BACTERIOPHAGE P35 AND ITS RELATIONSHIP TO THE PLASMID DNA OF SALMONELLA PULLORUM

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ABSTRACT

BACTERIOPHAGE P35 AND ITS RELATIONSHIP TO THE PLASMID DNA OF SALMONELLA PULLORUM

By

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Salmonella pullorum is closely related to the frequently studied species Salmonella typhimurium but differs significantly in ease in which genetic analysis can be performed. Recent F-prime mediated recombination experiments indicated the possibility of the presence of both prophage and plasmid DNA in S. pullorum. Experiments were, therefore, performed to analyze the prophage and extrachromosomal DNA in S. pullorum and their interrelationships.

S. pullorum was demonstrated to harbor several prophage and other inducible elements. One of these, the temperate phage P35, was characterized and shown to closely resemble phage P22 in its morphology (a hexagonal capsid 60 nm in diameter with a short tail and spikes), the size of its DNA molecule (28×10^6 daltons), its immunity properties and growth characteristics. Phage P35 spontaneously produced virulent mutants, one of which was

isolated and labeled P35c. RNA-DNA hybridization experiments indicated that phage P35 has some nucleotide sequences similar to P22.

It was demonstrated that S. pullorum contains two distinct plasmid molecules. These were isolated by lysis of lysozyme-EDTA treated cells with Brij 58 and deoxycholate and centrifugation of the lysate in a cesium chloride-ethidium bromide dye buoyant density gradient. The plasmid fractions isolated from this gradient were subsequently analyzed by neutral and alkaline sucrose gradient centrifugation. The results indicated the presence in the gradient of two species of covalently-closed, circular DNA molecules. Cosedimentation in neutral sucrose with colicin E1 DNA showed that the smaller molecule, labeled plasmid PO-1, had a molecular weight of 1.5×10^6 daltons and that the larger molecule, labeled plasmid PO-2, had a molecular weight of 45×10^6 daltons. It was calculated that there are 150 copies per host chromosome of plasmid PO-1 while there is only 1 or 2 copies of the PO-2 plasmid per chromosome. Electron micrographs of the plasmid DNA showed circular molecules with no unusual structures.

When S. pullorum MS53 was made lysogenic for phage P35 and the plasmid molecules isolated, it was found that plasmid PO-2 had been excluded. The plasmid was not regained upon curing the lysogen of the prophage. To determine if a relationship existed between the phage and

plasmid, ^{3}H-RNA was synthesized *in vitro* with RNA polymerase isolated from *Pseudomonas putida* using P35c DNA as a template. The RNA was then used in RNA-DNA hybridization experiments with isolated plasmid DNA. The results of these experiments indicated the presence of similar nucleotide sequences in the DNAs of phage P35 and plasmid PO-2. It was postulated then that the PO-2 plasmid contains phage nucleotide sequences and is excluded from lysogenic cells either by competition with the superinfecting phage for a single membrane attachment site or when the phage complements a defective plasmid function allowing it to integrate into the host chromosome.

During the course of this study it was also determined that wild type *S. pullorum* does not modify nor restrict the DNA of phage propagated on *S. typhimurium*.

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INTRODUCTION

Salmonella pullorum is a non-motile, slow-growing bacterium closely related to the extensively studied species Salmonella typhimurium. It has been demonstrated that all wild type strains of S. pullorum possess two genetic blocks inhibiting the use of inorganic sulfate (51,52) and that upon induced reversion to sulfate prototrophy, the now functional sulfite reductase enzyme is temperature sensitive (42). There has been established in S. pullorum a conjugation system which uses F' factors originating in S. typhimurium to mobilize chromosomal genes (35). During mating experiments using S. pullorum donor cells and S. typhimurium recipients, a phage was isolated which had arisen by zygotic induction. This phage was labeled P35 since it was believed to have originally existed as a prophage in S. pullorum strain MS35. Since it had been assumed that S. pullorum MS35 was non-lysogenic, it was decided that the origin and characteristics of P35 should be investigated and its relationship to Salmonella phage P22 determined.

Also, to understand fully the genetic structure of S. pullorum it would be advantageous to know if the cell

contained any genetic information existing in an extra-chromosomal state. It was, therefore, decided to undertake a study of the physical nature of the S. pullorum DNA complement, examining the cell for the presence of plasmid DNA and, if present, its relationship to the suspected prophage P35.

LITERATURE REVIEW

Part I

Plasmids and Extrachromosomal DNA

Since the initial genetic determinations of the existence in bacterial cells of extrachromosomal genetic elements, DNA corresponding to these plasmids has been isolated from many organisms by several techniques. Plasmids are now found so frequently that it may be only the exceptional cell which does not contain one. Since plasmids were assumed to consist of DNA similar to the chromosomal DNA as determined by buoyant density in CsCl, it was often necessary to transfer the plasmid first to a different host where it could be separated from the larger amount of chromosomal DNA by its density difference. This type of procedure, however, does not provide information about the structure and replication of the plasmid in its natural host. A direct procedure for the demonstration and isolation of plasmid DNA was developed by Radloff, Bauer and Vinograd (66) using the intercalating dye ethidium bromide in a preparative CsCl density gradient. Each ethidium bromide molecule which intercalates between the base pairs

of a duplex DNA molecule causes a 12 degree unwinding of the helical structure (5). As long as the ends of the duplex are free to rotate, the dye molecules can continue to bind until there is a maximum of one dye molecule bound for every four or five base pairs (88). However, if, as in the closed circular, plasmid DNA molecules, the ends are not free to rotate, then the unwinding due to the binding of the dye will be limited by the extent of superhelical structure of the molecule. The buoyant density of the DNA-dye complex is decreased as the amount of bound ethidium bromide is increased. Since the closed circular molecule cannot bind as much dye per unit length as can the open circular or linear molecules, at saturating concentrations there will be a density difference between the two forms. The closed circular molecule will be more dense than the open circular and linear forms. If these DNA-dye complexes are centrifuged to equilibrium in a CsCl density gradient, the closed circular plasmid molecules will band lower in the tube at a density greater than the open circular and linear forms.

Radloff *et al.* (66) had developed the dye-buoyant technique to isolate mitochondrial DNA from Hela cells but it was adapted for the isolation of bacterial plasmid DNA by Bazaral and Helinski (9). Using this procedure, they were able to isolate directly from crude lysates of *E. coli* plasmid DNAs which carried the genetic determinants for colicins E1, E2, and E3. These were subsequently

analyzed by sucrose gradient centrifugation and electron microscopy. In contrast to the results obtained when R factors (31,71) and colE1 DNA (36,70) were isolated from Proteus mirabilis, colE1 DNA isolated from its native host, E. coli, by the dye-buoyant density gradient procedure does not appear to be present in aberrant forms (dimers, trimers, etc.). Also, there is only a small number of copies per host chromosome rather than the multiple copies found in P. mirabilis.

ColE1 supercoiled DNA has a molecular weight of 4.6×10^6 and sediments through a neutral sucrose gradient with a sedimentation coefficient of 23s. The open circular form sediments at 17s (8).

Alkaline sucrose gradient sedimentation. When covalently closed circular plasmid DNA is centrifuged at pH values higher than 12, the molecules sediment through the gradient at a rate much faster than at neutral pH values. Vinograd et al. (86) proposed that this was due to inability of the strands of the denatured covalently-closed molecule to separate, leading, therefore, to a more compact structure of the same molecular weight. This phenomenon was utilized by Freifelder (32) in devising a method to identify the presence of plasmid DNA molecules in hosts whose chromosomal DNA has the same buoyant density as the plasmid. A lysate of E. coli containing an F'lac plasmid was vortexed to shear the large

chromosomal DNA and then layered directly on an alkaline sucrose gradient. After centrifugation the plasmid DNA was seen as a distinct peak much further down the tube than the bulk of the denatured chromosomal (single stranded) DNA. It was also demonstrated that the fast sedimenting form was converted to the slower sedimenting open circular form by a dose of X-irradiation sufficient to produce only one single strand break per molecule. This method could, therefore, be used both to verify that a DNA molecule is indeed a covalently closed circular structure and to isolate plasmid DNA.

Electron microscopy of plasmid DNA. The use of electron microscopy to visualize the plasmid DNA molecules has proven valuable in determining the topographical structure of these molecules. The most popular technique for preparing DNA molecules for electron microscopy is the protein monolayer technique of Kleinschmidt (53). The DNA is spread and adsorbed to a surface layer of denatured cytochrome c which can then be picked up on an electron microscope grid. The grids are then shadowed with heavy metals to give greater width to the DNA molecules. This technique has recently been modified by the development of a microversion (54) in which the protein film is made on the surface of a droplet containing only 40ul of solution. With this procedure as little as 4 ng of DNA are required.

Using the Kleinschmidt procedure, Hudson and Vinograd (43) have found unusual DNA structures present in the

plasmid band of a CsCl-EtBr gradient. The DNA in the gradient was isolated from Hela cell mitochondria. These molecules are multiples of the monomer form linked together as in a chain. These catenated molecules probably arise by recombination-like events between mature molecules and differ from the linear concatenated molecules which contain multiple genomes linked end to end. The latter have been proposed to arise during replication of the DNA of phages P22 and T7 (85).

Plasmids in β-hemolytic E. coli. Multiple species of plasmid DNA molecules have been found in beta-hemolytic strains of E. coli by Goebel and Schrempf (37). Three molecules were found each differing in molecular weight as shown by sucrose gradient centrifugation of the plasmid band from a CsCl-EtBr gradient and by electron microscopy. The larger transmissible molecule which has a molecular weight of 58×10^6 daltons and a sedimentation coefficient equal to 72s was tentatively associated with the production of the beta hemolysin. A molecule sedimenting at 23s (4.2×10^6 daltons) was assumed to be related to the colicinogenic character of the strain due to its non-transferability and size similarity to colE1 DNA. The third molecule was present in two forms, one sedimenting at 66s and the other at 45s. These were demonstrated to be the closed and open circular forms of a 46×10^6 MW plasmid. It sedimented at 159s in alkaline sucrose

gradient. No definite biological function could be described for this molecule though it may be part of the hemolytic factor which has dissociated from the transfer determinants.

The minute plasmid of E. coli 15. Recently Cozzarelli et al. (26) have isolated minute plasmid molecules from E. coli strain 15. They have a molecular weight of 1.5×10^6 daltons and were assumed to be the smallest known, autonomous, replicating DNA species yet observed. These molecules are extremely interesting since they could carry sufficient information to code for a polypeptide of only about 75,000 molecular weight or only a few cistrons. There were found to be approximately 15 copies of the plasmid per chromosome in exponentially growing cells. This is the greatest amount of redundancy found for any plasmid in its native host. No biological function was identified with the presence of the plasmid.

Part II

Bacteriophage P22

General description. Phage P22 was originally isolated in 1952 by Zinder and Lederberg (96) from S. typhimurium strain LT22. They found that it was active on strain LT2 and that it was identifiable with their filterable agent which mediated genetic recombination or transduction. P22 is a temperate phage which when it

enters the prophage state, integrates into the chromosome of S. typhimurium between the pro A and pro C loci (76,79). Gough and Levine (40) first demonstrated the circularity of the vegetative genetic map and ordered the known genes in a single circular linkage group. The latest genetic map of P22, published by Calandar (21) in 1970, includes 27 genes located at known distances around the map. The prophage map is a linear permutation of the circular vegetative linkage map.

Levine in 1957 (55) made an extensive study of the genes involved in lysogeny by P22. He demonstrated that by increasing the multiplicity of infecting phage, the frequency of lysogenization was increased. At a multiplicity of infection above 10 there was nearly 100% lysogenization. P22 produced the typical turbid plaque of a temperate phage but each stock lysate contained at a frequency of 0.1% spontaneous mutants which produced clear plaques. By complementation analysis Levine grouped these into three classes: C1, c2 and c3. These all mapped close together. Revertants of the virulent mutants were not seen. Another class of mutants comprising a separate, single complementation group involved in lysogeny has been isolated (74,78). Although the c genes function normally, these int mutants (originally called L mutants) cannot complete the lysogenic response due to an inability to integrate into the host chromosome. These mutants produce turbid plaques which resemble the

wild type but the phage genome is progressively diluted out among the segregating progeny cells. The int gene is also necessary for normal detachment from the chromosome during induction. Additional virulent mutants have recently been isolated by their ability to form plaques on P22 lysogens (20). Several different classes have been defined and the mutant locus of some have been mapped to lie within the c2 gene.

According to Calandar (21) whether an infection by P22 leads to a lytic or to a lysogenic response is determined by four genes: c1, c2, c3 and mnt. C1 is responsible for repression of the phage DNA synthesis. C2 produces the immunity repressor analogous to the c1 gene product which exerts a negative control on the transcription of the remaining genes. C3 and mnt are necessary for a high frequency of lysogenization but their exact function is not known. However, it has been shown that the mnt locus produces a cytoplasmic product which is necessary for the stable maintenance of lysogeny (38).

Phage related to P22. Yamamoto and Anderson (93) isolated from stocks of P22 a serologically and morphologically unrelated phage which they labeled P22l. The P22 phage particle is approximately 60 nm in diameter with a short non-contractile tail containing a hexagonal base plate with 6 spikes (47,93). P22l was found to have a long flexible tail with no base plate. A genetic

relationship between P22 and P221 was demonstrated by the formation of recombinants. The origin of P221 was determined when it was found that the strains of S. typhimurium on which the P22 stocks containing P221 were made were lysogenic for two prophages: Fels1 and Fels2 (91). Fels1 is serologically and morphologically related to P221. But while P221 is homoimmune to P22, Fels1 is heteroimmune. Fels2 appeared to be unrelated. UV induction experiments of Fels1 during infection with P22 lead to the hypothesis that P221 arose as a consequence of an infrequent recombinational event between P22 and Fels1 or its defective genome. Independent preparations of P221 contain varying amounts of P22 genetic material. Most of the P221 DNA is derived from Fels1 with P22 supplying the C gene immunity region plus various amounts of other genes. P221 also has a different chromosomal attachment site than P22 (94). Later results have indicated that P22 can infect Fels2 lysogens and upon vegetative growth can recombine with the Fels2 genetic material and produce another hybrid, F22 (92).

Since the isolation of P221 it has been shown that infection of S. typhimurium LT2 by P22 or P221 induces the formation of a large number of different phage types which can be found in the lysates (95). These differ in morphology, serology, immunity, buoyant density, host range and even in the length of the latent period and the

number of infective centers produced from a single burst. P22 has a latent period of 33 minutes when infecting S. typhimurium in an aerated nutrient broth and a burst size of about 160. Some of the other phage isolated from LT2 have latent periods up to 40 minutes and produce as few as ten phage per infected cell.

Phage L, which is genetically related to P22, has also been induced by UV light from a substrain of S. typhimurium LT2 (10). The phage is morphologically and serologically similar to P22 and shows the same high resistance to heat inactivation. There are significant differences in their immunity characteristics. P22 clear mutants form turbid plaques on strains lysogenic for phage L. Both P22 and L phage can be isolated from these turbid plaques. Also, P22 is found to abortively infect strain 1559 (the cells are killed without phage production) but this does not occur with phage L. The abortive infection was later found to be due to the presence or absence of alleles of gene H which controls the reestablishment of protein synthesis in the infected cell (2). Allele $^{L}H1$ in P22 cannot efficiently reestablish protein synthesis unless phage L carrying an active H gene is present.

A phage which may be related to P22 was isolated from chicken feces in 1944 (7). This phage was found to infect S. pullorum leading to cell lysis. Electron micrographs

identified the phage a particle of about 40-45nm in diameter.

Superinfection exclusion determined by prophage P22. When a P22 lysogen is induced by exposure to UV light and then superinfected with another strain of P22, the superinfecting DNA is excluded (67). Even though the immunity has been lifted, the superinfecting DNA is still excluded and complementation will not occur. The superinfection exclusion was demonstrated to be due to the sie locus on the P22 genome and shown not to involve degradation of the superinfecting DNA. The exclusion is effective also against the heteroimmune phage L. The mechanism for this type of exclusion is presently unknown although there are two indications that it may be localized at or near the cell surface. First, the exclusion is dependent upon the method by which the DNA molecule enters the cell. The exclusion does not operate if the phage DNA enters as a prophage during conjugation. Second, non-excluding P22 mutants were found not to produce the somatic antigen 1 which is normally produced by lysogenic conversion of the host upon P22 lysogenization (87).

P22 DNA: molecular structure and replication. The DNA of P22 is a circularly permuted collection of linear duplex molecules of 13.7 u in length and with a molecular weight of 27×10^6 (68). A double stranded terminal redundancy accounts for about 3-5% of the molecule (about

1500 nucleotide pairs). When isolated from the mature phage particles, the DNA sediments through a neutral sucrose gradient of 1.0 M NaCl at 35s. The circular permutation of the P22 DNA molecule agrees with the evidence that the vegetative genetic map is circular (40). The fact that P22 DNA is circularly permuted whereas the λ DNA molecule is unique (nonpermuted) with a very short single strand terminal redundancy may be related to P22 being able to perform generalized transduction while λ can only perform specialized transduction. If, in the maturation process, P22 is capable of incorporating any piece of DNA of the correct size irrespective of nucleotide sequence, then any sufficiently large piece of chromosomal DNA could also be incorporated into the phage head (68).

Smith and Levine (75) have shown that immediately after infection of S. typhimurium LT2 by P22, there was a depression of the rate of DNA synthesis regardless of whether the infection led to a lytic or a lysogenic response. After 3 minutes there was a sharp rise in the rate of DNA synthesis which was correlated with one round of replication of the phage DNA. In the lysogenic infection by wild type P22, DNA synthesis then ceases while the phage molecule is integrated into the bacterial chromosome after which DNA synthesis reinitiates. Synthesis procedes at a high rate until it nearly returns

to the level in the uninfected cell. Lytic infections with c1 or c2 mutants showed altered patterns of incorporation of ^{3}H-thymidine leading to extensive replication of the phage DNA and eventually production of mature phage. It has recently been shown that even in lytic infections with P22 there is no breakdown of the host chromosome into oligonucleotides or even into fragments larger than the size of phage DNA (72). After infection, in conditions leading to lysogeny, the parental linear molecules are converted to covalently-closed, supercoiled molecules (69,85). The conversion occurs after the initial phage replication demonstrated by Smith and Levine (75,77). Later in the infection up to 14 copies of the supercoiled monomer were found. Also, catenates of two interlocked, supercoiled molecules were found. These were similar to those observed by Hudson and Vinograd (43) in Hela cell mitochondria. It is possible that, due to the circular permutation of P22 DNA, the end of one molecule could recombine with the middle of another to produce linear concatemers of fractional genome sizes.

Botstein and Levine (13,14) have also studied the intracellular replication of P22 DNA. Soon after infection or UV induction, they found that the newly labeled phage DNA is in a form called intermediate I. This has a high sedimentation value (1000s), is probably associated with some cellular structure (membrane) and appears to be

the replicating form of the phage DNA. A phage specific function is required for the formation of intermediate I. The immunity repressor may play a physical role by inhibiting phage DNA from associating with the membrane (56). When cells undergoing phage replication were pulse labeled with ^{3}H-thymidine early in infection and then chased with unlabeled thymidine for varying lengths of time, it was found that the label originally in intermediate I was converted to a slower sedimenting form. This form, intermediate II, possessed a sedimentation coefficient 1.3 to 1.7 times greater than that of the mature phage DNA, i.e., 43 to 56s. The DNA in intermediate II did not replicate but appeared to be linear and of lengths greater than mature phage DNA. It probably contained multiple phage genomes. Intermediate II is the immediate precursor of the DNA in the mature phage particles. It has been found that even with a temperature sensitive cI mutant which does not make phage DNA, the parental DNA was incorporated into intermediate I (15). If intermediate II is a linear concatenate, then maturation of the mature phage particle may involve a mechanism related to the "headfull" hypothesis as proposed for phages T7 and T4 (83). This would also provide a means for production of the general transducing particles.

Part III

Phage Related Plasmids

Phage P1. Genetic studies by Boice and Luria (11) on P1, the general transducing phage of E. coli, had indicated that the prophage was not linked to the host chromosome during Hfr($P1^+$) x F^-($P1^-$) matings. These results stimulated two subsequent investigations into the physical relationships of the prophage DNA molecule to the host chromosome. Inselburg (46) isolated the total cellular DNA from both P1kc lysogens and non-lysogens and sedimented the purified DNAs in neutral sucrose gradients. Pooled fractions from the gradients were then hybridized to P1 DNA immobilized in agar. He found that the P1 specific-binding DNA was distributed throughout the gradient at a constant proportion relative to the amount of chromosomal DNA. Isolated P1 DNA with a molecular weight of 60×10^6 sedimented in a single peak and when it was hybridized to the P1 DNA, the fraction with the maximum specific hybridization corresponded to the peak of P1 DNA. He concluded from this that P1 prophage DNA does not exist in the cell as a separate entity but, in contrast to the genetic data, is integrated.

