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Bacteriophages of *Bacillus subtilis*

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INTRODUCTION

Until relatively recently, studies of *Bacillus subtilis* bacteriophages have been almost inseparable from investigations of the genetics and physiology of the host, and much experimental work has been only tangentially related to the structure, development, and molecular biology of the viruses themselves. Thus, bacteriophage infections of sporulating cells have been used to assay possible changes in the structure and template specificity of deoxyribonucleic acid (DNA)-directed ribonucleic acid (RNA) polymerase during sporulation. Studies of trapping of viral genomes in spores has afforded insight into the nature of dormancy and may ultimately contribute to our understanding of DNA replication and transcription during outgrowth.

Genetic investigations of *B. subtilis* are dependent on transduction and transformation, and exploitation of the former has required a knowledge of the biology of transducing phages. Moreover, the DNA of most *B. subtilis* phages can be used in transfection, and analysis of this phenomenon has contributed significantly to studies of competence, DNA uptake, and recombination. As with other viral infections, phage-resistant mutants of the host are being used to elucidate the structure of the bacterial cell envelope.

It has become apparent in recent years, however, that some of the *B. subtilis* phages are uniquely interesting in their own right. In size, they range from PBS1 and SP15, which are among the largest phages, to φ29 and φ15, which are the smallest known double-stranded DNA phages. A number of unusual DNA bases are found in these phages, including substitutions of uracil, hydroxymethyluracil (HMU), and 5-(4′,5′-dihydroxypentyl) uracil (DHPU) for thymine; at least one phage contains glucosylated DNA. In addition to a variety of virulent and temperate phages, there are also pseudotemperate (pseudolysogenic) phages and most strains of *B. subtilis* host one or more defective phages. Several *Bacillus* phages are thermophilic, and others prefer asporogenous hosts. Thus, the *B. subtilis* phages encompass a diverse group with widely varying properties and offer many advantages for biochemical studies of DNA replication, control of transcription and perhaps also for phage assembly.

The genetics of *B. subtilis* (371), the methodology of transduction (372), and the molecular biology of transfection (210, 333) have been summarized or intensively reviewed in the last few years and the present paper will concentrate on the biology and molecular biology of the bacteriophages themselves. In the last decade, most research has focused on the bacteriophages which infect *B. subtilis* and we have restricted ourselves to these viruses.

MAJOR GROUPS OF B. SUBTILIS PHAGES

Virulent Phages

HMU-containing phages. Among the most frequently isolated and most thoroughly studied *B. subtilis* phages are a group of large virulent viruses which contain the DNA base substitution HMU in place of thymine (151). These phages are apparently widespread in soil, having been isolated from this source in California (256), Ohio (B. E. Reilly, Ph.D. thesis, Western Reserve Univ., Cleveland, Ohio, 1965), Massachusetts (103), and The Netherlands (14). The most commonly studied members of this group are SP01, φe, H1, φ25, 2C, SP8, and SP82G. (Note: SP82G is referred to as SP82 in some of the literature, but the nomenclature has been changed to avoid confusion with another phage [106].)

Most of the HMU-containing phages are very similar in morphology and size (Fig. 1–3), with the dimensions of SP8 perhaps being typical (60). This virus has an icosahedral head 100 nm in diameter and a 165-nm tail which terminates in a plate (85 nm in diameter) bearing specialized structures similar to the pins on the T2 phage tail plate, and tail fibers. Very frequently, the tail sheaths of SP8 are seen in a contracted state (70 nm long, 26 to 30 nm wide)

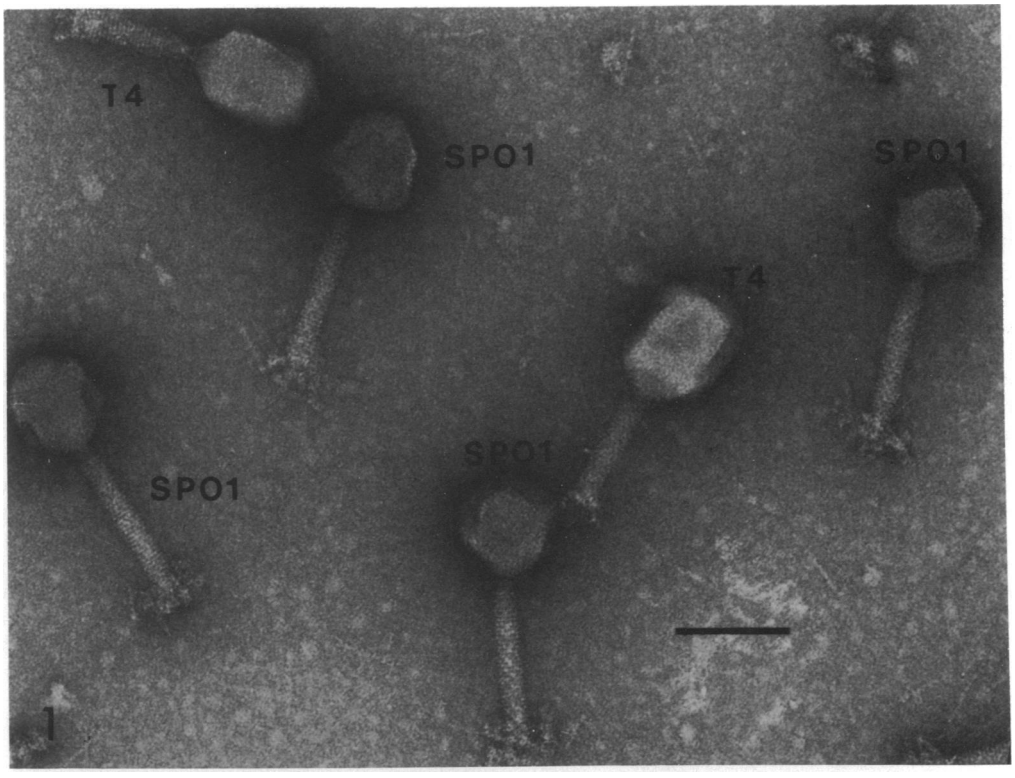

FIG. 1. *Electron micrograph of B. subtilis phage. Mixture of SP01 and coliphage T_4, ×110,000, courtesy of M. Parker. Reference bar represents 100 nm.*

and having a protruding inner tube (165 nm long, 8 nm wide) which apparently forms the core of the tail. Isolated capsomers appear hollow and resemble doughnuts 7.5 nm in diameter and 4 nm deep.

Several lines of evidence besides their similar structure suggest the various separately isolated HMU-containing phages are closely related to each other. The sedimentation coefficients, the contents of guanine and cytosine, the molecular weights, the melting points, and the buoyant densities of the DNAs of most HMU-containing phages are similar (Table 1). DNA/DNA hybridization studies (339) indicate considerable sequence homology among these phages. Thus, 2C DNA binds equally well to 2C, SP82G, and SP01 DNAs and binds to SP8 and ϕe DNAs 92% and 83%, respectively, as efficiently as to 2C DNA. Only ϕ25 shows physical parameters which differ significantly from other HMU-containing phages; it is somewhat smaller and has a lower DNA molecular weight (175).

Phages of the HMU group are easy to grow, yield lysates having high titers, and produce large, easily counted plaques. The presence of HMU in the phage DNA offers the experimental advantages noted for hydroxymethylcytosine in T-even phages; the phage DNA can be recognized in the presence of host DNA due to the odd base and the fact that the buoyant density of the viral DNA (1.742 g/cm^3) is markedly different from *B. subtilis* DNA (1.703 g/cm^3). As might be expected, the developing phage must direct the synthesis of a number of new enzymes required for the production and utilization of HMU, and these enzymes are reliable indicators of temporal development of the phage (150, 226, 304). Interestingly, there is no convincing evidence that the DNAs of these phages are extensively glucosylated as are those of the T-even phages (1).

Centrifugation of denatured DNA from HMU-containing phages on CsCl yields two bands having different buoyant densities (1.752 and 1.762 g/cm^3). Even better separation of the two fractions can be achieved by complexing the denatured DNA with polyribonucleotides prior to the centrifugation step (285). This useful property will undoubtedly be of increasing importance as efforts are made to elucidate the programs of transcription and to determine the

mechanisms controlling the synthesis of various classes of RNAs during phage development (168).

SP50, ϕ1, ϕ2, and ϕ14. Representatives of a second group of serologically related phages have been isolated by Földes and Trautner (80) and by Reilly and Spizizen (240); these phages have no base substitutions in their DNA. The four viruses of this series (ϕ1, ϕ2, ϕ14, and SP50) have identical DNA buoyant densities and exhibit the same content of guanine plus cytosine (G + C) in their DNA (311), but to date the base sequence homology has not been studied. Members of this group can be partially distinguished by host range. Bacteriophages ϕ2 and ϕ14 cannot productively infect wild-type *B. subtilis* 168 strains which do support the growth of ϕ1 and SP50 (140). On the other hand, ϕ1 cannot form plaques on lysogens of the temperate phage SP02 which permit development of ϕ2 (243; H. E. Hemphill, unpublished observations). As will be discussed later, SP50 and, to a lesser extent, ϕ1 have been extensively used in transfection studies.

The most comprehensively studied of the phages of this group is SP50 (Table 1). This virus is a tailed phage with a hexagonal head and overall dimensions slightly smaller than phages of the HMU-containing group. Genetic (120) and physical studies (24, 245) indicate the genome is a nonpermuted, linear DNA molecule which normally contains randomly located single-strand nicks, with the number and distribution of these breaks depending on the host bacterium in which SP50 is grown. As in the case of HMU-containing *B. subtilis* phages, denatured SP50 DNA can be separated into "light" and "heavy" fractions on CsCl (24). One important property of the SP50 genome is that the native DNA molecule can be broken approximately in half by shearing and the separate pieces can be used in transfection-marker rescue studies to examine the physical location of various markers on the phage DNA (121).

β22. Bacteriophage β22 has been the focus of investigations on phage suppression of host functions (125, 365), phage-directed transcription (124) and mixed phage infections (161, 345). β22 is a large, virulent phage (Fig. 4) with no known base substitutions in its DNA and it has one of the broadest host ranges of any *B. subtilis* phage (discussed in section 11). The molecu-

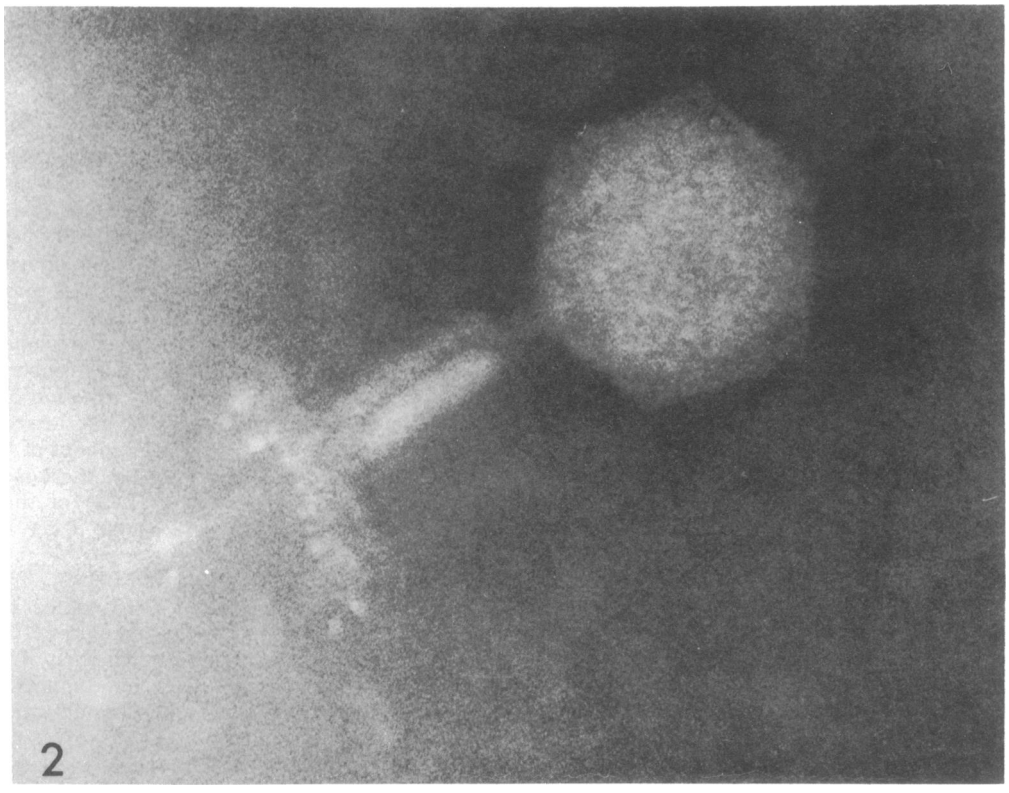

FIG. 2. *Micrograph of phage SP82G, ×450,000, courtesy of J. Lara.*

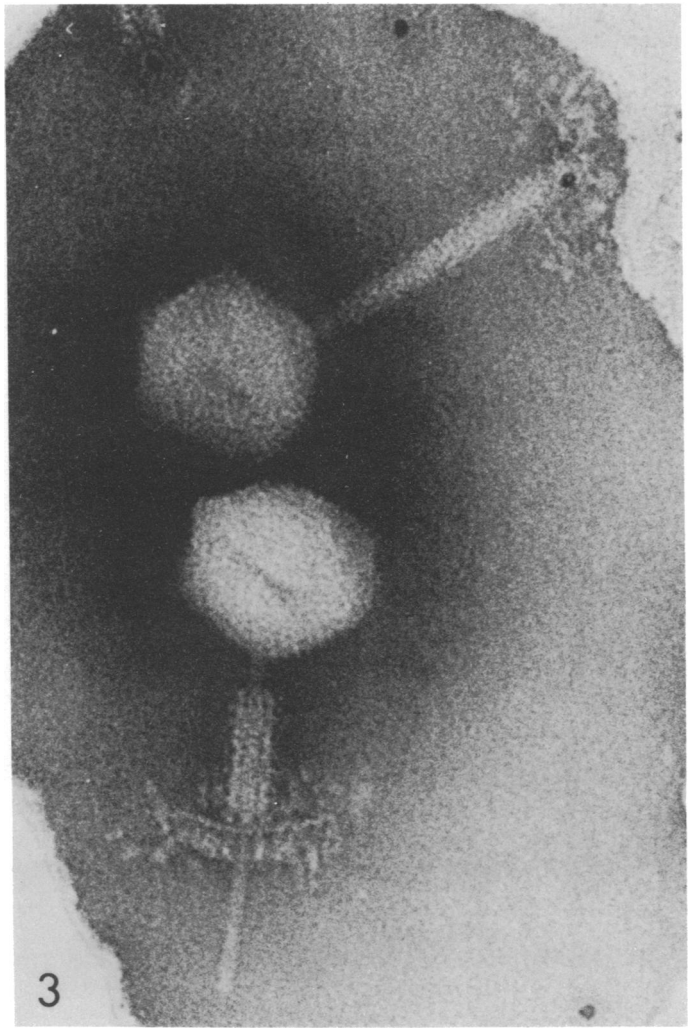

FIG. 3. *Micrograph of phage φ25, ×310,000, courtesy of D. L. Anderson (175).*

lar weight of β22 DNA has been reported to be 30×10^6 by sedimentation velocity measurements (365), but estimates from renaturation kinetics (125) and measurements of the size of the virion head suggest that the molecular weight is closer to 100×10^6. β22 is the only bacteriophage which has been reported to be able to grow in cells committed to sporulation (365). One factor which has limited the usefulness of β22 is that the plaque-forming efficiency is variable and the plaques are small, which in turn may be related to the long latent period (60 to 70 min) of this virus.

