and -10 RNA polymerase recognition sites. It has been proposed that features in addition to the -35 and -10 sites, such as the base composition of the region upstream of the -35 site and the spacing between the consensus -35 and -10 sites may be needed for specific promoter recognition by B.subtilis RNA polymerase (LeGrice and Sonenshein, 1982).

The initiation of RNA synthesis in E.coli generally occurs some 6 to 9 bases beyond the last T residue of the -10 region (Rosenberg and Court, 1979), while in B.subtilis it is generally some 7 to 8

ases beyond this T residue (Moran et al., 1982). The starting nucleotide, in both organisms, is generally a template-specified purine, adenine(A) more often than guanine(G) (Rosenberg and Court, 1979; Moran et al., 1982). In E.coli promoter regions, some homology has been found in the bases immediately preceding and following the mRNA start points. In approximately 50% of the E.coli promoter sequences which have been examined (Rosenberg and Court, 1979), a three base pair sequence, CAT, is found 4 to 7 bases downstream of the last T residue in the TATAAT Pribnow sequence, while a T or A residue is found immediately following the initiating purine of the transcribed RNA. At present very few of the starting nucleotides of B.subtilis mRNAs have been determined and thus the degree of homology which may exist in these regions is unknown.

Efficient translation of prokaryotic mRNAs is dependent on many factors, one of which is the initial interaction of the ribosome with the RNA template. The E.coli ribosome 30S particle, which is made up of the 30S protein S12 and 16S rRNA, directly participates in the selection of mRNAs for translaton (Gold et al., 1981). Shine and Dalgarno (Shine and Dalgarno, 1974) observed that immediately preceding the translation initiation codon in all E.coli mRNAs, there are sequences complementary to sequences within the 3'-terminus of the 16S rRNA:OH-AUUCCUCCACUAG(5').

Although the degree of homology found in E.coli mRNAs is variable, it appears that a minimum of three to five bases are conserved with the sequence GGAGG (Gold et.al, 1981). B.subtilis mRNAs also possess sequences complementary to the 3' terminus of 16S rRNA of B.subtilis: OH-AUCUUUCCUCCACUAG(5') (Moran et.al, 1982). The mRNAs from B.subtilis generally exhibit a stronger sequence complementary to the 3' end of their 16S rRNA than do the E.coli mRNAs (McLaughlin et.al., 1981a). This conservation of sequences complementary to the 16S rRNA sequence may be required for efficient translation of mRNAs by B.subtilis ribosomes as suggested by the fact that B.subtilis ribosomes are unable to translate mRNA from E.coli in vitro (McLaughlin et al., 1981b).

It has been suggested by McLaughlin et al. (McLaughlin et al., 1981a) that initiation of translation by ribosomes from B.subtilis requires a stronger homology to the Shine-Dalgarno sequence, including more G-C base pairing, than that needed by E.coli. This suggestion was based on the differences in the calculated free energies of base pairing between rRNA and the Shine-Dalgarno sequences found in B.subtilis and E.coli mRNAs which are -11.6 to -21.0 Kcal/mol and -4 to -22 Kcal/mol, respectively (McLaughlin et al., 1981a).

The ribosome binding site, as identified by the Shine-Dalgarno complementary sequence, is located 5 to 9 nucleotides before the first base of the initiation codon. The spacing between the Shine-Dalgarno sequence and the initiation codon appears to be similar in both E.coli and B.subtilis (Gold et al., 1981; Moran et al., 1982).

The nucleotide triplet which is most frequently used as an initiation codon for RNA translation in both B.subtilis and E.coli is AUG. The frequency of initiation codons other than AUG is higher in B.subtilis (3/12) than in E.coli (4/123) (McLaughlin et al., 1981a). The additional initiation codons which are utilized by B.subtilis and E.coli ribosomes are GUG, which is found in both species, and UUG, which has been found only in gram-positive species (McLaughlin et al., 1981a). It has been suggested that the reduced base-pair stability of GUG and UUG with tRNA-fmet, relative to AUG with tRNA-fmet, is offset in B.subtilis by the more extensive Shine-Dalgarno complementarity with the 16S rRNA (McLaughlin et al., 1981a).

IV. GOALS OF RESEARCH

The goal of this thesis is to further our understanding of the mechanism of repression in the temperate B.subtilis phage ø105. In order to examine the repression of lytic growth in ø105 the following projects were proposed:

1) determine the region of the ø105 genome which contained the gene(s) responsible for repression during the maintenance of ø105 lysogeny;

2) identify the ø105 polypeptides encoded by this region and surrounding areas;

3) determine the DNA sequence of the immunity region of the wild type phage as well as clear plaque deletion mutants; and

4) determine the region transcribed during the maintenance of lysogeny.

These projects have been completed and their results have contributed to our knowledge about the genes and gene products of ø105, in particular those responsible for the regulation of lysogeny. This new information hopefully will contribute to our basic understanding of gene organization and regulation in gram-positive bacteria in general.

MATERIALS AND METHODS

I. MEDIA AND BUFFERS

Minimal medium (MM) contains Spizizen salts (Anagnostopoulos and Spizizen, 1961) supplemented with 0.5% (w/v) glucose and 25 µg/ml aspartic and glutamic acids. KS medium contains MM supplemented with 0.1% (w/v) Difco yeast extract. CII medium is Spizizen minimal salts supplemented with 0.5% (w/v) glucose, 0.05% (w/v) yeast extract, 0.01% (w/v) casamino acids, 0.5 mM spermine tetrahydrochloride, 1 mM CaC12, 2.5 mM $MgCl_2$, and 50 µg/ml required amino acids. GM-1 medium contains Spizizen minimal salts supplemented with 0.5% (w/v) glucose; 0.1 mM CaC12; 10^{-5} M $ZnCl_2$, $FeCl_2$, and $MnCl_2$; 100 µg/ml thymidine and methionine. GM-2 medium consists of GM-1 media without glucose and methionine. M-9 medium contains M-9 buffer (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 20 mM NH_4Cl) supplemented with 0.4% (w/v) glucose, 0.1 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.4% (w/v) casamino acids, and 2 µg/ml thiamine. M-9 medium without sulfate consists of M-9 buffer supplemented with 0.4% (w/v) glucose, 0.1 mM $CaCl_2$, 2 µg/ml thiamine, 50 µg/ml threonine and proline, 100 µg/ml arginine, and 1.0 mM leucine. VY nutrient broth consists of 2.5% (w/v) Difco Veal Infusion plus 0.5% (w/v) yeast extract. VY-NaCl broth is VY nutrient broth with 0.01 M NaCl. LB nutrient broth consists of 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl.

TBAB plates (Difco Tryptose Blood Agar Base) and LB broth solidified with 1% (w/v) Bacto agar were used for routine maintenance of *B.subtilis* and *E.coli* respectively. MB plates and MB top agar contain 1% (w/v) Bacto tryptone, 0.5% (w/v) NaCl, 10 mM $MgCl_2$, 0.2% (w/v) maltose, and 1% (w/v) or 0.6% (w/v) of Bacto agar respectively.

NTE buffer contains 20 mM Tris-HCl, 1 mM EDTA, and 10 mM NaCl pH 7.2. TMK buffer contains 10 mM Tris-HCl, 5 mM $MgCl_2$, and 300 mM KCl pH 7.2. TES buffer contains 30 mM Tris-HCl pH 7.5, 50 mM NaCl, and 5 mM EDTA. TE buffer contains 10 mM Tris-HCl pH 7.5 and 1 mM EDTA.

II. BACTERIAL STRAINS

The bacterial strains used in this study are listed in Table 1.

III. PHAGE

The phage strains used in this study are listed in Table 2.

A) PREPARATION OF PHAGE STOCKS

Wild type ø105 and ø105Jsus11 were prepared by induction of the respective B.subtilis lysogens. The lysogenic bacteria were grown in VY broth to early exponential phase (approximately 4 x 10^6 cfu/ml) and mitomycin C (MC, Sigma Chemical Co.) was added to a final concentration of 0.4 µg/ml. The culture was shaken vigorously at 37°C for 120 min by which time cell lysis had occurred. The lysates were incubated for 30 min at 37°C with 1 µg/ml of both pancreatic DNase and pancreatic RNase (Worthington) before removal of cell debris by low speed centrifugation (7,000 x g, 10 min). The cleared lysates were filtered through a 0.45 µm membrane filter (Millipore Corp.) and stored at 4°C.

Stocks of ø105 clear-plaque mutants were prepared by infection of B.subtilis 44AO with a seed stock of the phage. Cells were grown to exponential phase (approximately 5 x 10^7 cfu/ml) in 25 ml of VY-

TABLE 1 List of Bacterial Strains and Their Characteristics

Strain	Relevant Phenotype or Genotype	Source
A. B.subtilis		
BD99	Ø105^{S}	D.Dubnau[a]
BD99(Ø105)	Ø105^{r}, lysogenic for 0105	A.Garro[b]
BD99(pAG101)	Ø105^{r}, Km^{r}	D.Cully[bc]
BD99(pCM194)	Ø105^{s}, Cm^{r}	D.Cully[ad]
BD99(pUB110)	Ø105^{s}, Km^{r}	D.Cully[ac]
BD99(pDC$_4$)	Ø105^{r}, Cm^{r}	D.Cully[bd]
GB43	su^{3+}	C.P.Georgopoulis[e]
GB43(Ø105Jsus11)	su^{3+}, lysogenic for Ø105Jsus11	A.Garro
CU474	divIVB1, thyA, thyB, metB, Ø105^{S}	D.Dubnau[af]
CU474(pUB110)	" " " " " , Km^{r}	D.Dubnau[acf]
CU474(pAG101)	" " " " , Ø105^{r}, Km^{r}	D.Cully[bcf]
44AO	Str^{r}, Spo^{-}, Su^{-}	K.Bott[g]
B. E.coli		
C600		V.Racaniello
C600(pBR322)	amp^{r}, tet^{r}	P.Palese[h]
CSR603	uvrA, recA	D.Calhoun[i]
CSR603(pBR322)	uvrA, recA, amp^{r}, tet^{r}	D.Calhoun[hi]
CSR603(pDC1)	uvrA, recA, amp^{r}	D.Cully[hi]
CSR603(pDC2)	uvrA, recA, amp^{r}	D.Cully[hi]

a. Ø105^{s} cells are sensitive to Ø105 and are killed or lysogenized following Ø105 infection.

b. Ø105^{r} cells are resistant to Ø105 and are not killed following Ø105 infection.

c. Km^{r} cells are resistant to 5 ug/ml of kanomycin sulfate or 5 ug/ml of neomycin sulfate.

d. Cm^{r} cells are resistant to 5 ug/ml of chloramphenicol.

e. B.subtilis cells which carry the su^{3+} mutation support the replication of Ø105 sus mutants such as Ø105Jsus11.

f. B.subtilis cells carrying the div IV B1 mutation produce small cytoplasmic bodies called minicells which are devoid of chromosomal DNA.

g. Str^{r} cells are resistant to 1 ug/ml of streptomycin sulfate.

h. Amp^{r} and Tet^{r} cells are resistant to 50 ug/ml of ampicillin and to 25 ug/ml of tetracycline respectively.

i. The DNA of cells carrying the uvrA and recA mutations is degraded following exposure of the cells to doses of ultraviolet irradiation.

TABLE 2 List of Bacteriophage and Their Phenotypes

Bacteriophage	Phenotype	Source
Ø105	lysogenizes B.subtilis 168	L.Rutberg
Ø105 DI:1C	clear plaques[a]	I.Flock
Ø105 DI:2C	clear plaques[a]	I.Flock
Ø105 DI:4C	clear plaques[a]	I.Flock
Ø105 DII:6C	clear plaques[a]	I.Flock
Ø105 DI:29t	hazy plaques[a]	I.Flock
Ø105 DI:1t	hazy plaques[a]	I.Flock
Ø105csi-6	clear plaques[b]	L.Rutberg
Ø105Jsus11	turbid plaques[b]	A.Garro
SPO2c	clear plaques[a]	A.Garro

a. The B.subtilis 168 strain 44AO is used as a bacterial lawn for these plaque assays.

b. The B.subtilis 168 strain GB43 is used as a bacterial lawn for these plaque assays.

NaCl broth, pelleted by centrifugation, and resuspended in 0.1 ml of phage lysate in VY-NaCl at a multiplicity of infection (MOI) of approximately 0.05. The cell-phage suspension was incubated at 37°C for 15 min to allow for adsorption and then diluted 100-fold wth VY-NaCl and shaken at 37°C for 120 min. The lysates were cleared of cell debris, filtered, and stored as described for the wild type phage.

B) PREPARATION OF RADIOACTIVELY-LABELED PHAGE

Radioactively-labeled phage were prepared by the MC induction procedure with the following modifications. Thirty minutes after the addition of MC the cells were harvested by centrifugation and resuspended in an equal volume of prewarmed KS medium supplemented with 0.4 µg/ml MC. The cultures were shaken at 37°C for 120 min with 1.5 µCi/ml of [^{14}C]-amino acid mix (>50 mCi/ml-gm-atom of C., Amersham Co.) or 5 µCi/ml L-[^{35}S]-methionine (600-1400 Ci/mmol, Amersham Co.). The lysates were cleared of cell debris and the phage concentrated and purified as described in the next section.

C) CONCENTRATION AND PURIFICATION OF PHAGE

Phage particles were concentrated by polyethylene glycol precipitation and purified in CsCl step gradients using the procedure of Yamamoto et al. (Yamamoto et al., 1970). After centrifugation to equilibrium the bands of ø105 and PBSX which could be visualized in the centrifuge tubes by high intensity light were collected and had the expected densities of 1.484 g/cm^3 and 1.375 g/cm^3 respectively.

Phage preparations which were to be used for protein studies were further purified by rebanding in a CsCl gradient of 1.45 g/cm^3 average density. The CsCl purified phage preparations were dialyzed against NTE or TMK buffer.

D) PHAGE ASSAYS

Phage preparations were titered by plaque assay. Aliquots of serially diluted phage preparations were mixed with 2.5 ml of MB top agar which had been seeded with ø105-sensitive B.subtilis cells and overlayed onto MB agar plates. The B.subtilis strain routinely used for the plaque assay was 44AO. The overlayed plates were incubated at 37°C and the plaques scored the following day.

B.subtilis cells carrying recombinant plasmids were tested for immunity to ø105 by cross-streaking against the clear-plaque mutant ø105 DII:6C on TBAB plates. Clones which appeared immune or resistant by this procedure were subsequently tested for their ability to plaque ø105 DII:6C and a clear-plaque mutant of the heteroimmune phage SP02c. B.subtilis isolates which produced plaques when infected with SPO2 but not with ø105 were referred to as being resistant to ø105 infection ($ø105^r$).

A quantitative killing assay was used to measure the relative sensitivity of BD99(pAG101) and BD99(pUB110) to ø105. In this assay the bacteria were grown to exponential phase in VY-NaCl broth and 1 ml samples containing 7-9 x 10^7 cfu of BD99(pUB110) or BD99(pAG101) were exposed to various levels of the clear-plaque mutant ø105csi-6 for 15 min at 37°C. The samples were diluted 10-fold with cold VY-NaCl broth and 0.1 ml samples were plated for surviving cfu on TBAB plates.

The ability of ø105 to adsorb to BD99(pAG101) and BD99 (pUB110) was assayed using the following protocol: Serially diluted cell suspensions were mixed with a constant amount of ø105 (7 x 10^4 pfu) in 0.2 ml of VY-NaCl broth, and incubated for 6 min at 37°C. Cells and phage were immediately diluted 5-fold with cold medium and filtered through 0.45 µm membrane filters (Millipore Corp.). The sterile filtrates were assayed for unadsorbed pfu by a phage plaque assay.

Marker rescue of ø105Jsus11 by pAG101 DNA was assayed as follows: Competent GB43 cells were infected with ø105Jsus11 at a mul-

tiplicity of approximately 5 and incubated at 37°C for 15 min. The infected cells were pelleted, washed with CII medium, and then incubated at 37°C for 30 min either with pAG101 or pUB110 DNAs at a final concentration of 2 µg/ml. The cells were pelleted, suspended in VY broth, and incubated with shaking at 37°C for 240 min. The cultures were assayed for $\phi 105J^+$ pfu on a lawn of 44AO (su^-).

IV. B.SUBTILIS MINICELLS

The examination of phage and plasmid-coded proteins was facilitated by the use of the B.subtilis minicell system developed by Reeve (Reeve, 1977) and Shivakumar et al. (Shivakumar et al., 1979). The B.subtilis strain CU474 produces cytoplasmic organelles, lacking chromosomal DNA, which are referred to as minicells. Minicells were isolated from CU474, CU474(pAG101), and CU474 (pUB110) as described below. One liter cultures of cells were grown in GM-1 medium to early stationary phase (approximately 4×10^6 cfu/ml) at 30°C. The cells were harvested by centrifugation at 12,000 x g for 15 min and resuspended in 10 ml of GM-2 medium. Minicells then were separated from the normal cells by zonal sedimetation in sucrose density gradients. The cell suspension was layered onto two 33 ml, 5-30% (w/v) sucrose gradients and centrifuged at 0°C in a SW27 rotor at 5000 rpm for 20 min. The turbid minicell band was removed from the center of the tubes and then pelleted by centrifugation at 15,000 x g for 10 min. This pellet was resuspended in 5 ml of GM-2 medium and the sucrose banding and pelleting steps were repeated 2 more times. The purified minicell pellet was washed twice with cold GM-2 medium, resuspended in 1-1.5 ml of GM-2 medium containing 10% (v/v) glycerol, and frozen at -70°C. The final minicell concentration was estimated either with a hemocytometer using a phase contrast microscope (conversion factor: # minicells in 1 mm^2 x 4 x 10^6 = # minicells/ml) or by measuring the cell turbidity at 660 nm (A660 of 0.2 equals approximately 5×10^8 minicells/ml).

A) PHAGE-CODED PROTEINS

B.subtilis minicells isolated from the strains CU474 or CU474(pAG101) were infected with CsCl purified phage using the method of Reeve (Reeve, 1977). Phage was added to the minicells (MOI approximately 2) in the presence of 20 ug/ml cycloserine. Cycloserine was added to prevent the incorporation of amino acids into mucopeptides (Mertens and Reeve, 1977). Aliquots containing 2 x 10^9 minicells were placed at 30°C for 0, 10, 25, 45, or 75 min. A mixture of [^{14}C]-amino acids was then added to a final concentration of 20 uCiml and the samples were incubated for an additional 10 min at 30°C. The samples were pelleted (10,000 x g, 2 min) and washed with water to remove unincorporated label. The minicell pellets were resuspended in 10 µl of a 1 mg/ml lysozyme solution in H_2^0 and incubated at 37°C for 2 min. Samples to be examined on SDS-polyacrylamide gels were boiled for 5 min, immediately before applying to gels in a "cracking" buffer (50 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 2 mM EDTA, 1% (v/v) B-mercaptoethanol 10% (v/v) glycerol).

B) PLASMID-CODED PROTEINS

Minicells containing the plasmids pUB110 and pAG101 were isolated using the procedures described above. Proteins coded by these plasmids were radioactively labeled with [^{14}C]-amino acid mix or L-[^{35}S]-methionine using the methodology reported by Shivakumar et al. (Shivakumar et al., 1979) for labeling pUB110 proteins. In general CU474(pAG101)and CU474(pUB110) minicells were preincubated at 30°C for 30 min in the presence of 20 µg/ml cycloserine. Approximately 5 x 10^9 cells were incubated with either 20 µCi/ml of [^{14}C]-amino acid mix or a total of 25 µCi of L-[^{35}S]-methionine at 30°C for 30 min. Cells were washed to remove unincorporated radioactivity and lysed with lysozyme. Samples were boiled for 5 min in cracking buffer immediately before SDS-polyacrylamide gel electrophoresis.

V. E.COLI "MAXICELL" ANALYSIS OF PLASMID-CODED PROTEINS

Plasmid-coded proteins were studied using the "maxicell" technique developed by Sancar et al. (Sancar et al., 1979) and Roberts et al. (Roberts et al., 1979). This procedure utilizes the E.coli strain CSR603 which carries both the recA and uvrA mutations. Following exposure to low doses of UV irradiation this strain preferentially degrades chromosomal DNA, relative to plasmid DNA, thus allowing the preferential labeling of plasmid-coded proteins in UV irradiated cells. Strains of CSR603 carrying either pBR322, pDC1, or pDC2 were grown to early exponential phase in M9-medium at 37°C and irradiated at approx- imately 10 ergs/sec/mm^2. Following irradiation, the cultures were incubated 3 hours at 37°C at which time 200 µg/ml of cycloserine was added and the culture incubated for an additional 12 hours at 37°C with shaking. Aliquots of 1.5 ml were pelleted, washed in M9-medium, and preincubated in 0.75 ml of sulfate-free M9-medium for 1 hour at 37°C. Plasmid-coded proteins were labeled by incubating the cells with 16.7 µCi/ml of L-[^{35}S]-methionine for 1 hour at 37°C. The samples were washed in M9-buffer to remove unincorporated label, resuspended in cracking buffer, and boiled for 5 min before applying to SDS-polyacrylamide gels.

VI. BIOCHEMICAL TECHNIQUES

A) ELECTROPHORESIS

1) Protein gels

Proteins were examined on SDS-polyacrylamide gels using the discontinuous buffer system of Maizel (Maizel, 1971). The resolving gels consisted of either a 10-25% (w/v) linear gradient of polyacrylamide (acrylamide:bis ratio of 30:0.08) or a 15% (w/v) polyacrylamide (acrylamide:bis ratio of 200:1). Both resolving gels also contained 0.38M Tris-HCl pH 9.0, 0.1% (w/v) SDS, 0.005% (v/v) TEMED and 0.02% (w/v) $(NH_4)_2S_2O_8$. The stacking gel was 3% (w/v) polyacrylamide (acrylamide:bis ratio of 30:0.08), 60 mM Tris-base, 32 mM H_3PO_4, 0.1% (w/v) SDS, 0.05% (v/v) TEMED, and 0.1% (w/v) $(NH_4)_2S_2O_8$. The running buffer used was 50 mM Tris-base, 0.38 M glycine, and 0.1% (w/v) SDS. Protein samples were boiled in cracking buffer for 5 min immediately before applying to the gels. The samples were electrophoresed at 100 V for various amounts of time.

After electrophoresis the gels were removed from the glass plates and soaked in 45% (v/v) methanol and 12% (v/v) acetic acid to fix proteins and remove SDS. Following this fixation step the gels were treated in one of several ways. Gels containing isotopically labeled proteins were examined by autoradiography. Gels were fluorographed for autoradiography by treating with the commercial fluorographic material Enhance (NEN) according to the manufacturers specifications. After drying under vacuum, gels were exposed to X-ray film at -70°C. Gels containing nonradioactive proteins were stained with 0.2% (w/v) Coomassie brilliant blue in 45% (v/v) methanol and 12% (v/v) acetic acid and then destained in 45% (v/v) methanol and 12% (v/v) acetic acid. Non-radioactive proteins were also visualized by subsequently staining the destained gel with silver according to the protocol described by Merrie et al. (Merrie et al., 1979).

2) DNA gels

Plasmid and phage DNAs were examined on both neutral agarose (Studier, 1973) and polyacrylamide gels (Maxam and Gilbert, 1980). Neutral 0.7% (w/v) agarose gels were run in a Tris-acetate buffer system (TA) consisting of 40 mM Tris-base, 20 mM acetic acid, and 2 mM EDTA pH 8.1. Gels of 5% (w/v) polyacrylamide (acrylamide:bis: 30:1) were run in a Tris-borate buffer system (TBE) consisting of 89 mM Tris-base, 89 mM boric acid, and 2.5 mM EDTA pH 8.3. After electrophoresis, the gels were stained with 1 ug/ml ethidium bromide (EtBr) and the fluorescence of the DNA-EtBr complex visualized by ultraviolet (uv) illumination.

Radioactively-labeled DNA was electrophoresed on preparative 5% (w/v) and 8% (w/v) polyacrylamide gels in TBE buffer as described by Maxam and Gilbert (Maxam and Gilbert, 1980). If the DNA fragments were to be recovered from the gels (see DNA isolation), the gels were covered with Saran wrap and exposed to X-ray film at 4°C. Test polyacrylamide gels, which contained small amounts of ^{32}P-labeled DNA (200-300 counts per lane) were dried onto 3 MM Whatman paper and exposed to X-ray film at -70°C with an intensifying screen (Dupont Cronex).

3) Molecular weight estimates

Protein molecular weights were determined using the method of Weber and Osborn (Weber and Osborn, 1969). The marker proteins used on the SDS-polyacrylamide gels and their molecular weights were as follows: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 43(45),000; carbonic anhydrase, 31,000; α-chymotrypsin, 25,700; soybean trypsin inhibitor 21,500; β-lactoglobulin, 18,400; lysozyme, 14,300; cytochrome C, 12,300; bovine trypsin inhibitor, 6,200; and insulin A and B 3,000 (Bethesda Research Labs and BioRad).

Size estimates of DNA molecules were performed as described previously (Studier, 1973). The marker DNAs used on agarose, polacrylamide, and urea-polyacrylamide gels, and their size, in base pairs are as follows: HinfI digested pSV40 (gift of J.Zazra); 1845, 1666, 1056, 766, 543, 517, 506, 396, 344, 298, 239, 221, 220, 154, 109, 83, 75, 24; and EcoRI digested ø105 DNA, 16600, 10900, 8800, 7900, 5300, 3000, 1088, 894. (Scher et al., 1977).

B) RNA AND DNA ISOLATION

1) Phage DNA

DNA was extracted from CsC1-purified phage particles by vigorously mixing phage in TMK buffer with NTE-saturated phenol and CHCl3 (phage:phenol:$CHCl_3$; 1:0.5:0.5; v/v/v) until protein no longer remained at the interface (6-7 extractions). The aqueous phases were dialyzed 2-3 times against NTE buffer. Phage DNA to be used for DNA sequencing was further purified by isopycnic banding in a CsCl gradient of 1.658 g/cm^3 average density in the presence of 100 µg/ml EtBr. The DNA band was extracted with H_2O-saturated isopropanol, until all the EtBr was removed from the aqueous phase, and then dialyzed against NTE.