Contrasting results were obtained by Ikeda and Tomizawa (45) in a more extensive analysis utilizing DNA-DNA hybridizations on membrane filters coupled with cesium

chloride-ethidium bromide density gradient centrifugation and electron microscopy. They found that there was an average of one copy of Plkc per chromosome. The total cellular DNA was extracted, fragmented into pieces with an average size distribution of twice the size of Pl DNA molecules and then centrifuged in a $CsSO_4$ gradient containing $HgCl_2$. Since Pl DNA has a different GC content than that of the E. coli chromosome and if it is not physically associated with the chromosome, it will band at a position of slightly greater density than the chrosomal DNA. If the Pl DNA were associated with chromosomal DNA, it would be distributed in the gradient between the position of pure chromosomal DNA and pure Pl phage DNA. They then hybridized the DNA in each fraction to Pl DNA on nitrocellulose membrane filters and found that Pl prophage DNA banded at a position distinct from the bulk of the cellular DNA. The sucrose gradient experiment of Inselburg was also repeated by Ikeda and Tomizawa except that they were not forced to pool fractions but could test the hybridization efficiency in each fraction. By this method they were able to detect a separation of prophage Pl DNA from the bacterial DNA.

As added proof of the plasmid nature of Pl prophage DNA, Ikeda and Tomizawa isolated a satellite peak of DNA in a CsCl-EtBr gradient and demonstrated by hybridization that it was composed of 79% Pl-specific DNA. Electron microscopy of the satellite peak showed it contained

circular DNA molecules of an average length of 31.5 u which is slightly shorter than the 35.5 u observed for the phage DNA molecule. The terminal redundancy of the phage molecule could account for its extra length.

It has recently been shown that the bacterial lon allele is necessary for plasmid formation by P1 (82). The lon^+ gene function is necessary for plasmid formation although P1 can grow normally on lon^- strains. Mutants containing lon^- also have pleiotropic effects on UV sensitivity, septum formation and regulation of capsular polysaccharide formation. This effect on plasmid formation by lon is restricted to phages P1 and λ (N^-) since both F and R factors can form plasmids in lon^- strains and is assumed to involve the membrane (plasmid membrane binding sites).

The strict replication control which allows only one copy of P1 prophage per chromosome may also relate to the exclusion observed by Luria et al. (59) when P1 vir^+ superinfects a lysogen containing P1dl prophage. After P1 vir^+ infection the "quasi-stable lac^+" strains segregate lac^- segregants as opposed to those strains in which the lac characteristic had become associated with the chromosomes. Lac-segregants no longer contained the P1dl prophage.

P1-like plasmid. An extremely interesting finding was made by Ideda, Inuzuka and Tomizawa (44) when they studied the plasmid DNA in E. coli strain 15. In addition

to the small, supercoiled DNA molecule previously described by Cozzarelli et al. (26), they found two additional plasmid molecules of 63 x 10^6 and 104 x 10^6 daltons which they labeled P15B and P15C respectively. By DNA-DNA hybridization it was found that nearly all of the P15B DNA was homologous to phage P1 DNA. Induction of strain 15 with mitomycin c produces two phage-like particles neither of which carry DNA homologous to the DNA of plasmid P15B. An explanation for the absence of P15B DNA in the phage particles is the defectiveness of the P15B plasmid. Upon further examination it was found that the presence of plasmid P15B confers upon the cell a different restriction and modification pattern than does the presence of P1 prophage. P15B DNA also has a buoyant density in CsCl of 1.708 gm cm^{-3} while that of P1 DNA is 1.706 gm cm^{-3}. Although there appear to be differences in the modification-restriction system, P1-specific immunity is expressed by P15B. When a cell containing the plasmid P15B was infected with P1CM (a derivative of P1 carrying the chloramphenicol resistance determinant of an R factor; P1CM can form a stable lysogen) and plated on agar plates containing chloramphenicol, only one cell in 10^7 survived. These resistant cells were found to have simultaneously acquired the P1 modification and restriction system but were found not to have increased the amount of P1 specifically hybridizable DNA which they contained. Chloramphenicol sensitive segregants were isolated and these

were found to be lacking in the P1-like DNA. Therefore infection of E. coli 15 with P1CM can lead to exclusion of the P15B plasmid. Although P15B is about 5% larger than the P1 prophage molecule, the authors assumed that the exclusion may be due to a competition between P15B and P1 for a limited number of intracellular sites. Previous data (45) had indicated a strict replication control for the P1 prophage.

Phage λ: plasmid mutants. It has recently been shown that mutants of the λ prophage can exist as autonomously replicating plasmids. Two reports indicate that mutants in the N gene lead to plasmid formation (57,73). The N gene product may be necessary for the association of supercoiled λ molecules with the membrane (41) as well as being required for transcription of the early phage genes involved in DNA replication (28). Signer (73) postulated that without the product of the N gene, phage DNA replication could continue but since most other λ genes were repressed, no mature phage particles would be produced and the cells would not be killed. The replicated phage DNA would remain in the cell as a plasmid. The λ N^- plasmids were found to be present in the cell at 10-20 copies per host chromosome.

Matsubara and Kaiser (61) isolated from λ vir a deletion mutant which had lost most of the phage genome except that part required for phage DNA replication and

its regulation. This molecule of 8.6×10^6 daltons was also shown to exist in the host cell as a plasmid with 20 copies per bacterial chromosomes. The molecular weight of mature λ DNA as determined by electron microscopy is 33×10^6 daltons (23,60).

Also, when λ infects a hybrid cell containing genetic material of E. coli K12 and S. typhosa, it is adsorbed but cellular lysis does not occur (6). It was proposed that this diploid Salmonella hybrid inhibits the function of the N gene product and was later demonstrated that, like λ N^- mutants in E. coli, λ does exist in the hybrid as a plasmid (30).

Part IV

RNA-DNA Hybridization

DNA-RNA and DNA-DNA hybridizations have been used to study the relatedness of many nucleic acids and several methods have been developed to perform and analyze the products of the hybridization reactions. When it is possible, it is advantageous to immobilize one of the components (usually the unlabeled DNA) in agar (12) or on nitrocellulose filters (27,34). This procedure reduces the probability of the denatured unlabeled strands recombining and, therefore, no longer being available for hybridization with the labeled test DNA or RNA. But these procedures do require that the immobilized unlabeled

DNA be present in much higher concentrations than the labeled mobile component since any reaction of this component with itself will inhibit its hybridization with the immobile component. Greatly lowering the relative concentration of the mobile component will, therefore, favor its reaction with the immobile component. If high concentrations of the unlabeled DNA are not available, it is necessary to perform the hybridization reaction with both components in solution. Several methods to detect the resulting DNA-RNA complexes are available. The most convenient method was developed by Nygaard and Hall (63) who collected the complexes on nitrocellulose filters. They found that at moderate salt concentrations nitrocellulose filters will adsorb denatured DNA and DNA-RNA complexes but that free RNA will pass through the filter. By presoaking the filters before collection of the complexes and then extensive washing of the filters, the background can be greatly reduced so that less than 0.5% of the free, labeled RNA will be trapped by the filter.

Labeled RNA for hybridization can be isolated from bacteria but it possibly may not have a sufficiently high specific activity. ^{3}H-RNA of high specific activity can be synthesized *in vitro* using DNA and RNA polymerase. RNA polymerase was first identified and isolated in the early 1960's from *E. coli* (33,81) and from *Micrococcus lysodeickticus* (89,90) and since has been extracted from

several bacterial sources. Burgess (16) has published a procedure for the assay and large scale purification of E. coli RNA polymerase. Characterization showed that core enzyme was composed of four subunits: α_2BB' (17). A similar enzyme has been isolated and characterized from Pseudomonas putida (48). It was found to be present in two forms; one with the subunit structure α_2BB' and the other with the subunit structure of α_2BB'σ. This enzyme can transcribe DNA from the Pseudomonas phage gh-1 as well as bacterial DNA (J. Boezi personal communication).

Part V

Modification and Restriction

Arber and Lin (4) have recently reviewed the literature on the modification and restriction systems of bacteria and phage. They indicate that the DNA molecule of a phage is "strain-specifically marked by the host cell during vegetative phage growth. A particular bacterial host strain can be successfully infected only with bacteriophage carrying the DNA modification produced by that strain, a property obtained through prior growth on the same strain: the inappropriately or unmodified DNA is broken down upon penetration into the host cells, these being restrictive for this DNA." Modification in the E. coli K12 and λ phage system has been shown to involve enzymatic methylation of DNA at strain-specific sites (39)

while restriction is caused by an endonucleolytic scission of the entering DNA molecule presumably at an unmethylated (modified) strain-specific site (29). Restriction is never complete and the unmodified infecting phage DNA does have a low but consistent probability of escaping the restriction and undergoing vegetative phage growth. During this process the phage DNA will be modified to the specificity of its new host and will now be able to reinfect this strain at high efficiency.

Modification and restriction capabilities can also be carried by temperate phages such as P1 which will when it lysogenizes a cell, confer this property on the new host (4). Plasmids such as R factors (3) can also confer upon their hosts specific modification and restriction abilities.

Modification and restriction in Salmonella. Salmonella typhimurium is an inefficient recipient of DNA transferred to it from E. coli. This was demonstrated by Okada et al. (65) to be due to a host specific modification and restriction system in S. typhimurium strains LT7 and LT2. They succeeded in isolating from strain LT7 a mutant, LT7 fer, which had an increased ability to act as a recipient of foreign DNA. It was also found that when P22 was propagated on LT7 fer, it showed a greatly reduced efficiency of plating on other S. typhimurium strains. However, P22 grown on the other strains plated

at an equal efficiency on LT7 fer and on the other non-fertile strains. These results were interpreted as indicating that LT7 fer had lost the ability for host-controlled modification and restriction.

Okada and Watanabe (64) subsequently isolated from S. typhimurium LT2 by two steps of mutagenesis mutants which were able to act as high efficiency recipients for R factor DNA from E. coli. Mutant Rferl had a one hundred fold increase in its ability to act as a recipient while mutant Rfer2 had approximately a ten thousand fold increased ability. That both mutant strains had alterations in their modification and restriction systems was seen by comparison of the efficiencies of plating of P22 grown on the mutant strain to the efficiencies of phage grown on the wild type parent strain. Rfer2, the strain with the greatest recipient ability, also apparently had undergone an alteration in the cell wall composition since it was now sensitive to E. coli phages T3, T7, and P1, was resistant to P22, and produced a rough colonial morphology. Rferl, while not quite as good a recipient as Rfer2, remained sensitive to P22.

MATERIALS AND METHODS

Bacteria and growth conditions. Salmonella pullorum strains MS35, MS53 and MS6-18 were from our laboratory stock collection. Salmonella typhimurium LT2 was originally received from P. E. Hartman and has been maintained in our laboratory. Escherichia coli JC411 (colE1$^+$) was obtained from D. E. Helinski.

The colicinogenic character of E. coli JC411 (colE1$^+$) was tested on Escherichia coli ψ which was obtained from R. R. Brubaker. Salmonella typhimurium LT2 Rfer1 and Rfer2 were received from A. Laskin and Salmonella montevideo from E. Sanders.

For routine cultivation, L broth containing 10 g of tryptone (Difco), 5 g of yeast extract and 10 g of NaCl per liter were used. For solid media, Difco agar was added to a final concentration of 1.5%.

The purity of the S. pullorum cultures were routinely determined by their reaction with antisera (Difco, Salmonella O antiserum group D factor 9) and in SIM agar.

For radioactive labeling, the bacteria were grown in TCG broth which contains 0.1 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride pH 7.4, 0.4% vitamin-free

casamino acids, 250 ug/ml of deoxyadenosine. Glucose (40%) was autoclaved separately and added just before use to a final concentration of 0.4%.

Bacteriophage. P35 is a temperate phage originally isolated from S. pullorum MS35 by zygotic induction. P35c was isolated as a spontaneous, clear plaque mutant of P35. P22 and P22c2 were originally obtained from M. Levine.

Phage were propagated and titered as described by Adams (1). Stock lysates were prepared by infecting log phase bacterial cells in aerated L broth with phage at a multiplicity of infection (m.o.i.) of 0.1 and incubating for 4-5 hours at 37C. Chloroform was added to the culture and the bacterial debris was removed by centrifugation for 10 min at 8000xg. The phage in the supernant fluid were stored at 4C over a drop of chloroform. The phage were titered by assay of the number of plaque forming units (pfu) per ml by the soft agar overlay method.

Chemicals. All general chemicals were reagent grade and purchased from standard commercial sources. Other chemicals are listed in Table 1.

Buffers and dialysis. Two general buffers made in deionized, distilled water were used in many of the experiments reported here. TES:0.05 M tris (hydroxymethyl) aminomethane (Tris)--hydrochloride, 0.005 M (ethylenedinitrilo) tetraacetic acid (EDTA), 0.05 M NaCl, pH 8.0. SSC (standard saline citrate):1x concentrated SSC contains

Table 1. Chemicals and sources

Chemicals	Source
Pronase B grade, ribonuclease (RNase) 5x cryst A grade, ethidium bromide (EtBr), mitomycin c, adenosine triphosphate (ATP), guanine triphosphate (GTP), uridine triphosphate (UPP) and cytosine triphosphate (CTP)	Calbiochem San Diego, Calif.
Lysozyme (egg white)	Armour Pharmaceutical Co., Kankakee, Ill.
Cesium chloride (CsCl)	Mann Research Laboratories, New York
Bovine serum albumin (BSA)	Pentex Biochemicals, Kankakee, Ill.
Brij 58	Emulsion Engineering Co., Elk Grove, Ill.
Dithiothreitol	P & L Biochemical, Milwaukee, Wis.
Antisera	Difco Laboratories, Detroit, Mich.
2,5 diphenyloxazole (PPO) 1,4,-bis 2(4-methyl-5-phenyloxazole)-benzene (POPOP)	Packard Instrument Co., Downers Grove, Ill.

0.15 M NaCl, 0.015 M sodium citrate, pH 7.0. The 0.1x and 2x solutions are one tenth and twice concentrated, respectively.

All dialysis was performed using sterile dialysis tubing which had been boiled in 0.5 M EDTA for 10 min and then autoclaved in 0.05 M Tris, pH 8.0.

Radioactive labeling and counting. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham. Cells growing in TCG broth containing deoxyadenosine were labeled with ^{3}H-thymidine (15 Ci/m mole) or ^{14}C-thymidine (60 mCi/m mole) at 1 uCi/ml of broth for ^{3}H and 0.05 u Ci/ml for ^{14}C. The radiochemicals were added to the culture and were allowed to be incorporated by the cells for at least five generations. This was necessary because S. pullorumincorporates thymidine very inefficiently from the media.

All radioactive counting was done in a scintillation mixture containing 1.35 g of 2,5-diphenyloxazole (PPO) and 27 mg of 1,4,-bis 2(4-methyl-5-phenyloxazole)-benzene (POPOP) per liter of toluene. Ten ml of this mixture was added to vials containing the dried radioactive samples on filter paper or nitrocellulose filters and these were counted either in a Nuclear Chicago Mark I liquid scintillation counter or a Packard Model 2002 Tricarb liquid scintillation counter.

Preparation of bacterial lysates. Lysates for the isolation of plasmid DNA were prepared by a modification

of the procedure of Clewell and Helinski (25). Radioactively-labeled cells were harvested in late log phase from a 10 ml TCG broth culture by centrifugation for 10 min at 10,000xg in the Sorval RC2 centrifuge. The cell pellet was resuspended in 0.3 ml of 25% sucrose in 0.05 M Tris pH 8.0, and 0.1 ml of a lysozyme solution was added (5 mg/ml in 0.25 M Tris pH 8.0). This mixture was incubated for 5 min in an ice bath, and then 0.1 ml of 0.25 M EDTA pH 8.0 was added and the mixture incubated on ice for another 5 min. The cells were then lysed by addition of 0.5 ml of a "lytic-mix" containing: 1% Brij 58, 0.4% sodium deoxycholate, 0.0625 M EDTA, and 0.05 M Tris pH 8.0. The cell preparation was incubated in an ice bath for 15-30 min after which lysis was completed by subjecting the cells to 4-5 cycles of freeze-thawing. The cell suspension was placed in a 5/8 x 2 1/2 inch polyallomer tube which was alternately placed in an ethanol-dry ice bath and a 45C water bath. The chromosomal DNA was removed by centrifuging the lysate at 48,000xg (20,000 RPM) in the RC-2B centrifuge for 25 min. This step normally pelleted 95% of the chromosomal DNA leaving the plasmid DNA in the supernatent fluid. This fluid henceforth will be referred to as the cleared lysate.

Sucrose density gradient centrifugation. The DNA sample in 0.1-0.3 ml of TES buffer was layered on a linear 20-31% sucrose gradient (5.0 ml) made in 0.05 M Tris,

0.005 M EDTA, 0.5 M NaCl, pH 8.0. The gradient was centrifuged in a Beckman Model L or Model L3-50 ultracentrifuge at 15C. Thirty to thirty-two fractions of 0.17 ml (8 drops) were collected from the bottom of the tube using a Beckman fraction recovery system and dropped directly on to 0.75 inch squares of #1 filter paper. The numbered filter papers were dried under a heat lamp and washed successively in 250 ml of cold 5% TCA, 70% ethanol and anhydrous ether. The filter papers were then dried, placed in a vial containing 10 ml of toluence scintillation fluid and counted in a scintillation counter.

Alkaline gradients were made as described by Freifelder (32). Linear gradients of 20-31% sucrose in 0.3 M NaOH, 1.0 M NaCl, 0.01 M EDTA, pH 12.0 were made in 1/2 x 2 inch nitrocellulose tubes which had been soaked with 100 ug/ml denatured calf thymus DNA and 1.0 mg/ml BSA in TES for 1 hour before use. Centrifugation and fractionation were carried out as above.

Recoveries on all gradients were greater than 90% of the added radioactivity.

Sedimentation coefficients (S) were calculated from the distances sedimented (D) during cosedimentation with molecules of known S values by the equation of Burgie and Hershey (18).

$$(D_2/D_1) = (S_2/S_1)$$

Molecular weights of the supercoiled DNA were then determined using the relationship of S to the molecular weight shown by Bazaral and Helinski (8) and Clayton and Vinograd (24).

The linearity of the gradients was occasionally checked by determining the refractive index of the fractions in a Bausch and Lomb refractometer.

Dye-buoyant density gradient equilibrium centrifugation. Isolation of plasmid DNA was performed in cesium chloride-ethidium bromide (CsCl-EtBr) dye-buoyant density gradients following the procedure of Bazaral and Helinski (9). Three ml of a cleared lysate from a 30 ml TCG culture, 2.7 ml of TES buffer, 0.5 ml of EtBr (5 mg/ml TES) and 6 g anhydrous CsCl (final density 1.54 g/ml) were mixed in a vial and poured into a pretreated polyallomer centrifuge tube. Tubes were pretreated by boiling 15 min in TES buffer and then soaking with 100 ug BSA/ml TES buffer for 1 hour. The contents of the tube were overlaid with light mineral oil. The tube was capped and centrifuged in a Type 50 rotor for 44 hours at 44,000 RPM in the Model L or L3-50 ultracentrifuge at 15C.

The gradient was fractionated into approximately 60 fractions of 0.1 ml each by collecting 12 drops from a #24 gage needle punctured into the bottom of the tube. The fractions were collected in autoclaved 12 x 75 mm polypropylene tubes, and 5 ul aliquots of each fraction

were then spotted on filter paper squares which were washed, dried, and counted as before.

The plasmid peak fractions were pooled and dialyzed in the dark against TES buffer to remove the EtBr and CsCl.

Extraction and purification of bacterial DNA. Bacteria were grown overnight to 2 x 10^9 cells/ml in L broth, harvested by centrifugation and washed once with TES buffer. Cells were resuspended in 3 ml of TES buffer and 0.5 ml of lysozyme (5 mg/ml in TES buffer) was added plus 0.5 ml of 0.25 M EDTA (pH 8.0). The cell suspension was incubated in an ice bath for 20 min after which 0.3 ml of 10% SDS plus 0.3 ml of the lytic mix were added and incubated for an additional 30 min on ice. The lysate was then subjected to 3-4 cycles of freeze-thawing after which 1 ml of self-digested pronase (4 mg/ml TES buffer) was added. The pronase was pre-incubated at 60C for 2 hr. The lysate was incubated with the pronase for 6 hr at 37C and then deproteinized by 3 extractions with TES saturated-redistilled phenol. The aqueous layer containing the DNA was then mixed with an equal volume of ether, the layers allowed to separate and the lower aqueous layer dialyzed against a buffer containing 1 M NaCl, 0.01 M Tris and 0.01 M EDTA pH 8.0, to remove the remaining phenol and then against TES buffer over night at 4C. The DNA was then treated with 3 ug/ml of RNase which had been

pre-incubated at 85C for 10 min. After 10 min of incubation with RNase at 37C, the DNA was reextracted with phenol 3 times, dialyzed as above and stored frozen at minus 30C.

The purity and concentration of the isolated DNA was determined by analysis of its UV absorbance spectrum.