φ29 and related phages. Bacteriophage φ29 and related viruses φ15 and Nf are the smallest *B. subtilis* phages so far isolated and are among the smallest known phages containing double-stranded DNA. The virion of φ29 (Fig. 5) has a molecular mass of $18 \pm 1 \times 10^6$ daltons (262) and is structurally quite complicated. The hexagonal head has a flattened base which is connected through two collars to the tail. Twelve spindle-shaped appendages are attached to the lower collar and numerous projections (2 by 14 nm) radiate from the head (5). Studies have been made of the polypeptides isolated from intact phage, ghosts from infected cells, and purified components of disassembled virus. Hawley and co-workers (122) reported that the virion head contains two proteins (molecular weights 45,000 and 28,500), that the neck, having two collars and twelve appendages, is formed by three polypeptides (molecular weights 75,300, 36,400 and 35,200), and that the

TABLE 1. *Physical and biological characteristics of B. subtilis phages*[a]

Phage	Example	Size (nm)		Molecular wt. of DNA ($\times 10^6$)	DNA base composition (% G + C)	Unusual bases	Latent period at 37 C (min)	Burst size	Other phages in group
		Head	Tail						
Virulent phages									
Group 1	SP01	100 × 100	200 (219)	100 (219)	43 (311)	HMU	50 (219)		
	SP82G	100 × 100	20 × 165 (333)	130 (103)		HMU	40 (103)	200 (103)	φe, 2C
	φ25	75 × 75	13 × 130 (175)	96 (175)	42 (175)	HMU	45 (240)	100 (240)	H1
	SP8	100 × 100	165 (60)	130 (60)	43 (311)	HMU			
Group 2	SP50	80 × 80	25 × 170 (80)	97 (24)	44 (24)		45 (80)	200 (80)	φ1, φ2, φ14
Group 3	β22			33 (365) or 100 (124)	36 (365)		50 (365)	115 (365)	
Group 4	SP3	110 × 115	22 × 260 (75)	150[b]	35 (311)				
Group 5	SPP1	45 × 45	6.5 × 140 (252)	25 (252)	43 (252)		60 (159)	200 (159)	
Group 6	φ29	41.5 × 31.5	6.0 × 32.5 (5)	11 (6)	34 (311)		45 (275)	570 (275)	φ15, NF, GA-1, SF_5
Group 7	TSP-1	90 × 90	200 (162)	56 (162)	44.7 (162)		45–50 (162)	55 (162)	
Temperate phages	SP02	55 × 55	6 × 200 (255)	26 (255)	43 (255)		55–60 (255)	40 (255)	φ3T
	φ105	52 × 52	10 × 220 (23)	25 (23)	43.5 (23)		40 (23)	30–50 or 100–200[c] (23)	
Pseudotemperate phages									
Group 1	SP10	90 × 90	160 (218)		44 (311)	?	55 (218)	80 (218)	
	β3			59 (365)	36 (365)	?	35 (365)	25 (365)	
Group 2	PBS1	120 × 120	240 (74)	190 (135)	28 (311, 316)	Uracil (315)	50 (313)	23 (313)	PBS2, 3 NT, I 10
Group 3	SP-15	120 × 120	250 (341)	250 (341)	42.2 (190)	DHPU (190)			

[a] Numbers in parentheses are references.
[b] P. D. Beard, Ph.D. thesis, University of California at Los Angeles, Los Angeles, 1966.
[c] Burst size of φ105 depends on medium.

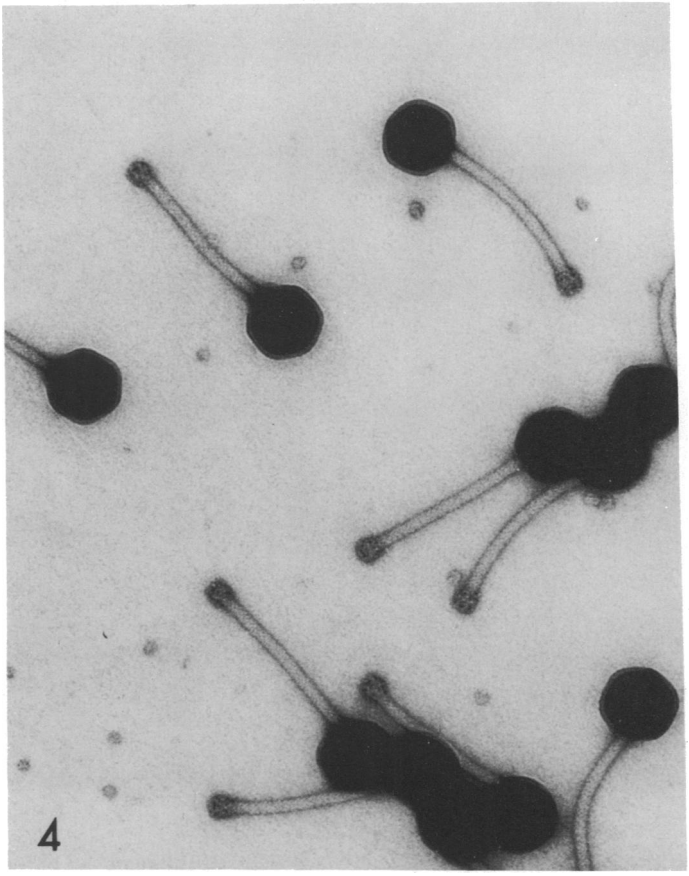

FIG. 4. *Micrograph of phage β22, ×100,000, courtesy of L. R. Brown.*

tail contains one polypeptide with a molecular weight of 62,300. Similar results were reported by Viñuela et al. (196, 238, 270), although their determinations of the molecular weights of various virion proteins differ somewhat from those given above. In addition they found evidence of a third polypeptide component of the head.

Tosi and Anderson (331) have reported that the antigenicity of the intact phage apparently involves at least three of the φ29 structural proteins: head fibers, neck appendages, and head surface. The main serum blocking power is associated with the 12 small neck appendages, and they also found evidence that these structures are in contact with the host cell wall during absorption. This suggests that the appendages might anchor the phage so that the small tail knob of the virion could be inserted into the cell wall.

Most DNA preparations made from φ29 show the molecule to be a linear duplex, nonpermuted, with a DNA molecular weight of 11×10^6 (6). The denatured strands of the DNA can be separated into light and heavy fractions by poly(UG) binding and buoyant density centrifugation (200). Two reports have now been published, however, which indicate that a protein is closely associated with the DNA of φ29. Hirokawa (127) found that treatment of φ29 DNA with protease greatly reduced its transfecting activity, although he could not ascertain any change in molecular weight. Ortin et al. (223) found that DNA which was prepared from φ29 by sarkosyl treatment rather than by phenol extraction was circular, and that trypsin treatment converted such molecules into linear duplexes. They were able to copurify a protein with φ29 DNA, which, when isolated on polyacrylamide gels, was shown to have a molecular weight of 54,000. Evidence for DNA-associated protein has also been reported for phage GA-1 (15).

φ29 forms tiny plaques on lawns of *B. subtilis* 168 even though it productively infects this bacterium in liquid medium. Large plaques are formed, however, on asporogenous variants of

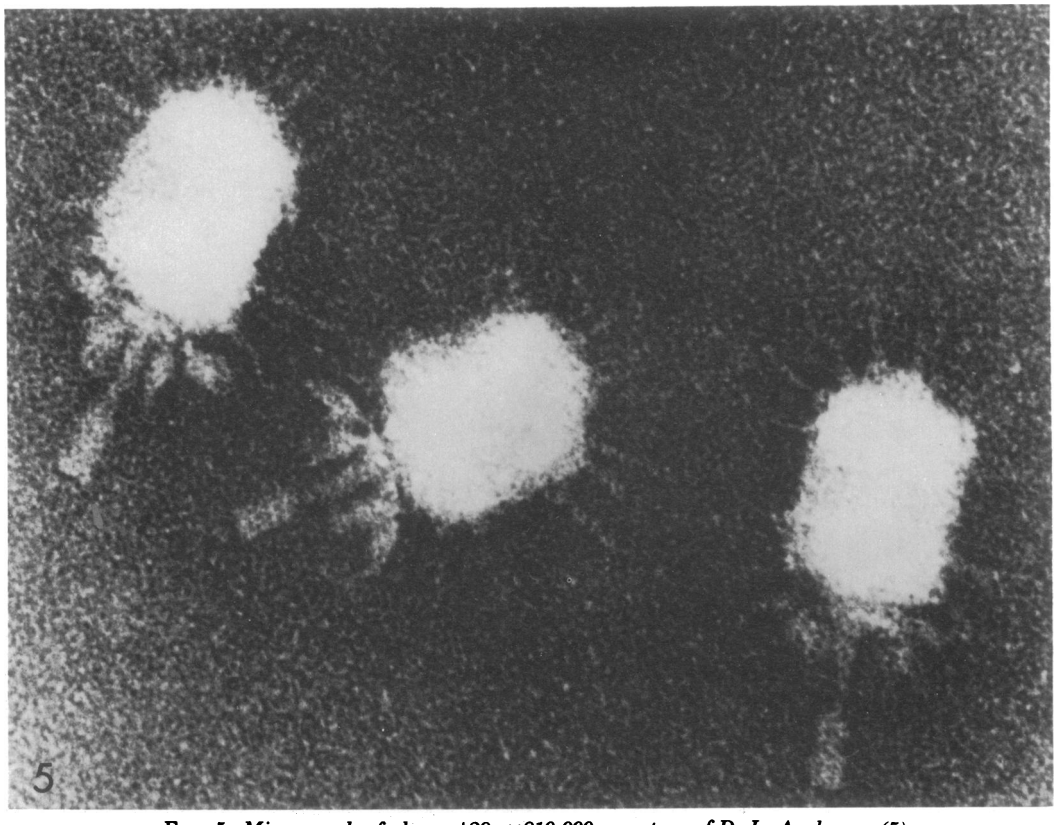

FIG. 5. *Micrograph of phage ϕ29, ×810,000, courtesy of D. L. Anderson (5).*

strain 168 carrying the A12 mutation, which is a lesion in the *spo*A gene whose product is required for the very first stages of sporulation (141). ϕ29 does not grow well in cells preparing to sporulate, and it is possible that the large plaques observed on *spo*A mutants indicate this host remains indefinitely permissive for productive infections. Some investigators produce lysates of ϕ29 on *B. amyloliquefaciens* H and titer the phage on the asporogenous strain to maximize plating efficiency (200).

Morphologically, ϕ15 (139) and Nf (288) are very similar to ϕ29 but ϕ15 differs from ϕ29 in its growth properties and in DNA base sequences (138). Although ϕ29 can produce visible, albeit very small, plaques on wild-type *B. subtilis* 168 and on late-blocked asporogenous mutants, ϕ15 forms plaques only on early-blocked asporogenous mutants (131, 139; B. E. Reilly, Ph.D. thesis, Western Reserve Univ., Cleveland, Ohio 1965). More recently, Ito et al. (139) found that ϕ15 can replicate in the wild-type bacterium for a limited time after outgrowth of germinated spores, but phage growth is markedly decreased during later stages of cell development.

Studies of the physical properties of ϕ15 DNA (139) revealed slightly different values for the G+C content obtained by CsCl centrifugation (35%) and by thermal denaturation (40.2%). Methylation or the presence of small amounts of unusual bases in the DNA may account for these differences. Similar discrepancies in the G+C content were reported for ϕ29 DNA (Reilly, Ph.D. thesis, 1965) and for Nf (288). The molecular weights of ϕ15 and Nf DNA as determined by sedimentation in neutral and alkaline sucrose gradients and by electron microscopy (the average length of the DNA was 6.1 to 6.4 μm) were the same, 12×10^6. Both circular and linear forms of DNA were found in preparations of Nf phage gently lysed with low concentrations of sodium dodecyl sulfate (288), whereas only linear forms were found when higher concentrations of detergent were used. No evidence supporting the existence of cohesive ends was obtained. Seven structural proteins of ϕ15 have been identified by sodium dodecyl sulfate-acrylamide gel electrophoresis; the sum of the molecular weights of the polypeptides (354,000) would account for 59% of the coding capacity of the DNA (139).

Two other very small *B. subtilis* phages, GA-1 (33, 34) and SF_5 (246), have been described, although their relationship, if any, to the other members of the ϕ29 group is not clear. The virion of GA-1 is similar in overall morphology and size to ϕ29 but it lacks the head appendages of the latter. SF_5 is reported to have a short noncontractile tail, but it, too, lacks the head projections of ϕ29 and has a different collar structure. The host ranges of GA-1 and SF_5 differ from that of ϕ29 (246).

SPP1. SPP1 is an intermediate-sized phage with a hexagonal, presumably icosehedral head, containing DNA with a molecular weight twice that of ϕ29. Mapping studies indicate that the SPP1 genome contains a linear, unique, nonpermuted sequence of markers (157, 297). This virus has been of increasing importance in analyses of host competence, phage recombination, and gene conversion phenomena during transfection (251, 296, 297). These latter studies have been aided by the finding that denatured SPP1 DNA can be separated on CsCl gradients into fractions of unusually disparate buoyant densities (1.713 g/cm^3 and 1.725 g/cm^3); thus, it is relatively easy to obtain pure light and heavy single-stranded DNA. Moreover, as in the case of SP50, shearing of SPP1 DNA causes breakage of the genome in a specific region, allowing one to separate genes on one portion of the chromosome from those on another.

SPP1 has a burst size of about 200 (159) and produces large, easily counted plaques which enhance its usefulness in experiments requiring precise measurements of infected cells and progeny. It has recently been found that SPP1 can carry out generalized transduction (362).

SP3. Bacteriophage SP3 played an important role in several early studies of *B. subtilis* phages but has been little used in recent years. It is a large phage with a hexagonal head and a tail bearing a contractile sheath which exposes a core about 10 nm by 240 nm (Fig. 6) (75). SP3 has no antigenic similarity to a wide range of *B. subtilis* phages (Reilly, Ph.D. thesis, 1965) although it has not been compared to β22, which it somewhat resembles in morphology and size. Transfection of *B. subtilis* was first demonstrated with DNA from this phage (254) and the first temperature-sensitive mutants of *B. subtilis* phages were isolated in SP3 (208). SP3 has also been used in studies of phage resistance in *B. subtilis* since it apparently recognizes a different host receptor site than most other *B. subtilis* phages (368; Reilly, Ph.D. thesis, 1965).