2) Plasmid DNA

Plasmid DNA was isolated from B.subtilis and E.coli using two different modifications of the cleared lysate technique developed by Guerry et al. (Guerry et al., 1973). B.subtilis cells containing plasmid DNA were grown in 200 ml of VY broth to late exponential phase (approximately 7-9 x 10^7 cfu/ml) and harvested by low speed centrifugation (5000 x g, 10 min). Cell pellets were resuspended in 20 ml of a solution containing 25% (w/v) sucrose, 0.1 M NaCl, and 0.05 M Tris-HCl pH 7.5. Lysozyme was added to a final concentration of 0.5 mg/ml

and the suspended cells incubated at 37°C for 15 min. The following solutions then were added to the cell suspension in the following order: 4.8 ml of 5 M NaCl, 1.2 ml of 0.5 M EDTA pH 8.5, and 26 ml of 2% SDS-0.7 M NaCl. The sample was gently inverted once and placed on ice at 4°C for approximately 18 hours. This lysate was centrifuged at 15,000 x g for 45 min and the supernatant collected and made 0.3 M with respect to Na acetate. The supernatant was mixed with two volumes of cold 95% ethanol and kept at -20°C for approximately 2 hours. The precipitate which formed was collected by centrifugation (5000 x g, 30 min) and dissolved in 5 ml of TES buffer. This solution was incubated with 50 µg/ml of pancreatic RNAse and 1 unit/ml of T1 RNAse for 30 min at 37°C and then treated with 500 µg/ml of predigested pronase (predigested 10 min at 65°C) for one hour at 37°C. The plasmid DNA was purified from the cleared lysate by isopycnic banding in a CsCl gradient of 1.62 g/cm^3 average density in the presence of 200 µg/ml EtBr. The bottom DNA band was collected and extracted with H_2O-saturated isopropanol until all the EtBr was removed from the aqueous phase and dialyzed against TE buffer.

E.coli plasmid DNAs were extracted and purified as described (Bothwell et al., 1981). Plasmid containing E.coli cells were grown in one liter of LB broth to stationary phase and harvested by centrifugation at 5000 x g for 10 min. Cell pellets were resuspended in 12.5 ml of a solution containing 25% sucrose and 50 mM Tris-HCl pH 7.5. Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was kept on ice for 5 min and then placed at 37°C for an additional 2 min. The cell suspension then was mixed with 4.6 ml of 0.2 M EDTA pH 7.5, held on ice for 5 min, and then mixed with 18 ml of Triton solution (0.1% (v/v) Triton X-100, 62.5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0), and kept on ice for an additional 15 min. The lysate was centrifuged at 15,000 x g for 45 min, the supernatant collected, and its volume adjusted to 34.5 ml with H_2O. Plasmid DNA was purified from the cleared lysate by isopycnic banding in a CsCl gradient of 1.62 g/cm^3 average density in the presence of 200 µg/ml EtBr. The bottom DNA band was collected and extracted with H_2O-saturated

isopropanol until all the EtBr was removed from the aqueous phase and dialyzed against TE buffer.

3) Isolation of DNA fragments from gels

DNA fragments were recovered from agarose gels using a modification of the glass powered technique described by Vogelstein and Gillespie (Vogelstein and Gillespie, 1979). Gel slices containing DNA fragments were dissolved in NaI-saturated water mixed with glass powder (1 mg glass per µg of DNA, and gently mixed for 12 hours at 4°C. The glass powder with bound DNA was collected by centrifugation (10,000 x g, 5 min) and washed first with the saturated NaI solution and then with a cold ethanol wash solution (50% [w/w] ethanol, 0.1 M NaCl, 10 mM Tris-Hcl pH 7.5, 1 mM EDTA). The DNA was eluted from the glass pellet by incubating in 2-3 volumes of TE buffer at 37°C for 30 min. The glass was removed by centrifugation at 10,000 x g for 5 min, the elution repeated, and the collected samples pooled.

DNA was recovered from polyacrylamide gels by electroelution. Polyacrylamide gel slices were placed in dialysis tubing containing Tris-borate buffer, and secured at both ends. The dialysis bags then were floated in Tris-borate buffer and 100 mA of current passed through the solution for 12 hours. The buffer was then changed and the elution repeated for 1 hour. The samples were pooled and the DNA was concentrated by ethanol precipitation.

4) DNA precipitation

DNA solutions which were to be ethanol precipitated first were made 0.3 M with respect to sodium acetate (NaOAc). They then were mixed with three volumes of 100% cold ethanol (-20°C), and frozen in crushed dry ice. The precipitated DNA was pelleted by centrifugation at 10,000 x g for 15 min and the pellets resuspended in 0.3 M NaOAc. The samples were reprecipitated with three volumes of ethanol, washed twice with 70% (v/v) ethanol, dried under vacuum, and finally resuspended in TE buffer.

5) Bacterial RNA

RNA was isolated from BD99 and BD99(ø105) according to the procedure of Gryczan et al. (Gryczan et al., 1980). Cells were grown in VY media to early stationary phase, ruptured in a french pressure cell at 16,000 lbs/in2, and extracted with buffer (10 mM NaOAc, 100 mM NaCl pH 5.0)-saturated phenol. The aqueous phase then was made 0.4 M with respect to NaCl, and the nucleic acids were precipitated by mixing the sample with 2 volumes of cold ethanol (-20°C). The samples were centrifuged at 10,000 x g for 30 min and the pelleted nucleic acids were rinsed in cold acetone. Air dried pellets were dissolved in a solution of 0.3 M NH4-acetate and 0.5% (w/v) SDS and reprecipitated with 2 volumes of ethanol. Pellets were rinsed in 70% (v/v) ethanol, dried under vacuum, and redissolved in TE buffer. The redissolved pellet was treated with 0.1 mg/ml of RNAse free DNAse (Miles #36-150-1) in the presence of 5 mM MgCl2 for 30 min at 37°C. The reaction was terminated by making the sample 0.5% (w/v) SDS and mixing with TE-saturated phenol. The aqueous phase was made 0.3 M with respect to Na acetate, mixed with two volumes of ethanol, and centrifuged at 10,000 x g for 15 min. The RNA pellet was dried under vacuum and stored at -70°C.

C) ENZYMATIC REACTIONS

1) DNA restriction endonucleases

DNA restriction endonuclases were purchased from New England Biolabs (NEBL). Restriction digests were carried out according to the reaction conditions specified (Davis et al., 1980) in the Cold Spring Harbor Manual on Advanced Bacterial Genetics. Three different buffer systems were used which consisted of low salt (10 mM Tris-HCl pH 7.4; 10 mM $MgSO_4$, 1 mM dithiothreiotol[DTT]); medium salt (50 mM Tris HCl

pH 7.4, 10 mM Mg SO_4, 50 mM NaCl, 1 mM DTT), and high salt (50 mM Tris-HCl pH 7.4, 10 mM MgS04, 100 mM NaCl). Reactions were conducted at the optimum reaction temperatures specified by the manufacturer for a minimum of 1 hour. Approximately 1 unit of enzyme was added per ug of DNA.

2) Calf alkaline phosphatase (CAP)

Calf alkaline phosphatase was purchased from Worthington as an ammonium sulfate precipitate. Prior to use the precipitate was resuspended in and dialyzed against a buffer consisting of 20 mM Tris pH 8.3 and 100 mM KCl. The enzyme was made 50% (v/v) glycerol before storing at -20°C. DNA was treated with CAP in the medium or high salt restriction buffers for 30 min at 45°C. Phosphatase was removed from the DNA sample by two extractions with an equal volume of TE-saturated phenol. The phenol was removed from the DNA by three extractions with anhydrous ether following which the DNA was concentrated by ethanol precipitation.

3) T4 DNA ligase

DNAs were treated with T4 DNA ligase (NEBL) according to the manufacturers suggested reaction conditions. In general DNA fragments were reacted with T4 DNA ligase (approximately 5.6 x 10-2 nanomolar units of ligase per µg of DNA) at 14°C for a minimum of 12 hours, in ligase buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, 1 mM adenosine triphosphate (ATP), and 5 ug/ml bovine serum albumin (BSA).

4) T4 polynucleotide kinase

The 5' ends of DNA fragments were radioactively labeled with ^{32}P using T4 polynucleotide kinase (PNK) according to the procedure developed by Maxam and Gilbert (Maxam and Gilbert, 1980). CAP-treated DNA was incubated with PNK (approximately 2 units of PNK per reaction)

for one hour at 37°C in the presence of gamma-[^{32}P]-ATP (150 pmoles, > 1000 Ci/mM). The PNK buffer used in the reaction consisted of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA.

5) AMV reverse transcriptase

Avian myeoblastosis virus reverse transcriptase (AMVRT) was obtained from J.Beard at Life Sciences Inc., St. Petersberg, Fla. RNA-DNA hybrids were reacted with reverse transcriptase in RT buffer for 60 min at 42°C. The RT buffer used for the AMVRT reaction was 50 mM Tris HCl pH 8.3, 50 mM KCl, 10 mM DTT, 10 mM $MgCl_2$, 40 µg/ml Actinomycin D, and 0.6 mM of each of the deoxynucleotide triphosphates: dGTP, dCTP, dATP, dTTP.

VII. RECOMBINANT DNA

A) CLONING VECTORS

The cloning vectors used in this study are listed in Table 3. Included in this table is some relevant information about the physical and genetic traits of these plasmids

B) CONSTRUCTON OF SPECIFIC CHIMERIC PLASMIDS

The cloning vectors listed in Table 3 were used to construct hybrid plasmids which contain restriction fragments of the ø105 genome. The construction of the ø105 hybrid plasmids used in this study is described in detail below.

TABLE 3 List of Plasmid Vectors and Their Characteristics

Plasmid	Host Source	Plasmid-Specified Trait	Size (bp)	Relevant Restriction Sites	Reference
pUB110	S.aureus[a]	Km^r	4390	EcoRl 1 HindIII 0	Gryczan et al., 1978
pCM194	S.aureus[a]	Cm^r	2730	HindIII 1 PvuII 1 EcoRl 0	Ehrlich, 1977
pPL1004	B.subtilis	Cm^r Ø105^r	5160	EcoRl 2 HindIII 2 PvuII 2	Marrero and Lovett, 1980
pBR322	E.coli	Amp^r Tet^r	4362	EcoRl 1 HindIII 1 PvuII 1	Sutcliff, 1979

a) Plasmid can be stably maintained in B.subtilis.

b) Construct containing Ø105 HindIII fragment F inserted into the HindIII site of pCM194.

1) pAG101

One µg of purified ∅105 DNA was mixed with 8 µg of purified pUB110 DNA and incubated for 5 hours at 37°C with 18 units of EcoRl in high salt restriction buffer. The reaction was terminated by heating at 65°C for 10 min, and the EcoRl-generated fragments were ligated with T4 DNA ligase. The ligase reaction was terminated by heating at 68°C for 3 min.

2) pDC1

One µg of purified pBR322 and 2 µg of purified pPL1004 were separately incubated for 1 hour at 37°C wth 4 units of HindIII in medium salt restriction buffer and then incubated an additional hour at 37°C with 4 units of EcoRl in high salt restriction buffer. The HindIII-EcoRl digested pBR322 and pPL1004 samples were electrophoresed on 0.7% agarose gels and a 4300 bp pBR322 fragment and a 1050 bp pPL1004 fragment were recovered from the gels using the glass powder technique. Approximately 0.44 µg of the HindIII-EcoRl pBR322 fragment was mixed with 0.25 µg of the HindIII-EcoRl pPL1004 fragment and ligated with T4 DNA ligase.

3) pDC2

The hybrid plasmid pDC2 was constructed as follows: 2.5 µg of purified pBR322 and 10 µg of purified pDC1 were separately incubated for 1 hour at 37°C with 10 units of HindIII and 5 units of PvuII in medium salt restriction buffer. HindIII-PvuII digested pBR322 was treated with 10 units of calf alkaline phosphatase for 30 min at 45°C. The CAP was removed by phenol extraction as previously described. HindIII-PvuII digested pDC1 was run on a 5% (w/v) polyacrylamide gel. A 740bp pDC1 fragment was recovered from the gel by electroelution. The 740bp fragment was mixed with 0.6 µg of HindIII-PvuII digested pBR322 and ligated with T4 DNA ligase.

4) pDC4

The hybrid plasmid pDC4 was constructed as follows: approximately 100 ng of HindIII digested pDC2 was incubated with 5 units of CAP for 30 min at 45°C. The CAP was removed by phenol extraction. Approximately 7 µg of purified pCM194 was incubated for 1 hour at 37°C with 10 units of HindIII. The digestion was terminated by heating at 65°C for 10 min and then the HindIII generated fragments were ligated with T4 DNA ligase.

C) TRANSFORMATION AND SCREENING

Ligated DNAs were used to transform the appropriate competent B.subtilis or E.coli cells for antibiotic resistance. Competent B.subtilis cells were prepared using the two step growth procedure described by Rudner and Remeza (Rudner and Remeza, 1973) and frozen at -70°C for storage. Thawed cells were diluted 5-fold in cold CII medium immediately before incubation with transforming DNAs. Aliquots of the cells (0.5 ml) were mixed with 1-100 ul of plasmid DNA (1-100 ng) for 30 min at 0°C and gently shaken at 37°C for 1 hour. Transformed B.subtilis cells were selected on TBAB plates containing 5 µg/ml kanomycin or chloramphenicol.

Competent E.coli cells were prepared using the $CaCl_2$ technique developed by Dagert and Ehrlich (Dagert and Ehrlich, 1979). Freshly prepared 0.1 ml aliquots of competent cells in 100 mM $CaCl_2$ were mixed with 1-100 µl of plasmid DNA (1-100 ng) for 45 min at 0°C, incubated at 37°C for 5 min, and then gently shaken with 1 ml of LB nutrient broth for 1 hour at 37°C. Transformed E.coli cells were selected on LB plates containing 50 µg/ml ampicillin.

E.coli and B.subtilis colonies which grew on the selective media were screened for the presence of recombinant plasmids using a rapid plasmid isolation technique developed by Klein et al. (Klein et

al., 1980). In this procedure small volumes of cells are successively extracted with phenol and ether, followed by two ethanol precipitations of the DNA. The plasmid DNA preparations were cut with appropriate restriction enzymes and analyzed by agarose gel electrophoresis.

VIII. DNA SEQUENCING

The DNA sequences of phage and plasmid DNAs were determined using the chemical DNA sequencing method developed by Maxam and Gilbert (Maxam and Gilbert, 1980).

A) DNA PREPARATION

The chemical DNA sequencing method requires that the DNA to be sequenced be labeled with a radioactive phosphate at a single terminus. To accomplish this purified plasmid or phage DNAs were treated with restriction enzymes which produced 5'-cohesive termini. The 5'-terminal phosphates were removed with CAP and replaced with radioactively labeled phosphates using PNK and gamma-[^{32}P]-ATP. The two 5'-labeled ends were separated from each other by one of two methods: strand separation or recutting with restriction enzymes. To strand separate the labeled molecules the DNA first was denatured by heating in SS buffer (30% (v/v) DMSO, 1 mM EDTA pH 7.5, 0.05% (w/v) xylene cyanole (XC), 0.05% (w/v) BPB and immediately freezing in powdered dry ice.

The denatured DNA strands were separated on an 8% (w/v) polyacrylamide gel and recovered by electroelution. When the labeled ends were separated by recutting the double-stranded DNA with a restriction enzyme, the subsequent fragments were separated on a 5% (w/v) polyacrylamide gel. The labeled fragments were recovered from the gels by electroelution.

B) DNA SEQUENCING REACTIONS

The chemical sequencing method of Maxam and Gilbert consists of a series of limited DNA modification reactions which are followed by a quantitative cleavage of the DNA at the modified bases. The chemical procedures which are used partially modify and cleave DNA at the following bases: guanine(G), guanine and adenine(G A), cytosine and thymine(C T), cytosine(C), and adenine more than thymine(A>T). The extent of modification at these bases depends on the temperature and duration of the reaction and the concentration of dimethylsulfate for G; formic acid for G+A; hydrazine for C+T and C; or sodium hydorxide for A>C. Subsequent strand cleavage at the modified base is accomplished by treating the modified DNA samples with 100 mM piperidine at 90°C for 30 min. Piperidine is removed from the DNA samples by ethanol precipitation or by lyophylization. Samples are resuspended in formamide dye buffer (80% (v/v) formamide, 90 mM Tris-borate pH 8.3, 4.5 mM EDTA, 0.06% xylene cyanol) and stored at -20°C. Prior to electrophoresis the samples are boiled for 2 min.

C) DNA SEQUENCING GELS

The five sets of DNA cleavage products were electrophoresed through denaturing 7M urea-polyacrylamide gels as described by Maxam and Gilbert (Maxam and Gilbert, 1980). The concentration of acrylamide and the distance the molecules are electrophoresed determined the number of DNA bases resolved on the gel. Approximately 350 bases proximal to the labeled end could be sequenced if the five DNA samples were run on three different gels. The acrylamide concentrations of these gels and the approximate bases resolved were: 1) 15% (w/v) polyacrylamide (acrylamide:bis, 38:1), bases 1-30; 2) 8% (w/v) polyacrylamide (acrylamide:bis, 20:1), bases 30-100; 3) 6% (w/v) polyacrylamide (acrylamide:bis, 20:1), bases 80-350 (sample electrophoresed on same gel with two consecutive applications). The gels also contained

7M urea, 90 mM Tris-borate pH 8.3, 2.5 mM EDTA, 0.1% (w/v) $(NH_4)_2S_2O_8$ and 0.04% (v/v) TEMED. After electrophoresis the sequencing gels were covered with Saran wrap and exposed to X-ray film at -70°C using an intensifying screen.

IX. PRIMER EXTENSION

An RNA-DNA hybrid was prepared and used as a template for cDNA synthesis by the RNA-dependent DNA polymerase, AMV reverse transcriptase. This method was used to map B.subtilis ø105 lysogenic RNA to a specific region of the ø105 genome.

A) PRIMER ISOLATION

A restriction fragment of ø105 DNA representing bases 299-561 of the immunity region, was isolated from clone pDC4 and 5'-end labeled with gamma-[^{32}P] ATP and PNK. This double-stranded HinfI-DdeI fragment was denatured and strand separated on an 8% (w/v) polyacrylamide gel. The separated strands, which were to be used as primers for the reverse transcriptase reactions, were recovered by electroelution and their DNA sequences determined. The separated strands are referred to below as primer A and B.

B) DNA-RNA HYBRIDIZATION

Purified RNA isolated from B.subtilis BD99 and BD99(ø105) were each hybridized to primer A (5'-end labeled at position 561) or primer B (5'-end labeled at position 299). Approximately 200,000 counts of each primer was mixed with 50 µg of RNA in 25 µl of DDW and heated at 100°C for 2 min. An equal volume of 2x hybridization buffer (20 mM

Tris-HCl pH 7.7, 600 mM NaCl, 2 mM EDTA and 0.2% SDS) was added and the reaction was placed at 68°C for 24 hours. A thin layer of mineral oil was layered on the sample to minimalize evaporation during hybridization. After incubation the sample was ethanol precipitated twice and washed once with 70% (v/v) ethanol to remove salt and SDS.

C) PRIMER EXTENSION WITH REVERSE TRANSCRIPTASE

The RNA-DNA hybrids were used as templates for cDNA synthesis with AMV reverse transcriptase (RNA-dependent DNA polymerase). Hybrid pellets were resuspended in 50 ml of RT buffer and incubated with reverse transcriptase for 1 hour at 42°C. The RT reaction was terminated and the RNA template destroyed by incubating in 4 mM EDTA and 0.1 N NaOH for 2 hours at 37°C. The samples were neutralized by the addition of 100 ul of 1M Tris-acetate pH 5.8 and phenol extracted with an equal volume of TE-saturated phenol. The DNA was ethanol precipitated with 10 µg of carrier tRNA, washed with 70% (v/v) ethanol, and dried under vacuum. DNA pellets were resuspended in formamide-dye buffer and stored at -20°C. Prior to gel electrophoresis the samples were boiled for 2 min.

D) ELECTROPHORESIS OF EXTENDED DNA

The products of primer extension by reverse transcriptase were examined by electrophoresis on a denaturing 7M urea-8% (w/v) polyacrylamide gel. After electrophoresis the gel was covered in Saran wrap and exposed to X-ray film at -70°C with an intensifying screen.

RESULTS

I. CONSTRUCTION AND ANALYSIS OF PLASMIDS CONTAINING THE ø105 IMMUNITY REGION

Restriction fragments of the ø105 genome were cloned into B.subtilis and E.coli vectors in order to identify the DNA fragment(s) which coded for the ø105 repressor.

A) CONSTRUCTION AND ISOLATION OF BD99(PAG101)

The ø105 EcoRl fragment F was cloned into the EcoRl restriction site of the vector plasmid pUB110 by standard "shotgun" cloning methods as illustrated in Fig.2. The resulting hybrid plasmid, pAG101, rendered B.subtilis BD99 cells resistant to infection by ø105, as tested by cross-streaking against the clear-plaque mutant ø105 DII:6C. Plasmid DNA isolated from the ø105 resistant cells was shown to contain the ø105 EcoRl fragment F (see below).

1) Agarose gel analysis of pAG101

Plasmid DNA isolated from the B.subtilis strains BD99 (pAG101) and BD99(pUB110) was analyzed by agarose gel electrophoresis (Fig.3). Lanes 1 and 2 of Fig.3 show the three structural species of the plasmids pAG101 and pUB110, which in their order of migration, fastest to slowest, are covalently closed circular, linear, and nicked circular, respectively. Digestion of pAG101 DNA with EcoRl (Fig.3, lane 3) converted all three species into two fragments, one of which migrated with linear pUB110 (top band) and the other of which migrated with the

FIGURE 2

Construction and isolation of pAG101

ø105 EcoRl fragments were inserted into the EcoRl site of the B.subtilis vector pUB110 as described in Materials and Methods. B.subtilis competent cells were transformed with ligated DNAs and a kanomycin (Km^r resistant colony was isolated which was resistant to ø105 killing as tested by cross-streaking against the clear-plaque ø105 deletion mutant DII:6C. The plasmid contained in these resistant cells was found to possess the ø105 EcoRl fragment F by gel analysis (Fig.3) and is called pAG101.

Figure 2

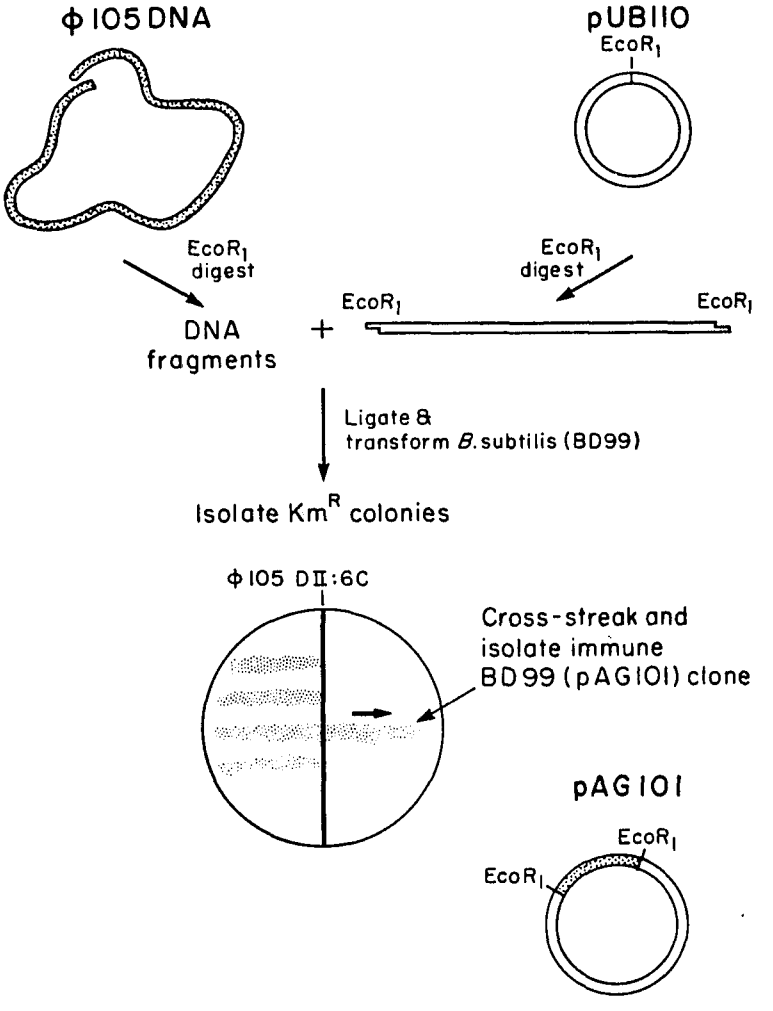
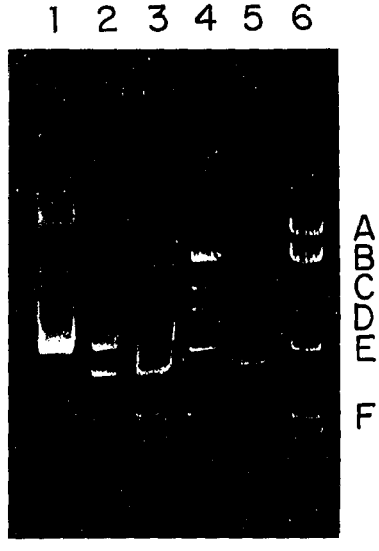

ϕ105 DNA
pUB110
EcoR1
EcoR1 digest
EcoR1 digest
EcoR1
EcoR1
DNA fragments
+
Ligate & transform B. subtilis (BD99)
Isolate Km^R colonies
ϕ105 DII:6C
Cross-streak and isolate immune BD99 (pAG101) clone
pAG101
EcoR1
EcoR1

FIGURE 3

Electrophoresis of ø105 and plasmid DNAs in 0.7% agarose

Lane 1, pAG101; lane 2, pUB110; lane 3, EcoRl-digested pAG101; lane 5, EcoRl-digested pUB110; lanes 4 and 6, EcoRl-digested ø105 DNA. The previously determined sizes of the ø105 DNA fragments in base pairs are as follows: A, 16,000; B, 10,800; C, 8,800; D, 7,900; E, 5,300 (Scher et al., 1977). Fragment A consists of the terminal fragments C and D associated, noncovalently, through their complementary single-stranded ends (Scher et al., 1977). In the digest shown in lane 4, fragment A has been totally dissociated. The previously determined size of the plasmid pUB110 is 2.9×10^6 base pairs (Gryczan et al., 1978).

Figure 3



EcoRl fragment F of ø105. The smaller pAG101 fragment also comigrated with covalently closed circular species of pAG101.

2) Marker rescue of ø105Jsus11 by pAG101 DNA

In order to confirm that pAG101 carried the ø105 EcoRl fragment F, the plasmid was tested for its abilty to rescue ø105 carrying the conditional lethal mutation Jsus11. This mutation was shown by Scher et al. (Scher et al., 1978) to map on EcoRl fragment F (Fig.1). Following marker rescue with pAG101 DNA the number of infectious centers observed on a lawn of su^- cells is approximately 10^4 higher than the background due to spontaneous reversion of this mutation (Table 4). This result genetically confirmed that pAG101 contained the EcoRl fragment F of ø105.