Preparation of phage DNA. Bacteriophage were purified and the DNA extracted using modifications of the procedures described by Thomas (50,68,84). Bacteria were grown in 1 liter of aerated L broth to a concentration of approximately 1 x 10^8 cells/ml and infected with phage. After lysis the remaining cells were removed by centrifugation at 8,000xg using the GSA rotor in a Sorval RC-2 centrifuge at 4C. The phage were then pelleted in a Type 30 rotor which was centrifuged at 30.000 RPM and 4C in the Model L or L3-50 ultracentrifuge. The phage were then resuspended over night at 4C in 1 ml of phage buffer (0.5 M NaCl, 0.001 M $MgSO_4$, 0.01 M Tris, pH 7.4). The resuspended phage were then recentrifuged at 7,000xg in the Sorval RC-2 centrifuge to remove contaminating cell debris. Two ml of the supernatant fluid were gently layered on a preformed CsCl step gradient (0.5 ml steps of 80%, 70%, 60%, 50%, 40% and 30% saturated CsCl in distilled water) and centrifuged at 35,000 RPM and 15C in a SW39 or SW50L rotor in the Model L or L3-50 ultracentrifuge for 1 hr. The phage, which form a dense white band at

about the middle of the tube, were collected by drop collecting from the bottom of the tube and immediately dialyzed against a buffer containing 1 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 7.0, and then successively against 5x and 1x SSC. DNA was extracted from the purified phage by gently shaking the phage suspension with an equal volume of TES-saturated redistilled phenol. The phenol and aqueous layers were separated by a brief centrifugation in an International clinical centrifuge and the upper aqueous phase removed, fresh phenol added and the extraction repeated twice more. The extracted DNA was then dialyzed against a buffer containing 1 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 8.0 and then against the appropriate buffer for the experiment in which it was to be used.

Purity and concentration of the isolated DNA were determined by analysis of its UV absorbance spectrum determined on a Beckman DB-G spectrophotometer.

In vitro synthesis of ^{3}H labeled RNA. ^{3}H-RNA was synthesized using P35c DNA as a template and the RNA polymerase purified from Pseudomonas putida (48) which was generously supplied by Dr. John Boezi. A modification of the assay procedure of Burgess (16) was used in the assay and synthesis.

The specific activity of the RNA polymerase using P35c DNA as a template was assayed in sterile 0.4 ml reaction mixtures containing 0.04 M Tris, pH 7.9, 0.01 M $MgCl_2$, 0.05 M KCl, 0.15 mM ATP, GTP, CTP and ^{3}H-UTP

(10 uc/u moles), 50 ug P35c DNA/ml and RNA polymerase at 0, 25, 50 and 100 ug/ml. The RNA polymerase was diluted in buffer (0.01 M Tris, pH 7.9, 0.01 M $MgCl_2$, 10^{-4} M EDTA, 10^{-4} M dithiothreitol and 5% glycerol) and added last to start the reaction. The reaction-mixture was incubated at 37C for 10 min. The reaction was then stopped by addition of 0.05 ml BSA (1 mg/ml) plus 1 ml of 5% TCA and it was then placed in an ice bath. The precipitated ^{3}H-RNA was collected on nitrocellulose filters, washed with 15 ml cold 5% TCA, dried and counted in a scintillation counter.

The synthesis was performed in a sterile reaction mixture containing the following: 0.04 M Tris-Cl, pH 7.9, 0.01 M $MgCl_2$, 0.05 M KCl, 0.15 mM GTP, ATP and CTP plus 0.09 mM ^{3}H-UTP (81.5 uc/u mole), and 50 ug P35c DNA per ml. This was made up in a 2x concentrated solution and placed at 37C. The reaction was started by addition of RNA polymerase to a final concentration of 41 ug protein/ml plus sterile distilled water to a final volume of 3 ml. The reaction mixture was incubated for 10 min at 37C. It was stopped by addition of 0.3 ml of 10% SDS. 1 M sodium acetate pH 5.2 was then added to a final concentration of 0.2 M and the reaction mixture placed in an ice bath.

The ^{3}H-RNA was extracted three times with water saturated phenol. An equal volume of phenol plus 0.015 ml of 1.0 M $MgCl_2$ (final concentration of 0.005 M) were added to the reaction mixture which was then heated to 65C

with occasional shaking. After chilling in an ice bath, the phases were separated by a short centrifugation at 2,000xg at 4C. The upper aqueous layer was removed carefully avoiding the interface. The aqueous layer was then extracted twice more.

The ^{3}H-RNA was then dialyzed against a buffer containing 1 M NaCl, 0.01 M Tris, pH 7.9 for 8 hours and then against 0.01 M Tris pH 7.9 over night and stored frozen. The extraction was followed at each step by removing 0.05 ml samples which were mixed with BSA and precipitated with TCA. The amount of precipitated ^{3}H-RNA was determined as above.

Determination of the concentration and specific activity of the ^{3}H-RNA. The amount of ^{3}H-UTP incorporated into RNA was determined by precipitating duplicate 0.05 ml samples of the RNA plus 0.05 ml of carrier BSA (1 mg/ml) with 1 ml of 5% TCA. This was then collected on a glass fiber filter (Whatman glass fiber paper, GF/A), dried, placed in a vial and solubilized by addition of 1 ml of Soluene-100 (Packard Instruments) and allowed to stand over night at room temperature. After the RNA had solubilized, 10 ml of toluene-scintillation fluid was added and the radioactivity counted in a scintillation counter. Duplicate samples of the ^{3}H-UTP used were also counted in the same way to ensure equal counting efficiency for each sample. The determined specific activity of the ^{3}H-UTP

(as cpm/u mole) was then used to calculate the amount of ^{3}H-RNA which was synthesized.

RNA-DNA hybridization. The DNA-RNA hybridization procedure of Nygaard and Hall (63) was used. A sample of the test DNA was denatured by boiling for 10 min in 0.1x SSC followed by rapid cooling in an alcohol ice bath. The denatured DNA was then mixed with the appropriate concentration of ^{3}H-RNA in a 1 ml solution of 2x SSC. Annealing was carried out at 60C for 6 hr after which the DNA-RNA complexes were collected on nitrocellulose membrane filters (Type B6 25 mm from Schleicher and Schuell, Inc., Keene, N.H.). Filters were washed with 2x SSC, dried, placed in vials, covered with scintillation fluid and counted in a scintillation counter.

Electron microscopy. Plasmid DNA was prepared for electron microscopy by the microversion of Lang and Mitani (54). A droplet of 40 ul was prepared in a sterile, disposable polycarbonate petri plate. Thirty-five ul of 0.15 M ammonium acetate, pH 6.0, containing 1×10^{-3} to 1×10^{-4} ug of plasmid DNA was first placed on the hydrophobic surface and to this was added 4 ul of cytochrome c (0.13 mg/ml of water) and 1 ul of 8% formaldehyde. The drops were allowed to set for 5 to 60 min which allowed the protein and DNA to diffuse to the surface where the cytochrome c denatured and formed a protein monolayer which absorbed the DNA molecules. The protein film was

picked up on a 300 mesh carbon coated collodion grid by touching the surface of the droplet with the grid and lifting it off at a 45 degree angle. The grid was then touched to the surface of a 95% ethanol solution for 1 sec and dried face down on filter paper. The grids were shadowed with platinum-palladium (80-20%) from two directions 90 degrees apart at an angle of 7 degrees. The grids were viewed in a Phillips 300 electron microscope and photographs taken on an instrument magnification of 7000-9000x.

Phage were prepared for electron microscopy by placing a drop of a CsCl step-gradient purified phage preparation on a 300 mesh grid coated with collodion, air-dried and stained with 0.5% phosphotungstic acid (PTA). Photographs were taken at an instrument magnification of 67,000-108,000x.

RESULTS

Part I

Isolation and Characterization of Phage P35

During experiments in conjunction with the development of a mating system in S. pullorum, Godfrey (35) occasionally observed plaques which arose in the bacterial lawns produced by S. typhimurium recipient strains after they had been mated with S. pullorum MS35 donor cells. One of these plaques was picked and found to have resulted from the presence of a phage. This phage was labeled P35 since it was assumed to have arisen by zygotic induction from S. pullorum MS35. Phage P35 produced the turbid plaques which are typical of a temperate phage. The exact origin of this phage remains obscure since it was found that P35 was able to be propagated and to produce plaques on F^- strains of MS35 and MS53. Phage P35 was shown to be antigenically similar to the S. typhimurium phage P22 and to be able to carry out generalized transduction.

Relationship of P35 to P22. The close relationship of P35 to P22 was demonstrated by finding that both require the same cell surface site for adsorption. Phage P22 is

known to require antigen 12 for adsorption to S. typhimurium (96). P22 and P35 adsorb to S. pullorum and to S. typhimurium (Figure 1) both of which have antigen 12 on the cell surface (19). S. montevideo has a similar surface antigen structure but does not produce antigen 12 (19). When P35 and P22 were added to a culture of S. montevideo, the phage were not adsorbed to the cells indicating that antigen 12 may be required for adsorption of both P22 and P35.

The latent period and burst size of P35 were determined by a one step growth experiment (Figure 2). It can be seen that when P35 infects S. pullorum growing in aerated L broth, there is a latent period of approximately 40 min and a burst yielding 300 pfu per infected cell.

Although P35 can be propagated on strains of S. pullorum, it was thought advantageous to determine if S. pullorum could be induced to liberate phage or bacteriocins. Log phase cultures of MS35 and MS53 were treated with mitomucin c (1 ug/ml for 10 min) or irradiated with UV light and subsequently tested for the presence of phage or bacteriocin activity by spotting chloroform treated samples on fresh lawns of S. typhimurium LT2. The response to these treatments was highly variable. On several occasions plaques were observed on the indicator plates. Each plaque was picked and reinoculated into

Figure 1. Adsorption of P35 and P22 to S. pullorum MS35 and to S. typhimurium LT2. Log phase (1 x 10^8 cells/ml) cultures in aerated L broth were infected with phage at an m.o.i. = 0.1. At intervals 1.0 ml samples were removed and treated with chloroform. After removal of the cells by centrifugation the phage remaining in the supernant were titered.

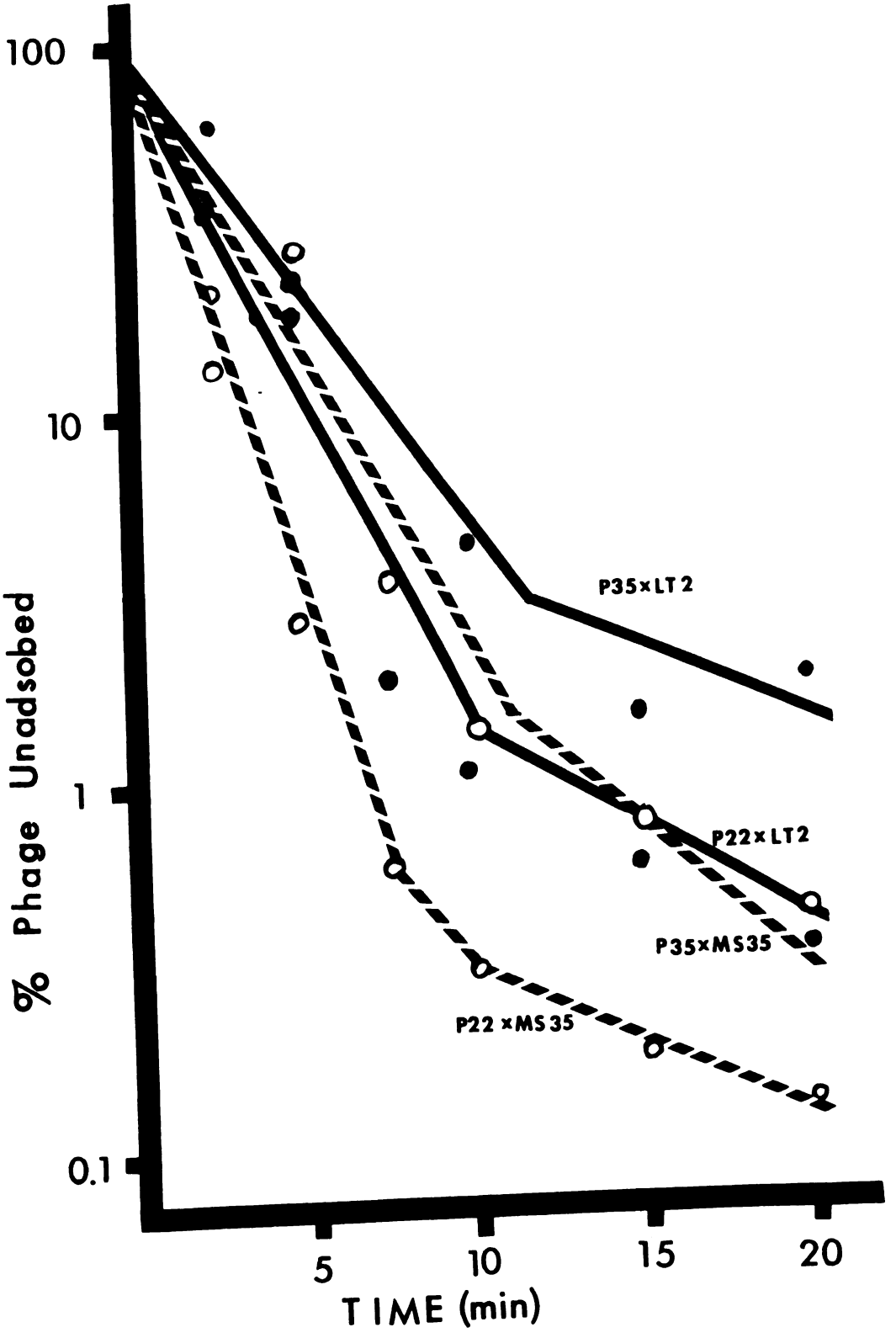
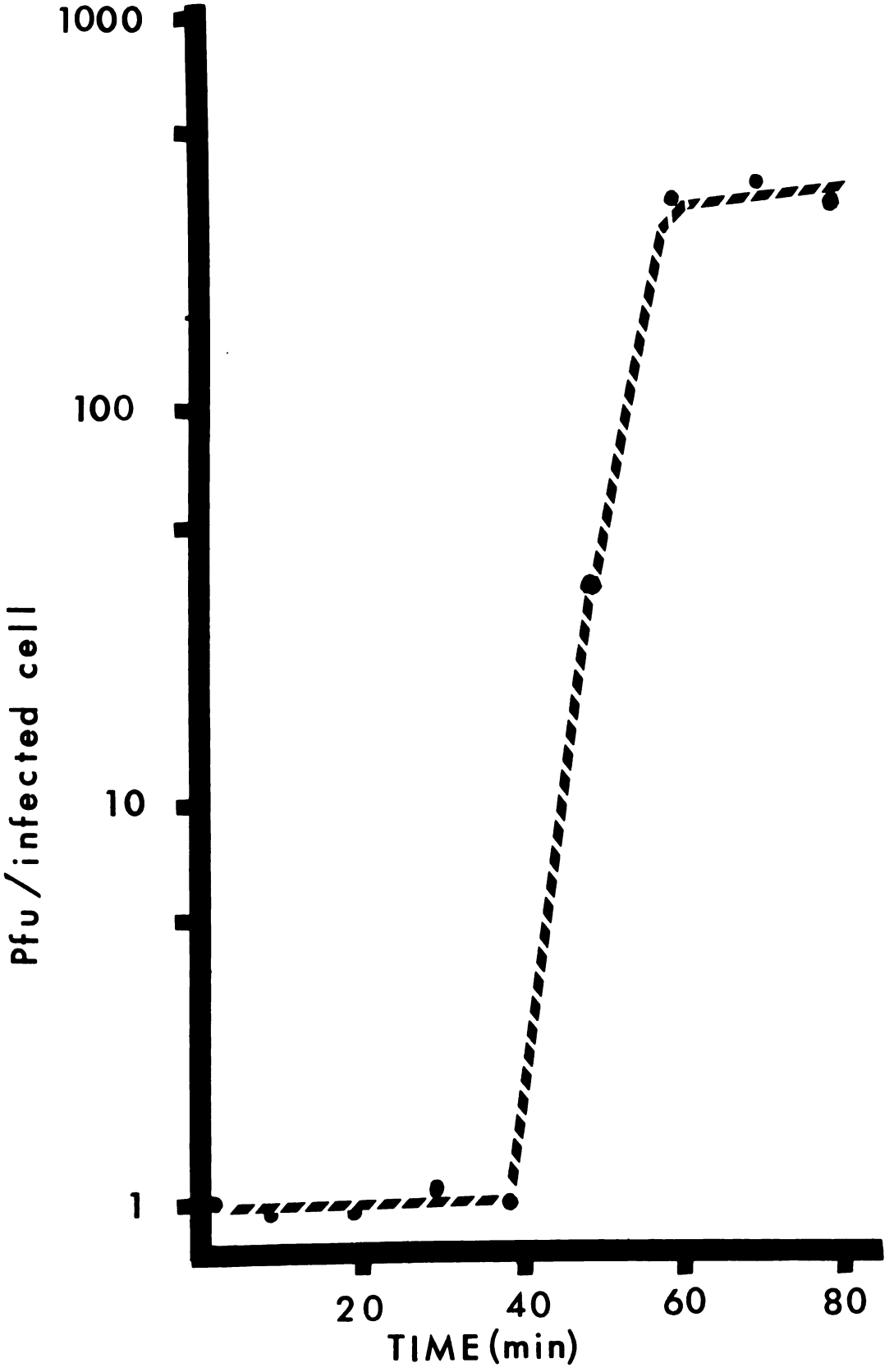

100
10
1
0.1
% Phage Unadsobed
5
10
15
20
TIME (min)
P35×LT2
P22×LT2
P35×MS35
P22×MS35

Figure 2. Single step growth curve of P35. A log phase culture (1 x 10^8 cells/ml) of S. pullorum MS35 growing in aerated L broth was infected with P35 at an m.o.i. = 0.1. At intervals samples were removed and the plaque forming units per ml were immediately titered.


1000
100
10
1
Pfu/infected cell
20
40
60
80
TIME (min)

3 ml of L broth containing a log phase culture of S. typhimurium LT2. The ability to increase the titer of plaque forming units after several hours incubation was taken as a presumptive indication of the presence of phage particles. The presence of no plaque forming units would indicate the original plaque was possibly produced by a bacteriocin or was an artifact. The assay was performed on many plaques but only three indicated the presence of phage. They were derived from mitomycin c induced cultures of MS35. The identity of these phage with the originally isolated P35 could not be immediately inferred due to the known variety of phage inducible from Salmonella (95). Comprehensive genetic, serological and morphological comparisons have not been performed on the separate phage isolates.

Isolation of P35c. Occasionally a clear, sharp-edged plaque would appear on a plate which contained many plaques of P35. One of these plaques, which was assumed to be caused by spontaneously occurring mutations in the phage, was picked and the phage purified by several plaque isolations on S. pullorum MS35. The phage was labeled P35c. The frequency of the occurrence of the mutation(s) leading to the clear plaque phenotype was calculated by using the equation presented by Stent (80) where M is the chance per round of phage DNA replication of the mutation event occurring (mutation frequency) and π_o and p_o are

the proportion of mutants and the total number of phage in the culture at time equal to zero. π and p are then the proportion of mutants and total phage in the culture after some fixed time interval.

$$M = \frac{\pi - \pi_o}{\ln(p/p_o)}$$

M for the mutation(s) to the clear plaque phenotype was calculated to equal 1.2×10^{-4} when P35 was grown in S. pullorum MS35 and 1.4×10^{-4} when P35 is propagated in S. typhimurium LT2. Spontaneous revertants of P35c were not observed.

P35c is a virulent mutant which has apparently lost the ability to enter into the prophage state. Superinfecting P35c are adsorbed by lysogenic strains of S. pullorum but are not propagated indicating that the mutant is sensitive to and inhibited from replication by the immunity repressor produced by the resident prophage genome (67). When P35c was simultaneously infected at an equal multiplicity of infection with P22c2, no complementation was observed (i.e., no turbid plaques were produced) indicating that P35c probably has a defect in the gent for production of the repressor protein which is necessary for the establishment of lysogeny (75).

P35c grows at the same rate as wild type P35 but produces maximum titers of 2×10^{10} - 3×10^{10} pfu/ml in contrast to the 6×10^{9} pfu/ml produced in P35 lysates.

Isolation of S. pullorum MS53(P35). A log phase (1×10^2 cells/ml) culture of S. pullorum MS53 in aerated L broth was infected with P35 at an m.o.i = 10.0. After 3 hours growth the surviving cells were streaked for isolated colonies on L agar. After overnight incubation at 37C many of the colonies had a rough appearance instead of the typical smooth colony of S. pullorum. Several of these were picked and purified by three more clonal isolations. Each of these when tested was found to be lysogenic as indicated by spontaneous phage liberation and an inability to produce plaques when superinfected with P35 or P35c. Antisera and reactions in SIM agar were positive for S. pullorum. The lack of plating of the phage was due not to an inability to adsorb the superinfecting phage (Figure 3) but apparently to immunity due to the presence of the prophage.

When grown in either L broth or TCG broth, a culture of MS53(P35) will contain 1.5×10^{-2} free pfu/cell.