TSP-1. Although *B. subtilis* is usually considered to be mesophilic, some strains of this bacterium can grow at temperatures as high as 50 to 55 C. LaMontagne and McDonald (162) have reported the isolation of a bacteriophage, TSP-1, which can productively infect *B. subtilis* only at these elevated temperatures. The obligate

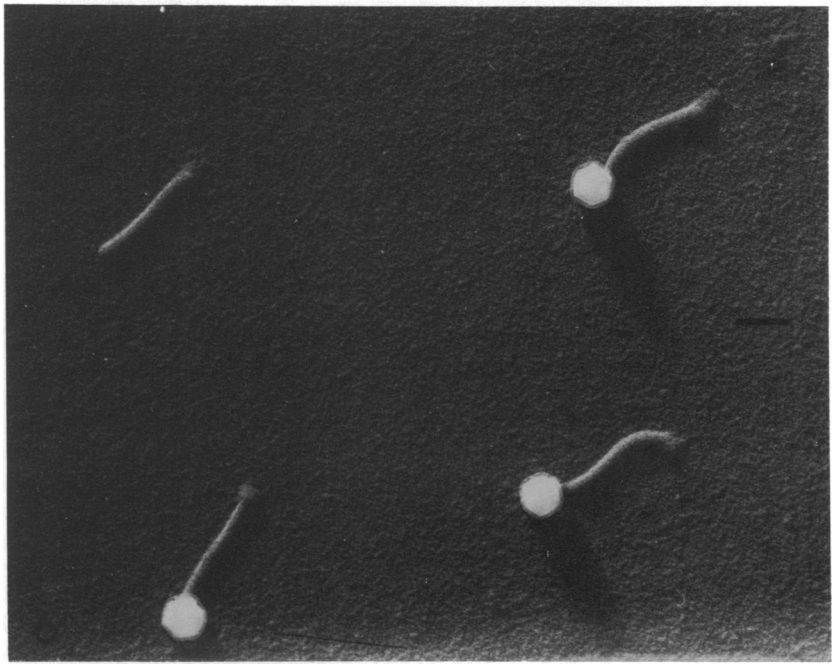

FIG. 6. *Micrograph of phage SP3, ×68,000, courtesy of F. A. Eiserling. Reference bar represents 100 nm.*

requirement for high temperature appears to involve two factors. First, the host bacterium does not possess TSP-1 receptor sites when grown at lower temperatures, although cells grown at 53 C and then shifted to 37 C retain the ability to adsorb phage for a short time (163). Secondly, DNA replication appears to involve a thermophilic process as indicated by the fact that cells infected at 53 C and then shifted to 37 C immediately stop synthesis of phage DNA (164). If the period of exposure to 37 C is short (2 min), DNA replication will resume when the cells are returned to 53 C, but longer incubation at the nonpermissive temperature results in abortive infection.

Aside from these obligately thermophilic properties, TSP-1 does not demonstrate any unusual physical characteristics nor odd bases in its DNA (Table 1). The G+C content of its DNA is higher than that of most *B. subtilis* phages but cannot be considered to be especially elevated. TSP-1 will grow on several *B. subtilis* strains and on *B. licheniformis* at 53 C, but it does not productively infect the *B. stearothermophilus* strains thus far tested.

Other virulent phages. In the foregoing we have described the best characterized virulent phages which infect *B. subtilis*. Others have been isolated, however, and reference to many of these lesser known phages may be found in reference 333 and in two doctoral theses (Reilly, Ph.D. thesis, 1965; and S. Neubort, Ph.D. thesis, Albert Einstein College of Medicine, Yeshiva University, New York, 1972).

Temperate B. subtilis Phages

Two temperate phages, ϕ105 and SP02, which lysogenize *B. subtilis* 168 have been isolated and well characterized. These phages show some antigenic similarity (27), demonstrate about 14% base sequence homology in their DNAs (53), and are morphologically identical with hexagonal, probably iscosahedral, heads and flexible tails (Fig. 7). As noted in Table 1, the DNA molecular weights and dimensions of virion structural components in the two phages are very similar. Vegetative ϕ105 and SP02 DNAs have linear sequences that are not circularly permuted, but their DNAs do have cohesive ends (53, 54).

In spite of their structural similarities, the two phages do demonstrate significant differ-

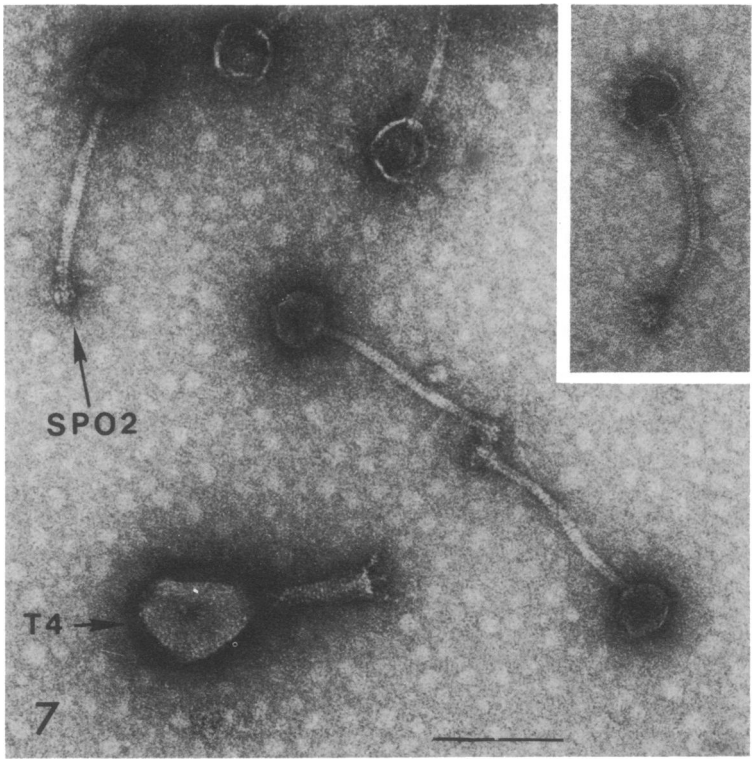

FIG. 7. *Mixture of SP02 and coliphage T_4, ×175,000; insert shows a ghost particle with a longitudinal tail channel common in SP02 particles which have lost DNA from the head. Electromicrograph courtesy of L. B. Boice. Reference bar represents 100 nm.*

ences, however. They integrate at different positions on the host genome (136, 263), and have dissimilar immunities as evidenced by the fact that double lysogens are easily prepared (242). Moreover, mutants of SP02 and ϕ105 do not complement one another in appropriate mixed infections, and they do not undergo heterologous genetic recombination (267). *eco*R1 restriction endonuclease fragments ϕ105 DNA into 11 and SP02 DNA into 7 pieces, thus indicating the recognition palindromes for the enzyme are located at different sites on the genomes of the two phages (353). ϕ105 and SP02 apparently adsorb to different host receptor sites (26).

Until recently, neither generalized nor specialized transduction had been demonstrated with either ϕ105 or SP02. However, Shapiro and co-workers (284) have now reported a low frequency of transduction of the *leu*B, C, and *ilv*B markers by ϕ105. These loci are known to be close to the prophage insertion locus, and more distal markers are not carried; thus, the transduction is apparently specialized. High-frequency transducing phage have not yet been isolated.

A third temperate *B. subtilis* phage, originally designated ϕ3 but now called ϕ3T, which infects strain 168 has been isolated (R. G. Tucker, Proc. Biochem. Soc. 92:58–59p, 1974) but has not been well characterized. The G+C content of its DNA (35.9%) is well below that of the other temperate *B. subtilis* phages (43%), and ϕ3T is heteroimmune to both SP02 and ϕ105 (353). The most unusual known property of this virus is that it carries a structural gene for thymidylate synthetase, and thymine-requiring mutants of *B. subtilis* which have been lysogenized with ϕ3T contain the same amount of the enzyme as do wild-type bacteria. Moreover, phage DNA is able to transform thymine auxotrophs to thymine independence, suggesting the ϕ3T thymidylate synthetase gene is closely related to and may have originated from the bacterial gene (340). *eco*R1 restriction endonuclease digestion of the DNA of ϕ3T yields about 24 fragments (353).

Transducing Phages and Pseudolysogeny

Until the recent successes with temperate phage ϕ105 (284) and virulent phage SPP1 (362), virtually all transduction studies in *B. subtilis* utilized phages SP10, PBS1, or SP15 (312). These phages make turbid plaques as do temperate phages, but do not form stable lysogens nor does the viral DNA integrate into the host genome. Thus, they probably should be classified as virulent phages. However, under some circumstances, these viruses establish a relatively long-term "pseudolysogenic" relationship with the host which mimics true lysogeny, and as a result are referred to as pseudolysogenic, or more correctly "pseudotemperate" phages. The basis of this unusual virus-host relationship has been examined most thoroughly in SP10.

SP10. The virion of SP10 is of intermediate size relative to other *B. subtilis* phages (Table 1) and is considerably smaller than the other pseudotemperate phages, PBS1 and SP-15. The DNA molecular weight has not been reported in the literature, but is assumed to be similar to that of related phage β3 (K. Bott, personal communication) SP10 DNA is thought to contain either some base substitution or to be partially methylated because the G+C content indicated by thermal denaturation does not agree with the G+C content calculated from density measurements (218). The hypothetical base has not been identified but the thymine content appears to be somewhat low, suggesting a partial replacement of this pyrimidine. The substitution is thought not to be HMU, uracil, or hydroxymethylocytosine, and the DNA is apparently not glucosylated (K. Bott, personal communication). It seems unlikely that SP-10 contains 5-(4',5'-dihydroxylpentyl)-uracil-(DHPU) as does SP-15 (35), since SP10 DNA does not display the low melting temperature T_m characteristic of DNA containing this base. Another peculiar feature is that the buoyant density of SP10 DNA, as measured in CsCl, changes if the DNA is stored for long periods (311).

When plated on a permissive host such as *B. subtilis* strain W23, SP10 gives turbid plaques resembling those of temperate phages (29, 324). Cells recovered from these turbid plaques produce colonies that release free infectious particles. About 60% of the spores derived from these pseudolysogenic cultures yield clones that release phage, even if the spores are heated or treated with SP10 antiserum (29).

However, the association of SP10 with the host differs markedly from true lysogeny. First, cultures carrying SP10 produce very high titers of free phage (up to 10^{10}/ml) compared to the titers obtained with true temperate phages such as SP02 (10^5 infectious centers per ml). Second, 30 to 40% of the total DNA extracted from pseudolysogenic cells and banded to equilibrium on CsCl can be shown to be free SP10 DNA, implying that most, if not all, of the phage DNA is not integrated into the host genome. Third, cultivation of pseudolysogenic cells in the presence of SP10 antiserum leads to loss of phage, indicating that continuance of the carrier state requires frequent reinfection of cells in the growing culture.

Because it lacks the stability and precise regulation of true lysogeny, pseudolysogeny might be viewed as an incidental digression from the normal life cycle of the phage. However, there is evidence that maintenance of the carrier state involves specific phage products. Clear-plaque mutants of SP10 which do not establish a carrier relationship with the host occur spontaneously at high frequency, and this implies that some viral gene product is involved in establishing pseudolysogeny. Moreover, carrier cells are immune to superinfection with SP10 or its clear-plaque mutants (152). The physiological state of the bacterium may also be important in determining the stability of the phage association. Pseudolysogenic cultures grown in poor medium show less spontaneous induction than do bacteria grown in rich medium and cells reaching stationary phase show very little induction (152).

PBS1 and PBS2. Bacteriophage PBS1 and its clear-plaque variant, PBS2, are among the largest phages known (Table 1); the iscoshedral head of the former has an estimated internal volume twice that of T4 (74). PBS1 possesses a contractile tail of unusual complexity which, in addition to a sheath, has two unique filamentous structures (Fig. 8). The first of these is several helical fibers (8 by 125 nm) attached to the base plate; generally three of these are visible per particle. The second unusual structure is a number of "contractile fibers" (2 by 80 nm) which project outward from the base of the contracted sheath but are not visible in the uncontracted sheath (74).

PBS1 and PBS2 are particularly noted for the fact that uracil completely replaces thymine in their DNAs (315). Despite this substitution, the X-ray diffraction pattern of PBS2 DNA is typical of double-stranded DNA in the β configuration (165); the viral genome is also thought to have single-strand interruptions (354). PBS1 DNA was reported to be glycosylated (316) but more recent evidence (1) indicates that this conclusion, which was based on discrepancies between the chemically determined composition of PBS2 DNA and values calculated from density of denaturation profiles, is erroneous; the unusual properties of the DNA relate to the uracil substitution (P. Cassidy, F. Kahan, and A. Alegria, Fed. Proc. 24:266, 1965). Bacteriophages PBS1 and PBS2 adsorb to the flagella of host cells and cannot infect mutants of *B. subtilis* which lack flagella (147). (A more detailed discussion of the mechanism of PBS2 infection is given in a later discussion of phage receptor sites.)

Two other phages, 3 NT and I 10, with physi-

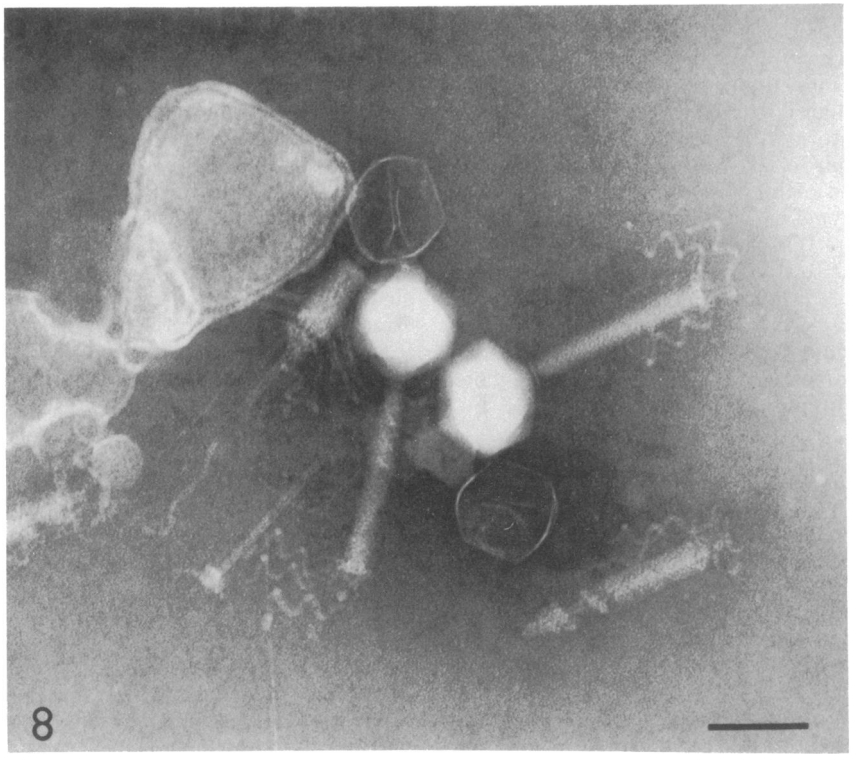

FIG. 8. *Micrograph of phage PBS1, ×130,000, courtesy of F. A. Eiserling. Reference bar represents 100 nm.*

cal properties very similar to PBS1 have been described. Both phages are capable of carrying out generalized transduction (58, 248).