3) Resistance of BD99(pAG101) to ø105

From the initial screening protocol used to isolate BD99(pAG101), it was concluded that the presence of pAG101 enabled cells to survive infection with a clear-plaque mutant of ø105 which normally kills essentially all infected cells. This was examined in more detail by quantitatively assaying the ability of cells carrying either pUB110 or pAG101 to survive exposure to a ø105 clear-plaque mutant. The results shown in Fig.4 demonstrated that BD99(pAG101) was able to survive exposure to a level of ø105csi-6 which caused a 99% reduction in the viability of BD99(pUB110).

4) Adsorption of ø105 to BD99(pAG101) and BD99(pUB110)

The ability of BD99(pAG101) to survive ø105 infection was not due to an alteration in the capacity of the cells to adsorb ø105. This was shown by measuring phage adsorption to BD99(pAG101) and BD99(pUB110). The cells were indistinguishable with respect to their ability to adsorb the phage as shown in Fig.5.

TABLE 4

Marker Rescue of ø105J*sus*11 by pAG101 DNA

DNA	No. of INFECTIOUS CENTERS
pAG101	5.8×10^7
pUB110	3.4×10^3
no DNA	3.4×10^3

a) Competent Su^- bacteria were infected wth ø105J*sus*11 and then transformed either with pAG101 or pUB110 DNA as described in Materials and Methods. The cultures were assayed for $ø105J^+$ PFU on a lawn of Su^- cells.

FIGURE 4

Effect of exposure to ø105csi-6 on the survival of BD99(pUB110) or BD99(pAG101)

Exponential phase cultures of BD99(pUB110) or BD99(pAG101) were exposed to the indicated levels of phage as described in Materials and Methods. The samples were diluted with ice-cold medium and plated on TBAB plates for surviving colony-forming units. Symbols: ▲ , BD99(pUB110); ● , BD99(pAG101).

Figure 4

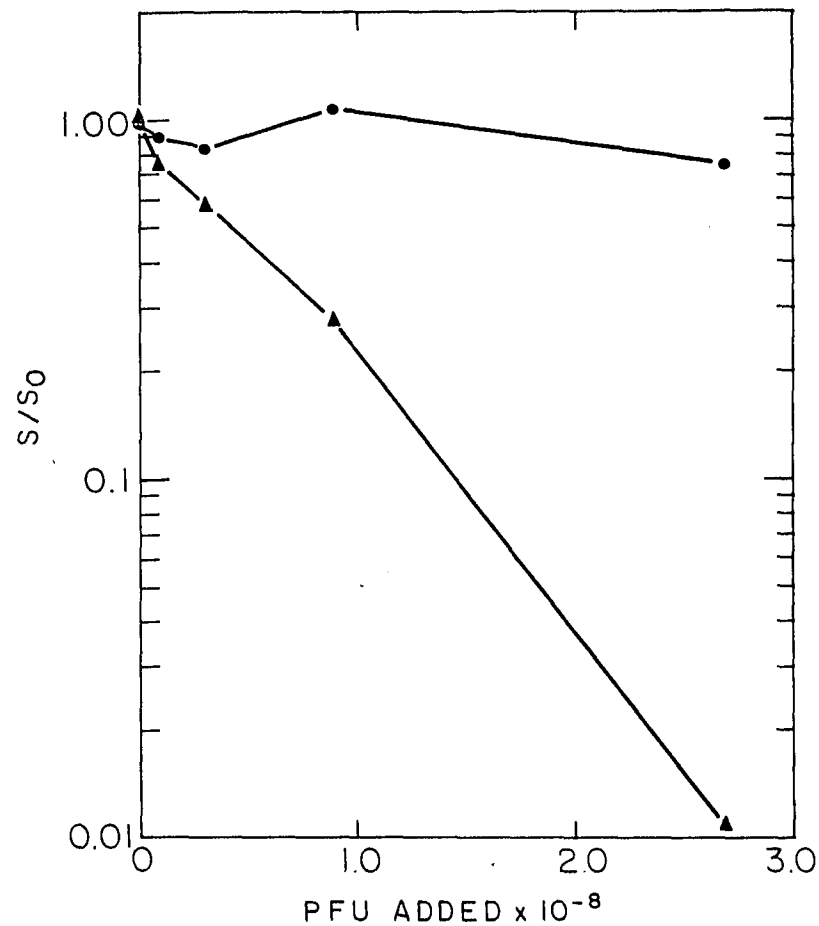
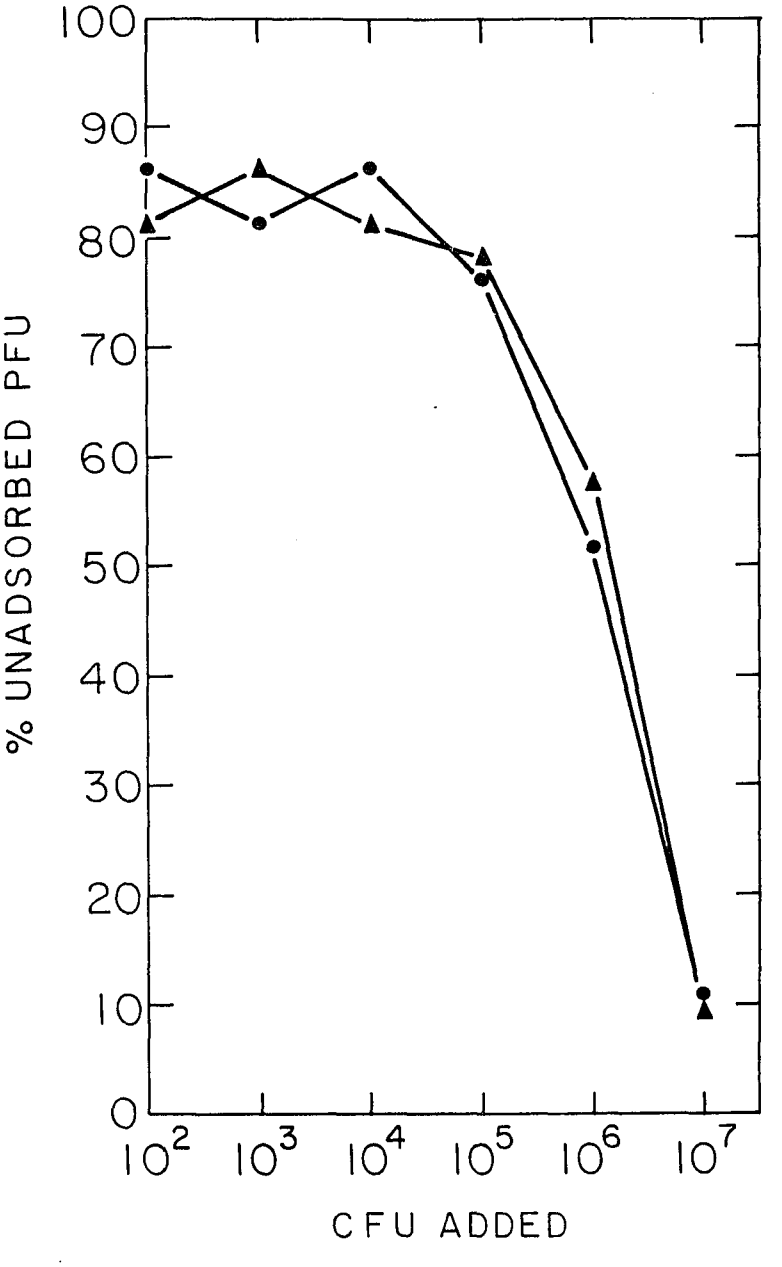


FIGURE 5

Adsorption of ø105 to BD99(pUB110) and BD99(pAG101)

Approximately 7 x 10^4 PFU of ø105 was incubated with the indicated levels of bacteria for 6 min at 37°C. The infected cultures were immediately diluted and sterile filtered as described in Materials and Methods. The sterile filtrates were assayed for unadsorbed PFU. Symbols: ▲ , BD99(pUB110); ● , BD99(pAG101).

Figure 5


100
90
80
70
60
50
40
30
20
10
0
% UNADSORBED PFU
10^2 10^3 10^4 10^5 10^6 10^7
CFU ADDED

5) EcoRl and HindIII restriction analysis of pAG101 and pPL1004

Analysis of ø105 deletion mutants by Scher et al. showed that the ø105 immunity region was associated with the ø105 EcoRl restriction fragments F, H, and B (Scher et al., 1978) (See Introduction). Similar analysis of the ø105 clear-plaque deletion mutant DI:1C indicated that the HindIII fragment F also was associated with the ø105 immunity region (Scher et al., 1978). A chimeric plasmid which contained the ø105 HindIII fragment F subsequently was isolated and characterized by Marrero and Lovett (Marrero and Lovett, 1980). This plasmid, pPL1004, resembled pAG101 in that it was able to render B.subtilis cells immune to killing by ø105 (Marrero and Lovett, 1980).

To determine if pAG101 ad pPL1004 had a ø105 DNA fragment in common which carried the gene or genes responsible for immunity to superinfection, the plasmids were analyzed with the restriction enzymes HindIII and EcoRl. HindIII digestion of ø105 DNA resulted in 14 fragments as visualized by agarose gel electrophoresis (Fig.6A). Digestion ofpAG101 DNA with HindIII produced two fragments (Fig.6B, lane 1) of approximately 5200 and 1850 base pairs. The 5200 bp fragment most probably contained the pAG101 vector plasmid pUB110 (4390 bp) which does not possess a HindIII cleavage site (Gryczan et al., 1978) as well as portions of the ø105 EcoRl fragment F. The smaller HindIII fragment of pAG101 comigrated with the 1850 bp HindIII fragment G of ø105 (Fig.6B, lane 2). Thus, the 3000 bp EcoRl fragment F contains the two HindIII sites which generate the ø105 HindIII fragment G plus additional DNA. This observation agreed with that of Lovett and coworkers, who had reported that the ø105 EcoRl fragment F contained within it a HindIII fragment of approximately the size of HindIII fragment G (Williams et al., 1981). Lovett estimated the size of the DNA fragments bordering the 1820 bp HindIII fragment to be approximately 270 and 940 base pairs. HindIII-EcoRl double digestion of pAG101 essentially confirmed the presence of these two small fragments on EcoRl fragment F (date not shown). However, we have deter-

FIGURE 6

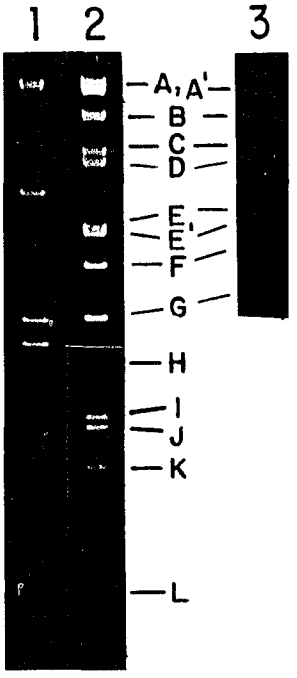
Electrophoresis of restriction endonuclease digested pAG101 and ø105 DNAs in 0.7% agarose

Lane 1 contains as DNA size markers EcoRl digested ø105 and HinfI digested pSV40 DNA; the fragment sizes in base pairs are (from top to bottom) 5300, 3000, 1845, 1666, 1090, 1056, 890, 766, 543, 517, 506, 396, and 380. Lanes 2 and 3 contain HindIII diested ø105 DNA. The largest (top) HindIII fragment consists of two DNA fragments, A and A', which are shown separated in the gel presented in lane 3. Analysis of ø105 deletion mutants (Results, section III B) supports the presence of two A fragments. ø105 HindIII fragment E also can be resolved (lane 3) into two fragments, E and E'. The size of the ø105 HindIII fragments as determined from the DNA standards in base pairs are as follows: (A, A'), 5200-5100; B, 4600; C, 3900; D, 3700; E, 2800; E', 2700; F, 2430; G, 1850; H, 1500; I, 1100; J, 1050; K, 900; and L, 540.

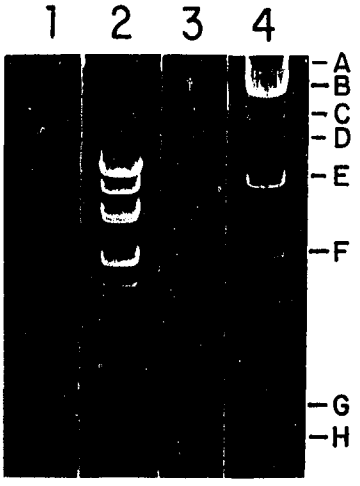
B) Electrophoresis of HindIII and EcoRl digested pAG101 and ø105 DNAs in 0.7% agarose. Lane 1 contains HindIII digested pAG101 DNA; lane 2 contains HindIII digested ø105 DNA; lane 3 contains EcoRl digested pAG101 DNA; lane 4 contains EcoRl digested ø105 DNA; and the size of the ø105 EcoRl DNA fragments in base pairs are: A, 16,000; B, 10,900; C, 8800; D, 7900; E, 5300; F, 3000; G, 1090; and H, 890 (Scher et al., 1977); size of fragment H, this thesis). The conditions for DNA digestions and gel electrophoresis are given in Materials and Methods.

Figure 6

A



B



mined, on the basis of DNA sequence analysis which is presented in Results, section IIIA, that the fragment reported to be 940 bp in length is actually 10^88 bp. It should be noted that we originally estimated the size of this fragment, by agarose gel electrophoresis, to be approximately 1050 bp and this is the value which is used in the discussion below.

In order to determine if either the 240 bp or 1050 bp fragments, present in EcoRl fragment F, also were present in the HindIII fragment F, pPl1004 DNA was subjected to restriction analysis with HindIII and EcoRl.

Digestion of pPl1004 DNA with HindIII resulted in two fragments as expected: linear pCM194 (2730 bp) and ø105 HindIII fragment F (2430 bp) (Fig.7, lane 3) (Marrero and Lovett, 1980). EcoRl digestion of pPL1004 resulted in two DNA fragments (Fig.7, lane 2), one of which comigrated with ø105 EcoRl fragment H (890 bp). The second larger fragment is approximately 4270 bps and most likely containes the vector plasmid pCM194 (2730 bp), which is known not to possess an EcoRl restriction site (Gryczan et al., 1978), plus additional DNA. Digestion of pPL1004 DNA with both EcoRl and HindIII resulted in four fragments (Fig.7, lane 7) which are approximately 2730, 1050, 890, and 490 base pairs in length. The largest fragment is the vector pCM194 which contains a single HindIII restriction site and lacks an EcoRl cleavage site (Table 3). The 890 bp fragment was present in the EcoRl digest of pPL1004 and is most likely the EcoRl fragment H. Thus, the remaining fragments of 1050 bp and 490 bp are the terminal HindIII-EcoRl fragments of the ø105 HindIII fragment F.

The HindIII-EcoRl 1050 bp fragment of pPL1004 was also present in the HindIII-EcoRl digest of pAG101. The ø105 EcoRl fragments F and H have been mapped and lie next to each other as depicted in Fig.8 (Scher et al., 1978). Thus, it appears that the 1050 bp fragment contained in pAG101 and pPL1004 is the only piece of the ø105 genome present in both plasmids (Fig.8). The 1050 bp fragment and the EcoRl fragment H were further studied by DNA sequence analysis (Results,

FIGURE 7

Electrophoresis of pPL1004 DNA in 0.7% agarose

Lane 1 and lane 6 contain EcoRl digested ø105 DNA which are used as size markers. Lane 2 contains EcoRl digested pPL1004 DNA. Lane 3 contains HindIII digested pPL1004 DNA. Lane 4 contains undigested pPL1004 DNA. Lane 5 contains HindIII digested ø105 DNA which are used as size markers. Lane 7 contains pPL1004 DNA which has been digested with both EcoRl and HindIII. The molecular weights of ø105 EcoRl and HindIII fragments are listed in the legend to Fig.6 A and B. The conditions for restriction enzyme digestion of DNAs and gel electrophoresis are as described in Materials and Methods.

Figure 7

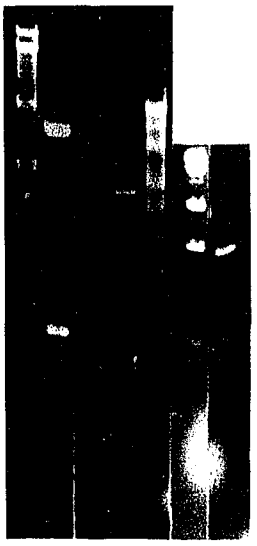


FIGURE 8

Restriction map of ø105 contained in the plasmids pAG101 and pPL1004

The HindIII and EcoRl restriction map of the ø105 immunity region is shown on this schematic diagram (bold line). The map is positioned with the J gene to the left and the H gene to the right. The bars which are labeled pAG101 and pPL1004 represent the region of the ø105 DNA which is contained in these plasmids.

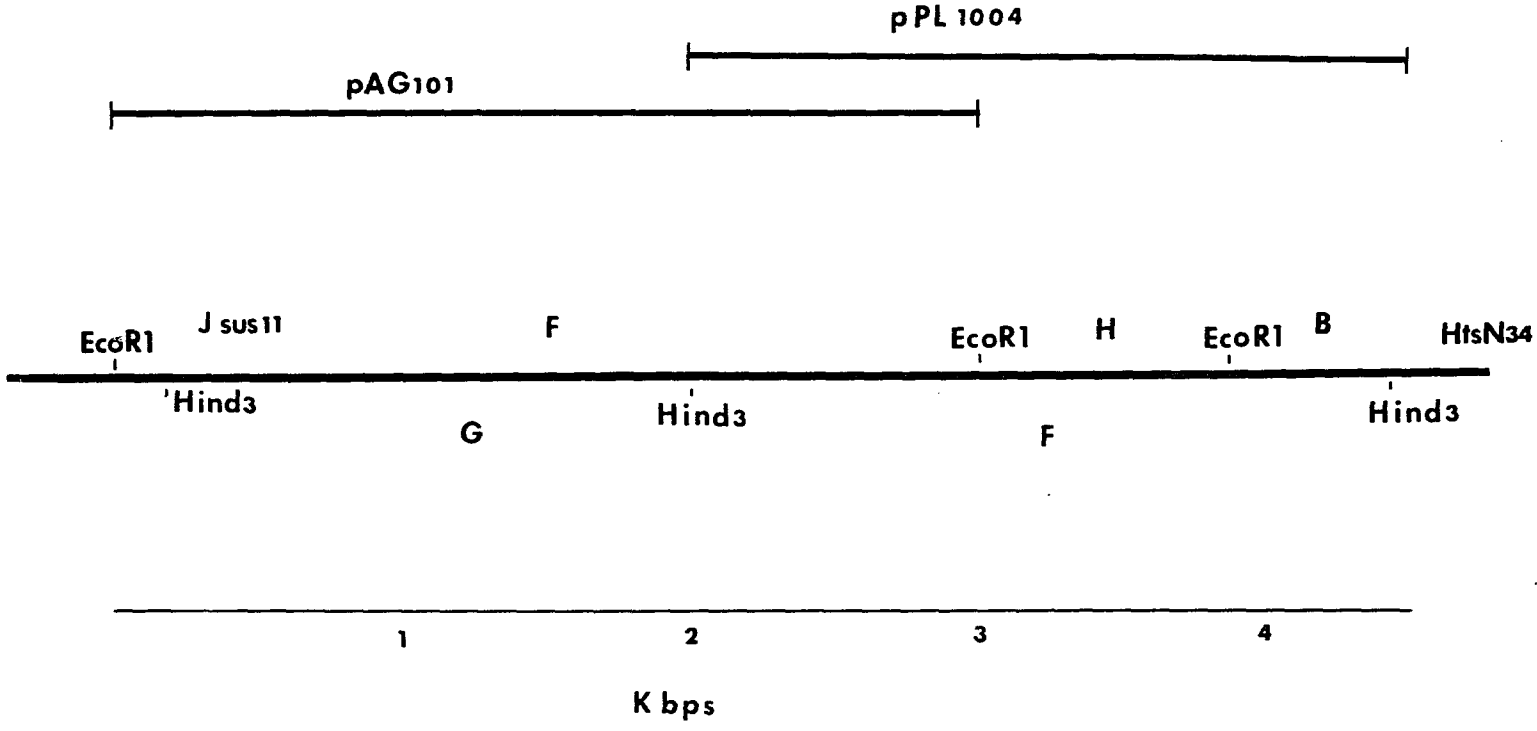

pPL 1004
pAG101
EcoR1
J sus11
F
EcoR1
H
EcoR1
B
HtsN34
'Hind3
G
Hind3
F
Hind3
1
2
3
4
K bps

Figure 8

section III A) which confirmed the map present in Fig.8. It should be noted that the estimated sizes of the fragments given above, as determined by gel electrophoresis, were found to vary slightly from their actual size as determined by DNA sequencing (Results, section III A).

B) CONSTRUCTION AND ANALYSIS OF PDC1, PDC2, AND PDC4

In view of the above findings it became of interest to examine the ø105 immunity region in order to determine the location of the region coding for the ø105 repressor. The methodology used to study the immunity region was to subclone ø105 restriction fragments into plasmid vectors which could then be used to examine ø105 proteins and DNA sequence.

1) pDC1

A derivative of the E.coli plasmid pBR322 was constructed wich contained the 1050 bp HindIII-EcoRl fragment present in both pAG101 and pPL1004. This plasmid, pDC1, was used in E.coli maxicells to identify proteins coded by the ø105 immunity region.

pDC1 was constructed as described in Materials and Methods and as outlined in Fig.9. The 1050 bp HindIII-EcoRl fragment was isolated from pPL1004 and ligated into HindIII-EcoRl cleaved pBR322. The ligated DNA was used to transform competent E.coli C600 cells and ampicillin resistant colonies were isolated. In order to identify transformants which had acquired the desired chimeric plasmid, plasmid DNAs isolated from Amp^r C600 transformants were cleaved with both EcoRl and HindIII and screened for the appropriate size fragments. A plasmid which yielded the expected fragments was isolated and its restriction analysis is shown in Fig.10. Lanes 2 and 4 of Fig.10 show the results of a HindIII-EcoRl double digestion of the plasmid. The largest DNA fragment is approximately 5000 bp and may represent linear pDC1 DNA (5362 bp) which would have resulted from incomplete digestion

FIGURE 9

Construction of pDC1

A plasmid was constructed which contains the HindIII-EcoRl 1050 bp fragment of the ø105 HindIII fragment F inserted into the HindIII-EcoRl site of pBR322 and is called pDC1. The experimental details for this construction are outlined in Materials and Methods.

Figure 9

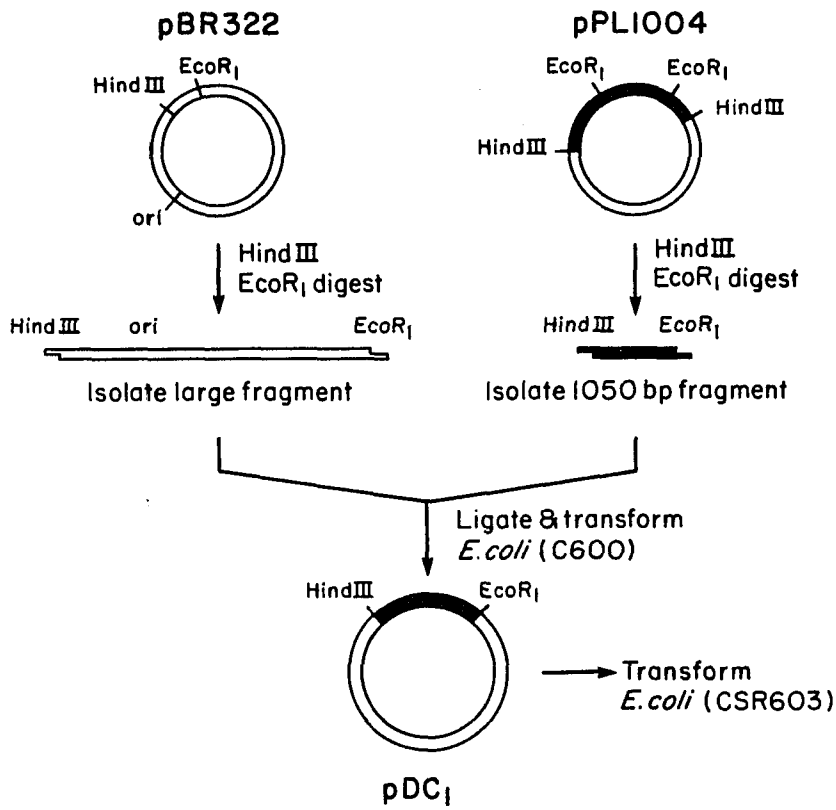
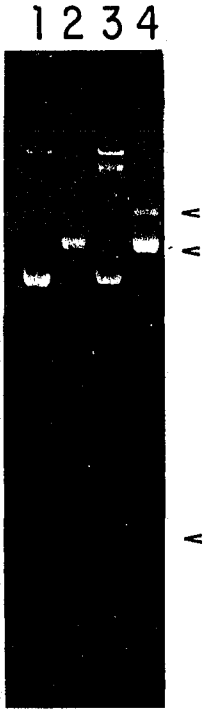

pBR322
EcoR$_I$
Hind III
ori
Hind III
EcoR$_I$ digest
Hind III
ori
EcoR$_I$
Isolate large fragment
pPL1004
EcoR$_I$
EcoR$_I$
Hind III
Hind III
Hind III
EcoR$_I$ digest
Hind III
EcoR$_I$
Isolate 1050 bp fragment
Ligate & transform
E.coli (C600)
Hind III
EcoR$_I$
Transform
E.coli (CSR603)
pDC$_1$

FIGURE 10

Electrophoresis of pDC1 DNA in 0.7% agarose

Plasmid DNAs were isolated from Amp^r C600 cells which were transformed with ligated DNAs as shown in Fig.9. DNA was isolated by the rapid plasmid DNA isolation procedure described in Materials and Methods (see section III C). Lanes 1 & 3 contain the uncut plasmid DNA (pDC1). The top bands are probably various forms of nicked circular DNA while the faster migrating species is covalently closed circular DNA. Lanes 2 & 4 contain plasmid DNA (pDC1) which has been digested with both EcoRl and HindIII. Arrows from top to bottom represent, in order, linear pDC1 DNA, pBR322 DNA and the 1050bp pPL1004 HindIII-EcoR1 DNA fragments described in the text.

Figure 10



of the plasmid DNA. The remaining DNA fragments are 4312 bp and 1050 bp and represent the HindIII-EcoRl linear pBR322 and the pPL1004 HindIII-EcoRl fragments as expected. Thus, these Amp^r C600 transformants contained the plasmid pDC1 as shown in Fig.9.