This lysogenic strain, MS53(P35), was used to further investigate the relationship of P35 to P22. It was found that upon superinfection of the lysogen by P22, although the phage were adsorbed, no plaques were produced. This indicated that P22 and P35 have similar immunity systems (i.e., are homoimmune).

Morphology of P35. To further characterize P35, electron micrographs were taken of PTA stained phage preparations. Figure 4 shows a field containing many

Figure 3. Adsorption of P35c to S. pullorum 53 and 53 (P35). Log phase cultures (1×10^8 cells/ml of S. pullorum 53 and 53 (P35) were infected with P35c at an m.o.i. = 0.1. At intervals 1 ml samples were removed and treated with chloroform. After removal of the cells by centrifugation, the phage in the supernant fluid were titered on sensitive indicator lawns of MS53. Only the clear plaques of P35c were counted. P35 which is spontaneously liberated from 53 (P35) produces a turbid plaque. The titer of P35 remained constant at 2×10^6 pfu/ml throughout the time of the experiment.

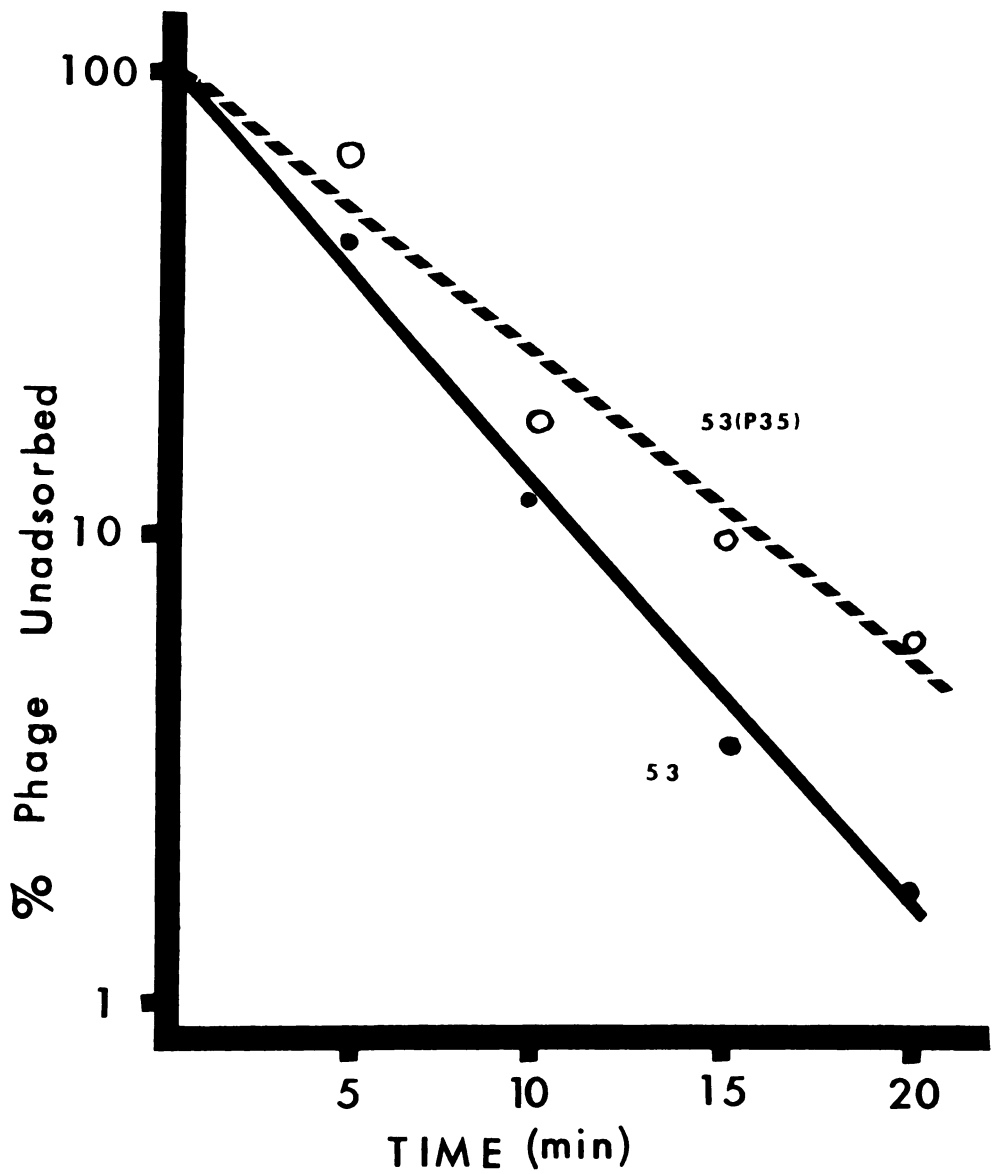
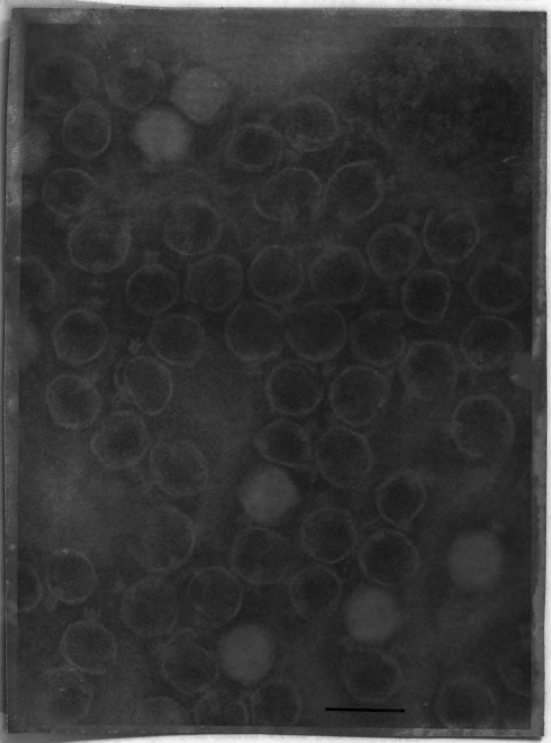
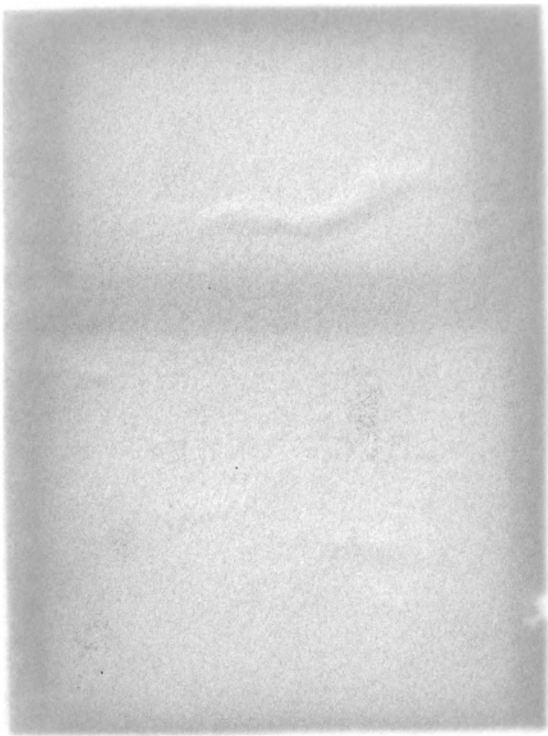

100
10
1
% Phage Unadsorbed
53(P35)
53
5
10
15
20
TIME (min)

Figure 4. Electron micrograph of P35 phage particles. P35 was purified on a CsCl step gradient. The electron micrograph was taken after negative staining with 0.5% PTA. Magnification is 201,000x. The bar represents 100nm.



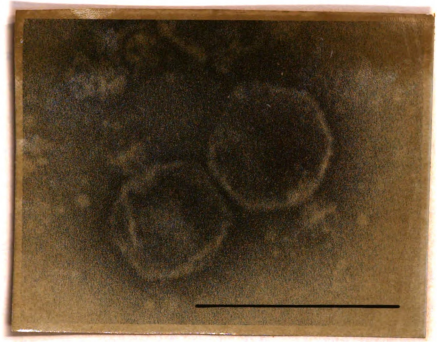


phage particles isolated after propagation on MS53. Many of the phage heads appear to be devoid of a core (nucleic acid). This is believed to be an artifact of the preparation since a similarly treated sample of E. coli phage T4 also appeared to be empty headed. Figure 5A is an enlarged view of two P35 particles. It can be seen that the phage head is hexagonal in shape and approximately 60-70 nm in diameter. There is a short tail and a base plate with spikes. Very occasionally in the lysates prepared on S. pullorum MS53 there were observed phage-like particles with an unusual structure. Figure 5B shows such a particle with a long tail (150 nm) and triangular shaped head. These particles may have been contaminants or have been induced from S. pullorum upon infection with P35. Since different phages have not been detected by plaque morphology or growth characteristics these particles may be defective phage or bacteriocins.

The DNA of P35. To determine the size of the P35 DNA molecule labeled phage DNA was analyzed on a sucrose gradient. A phage lysate was prepared on S. pullorum MS53 grown in 30 ml of TCG broth containing ^{3}H-thymidine (1 uCi/ml). The ^{3}H labeled phage were harvested and purified on a CsCl step gradient. DNA was then extracted from the phage by treatment with phenol. A 0.1 ml sample of the purified P35 ^{3}H-DNA was then mixed with 0.05 ml of ^{14}C labeled colE1 DNA. This mixture was then layered

Figure 5. Electron micrographs of indicidual phage particles. A. PTA stained preparation of phage P35. Magnification is 540,000x. The bar represents 100 nm. B. An example of a particle with unusual morphology found in a PTA stained preparation of P35. Magnification is 540,000x. The bar represents 100 nm.

A.



B.

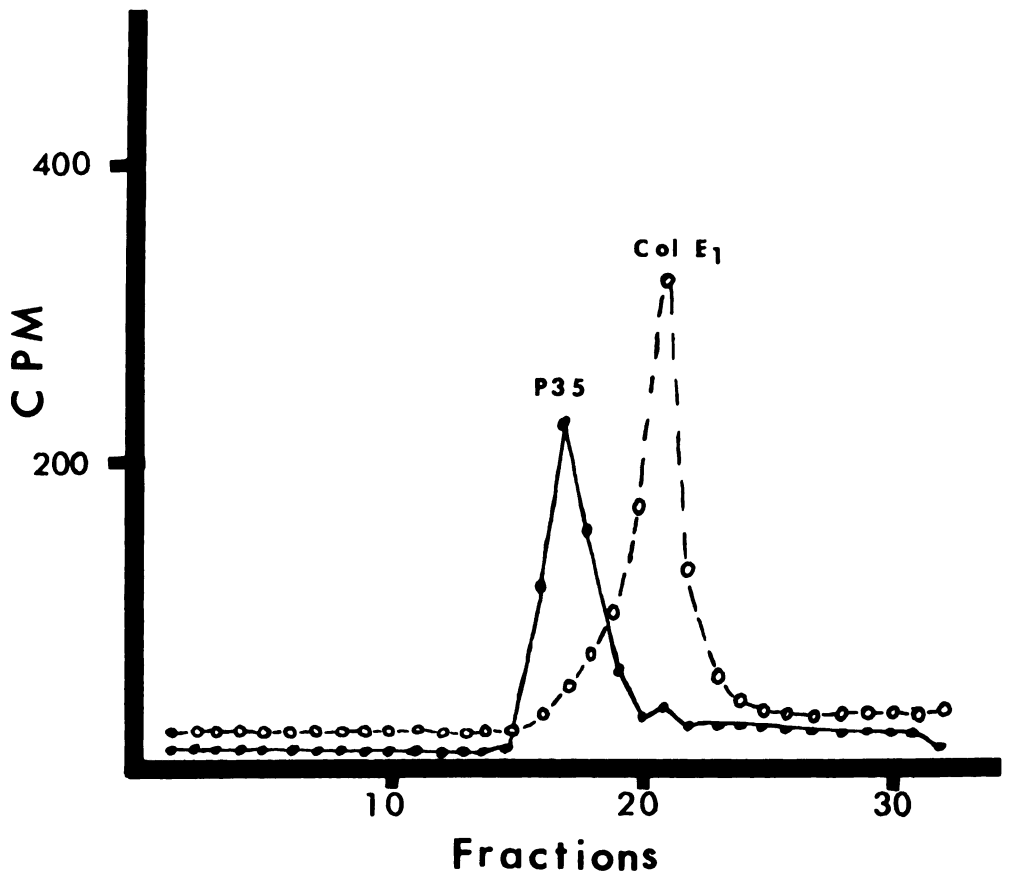
on a neutral 20-31% sucrose gradient. The colE1 DNA had been extracted from E. coli JC411 (El^+) and purified in a CsCl-EtBr dye buoyant density gradient. When prepared in this manner, colE1 DNA is a supervoiled molecule with a sedimentation coefficient of 23s (9). The gradient was centrifuged for 115 min at 50,000 RPM and then fractionated by collecting drops from a needle that had been forced through the bottom of the tube. Figure 6 shows that the ^3H labeled P35 DNA moved slightly further in the gradient than the ^{14}C labeled colE1 DNA. By comparison with the known s value of colE1 DNA, it was calculated that P35 DNA has a sedimentation coefficient in neutral sucrose of 33s. If the phage DNA molecule is a linear structure, as is P22 DNA (68), then from the relationship of Bazaral and Helinski (8) it can be determined that the molecular weight of the P35 DNA molecules is 28×10^6 daltons.

Part II

Isolation and Characterization of Plasmid DNA from S. pullorum

Isolation of plasmid DNA. To determine if S. pullorum contains plasmid DNA a lysate was layered on CsCl EtBr solution and centrifuged to equilibrium. Cells were labeled with ^3H-thymidine (1 uCi/ml) in 30 ml of TCG and were lyxed with lysozyme-EDTA and Brij 58-deoxycholate treatment followed by freeze thawing. After removal of

Figure 6. Sucrose gradient of P35 DNA. An 0.15 ml sample containing ^{3}H labeled P35 DNA and ^{14}C labeled colE1 DNA was layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW50L rotor at 50,000 RPM for 115 min. Fractions were collected directly on to filter paper squares, dried, washed with TCA and counted in a scintillation counter. ^{3}H ———, ^{14}C -o--o-. Sedimentation in this and all subsequent sucrose gradients is from right to left.


400
200
CPM
Col E1
P35
10
20
30
Fractions

most of the chromosomal DNA by centrifugation in the RC-2B centrifuge, the cleared lysate was layered on a CsCl-EtBr dye-buoyant solution and centrifuged to equilibrium. At equilibrium two fluorescent bands could be observed when the tube was illuminated with a mineral lamp. The gradient was fractionated into approximately 60 fractions by collecting drops from a needle placed through the bottom of the tube. Aliquots of 10 ul from the fractions containing the fluorescent bands were spotted on filter paper squares, washed with TCA, dried, and the radioactivity counted in a scintillation counter.

Figure 7A shows that there were two peaks of radioactivity in the CsCl-EtBr gradient of the cleared lysate from S. pullorum MS53. The denser peak in fractions 17-21 represented covalently-closed circular plasmid DNA molecules as demonstrated by Radloff, Bauer and Vinograd (66). The lighter peak was composed of open-circular plasmid and linear chromosomal DNA. From a comparison of the total amount of radioactivity in the satellite fraction (7.36×10^6 cpm) to the total radioactivity taken up by the cells (4.5×10^7 cpm), it was calculated that about 16% of the total cellular DNA existed as extrachromosomal plasmid DNA.

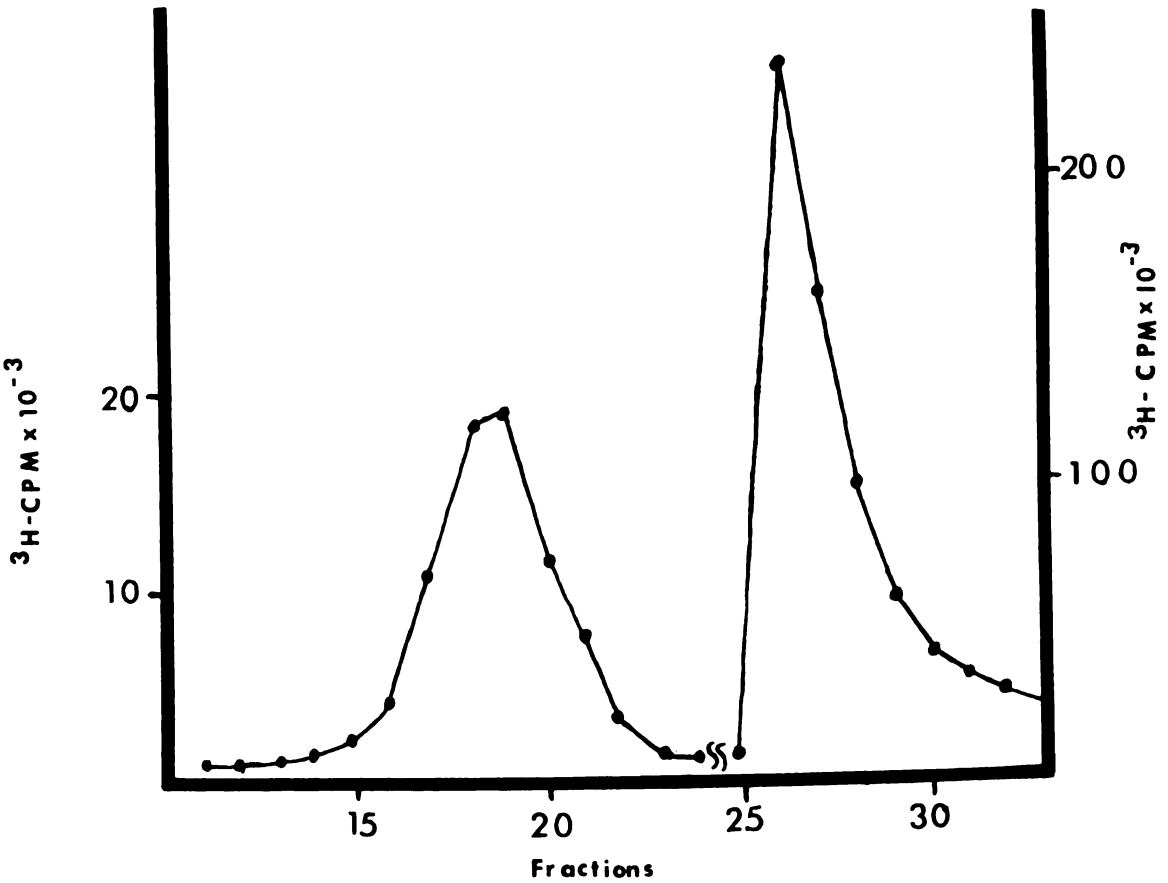
The plasmid fractions were pooled, dialyzed against TES buffer and a 0.1 ml sample was layered on a neutral 20-31% sucrose gradient. After centrifugation the

Figure 7. Isolation of plasmid DNA from S. pullorum MS53.

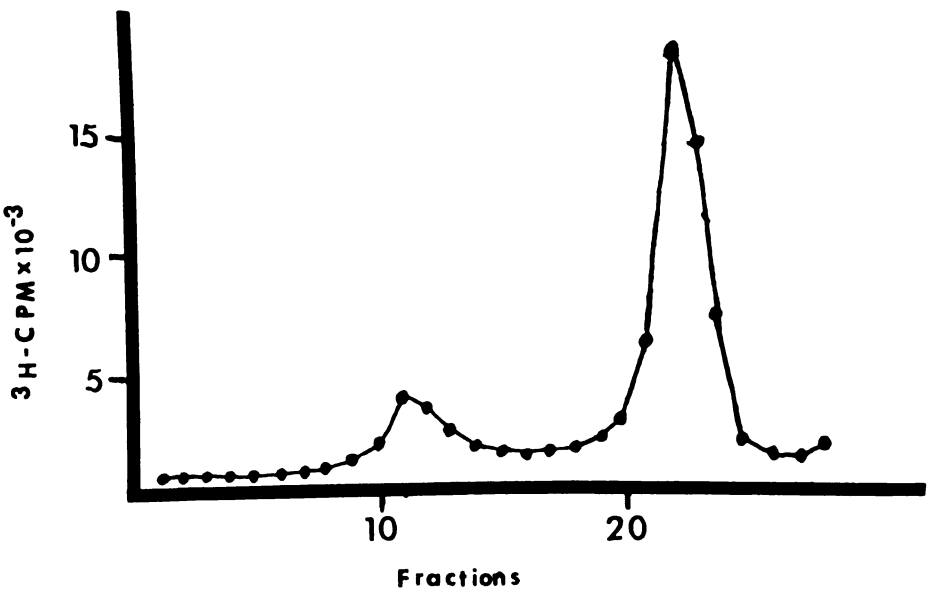
A. 3.0 ml of a cleared lysate of S. pullorum MS53 labeled with ^{3}H-thymidine were centrifuged in a CsCl-EtBr dye-buoyant density gradient for 30 hours at 44,000 RPM in a type 50 rotor. Fractions were collected and 10 ul samples spotted on filter paper squares, TCA washed and counted for radioactivity. Fractions 16-21 containing plasmid DNA were pooled and dialyzed in the dark against TES buffer.