SP-15. *B. subtilis* phage SP-15 was isolated by Taylor and Thorne from the same soil sample which yielded SP10 (320), but is serologically unrelated to either of the other transducing phages. SP-15 is a large phage (Table 1) equal in size to PBS1 and, like the latter, it adsorbs to flagella (341). SP-15 is much less commonly used in genetic studies that is SP10 or PBS1 because it cannot productively infect or transduce *B. subtilis* 168 (320).

Recent interest in SP-15 has centered on the discovery that the DNA of this phage contains a unique pyrimidine, DHPU, which partially replaces thymine (35, 190). Base analyses of SP-15 DNA indicates it contains equal molar ratios of guanine and cytosine (28.1%), while the molar percentage of adenine (28.9%) is approximately equal to the sum of thymine (16.9%) plus DHPU (12.0%). SP-15 DNA has an unusually low melting temperature (T_m of 61.5%) and, in addition, has a very high density in CsCl (1.761 g/cm^3). Treatment of the DNA with alkali reduces the buoyant density and releases a phosphorylated sugar which is thought to be linked to the 4'- and/or 5'-hydroxyl group of the side chain of DHPU by means of a phosphodiester linkage (204). Investigations of the mode of synthesis of SP-15 DNA indicate that uracil serves as the precursor of both DHPU and thymine. The former is thought to be incorporated into DNA via deoxyuridine triphosphate and then converted to the bases found in mature DNA (204).

Transduction with SP10, PBS1, and SP-15. The methodology of transduction has recently been reviewed by Young and Wilson (372) and will not be detailed here. However, several general features of the process as they relate to specific phages should be noted. As mentioned above, virtually all transduction work has been done with pseudotemperate phages and these viruses carry out general transduction. Studies on PBS1 (355) show that the transducing particles in a lysate can be separated from infectious particles in CsCl, and the former contain only host DNA. Similarly, investigations of SP10 indicate transducing DNA is not covalently bound to phage DNA, and most likely resides in different virions (218). Probably some maturing PBS1 and SP10 phage particles inadvertently package pieces of host DNA.

The three common transducing bacteriophages differ considerably in host range (Table 2); however, it can be seen that the inability to support productive infection of a given phage does not preclude being transduced by that virus. Thus, although SP10 cannot grow on *B. subtilis* 168, lysates of that phage prepared on strain W23 can transduce mutants of the nonpermissive host. It should be noted that DNA from strain W23 can also be used to transform strain 168. PBS1 can adsorb to *B. licheniformis* 9945A, although it does not form plaques on this bacterium (341). Heterologous transduction has not been shown in this instance, possibly because there is insufficient DNA homology between potential donors such as strain 168 and W23 and the recipient.

TABLE 2. *Comparison of host and transducing ranges of bacteriophage PBS1, SP10, and SP-15*[a]

Range	Bacteriophage		
	PBS1	SP10	SP-15
Host range (plaque formation)			
B. subtilis W23	+	+	+
B. subtilis 168	+	−	−
B. licheniformis 9945A	−	+	+
Transducing range			
B. subtilis W23	+	+	+
B. subtilis 168	+	+[b]	−
B. licheniformis 9945A	−	+	+

[a] After Taylor and Thorne (320) and Tyeryar et al. (341).

[b] Transduction of *B. subtilis* strain 168 by SP10 is carried out with lysates prepared on *B. subtilis* W23.

Bacteriophage PBS1 can productively infect *B. subtilis* strains 168 and W23 but lysis is often delayed and irregular, and titers are variable. Many investigators use a complicated procedure to prepare stocks in which lysates are prepared on *B. pumilis* (185, 186) and then used to infect the proposed donor strain for transduction; the latter is allowed to lyse overnight, clarified, and then used to infect the recipient. Under optimal conditions, PBS1 carries very large fragments of donor DNA which have been estimated to be 5 to 6% of the host genome (371). SP-15 can transduce DNA fragments of even larger size (341). Since *B. subtilis* does not have a mating system, mapping by means of PBS1 transduction has been used to bridge the gaps in linkage groups established by transformation.

Bacteriophage SP10, on the other hand, carries transducing fragments which are approximately the same size as transforming DNA (341, 371). Thus, this virus is useful in studies of closely linked markers and for transductions between heterologous *Bacillus* strains. Interestingly, a lower level of heterotransduction between strains W23 and 168 is observed in experiments with PBS1 than in transduction by SP10 or in transformation, suggesting that the recipi-

ent cells have a mechanism which discriminates against large pieces of heterologous donor DNA (67). Bacteriophage PBS2 will also carry out transduction but at a lower efficiency than PBS1 (313).

Defective Phages

All strains of *B. subtilis* so far examined have been found to be lysogenic for one or more prophages. Phage particles resulting from spontaneous induction of these prophages can be observed by electron microscopic examination of culture supernatants in which these bacteria have been grown. If *B. subtilis* cells are treated with mitomycin C, bromouracil, ultraviolet (UV) light, or nalidixic acid, or if thymine auxotrophs are starved for thymine, much larger numbers of these particles are released into the medium (113, 128, 308). These phages are defective, however, and cannot be propogated on new host cells.

F. A. Eiserling (Ph.D. thesis, Univ. of California at Los Angeles, Los Angeles, 1964) made the first detailed study of the structure of the defective phages of *B. subtilis*, and he reported that derivatives of *B. subtilis* strain 168 released two morphologically different viruses which he named SPα and SPβ. The former has a small head (about 45 nm in diameter), a cylindrical, contractile tail 18 nm wide and 200 nm long, and tail fibers approximately 3 by 50 nm (Fig. 9). SPβ occurs at a frequency 100 times less than SPα; it has a larger head (82 by 88 nm)

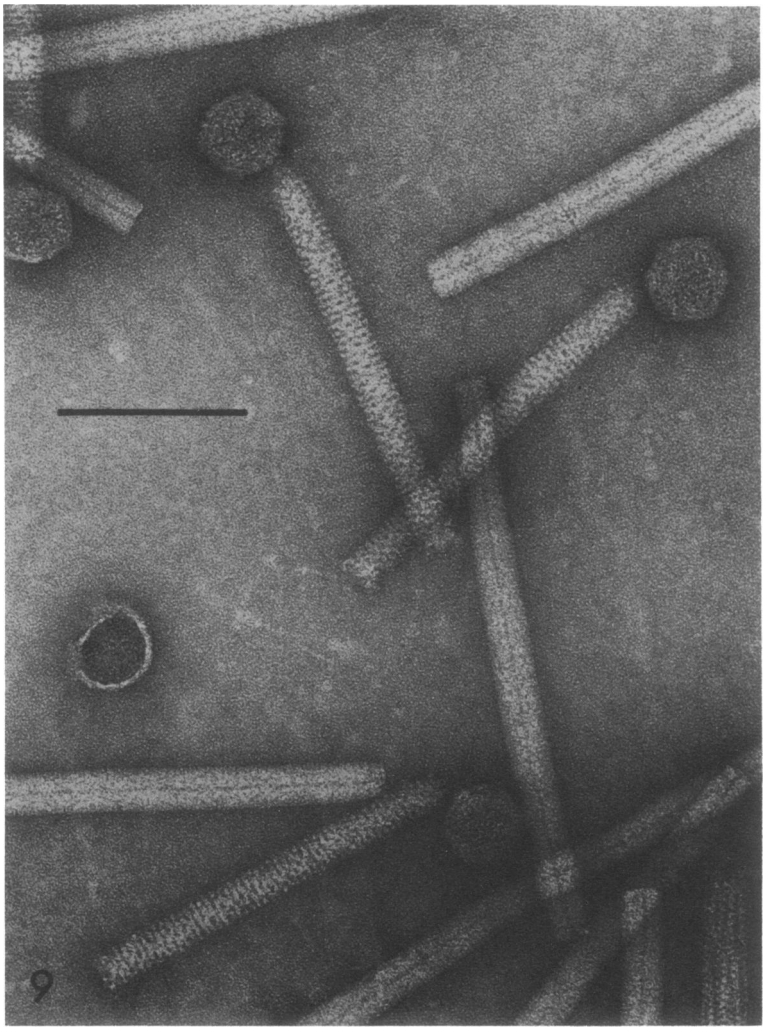

FIG. 9. *Mixture of defective phage PBSX and tobacco mosaic virus, ×250,000, courtesy of F. A. Eiserling. Reference bar represents 100 nm.*

and a longer (12 by 320 nm), more flexible tail which is not contractile.

In an independent study, Seaman and co-workers (281) named the more common defective phage of *B. subtilis* 168, PBSX, and this nomenclature has superceded the term SPα. Subsequently, Marmur and co-workers (213, 308) demonstrated that *B. subtilis* strains S31 and W23 also release defective phages which they designated, respectively, PBSY and PBSZ. Although PBSX, PBSY, and PBSZ have similar morphologies, they can be distinguished because each has bacteriocidal activity against the two heterologous bacteria but not against the strain which carries the homologous prophage (213). Defective phages have been found by other investigators and named *mu* (137), GA-2 (33, 34), and PBSH (113); the latter is identical to PBSX and SPα. Apparently, no further studies of SPβ have been made.

Subsequent investigations of the structure of PBSH (PBSX) confirm Eiserling's original description of SPα (113, 213). In addition, Thurm and Garro (325) have resolved seven structural proteins of the PBSX virion; five of these comprise the phage tail and two polypeptides are associated with head structure. Haas and Yoshikawa (113) reported the burst size of PBSH after mitomycin C induction to be between 100 and 400. The virion does not contain DNA which is distinguishable as being of viral origin, but, rather, incorporates DNA which resulted from random fragmentation of the host genome (114, 214).

The mechanism by which the defective phages kill sensitive strains of *B. subtilis* is not understood. The killing of *B. subtilis* W23 by PBSX shows single-hit kinetics (213). Within 5 min after the addition of PBSX, the syntheses of host protein, DNA, and RNA are almost completely arrested. However, the defective particle does not inject the DNA it carries, and, thus the killing action may not be due to mechanical disruption of the cell surface. Most *B. subtilis* strains lysogenic for PBSX, PBSY, or PBSZ do not adsorb the homologous mature phage particle and thus are immune to superinfection. However, a few strains of *B. subtilis* carrying PBSZ prophage do adsorb the homologous phage but are nevertheless resistant (213).

There has been considerable controversy as to exactly where the PBSX prophage is located on the genome of *B. subtilis* 168, and it has even been suggested that the viral genes might be scattered throughout the host chromosome. Recently, however, Garro et al. (93, 326) have isolated mutants of *B. subtilis* 168 with lesions in prophage genes controlling structural proteins in the virion capsid, and these mutations appear to be clustered between the *arg*C and *met*C markers on the host chromosome. They also found that during induction of PBSX, DNA carrying the *met*C marker is specifically synthesized in multiple copies, which may mean that the prophage DNA replicates in situ with the concomitant amplification of adjacent host markers. It should be noted in this regard that the prophage of nondefective temperate phage ϕ105 is replicated several times before excision (10). In the case of the PBSX, it may be suggested that such in situ amplification provides multiple copies of genes needed to synthesize large quantities of capsid proteins after induction. However, this explanation is not completely satisfactory since the induction and release of the defective phage can be achieved even under conditions where DNA synthesis is inhibited by mitomycin C (213, 214).

Haas and Yoshikawa (114, 115) noted that after PBSX (which they called PBSH) induction there was a 30- to 100-fold increase in the *B. subtilis ade*-16 marker, which is close to the origin of the bacterial chromosome. Presumably this observation is not directly related to replication of the prophage genome, however, as the *ade*-16 gene is not close to the *met*C and *arg*C loci which bracket the known PBSX functions. Moreover, there is evidence that multiple initiation of the bacterial chromosome may result from use of mitomycin C as an inducing agent rather than being directly related to induction of the prophage (326).

HOST-RELATED FUNCTIONS

Common Hosts

Perhaps no facet of the many studies of *B. subtilis* phages has created so much confusion as the precise definition of host strains. The strain designations have been changed so frequently that some investigators probably do not know the origin of the hosts they are using. Moreover, in early genetic studies, *B. subtilis* W23 was frequently used as a source of DNA to transform *B. subtilis* 168 and, thus, at least some genetically useful strains of the latter are heterogenetic (E. W. Nester, personal communication).

The name *Bacillus subtilis* was first used by W. Cohn in 1875 to designate a small sporulating bacterium that he probably observed only in mixed cultures. Cohn's descriptions were not very precise and a number of strains purported to be derived either from Cohn's original culture or obtained by reisolation were eventually shown to be different bacteria (57). A careful

survey of available strains led Conn (57) to conclude that a bacterium obtained from the University of Marburg collection most closely resembled early descriptions of *B. subtilis*. This conclusion was eventually accepted by the International Congress of Microbiology (Int. Congr. Microbiol., 2nd, London, 1937, Resolutions of the nomenclature committee, Rpt. Proc., 1936, p. 28-29) and the Marburg culture became the type strain. This strain is designated by the American Type Culture Collection as ATCC 6051 and by the National Collection of Type Cultures of London as NCTC 3610.

In 1947, Burkholder and Giles (41) reported the isolation of a variety of biochemical mutants from the "Marburg strain" of *B. subtilis*, although it is not certain whether their parent strain was derived from ATCC 6051 or another organism. A tryptophan auxotroph, *B. subtilis* 168, one of the mutants surviving from these studies, was shown by Spizizen (300) to develop competence for transformation and it was the origin of most currently important transformable strains of *B. subtilis*. This bacterium is by far the most commonly used host for *B. subtilis* phages, and offers the dual advantages of being the best genetically characterized strain and being susceptible to transfection as well as transformation. Strain 168 comes under a number of pseudonyms in the literature; *B. subtilis* 168M is a highly transformable derivative of 168 which originated in the laboratory of J. Marmur (Reilly, Ph.D. thesis, 1965), whereas strains given the prefix SR or SB are usually mutants of 168 which carry additional biochemical markers. Cultures bearing the prefix BR, for "*Bacillus* recombinant," are recombinants of *B. subtilis* which were constructed in an effort to develop an isogenic collection of recipients to be used in marker location. One important exception to the above generalizations is strain SB11, which is a derivative of *B. subtilis* W23. *B. subtilis* 168 is also occasionally referred to as "*B. subtilis* 168 Marburg," a point which is discussed below.

A prototrophic bacillus, *B. subtilis* W23, was once widely used as a source of DNA for transformation experiments, possibly because some investigators erroneously believed it was closely related to, or perhaps was, the wild-type progenitor of 168. The precise origin of strain W23 is unknown but it probably originated in the culture collection of Western Reserve University (B. Reilly, personal communication). It is now clear that strain W23 differs significantly from *B. subtilis* 168: (i) the two strains support different spectra of phages (Table 3); (ii) the cell wall of W23 contains polyribitol and glucosyl-polyribitol phosphate in the teichoic acid, whereas the teichoic acid of 168 contains glycosylated polyglycerolphosphate (52, 97); (iii) the DNA of W23 shows only 89% homology to the DNA of strain 168 (184); and (iv) even though DNA from W23 can be used to transform *B. subtilis* 168, strain W23 is not transformable.

Although the differences between *B. subtilis* 168 and W23 are now well established, the precise relationship between either of these and Conn's Marburg strain is not absolutely clear. Confounding this problem are several references in the phage literature to host strains simply designated "*B. subtilis* Marburg" (204, 218, 313). In some instances, these citations may refer to strain 168 but this cannot be the case in all studies. For example, SP10 and SP-15 cannot grow on *B. subtilis* 168 but are reported to grow on *B. subtilis* Marburg (207, 218).