2) pDC2

The DNA sequence of the 1050 bp HindIII-EcoRl fragment present in pDC1 (Results, section III A) provided information on the open reading frames in this fragment and also showed the location of additional restriction enzyme cleavage sites. It thus was possible to subclone a HindIII-PvuII 740 bp fragment which would leave only three open reading frames and would eliminate 30% of the DNA present in the 1050 bp HindIII-EcoRl fragment cloned in the pDC1 (Fig.22).

A derivative of the E.coli plasmid pBR322 therefore was constructed as shown in Fig.11 which contains the 740 bp HindIII-PvuII fragment that is present in the plasmid pDC1, pAG101, and pPL1004. The resulting plasmid pDC2 was isolated from Amp^r E.coli C600 transformants and its composition verified by agarose gel analysis of restriction digested plasmid DNAs (data not shown). This plasmid was used to study ø105-immunity region protein synthesis in E.coli maxicells (Results, section II C) and also was used to construct pDC4, a plasmid which replicates in B.subtilis.

3) pDC4

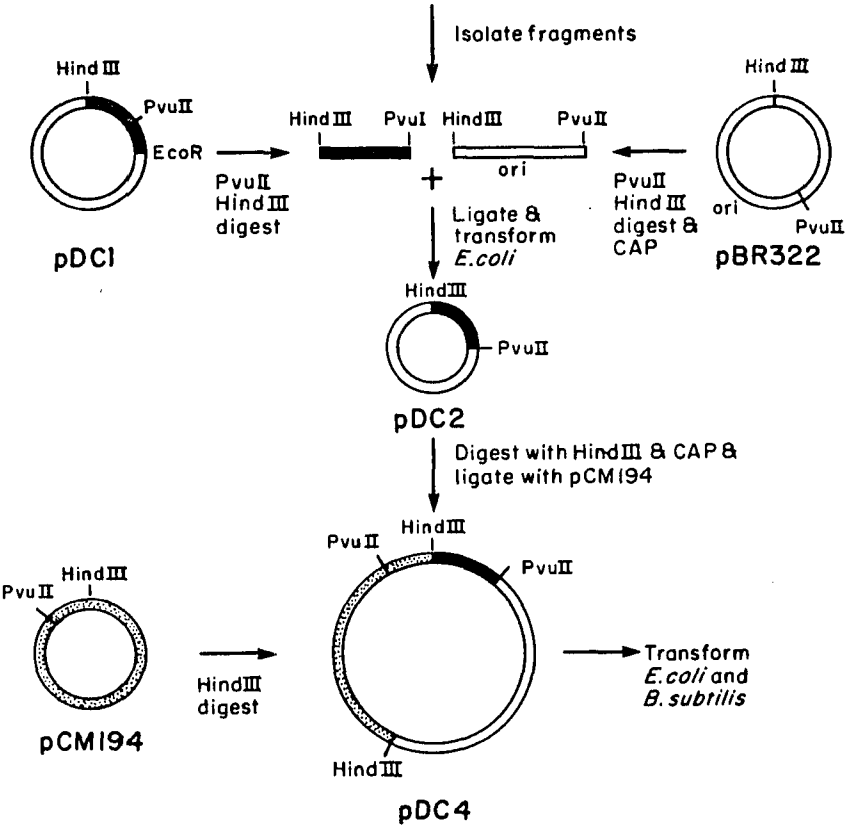
In order to determine whether B.subtilis cells which carried a plasmid containing the 740 bp HindIII-PvuII fragment were immune to ø105 infection it was necessary to introduce this DNA into a plasmid which could replicate in B.subtilis. This was done by joining pDC2 to pCM194 through their HindIII sites. Purified pDC2 and pCM194 DNAs were linearized with HindIII and treated with T4 DNA ligase as described in Materials and Methods and as shown in Fig.11.

FIGURE 11

Construction of pDC2 and pDC4

A plasmid was constructed which contained the HindIII-PvuII 740 bp fragment of the HindIII fragment F inserted into a HindIII-PvuII fragment of pBR322 and is called pDC2. This plasmid can replicate in E.coli. The plasmid pDC4 was constructed from pDC2 by inserting the B.subtilis vector at the HindIII sites. The new plasmid pDC4 can replicate in B.subtilis. The experimental details for this construction are described in Materials and Methods.

Figure 11


Isolate fragments
Hind III
Pvu II
EcoR
pDC1
Pvu II
Hind III
digest
Hind III
Pvu I
Hind III
Pvu II
ori
+
Ligate &
transform
E.coli
Pvu II
Hind III
digest &
CAP
Hind III
ori
Pvu II
pBR322
Hind III
Pvu II
pDC2
Digest with Hind III & CAP &
ligate with pCM194
Pvu II
Hind III
Pvu II
Hind III
pCM194
Hind III
digest
Pvu II
Hind III
Pvu II
Hind III
pDC4
Transform
E.coli and
B. subtilis

Ligated DNAs were used to transform competent C600 cells. A plasmid, pDC4, was identified by rapid screen restriction analysis of plasmid DNA isolated from the resulting Amp^r C600 cells. When pDC4 was digested with HindIII and PvuII (Fig.12, lane 2) three DNA fragments were seen by gel electrophoresis. These fragments had the respective sizes of 2380 bp, 2310 bp, and 740 bp and represent the large HindIII-PvuII fragment of pCM194, the HindIII-PvuII fragment of pBR322, and the HindIII-PvuII ø105 fragment of pPL1004 (Fig.11). The small 350 bp HindIII-PvuII fragment of pCM194 (see Fig.11) which is not visible in lane 2 of Fig.12, has been observed on gels which have been overloaded with restricted pDC4 DNA (data not shown).

4) Resistance of BD99(pDC4) to ø105 Infection

Purified pDC4 DNA was used to transform competent cells of the B.subtilis BD99 and chloramphenicol resistant cells which were shown to carry pDC4 by gel analysis, were isolated. These cells, BD99(pDC4), were tested for immunity to ø105 killing by cross-streaking across ø105 DII:6C as described in Materials and Methods. Cells containing pDC4 were immune to ø105 killing in this test as well as in a plaque assay with the clear-plaque mutant ø105csi-6 (Table 5). BD99 cells containing pCM194, the vector plasmid, were killed by ø105 in these assays. This suggests that the 740 bp HindIII-PvuII fragment present in pDC4 coded for a product which was inhibitory to ø105 replication.

II. IDENTIFICATION OF PROTEINS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

The hybrid plasmids described above were used to examine the proteins produced from the ø105 immunity region. Our reasons for examining proteins produced from these plasmids were two-fold: first,

FIGURE 12

Electrophoresis of pDC4 DNA in 0.7% agarose

Lane 1 contains HinfI digested pSV40 which was used as DNA size markers. The molecular weights of these fragments are listed in Materials and Methods. Lane 2 contains pDC4 DNA which has been digested with both HindIII and PvuII. Lane 3 contains EcoRl digested ø105 DNA as DNA size markers. The sizes of these markers are listed in Materials and Methods.

Figure 12

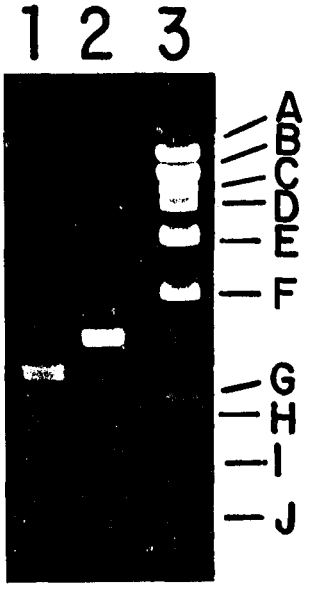


TABLE 5

Immunity of BD99(pDC4) to Killing by ø105*csi*-6

Lawn	pfu/plate
BD99	100
BD99(pCM194)	100
BD99(pDC4)	0

a) The bacterial strains listed were used as lawns in a standard plaque assay with the clear plaque ø105 mutant *csi*-6 as described in Materials and Methods.

to identify a protein or proteins responsible for the immunity to superinfection in cells containing pAG101 and pDC4, and second, to examine the ability of the gram-negative organism, E.coli, to recognize and utilize gram-positive regulatory signals at both a transcriptional and translational level.

A) PROTEINS PRODUCED IN B.SUBTILIS MINICELLS CONTAINING PAG101 OR PUB110 DNA

The B.subtilis minicell producing strain, CU403, was used to examine proteins produced from pAG101 and pUB110. Minicells were purified from CU403(pAG101) and CU403(pUB110) by density gradient centrifugation, and their protein synthesis analyzed using [^{35}S]- methionine as described in Materials and Methods. The labeled proteins were examined by SDS-polyacrylamide gel electrophoresis (Fig.13). Four polypeptides with approximate molecular weights of 53,000; 38,000; 25,000, and 11,000 were detected in the extracts of the CU403(pUB110) minicells. Dubnau and co-workers (Shivakumar et al., 1979) using a [^{3}H]-leucine label previously had identified five pUB110 specific polypeptides, with approximate molecular weights of 58,000; 38,000; 26,000; 25,000 and 11,000. However, in their study the 26,000 and 25,000 dalton polypeptides were not easily detected in CU403(pUB110) minicells that were labeled with [^{35}S]-methionine and the results shown in Fig.13 are consistent with this observation.

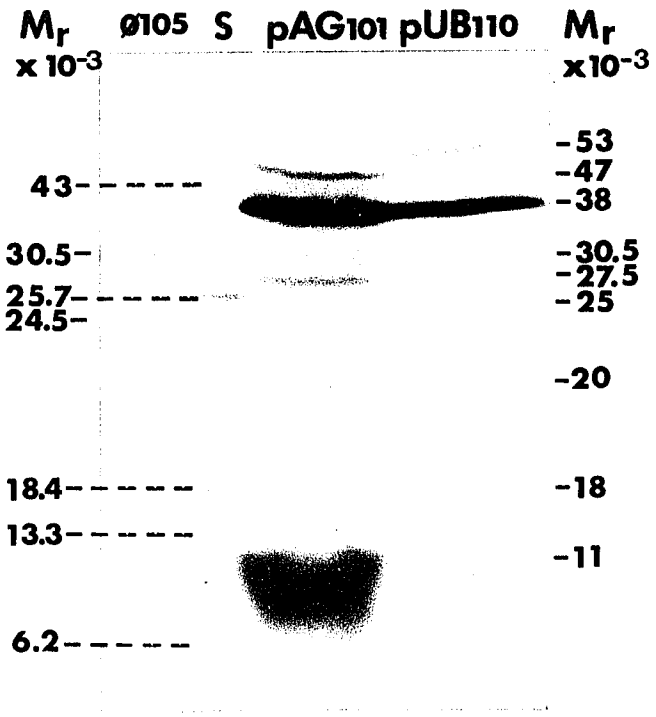
A total of eight polypeptides were detected in the extracts of the CU403(pAG101) minicells (Fig.13). Three of these, namely the 38,000; 25,000; and 11,000 dalton polypeptides were also produced by the pUB110-containing minicells (Shivakumar et al., 1979). The new pAG101 coded polypeptides had the molecular weights of 47,000; 30,500; 27,500; 20,000, and 18,000. Considering the immune phenotype of pAG101 containing cells, it was reasonable to assume that one or more of these polypeptides was involved in superinfection immunity and the regulation of ø105 lysogeny.

FIGURE 13

Electrophoresis of extracts of B.subtilis minicells containing pAG101 and pUB110 in SDS-polyacrylamide gels

CU403(pAG101) and CU403(pUB110) minicells were labeled with L-[^{35}S]-methionine as described in Materials and Methods. Labeled cell extracts were electrophoresed on a linear 10-25% SDS-polyacrylamide gel as described in Materials and Methods. The molecular weights of the protein markers in lane S are noted on the gel. ø105 virion proteins were electrophoresed in the lane noted. The ø105 structural proteins are analyzed in the following sections.

Figure 13


Mr x 10-3
ø105
S
pAG101
pUB110
Mr x10-3
43
30.5
25.7
24.5
18.4
13.3
6.2
53
47
38
30.5
27.5
25
20
18
11

The identification of some of these pAG101 unique polypeptides was aided by the knowledge that the EcoRl fragment F carried an essential gene, namely J, which was identified by conditional lethal mutant analysis and marker rescue (Scher et al., 1978). Also, as evident in the gel shown in Fig.13, a ø105 virion polypeptide with a molecular weight of 30,500, comigrated with a pAG101 polypeptide.

It was possible that the gene for this ø105 structural protein also was present on the EcoRl fragment F. Thus, to identify the regulatory proteins coded by EcoRl fragment F, it was necessary first to identify the ø105 structural proteins and the ø105 J gene product.

B) ANALYSIS OF ø105 PROTEINS

1) ø105 structural proteins

The proteins contained in the ø105 virion are by definition the phage structural proteins. The molecular weights of these polypeptides were determined by electrophoresis in SDS-polyacrylamide gels with appropriate protein size markers. Two methods were used to visualize the ø105 structural proteins which were separated in gels. Gels which contained phage proteins labeled with radioactive amino acids were visualized by autoradiography while gels containing unlabeled phage proteins were visualized by silver stain. Using these techniques, nine virion polypeptides were identified on SDS-polyacrylamide gels (Fig.14). Minor polypeptide bands were occasionally observed on these gels with sizes ranging from 12,000 to 24,500 daltons (Fig.14, lane B; Fig.16, lane ø105). These minor bands are believed to be breakdown products of the larger virion polypeptides since they are not consistently observed and their appearance increases upon storage of the virus. The molecular weights of the structural polypeptides as well as the relative amounts of each peptide in the virion are listed in Table 6. The relative amounts of each peptide were determined by

FIGURE 14

Electrophoresis of ø105 Virion Polypeptides

CsCl purified phage was electrophoresed on 15% SDS polyacrylamide gels. Lane A is an autoradiograph of a gel on which [^{35}S]-methionine labeled ø105 polypeptides have been separated. Lane B is a gel on which unlabeled ø105 polypeptides have been separated and subsequently silver stained. Protein standards were used to determine the M_r of the virion polypeptides as described in Materials and Methods.

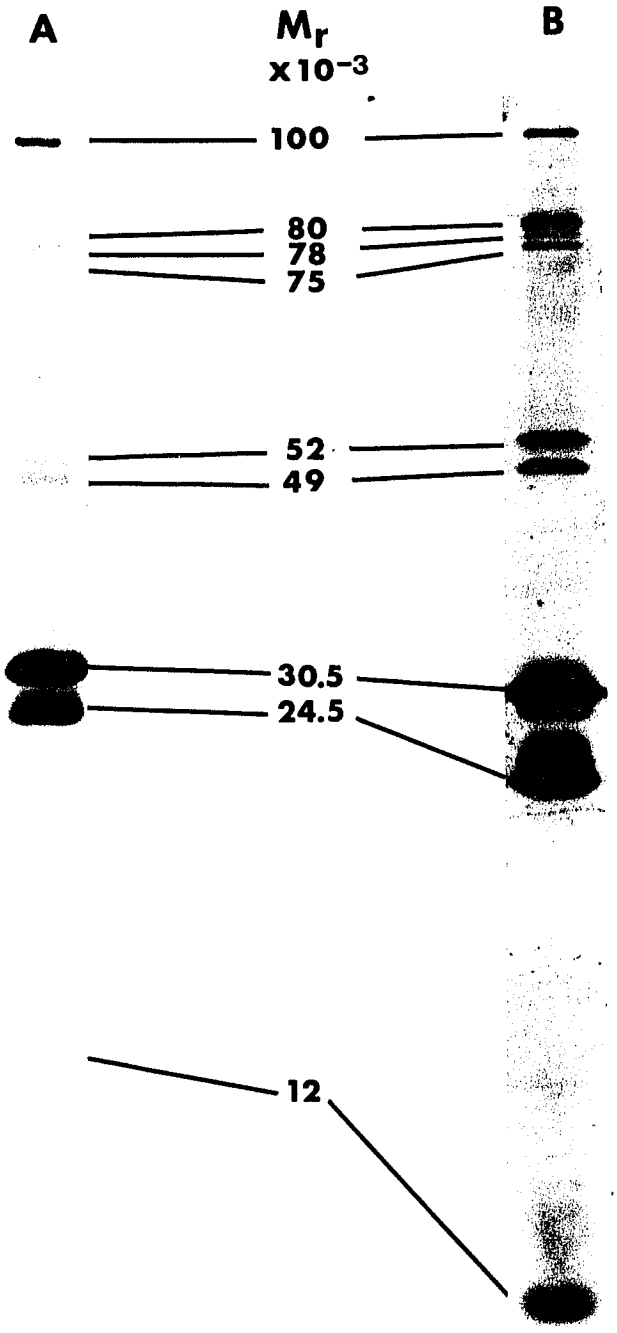

A
M_r
x10^{-3}
B
100
80
78
75
52
49
30.5
24.5
12

FIGURE 15

Quantitative analysis of ø105 structural polypeptides labeled with [^{14}C]-Amino Acids

A polyacrylamide gel in which [^{14}C]-labeled ø105 had been electrophoresed was cut into 1 mm slices. The gel fractions were placed in in aqueous liquid scintillation fluid and the radioactivity quantitated in a Beckman liquid scintillation spectrophotometer (LS9000).

Figure 15

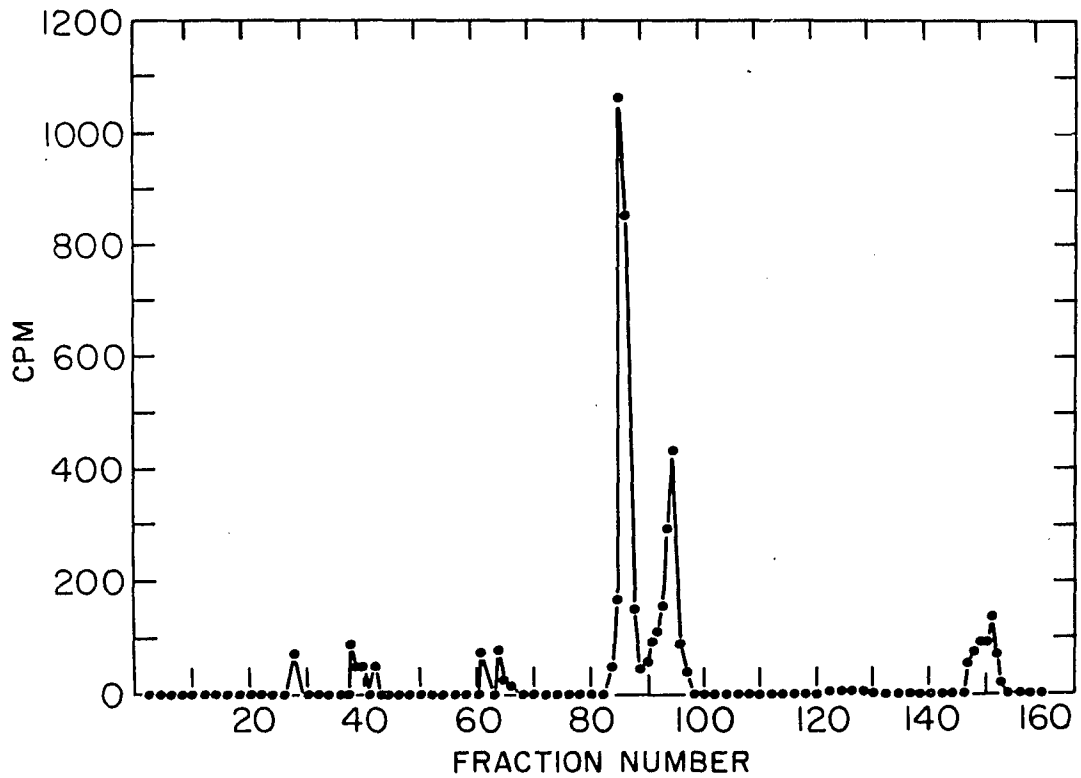

1200
1000
800
600
400
200
0
CPM
20
40
60
80
100
120
140
160
FRACTION NUMBER

TABLE 6

Structural Proteins of ø105

ø105 Protein	M_r [a]	%Total Protein in Phage [b]
S1	100,000	1.10
S2	80,000	1.80
S3	78,000	1.30
S4	75,000	0.70
S5	52,000	1.70
S6	49,000	1.60
S7	30,500	56.90
S8	24,500	27.10
S9	12,000	7.80

a) The molecular weights of the phage polypeptides were determined on SDS-polyacrylamide gels with protein size standards (see text and Fig. 14).

b) The relative amounts of ø105 polypeptides in the virions were determined as described in the legend to Fig.15.

measuring the amount of radioactivity in 1 mm slices of a SDS-polyacrylamide gel in which purified [^{14}C]-labeled ø105 had been electrophoresed (Fig.15). The ø105 S_2 and S3 proteins migrated very close to each other in this gel system as shown in Fig.14. Thus, to determine the relative amounts of these proteins the total amount of radioactivity in the gel fraction 38 was attributed to the S_2 protein and fraction 39 to the S3 protein.

2) Identification of the ø105 J protein

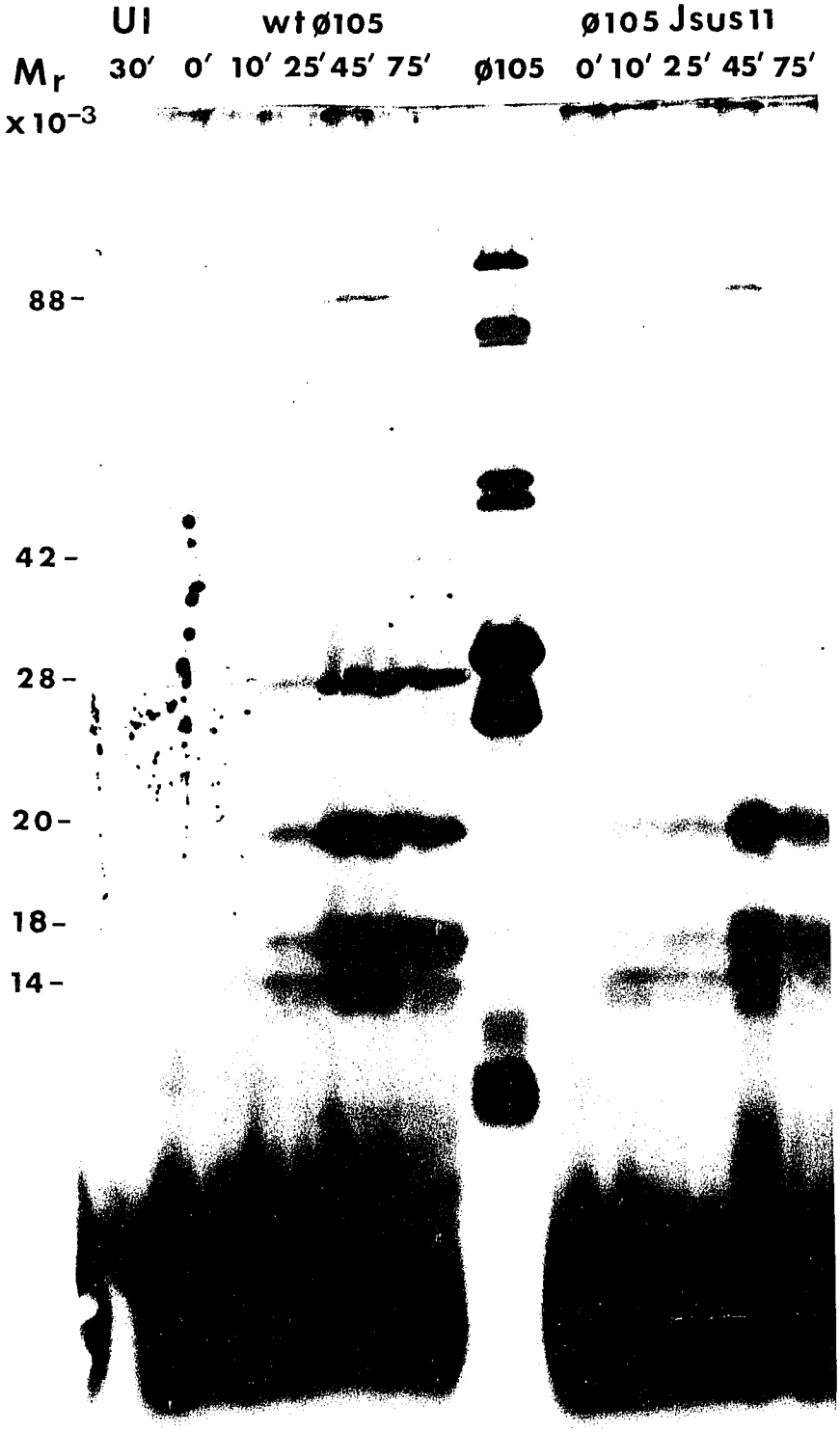
The J gene product was identified by examining the polypeptides produced in B.subtilis CU403 su$^-$ minicells following infection with either wt ø105 or ø105Jsus11. This method was used to identify the J gene product since it is known that cells of the su$^-$ genotype are unable to support the synthesis of a functional J gene product from ø105 carrying the Jsus11 mutation (Scher et al., 1978). These su$^-$ cells, however, can synthesize a functional J gene product from wt ø105. To compare mutant and wild type phage proteins, infected minicells were labeled with [^{35}S]-methionine and separated on SDS-polyacrylamide gels as shown in Fig.16. Six ø105 proteins were detected in B.subtilis minicells during a 75 min infection by wt phage. Their molecular weights were 88,000; 42,000; 28,000; 20,000; 18,000 and 14,000. The only one of these proteins which was not seen in the ø105Jsus11 infected cells was the 28,000 dalton protein which most likely is the J gene product. The size of this protein corresponds reasonably well with the 27,500 dalton protein coded by pAG101 (Results, section IIA). The J protein did not appear to be a ø105 structural protein because the molecular weight on SDS-polyacrylamide gel electrophoresis did not coincide with any of the ø105 structural proteins present in CsCl purified phage. In fact, no structural proteins appear to be made during a 75 min incubation period with the infected minicells.

The 20,000 and 18,000 dalton proteins detected in B.subtilis minicells during wt ø105 or ø105Jsus11 infection may correspond to the 20,000 and 18,000 dalton proteins detected in pAG101 containing mini-

FIGURE 16

SDS-polyacrylamide gel analysis of phage specified proteins produced in B.subtilis minicells

The infected cells were labeled for 10 min with [^{35}S]-methionine at 0, 10, 25, 45, and 75 min post-infection and cell extracts electrophoresed on a 15% SDS-polyacrylamide gel. CsCl purified [^{35}S]-methionine labeled ø105 polypeptides were used as molecular weight standards.