B. 0.1 ml of the pooled plasmid DNA from the CsCl-EtBr gradient was layered on a neutral 20-31% sucrose gradient. The gradient was centrifuged for 180 min in an SW39 rotor at 38,000 PRM. Fractions were collected directly onto filter paper squares, TCA washed and counted for radioactivity.

A.


^{3}H-CPM x 10^{-3}
20
10
200
100
^{3}H- CPM x 10^{-3}
15
20
25
30
Fractions

B.

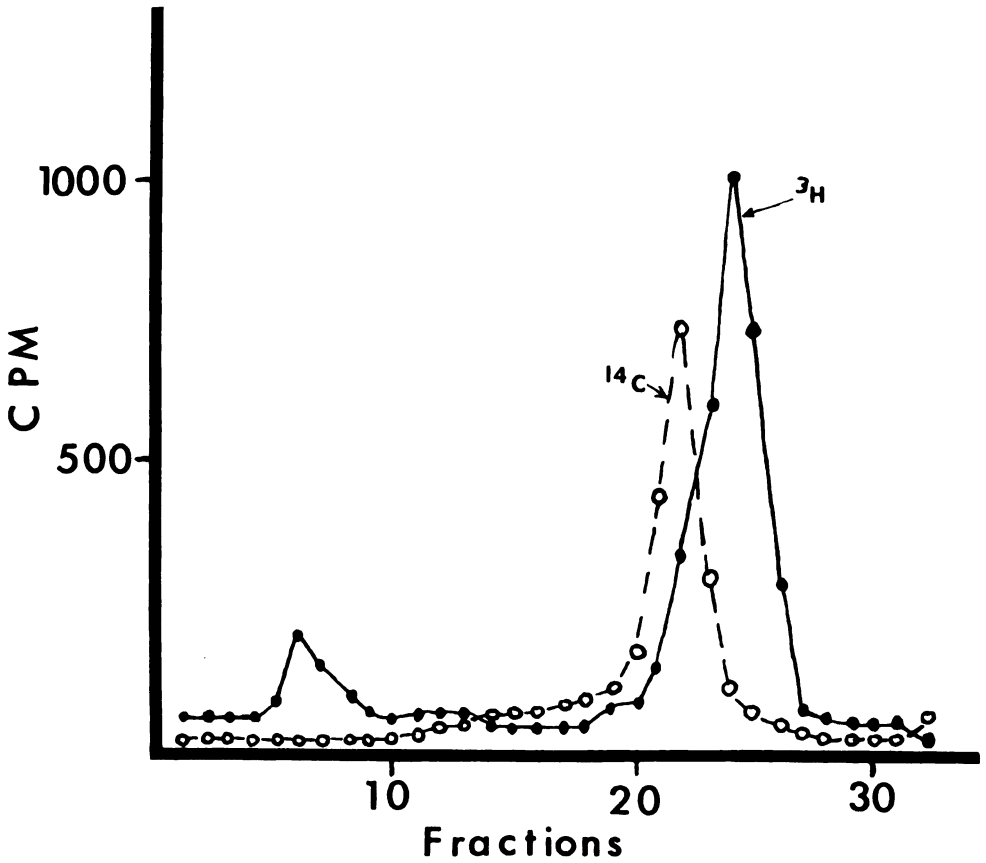

^{3}H-CPM x 10^{-3}
15
10
5
10
20
Fractions

gradient was fractionated and the results shown in Figure 7B indicate that S. pullorum MS53 contains two species of plasmid DNA of greatly different sedimentation values. Identical results were also obtained with S. pullorum MS35.

Sedimentation coefficients and molecular weights of the plasmid DNA. To determine the size and approximate molecular weights of the supercoiled DNA from S. pullorum MS53 the sedimentation coefficients were calculated by cosedimenting the plasmids with colE1 DNA. S. pullorum plasmids labeled with ^{3}H and ^{14}C labeled colE1 DNA from E. coli JC411 ($E1^{+}$) were first isolated on CsCl-EtBr dye-buoyant density gradients and then cosedimented in a neutral sucrose gradient. It has been shown by Bazaral and Helinski (9) that when colE1 DNA was isolated from a CsCl-EtBr gradient that it was a supercoiled molecule of 4.6×10^{6} daltons with a sedimentation coefficient of 23s.

From the results shown in Figure 8 it can be calculated that the faster sedimenting plasmid of strain MS53 has a sedimentation coefficient of approximately 65s while the smaller plasmid is about 17s. From the relationship given by Bazaral and Helinski (8) it can be computed that the 17s plasmid corresponds to a supercoiled DNA molecule of approximately 1.5×10^{6} daltons and the 65s supercoiled plasmid has a molecular weight of 45×10^{6} daltons.

Figure 8. Neutral sucrose gradient of plasmid DNA from MS53 and colE1 DNA. A 0.15 ml sample containing 0.1 ml of ^{3}H labeled MS53 plasmid DNA and 0.05 ml of ^{14}C labeled colE1 DNA were layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW50L rotor for 95 min at 50,000 RPM. ^{14}C -o-o-, ^{3}H ———.


1000
500
CPM
^{3}H
^{14}C
10
20
30
Fractions

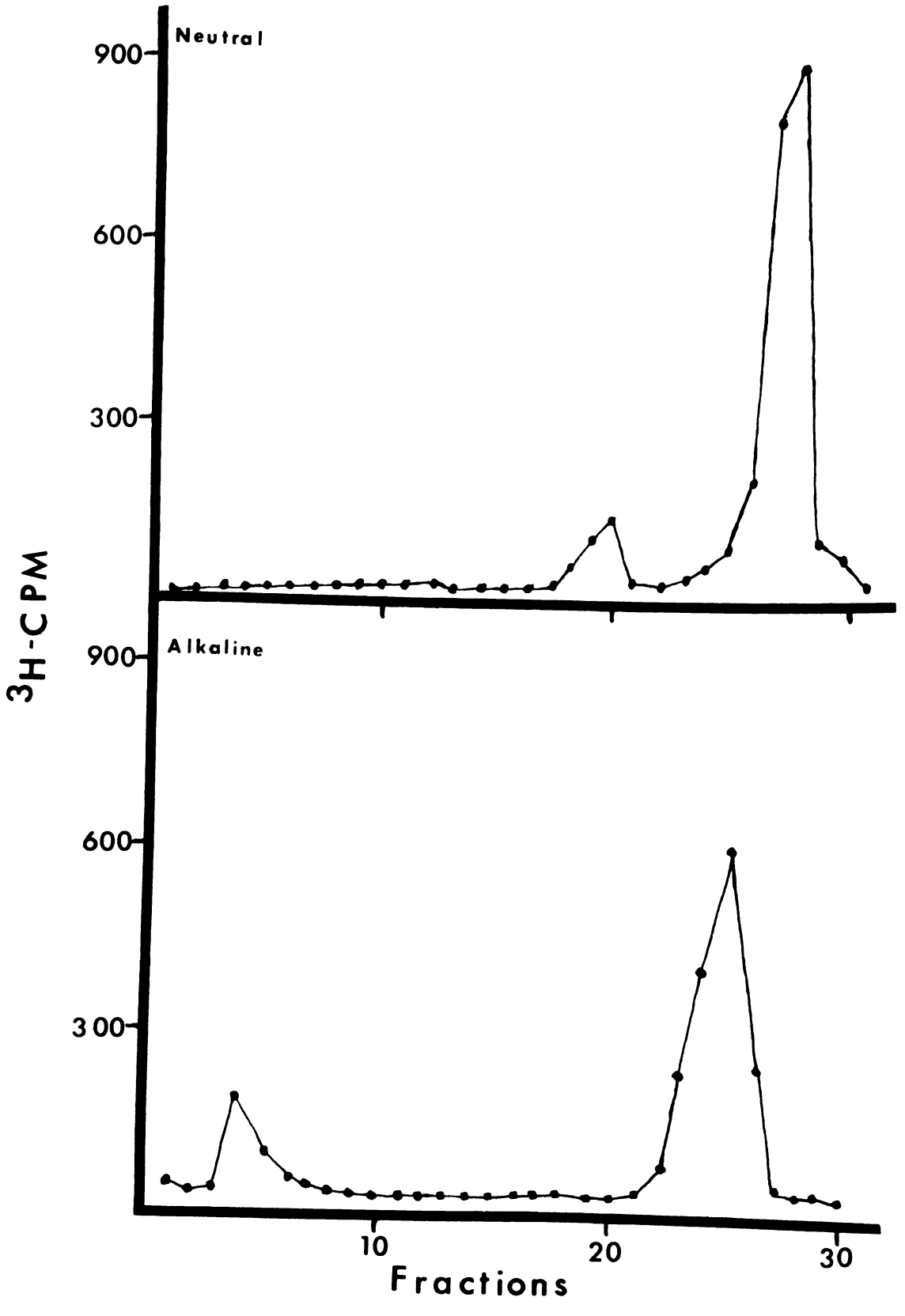
The 17s, 1.5 x 10^6 daltons molecule will hereafter be referred to as the PO-1 plasmid and the 45 x 10^6 daltons molecule as the PO-2 plasmid of S. pullorum

Alkaline sucrose gradient sedimentation of plasmid DNA. To verify that these plasmids are indeed supercoiled DNA, samples of the plasmid fraction from a CsCl-EtBr gradient were centrifuged in an alkaline sucrose gradient. It has been shown by Clayton and Vinograd (24) that when covalently-closed circular DNA is denatured and sedimented in an alkaline gradient, the s value is increased by approximately 2.5 fold.

Figure 9 shows that when the plasmid DNA from S. pullorum MS53 is centrifuged in an alkaline sucrose gradient, both peaks move a greater distance than in the neutral gradient. The data of a previous section indicated that the smaller PO-1 plasmid sedimented at 17s and the larger PO-2 plasmid at 65s in neutral sucrose. By comparison the plasmids have sedimentation values of 35s and 160s respectively in alkaline sucrose which would be the approximate values expected for supercoiled DNA. These data, therefore, verified the condlusion that the DNA observed in the neutral sucrose gradient was supercoiled as would be expected by their isolation from CsCl-EtBr gradients.

Sedimentation of a cleared lysate on a neutral sucrose gradient. The isolation of plasmid DNA from a

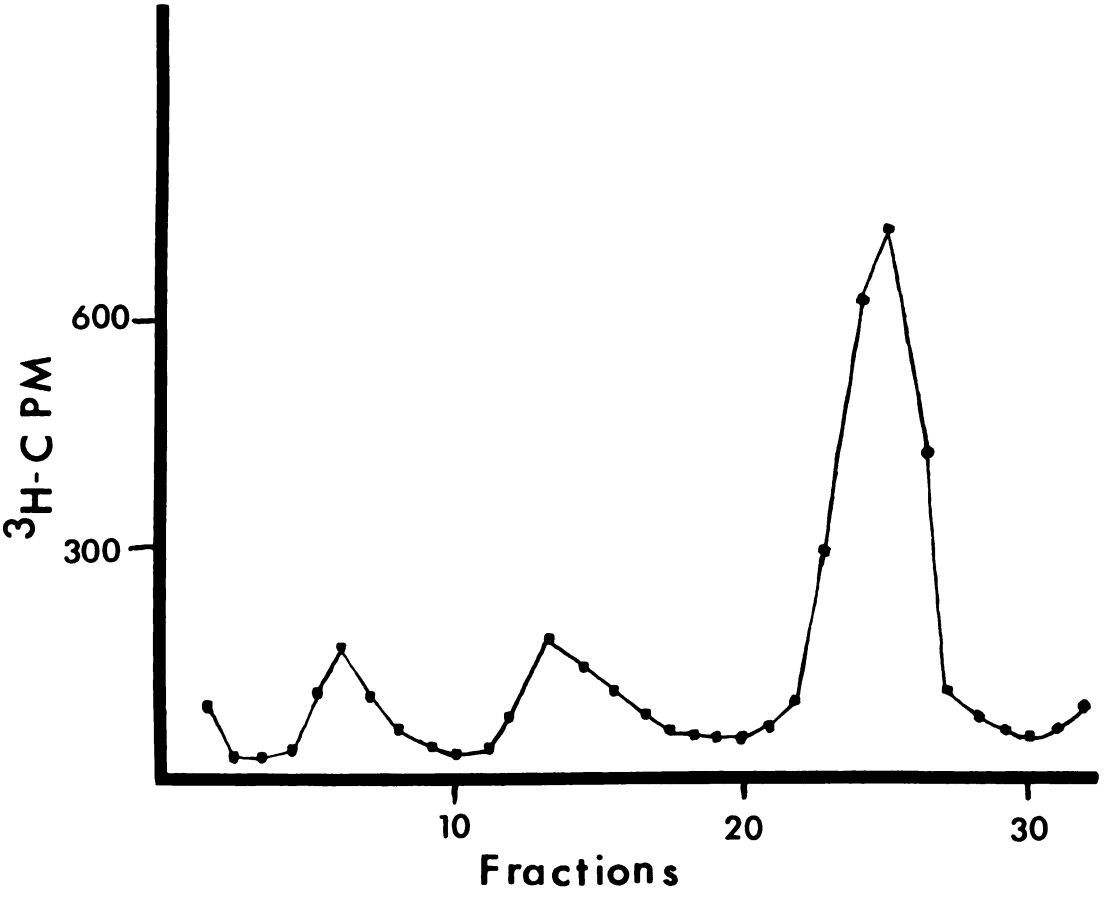
Figure 9. Alkaline and neutral sucrose gradients of S. pullorum 53 plasmids. 0.1 ml samples of ^{3}H labeled plasmid DNA isolated from a CsCl-EtBr gradient were layered on neutral and alkaline 20-31% sucrose gradients. The gradients were centrifuged simultaneously in an SW50L rotor at 50,000 RPM for 45 min.


Neutral
900
600
300
Alkaline
900
600
300
^{3}H-CPM
10
20
30
Fractions

Cs-Cl-EtBr gradient excluded the possibility of identifying any species of plasmid DNA that may have been nicked to the open circular form. To determine if any open circular molecular forms are present, a cleared lysate was layered directly on a neutral sucrose gradient and centrifuged. Three peaks of radioactivity were observed instead of two observed in the CsCl-EtBr purified preparation. As shown in Figure 10 this third peak sediments between the 17s and the 65s peaks in a region approximately corresponding to 45s. According to Clayton and Vinograd (24), this is the expected sedimentation value of the open circular form of a 65s supercoiled DNA molecule. Another indication that the 45s peak represents the open circular form of the PO-2 supercoiled molecule is the quantitative conversion of the material isolated from a CsCl-EtBr gradient from 65s to 45s during storage at room temperature or upon repeated thawings of a frozen sample. Probably due to its much greater size, the PO-2 plasmid was more likely to be nicked than was the PO-1 plasmid. Plasmid PO-1 showed no open-circular or linear forms in any cleared lysate produced during this work.

From three similar gradients of cleared lysates of S. pullorum 53 it was calculated that the larger PO-2 plasmid molecule (both closed and open circular forms) accounted for approximately 16% of the total plasmid DNA. Therefore, since there is five times as much DNA in the

Figure 10. Neutral sucrose gradient of a cleared lysate. A 0.2 ml sample of a cleared lysate of S. pullorum 53 labeled with ^{3}H-thymidine was layered directly on a neutral 20-31% sucrose gradient which was centrifuged for 220 min at 39,000 RPM using an SW39 rotor.


600
300
^{3}H-CPM
10
20
30
Fractions

PO-1 peak as there is in the PO-2 peaks, there is 5 x 45 x 10^6 daltons = 225 x 10^6 daltons of PO-1 DNA for each copy of the PO-2 molecule. There is, therefore, 225 x 10^6/1.5 x 10^6 = 150 copies of the smaller PO-1 plasmid per copy of PO-2. At one copy of the large molecule per cell there would be 225 x 45 x 10^6 = 270 x 10^6 daltons of plasmid DNA per cell. Assuming a molecular weight of the S. pullorum chromosome close to that of E. coli which is 2 x 10^9 (22) then the plasmid DNA would account for 14% of the total. This is in close agreement with the amount of plasmid DNA calculated from the original CsCl-EtBr gradient.

Electron microscopy of plasmid DNA. The previous sections have demonstrated that S. pullorum contains two distinct species of supercoiled plasmid DNA molecules. To observe the structure of these molecules, samples from the plasmid fraction of a CsCl-EtBr density gradient were prepared for viewing in the electron microscope. Cytochrome c protein monolayers containing the plasmid DNA were prepared by the microversion of Lang and Metani (54) and picked up on carbon-coated collodion grids. These were then viewed in the electron microscope and pictures taken of representative fields containing the circular DNA molecules. Figure 11A shows one of the many small circular structures seen in the preparation. From its size it was assumed that this probably is a molecule of the PO-1 plasmid in the open circular configuration.

Figures 11B and 11C show a much larger molecular species which is assumed to be the PO-2 plasmid. Both molecules show some interraveling of the strands and areas in which the strands appear to be discontinuous but these may be due to some supercoiled structure remaining in the open circular forms. No evidence for interlocked catenated molecules was observed in the PO-2 molecules.

Part III

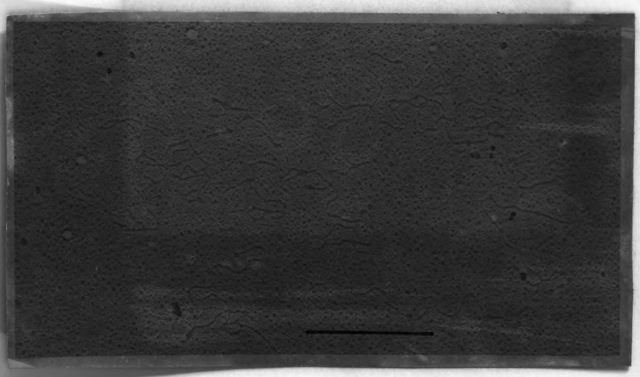
Exclusion of Plasmid DNA in S. pullorum by Lysogeny with P35

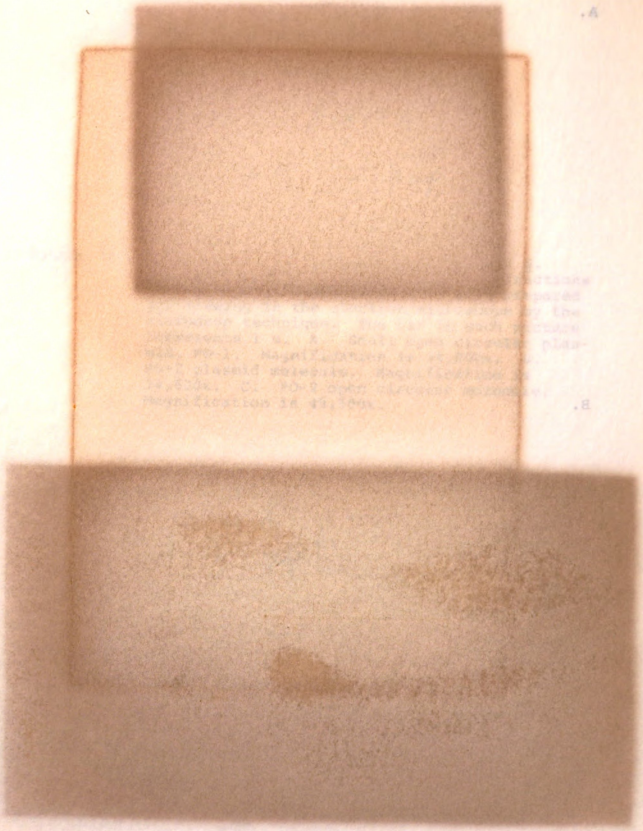
Plasmid DNA in S. pullorum MS53(P35). To determine if lysogenization by P35 had affected the plasmids which were already present in S. pullorum MS53 the lysogenic strain MS53(P35) was labeled with ^{3}H-thymidine, lysed and the supercoiled DNA isolated in a CsCl-EtBr dye-buoyant density gradient. Centrifugation of the pooled plasmid fractions in a neutral 20-31% sucrose gradient revealed that now, after lysogeny of this strain, the PO-2 plasmid was no longer present (Figure 12). The PO-1 plasmid was still present and sedimentation in an alkaline gradient indicated that it remained in the covalently-closed circular configuration. The loss of the plasmid was an unexpected finding since it had already been demonstrated that the DNA isolated from phage P35 has a molecular weight of about 28×10^{6} and plasmid PO-2 is 1.6 times larger than this.

Figure 11. Electron micrographs of plasmid DNA of S. pullorum MS53. DNA from the plasmid fractions of a CsCl-EtBr density gradient were prepared for viewing in the electron microscope by the microdrop technique. The bar in each picture represents 1 u. A. Small open circular plasmid, PO-1. Magnification is 34,000x. B. PO-2 plasmid molecule. Magnification is 34,600x. C. PO-2 open circular molecule. Magnification is 49,500x.

A.

B.





c.