In an attempt to clarify this situation, we asked a few investigators to provide strains from their collections which they believed were 168, W23, and/or Marburg; we also obtained the ATCC strain 6051 which is Conn's Marburg strain. These cultures were than compared with respect to their ability to support the growth of several *B. subtilis* phages and host range mutants of such phages. The results (Table 3) show that the ATCC 6051 strain and *B. subtilis* 168M had identical sensitivities and resistances to various phages. In particular, SP02c$_1$, ϕ105c$_1$, and SPP1, which are known to have a strong preference for strain 168, also grew on ATCC 6051; in contrast, two host range mutants, β22h and ϕ1*m*, which were selected for their inability to grow on strain 168M, also could not grow on ATCC 6051. Finally, the defective phage carried by ATCC 6051 has the same activity spectrum as does PBSX, the defective phage associated with *B. subtilis* 168 (A. Garro, personal communication).

On the other hand, three W23 cultures and three strains called "Marburg" were identical with respect to the spectrum of phages they supported. Though not conclusive, these data suggest that *B. subtilis* 168 is similar to, if not identical with, the Conn Marburg strain of *B. subtilis* (ATCC 6051). The origin of the other Marburg strains is still not known, but they may be related to W23 and probably could not have originated from the Conn Marburg strain. A more complete discussion of various strains and species of the genus *Bacillus* is presented by Gordon et al. (101).

Table 4 presents the results of our more extended examination of the host range of a variety of *B. subtilis* phages. It can be seen that some phages such as β22 have a very broad host

TABLE 3. *Comparison of the spectra of phages able to grow on several strains of B. subtilis*

Strain of *B. subtilis* tested	Bacteriophage[a]							
	β22	β22h	SPP1	SP10	φ1	φ1*m*	SP02c$_1$	φ105c$_1$
B. subtilis Marburg (ATCC 6051)	+	0	+	0	+	0	+	+
B. subtilis 168	+	0	+	0	+	0	+	+
B. subtilis W23[b]	+	+	0	+	+	+	0	0
B. subtilis Marburg[c]	+	+	0	+	+	+	0	0

[a] In each instance, about 10^4 phage particles were spotted on a plate seeded with the desired indicator. In all tests + = phage growth, and 0 = no phage growth.
[b] Including one strain from E. W. Nester and two strains obtained from K. Bott.
[c] Including one strain from *A. Garro* and two obtained from K. Bott.

TABLE 4. *Host ranges[a] of several B. subtilis phages*

Host bacterium	Bacteriophage						
	β22	SP10	φ29	SP82G	φ1	SP02c$_1$	SPP1
B. subtilis 168	+	0	+	+	+	+	+
B. subtilis W23	+	+	0	+[b]	+	0	0
B. natto	+	+	0	0	0	0	0
B. pumilus	+	0	0	+	+	0	0
B. subtilis var. Niger	+	+	+	0	0	0	0
B. amyloliquefaciens H	+	+	+	+	+	0	0
B. brevis	0	+	0	0	0	0	0

[a] Determined by spotting 10^4 phage particles on plates seeded with the desired indicator.
[b] Although SP82G grows on strain W23, its plaque-forming efficiency is much lower on this bacterium (242).

range and others are limited to only one strain, often the bacterium used in their primary isolation. The data also emphasize that species designations have little meaning in predicting host range. For example, SP10 did not productively infect strain 168, but grew on *B. subtilis* W23, *B. amyloliquefaciens* H, and a strain each of *B. brevis* and *B. natto*. A more detailed examination of phage host ranges was made by B. E. Reilly (Ph.D. thesis, 1965.).

Phage Receptor Sites

In view of the relative simplicity of the cell envelope of *B. subtilis* (370) compared to that of gram-negative bacteria, it is rather surprising that few investigations have been made of phage receptor sites in this organism. It is known that the host ranges of some *B. subtilis* phages are limited, at least in part, by a lack of appropriate adsorption sites (224), and many phage-resistant variants as well as poorly transformable mutants are altered in the composition of the wall (368). Reilly (Ph.D. thesis, 1965) found that mutants of *B. amyloliquefaciens* H which were selected for resistance to φ15 simultaneously gained resistance to the related phage φ29 and also to many unrelated phages including φ25, SP8, and SP10. However, phage SP-3 and one group of phages, including related phages φ1, φ2, and φ14, were still able to yield progeny in φ15-resistant mutants. These findings suggest either there is a limited variety of phage receptor sites on the cell wall of *B. amyloliquefaciens* or that different kinds of sites share some common mutable element.

Young (368) initiated a detailed study of mutants of *B. subtilis* 168 which are resistant to φ29. These variants, like those of *B. amyloliquefaciens*, are also resistant to unrelated phages φ25, SP02, SP10, as well as φ29, and cannot be productively infected by φ1 and SP3 in liquid medium, although these latter two phages can parasitize some φ29-resistant strains in semisolid or solid media. It is now known that *B. subtilis* mutants resistant to φ29 share in common an inability to glucosylate polyglycerol teichoic acid, but it is not certain whether the glucose is directly involved in the primary binding site for the phage or whether the absence of glucose alters the topography of the cell wall so as to distort the true binding sites. At least three genes located in a cluster (369) on the *B. subtilis* genome are involved in the glucosylation of teichoic acid. Mutants in *gta*A locus are deficient in uridine diphosphate (UDP) glucose polyglycerol teichoic acid transferase, contain

N-acyl galactosamine in the cell wall, and can grow on galactose as a carbon source. Mutants in the *gta*B gene do not have marked deficiencies in any enzymes known to be involved in the glucosylation of teichoic acid, have less *N*-acyl galactosamine in the cell wall than do wild-type cells, and cannot utilize galactose as a carbon source. ϕ29-resistant mutants in the *gta*C locus are deficient in phosphoglucomutase, have relatively little *N*-acyl galactosamine in their cell walls, and lyse when grown on galactose as the sole carbon source (368).

Temperature-sensitive mutants in the *tag*-1 gene (32), which is closely linked to the three genes involved in the glucosylation of teichoic acid, have only 9 to 20% as much teichoic acid when grown at 45 C as they do at 37 C. This loss of teichoic acid results in a change in morphology from normal rods at the lower temperature to irregular spheres at 45 C; the mutant also loses the ability to adsorb ϕ25 at the higher temperature.

A pleiotropic mutant of *B. subtilis* 168, designated Cbl-1, which was first noted for containing DNA with an atypically high buoyant density (310), has been found to lack teichoic acid (342). Teichoic acid normally makes up about 50% of the dry weight of the *B. subtilis* cell wall but the mutant contained abnormally large quantities of protein in its wall. Indeed, almost 50% of the material solubilized from the mutant wall consisted of a single protein with a molecular weight of 255,600 (342). Not unexpectedly, Cbl-1 is also resistant to those phages which require glucosylated teichoic acid for adsorption.

The primary site of adsorption of transducing phage PBS1 is the flagellum of the host bacterium (82, 147). Evidence for this conclusion includes the inability of the phage to attach to mutants of *B. subtilis* lacking flagella or to freshly deflagellated bacteria; cells treated with antiflagellar antibody are also protected. The actual mechanism of adsorption and penetration of PBS1, however, is still not clear (237). Electron micrographs show that PBS1 tail fibers wrap around the bacterial flagellum after adsorption, although there does not appear to be a single specific site for adsorption since the entire length of the flagellum can be covered at high multiplicities of infection. PBS1 can productively infect paralyzed mutants of *B. subtilis* 168 whose flagella lack the long period helix, suggesting movement of the flagellum has nothing to do with the process of penetration. However, these phages do not adsorb to free flagella. Irreversible adsorption and/or penetration apparently involves an energy-requiring step, since cells infected in the presence of cyanide do not yield progeny virus if they are deflagellated prior to plating.

Consideration has been given to the possibility that PBS1 may inject its DNA directly into the flagellum, but there is no evidence that flagella have a central canal of proper dimension (237). Moreover, phage with contracted sheaths and empty heads are rarely seen attached to the flagella. It has also been suggested that the viral genome might enter the host via a modified transfection mechanism but this seems unlikely since the infection is deoxyribonuclease (DNase) resistant. One remaining hypothesis is that the phage particles slide down the flagellum to an adsorption site on the wall or at base of the flagellum. A mechanism of infection similar to this has been proposed for the χ phage of *Escherichia coli* (277). If this is the case, the proposed surface site must be different from that utilized by other phages, since PBS1 can infect flagellated protoplasts of *B. subtilis* which cannot adsorb wall-specific phage SP8. Moreover, PBS1 can infect variants of the host which have modified teichoic acids or lack teichoic acid altogether (342). One other observation of interest is that apparently the adsorption of a phage particle to one flagellum of *B. subtilis* renders the entire complement of 15 to 20 flagella per cell nonmotile in less than 30 s. This paralysis can also be obtained with ghosts of PBS1 prepared by osmotic shock (237).

PBP1, a flagella-specific bacteriophage mediating transduction in *B. pumilis*, has been studied by Lovett (183). Comparison of host ranges of this phage and PBS1 on some 32 strains of *B. pumilis* indicates there are three classes of hosts. One group is sensitive to both PBP1 and PBS1, another is sensitive only to PBP1 and the third is resistant to both. It has been suggested that such studies may be useful in distinguishing different types of flagella in *Bacillus* but it is not certain whether differences in host range actually are due to differences in flagella, in the proposed second binding site at the base of the flagellum or to an interference mechanism (see below).

Abortive Infection

A number of instances have been described in which phage adsorb to a host bacterium but the infection is abortive and few or no progeny are released even though the cell may be killed and/or lysed. Little is known about the mechanisms which bring about many of these abortive infections, but there are at least two catagories of phenomena involved. First, classical restriction and modification has been observed in the *Bacillaceae*, and, secondly, there are ill-defined "interference" systems which are distin-

guishable from the former by the fact that the viral genome is not degraded and no modification is found.

Convincing evidence for restriction and modification in *B. subtilis* has only recently been obtained. Trautner and co-workers (332) found that phages SPP1, ϕ105, and SP02 which had been grown on *B. subtilis* 168 or its derivatives had a greatly reduced efficiency of plating on *B. subtilis* strain "R" (respectively, 1.1×10^{-5}, 2.2×10^{-4}, and 3.3×10^{-6}) compared to that on strain 168 itself. Phage recovered from those plaques which did appear on strain R were modified and plated with almost equal efficiency on strains 168 and R. Restriction and modification were also observed in transfection, with DNA from the phages grown on strain 168 being virtually unable to transfect the restricting host.

An endonuclease which inactivates transfecting SPP1, SP02, and ϕ105 DNAs lacking R-specific modification in vitro has now been purified 400-fold (S. Bron, K. Murray, and T. A. Trautner, personal communication). As expected, the corresponding DNAs carrying R-specific modification are resistant to this enzyme. The restriction endonuclease cleaves DNA at specific sites and the recognition palindrome has now been identified as: 3'-N-C-C-G-G-N-5' and 5'-N-G-G-C-C-N-3', where the arrows indicate the points of strand scission (S. Bron and K. Murray, personal communication).

Many *B. subtilis* phages, including ϕ29, SP82G, SP50, PBS1, and H1, do not demonstrate sensitivity to the restriction system in *B. subtilis* R, but the mechanism by which their viral genomes are protected is not known. Curiously, although auxotrophs of strain R cannot be transduced by PBS1 lysates prepared on *B. subtilis* 168, they can be transformed by DNA from the latter. This finding is difficult to explain since the purified restriction endonuclease discussed above inactivates DNA from 168 cells in vitro.

Arwert and Rutberg (11) also reported that SP02 phage grown on *B. subtilis* 168 were restricted on *B. subtilis* 5GR, a derivative of strain 168 into which the r^+m^+ genes of *B. subtilis* strain "R" have been introduced by transformation (332). In addition, they found that SP02 lysates prepared at high temperature on a strain of 168 which is temperature inducible for the defective PBSX prophage had a higher efficiency of plating on *B. subtilis* 5GR than did phage grown under noninducing conditions. A similar increase in efficiency of plating was found in SP02 grown on *B. subtilis* 168 cells pretreated with mitomycin C. They suggest the genetic determinants for modification of SP02 may be associated with the defective phage PBSX. It is not clear, however, just how this hypothetical PBSX-mediated modification would work. Possibly when, and only when, PBSX is induced it produces a modification enzyme which alters SP02 DNA such that the latter is then resistant to restriction endonuclease in *B. subtilis* 5GR.

A second series of host-controlled restriction and modification systems, so far shown to affect only ϕ105c (a clear plaque mutant of ϕ105), has been described by Shibota and Ando (287) in *B. subtilis* 168 and *B. amyloliquefaciens* strains N and H. Phages carrying the 168-specific modifications (ϕ105c·168) plated on *B. amyloliquefaciens* N and H with efficiencies of 10^{-5} and 10^{-2}, respectively. ϕ105c phages previously grown on strain H (ϕ105c·H) plated with efficiencies of 10^{-1} to 10^{-2} on strain 168 and 10^{-5} on strain N. Finally, phages prepared on strain N plated with equal efficiency on both strains of *B. amyloliquefaciens* but with an efficiency of only 4×10^{-2} on *B. subtilis* 168.

It has been noted previously that bacteriophage SP10 cannot productively infect *B. subtilis* 168, although it can transduce this strain. Gwinn and Lawton (111) reported that if strain 168 was grown at high temperature, cells became permissive for SP10 (and also for the related phage SP20), and they suggested that heat might inactivate some component of a restriction system. However, they found that the progeny released after heat treatment of the host were not modified as would be expected in a classical restriction-modification system. Noting that restriction systems in *Enterobacteriaceae* are often associated with prophages, Goldberg and Bryan (100) pretreated *B. subtilis* 168 cells with UV or mitomycin C to induce the defective prophage carried by this bacterium; such cells became permissive for SP10. Several mechanisms could be proposed to explain the results cited above, e.g., the defective prophage may control a restriction system, or the presumed repressor for maintaining the defective prophage in the lysogenic state may serendipitiously bind to SP10 DNA. The possibility that SP10 DNA may be degraded under nonpermissive conditions has not been investigated in detail. However, the observation that intact SP10 or SP20 genomes could be rescued from *B. subtilis* 168 by superinfection with a helper phage (112) suggests that rapid degradation by nuclease is not involved.

It has recently been found that two unrelated

phages, ϕe and $\phi 1$, abortively infect a strain of *B. subtilis* which is lysogenic for the temperate phage SP02 (242, 356). The plating efficiency of ϕe on lawns of *B. subtilis* 168 (SP02) is a thousand times less than on strain 168. In liquid, the yield of progeny ϕe from infections of lysogenic host is 1% of that from infection of permissive bacteria. Phage isolated from occasional plaques which are produced on lawns of 168 (SP02) are not phenotypically modified, but rather include mutants (ϕeI^r) which can develop normally on either the lysogenic host or strain 168. If these mutants are cycled through *B. subtilis* 168, they continue to plate with high efficiency on strain 168 (SP02). Interestingly, the SP02-mediated interference does not affect phage SP82G and has only a slight effect on SP01, even though these HMU-containing phages are related to ϕe. However, mutants of SP82G (called SP82GI^s) which are sensitive to the SP02-mediated interference have been isolated. In mixed infections of SP82G and SP82GI^s or ϕeI^r and ϕe the resistant phage can rescue the genome of the sensitive partner, suggesting the interference mechanism does not involve a restriction endonuclease (S. Friedman and H. E. Hemphill, unpublished observations).