UI
wt ø105
ø105 Jsus11
Mr
30′ 0′ 10′ 25′ 45′ 75′
ø105
0′ 10′ 25′ 45′ 75′
x 10-3
88-
42-
28-
20-
18-
14-

cells. Thus, if one of these proteins was the nonstructural protein responsible for the immunity to superinfection, it was produced following phage infection of B.subtilis minicells.

Of the eight pAG101 coded proteins which were seen in Fig.13, three, namely the 38,000; 25,000; and the 11,000 dalton polypeptides, are coded by the plasmid pUB110. In this section we have attempted to show that of the five remaining proteins, which are apparently pAG101 specific, the 27,500 dalton protein appears to be the J gene product and the 30,500 dalton protein appears to be a virion polypeptide. The protein responsible for superinfection immunity is therefore probably one of the remaining three, the 47,000; 20,000; or 18,000 dalton proteins.

C) PROTEINS PRODUCED IN E.COLI MAXICELLS CONTAINING PDC1, PDC2, OR PBR322 DNA

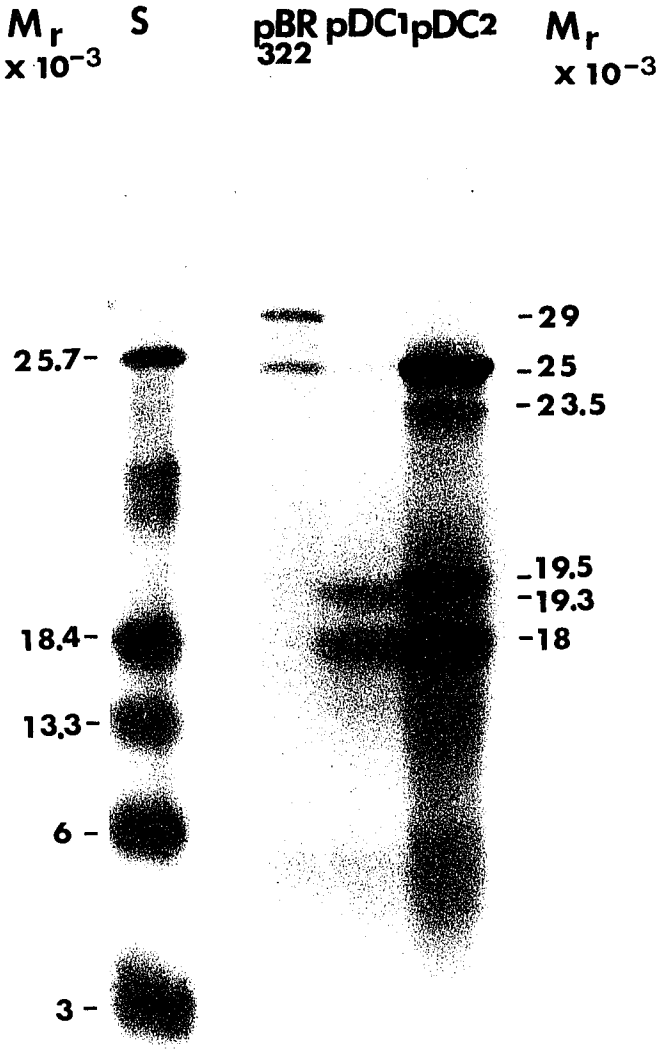
To determine which of the three unidentified pAG101-specific proteins was responsible for superinfection immunity the proteins synthesized from the plasmids pDC1 and pDC2 were examined using E.coli CSR603 maxicells. These plasmids, as described in Results, section IB, contained smaller regions of the ø105 EcoRl fragment F but were still capable of rendering cells resistant to ø105. CSR603 cells which contained the plasmids pDC1, pDC2, or pBR322, were labeled with [^{35}S]-methionine according to the procedure for labeling plasmid-coded proteins in maxicells (Sancar et al., 1979). The labeled proteins were analyzed by electrophoresis on SDS-polyacrylamide gels (Fig.17). As expected, the vector pBR322 coded for the products of the tet and bla genes which are polypeptides with approximate molecular weights of 29,000 and 25,000 respectively (Sutcliffe,1979) (Fig.17). The synthesis of the tet gene polypeptide was interrupted by the insertion of ø105 DNA fragments into either the HindIII-EcoR1 site of pBR322 as in pDC1, or the HindIII-PvuII site of pBR322 as in pDC2. This was expected since the tet gene is located near the HindIII site of pBR322

FIGURE 17

SDS-polyacrylamide gel electrophoresis of extracts of E.coli maxicells containing pDC1, pDC2, or pBR322

E.coli CSR603 maxicells which contain pDC1, pDC2, or pBR322 were labeled with [^{35}S]-methionine and extracts electrophoresed on a 15% SDS-polyacrylamide gel as described in Materials and Methods. [^{14}C]-labeled proteins were used as molecular weight standards.

Figure 17


Mr x 10-3
S
pBR 322
pDC1
pDC2
Mr x 10-3
25.7
18.4
13.3
6
3
29
25
23.5
19.5
19.3
18

and insertion or deletion of DNA in this region interferes with the production of this polypeptide (Gray et al., 1981). The bla gene 25,000 dalton polypeptide is occasionally processed into a smaller 23,500 dalton peptide (Sutcliffe, 1978) and this is seen in the pDC2 cell extracts (Fig.17).

Both pDC1, which contains a 1050 bp ø105 insert, and pDC2, which contains a 740 bp ø105 insert, code for two proteins which are not attributable to the vector pBR322. The molecular weights of these novel polypeptides, as determined by gel electrophoresis, are 18,000 and 19,300 for pDC1; and 18,000 and 19,500 for pDC2. It is interesting to note that all three plasmids, pAG101, pDC1, and pDC2, which contain the same 740 bp HindIII-PvuII region of the ø105 genome, code for what appears to be, by gel electrophoresis, an identical protein of 18,000 daltons. All three plasmids encode a second protein which are approximately the same molecular weight: 20,000 (pAG101); 19,300 (pDC1); and 19,500 (pDC2). Although similar in size, these proteins are clearly distinguishable when electrophoresed on the same SDS-polyacrylamide gel.

It is possible that the gene which codes for the 18,000 dalton protein is located within the 740 bp ø105 HindIII-PvuII fragment and thus remains the same size in all the plasmid constructions discussed. Since the other proteins of 20,000; 19,300; and 19,500 daltons are not detected in cells which contain the vector plasmid alone, they could possibly result from the fusion of plasmid and phage DNA sequences.

D) PROTEINS PRODUCED IN ø105 INFECTED B.SUBTILIS MINICELLS CONTAINING PAG101

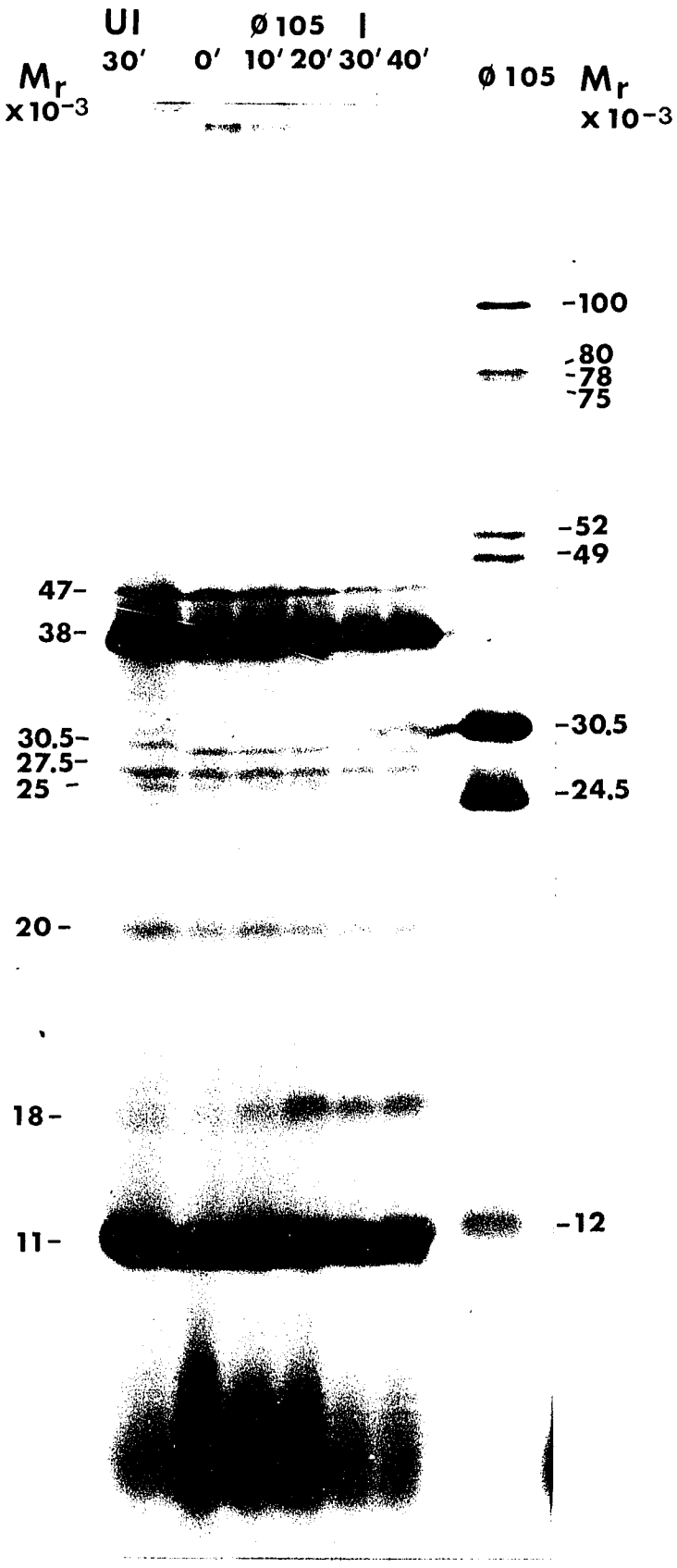
B.subtilis minicells which contained pAG101 were infected with wt ø105 to determine the effect of pAG101-encoded proteins on ø105 protein synthesis. Minicells isolated from CU403 (pAG101) were infected with wt ø105 and labeled for 10 min with [^{35}S]-methionine at

FIGURE 18

SDS-polyacrylamide gel electrophoresis of extracts of wt ø105 infected B.subtilis minicells containing pAG101

Purified wt ø105 was used to infect CU403(pAG101) minicells as described in Materials and Methods. The infected cells were labeled for 10 min with L-[^{35}S]-methionine at 0, 10, 20, 30, and 40 min post infection and cell extracts were prepared and electrophoresed on a 15% SDS-polyacrylamide gel. CsCl purified [^{35}S]-methionine labeled ø105 was used as protein standards.

Figure 18


UI
Ø 105 I
30'
0' 10' 20' 30' 40'
Ø 105
M_r x 10^{-3}
M_r x 10^{-3}
-100
-80
-78
-75
-52
-49
47-
38-
-30.5
30.5-
27.5-
25 -
-24.5
20 -
18 -
11 -
-12

0, 10, 20, 30, and 40 min post-infection. The labeled proteins were analyzed in a 15% SDS-polyacrylamide gel (Fig.18). The only polypeptides observed in phage infected CU403(pAG101) minicells were the pAG101-encoded proteins seen in uninfected cells (cf. Fig.13 and Fig.18). It then appears that the minicells isolated from CU403(pAG101) cells do not permit the synthesis of ø105 coded proteins other than those coded by the plasmid pAG101. It may be of some significance, however, that the 18,000 dalton polypeptide, which appears to be a nonstructural phage coded protein (Fig.16) actually increases in its rate of synthesis in the infected cells. It is not possible to determine from this experiment whether the increased synthesis of this protein is directed from plasmid DNA or from incoming phage DNA. Nevertheless, the increased synthesis of this protein in phage infected immune cells would be consistent with its being involved in repression and superinfection immunity.

III. DNA SEQUENCE ANALYSIS OF THE ø105 IMMUNITY REGION

To this point we have shown that the ø105 HindIII fragment F, which spans the EcoRl fragments F, H, and B, contains a HindIII-PvuII fragment which is capable of conferring immunity to ø105 (Results, section I; see also Fig.19). Two protein products encoded by this HindIII-PvuII fragment were identified by gel electrophoresis, with approximate molecular weights of 18,000 and 19,500 (Results, Sestion II). The DNA sequence of the ø105 HindIII fragment F was determined in order to identify the regions which potentially coded for these proteins. In addition, we hoped that the DNA sequence of this region would provide information on the use of regulatory sequences which are known to be involved in prokaryotic transcription and translation.

Early genetic studies had shown that mutations which interfered with the establishment or maintenance of lysogeny mapped in the vicinity of the HindIII fragment F. ø105 deletion mutants were examined,

as described below, by DNA restriction and sequence analysis in order to delineate the functions of the proteins predicted from the DNA sequence of the wild-type ø105 immunity region. The clear and hazy-plaque deletion mutants which were used for this study were previously isolated by Flock and are described in the legend to Fig.19 (Flock, 1977).

A) DNA SEQUENCE OF THE ø105 HINDIII FRAGMENT F

The HindIII fragment F which was used for all DNA sequence determinations was obtained from the hybrid plasmid pPL1004. The restriction enzymes which were used to produce the DNA fragments for polynucleotide kinasing and sequencing are shown in Fig.20. As Fig.20 depicts, the DNA sequence of both strands was determined for most of these fragments. There were regions in which only one strand was sequenced; however, the sequences of these regions were unambiguous and did not require the confirming sequence of the complementary strand. Fig.21 shows a sample of the DNA gels which were used to determine the sequence of the HindIII fragment F.

The DNA sequence of the HindIII fragment F is presented in Fig.22. The sequence as presented is oriented with respect to the ø105 genome as follows: the HindIII site which comprises bases 1-6 is located in the EcoRl fragment F while the HindIII site at position 2429-2434 is located within the EcoRl fragment B (see Fig.19). The two EcoRl sites which border the ø105 EcoRl fragment H are found within the HindIII fragment F at positions 10^{88} and 1980. The base composition of the HindIII fragment F as determined from the DNA sequence of the top strand in Fig.22 is 35.0% Adenine, 18.1% Cytosine, 21.7% Guanine, and 25.2% Thymine.

The predicted protein products from both DNA strands of the HindIII fragment F were determined through the use of an IBM computer equipped with Staden translation programs (Staden, 1977,1980). Com-

FIGURE 19

Restriction map of the ø105 immunity region

The EcoRl map of the ø105 immunity region was determined by Scher et al. (Scher et al., 1978). The ø105 clear-plaque mutants, DI:1C, DI:2C, and DI:4C, and the hazy-plaque deletion mutants DI:29t and DI:1t were obtained from I. Flock (Flock, 1977). The regions which are deleted in these mutants are noted by the solid lines above the ø105 map. The location of these deletions were roughly mapped on the ø105 genome by I.Flock using heteroduplex analysis. The DI:1C, DI:2C, and DI:4C deletions were more precisely determined in this study from DNA restriction restriction and sequence analysis. The HindIII and PvuII map of this region was determined by restriction and sequence analysis of the clear-plaque deletion mutants (Results, Section III), and the hybrid plasmids pAG101 and pPL1004 (Results, Section I).

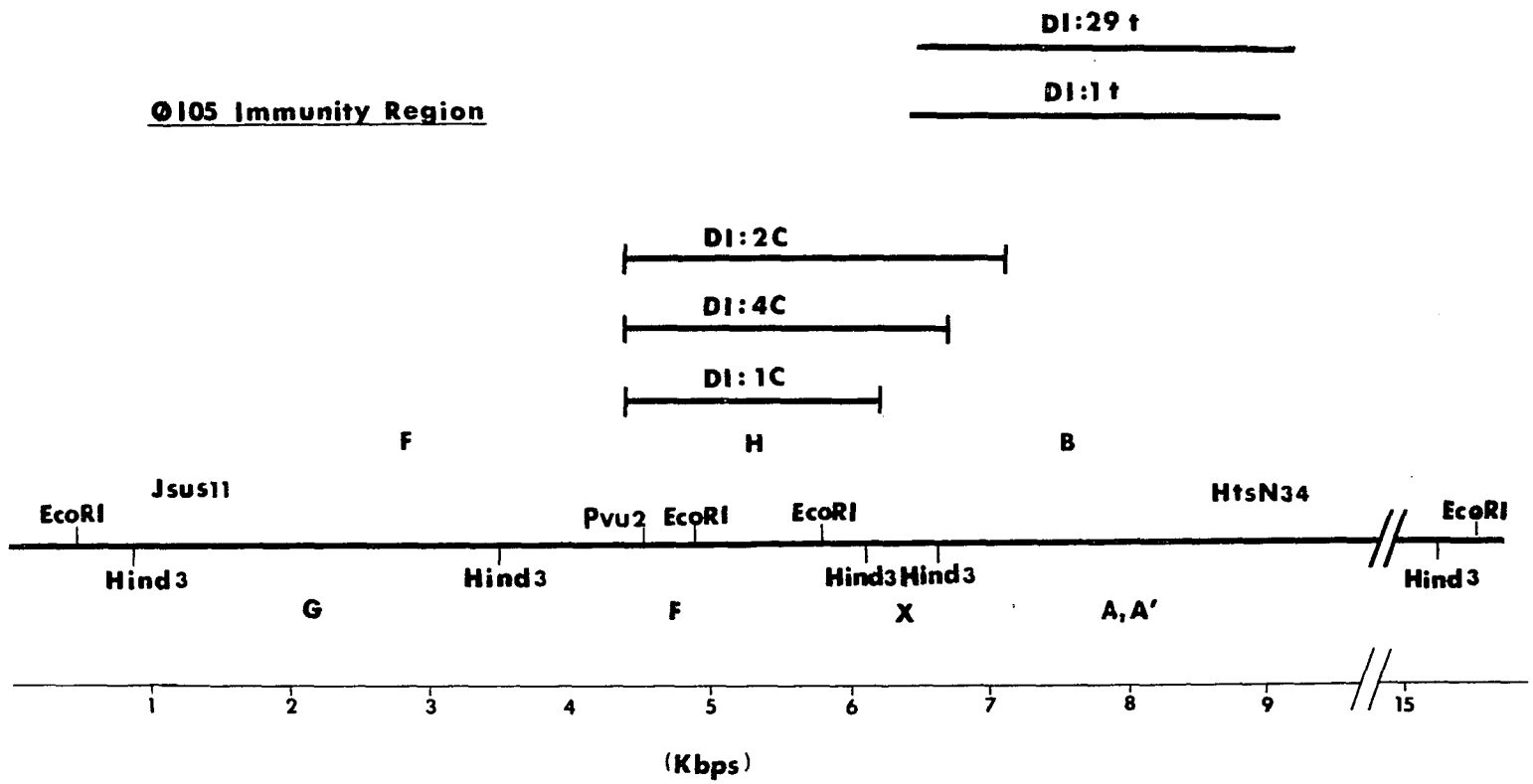

Ø105 Immunity Region
DI:29 t
DI:1 t
DI:2C
DI:4C
DI:1C
F
H
B
Jsus11
EcoRI
Pvu2
EcoRI
EcoRI
HtsN34
EcoRI
Hind3
Hind3
Hind3
Hind3
Hind3
G
F
X
A,A'
1
2
3
4
5
6
7
8
9
15
(Kbps)

Figure 19

FIGURE 20

Sequencing strategy for the ø105 HindIII fragment F

The ø105 HindIII fragment F is depicted as the bold line in the center of of the figure. The symbols which lie on this line represent the respective restriction sites found in the DNA sequence. The legend for these symbols is at the lower right corner of the figure. The lines with arrowheads represent the DNA fragments which were sequenced and the direction in which the sequencing was done (5' to 3'). All sequencing was done by the chemical method developed by Maxam and Gilbert for sequencing 5'-end labeled DNAs (Maxam and Gilbert, 1980).

Figure 20

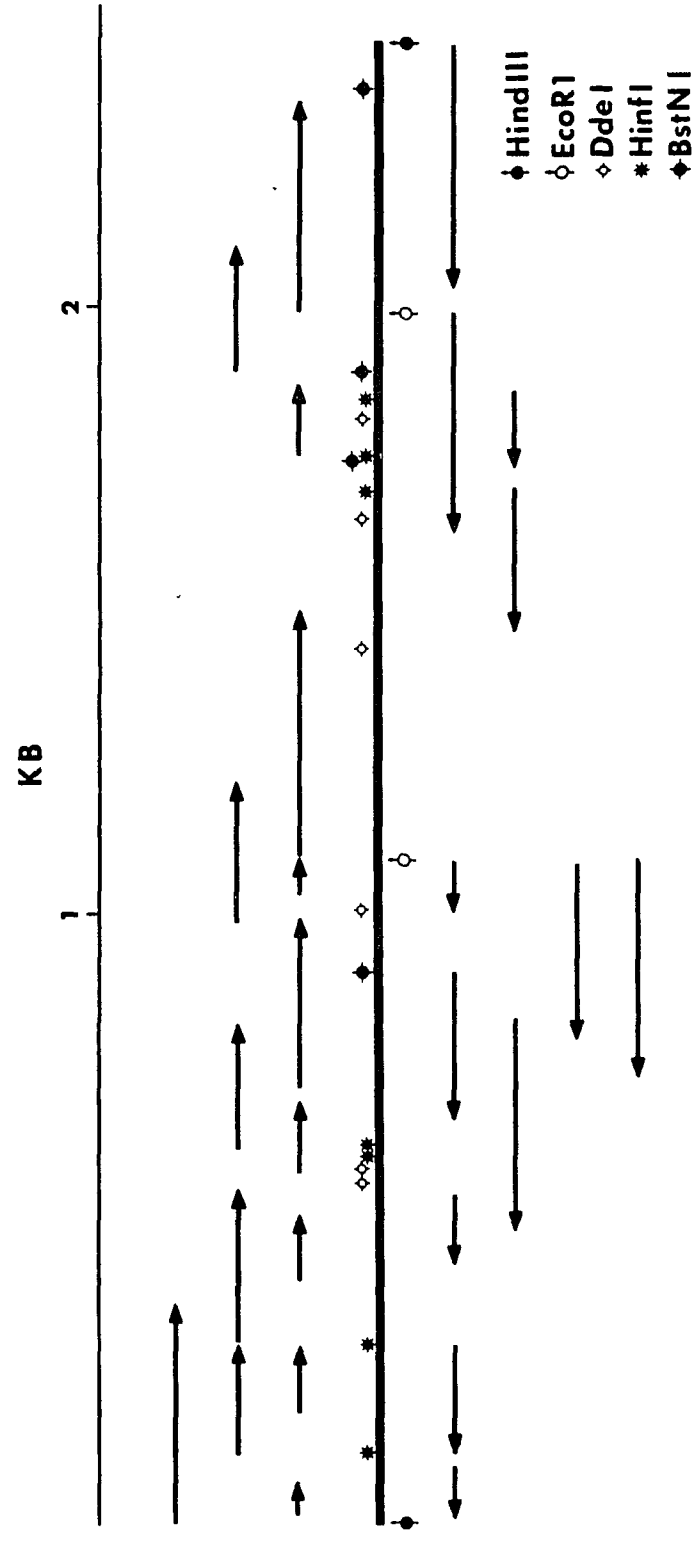


FIGURE 21

Autoradiograph of chemically sequenced ø105 DNA on a sequencing gel

DNA fragments from the HindIII fragment F which were 5'-end labeled with ^{32}P were sequenced by the chemical procedure of Maxam and Gilbert (Materials and Methods). Five separate reactions were employed which specifically cleave the end labeled DNAs at G, G+A, C+T, C or A>C. These five DNA samples, each containing approximately 50,000 to 100,000 counts were loaded in sample buffer onto a 5% polyacrylamide-7M urea gel and electrophoresed at 15-20 mA until the XC dye reached the bottom of the gel. After electrophoresis the wet gel was covered with saran wrap and exposed to X-ray film at -70°C, in the presence of an intensifying screen. As an example, a stretch of 14 nucleotides are identified at the right.

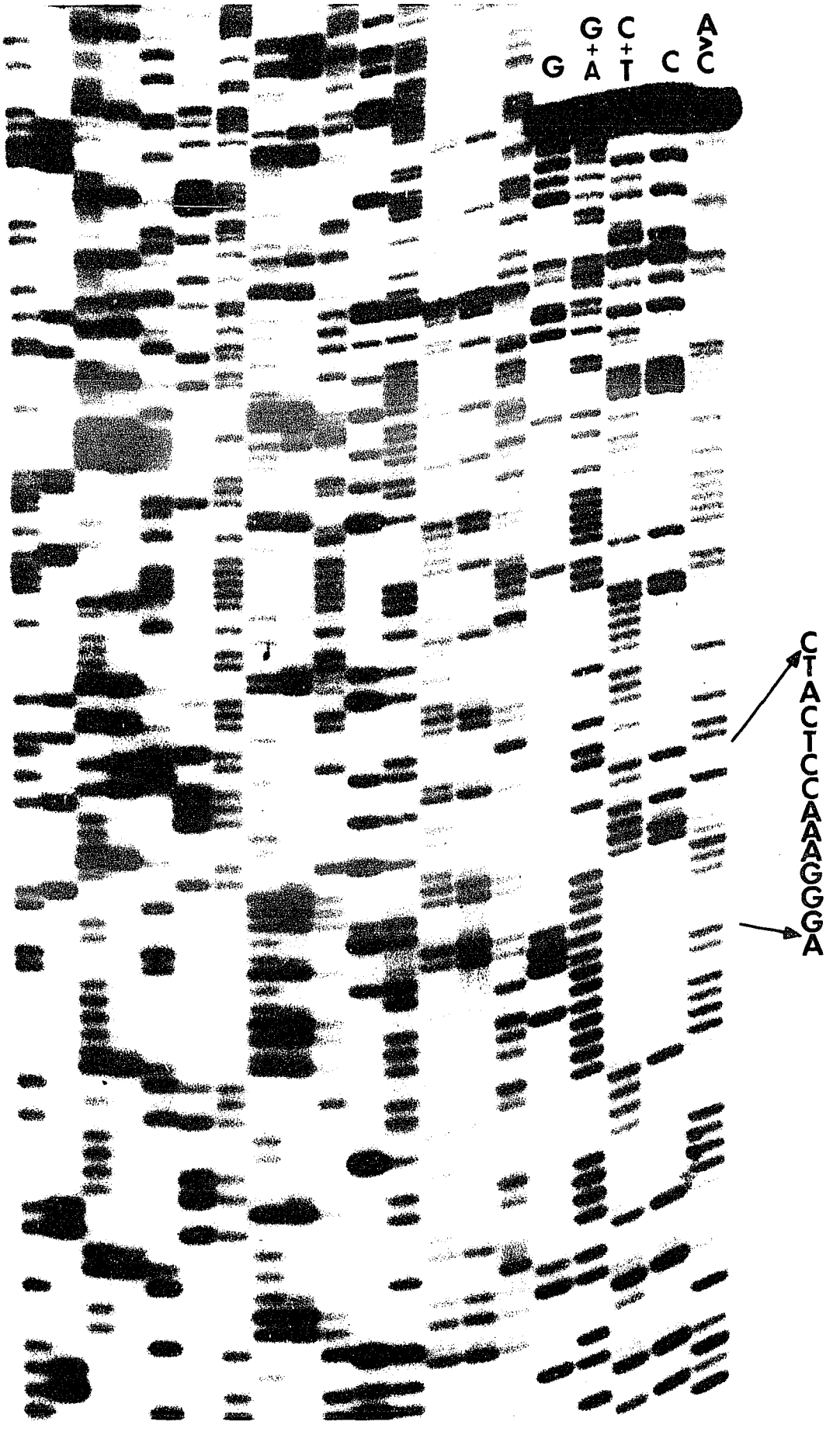

G+A
C+T
C
A>C
CTACTCCAAAGGGA

FIGURE 22

DNA sequence of the ø105 HindIII fragment F as determined by the Maxam and Gilbert procedure

The DNA sequence presented in this figure shows the HindIII, PvuII, and EcoRl restriction sites which were used for the plasmid constructions previously discussed (Results, Section I). The start codons for the putative proteins shown in Fig.23 are underlined and numbered. Also shown are the recognition signals which correspond to the 16S ribosome binding sites (dark solid lines) and the -10 (small dotted lines) and -35 (large dotted lines) RNA polymerase binding sites. The location of the termination codons for the predicted proteins are listed in Table 7.