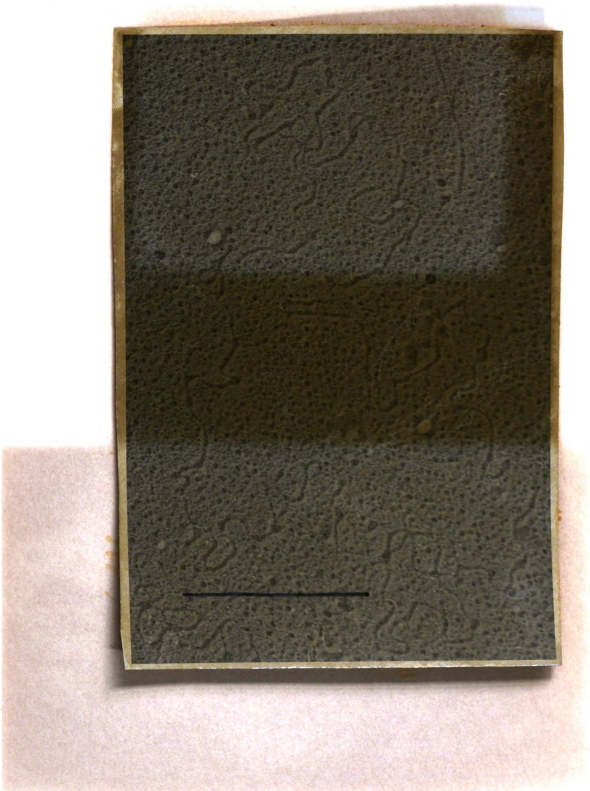
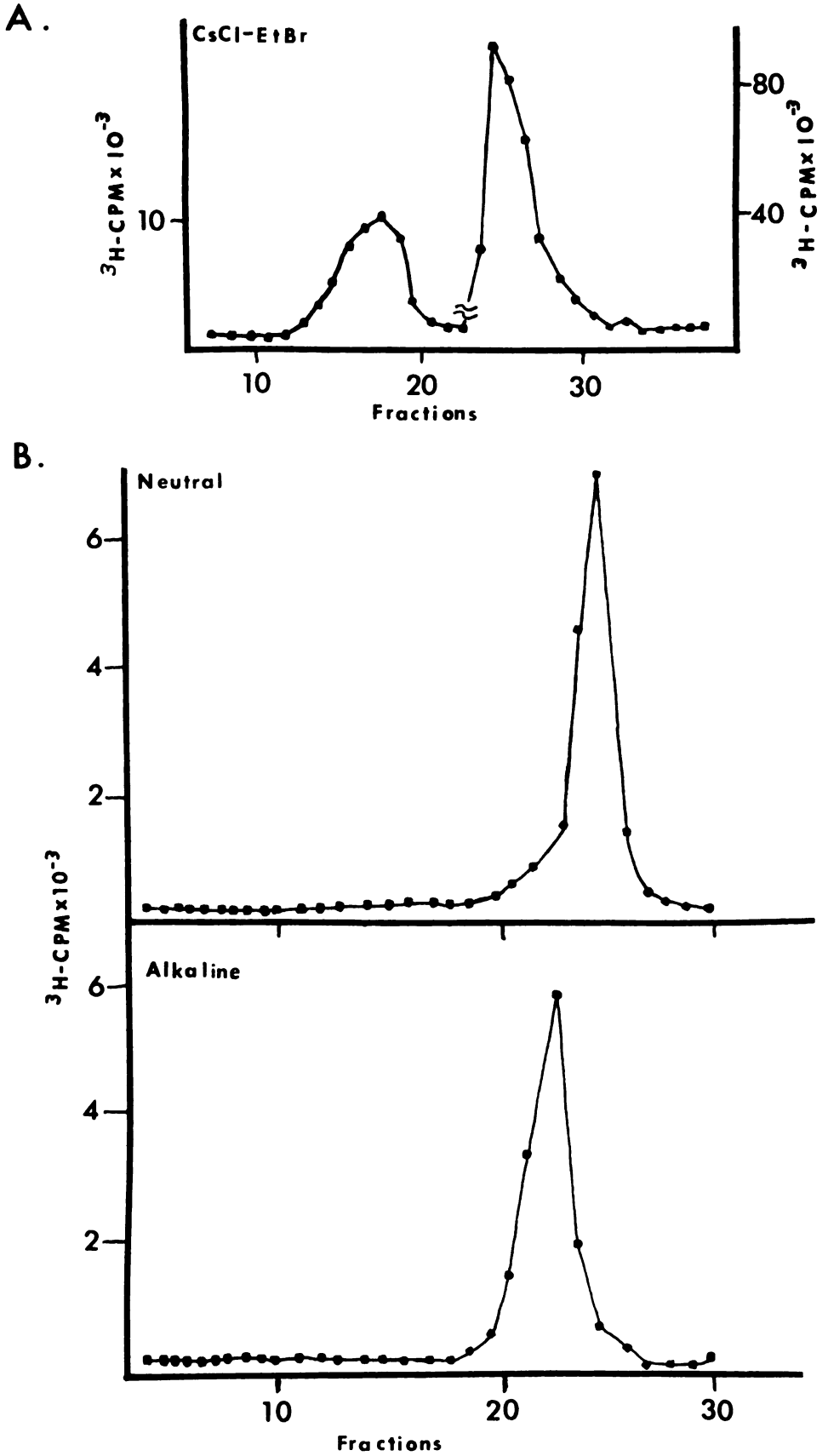


Figure 12. Plasmid DNA of 53(P35).

A. 3.0 ml of a cleared lysate of 53(P35) labeled with ^{3}H-thymidine were centrifuged in a CsCl-EtBr gradient for 36 hours at 44,000 RPM in a type 50 rotor. Fractions were collected, and the radioactivity in 5 ul samples counted. The plasmid DNA in fractions 14-19 was pooled and dialyzed against TES buffer.

B. 0.1 ml samples of the pooled plasmid DNA from the CsCl-EtBr gradient were layered on neutral and alkaline 20-31% sucrose gradients. The gradients were centrifuged for 50 min at 50,000 RPM in an SW50L rotor.


A.
CsCl-EtBr
^{3}H-CPM x 10^{-3}
10
80
40
10
20
30
Fractions
B.
Neutral
6
4
2
Alkaline
6
4
2
^{3}H-CPM x 10^{-3}
10
20
30
Fractions

Isolation of a cured derivative of S. pullorum MS53(P35). Since it has been demonstrated that when S. pullorum MS53 is made lysogenic for P35, the PO-2 plasmid is excluded, it was of interest to find out what would happen when the strain was cured of the prophage. Two possibilities existed: the plasmid would not be regained or the plasmid might be regained if it originated from the chromosome and was continuously and randomly being excised.

Approximately 50 isolated clones of S. pullorum 53 (P35) were picked and grown in L broth. After 8 hours of incubation the supernatant fluids were tested for the presence of free phage particles by spotting chloroform treated aliquots on to fresh lawns of sensitive cells. Four isolates were found not to liberate phage and were purified by three cycles of clonal isolation and then retested for phage production. These isolates were then tested for their ability to propagate phage P35. All were able to adsorb and propagate the phage. The isolates were then tested for their ability to produce phage upon treatment with mitomycin c or UV light. One, S192, was found to be non-inducible and also to be sensitive to P35, P35c, and P22.

To determine if S192 had regained the PO-2 plasmid a sample of a cleared lysate was analyzed directly on a neutral sucrose gradient. This allowed detection of both

the closed and open circular forms of the PO-2 molecule. The results shown in Figure 13 indicated that S192 like its parent 53(P35) did not contain the larger PO-2 plasmid molecule.

Attempt to follow the fate of plasmid PO-2 following infection with P35. Two experiments were performed to determine the fate of the PO-2 plasmid subsequent to infection of S. pullorum MS53 with phage P35. In the first experiment log phase cells were infected with P35 at an m.o.i. of 10. ^{3}H-thymidine was then added to the culture which was then incubated at 37C. At intervals after infection 10 ml samples were removed and the DNA replication quickly stopped by addition of KCN (to a final concentration of 0.002 M) and EDTA (to a final concentration of 0.05 M) and by chilling the cells in an ice water bath. The cells were then lysed and the cleared lysates centrifuged in neutral sucrose gradients. The results shown in Figure 14 indicate that by 30 min after infection much of the newly made DNA is in a form which sediments at a rate about equal to phage DNA. At 50 min the bulk of the DNA sediments in an extremely broad peak and at 90 min most of the DNA remaining in the cells is of phage DNA size. It also can be seen that by 90 min there is no indication of a peak at 65s. The PO-1 plasmid was present in each gradient and served as a reference marker for sedimentation.

Figure 13. Neutral sucrose gradient of cleared lysate of S192. 0.1 ml of a ^{3}H labeled cleared lysate of S192 was layered on a 20-31% neutral sucrose gradient centrifugation was in an SW39 rotor for 220 min at 39,000 RPM.

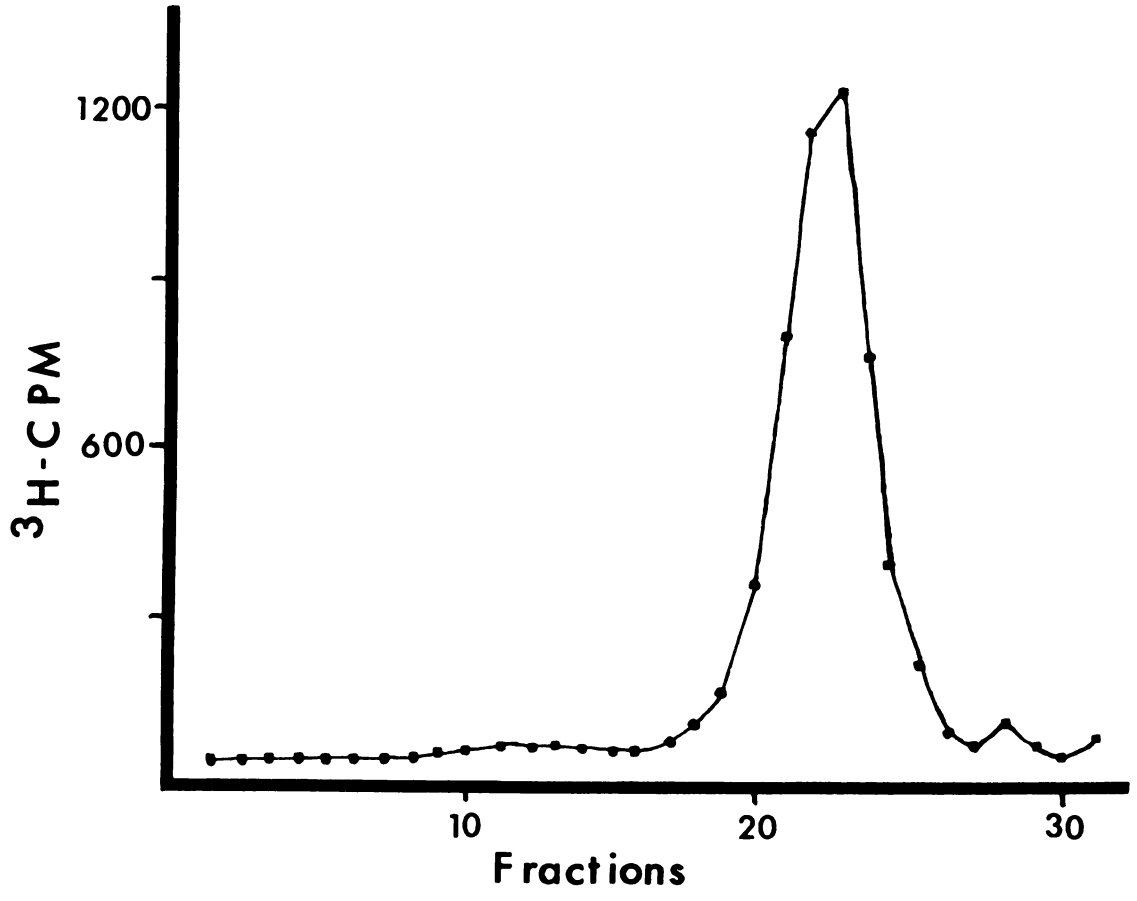
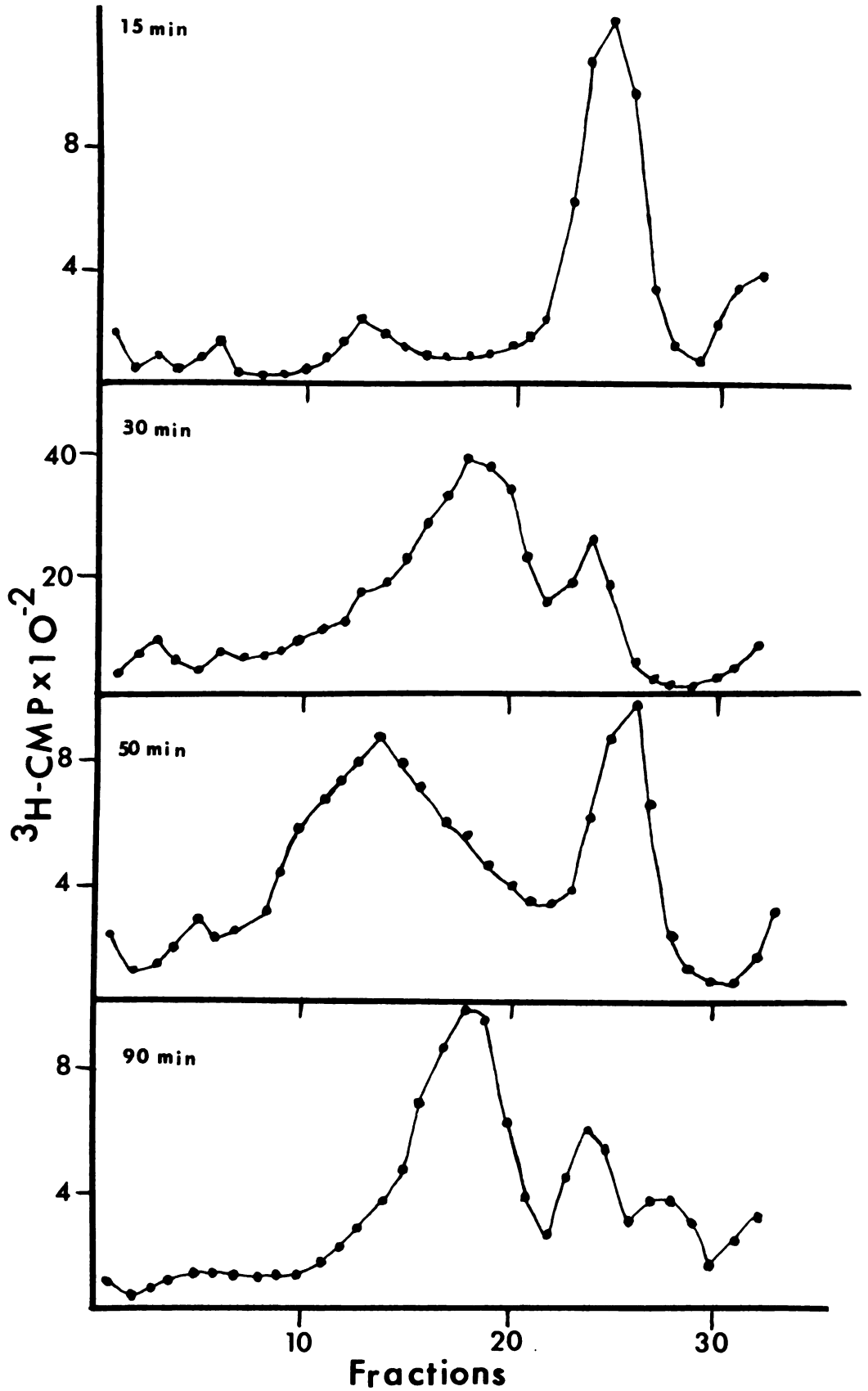

1200
600
^{3}H-CPM
10
20
30
Fractions

Figure 14. DNA synthesis in P35 infected S. pullorum MS53. 10 ml cultures of MS53 were grown to log phase in aerated TCG broth and labeled with ^{3}H-thymidine. The cultures were infected with P35 (m.o.i. = 10) and at the times indicated after infection a culture was chilled in an ice bath and 0.1 ml of KCN (0.2 M) plus 1.0 ml of EDTA (0.5 M) was added. Cleared lysates were made of each culture and 0.15 ml samples were layered on 20-31% neutral sucrose gradients which were centrifuged at 38,000 RPM for 220 min in an SW39 rotor.

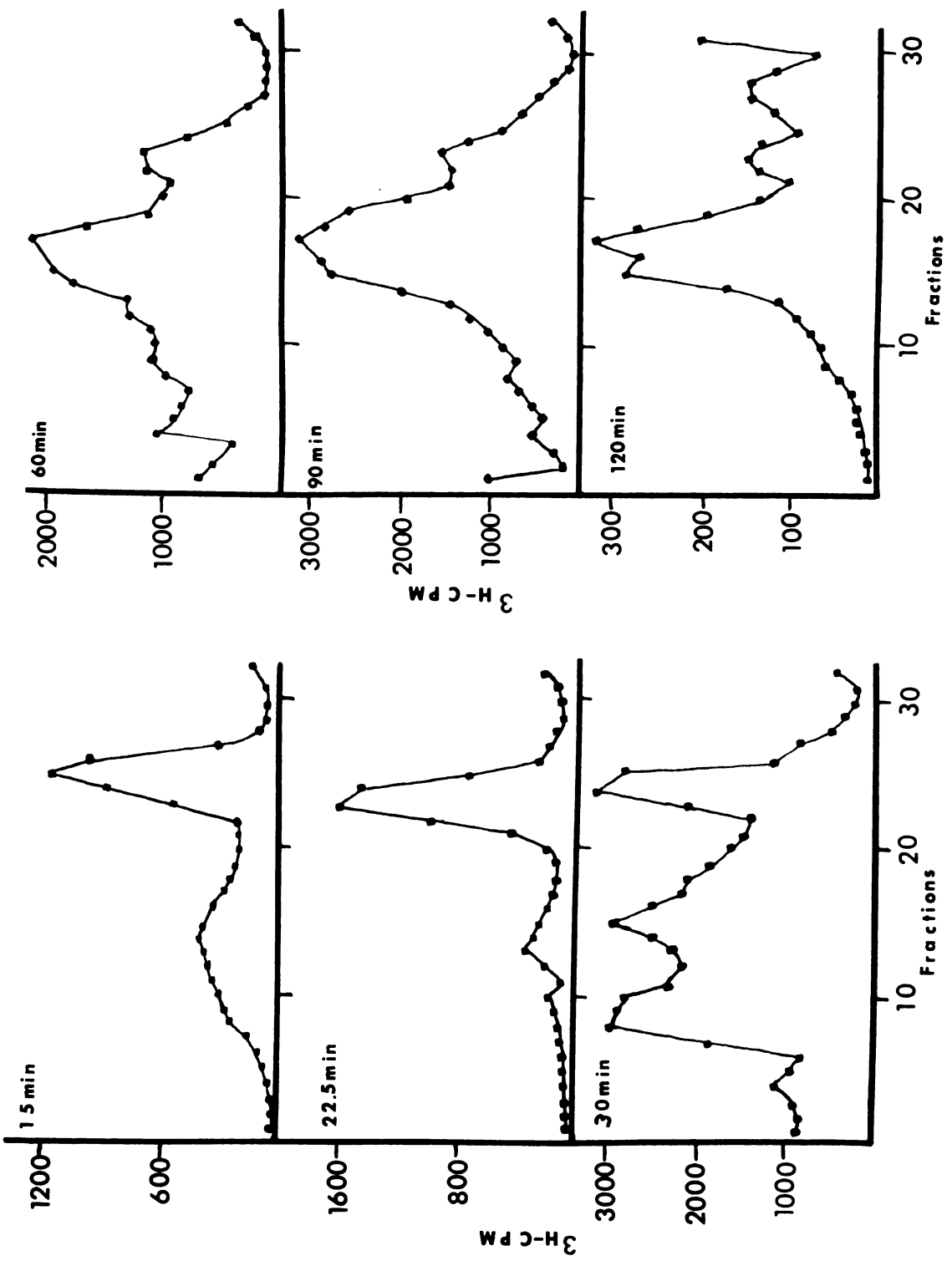

15 min
30 min
50 min
90 min
8
4
40
20
8
4
8
4
^{3}H-CMP x 10^{-2}
10
20
30
Fractions

In the second experiment cells were first labeled with ^{3}H-thymidine, harvested and resuspended in TCG broth without ^{3}H-thymidine. They were then infected with P35 at an m.o.i. of 10. As before, samples were removed at intervals, treated with KCN and EDTA, chilled and lysed. When these cleared lysates were centrifuged in neutral sucrose gradients, it was seen that soon after infection the cells contained a very broad distribution of labeled DNA (Figure 15). Apparently not all of this label could have come from the amount originally present as plasmid DNA but was probably derived from chromosomal DNA. Again late in infection there does not appear to be 65s peak present.