The mechanism of interference in *B. subtilis* 168 (SP02) has been most extensively studied with bacteriophage $\phi 1$, which belongs to a completely different group of phages than does ϕe (243). Bacteriophage $\phi 1$ is very strongly affected by the interference mechanism; no plaques are formed on lawns of the lysogenic host and no progeny are released in liquid. The phage genome is not replicated in the nonpermissive host, but is apparently not degraded. On the other hand, transcription of the DNA is initiated in *B. subtilis* 168 (SP02), but about 10 to 15 min after the infection begins, there is a sudden cessation of all phage and host-related macromolecular syntheses. Mixed infections of SP02c$_1$, a clear plaque mutant of SP02, and $\phi 1$ result in a similar suppression of the biosynthetic activities of the latter, suggesting the involvement of some SP02-related product which is made early in lytic infection and continuously by the prophage.

Mutants of SP02, lysogens of which can be productively infected by ϕe, $\phi 1$ and SP82GI^s, have been isolated (C. W. Rettenmier and H. E. Hemphill, unpublished observations). It now seems likely that the inhibitory mechanism affecting sensitive phages is controlled by a gene on the prophage similar to the *rex* gene of *E. coli* phage lambda (110).

Another abortive infection system have been described which blocks development of $\phi 2$. This virus is unable to infect *B. subtilis* 168 productively, although this bacterium is an excellent host for related phage $\phi 1$ (140; Reilly, Ph.D thesis, 1965). *B. amyloliquefaciens* H serves as a common host for both of these phages, so the ability to serve as host for $\phi 1$ and $\phi 2$ is not mutually exclusive in all bacteria. The interference mechanism in *B. subtilis* 168 is unknown, but Ito and Spizizen (140) have reported that phage DNA and RNA synthesis occur after infection of the nonpermissive host. The $\phi 2$ DNA produced in strain 168, however, is smaller in size than mature $\phi 2$ DNA and is not biologically active in transfection. These same investigators reported that certain asporogenous mutants of *B. subtilis* 168 can support the growth of $\phi 2$; all such mutants map either to the *spo*A or the *spo*B locus, while asporogenous variants in other cistrons are not permissive for $\phi 2$. The precise nature of the defects in the asporogenous mutants, both of which are involved in the early stages of sporulation, is unknown although it has been suggested that they may have altered RNA polymerase or altered membrane components (131, 140, 141).

A mutant of $\phi 1$ has been isolated which cannot productively infect *B. subtilis* 168 but which can be grown on the asporogenous host strains capable of supporting the growth of $\phi 2$ (244). This phage, $\phi 1m$, is not precisely equivalent to $\phi 2$, however, because it can grow on other strains of *B. subtilis* which do not support infection by the latter. Reduced host range mutants (*f* mutants) of SP50 which can no longer infect *B. subtilis* 168 have also been reported (261). SP50 is related to $\phi 1$ and the *f* mutants, like $\phi 1m$, adsorb to and penetrate the nonpermissive host. At least three groups of such host range mutants of SP50 have been reported, each mapping to a different position on the genome (120, 261).

An interesting system of abortive infection involving phages $\phi 29$ and $\phi 15$ has been reported for certain early-blocked (*spo*A) asporogeneous mutants of *B. subtilis* 168. Both $\phi 29$ and $\phi 15$ can infect the *spo*A strain, but only $\phi 29$ forms plaques on wild-type 168 (139). Two classes of mutants, neither of which is a revertant or suppressor of *spo*A, were isolated from *spo*A cultures (138); the first group, *tol*A mutants, were selected for tolerance to $\phi 15$ infection. These mutants adsorb $\phi 15$ but are not killed by this phage; curiously, they remain susceptible to $\phi 29$. The second class of mutants, *tol*B, were selected for resistance to $\phi 29$ and they adsorb both $\phi 29$ and $\phi 15$ but are not killed by either. Phage sensitivity, the levels of extracellular ribonuclease and protease, the production of antibiotics, and the stability of the mem-

brane to autolysis have been compared in the phage-tolerant mutants and the original asporogenous mutants. The results of the latter studies suggest that the tolerant mutants may have alterations in the cell envelope.

Jonasson et al. (146) have noted another type of lysogenic conversion affecting phage infection. Strains of *B. amyloliquefaciens* H which are lysogenic for PK prophage have altered surface properties so that superinfecting PK phage can no longer adsorb, although the unrelated phages ϕ15 and ϕ29 can be propagated. The biochemical basis for the PK-mediated change in receptor sites is unknown; the molar ratios of most of the common cell wall constituents appears to be unchanged in the lysogenic strain.

Physiological State of the Host and Entrapment

B. subtilis phages, like those of other species, have difficulty in productively infecting cells which are in the late log or the stationary-growth phase. The precise reason why phages do not develop in such cells is not well understood, but it is reasonable to assume that the declining metabolic activities of the cell affect the biosynthetic activities of the virus. The late stages of growth in the bacilli, however, are unique in that they are associated with the first stages of sporulation. When phages are added to *B. subtilis* prior to sporulation, few viral products are made and the phage genome may be "trapped" in the developing endospore. Bacteriophage functions are subsequently expressed and mature infectious particles are made during outgrowth (180, 295, 314, 365).

The genomes of pseudotemperate phages such as SP10 or PBS1 in the carrier state frequently become entrapped when their host sporulates. In fact, the majority of spores derived from such pseudolysogenic cultures have at least one copy of the viral DNA (29, 314, 321). Entrapment of more virulent phages can, with varying efficiency, be achieved simply by adding the desired phage to a stationary phase culture. However, there appear to be different optimum times in the sporulation sequence for the incorporation of the DNA of certain phages. In the case of ϕe, this is 5 to 6 h after the end of logarithmic growth (295); ϕ15, on the other hand, is maximally trapped when cells are infected at the end of logarithmic growth (139). As noted earlier, a variety of asporogenous mutants of *B. subtilis* 168 which are blocked at various stages in the sporulation process have been isolated, and some of these retain the ability to support phage growth beyond the time that trapping begins in wild-type cells (139, 141).

In recent years, studies of early biochemical changes related both to sporulation and trapping have emphasized possible modification of RNA polymerase. Losick and Sonenshein (179) reported that polymerase isolated from sporulating cells had a greatly reduced capacity to transcribe phage ϕe DNA in vitro, although it was still active on a poly(dAT) template. Such changes would, of course, be sufficient to explain why ϕe DNA is not transcribed in vivo in sporulating cells and also might be correlated with a shut-off of host vegetative functions. Presumably, further changes in the polymerase would then cause the enzyme to transcribe sporulation-specific genes. Examination of RNA polymerase isolated from cells at early stages of sporulation indicated that the loss of ϕe templating activity might be caused by loss of the sigma subunit (36, 177, 327) and/or association of two new polypeptides having molecular weights of 90,000 and 70,000 with the core enzyme (109). (Note: The structure of *B. subtilis* RNA polymerase is discussed in detail in the section on synthesis of macromolecules during the development of phages.) Addition of chloramphenicol to sporulating *B. subtilis* was found to prevent loss of sigma from the core subunits and to permit transcription of ϕe (282).

It has also been proposed that loss of ϕe templating activity was due to cleavage of the β' subunit of the enzyme by serine protease (170, 180). Another line of evidence indicating the importance of changes in polymerase during sporulation is the finding that some rifampicin-resistant mutants of *B. subtilis* are asporogeneous and do not undergo the alterations in template specificity characteristic of sporulating wild-type cells (64, 294). Lastly, polymerase isolated at a later time during sporulation has been reported to contain two small polypeptides (85; R. Fukuda, J. Sanchez-Anzaldo, and R. H. Doi, Fed. Proc. 34:649, 1975), further suggesting that the enzyme may undergo a series of changes prior to spore formation.

The relationship between these changes in the properties of polymerase and sporulation and trapping has been challenged by studies suggesting that the structural modifications may, at least in part, be artifacts of culture conditions and/or enzyme isolation. Linn and co-workers (177) presented evidence that modification of the β' subunit was the result of proteolysis during enzyme purification and did not occur in vivo. Ito et al. (139) found that decreases in the burst sizes of phages ϕ15 and ϕ29, which are trapped during sporulation at different times in the growth cycle, were not

accompanied by changes in relative templating efficiency of the DNA of the two phages. Furthermore, transcription of the phage genome apparently depends on the phage, since expression of $\phi 29$ is blocked early in sporulation, whereas transcription of ϕe continues until a later time (153). Murray et al. (202) compared polymerase activities of cells sporulating in a defined medium and in an enriched medium and report that loss of specificity for phage DNA occurred only in the latter. Others (156, 222) have proposed that the loss of ϕe template activity is completely unrelated to polymerase modification. Thus, although changes have been reported in the subunit structure of polymerase isolated from sporulating cells, it is not clear what relationship these changes have to entrapment or whether these modifications are obligate requirements for sporulation.

Mixed Bacteriophage Infections

Investigations of mixed infections involving the *E. coli* T phages (61, 62, 63) revealed that: (i) in most instances, only one partner is successful in producing progeny ("mutual exclusion"); (ii) with some exceptions, the same phage is always dominant if the cells are infected simultaneously, but a subordinate partner may produce progeny if given sufficient "lead time;" (iii) closely related phages can productively infect the same bacterium but often one partner produces more than half the progeny ("partial exclusion;" [70]); and (iv) in mixed infections involving T_2 and T_4, the first phage quickly induces changes in the surface of the host cell so that DNA of the second phage does not penetrate beyond the cell envelope ("superinfection exclusion;" [4, 83, 343]).

Some of these effects have also been observed in mixed infections of *B. subtilis* but superinfection exclusion has not been found. Studies of progeny released from *B. subtilis* mixedly infected with pairs of unrelated phages (224), revealed that SP82G, $\beta 22$, and $SP02c_1$ can be ranked in a hierarchical order in terms of their ability to dominate infections. Thus, SP82G blocks the development of $\beta 22$ or $SP02c_1$ and $\beta 22$ can inhibit the development of $SP02c_1$. In all instances, however, the yield of the dominant phage is reduced compared to single infections.

At the molecular level, the dominant phage apparently suppresses development of the subordinate phage by inhibiting synthesis and transcription of the DNA of the subordinate phage (161). For example, if $\beta 22$ is added 5 min before $SP02c_1$, negligible amounts of $SP02c_1$ DNA and RNA are made and the only effect of superinfection is a reduction in the yield of the dominant phage. The binding of $SP02c_1$ DNA to the membrane in this infection is significantly less than the binding in single infections or in mixed infections where $SP02c_1$ is added first. When the conditions of the experiment are reversed, $SP02c_1$ retains control over the time of lysis but the two phages interfere with one another and the infections are abortive in the sense that very little of either phage is produced. Studies of the synthesis of nucleic acids in mixed infections in which $SP02c_1$ precedes $\beta 22$ by 5 min indicate that the latter almost completely suppresses $SPO2c_1$ transcription although apparently both phages replicate their DNAs.

Similar effects on nucleic acid synthesis can be observed in mixed infections of SP82G and $\beta 22$, but here dominance is at least partially dependent on host (345). In infections of *B. subtilis* SB11 (a derivative of W23), SP82G is clearly subordinate and $\beta 22$ can interfere with the development of SP82G even when added to cells preinfected 5 min with the latter. Again dominance can be correlated with the synthesis of nucleic acids specific for each phage. However, in *B. subtilis* 168, SP82G is dominant and suppresses $\beta 22$-directed nucleic acid synthesis when added before or simultaneously with the latter. Strain dependency in this mixed infection can be at least partially explained by the fact that SB11 is not a preferred host for SP82G. However, at the high multiplicities of infection used in these experiments, SP82G produces high yields of progeny in single infections of SB11, and yet $\beta 22$ dominates even when SP82G is given a lead time of 5 min in mixed infections in this bacterium.

In contrast, SP82G blocks the development of $SP02c_1$ (161) and also $\phi 29$ (D. Linemeyer and H. R. Whiteley, unpublished observations) if added first, simultaneously, or 5 min after either of the latter two phages. More surprisingly, SP82G can interfere with the infection by $SP02c_1$ even if addition of the former is delayed for 15 min after infection with $SP02c_1$. In the latter instance, SP82G begins a qualitatively normal latent period in which early RNAs are synthesized and this is followed by SP82G DNA replication. However, SP82G cannot completely suppress $SP02c_1$ development since the latter lyses the cells before SP82G progeny are produced. It is possible that appropriate conditionally lethal mutants of $SP02c_1$ which lyse late might be used to determine how changes directed by this phage affect SP82G morphogenesis. Investigation of membrane-associated DNA indicate that secondary infection with SP82G affects the interaction of $SP02c_1$ DNA with

membrane. Thus, if SP82G is added 5 min after $SP02c_1$, $SP02c_1$ DNA is replicated but it is not released from the membrane.

The mechanisms involved in establishing dominance of any one of these phages over another are not known at the present time. It is clear, however, that in some mixed infections the DNA of the subordinate phage is not degraded although its interaction with the membrane may be altered in comparison with the sequence observed in single infections (161). These changes may, in turn, reflect alterations in the capacity of the cell to synthesize phage DNA. The synthesis of phage-specific RNAs in mixed infections is of particular interest since modification of the host RNA polymerase has been observed after infection by each of the phages listed above (discussed in section on synthesis of macromolecules during phage development). Thus, mixed infections might be used to determine the relevance of the changes produced by the first phage in suppressing host functions and in controlling the development of the second phage.

GENETICS OF B. SUBTILIS PHAGES

Types of Mutants

Plaque morphology and host range mutants. Plaque morphology and host range mutants have played a less important role in the genetics of *B. subtilis* phages than in coliphages. This is partly because some of these mutants, particularly host range variants, are difficult to find and partly because the great potential of conditional lethal mutants was recognized just as studies of *B. subtilis* phages began in earnest.

Two plaque morphology mutants of SP01 have been used in transfection studies; one of these gave a clearer plaque than the wild type and the other was distinguishable by a "black" halo inside the plaque (219). Phage SPP1 produces 3-mm plaques having a wide halo on the bacterial lawn. Mutants lacking this halo have been isolated and used in investigations of transfection and gene conversion (296). Clear and minute plaque mutants of SP50 have also been described and used in genetic and transfection studies of this virus. Little is known, however, about the physiological basis of the plaque morphology mutants of any of the virulent phages. On the other hand, clear-plaque variants of the temperate phages SP02 and ϕ105 have been isolated and are apparently defective in the production of prophage repressor (216, 263, 363). Clear-plaque mutants have also been reported in the pseudotemperate phages SP10 and PBS1 (152, 313).

Brodetsky and Romig (37) reported that many newly isolated *B. subtilis* phages can be propagated initially only on the host strain used in their enrichment and isolation. Upon subsequent propagation, "star" variants were isolated which had extended host ranges. In addition, variants of SP50, ϕ1, and β22 which are unable to infect *B. subtilis* 168 productively, but which can grow on other strains of this bacterium, have been found (244, 261).