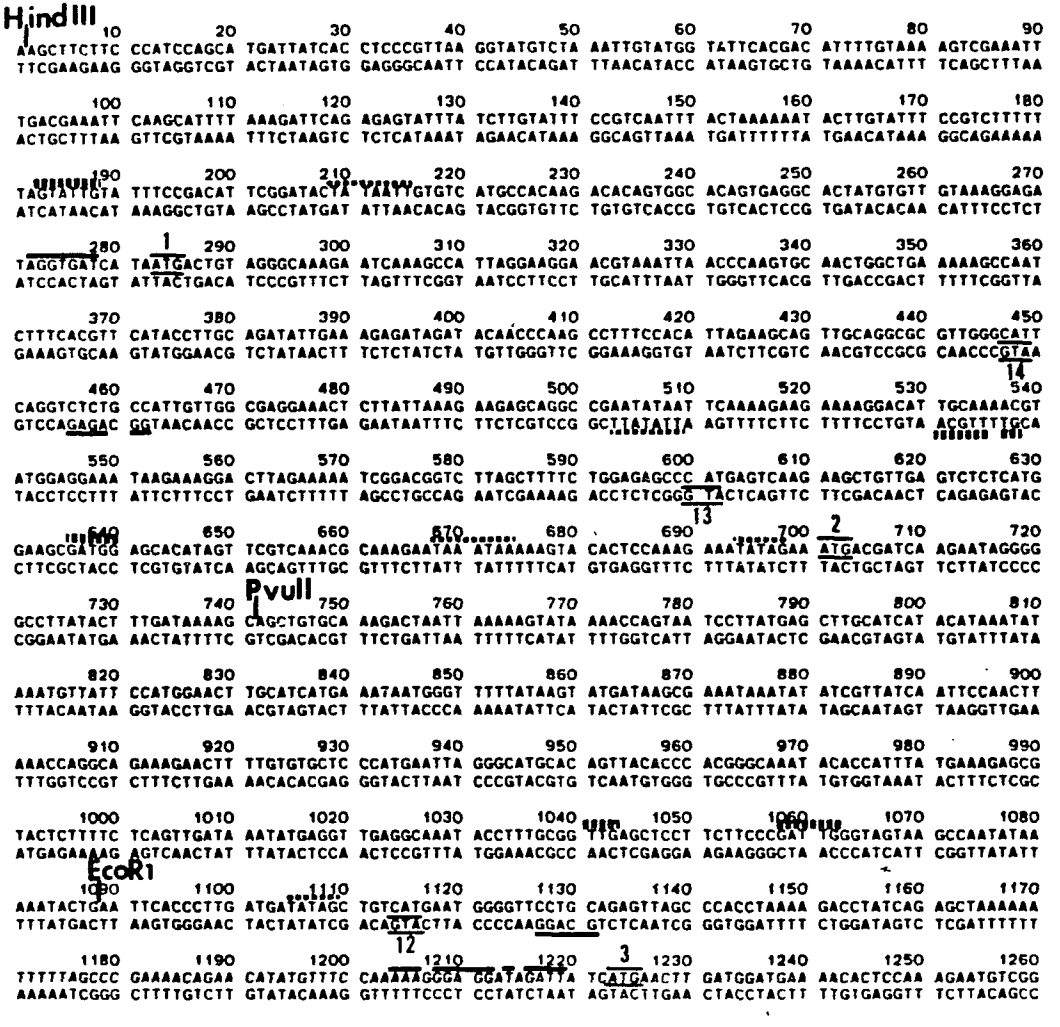


Figure 22

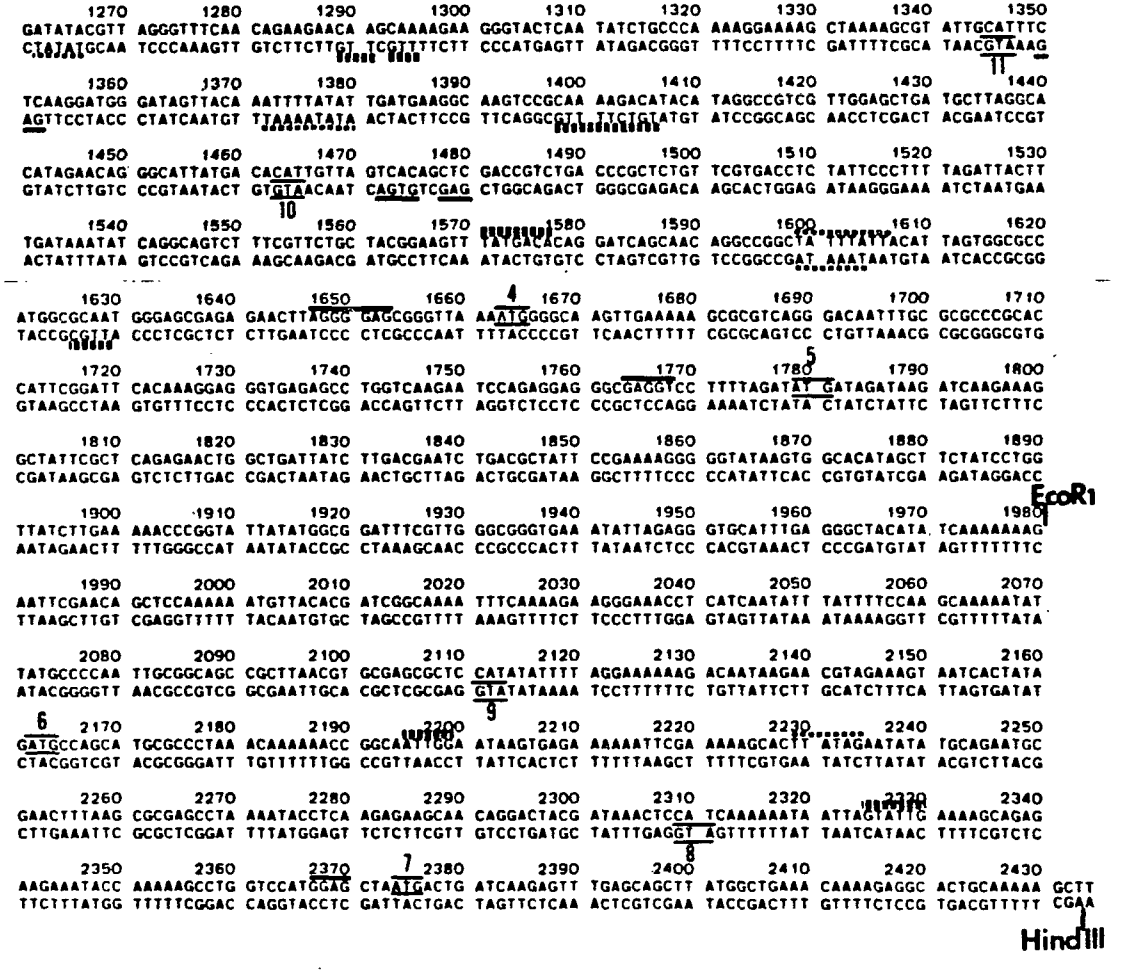


Figure 22

puter analysis revealed several open reading frames which predicted the protein map shown in Fig.23. The initiation codons which correspond to these predicted open reading frames are underlined and numbered in Fig.22. Preceding many of these open reading frames are the DNA sequences which correspond to the 16S ribosome binding site (GGAGGTGATT) and to the -10 (TATAATT) and -35 (GTATTG) RNA polymerase binding sites (Gold et al., 1981; Rosenberg and Court, 1979). The location of these recognition signals in the HindIII fragment F and the proteins associated with them are listed in Table 7. Thus, some of the DNA sequences which correspond to these predicted proteins appear to possess all the necessary recognition signals for efficient transcription and translation in both E.coli (Rosenberg and Court, 1979; Gold et al., 1981) and B.subtilis (Moran et al., 1982).

The open reading frames which are found on the 740 bp HindIII-PvuII fragment predict three proteins with molecular weights of 16,521; 7,332; and 5,516. The plasmids which contain this fragment, pAG101, pDC1, and pDC2, appear to produce a common polypeptide with the molecular weight which was estimated to be 18,000 by gel electrophoresis (Results, section II). This protein was suspected to be involved in ø105 repression and superinfection immunity. It is probable that this 18,000 molecular weight polypeptide actually corresponds to the 16,521 dalton sequence-predicted protein since the molecular weight of a protein as determined in SDS-polyacrylamide gels does not always agree with the molecular weight as predicted by DNA sequencing. An example of this discrepancy is the B-lactamase protein encoded by pBR322 which is predicted to be 31,480 daltons from the DNA sequence but is estimated on SDS-polyacrylamide gels to have a molecular weight of 27,000 (Sutcliffe, 1978).

FIGURE 23

Predicted protein map of the ø105 HindIII Fragment F

The proteins predicted from the DNA sequence of the ø105 HindIII fragment F are depicted as solid lines with arrowheads. The arrows are pointing in the direction of the predicted 5' to 3' synthesis and translation of the RNAs. The arrowhead of each protein represents a translation termination codon in the sequence (Table 7). The molecular weights of the predicted proteins are listed in Table 7.

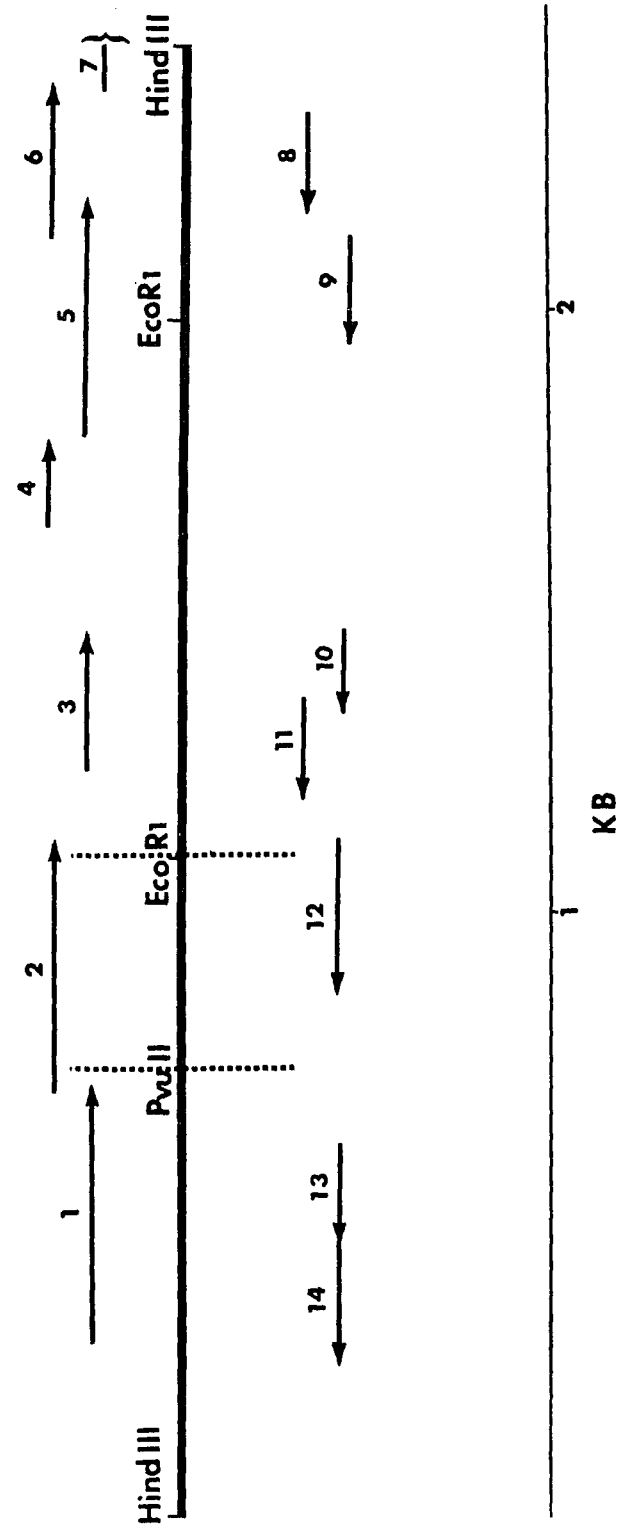

Hind III
7
6
5
4
3
2
1
Eco R1
Pvu II
Hind III
8
9
10
11
12
13
14
Eco R1
2
1
KB

TABLE 7

Location of Transcriptional and Translational Control Sequences in the ø105 Immunity Region [a]

Protein(M_r) [b]	RNA Polymerase Binding Sites [c]		Ribosome Binding Sites [d]	Translation Initiation & Termination Codons [e]
	-35	-10		
1 (16,521)	183	209	272	283- 715
2 (18,408)	636	668	none	701-1,175
3 (9,484)	1,058	1,105	1,204	1,223-1,469
4 (4,478)	1,571	1,599	1,647	1,663-1,774
5 (15,680)	none	none	1,764	1,779-2,178
6 (8,239)	none	none	none	2,162-2,372
7 (2,294)[f]	2,195	2,229	2,367	2,374- [f]
8 (5,574)	none	none	none	2,311-2,179
9 (5,863)	none	none	none	2,113-1,966
10 (5,255)	1,629	1,604	1,480	1,465-1,333
11 (6,636)	1,406	1,380	1,352	1,347-1,176
12 (9,970)	1,295	1,266	1,131	1,116- 855
13 (5,516)	none	none	none	602- 452
14 (7,332)	538	509	462	449- 254

a.) All nucleotides listed correspond to the ø105 HindIII fragment F sequence shown in Fig.22.

b.) Protein numbers correspond to those shown in Fig.23. The molecular weights (M_r) were determined from the exact amino acid composition of each predicted protein.

c.) Nucleotide position at which consensus sequences for the -35 and -10 RNA polymerase binding sites begin.

d.) Nucleotide position at which the consensus sequence for the 16S ribosome binding sites begin.

e.) Beginning nucleotides of the initiation (AUG) and termination (UAG,UAA,UGA) codons.

f.) The open reading frame for protein 7 extends past the region sequenced (see Fig.23).

B) ANALYSIS OF ø105 CLEAR-PLAQUE DELETION MUTANTS

In order to delineate the functions of the proteins predicted from the DNA sequence of the wild-type ø105 immunity region, ø105 clear-plaque deletion mutants were examined by DNA restriction and sequence analysis. The clear-plaque mutants are believed to be defective in the maintenance of phage repression while the turbid deletion mutants (see Fig.19), which actually produce hazy-plaques less turbid than wild-type phage, are believed to be defective in the establishment of lysogeny (Belfort and Wulff, 1974). Among the nonessential genes expected to be deleted in the clear-plaque mutants is the gene(s) which codes for the protein(s) responsible for the maintenance of repression. Thus, it was of interest to determine whether the clear-plaque deletions extended into the 740 bp HindIII-PvuII region which contained the open reading frames for the 16,521, 7,332, and 5,516 dalton proteins suspected to be involved in ø105 repression and superinfection immunity (see above).

Scher et al. previously had shown that the clear-plaque ø105 deletion mutant DI:1C had an altered EcoRl restriction pattern in that the EcoRl fragments F and H were missing and the EcoRl fragment B was increased in size (Scher et al., 1978). This pattern was due to the deletion of a segment of DNA that contained the two EcoRl sites which generated fragment H and the consequent fusing of the remaining portions of fragments F and B (see Fig.19). The other clear-plaque deletion mutants, DI:2C and DI:4C, and hazy-plaque deletion mutants, DI:29t and DI:1t, had been mapped by heteroduplex analysis by Flock (Flock, 1977). The approximate map positions of these respective deletions relative to the ø105 restriction map also are shown in Fig.19.

Gel analysis of EcoRl restricted DI:2C and DI:4C showed that the wt ø105 EcoRl fragments H and F are deleted in these mutants and that the EcoRl B fragments vary in size as Scher et al. had shown for DI:1C (Scher et al., 1978; Fig.24). The size of the various EcoRl

fragment Bs are as follows: wt ø105, 10,900 bp; DI:1C, 12,400 bp; DI:2C, 10,500 bp; and DI:4C, 12,000 bp. The relative sizes of the DNA deletions were calculated by determining the difference between the new B fragments and the combined sizes of the wt fragments H(909 bp), F(3,000 bp), and B(10,900 bp). DI:1C has the smallest deleted region of 2,400 bps while DI:4C has 2,800 bps deleted and DI:2C has 4,300 bps deleted.

Analysis of wild-type ø105 and the clear-plaque mutants by HindIII digestion showed that the HindIII fragment F is deleted in all three mutants (Fig.25). In the case of DI:1C a new small fragment of 1,300 bp (F_{1C}) is seen whereas the deletions in DI:2C and DI:4C result in the production of two new large fragments of 4,300 bp (B_{2C}) and 5,600 bp (A_{4C}) respectively. Since the HindIII fragment F (2434 bp) is smaller that either of these latter two fragments the DNA deletions in DI:2C and DI:4C must have resulted in the fusion of what is left of fragment F with a larger HindIII DNA fragment, namely A or A' (5,200-5,100 bp), one of which is clearly missing in the DI:2C and DI:4C patterns (Fig.25). In the case of DI:1C the migration of A and A' is not affected and a new fragment smaller than F appears (F_{1C}). To explain this and also take into account the fact that the deletion in DI:1C spans EcoRl fragment H as well as Flock's heteroduplex analysis, which showed that DI:1C, DI:2C, and DI:4C terminated at their left ends in approximately the same site (Flock, 1977), we postulated the existance of a heretofore undetected HindIII fragment designated X in Fig.19. The deletions in DI:2C and DI:4C span fragment X fusing the remaining portions of fragments F and A (A'). The deletion in DI:1C, however, terminates in fragment X producing the shortened F_{1C} fragment (see Fig. 19).

HindIII digestion of DI:2C also results in an additional HindIII band of approximately 2000 bp (Fig.25, lane 6), which appears between bands E and G. Interestingly, EcoRl digestion of DI:2C also results in a fragment of approximately 2500 bp (Fig.24, lane C). The relative intensity of these fragments do not appear to be equal in

FIGURE 24

Agarose gel electrophoresis of EcoRl digested wild-type ø105 and ø105 clear-plaque deletion mutant DNAs

EcoRl digested DNA from wild-type ø105 was electrophoresed in Lane A. The size of these fragments in base pairs are A, 16,000; B, 10,900; C, 8,800; D, 7,900; E, 5,300; F, 3,000; G, $10^9$0; and H, 909 (Scher et al., 1977). Also present in lane A (unlabeled bands) are HinfI-digested pSV40 DNA as size markers (Materials and Methods). Lanes B, C, and D contain EcoRl digested DNA from the clear-plaque deletion mutants DI:1C, DI:2C, and DI:4C, respectively. Incomplete digestion of ø105 DI:1C resulted in two partial digestion fragments which are visible above the fragments A and E.

Figure 24

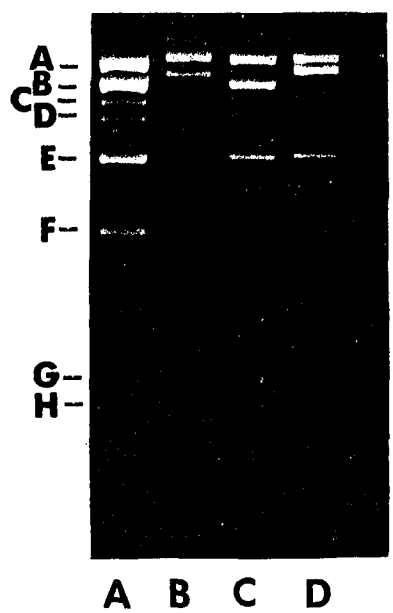
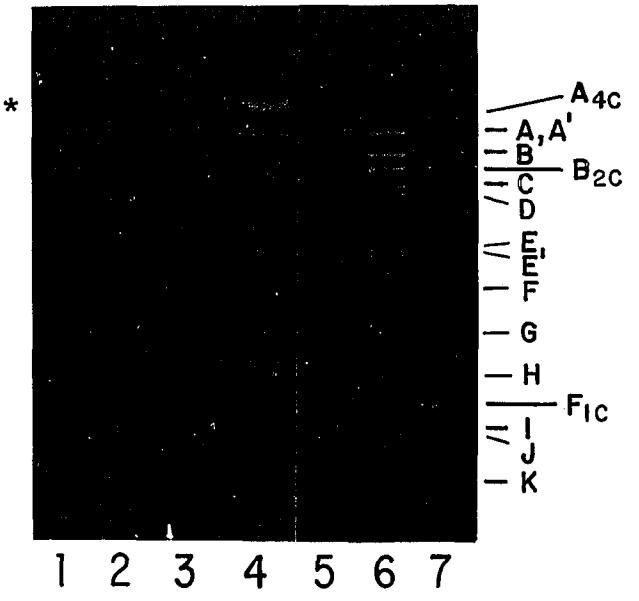


FIGURE 25

Agarose gel electrophoresis of HindIII digested wild-type ø105 and ø105 clear-plaque deletion mutant DNAs

HindIII digested DNA from wild-type ø105 was electrophoresed in Lane 4. The size of these fragments in base pairs are A,A', 5200-5100; B, 4600; C, 3900; D, 3700; E, 2800; E', 2700; F, 2430; G, 1850; H, 1500; I, 1100; J, 1050; and K, 900 (Results, Section I). Lanes 5, 6, and 7 contain HindIII digested DNA from the ø105 clear-plaque deletion mutants DI:1C, DI:2C, and DI:4C, respectively. Lanes 1, 2, and 3 also contain HindIII digested DI:1C, DI:2C, and DI:4C mutant DNAs; however, smaller amounts of DNA have been electrophoresed in order to more easily discern the large molecular weight fragments A and A' (*). The fragments which resulted from these digests are labeled at the right as they are referred to in the text.

Figure 25



molarity to the other DNA restriction fragments seen in these gels. However, the presence of these fragments can not be explained by incomplete digestion of DI:2C by EcoRl or HindIII since the 2000-2500 bp fragments were observed from several different digests. It is probable that the HindIII 2000 bp fragment is not from the immunity region because its DNA sequence does not correspond with any of the HindIII fragment F sequences (data not shown). It is possible that this 2000-2500 bp DNA fragment is copurified with ø105 DI:2C virions and is actually insensitive to both EcoRl and HindIII cleavage. At présent there is no explanation for the origin of this DNA.

The HindIII fragment F_{1C} (1300 bp) of ø105 DI:1C and HindIII fragment B_{2C} (4300 bp) of ø105 DI:2C were isolated from agarose and acrylamide gels and analyzed by DNA sequencing techniques. The DNA sequence of these fragments showed that the HindIII termini which is contained in the EcoRl fragment F (see Fig.19) has the same sequence as that found in wt ø105 DNA. The other HindIII termini of fragments F_{1C} and B_{2C} which are located in the EcoRl fragment B does not have the same sequence as the right termini of the wt ø105 HindIII fragment F. Thus, the right HindIII termini of the fragments F_{1C} and B_{2C} originate in the HindIII fragments X in DI:1C and A(A') in DI:2C (Fig.19).

The sequence of the ø105 DI:1C HindIII fragment F_{1C} showed that 497 bases of the wt ø105 HindIII fragment F sequence were conserved (Fig.26). The DI:2C ø105 HindIII fragment B_{2C} was found to conserve only 449 bases from the wt ø105 HindIII fragment F (Fig.26). Partial analysis of the ø105 DI:4C 5600 bp HindIII fragment A_{4C} showed that approximately 450 to 500 bases were also conserved from the wt ø105 sequence (data not shown). These clear-plaque mutants thus have deletions which extend into the 16,521 and 5,516 dalton proteins. The coding region of the 7,332 dalton protein is not deleted; however, the DI:2C deletion extends up to the initiation codon of this protein. The new sequence which is juxtaposed next to the 7,332 dalton protein because of this deletion is 5' to 3', GTAACCAAATGCATAATG. This sequence does not appear to be complementary to the 16S ribosome subu-

nit sequence (Moran et al., 1982) and therefore does not seem to be a functional ribosome binding site. Thus, none of the proteins predicted from the HindIII fragment F, with the possible exception of the 7,332 dalton protein, appear to be essential for ø105 lytic growth.

IV. IDENTIFICATION OF THE IMMUNITY REGION DNA STRAND TRANSCRIBED DURING ø105 LYSOGENY

The results presented above indicate that a protein is produced from the 740 bp HindIII-PvuII fragment of the ø105 immunity region which is nonessential for ø105 lytic growth and is involved in superinfection immunity and maintenance of repression during lysogeny. The molecular weights of the proteins predicted from this region are 16,521; 7,332, and 5,516 daltons. Since the coding sequence of the 16,521 molecular weight protein is on the strand complementary to the strand coding for the 7,332 and 5,516 dalton proteins, it was possible to determine which protein was produced in a stable ø105 lysogen. The phage RNA made is a stable lysogen, by analogy to other temperate phages (see Introduction), should be the mRNA for the phage repressor.

B.subtilis ø105 lysogenic RNA was mapped by hybridizing single-stranded DNA isolated from the 740 bp HindIII-PvuII fragment to RNA isolated from a ø105 lysogen. A 261 bp Hinf1-DdeI restriction fragment, which represented bases 299-561 of the immunity region, was prepared from pDC4 which was 5'-end labeled with ^{32}P, at both termini (Fig.27). The labeled DNA strands were separated and the DNA sequences of the isolated strands were determined to confirm the identity of the fragments (Materials and Methods). Fragment A was found to be 5'-end labeled at the DdeI site while fragment B was 5'-end labeled at the Hinf1 site (Fig.27).