These experiments gave very little information about the process of or the reason for the exclusion of plasmid PO-2. More definite results would have been obtained if if were possible to pulse label the infected cells for short time intervals with ^{3}H-thymidine but the poor uptake ability of S. pullorum for thymidine necessitated the lengthy exposures to the label.

To explain the apparent exclusion it was postulated that the plasmid must in some way be related to the phage. They may perhaps share common cellular replication sites or be sensitive to the same immunity system. Either of these would require that there be a degree of nucleotide similarity (homology) between the phage DNA and the plasmid DNA.

Figure 15. DNA synthesis in prelabeled MS53 infected with P35. Cultures of MS53 were grown and labeled as described in Figure 14 but before infection the cells were harvested, washed once and resuspended in fresh TCG broth without ^{3}H-thymidine. After infection with P35 (m.o.i. = 10) the cultures were treated at the indicated times with KCN and EDTA as before and lysed. 0.15 ml samples of the cleared lysates were layered on 20-31% neutral sucrose gradients and centrifuged for 220 min at 38,000 RPM in an SW39 rotor.


15 min
22.5 min
30 min
60 min
90 min
120 min
3H-C PM
Fractions

Part IV

Hybridization Studies with *S. pullorum* Plasmid DNA

The demonstration in Part III that plasmid PO-2 is excluded from cells lysogenized by phage P35 indicated a probable close relationship between the plasmid and phage DNAs. To determine if, in fact, they do contain homologous sequences it was decided to perform hybridization experiments between the phage and the plasmid. Initially, DNA-DNA hybridizations were attempted but these were unsuccessful due to an inability to isolate ^{3}H-labeled DNA of high specific activity and due to the low DNA yields from P35 phage preparations. It was, therefore, decided to synthesize *in vitro* ^{3}H-labeled RNA of high specific activity from a phage template and to use this ^{3}H-RNA to hybridize with the plasmid DNA. The DNA extracted from P35c was used as a template rather than DNA from P35 since the yield of purified DNA per liter of lysate was greater with P35c than with P35.

Assay of Pseudomonas putida RNA polymerase using P35c DNA as a template. Before using the RNA polymerase to produce a quantity of ^{3}H-RNA sufficient to use in hybridization studies, it was necessary to assay the enzyme's ability to incorporate nucleotide triphosphates into RNA when using P35c DNA as a template. The preparation of RNA polymerase provided by Dr. J. Boezi

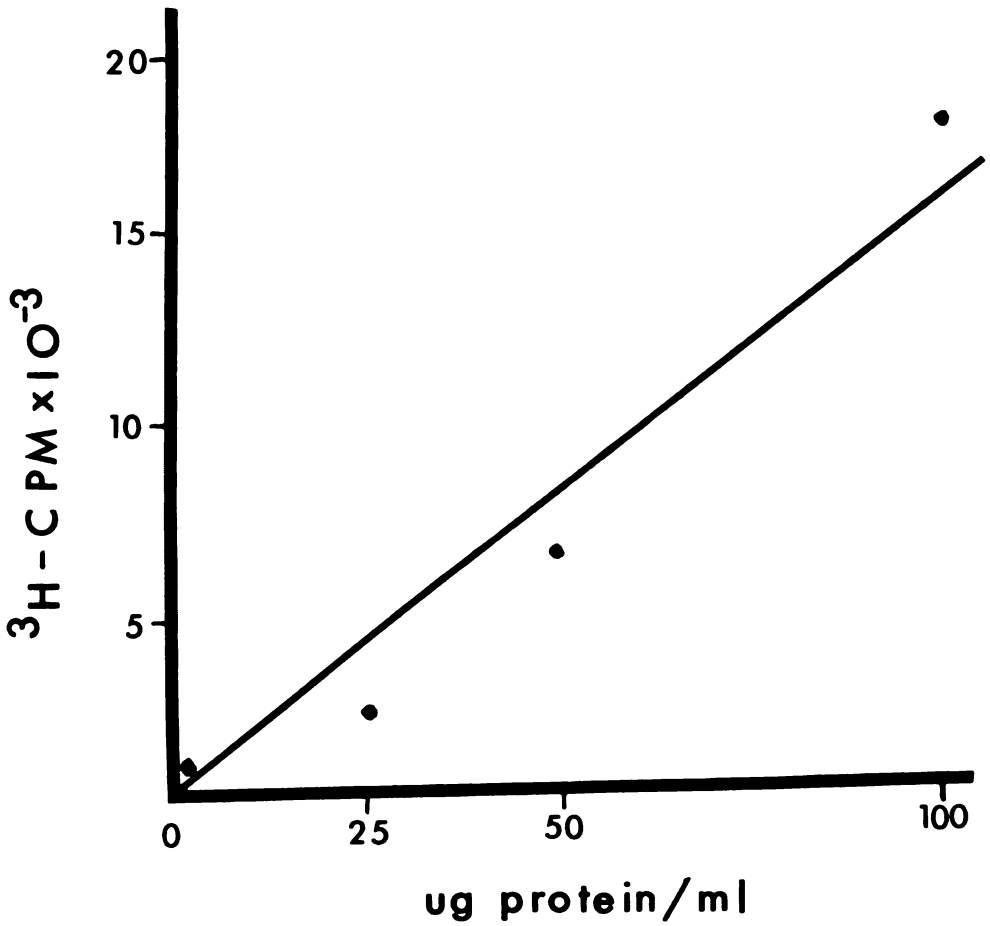
contained 14.4 mg of protein per ml which when assayed on phage gh-1 DNA had a specific activity of 2000 n moles of ^{3}H-CMP incorporated per hour per mg of protein.

The results of an assay using P35c DNA as a template are shown in Figure 16 and the specific activity calculated from the data is 450 n moles of ^{3}H-UTP incorporated per hr per mg of protein. This, therefore, indicated that the P. putida RNA polymerase could transcribe P35c DNA although at a rate one third that using gh-1 DNA and that the enzyme could be used for the in vitro synthesis of ^{3}H-RNA.

In vitro synthesis of ^{3}H-RNA. Since the assay had indicated that the P. putida RNA polymerase could use P35c DNA as a template, a procedure was set up to synthesize sufficient RNA for hybridization studies. To 3 ml of the reaction mixture were added 41 mg/ml of RNA polymerase, 50 ug/ml of P35c DNA and ^{3}H-UTP (81.5 uc/u mole) to a final concentration of 0.09 mM. This was incubated for 10 min at 37C. The reaction was stopped by addition of 0.3 ml of 10% SDS and the RNA extracted three times with water-saturated phenol. After dialysis to remove the phenol and any remaining unincorporated nucleotides, the amount of ^{3}H-RNA present was determined.

The concentration of ^{3}H-UTP which was added to the reaction mixture was determined from its UV absorbance at 269 nm to be 4.1 mM (the molar extinction coefficient of UTP at 269 nm is 10^{4}). The amount of radioactivity in the

Figure 16. Assay of RNA polymerase. Each assay mixture of 0.4 ml contained 50 ug of P35c DNA and 0.15 mM ^{3}H-UTP at 10 uc/u mole (2.09×10^{6} cpm-u mole) and was incubated for 10 min at 37C.


20
15
10
5
^3H-CPM x 10^{-3}
0
25
50
100
ug protein/ml

^{3}H-RNA and ^{3}H-UTP was determined by spotting samples on glass fiber filters and solubilizing them with soluene-100 for maximum and equal counting efficiency. The ^{3}H-UTP was diluted 1:20 and a 0.1 ml sample of the dilution containing 0.0205 u moles and was spotted and counted. It was found to have 784,795 cpm. The specific activity of ^{3}H-UTP, therefore, was 3.83×10^{7} cpm/u mole. A 0.05 ml sample of the synthesized ^{3}H-RNA was counted and found to have 6436 cpm. Therefore, in a 0.05 ml sample of ^{3}H-RNA there is 6436 cpm/3.83×10^{7} cpm/u mole ^{3}H-UTP or 0.168 n moles of ^{3}H-UTP as ^{3}H-RNA. Assuming equal molar amounts of the four nucleotide triphosphate incorporated into RNA and the average molecular weight of the nucleotide monophosphates is 411, then 4 x 0.168 n moles/0.05 ml equals 0.672 n moles of nucleotide monophosphate per 0.05 ml. And 411×10^{-9} gm/n mole nucleotide monophosphate x 0.672 n moles of nucleotide monophosphates as RNA/0.05 ml equals 272×10^{-9} gm per 0.05 ml. There is, therefore, 0.272 ug RNA/0.05 ml x 20 equals 5.45 ug ^{3}H-RNA/ml.

Hybridization efficiency. To test the efficiency of hybridization of the ^{3}H-RNA to its complementary DNA and to determine the optimal DNA concentrations to be used in subsequent experiments, varying concentrations of denatured P35c DNA were annealed to the RNA for 6 hours at 60C. After incubation the DNA-^{3}H RNA complexes

were collected on nitrocellulose filters. The results shown in Figure 17 indicate a linear increase in the amount of ^{3}H-RNA collected as the amount of test DNA added was increased up to a maximum concentration. At greater DNA concentrations it is likely that some DNA-DNA interaction may interfere with the DNA-RNA hybridization. In all further experiments, where possible, 5 ug of the test DNA per ml were used in the hybridization reactions.

It was also noted that the percentage hybridization varied from experiment to experiment. Therefore, in all subsequent experiments a control hybridization with P35c DNA was included and the results expressed relative efficiency to the control.

Hybridization of ^{3}H-RNA (P35c) to phage DNAs. To test the relatedness between phage P35c and its assumed parent P35 and between P35c and S. typhimurium phage P22, hybridizations were performed using the RNA transcribed from P35c and the DNAs isolated from phages P35, P22, and the unrelated E. coli phage T4. It was necessary to definitely determine that P35c and P35 are identical since P35 is the lysogenic phage which excludes the PO-2 plasmid but the RNA was made with P35c DNA as a template.

The data shown in Table 2 indicate that P35c and P35 each contain homologous DNA and likely are the same phage. The DNA isolated from P22, however, shows a

Figure 17. Hybridization efficiency of ^{3}H-RNA (P35c) to P35c DNA. Each hybridization was performed in 1 ml of 2x SSC which contained 0.126 ug of ^{3}H-RNA (9200 cpm/ug) and the indicated concentration of denatured P35c DNA. Annealing took place for 6 hours at 60C after which the DNA-^{3}H-RNA complexes were collected on nitrocellulose filters.

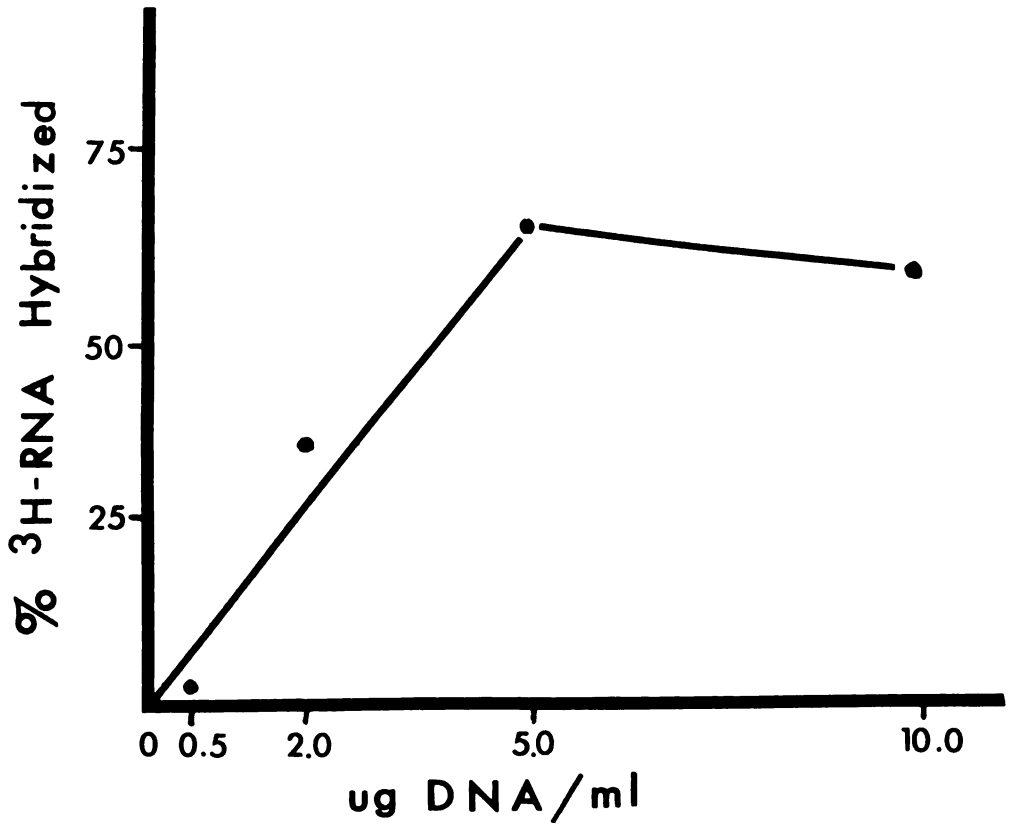

% 3H-RNA Hybridized
75
50
25
0
0.5
2.0
5.0
10.0
ug DNA/ml

Table 2. Hybridization efficiency of ^{3}H-RNA (P35c) to other phage DNAs

DNA added	CPM Specifically Bound	% of Input	Relative Efficiency
5.0 ug P35c	212	18.1	1.0
5.0 ug P35	232	20.0	1.1
5.0 ug P22	88	7.6	0.42
5.0 ug T4	2	0.17	0.009

Hybridization reactions were carried out in 1 ml of 2x SSC in the presence of 0.126 ug of ^{3}H-RNA (9200 cpm/ug) and were incubated for 6 hours at 60C. The data shown here are the average of either 2 or 3 experiments with duplicate reactions at each point. In each experiment controls containing no DNA were run simultaneously and this background level (always less than 0.6% of the input) was subtracted from the total cpm bound to the filters giving the cpm specifically bound.

somewhat lower efficiency of hybridization which may be due to poor quality of the DNA preparation or to an actual nonhomology between segments of the genomes of P22 and P35. T4 DNA did not hybridize to the RNA synthesized from P35c DNA.

Hybridization of S. pullorum MS53 plasmid DNA to ^{3}H-RNA (P35c). To determine if there is a relationship between the phage P35 and the PO-2 plasmid hybridizations were performed between the ^{3}H-RNA (P35c) and plasmid DNA. S. pullorum MS53 was grown in TCG broth and labeled at low specific activity with ^{3}H-thymidine (0.25 uc/ml), the cells were harvested, lysed and the total plasmid DNA

isolated from the cleared lysate by CsCl-EtBr dye-buoyant density gradient centrifugation (Figure 18A). Due to the poor uptake of thymidine by S. pullorum, the cells were not labeled with ^{14}C-thymidine since sufficient counts of ^{14}C would not be available to locate accurately the plasmid peaks in the final sucrose gradient. After centrifugation plasmid DNA was pooled and dialyzed against TES buffer. The concentration of DNA in the pooled plasmid fractions (total volume of 0.5 ml) was determined by its UV absorbance at 260 nm to be 31 ug/ml with a specific activity of 730 cpm/ug. A 0.3 ml sample of the plasmid preparation was then centrifuged in a neutral sucrose gradient to separate the PO-1 and PO-2 plasmids (Figure 18B). The two plasmid fractions were then separately pooled and each diluted with 0.1x SSC to a concentration of 0.1 ug DNA/ml. The supercoiled plasmid DNA was then sheared by vortexing for 10 min followed by heat denaturation. The sheared-denatured plasmid DNA was then hybridized with the ^{3}H-RNA.

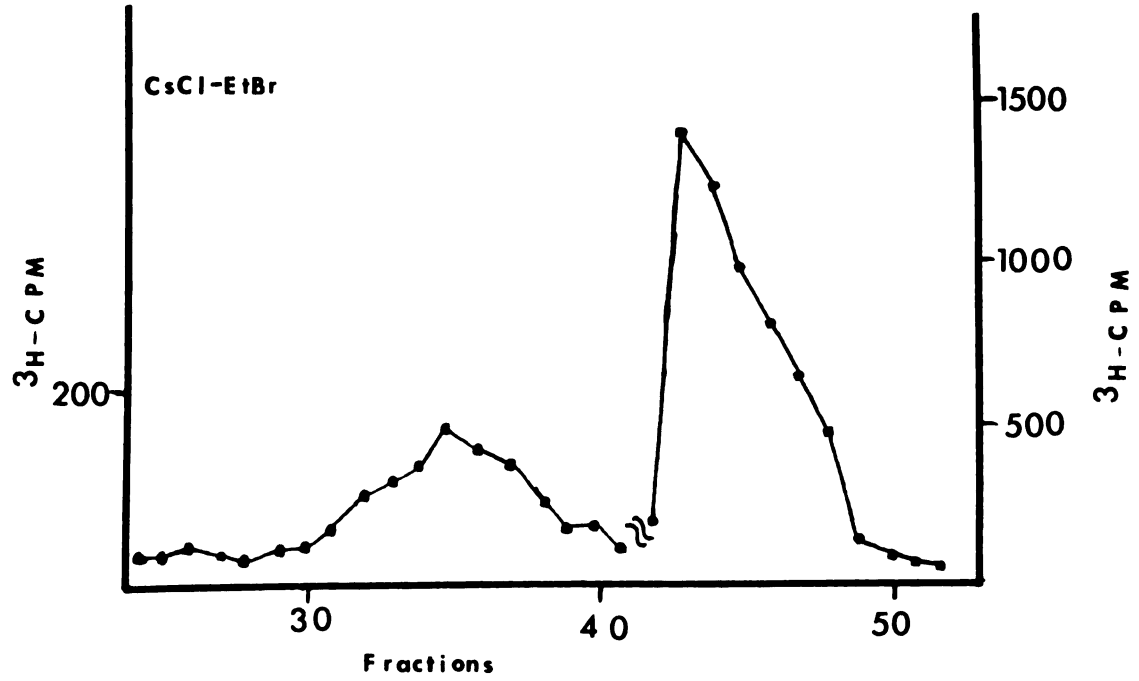
The results shown in Table 3 indicate that there is a high degree of sequence similarity (homology) between the PO-2 plasmid and phage P35. The high relative efficiency of hybridization indicates that even though the molecular weight of the 65s plasmid is approximately one and one-half times that of the phage DNA, most or all of its nucleotide sequences are similar to those of P35

Figure 18. Isolation of plasmid DNA for hybridization.

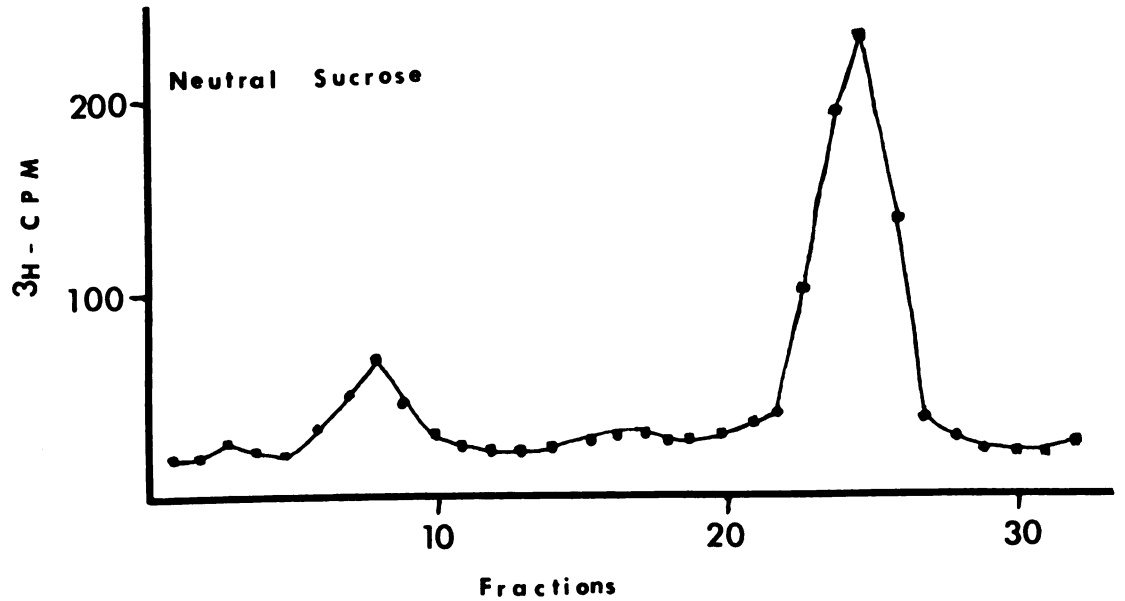
A. CsCl-EtBr gradient of a cleared lysate of S. pullorum 53. Centrifugation was for 42 hours at 44,000 RPM in a type 50 rotor. 5 ul of each fraction were spotted on filter paper squares and counted for radioactivity. Fractions 32-37 containing the plasmid DNA were pooled and dialyzed against TES.