Phage host range variants able to adsorb to and productively infect phage-resistant *B. subtilis* mutants have been looked for, but very few have been reported. Apparently, the changes which alter the phage receptor sites on the cell walls of resistant *B. subtilis* variants usually cannot be accommodated by corresponding changes in the virion. However, Okubo and coworkers (219) succeeded in isolating a host range mutant of SP01 which could produce progeny in a mutant of *B. subtilis* 168 which was resistant to wild-type SP01.

Conditional lethal mutants. Conditional lethal temperature-sensitive (*ts*) mutants of a *B. subtilis* phage were first reported for SP3 by Nishihara and Romig (208) and since have been isolated for SP82G (107, 148), ϕ29 (117, 317), ϕ105 (263), and others. Most such mutants can productively infect *B. subtilis* at temperatures below 37 C but are unable to develop above 42 to 45 C.

Two apparent nonsense suppressor mutants in *B. subtilis* 168 derivatives, *sup*-1 (220) and *sup*-3 (96), have gained wide use in recent years, and many genetic studies of *B. subtilis* phages now employ suppressor-sensitive (*sus*) mutants of these viruses. Such phage variants have been reported in ϕe (96), SP02 (363), SP01 (221), and ϕ29 (241). Although separately isolated, the two suppressor mutations show similarities in suppression pattern and are thus thought to be of the same type, but they are not mutations of the same gene (322). The precise mechanism of suppression in the *sup*-1 and *sup*-3 strains has not been completely elucidated, but both are thought to act at the level of translation (286) by recognition of a chain-terminating triplet. Bacteria carrying either suppressor produce high-molecular-weight isozymes of otherwise normal proteins (18), and *sus* mutants of ϕ29 produce short proteins under nonsuppressing conditions (45). A recent study (289) indicates the product of the *sup*-1 gene suppresses polypeptide chain termination with an efficiency of 25 to 30%.

Suppressor-sensitive phage mutants isolated on one of the two host strains vary in their ability to grow on the other suppressor-bearing strain; some grow quite well on either bacte-

rium and others have a strong preference for or can only grow on one strain. A reasonable explanation for this observation and the finding that *sup*-1 and *sup*-3 map to different loci is that both hosts suppress the same nonsense codon but a different amino acid is inserted in each case. In some instances either amino acid substitution leads to an active product, in which case the phage mutant can productively infect either host.

Many temperature-sensitive and suppressor-sensitive phages have been isolated from phage stocks treated with nitrous acid or hydroxylamine and then allowed one cycle of infection to eliminate heterozygotes (208, 318). Others have been obtained from progeny phage released from infected cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, bromodeoxyuridine, or aminopurine during the latent period (7, 148, 221, 263).

Genetic Maps

Recombination frequency of maps of temperature-sensitive and/or suppressor-sensitive mutants have been published for bacteriophages SP82G (105, 107, 148, 149), SP01 (221), SP02 (363), ϕ105 (263), and ϕ29 (7, 117, 195, 241, 317, 318, 319). In general, these maps indicate the phages have linear, nonpermuted genomes, although a circular map has been reported for ϕ105 (263). Both this phage and SP02 are reported to have cohesive ends (53), as might be expected in temperate phages which are inserted into the host chromosome by reciprocal recombination. There is also physical evidence that the virion DNA of ϕ29 is a supercoiled circle held by a protein linker (223); however, the recombination frequency map is linear. Since most of the cistrons have not been identified in the larger phages, only partial correlation of genetic defects and functions is possible at the present time. A significantly larger amount of information is avaliable for the small phage ϕ29 and identification of the function of each cistron can be anticipated in the near future.

HMU-containing phages. Temperature-sensitive mutants of SP82G have been isolated (107, 148, 149) and have been assigned to 27 complementation groups (about 20% of the probable total number of cistrons). The functions of some of the cistrons have been determined and the first structure-function map of this phage is beginning to emerge (107, 149). The genome of SP82G can be divided into three functional regions (Fig. 10): DNA synthesis, tail synthesis and head synthesis. A similar clustering of genes of related function has, of course, been found in the coliphages—e.g., T_4 (reviewed by Mathews, 192), lambda (201), and T_7 (307).

In SP82G, the earliest genes to function (cistrons 19, 20, 1, 21, 2, 3, 22, 18 and 4) are on the arbitrarily designated left end of the map and defects in these genes directly or indirectly affect DNA synthesis. Genes 2 and 3 are structural genes for dUMP-hydroxymethylase and dHMU kinase, respectively (149, 233); the functions of the other genes are not known but mutants in cistrons 1, 2, 3, and 18 are also defective in cell lysis (149). Mutations in cistrons 19 and 22, which are in the same general segment of the genome, permit nearly normal amounts of DNA replication under nonpermissive conditions and their role in phage development is unknown. An interesting sidelight to the finding that early functions are grouped near one end of the phage chromosome was made by McAllister (193). Using marker rescue techniques, he was able to demonstrate that the left half of the genome (which is thought to control early functions) is injected first and that complete transfer of the virion DNA requires several minutes (mean time = 2.3 min at 28 C; 4.0 min at 24 C).

Another adjacent group of genes (cistrons 5–10, 23, 11, and 12) are apparently concerned with tail formation and mutations at many of these loci result in the appearance of structures having filled heads but lacking tails. A mutant in gene 8 when grown at the nonpermissive temperature accumulates thin tubular structures whose diameter equals that of the tail core (107). Mutants in gene 5 do not produce either phage particles or phage antigen, suggesting a pleiotropic control of tail formation. Mutants in genes 24 and 13 do not produce phage antigen under nonpermissive conditions, although particles equal in density to whole SP82G phage appear in the lysates (107).

Finally, mutants in a third group of cistrons (genes 14, 26, 15, 25, 16, 17, and 27) are defective at high temperatures in the production of phage heads. They produce normal amounts of DNA and antigen but do not yield filled heads or complete phage particles.

Genetic studies have been initiated by Okubo and co-workers (221) on suppressor-sensitive mutants of the related phage, SP01. Some 36 cistrons have been recognized by complementation tests and evidence has been obtained that related functions tend to cluster together (Fig. 10). As in SP82G, most cistrons involved directly in DNA synthesis (presumed early functions) are concentrated toward one end of the genome, arbitrarily designated the right end in SP01. A point of some interest in both SP82G

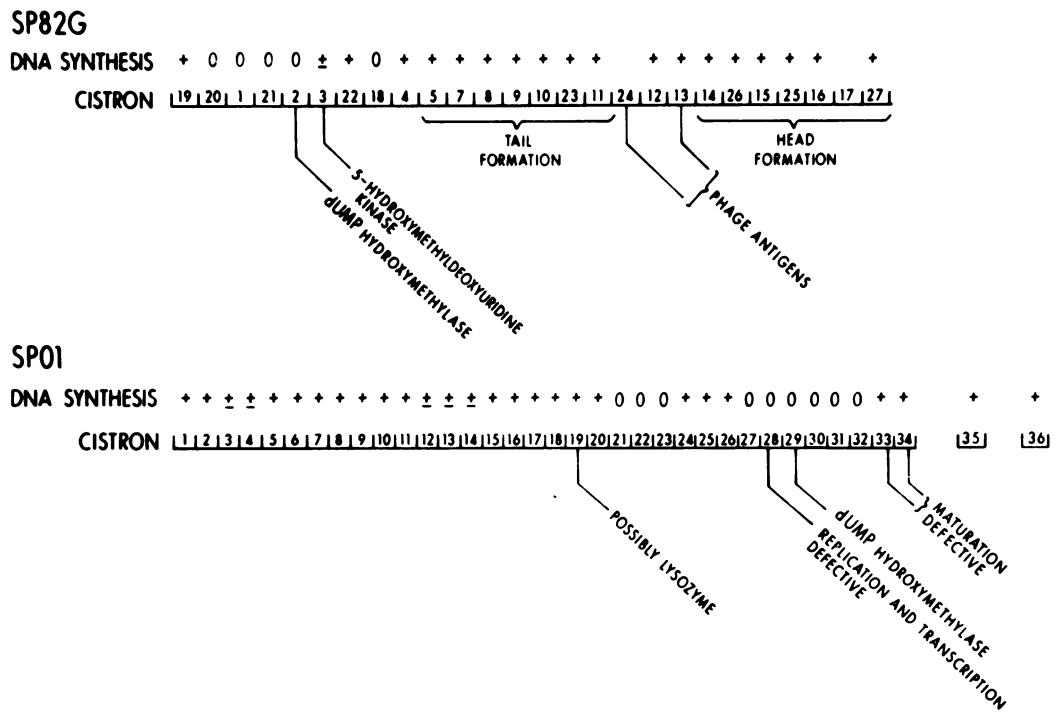

FIG. 10. *Genetic maps of SP82G ts mutants and SP01 sus mutants showing cistron orders. Recombination frequencies are not implied. In DNA synthesis, 0 = little or no viral DNA synthesized; ± = small amounts of DNA synthesized; and + = greater than 30% normal amount of DNA synthesized. Maps are based on data from references 84, 107, 148, 149, 221, and 232.*

and SP01 is the function of one or two cistrons located at the end of the genome immediately proximal to the genes controlling DNA synthesis. Despite their location, these genes (cistron 19 in SP82G and cistrons 33 and 34 in SP01) do not seem to be involved in DNA replication since *ts* or *sus* mutants in these loci make large amounts of viral DNA under nonpermissive conditions (107, 221). The function of cistron 19 in SP82G is not known but mutations in genes 33 and 34 in SP01 have multiple effects and a variety of late proteins are not synthesized (221). Fujita and co-workers (84) list these mutants as maturation defective and note they cannot synthesize several middle and late classes of viral RNA, and do not form protein subunits of the virion. They are also defective in their ability to shut off transcription of certain classes of viral RNA at the appropriate time in the latent period.

Mutants in gene 29 of SP01 are similar to those of gene 2 in SP82G in being defective in dUMP hydroxymethylase activity (221). SP01 phages lacking the gene 28 function are classified as replication and transcription defective by Fujita et al. (84). In nonpermissive hosts, these variants transcribe only those classes of viral RNA for which no protein synthesis in the infected cell is required, and almost entirely cease viral transcription 8 to 10 min after infection. Gene 28-deficient mutants lack both deoxyuridine monophosphate (dUMP) hydroxymethylase and deoxycytidine monophosphate (dCMP) deaminase, probably because both enzymes are translated from RNAs which are among those classes not synthesized in these phage variants (221). From their map position and the fact that they make viral DNA, it seems likely that genes 1 to 20 in SP01 are late functions such as head and tail proteins, but this has not yet been investigated. There is some evidence that gene 19 controls a viral lysozyme (221).

ϕ29. As noted earlier, bacteriophage ϕ29 with a DNA molecular weight of 11×10^6 is thought to contain sufficient genetic information for about 20 cistrons, and several recombination maps of *ts* or *sus* mutants have been published (117, 199, 241, 318). Unfortunately, the results of these independent genetic studies of ϕ29 have not yet been combined into a single definitive map, and it is not certain which mutants or cistrons are repetitious and precisely how many genes have been identified. The max-

imum number of complementation groups reported by an one laboratory is 17 (199); thus even if all cistrons reported by other groups are redundant, this appears to provide a minimum number of polypeptides whose synthesis is directed by ϕ29 DNA. In UV-irradiated *B. subtilis* infected with ϕ29, up to 23 phage-induced proteins have been separated by gel electrophoresis (48, 122, 195, 227). It is possible, however, that some of the proteins identified on gels may be precursor proteins which are cleaved to generate lower-molecular-weight products which are observed on the same gels (7).

The most complete map of ϕ29, that of Moreno and co-workers (199), integrates data from a group of *ts* mutants originally isolated by Talavera et al. (317) and newly isolated *sus* mutants. Using two- and three-factor crosses, these investigators arranged mutants in 16 of 17 known complementation groups into a linear map of 24.2 recombination units. The possible roles of only a few of these cistrons have been reported, however. Mutants in cistrons F and K, which are located at the extreme left of the recombination map, do not synthesize viral DNA and, on the basis of temperature-shift analysis, are thought to direct early functions (317, 318, 319). Cistron C, whose position on the ϕ29 chromosome has not yet been determined, is also blocked in DNA synthesis but is not an early function. Both *sus* and *ts* mutants in three cistrons (P, N, B) result in reduced plaque sizes, but the precise function of these genes is not known.

In a study of *ts* mutants of ϕ29 made by Hagen and co-workers (117), 13 cistrons were organized in a linear map of 10 recombination units. On the basis of temperature shift-up experiments (274), one gene (cistron 4) located near the left end of the map was found to be involved in an early function required in the first minute of the latent period. Mutants in this cistron are blocked in DNA synthesis, but since genome replication is not initiated until 12 to 15 min after infection, it is possible that cistron 4 is a regulatory gene. Cistron 5, which is required 10 min after infection, apparently directs the synthesis of a protein required for attachment of ϕ29 DNA to the bacterial membrane before DNA replication can be initiated (142); it corresponds to cistron 1 of McGuire et al. (195). The remaining 11 cistrons are all involved in late activities required 19 or more minutes after infection; most are of unknown function, although the product of gene 9 is required for DNA synthesis.

Reilly and co-workers (7, 241) have isolated suppressor-sensitive mutants in 14 complementation groups (labeled A through N) and by three-factor crosses have assigned an unambiguous order to 10 cistrons. Examination of the patterns of ϕ29-specific protein synthesis was made by irradiating nonpermissive host cells with UV and infecting with the various *sus* mutants in the presence of ^{14}C-labeled amino acids. Proteins were then isolated and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). Four cistrons (H, J, L, N) clearly code for single proteins: P(H), the tail protein; P(J), a precursor protein that is thought to give rise to the virion appendage protein and a low-molecular-weight polypeptide (47); and P(L) and P(N) which are nonstructural proteins of undetermined function. Two or more ϕ29-related proteins are missing from gels of proteins obtained from nonpermissive cells infected with *sus* mutants in complementation groups C, D, E, F, I, and M. In some instances, this may result from a missing precursor protein; in other instances, failure to produce a regulatory protein could explain the pleiotrophic effects observed on the synthesis of several proteins. Mutations were also detected in at least five genes coding for low-molecular-weight polypeptides. Only one of these genes contains a conditionally lethal mutation, however, suggesting some of these cistrons are not essential and may have a regulatory function.

A third independent study of 54 suppressible mutants of ϕ29 has been made by McGuire and co-workers (195). These investigators assigned mutants to eight cistrons, numbered I through VIII, which they ordered in a linear map by two-factor crosses. They proposed the following functions for the cistrons. (i) Cistrons I and II are concerned directly or indirectly with DNA synthesis (as noted above, the protein specified by cistron I is thought to be required for attachment of viral DNA to the membrane [142]). (ii) Cistrons III and VI play a regulatory role (mutants in these cistrons show pleiotrophic effects and either fail to synthesize several late proteins or produce them in reduced amounts). (iii) Cistrons IV and V code, respectively, for phage tail and collar proteins. (iv) The role of proteins synthesized by complementation groups VII and VIII has not been determined.