These 5'-end labeled single-stranded DNAs were hybridized to RNA isolated from either a B.subtilis ø105 lysogen (BD99[ø105]) or a non-lysogen (BD99) as described in Materials and Methods. The resulting

FIGURE 26

Deletion map of the ø105 clear plaque mutants DI:1C and DI:2C

The exact positions of the regions deleted in the ø105 clear-plaque mutants DI:1C and DI:2C were determined by DNA sequence analysis as described in the text. The dotted lines in the figure represent the regions which are deleted in these mutants. DI:1C contains the 497th base of the wild-type HindIII fragment F while DI:2C still contains the 449th base. The 7.3 K protein start codon begins at the 449th base (Table 7).

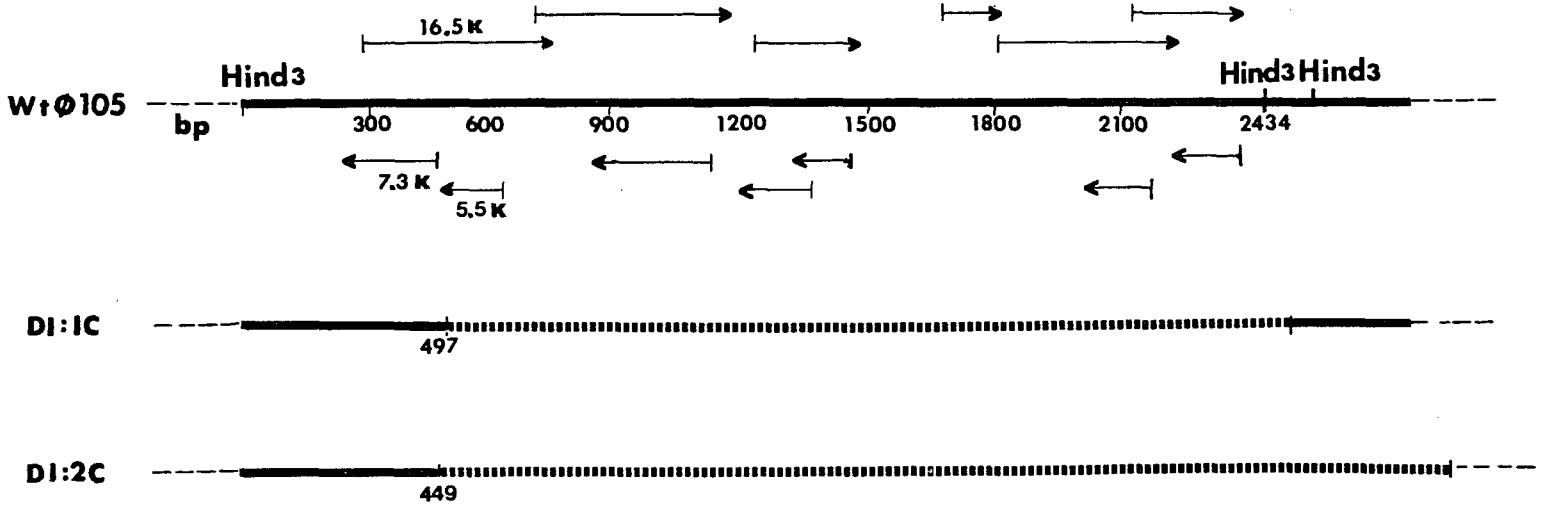

16.5 K
Hind3
Hind3 Hind3
WtØ105
bp
300
600
900
1200
1500
1800
2100
2434
7.3 K
5.5 K
DI:1C
497
DI:2C
449

Figure 26

RNA-DNA hybrids were used as templates for cDNA synthesis by reverse transcriptase. The extension products were examined on a denaturing 7M urea polyacrylamide gel (Fig.27). Extension of the hybrid which contained RNA from a B.subtilis ø105 lysogen and the 5'-end labeled fragment A DNA resulted in a DNA fragment of approximately 342 bps in length (Fig.27). Fragment A DNA did not prime cDNA synthesis when RNA from a nonlysogen was used. The 5'-end labeled fragment B did not serve as a primer for cDNA synthesis using RNA isolated either from a nonlysogen or a lysogen. Thus, it appears that B.subtilis ø105 lysogens contain RNA which can specifically hybridize to fragment A DNA and serve as a template for cDNA synthesis. Since fragment A DNA is the coding strand of the 16.5K protein, the results of this experiment suggest that the mRNA for this protein is produced in ø105 lysogenized cells and that this protein is responsible for repression.

From the data obtained from the primer extension experiment it was possible to locate the 5' terminus of the lysogen specific mRNA on the wt ø105 DNA sequence. The sequence and location of the primer A (thin line) is shown in Fig.28 as well as the DNA segment presumably generated by extension of this primer (thick line). Based on the size of the extended product (342 bp), the 3' terminus of the DNA synthesized onto the primer is estimated to lie between bases 217 to 224. Since the synthesis of the cDNA molecule most likely terminates when the reverse transcriptase reaches the 5' terminus of the RNA template, it is possible to predict that the ø105 lysogen mRNA originates at approximately base 217 to 224. In addition, the presence of an RNA polymerase binding site (promoter) at base 183 to 215 further supports the idea that transcription initiates in this region. The 5' terminal region of the mRNA would thus map proximal to the DNA sequence TATAAT (base 209), the consensus sequence for the -10 RNA polymerase binding site (Moran et al., 1982). Located 25 bases upstream from this -10 site is the DNA sequence GTATTG (base 183), which is the consensus sequence of the -35 RNA polymerase holoenzyme binding site (Moran et al., 1982). The ø105 specific lysogenic RNA then appears to be transcribed from the DNA region immediately following these RNA polymerase

FIGURE 27

Electrophoresis of products synthesized by primer extension of ø105 DNA on a 7M urea 8% polyacrylamide gel

Primers A and B were 5'-end labeled with ^{32}P, hybridized to RNA isolated from a B.subtilis ø105 lysogen (L) or nonlysogen (NL), and extended with reverse transcriptase as described in the text. The extension products were electrophoresed and detected by autoradiography. From left to right the lanes contain marker DNAs (S), primer A (-), products of the extension of primer A on lysogen (L) and nonlysogen (NL) RNA; primer B (-), and products of the extension of primer B on lysogen (L) and nonlysogen (NL) RNA. The top diagram shows the 740 bp HindIII-PvuII region of ø105 from which primers were prepared (see text). Primers A and B are complementary strands of the Hinf1-DdeI fragment.

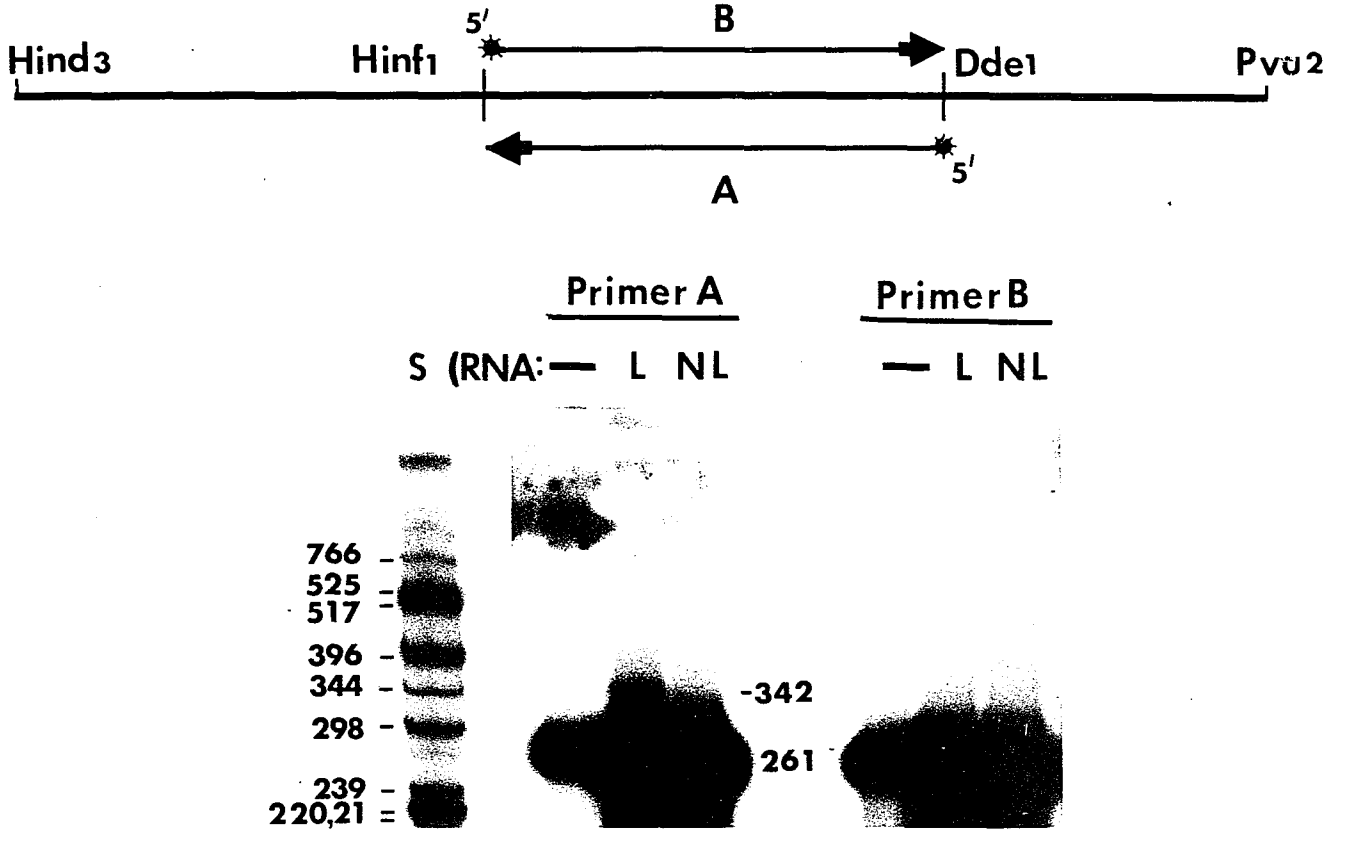

Hind3
Hinf1
5′
B
Dde1
Pvu2
A
5′
Primer A
Primer B
S (RNA: — L NL
— L NL
766
525
517
396
344
298
239
220,21
-342
261

Figure 27

recognition sites and thus this region (bases 182 to 215) appears to be serving as the promoter for the ø105 lysogen transcript.

Located throughout the region preceding this promoter-like area are sequences which show partial two-fold rotational symmetry (shadowed areas in Fig.28). Sequences which show similar types of symmetry have been found to be specific recognition sites for the phage lambda repressor (see Introduction). As in the case with the lambda repressor and the PRM promoter, the ø105 promoter-like area described above immediately precedes the region which encodes the 16,521 dalton protein (Fig.28).

The primary structure of the 16,521 dalton protein as well as control regions associated with its transcription and translation are summarized in Fig.29. The initiation codon (AUG) for this 144 amino acid protein is located at nucleotide 283. A single termination codon (UAG) is found at nucleotide 715. Preceding the initiation codon at nucleotide 272 is a stretch of eight nucleotides which are complementary to the 16S ribosomal RNAs of B.subtilis (Moran et al., 1982) and E.coli (Gold et al., 1981). Thus, it appears that an RNA transcript of this region could be efficiently translated in both B.subtilis and E.coli.

FIGURE 28

Map of the products synthesized by primer extension of ø105 DNA

The ø105 DNA sequence was taken from Fig.22. The sequence of the 5'-end labeled primer A is shown as a thin line which extends from base 564 to 302. The DNA segment generated by extension of this primer is shown as a thick line extending from base 302 to 225. The 3' terminus of this product (dotted line) is estimated to lie between base 218 and 224. The -10 (small dotted line) and -35 (thick dotted line) RNA polymerase binding sites are shown at base 219 and 183, respectively. Four regions which show two-fold rotational symmetry are shown in gray and are located at base 78, 110, 148, and 204. A map of the open reading frames found on this HindIII-PvuII fragment is shown below. The proposed 5' terminus for the mRNA which codes for the 16.5 K protein is shown as a dotted line.

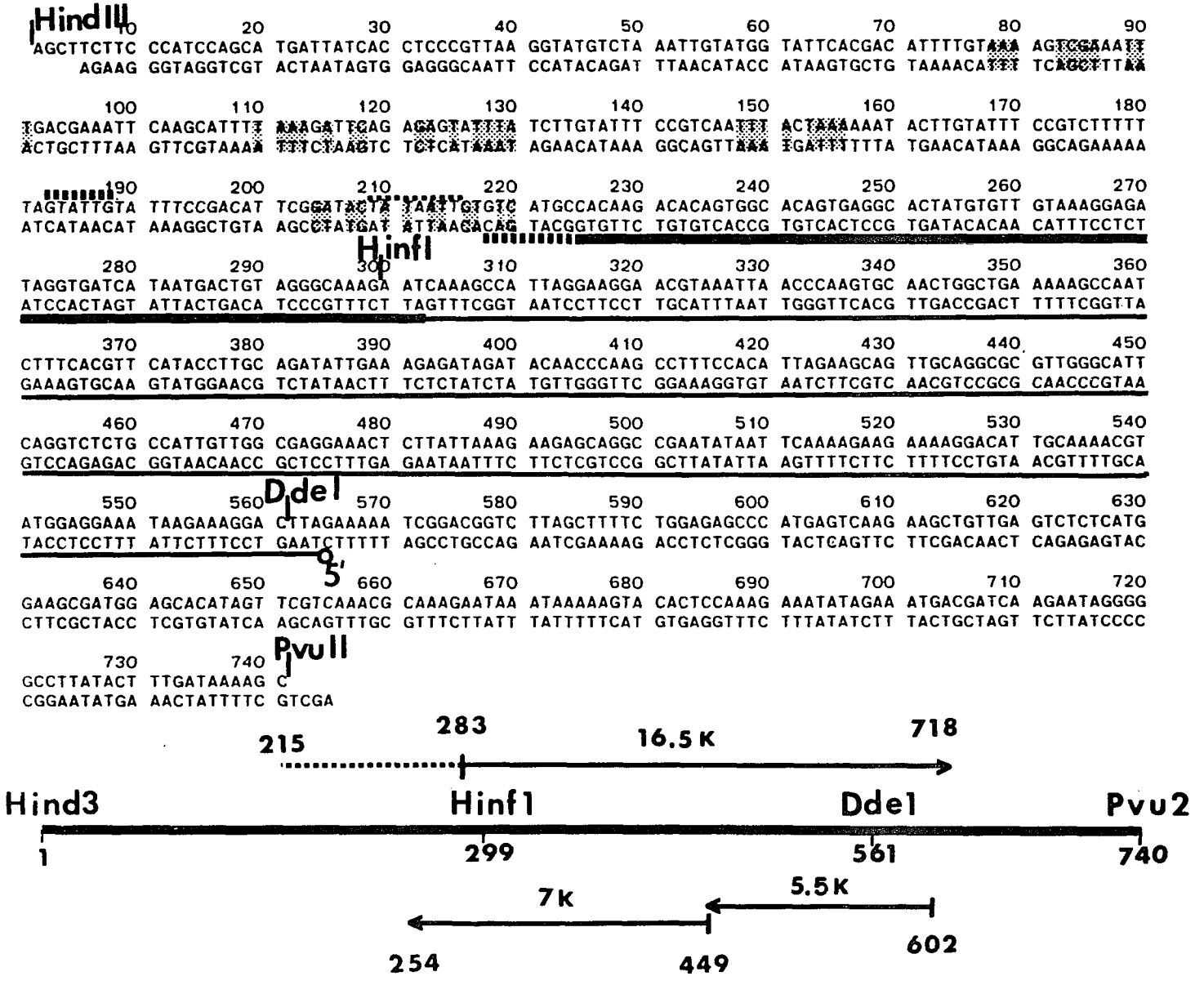

HindIII
Hinf I
Dde I
Pvu II
215
283
16.5 K
718
Hind3
Hinf1
Dde1
Pvu2
1
299
561
740
7K
5.5K
254
449
602

Figure 28

FIGURE 29

Amino acid sequence of the ø105 16.5K dalton protein

The ø105 DNA sequence was taken from Fig.22. The amino acid sequence of the 16.5K dalton protein is shown as a solid line. The initiating methionine is shown in a box at base 283 while the single termination codon, TAG, is shown thickly underlined at base 715. Eight nucleotides complementary to the E.coli and B.subtilis 16S ribosomal RNAs are shown as a thick line at bases 272 to 278. The -10 (small dotted line) and -35 (thick dotted line) RNA polymerase binding sites are shown at base 219 and 183, respectively.

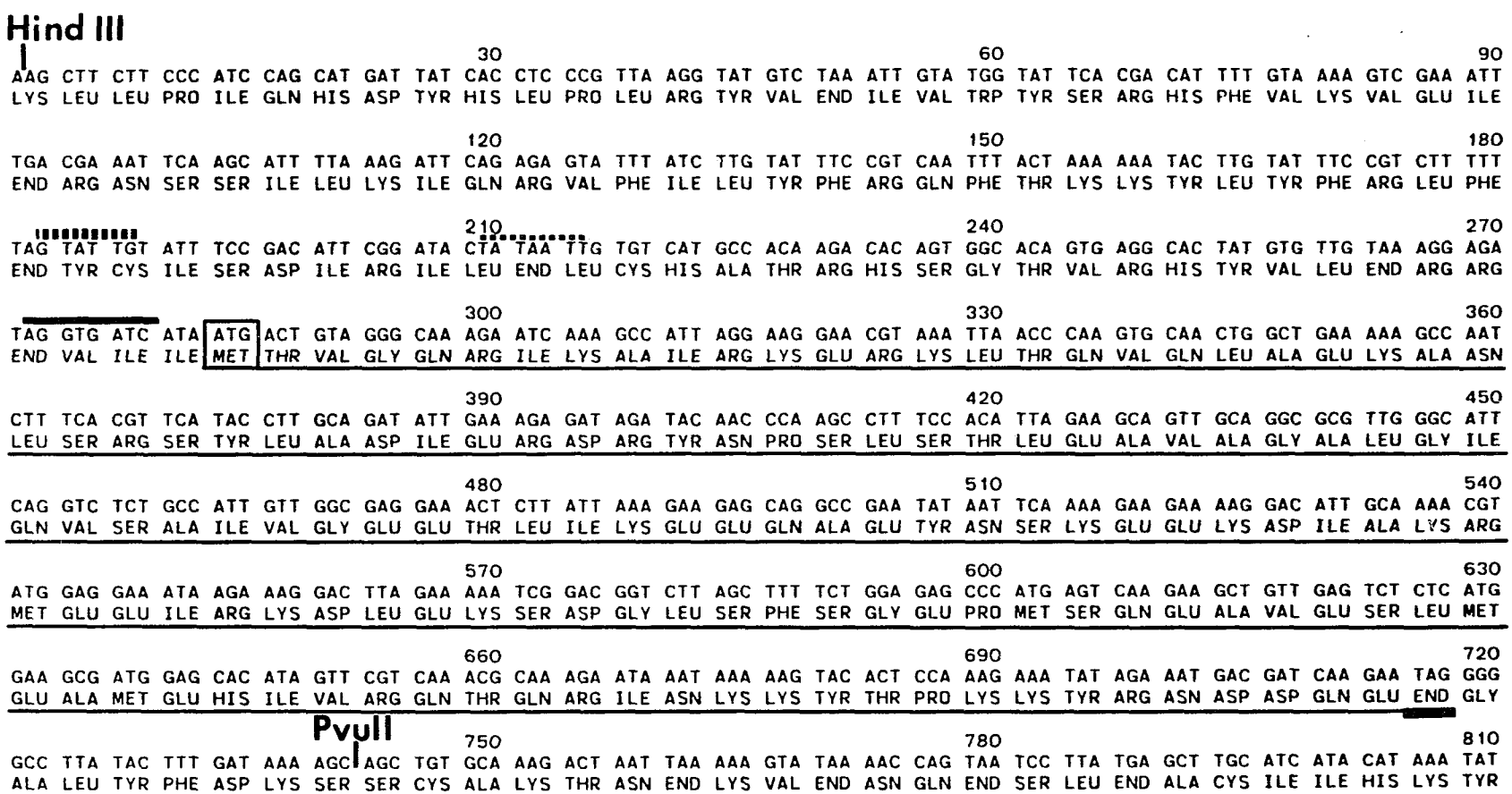

Hind III
30
60
90
AAG CTT CTT CCC ATC CAG CAT GAT TAT CAC CTC CCG TTA AGG TAT GTC TAA ATT GTA TGG TAT TCA CGA CAT TTT GTA AAA GTC GAA ATT
LYS LEU LEU PRO ILE GLN HIS ASP TYR HIS LEU PRO LEU ARG TYR VAL END ILE VAL TRP TYR SER ARG HIS PHE VAL LYS VAL GLU ILE
120
150
180
TGA CGA AAT TCA AGC ATT TTA AAG ATT CAG AGA GTA TTT ATC TTG TAT TTC CGT CAA TTT ACT AAA AAA TAC TTG TAT TTC CGT CTT TTT
END ARG ASN SER SER ILE LEU LYS ILE GLN ARG VAL PHE ILE LEU TYR PHE ARG GLN PHE THR LYS LYS TYR LEU TYR PHE ARG LEU PHE
210
240
270
TAG TAT TGT ATT TCC GAC ATT CGG ATA CTA TAA TTG TGT CAT GCC ACA AGA CAC AGT GGC ACA GTG AGG CAC TAT GTG TTG TAA AGG AGA
END TYR CYS ILE SER ASP ILE ARG ILE LEU END LEU CYS HIS ALA THR ARG HIS SER GLY THR VAL ARG HIS TYR VAL LEU END ARG ARG
300
330
360
TAG GTG ATC ATA ATG ACT GTA GGG CAA AGA ATC AAA GCC ATT AGG AAG GAA CGT AAA TTA ACC CAA GTG CAA CTG GCT GAA AAA GCC AAT
END VAL ILE ILE MET THR VAL GLY GLN ARG ILE LYS ALA ILE ARG LYS GLU ARG LYS LEU THR GLN VAL GLN LEU ALA GLU LYS ALA ASN
390
420
450
CTT TCA CGT TCA TAC CTT GCA GAT ATT GAA AGA GAT AGA TAC AAC CCA AGC CTT TCC ACA TTA GAA GCA GTT GCA GGC GCG TTG GGC ATT
LEU SER ARG SER TYR LEU ALA ASP ILE GLU ARG ASP ARG TYR ASN PRO SER LEU SER THR LEU GLU ALA VAL ALA GLY ALA LEU GLY ILE
480
510
540
CAG GTC TCT GCC ATT GTT GGC GAG GAA ACT CTT ATT AAA GAA GAG CAG GCC GAA TAT AAT TCA AAA GAA GAA AAG GAC ATT GCA AAA CGT
GLN VAL SER ALA ILE VAL GLY GLU GLU THR LEU ILE LYS GLU GLU GLN ALA GLU TYR ASN SER LYS GLU GLU LYS ASP ILE ALA LYS ARG
570
600
630
ATG GAG GAA ATA AGA AAG GAC TTA GAA AAA TCG GAC GGT CTT AGC TTT TCT GGA GAG CCC ATG AGT CAA GAA GCT GTT GAG TCT CTC ATG
MET GLU GLU ILE ARG LYS ASP LEU GLU LYS SER ASP GLY LEU SER PHE SER GLY GLU PRO MET SER GLN GLU ALA VAL GLU SER LEU MET
660
690
720
GAA GCG ATG GAG CAC ATA GTT CGT CAA ACG CAA AGA ATA AAT AAA AAG TAC ACT CCA AAG AAA TAT AGA AAT GAC GAT CAA GAA TAG GGG
GLU ALA MET GLU HIS ILE VAL ARG GLN THR GLN ARG ILE ASN LYS LYS TYR THR PRO LYS LYS TYR ARG ASN ASP ASP GLN GLU END GLY
PvuII
750
780
810
GCC TTA TAC TTT GAT AAA AGC AGC TGT GCA AAG ACT AAT TAA AAA GTA TAA AAC CAG TAA TCC TTA TGA GCT TGC ATC ATA CAT AAA TAT
ALA LEU TYR PHE ASP LYS SER SER CYS ALA LYS THR ASN END LYS VAL END ASN GLN END SER LEU END ALA CYS ILE ILE HIS LYS TYR

Figure 29

DISCUSSION

I. IDENTIFICATION OF THE ø105 REPRESSOR AND THE REPRESSOR GENE

From the results of the experiments described above it has been possible to identify a ø105 gene and gene product which appear to be responsible for the maintenance of repression during lysogeny. In summary, a segment of the ø105 immunity region was isolated by cloning restriction fragments from ø105 genomic DNA into B.subtilis cloning vectors. Analysis of cells which contained these hybrid plasmids showed that they were immune to superinfection by ø105. Examination of the DNA sequence of the phage DNA fragments responsible for this immune phenotype revealed several open reading frames. It was found that one of the proteins predicted from this DNA sequence roughly corresponded in molecular weight to a protein expressed by the hybrid plasmids in B.subtilis minicells. The hypothesis that the 16,521 dalton (16.5 K) protein so identified actually is repressor protein and responsible for the maintenance of lysogeny is supported by two lines of evidence. First, the DNA segment which contains the 16.5 K protein was found to be deleted in all clear-plaque ø105 deletion mutants examined, and second, a ø105 lysogen was shown to synthesize an RNA that hybridizes to the DNA strand which codes for this protein.

Initially, identification of the ø105 genes involved in the regulation of lysogeny was based on the genetic analysis of hazy and clear-plaque mutants believed to be defective in the establishment and maintenance of lysogeny, respectively. (Rutberg, 1969; M.F. Law, Ph.D. Dissertation, CUNY, NY,NY, 1977).

Mapping of these lysogen-defective mutants by heteroduplex and EcoRl restriction analysis enabled the construction of the map shown

in Fig.1. As indicated, the clear-plaque mutant ø105csi-6, which is believed to possess a defective phage repressor (see Introducton) carries a mutation which maps on the EcoRl fragment F (Scher et al., 1978). In addition, with the exception of ø105cng-2, all other ø105 clear-plaque mutants isolated to date fall into the same CI complementation group as ø105csi-6 and most probably contain mutations which map on the EcoRl fragment F (M.F. Law, Ph.D. Dissertation, CUNY, NY,NY, 1977). Furthermore, the mutations found in the ø105 clear-plaque deletion mutants also have been located on the EcoRl fragment F (Scher et al., 1978; Fig.1). Thus, the EcoRl fragment F appears to contain the gene or at least part of the gene which codes for a ø105 product that resembles the repressors found in temperate phage.