B. 0.3 ml of the plasmid DNA isolated from the CsCl-EtBr gradient was layered on a 20-31% neutral sucrose gradient and centrifuged in the SW50L rotor at 50,000 RPM for 90 min. Fractions were collected in autoclaved 12 x 75 polyethylene tubes and 50 ul of each fraction were spotted on filter paper and counted for radioactivity. Fractions 6-9 containing the PO-2 plasmid DNA and fractions 24 and 25 containing the PO-1 plasmid DNA were pooled separately and used for the hybridization experiment.

A.


CsCl-EtBr
^{3}H-CPM
200
1500
1000
500
^{3}H-CPM
30
40
50
Fractions

B.


Neutral Sucrose
^{3}H-CPM
200
100
10
20
30
Fractions

and that there is little if any non-homologous DNA associated with the plasmid. The data in Table 3 also indicate that the PO-1 plasmid has little or no homology with the phage DNA. The total cpm specifically bound in this experiment is relatively low. This is due to the much smaller amount of test DNA added for each hybridization than in previous experiments.

Table 3. Hybridization with S. pullorum 53 plasmid DNA

DNA Added	cpm Specifically Bound*	% of Input	Relative Efficiency
0.08 ug P35c	101.0	10.3	1.0
0.08 ug PO-2	98.0	9.9	0.96
0.08 ug PO-1	6.0	0.61	0.059

0.8 ml of the denatured plasmid DNA (0.1 ug/ml, 730 ^{3}H cpm-ug) were added to each vial. 0.105 ug of ^{3}H-RNA (9200 cpm/ug) were added and the hybridization mixture brought up to a volume of 1 ml with 10x SSC (final concentration 2x SSC). The mixture was incubated 6 hours at 60C and the DNA-RNA complexes collected on nitrocellulose filters. Data reported here are the average of 2 experiments with duplicate determinations at each point.

*Controls with no DNA or no RNA were run simultaneously. The counts bound to the filter without DNA were those due to any trapped ^{3}H-RNA, and the counts bound to the filter without RNA were due to denatured ^{3}H-DNA which binds to the filter. The average counts due to ^{3}H-RNA (2 cpm) and the average counts due to ^{3}H-DNA (47 cpm) were added, and this total was subtracted from the total counts bound to each filter to give the cpm specifically bound.

In a separate experiment the total cellular DNA of S. pullorum 53 was hybridized to the ^{3}H-RNA (P35c). At 5.0 ug of 53 DNA per ml there was no detectable hybridization.

Part V

Modification and Restriction in S. pullorum

During the course of this study it was noted that when P35 was propagated on S. pullorum, the phage would form plaques on S. typhimurium LT2 only at a greatly reduced efficiency. This led to the hypothesis that S. typhimurium may be restricting the foreign DNA made in S. pullorum. To test this possibility stock lysates were made of both P35 and P22 on strains of S. pullorum and S. typhimurium. In addition to the wild type strain LT2 the restrictionless strains of S. typhimurium isolated by Okada and Watanabe (64) were also used. These strains were derived from LT2 by two steps of mutagenesis and selected for their ability to receive R factors at an increased frequency from E. coli donor strains. The most efficient recipient (i.e., least restricting) strain, Rfer2, however, was also resistant to phage P22. It was also shown in this laboratory by the author that Rfer2 is resistant to P35 (i.e., does not adsorb the phage). Strain Rfer1 is not as efficient a recipient as Rfer2 but does adsorb and propagate P22 and P35. Phage lysates

were, therefore, also made on this non-restricting strain S. typhimurium and Rferl was also used as an indicator strain.

The phage lysates were then titered on the various strains to determine efficiency of plating on each. Table 4 shows that when either phage P22 or P35 are propagated on S. pullorum their plating efficiencies on wild type S. typhimurium LT2 are reduced by a factor of 10^5. However, they do plate at high efficiency on Rferl. The data also indicate that S. pullorum does not restrict

Table 4. Efficiency of plating of P35 and P22 on various Salmonella strains

Phage	Lysate prepared on	Efficiency of plating on			
		LT2	Rferl	MS6-18	MS35
P22	35	1.3×10^{-5}	0.1	1.0	1.0
	6-8	1×10^{-4}	1.0	1.0	1.0
	LT2	1.0	1.0	0.5	0.5
	Rferl	6×10^{-3}	1.0	1.1	1.0
P35	35	2.3×10^{-5}	0.35	1.0	1.0
	6-18	3.5×10^{-5}	0.34	1.0	1.0
	LT2	1.0	1.0	0.7	1.0
	Rferl	1.2×10^{-3}	1.0	1.0	0.8

Each of the phage lysates was titered on each of the indicator strains. The efficiency of plating was determined by comparison of the plaque forming units per ml on the test strain to the plaque forming units per ml when titered on the same strain on which the phage was propagated.

DNA made in S. typhimurium. Rferl DNA is also restricted by strain LT2 but not in S. pullorum.

DISCUSSION

Part I

Isolation and Characterization of Phage P35

Phage P35 was isolated from plaques in a lawn of S. typhimurium after apparent transfer from S. pullorum F' donor cells and zygotic induction in the recipient. The origin of P35 and its relationship to the common Salmonella phage P22 is uncertain. Characterization of the phage has demonstrated that it has many similarities with P22, such as: antigenic structure, cell adsorption site, growth cycle, size of its DNA molecule and morphology of the phage particle. P35 also appears to be homoimmune to P22 as seen by the inability of P22 to produce plaques on P35 lysogens. Recently in this laboratory it has been demonstrated that P35 has a chromosomal attachment site close to the pro locus of S. pullorum (P. Stiffler unpublished data) which is identical to the P22 attachment site in S. typhimurium (76). It does appear from these data that if P35 and P22 are not identical, they are at least very closely related. However, in the hybridization experiments reported in Table 2 there is an

indication of some non-homology between the DNAs of P22 and P35. This will be discussed more fully in Part IV.

The exact origin of P35 is also obscured by the variable results obtained during induction experiments with S. pullorum strains. S. pullorum MS35 and MS53 probably harbor several types of prophages, defective phages and/or bacteriocins. When P22 infects S. typhimurium, it can induce the formation of many other phage types (45) and even recombine with unrelated heteroimmune phage producing a class of similar but non-identical phage, P221 (91, 92). It is likely that in a culture of S. pullorum whether infected with P35 (or P22) or induced with UV light (or mitomycin c) several types of particles are liberated. One such particle may be that observed in the electron micrograph taken of a lysate of P35 propagated on MS53 (Figure 4B).

A spontaneous clear-plaque forming, virulent mutant, P35c, has been isolated from lysates of P35 grown on S. pullorum. It is similar to the spontaneous occurrence of the P22c2 mutant isolated by Levine (55). Lack of complementation between P35c and P22c2 indicates that the mutation in P35c is also in the c2 gene (immunity repressor).

A P35 lysogen was isolated after infection of MS53 with the wild type phage. Strain MS53 was utilized for this since it had been observed that after P35 infection

the colonies had a rough morphology. This colonial morphology was not readily discernible in MS35. The lysogen 53(P35) was not stable and segregated non-lysogenic progeny at a high frequency.

Part II

Isolation and Characterization of Plasmid DNA in S. pullorum

The demonstration of the presence of plasmid material in S. pullorum by centrifugation of ^{3}H labeled DNA in a CsCl-EtBr dye-buoyant density gradient was not unexpected since many bacterial species have recently been found to possess extrachromosomal DNA (62). However, it was not anticipated that 15% or more of the total cellular DNA would be non-chromosomal. The majority is composed of the 1.5×10^{6} daltons supercoiled plasmid molecule, PO-1, which is present in an extremely high number of copies (at least 150 per chromosome). This is a very large amount of redundant genetic information and its necessity or survival value to the cell is hard to imagine. Obviously there is a very stable association between the plasmid and the host cell since it was observed to be present in each of the separate S. pullorum cultures used in this study. Being present at such high multiplicities the plasmid need not, and probably does not, have a strict system for control of its replication or for its orderly

segregation at each cell division. The replication control system for PO-1 may be similar to the relaxed control of replication of R factors in P. mirabilis (49,71). Although the R factor is much larger (55 x 10^6 daltons) than plasmid PO-1, it is present in about 10-12 copies per chromosome equaling about 16% of the total cellular DNA. To maintain this number of copies, the R factor must be replicated several times during each cellular division cycle. It has been proposed that the individual R factor molecules replicate in a random order with no preference for which molecule is selected for replication from the multicopy pool (49). R factor DNA replication also continues during the stationary growth phase while the chromosome ceases to be replicated.

The only other autonomous replicating DNA molecule reported in the literature which is as small as the PO-1 plasmid is the minute plasmid isolated from E. coli 15 (26). These two elements are similar in that no biological function has yet been identified with their presence but the number of copies of PO-1 in S. pullorum is at least ten times greater than the number of E. coli 15 minute plasmids per cell. It would seem logical that the PO-1 plasmid would carry some necessary information, the gene product of which is needed by the host cell in large amounts since if it were non-functional, it would have been selected against and segregated out of the species during evolutionary development.

The larger plasmid, PO-2, of S. pullorum has a molecular weight of 45 x 10^6 daltons and is a fragile structure easily nicked and converted from the supercoiled 65s form to the slower sedimenting 45s open circular form. As seen by direct centrifugation of a cleared lysate on a neutral sucrose gradient (Figure 10) approximately one-half of the PO-2 molecules exist in the lysate in the open circular form. This may be a result of the lysis procedure; the open circular form is probably not found in the intact viable cell. The PO-1 plasmid was never found in an open circular form. Its stability is due to its very small size reducing the possibility of nicks in the DNA strands due to shear forces.

The PO-2 molecule appears to be present in a low number of copies, 1 or 2, per host chromosome indicating a more strict control over its replication than is apparent for the smaller PO-1 plasmid. No biological function could be identified with the PO-2 plasmid.

No unusual structures or features associated with the plasmid molecules were seen when samples were viewed with the electron microscope.

Part III

Exclusion of Plasmid PO-2 Upon Lysogeny of MS53 by P35

A very interesting finding was the apparent exclusion of plasmid PO-2 when S. pullorum MS53 was made lysogenic

for phage P35. When strain MS53(P35) was cured of the prophage, the resulting strain, S192, had not regained plasmid PO-2. This interaction of phage and plasmid resembles the exclusion observed when phage P1 vir infects a P1dl lysogen (59) or when P1 infects E. coli 15 (44). In these cases either the preexisting prophage is excluded and non-lysogenic cells segregate or P1 infection leads to exclusion of a plasmid, P15B, carrying P1 homologous DNA.

It is unlikely that plasmid exclusion observed in S. pullorum is related to the superinfection exclusion determined by the sie locus of P22 (67). The P22 controlled superinfection exclusion appears to involve a surface or membrane alteration prohibiting the infecting phage DNA from entering the cell and replicating. However, labeled P35 DNA can enter cells of MS53, replicate and be incorporated into progeny phage (unreported data).

Whether exclusion occurs in every infected cell cannot be determined from these initial observations since, of all the infected cells in a culture, only those which became lysogenic were isolated and examined. An attempt was made to follow the fate of the PO-2 plasmid after infection of MS53 with P35 by performing two experiments in which cells were labeled with ^{3}H-thymidine either before or during infection and the labeled DNA analyzed on neutral sucrose gradients. The distribution of DNA

in the gradients was quite complex and not easily interpreted. Clearer results might have been achieved if the cells had been pulse labeled for short intervals and the fate of the DNA replicated during the pulse followed. But, due to the poor ability of wild type S. pullorum to take up thymidine from the media, lengthy exposures to the radioactive label were necessary. (Several thymine mutants have been isolated in this laboratory but all have proven to be extremely unstable.)

The broad peaks of DNA isolated at times after infection (Figures 14 and 15), sedimenting between 35 and 55s, probably represent linear concatenates which eventually are incorporated into progeny phage particles [Intermediate II of Botstein and Levine (13,14)]. There does not appear to be any DNA with a sedimentation coefficient of 65s late in the infection. This may indicate that the PO-2 plasmid ceases to replicate in the infected cells or becomes incorporated into the structures comprising the broad peak. The major peaks present very late in the infection sediment at rates approximately equivalent to isolated P35 phage DNA.

The cured strain, S192, may be able to be used to identify a function associated with the presence of plasmid PO-2. Salmonella phages have been shown to cause a lysogenic conversion of the cellular antigenic structure (50) while some phages (4) and plasmids (3) alter the modification and restriction system of the host cell.

These along with virulence determinants are the possible types of altered characteristics which may be expected to be found in S192.

The most likely reasons for the exclusion of the PO-2 plasmid by P35 is that there are very close relationships between the phage and plasmid in DNA nucleotide homologies, immunity systems and/or requirements for intracellular maintenance sites.

Part IV

Hybridization of S. pullorum Plasmid DNA to ^{3}H-RNA (P35c)

A very probable explanation for the exclusion of plasmid PO-2 following lysogeny of S. pullorum by P35 is that the plasmid and phage share common nucleotide sequences. These sequences may contain the genetic information needed for the immunity system or the intracellular attachment site. To determine the degree of sequence homology between PO-2 and P35, ^{3}H labeled RNA was synthesized in vitro using P35 DNA as a template and this was hybridized with the isolated plasmid DNA. The DNA extracted from P35c was used since this virulent mutant of P35 produces higher titers in the lysates than P35 and this facilitated the isolation and purification of sufficient phage DNA.

Previously a similar experiment was attempted by performing DNA-DNA hybridizations on nitrocellulose

membrane filters. These were unsuccessful for two technical reasons. First, P35 does not produce high titer lysates and along with the small size of its DNA molecule it was very difficult to isolate and purify the large quantity of phage DNA which was needed for immobilization on the filters. Second, the poor ability of wild type S. pullorum MS53 to incorporate ^{3}H-thymidine led to the isolation of plasmid DNA with total counts of radioactivity much too low to yield reproducible results. This poor rate of incorporation of thymidine even necessitated the use of a single label in the RNA-DNA hybridization experiment since the ^{14}C labeled thymidine available did not have a sufficiently high specific activity even to be able to locate the plasmid fractions in a sucrose gradient.

The P. putida RNA polymerase generously supplied by Dr. J. Boezi was found to be efficient in the in vitro synthesis of ^{3}H-RNA using P35c DNA as a template. Although its specific activity when assayed on P35c DNA was only about one-third the activity when assayed on phage gh-1 DNA, it could be used to synthesize the ^{3}H-RNA required for the anticipated hybridization studies.

It was first necessary to prove that P35c and its supposed P35 parent were identical as judged by their relative hybridization efficiencies with ^{3}H-RNA (P35c). The data in Table 2 clearly demonstrate this and also indicate that P35 and P22 each contain a considerable

of similar nucleotide sequences. This was to be expected since the data presented in Part I had indicated a very close relationship of P35 and P22. From the limited hybridization data presented here it cannot be stated exactly what percentage of these genomes are homologous. The homology may be much greater than the 40% indicated since it is not known exactly to what extent the P22 DNA was actually available for hybridization. To be able to determine the precise amount of homology it would be necessary to also perform the complementary experiment; i.e., ^{3}H-RNA made from P22 DNA and hybridized to P35 DNA. However, the data does give slight support to the possibility that P35 is a unique phage which is closely related to P22.

That phage P35 and plasmid PO-2 do contain a high degree of sequence homology was demonstrated when ^{3}H-RNA OP35c) was hybridized at high efficiency to PO-2 plasmid DNA (Table 3). The data also indicate that P35 has no or few common sequences with the smaller, more abundant PO-1 plasmid and that when hybridized with the total cellular DNA of S. pullorum MS53, the amount of hybridized (if any) was below the level of detection. The high relative efficiency of hybridization of ^{3}H-RNA (P35c) to PO-2 DNA indicates that all or nearly all of the DNA of this plasmid is homologous to that of the phage or at least those segments of the phage DNA which were available for transcription by the RNA polymerase.

Part V

Relationship of Plasmid PO-2 to Phage P35

From the data of the hybridization experiments it appears that the DNA of plasmid PO-2 probably contains phage sequences. The plasmid may even be composed entirely of DNA homologous to phage P35 including those sequences which determine immunity and/or intracellular maintenance sites. The molecular weight of PO-2 is 45×10^6 daltons and when in the prophage state is integrated into the host chromosome. The contradiction in the intracellular location of the molecules may be explained by proposing that PO-2 is actually a defective phage genome containing sufficient information to replicate itself but does not have the phage att locus which is needed for attachment to the host chromosome. An alternate explanation would be a lack of a function similar to the N gene mutants in phage λ (57,73). The phage DNA may be able to replicate but without this phage-specific product the phage functions necessary for integration would be repressed. The contradiction in the molecular weights may be explained by proposing that in the process by which PO-2 became defective it lost by deletion some of the phage genome but also added other redundant sequences. An event such as this could occur by circularization of the linear concatenates present in

Intermediate II as described by Botstein and Levine (13, 14) to be present during replication of the P22 DNA molecule. It is also possible that PO-2, while being related to P35, also contains some extra genetic information which is used to code for the suspected defective phage or bacteriocins present in the strains of S. pullorum.

The exclusion of PO-2 upon lysogeny by P35 could occur if they possessed similar immunity systems and a competition occurred between the infecting phage and the resident plasmid. Selecting for the lysogenic strains then assures that the exclusion would be observed. The exclusion would also appear to occur if the plasmid were defective in any aspect of its integration system which could be complemented by the infecting phage. Again the isolation procedure would select against those non-lysogenic for P35 but which have now integrated the PO-2 plasmid. Perhaps the cured strain, S192, is similar to this. The experiments attempting to follow the fate of the PO-2 plasmid during P35 infection are too ambiguous to interpret meaningfully and to help explain the mechanism of or reason for the exclusion of PO-2.

It would be of interest to determine if infection of S. pullorum by P22 also causes the exclusion of the PO-2 plasmid. Since P22 and P35 are homoimmune, the exclusion would be expected if the phage immunity system were

involved. If by the use of strain S192, a phenotypic characteristic were found associated with the PO-2 plasmid, then it would be possible to isolate non-excluding phage mutants of P35 by selecting lysogens which have retained the plasmid-specific phenotype. If this were combined with the large number of known mutants of P22, it would allow a genetic approach to the solution of this problem.

Part VI

Restriction and Modification

The low efficiencies of plating observed when phage propagated on S. pullorum strains were plated on S. typhimurium LT2 suggested that there exists in S. pullorum a different modification and restriction system than in S. typhimurium. This hypothesis was tested by cross infecting each species with phage grown on the other and calculating relative efficiencies of plating. The restrictionless mutant, S. typhimurium Rferl, (14) was also used as a control. The results indicated that S. pullorum does not restrict foreign DNA and in this way is similar to the fertile mutants isolated from S. typhimurium which have lost their functional modification and restriction systems (64,65). It can be inferred from a comparison with Rferl that S. pullorum probably does not produce strain specific DNA methylases (39) nor the restriction specific endonucleases (29). The data do

not, however, entirely eliminate the possibility that modification enzymes may be present but this is unlikely since modification and restriction abilities are present or absent together in the fertile mutants of Salmonella (64,65).

SUMMARY

Bacteriophage P35, isolated by zygotic induction from Salmonella pullorum MS35, was characterized as a temperate phage closely related to P22. P35 was demonstrated to be homoimmune with P22. Electron micrographs show that P35 has a hexagonal capsid of 60 nm in diameter with a short tail. The mature phage DNA molecule was found to sediment in a neutral sucrose gradient at 33s corresponding to a molecular weight of 28×10^6 daltons. P35 spontaneously produces clear plaque mutants which are not complemented by P22c2. P35 and P22 were found to have a degree of nucleotide sequence homology as measured by RNA-DNA hybridization. However, identity between the two phage DNAs could not be established.

S. pullorum was demonstrated to contain two distinct, supercoiled DNA molecules by CsCl-EtBr dye-buoyant density gradient centrifugation followed by sucrose gradient analysis of the plasmid fractions. The plasmids are designated as PO-1 and PO-2. Plasmid PO-1 is a molecule of 1.5×10^6 daltons and is present in the cell in at least 150 copies per host chromosome. Plasmid PO-2 is a molecule of 45×10^6 daltons and is present in 1-2 copies

per host chromosome. Electron micrographs of the plasmids revealed no unusual structures associated with the DNA molecules.

When S. pullorum MS 53 was made lysogenic for P35, it was found that plasmid PO-2 was excluded and that the plasmid was not regained upon curing the lysogen of the prophage. A close relationship between the DNA of P35 and plasmid PO-2 was demonstrated by RNA-DNA hybridization. The ^{3}H-RNA was synthesized in vitro using P35c DNA as a template and the RNA polymerase of Pseudomonas putida.

To account for the exclusion of plasmid PO-2 and the nucleotide homology between the phage and plasmid, it is postulated that the PO-2 plasmid may be a defective phage genome carrying some redundant genetic information but which has lost the ability to integrate into the host chromosome. Infection with P35 causes exclusion of PO-2 by the action of its immunity repressor or an apparent exclusion by supplying to the plasmid a missing function which allows the plasmid to integrate.

It was also observed during the course of this work that S. pullorum neither restricts nor modifies DNA which is made in Salmonella typhimurium LT2.

LITERATURE CITED

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