The results presented here have shown that the EcoRl fragment F does indeed produce a gene product that can render B.subtilis cells immune to ø105 infection (Fig.2). The hybrid plasmid pAG101, which contains EcoRl fragment F, was constructed by randomly cloning ø105 EcoRl restriction fragments into the B.subtilis vector pUB110. This was done in order to isolate the EcoRl DNA fragment which contained a ø105 gene that rendered B.subtilis cells immune to ø105 infection in the same manner that a ø105 lysogen is immune. As shown in Figs. 4 and 5, cells which contained pAG101 behave like immune ø105 lysogens in that they are able to adsorb phage particles but are immune to killing by phage.

Scher et al. demonstrated that in addition to EcoRl fragment F, ø105 HindIII fragment F also is partially deleted in the ø105 clear-plaque deletion mutant DI:1C (Scher et al., 1978). By screening B.subtilis cells for their immunity to ø105 infection, Marrero et al. were able to isolate a plasmid, pPL1004, which contained the 2,436 bp ø105 HindIII fragment F which produced the same phenotype as pAG101 (Marrero et al., 1980). Restriction enzyme analysis of pAG101 and pPL1004 DNAs showed that both plasmids contained a common 1050 bp HindIII- EcoRl fragment (Figs. 6, 7, and 8). Thus, the gene responsible for the immune phenotype generated by the chimeric plasmids pAG101 and pPL1004 was localized to a 1050 bp HindIII-EcoRl fragment.

The potential coding regions present on the 1050 bp HindIII-EcoRl ø105 fragment were identified from the DNA sequence of the ø105 HindIII fragment F (Fig.23). Knowledge of the sequence of this region made it possible to subclone a 740 bp HindIII-PvuII fragment contained within the 1050 bp HindIII-EcoRl fragment (Fig.11), thus generating the plasmid pDC4 which also was capable of conferring ø105 immunity. The DNA sequence of the 740 bp HindIII-PvuII fragment (Fig.22) contains three open reading frames which predict proteins with molecular weights of 16,521 (16.5K); 5,516 (5.5K); and 7,332 (7.3K). Analysis of the DNA sequences immediately preceding these three open reading frames showed that only the 16.5 K and 7.3 K protein coding sequences were associated with the conserved consensus sequences for the initiation of transcription and translation (Table 7). Both open reading frames are preceded by DNA sequences which are almost identical to the conserved -35 and -10 sequences of an RNA polymerase binding site (Fig.22). In addition, sequences which are strongly complementary to Bacillus 16S rRNA (Shine-Dalgarno sequences) are found approximately 3 to 13 bases upstream from the predicted translation initiation codons of the 7.3 K and 16.5 K proteins (Fig.22). Thus, it appears possible that B.subtilis cells which contain the 740 bp HindIII-PvuII ø105 fragment may be able to synthesize both the 7.3 K and 16.5 K proteins. This idea is supported by the observation that B.subtilis minicells, which contain the 740 bp fragment, synthesize a polypeptide which most likely corresponds to the 16.5 K protein (see below for discussion). However, from the above information alone it was not possible to determine which of these predicted proteins was responsible for the immune phenotype of cells which contained the 740 bp fragment.

Analysis of ø105 clear-plaque deletion mutants defective in the maintenance of repression aided in delineating the function of the predicted 16.5 K, 7.3 K, and 5.5 K proteins. Through heteroduplex analysis Flock had shown that deletions present in the ø105 clear-plaque mutants DI:1C, DI:2C, and DI:4C all extend to what appears to be a conserved position in the EcoRl fragment F (Flock, 1977; Fig.19). Contrary to this left-hand terminus the right-hand termini of the

regions deleted are heterogeneous. The implications for the conservation of the left-hand terminus in these deletions will be discussed below. As a result of the deletions present in DI:1C and DI:2C new HindIII fragments were produced, namely F_{1C} and B2C, which were not present in the wild-type restriction digest (Fig.25). The DNA sequence of these new fragments showed that the region which corresponded to the 740 bp HindIII-PvuII fragment was partially deleted and that only the first 449 to 497 bases of this fragment was conserved (Fig.26). Partial analysis of the DI:4C deletion mutant showed that approximately 450 to 500 bases of this region also were conserved (data not shown). Thus, these clear-plaque mutants, presumably defective in the maintenance of represson, possess deletions which extend into the coding regions of the 16.5 K and 5.5 K proteins and up to the initiation codon of the 7.3 K protein. The new sequence which is juxtaposed next to the start codon for the 7.3 K protein in the DI:2C mutant is, 5' to 3', GTAACCAATGCATAATG. This sequence does not share any sequence complementarity with Bacillus 16S rRNA and therefore, the area preceding the 7.3 K coding region in the DI:2C mutant does not appear to contain a functional ribosome binding site which is believed to be required for efficient translation of mRNAs in B.subtilis (Moran et al., 1982). Thus, it appears that none of the proteins predicted from the HindIII fragment F, with the possible exception of the 7.3 K protein (discussed below), are essential for ø105 lytic growth.

The phage RNA made in a stable lysogen, by analogy with other temperate phages, should include the mRNA for the phage repressor. Since the coding sequence of the 16.5 K protein is on the strand complementary to the strand coding for the 7.3 K and 5.5 K proteins it was possible to determine whether the 16.5 K protein was transcribed in a stable lysogen. Through the use of single-stranded DNA probes derived from the 740 bp HindIII-PvuII region, a primer extension experiment was conducted in which it was possible to detect ø105 lysogen RNA that was complementary to the DNA strand which coded for the 16.5 K protein (Fig.27). Since the DNA strand which codes for the 7.3 K and 5.5 K proteins did not prime cDNA synthesis from ø105 lysogen

RNA it can be concluded that mRNA for these proteins either is not produced in a ø105 lysogen or is not detectable by these methods. The results of this experiment suggest that the mRNA for the 16.5K protein is produced in ø105 lysogenized cells and that this protein is responsible for repression and superinfection immunity in both ø105 lysogens and in cells which contain the ø105 740 bp HindIII-PvuII region.

A) PROMOTER FOR MAINTENANCE OF REPRESSION

The data obtained from the primer extension experiments also made it possible to locate the 5' terminus of the lysogen specific mRNA on the ø105 DNA sequence (Fig.28). From the size of the primer plus the extended DNA product, the 3' terminus of the newly synthesized DNA was estimated to be between bases 217 to 224 on the HindIII-PvuII fragment shown in Fig.28. This 3' DNA terminus most likely corresponds to the 5' terminus of the RNA template and suggests that synthesis of the ø105 mRNA analyzed originates approximately at bases 217 to 224. This transcription initiation site is just downstream from two DNA sequences which correspond to the conserved sequences found in the -35 and -10 RNA polymerase binding sites. Thus, it appears that at least one of the mRNAs synthesized in a stable ø105 lysogen is transcribed from a promoter located at approximately bases 182 to 215 in the ø105 HindIII fragment F (Fig.28). The function of this region as a true promoter can only be definitely established by experimental tests. However, its excellent sequence homology to known promoters, in conjunction with the results of the primer extension experiments shown in Fig.27, provides a reasonable degree of certainty.

The evidence discussed above suggests that this promoter is the site from which repressor RNA is synthesized in a stable lysogen and is thus functionally analogous to the lambda PM promoter. The ø105 PM promoter also is similar to the PM promoter of lambda in that throughout the region there are located sequences which show partial two-fold

rotational symmetry (Fig.28). In lambda such sequences have been found to be specific recognition sites for the lambda repressor and are the regions defined as OR1, OR2, and OR3 (see Introduction) which immediately precede the lambda repressor coding sequence. It should be possible to determine whether these ø105 regions function as repressor binding sites by examining the interaction of the purified 16.5 K protein with these DNA sequences.

B) ROLE OF THE PM PROMOTER AND THE 7.3K PROTEIN IN ø105 LYTIC REPLICATION

The ø105 PM promoter and the region coding for the amino terminal 55 amino acids of the 16.5K protein are conserved in the ø105 clear-plaque deletion mutants (Fig.26). At least three possible reasons for the retention of this region in the clear-plaque mutants can be envisioned. First, the promoter responsible for the synthesis of the repressor in a stable lysogen (PM) may also be functional during the lytic cycle of phage growth, as in the case of the PR and PM promoters of lambda. In the case of lambda this region actually contains two promoters which can initiate transcription in opposite directions, PM for the repressor and PR for the cro protein. The sequence of the 450 bases conserved in the ø105 clear-plaque mutants (Fig.26) shows an open reading frame, for the 7.3K protein, which would be coded by RNA transcribed in the opposite direction of that used for the 16.5K repressor (Fig.23). However, Williams et al. have tested the PM promoter for activity in the leftward direction by fusing a chloramphenicol resistance gene onto the HindIII end of the 1050 bp HindIII- EcoRI fragment contained in pDC1 and failed to detect any transcription (Williams et al., 1981).

The second potential reason for the conservation of the first 449 bases of the ø105 HindIII fragment may be due to the need for an efficient PM promoter to transcribe other genes required for lytic replication. In this case the PM promoter would fuse with ø105 late

genes which would in turn utilize this promoter for efficient transcription. This alternative seems unlikely however, since all three clear-plaque deletion mutants would, by virtue of their different size deletions, have selected for the transcription of three different regions each needed for efficient lytic growth. Nevertheless, it has been shown, by Williams et al. that a chloramphenicol resistance gene can be transcribed from a promoter located on the ø105 1050 bp HindII-I-EcoRI fragment contained in pDC1 when the gene is fused to the EcoRI terminus (Williams et al., 1981). The location of the promoter utilized in this experiment is not known but the DNA sequence of this HindIII-EcoRI fragment shows the presence of an open reading frame (protein 2 in Fig.23) which overlaps the repressor coding region and continues past the EcoRI site. The region immediately preceding this open reading frame also contains the conserved DNA sequences associated with RNA polymerase binding but does not contain a region complementary to the 16S ribosomal RNA. Thus, it it not clear whether the promoter utilized in the transcription of the chloramphenicol resistance gene is the PM promoter or the promoter preceding the second open reading frame.

A third possible explanation for conservation in clear-plaque deletion mutants of the first 449 bases of the HindIII fragment F proposes a function for the 7.3K protein. In the clear-plaque deletion mutants analyzed it appears that the region coding for the 7.3K protein remains intact. Thus, it is possible that the 7.3K protein is a ø105 product required for lytic phage replication. If this is the case then each fusion which resulted from the different deletions must, by virture of selective pressure, introduce a functional promoter in front of the 7.3K protein. Unfortunately, the DNA sequence of the new regions upstream from the 7.3K protein which resulted from these fusions were not determined. This information might reveal the presence of promoter consensus sequences which were donated from different regions of the ø105 genome. The DNA sequences which were obtained from the deletion mutant DI:2C does show, however, that the new fusion does not introduce a region which is complementary to the

16s rRNA. It might be possible to determine whether the putative 7.3K protein is essential for lytic growth by examining the DNA sequences of the CI mutants which map in this region. Since the mutations carried by these clear-plaque mutants do not block lytic replication, they would not be expected to affect the function of the 7.3K protein and therefore should be restricted to the region of the 16.5K coding region which does not overlap the 7.3K coding region. Such experiments should aid in elucidating the function of the 7.3K protein and the PM promoter region during ø105 lytic growth.

II. ANALYSIS OF PROTEINS SYNTHESIZED IN B.SUBTILIS MINICELLS AND E.COLI MAXICELLS

To identify polypeptides associated with ø105 superinfection immunity, proteins encoded by the appropriate recombinant plasmids, pAG101, pDC1, and pDC2 (Figs. 2,9,and 11) were examined by SDS-polyacrylamide gel electrophoresis. These plasmids all contain the 740 bp HindIII-PvuII ø105 fragment which is sufficient to render cells immune to ø105 infection and, as discussed above, encodes the 16.5K protein believed to be the ø105 repressor. By analyzing proteins encoded by these plasmids in B.subtilis minicells and E.coli maxicells it was possible to identify a single polypeptide of approximately 18K daltons which is common to all 3 plasmids. Since this protein is most likely responsible for the immune phenotype of cells which contain the 740 bp HindIII-PvuII region it thus appears that the 18K polypeptide detected by gel electrophoresis corresponds to the 16.5K protein, predicted by DNA sequence, which is believed to be the ø105 repressor.

The plasmid pAG101, which contains the 3000 bp EcoR1 fragment F and is able to confer superinfection immunity to B.subtilis cells, synthesizes eight polypeptides in B.subtilis minicells, three of which are coded by the vector plasmid pUB110 (Fig.13). Of the five pAG101-specific polypeptides, the 27.5K protein most probably is the

ø105 J gene product (Fig.16) and the 30.5K protein may possibly be the ø105 S7 virion polypeptide (Fig.13). The remaining unidentified pAG101-specific polypeptides, which are approximately 47K, 20K and 18K daltons, most likely include the ø105 protein responsible for superinfection immunity and other polypeptides which result from the fusion of plasmid and phage DNA sequences. Since the DNA sequence of the vector plasmid pUB110 and the complete EcoR1 fragment F are not available it is not possible to determine which of these polypeptides may be fusion proteins. However, the 1050 bp HindIII-EcoR1 region of the EcoR1 fragment F (Fig.9), which is present in the plasmid pDC1, has been sequenced and contains four open reading frames, one of which extends past the EcoR1 site (protein 2, Fig.23). Examination of proteins synthesized from pDC1 in E.coli maxicells (Fig.17) shows two pDC1-specific polypeptides of 19.3K and 18K daltons, which are not synthesized by the vector plasmid pBR322. In addition, the plasmid pDC2, which contains the 740 bp HindIII-PvuII immunity region discussed above, synthesizes two specific polypeptides of 19.5K and 18K daltons (Fig17). Thus, all three plasmids that contain the ø105 repressor gene synthesize a 18K protein which roughly corresponds in size to the 16.5K ø105 repressor. It is not unreasonable that the molecular weight of the repressor peptide appears to be slightly larger than that predicted by the DNA sequences, given the inaccuracies in measuring the size of proteins in SDS-polyacrylamide gels.

As mentioned above, the additional plasmid-coded polypeptides may result from the fusion of ø105 and vector plasmid DNAs. Williams et al. have shown that a chloramphenicol resistance gene can be transcribed from a promoter located on the 1050 bp HindIII-EcoR1 fragment when the gene is fused to the EcoR1 terminus (Williams et al., 1980). Thus, it is possible that the pAG101 47K and 20K polypeptides and the pDC1 19.3K polypeptide are products of a transcript which originates in ø105 DNA and extends into the vector plasmid pUB110 or pBR322. If this is the case then the fusion polypeptides could result from the attachment of a truncated protein 2 with amino acids coded by either pUB110 or pBR322. The actual molecular weight of the truncated pro-

tein 2 is 15,500 as determined from the first methionine to the last amino acid coded from the region immediately preceding the EcoR1 site. The DNA sequence of pBR322 reveals an open reading frame at the EcoR1 site which could produce an elongated protein 2 with a final size of 17,300 daltons (Sutcliffe, 1979). If this fusion protein is produced in pDC1-containing E.coli maxicells, it most likely corresponds to the 19.3K polypeptide seen on SDS-polyacrylamide gels (Fig.17). This conclusion is based on the fact that pDC2-containing cells, which no longer contain the coding region for protein 2, do not synthesize the 19.3K polypeptide The location of the region which codes for the 19.5K pDC2-specific polypeptide is not known. A possible fusion protein can be produced from pDC2, however, with only 14aa from protein 2 and 49aa from pBR322, resulting in a putative polypeptide of approximately 7K daltons as determined from the DNA sequence. This putative fusion protein would terminate at a single TGA nonsense codon in the pBR322 sequence which is followed 12 codons downstream by a second single TAA nonsense codon. Thus, the DNA sequence of both the ø105 and pBR322 HindIII-PvuII fragments do not contain open reading frames which predict a polypeptide of 19.5K daltons. It is possible that the pBR322 DNA used for these experiments does not contain the same sequence as that determined by Sutcliff and perhaps there is an open reading frame which could account for a larger fusion protein (Sutcliffe, 1979).

The analysis of ø105 directed transcription by Williams et al. has supported the hypothesis that the ø105 protein 2, or 18.4K protein, is transcribed in B.subtilis cells. The promoter active in the transcription of the 18.4K protein has not been identified. The region immediately preceding the 18.4K protein initiation codon contains DNA sequences which resemble an RNA polymerase binding site but lack a Shine-Dalgarno sequence required for the initiation of translation (Table 7). The genes which code for the 16.5K and 18.4K proteins are in different reading frames which overlap each other (Fig.23). This organization has been observed in other bacteriophages, in particular the DNA phage G4 (Godson et al., 1978), and the RNA phage MS_2 (Kastelein et al., 1982). The translation of the 2nd protein in the

MS_2 phage is dependent on the ribosome binding at the Shine-Dalgarno sequence of the 1st protein. The presence of a Shine-Dalgarno sequence for the 2nd protein appears to be dispensable for a translational restart. Thus, the PM promoter, which is active in a ø105 lysogen, could be used to synthesize RNA that would encode both the 16,5K and 18.4K proteins. Further detailed experiments are needed to determine if this translational mechanism is active in ø105.

The ø105 proteins synthesized in B.subtilis minicells following ø105 infection have been examined both by J. Reeve and this study (Reeve, 1977, Fig.16). While Reeve has shown that 23 phage-specific polypeptides are synthesized during a 55 min infection, this study has detected only six major polypeptides during a 75 min infection (Fig.17). However, the estimated size of the proteins observed in both experiments are similar. The difference in the number of ø105 proteins detected is most probably due to the variability seen in different B.subtilis minicell preparations. Six of the ø105 proteins observed by Reeve were identified as early gene products on the basis of their ability to be transcribed by non-modified host RNA polymerase (Reeve, 1977). These early proteins have estimated sizes of 102K, 29.5K, 22K, 20.3K, 18K, and 8.5K daltons. Among the early gene products of temperate phage are the proteins required for the establishment and maintenance of repression. It is likely, therefore, that some of the early ø105 gene products may possess these functions. Thus, it is not surprising that an 18K polypeptide is among the early ø105 proteins since the ø105 repressor has been identified as an 18K polypeptide on SDS-polyacrylamide gels (Fig.17). In addition, the 20.3K and 8.5K early gene products are similar in size to some of the predicted proteins coded by the ø105 HindIII fragment F (Table 7). Since the ø105 CII and CIII hazy-plaque mutants are located in this region (see Introduction) it is possible that these putative proteins are involved in the establishment of lysogeny.

The inhibition of protein synthesis during infection of a ø105 lysogen has been examined in B.subtilis minicells derived from either

a ø105 lysogen (Reeve, 1977) or from cells containing the immune plasmid pAG101 (this study). The ø105 lysogen-derived minicells support the synthesis of 10 phage-specific polypeptides; the most predominant having the molecular weight of 102K, 41K, 29.5K, 22K, 18K, and 7K. Some of these ø105 proteins synthesized in immune cells including the 18K repressor, are similar in size to the early ø105 proteins. It is surprising, however, that so many proteins continue to be produced in an immune cell. This may reflect the inability of residual repressor molecules to completely turn off transcription of the incoming phage genes. In comparison, B.subtilis minicells which contain the plasmid pAG101 do not permit the synthesis of any ø105 proteins other than those coded by the plasmid (Fig.18). Thus, the level of active repressor in these cells is probably sufficient to inhibit the transcription of the infecting phage genes.

The infection of pAG101-containing cells results in the stimulation of repressor synthesis, as is evident by the increased rate of 18K protein synthesis seen in Fig.18. A possible mechanism for this stimulation is that the incoming phage genomes titrate the free repressor in the cell, thus causing the repressor gene, present either on pAG101 or phage DNA, to derepress and increase its° rate of transcription. In addition, the results shown in Fig.18 suggest that other pAG101-coded proteins, such as the 30.5K and 20K proteins, may actually decrease in synthesis during phage infection. However, this observation must be examined more carefully before definitively making these conclusions.

III. COMPARISON OF ø105 WITH GRAM-NEGATIVE TEMPERATE BACTERIOPHAGE

The organization of the ø105 immunity region differs from that found in other temperate phage, such as lambda where the order of the genes located near the immunity region are CIII-N-rex,CI-CRO-CII. Both the N and cro genes required for lytic growth as well as

the CII and CIII genes responsible for the establishment of lysogeny, flank the CI repressor gene. Contrary to lambda, genetic and physical studies with ø105 clear and hazy-plaque mutants place the CI repressor gene on one side of the CII and CIII genes (see Introduction). Since the region spanning the CI and CII, CIII genes appears to be nonessential for lytic growth (Fig.19) it seems unlikely that a gene homologous in function to cro or N is located between these genes. Thus, the mechanisms by which ø105 regulates the establishment of a lysogen may differ from that found in gram-negative temperate phage.

Information on the interaction of DNA-binding proteins with DNA has been obtained largely from extensive studies on the lambda repressor and cro proteins. Since these proteins have been purified it has been possible to determine their three-dimentional structure which shows the presence in both proteins of an alpha helix-turn-alpha helix structure that is believed to play a role in the interaction of these proteins with DNA (Anderson et al., 1981; Pabo and Lewis, 1982). Other gram-negative DNA-binding proteins that show amino acid homologies with the CI and cro proteins especially in the alpha helix-turn-alpha helix region are shown in Fig.30 (Sauer et al., 1982). Four positions in these sequences, namely the lambda repressor residues 33, 37, 41, and 47, are highly conserved and are believed to be important in determining the relationship between the helical domains in the repressor protein. Sauer et al. believe that the gly at position 41 is required for the molecules to turn at this region(Sauer et al., 1982). In addition, the lambda repressor and these DNA-binding proteins in general, contain hydrophobic amino acids at residues 36, 40, 42, and 50 which are believed to form part of the hydrophobic core of the amino-terminal repressor domain, and hydrophilic amino acids at residues 33-35, 38-39, 43-46, and 48-49. Therefore, with these requirements as guidelines, the ø105 CI repressor protein was aligned with the alpha-helices of these DNA-binding protein sequences. As shown in Fig.30, the ø105 repressor possesses a region fairly homologous to these proteins which includes three of the four highly conserved residues at positions 37, 41, and 47. The residues also show

FIGURE 30

Alignment of amino acid sequences of DNA-binding proteins in regions corresponding to alpha helices of the lambda repressor and cro proteins.

The sequences of the top eleven proteins and their optimal alignments were taken from Sauer et al. (Sauer et al., 1982). The numbering at the top of the figure corresponds to amino acid residues of the lambda repressor. Underlined amino acids represent highly conserved residues. Residues marked with a dotted line are believed to be amino acids which contact DNA in proteins which recognize similar DNA binding sites (Sauer et al., 1982). The bottom numbering refers to the amino acid residues of the ø105 repressor. The alignment of the ø105 repressor was determined as described in the text.

	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
Lambda R	GLN	GLU	SER	VAL	ALA	ASP	LYS	MET	GLY	MET	GLY	GLN	SER	GLY	VAL	GLY	ALA	LEU	PHE	ASN
Lambda cro	GLN	THR	LYS	THR	ALA	LYS	ASP	LEU	GLY	VAL	TYR	GLN	SER	ALA	ILE	ASN	LYS	ALA	ILE	HIS
P22 R	GLN	ALA	ALA	LEU	GLY	LYS	MET	VAL	GLY	VAL	SER	ASN	VAL	ALA	ILE	SER	GLN	TRP	GLN	ARG
P22 cro	GLN	ARG	ALA	VAL	ALA	LYS	ALA	LEU	GLY	ILE	SER	ASP	ALA	ALA	VAL	SER	GLN	TRP	LYS	GLU
434 R	GLN	ALA	GLU	LEU	ALA	GLN	LYS	VAL	GLY	THR	THR	GLN	GLN	SER	ILE	GLU	GLN	LEU	GLU	ASN
434 cro	GLN	THR	GLU	LEU	ALA	THR	LYS	ALA	GLY	VAL	LYS	GLN	GLN	SER	ILE	GLN	LEU	ILE	GLU	ALA
Lambda CII	THR	GLU	LYS	THR	ALA	GLU	ALA	VAL	GLY	VAL	ASP	LYS	SER	GLN	ILE	SER	ARG	TRP	LYS	ARG
P22 CI	GLN	ARG	LYS	VAL	ALA	ASP	ALA	LEU	GLY	ILE	ASN	GLU	SER	GLN	ILE	SER	ARG	TRP	LYS	GLY
434 CII	THR	GLU	LYS	THR	ALA	GLU	ALA	VAL	GLY	VAL	ASP	LYS	SER	GLN	ILE	SER	ARG	TRP	LYS	ARG
GAL R	ILE	LYS	ASP	VAL	ALA	ARG	LEU	ALA	GLY	VAL	SER	VAL	ALA	THR	VAL	SER	ARG	VAL	ILE	ASN
LAC R	LEU	TYR	ASP	VAL	ALA	GLU	TYR	ALA	GLY	VAL	SER	TYR	GLN	THR	VAL	SER	ARG	VAL	VAL	ASN

	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
ø105 R	LEU	GLU	ALA	VAL	ALA	GLY	ALA	LEU	GLY	ILE	GLN	VAL	SER	ALA	ILE	VAL	GLY	GLU	GLU	THR

**********HELIX 2 ********* TURN **********HELIX 3 **********

conservation of the hydrophobic and hydrophilic residues discussed above. Thus, it is possible that the ø105 repressor, which by analogy to other phage repressors binds DNA, contains an alpha helix-turn-alpha helix structure similar to the gram-negative DNA-binding proteins. It is interesting to note that this alignment would place the alpha helix domains near the middle of the ø105 repressor which would be unique, since all the DNA-binding proteins shown in Fig.30 possess this domain closer to their amino termini.

The ø105 repressor is transcribed in a stable lysogen from the PM promoter which is located approximately 100 bases upstream from the 16.5K initiation codon (Fig.28). Surrounding the PM promoter are regions which exhibit two-fold rotational symmetry. Regions also showing two-fold symmetry are found near the lambda PM promoter and serve as the CI and cro protein binding sites. These three binding sites are each 17 bp long and are separated from each other by spacers 5 to 7 bp long. One side of such a binding site resembles the sequence 5°-TATCACCGC-3° which may differ by a few bases from TAACACCGT to TACCTCTGG. The spacers separating these regions are rich in adenine and thymine. Although the ø105 symmetrical sequences do not resemble the lambda sequences many AT rich regions are located throughout the PM region. Thus, the possibility that these regions also serve as binding sites for the ø105 repressor will only be determined through DNA-binding studies with purified ø105 repressor.

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