ϕ105 and SP02. Recombination maps of temperature-sensitive, suppressible, and clear-plaque mutants of the temperature phages ϕ105 and SP02 have been published, although only a modest amount is known about the functions of various cistrons (9, 263, 363). Considerable attention has been focused on cistrons possibly associated with lysogeny, and clear-plaque variants have been isolated in both SP02 and

φ105. Most such mutants show the expected pattern for repressor mutants; clear plaques are produced on nonlysogenic cells but no plaques are made on lysogens of the wild-type phage (216, 263, 363). The isolation of a mutant φ105 which is temperature inducible is compatible with the production of a protein repressor which functions in a manner analogous to the lambda repressor (10, 169). A suppressor-sensitive mutant in the repressor function of SP02 has been reported (363).

A mutant in a second φ105 gene, c4, also produces clear plaques, but this variant retains the ability to lysogenize at greatly reduced efficiency (90). Moreover, lysogens of φ105c4 exhibit a spontaneous induction rate 1,000-fold lower than wild-type φ105 and are not inducible by mitomycin C. The prophage can be induced, however, if lysogens are grown to competence and exposed to transforming DNA isolated from *B. subtilis* 168 or closely related strains (91). It is suggested that this DNA-stimulated induction may be related to a recombination at the prophage insertion site.

There is one reference (332) in the literature to a virulent mutant of SP02, but the precise phenotype is not given. Thus, it is not clear at the present whether any investigators have isolated virulent mutants of SP02 or φ105 which are capable of forming plaques on *B. subtilis* strains lysogenic for the homologous phage. Until such mutants have been definitively described, it will be difficult to ascertain whether control over lysogeny in these phages is truly analogous to that of the temperate coliphages.

Sixty suppressor-sensitive mutants of SP02 were isolated and classified into 18 cistrons by Yasunaka et al. (363), and a linear recombination map was constructed by two-factor crosses. The C_1 gene, which presumably directs the synthesis of the repressor, is located near the center of this genetic map, with 10 known cistrons on the left half and six on the right; two cistrons have not yet been positioned. Four genes surrounding the C_1 locus are involved in early function; three of these cistrons immediately contiguous to the right side are required for DNA synthesis, and one of the latter, gene L, codes for a DNA polymerase (266). The proximity of early genes to the repressor resembles the organization found in other temperate phages. Several late functions directing head or tail assembly and synthesis have been located on both halves of the genome. Both genetic (12) and physical studies (54) indicate the SP02 prophage is permuted relative to the vegetative map.

Using temperature-sensitive and suppressor-sensitive mutants, 11 essential genes have been identified in φ105 and a recombination map has been established by two- and three-factor crosses (9). The genes can be arranged in a unique linear order which is identical in the vegetative phage and in the prophage (9, 263). Assuming that φ105 integrates into the bacterial chromosome in the manner proposed by Campbell (46), this latter finding means the phage attachment site (*att*) must be located outside the presently known genes. As will be noted later, physical studies also suggest the *att* region is very close to or overlaps the ends of the mature phage genome (54). Evidence that the vegetative φ105 map is circular is found in the observation that mutants in the gene thought to direct the synthesis of the repressor are linked to markers on both the left and right end of the map (263).

The location of the SP02 and φ105 prophages on the *B. subtilis* genome and the order of genes in the integrated state have been investigated by means of transformation and PBS1 transduction. Inselburg and co-workers (136) found that the SP02 prophage co-transduced 60 to 65% with the erythromycin marker (*ery*-1) and 20% with a cysteine locus (*cys*A14); four-factor crosses demonstrated the gene order to be *sul-cys*A14-*ery*-1-SP02, where *sul* is sulfanilimide resistance. More precise mapping has shown that the SP02 attachment site is located between *spc*-2 and *lin*-2 which are, respectively, sites conferring resistance to the antibiotics spectinomycin and lincomycin (292). Linkage was 90% to *spc*-2 and 30% to *lin*-2. Heteroduplex mapping has revealed that the SP02 attachment site is 6,200 DNA base pairs from the 16S and 23S ribosomal RNA genes (54). (Note: A recent map of the *B. subtilis* chromosome may be found in reference 372.)

Similar PBS1 transduction studies showed that φ105 co-transduces 20% with an isoleucine, leucine, valine maker (*ilv*A1) and 20% with a phenylalanine marker (*phe*-1) and that φ105 is inserted between the two host markers (263). A *ts* mutation in the presumed repressor function (c gene) is at the end of the prophage proximal to *ilv*A1 host marker. Close association of the prophage φ105 with genes of the leucine-isoleucine pathway is also indicated by the fact that some of these markers can undergo specialized transduction with this phage (284).

Transfection

Release of progeny virus from competent *B. subtilis* cells infected with phenol-extracted DNA from a bacteriophage was first reported by Romig (254) in his studies of SP3. Subse-

quently, Földes and Trautner (80) coined the word "transfection" to describe "the infection of cells by the isolated nucleic acid from a virus, resulting in the production of a complete virus," and this terminology is now widely used. Transfection in *B. subtilis* and other bacteria has been the subject of recent reviews (210, 333), and our discussion here will be limited to an account of the mechanism of transfection and its uses in studying phage development.

Competence for transfection and transformation. Virtually all studies to date lead to the conclusion that cells which are competent for transformation are also competent for transfection. The same regimen is used for preparing bacteria for either process, conditions which alter the development of competence affect both transformation and transfection (30, 31, 351). Perhaps most convincingly, the number of transformed cells in a competent culture pretreated with bacterial DNA is greatly reduced by subsequent exposure to phage DNA (240, 252). Indeed even a heterologous viral DNA such as that of T2 can reduce the number of transformants when added 30 min after the transforming DNA (Reilly, Ph.D. thesis, 1965).

In transfection, however, the viral DNA must initiate the sequence of events leading to the production of mature phage in cells which differ in many respects from the log-phase bacteria typically used in bacteriophage infections. Only approximately 10% of the cells in a culture are competent at any given time, and these bacteria have reduced levels of metabolic activity (65, 203) and a lower buoyant density (43, 116). *B. subtilis* phages develop most rapidly in growing cells, and the altered biosynthetic capacity of the competent bacteria may contribute to the longer latent period, the lower burst size, and the low efficiency characteristic of transfection (103, 240, 301).

Entry of transfecting DNA. The mechanism by which transfecting viral DNA enters competent cells has been extensively investigated with SP82G (347). In these studies, competent *B. subtilis* cultures were preinfected with various *ts* mutants of this phage and then exposed to wild-type SP82G DNA. Uptake of DNA was subsequently interrupted at various time intervals by cold or heat shocks, shearing, or DNase treatment, and the penetration of various markers on the transfecting genome was measured by their ability to contribute to maker rescue with appropriate mutant phages. The results indicate there is an initial 4- to 5-min period during which DNA adsorption to the bacterial envelope can be reversed by heat shock, followed by irreversible complexing of one end of the molecule. Entry of DNA begins when the opposite end of the genome becomes bound to the cell some 6 min later. However, markers in the middle of the DNA molecule remain sensitive to shear for several additional minutes, indicating that portion of the transfecting genome is not as tightly bound to the cell envelope as the two ends. Ingression of the DNA is thought to be complete by 12 to 15 min, because all markers are resistant to DNase by that time (103, 347). The rate of penetration is comparable to that observed for injection of DNA by SP82G phage, but, surprisingly, the order of marker entry (and initial attachment to the cell envelope) is the reverse of that in phage infection (193).

Investigations by Spatz and Trautner (296) indicate that transfecting SPP1 DNA is probably taken up as double-stranded molecules. Similarly, phage DNA re-extracted from competent cells exposed to H1 transfecting DNA is still double stranded, although, as discussed below, it is degraded somewhat by various nucleases (14).

Intracellular inactivation. The processing of transfecting DNA from the time penetration is initiated until infective particles are synthesized has not been completely investigated and is the source of considerable controversy. Okubo and co-workers (219) made a study of the relationship between SP01 DNA concentration and the number of infectious centers formed in a competent *B. subtilis* culture, and reported that at very low DNA concentrations a 10-fold increase in bacteriophage DNA resulted in a 1,000-fold increase in plaque-forming units. From these observations they concluded that a recipient bacterium must be infected by two or more DNA molecules to yield a complete virus particle. Similar multiplicity effects have been noted in SP82G (103), in SPP1 and SP50 (297), in another recently described HMU-containing phage, H1 (14), and in ϕ1 and ϕ25 (240), although the exponent of DNA concentration varied from 2 to 5. On the other hand, transfection by DNAs from ϕ29, SP02, and ϕ105 show a more nearly direct proportionality between DNA concentration and plaque-forming units, a result similar to that found in transformation of single bacterial markers (268, 297).

It is now generally agreed that a slope of 2 or more, obtained in a log plot of infectious centers as a function of DNA concentration, indicates that a recombination between two or more phage genomes is required to produce a successful transfection, and this recombinational event has been termed "primary recombination" to distinguish it from recombinations occurring after replication of phage DNA has started (297). A slope of one in the above plot is

interpreted to mean that transfection may not require primary recombination. The need for several DNA molecules in most transfections might be attributed to damage done to transfecting DNA during its isolation, except that only intact DNA is able to transfect competent cells, although fragmented DNA can be used in marker rescue. This suggests that transfecting DNA is damaged during or immediately after uptake by competent cells.

A variety of experimental evidence indicates that molecules of transfecting DNA are degraded by bacterial nuclease(s). For example, the ability of viral DNA to participate in marker rescue declines dramatically as the time between exposure of cells to the transfecting DNA and addition of the superinfecting phage is increased (104, 194). Recombination frequencies in transfection crosses are higher than in corresponding phage crosses (104, 106, 108, 219, 297), and single-cell burst analyses of recombination in transfection show that a large percentage of bursts yield only one type of recombinant and no parental progeny (219, 297). This latter result can be interpreted as further evidence that primary recombination, presumably between two or more molecules of damaged DNA, is necessary to recover a single viable phage genome.

Green and Urban (108) made a detailed study of recombinant SP82G progeny released from cells transfected by mixtures of phage DNA molecules bearing independently isolated *ts* markers in gene 5. They detected a random assortment of surviving input alleles among the progeny derived from the mixedly infecting DNA molecules, even with marker pairs separated by distances corresponding to less than $^1/_{40}$ of the genetic map. From these results it was concluded that transfecting SP82G DNA suffers random introduction of about 40 unlinking hits per genome.

Investigations with H1, an HMU-containing phage whose transfection requires the cooperation of 4 to 5 DNA molecules, showed that the molecular weight of H1 DNA after uptake by competent cells is reduced by a factor of approximately 2.8 (14). After this initial fragmentation, however, the molecular weight of the transfecting genome remains constant up to 40 min. Arwert and Venema (14) suggest that the endonucleolytic breakdrown is associated with a cell membrane-bound enzyme which is involved in the irreversible fixation of the transfecting DNA. These investigators also noted that each H1 chromosome sustains about 23 single-strand breaks during the transfection, and they suggest this may be somehow related to the phenomenon of "unlinking" reported by Green and Urban in SP82G (108).

Epstein and Mahler (76, 77) reported that irradiation of competent cells with UV or exposure of cells to transfecting SP82G DNA together with UV-irradiated homologous or heterologous DNA leads to an enhancement of the efficiency of transfection, and causes a shift from approximately fourth-power dependence on DNA concentration to an approximately first-power dependence. They speculated that binding of intracellular nucleases to the damaged DNA could account for these results. Similarly, Arwert and Venema (14) found that UV irradiation of cultures prior to adding transfecting H1 DNA produced a 100-fold increase in the number of infective centers at nonsaturating concentrations of DNA, and a similar improvement was observed when competent cells were pre-incubated with UV-irradiated *E. coli* DNA before addition of H1 DNA. In their experiments, however, neither method of enhancement changed the slope of the DNA concentration dependence curves, and they speculate that the conflicting results obtained by Epstein and Mahler might be attributable to the use of excess DNA by the earlier investigators. Moreover, Arwert and Venema found that, in addition to endonucleolytic damage, irreversibly adsorbed transfecting DNA is subject to exonuclease attack. Since this exonucleolytic degradation is greatly reduced under enhancing conditions, they suggest that transfection enhancement results from trapping of an exo- rather than endonuclease by UV-irradiated DNA. More recent evidence (145) suggests this exonucleolytic breakdown is specifically related to the competent state, and may be mediated by a membrane-associated enzyme.

Overall, the results of the enhancement studies and the investigations of the fate of H1 and SP82G in competent cells suggest that transfecting DNAs showing multipowered dose response curves may be exposed to both exonucleolytic and endonucleolytic attack, with the former being a major factor in reducing the overall efficiency of transfection. Successful transfection, therefore, may require avoiding (or repairing) the damage due to exonuclease and primary recombination to restore a complete viral genome from the fragments produced by endonuclease digestion.

One finds conflicting evidence as to whether the more linear relationship between plaque-forming units and DNA concentration observed in transfection with DNAs from ϕ29, SP02, and ϕ105 really means that primary recombination is not required to yield progeny virus. There is some possibility that the slope of approximately 1 obtained with ϕ29 DNA (297) may be an arti-

fact, as it has been reported that the DNA of this phage has a tendency to aggregate (223). Thus, the incorporation of a second ϕ29 genome by a competent cell may not occur independently of the incorporation of the first DNA molecule. On the other hand, ϕ29 and SP02 DNAs are able to transfect recombination-deficient mutants of *B. subtilis* which are refractile to transfection by phage DNAs displaying multihit dose responses (297); this implies that transfection by the former is fundamentally different.

Flock and Rutberg (79) have made a careful analysis of the infectivity of mature and prophage ϕ105 DNA and concluded that primary recombination is, in fact, required for transfection with the former. In the first place, they note that while transfection marker rescue with mature phage DNA and infectivity of prophage DNA both demonstrated a linear relationship with DNA concentration, data from experiments with DNA isolated from mature ϕ105 yielded curves with a slope that varied between 1.1 and 1.6. Although these slopes are less than 2, these experiments suggest that there is a requirement for more than one DNA molecule. In addition, the rate of formation of infectious centers was constant per unit time for prophage DNA but increased with time when the transfecting DNA was isolated from mature virions. The latter observation also implies some cooperative relationship between mature ϕ105 DNA molecules during transfection.

Additional evidence that recombination, presumably primary recombination, is involved in transfection of mature ϕ105 DNA is seen in studies with mixtures of two DNA preparations genetically marked with *ts* mutations. Flock and Rutberg (79) noted that if recombination between two DNA molecules is essential for infectivity of mature phage DNA, the percentage of transfected cells which yield a certain type of recombinant should be relatively independent of DNA concentration. On the other hand, if recombination is not absolutely required to give an infectious molecule, the fraction of transfected cells yielding such recombinants should be dependent on DNA concentration. They found that for prophage ϕ105 DNA, there was a relationship between increasing DNA concentration and the percentage of infectious centers giving wild-type recombinants, and concluded that primary recombination between two prophage genomes was not required to obtain progeny. On the other hand, the percentage of transfected cells yielding recombinant wild-type phage when mature ϕ105 DNA was used (about 40 to 50% of the infectious centers) was independent of DNA concentration. This latter result, then, supports the hypothesis that primary recombination is essential for transfection with mature ϕ105 DNA.

Evidence from the ϕ105 studies also supports the view that transfecting DNA may be inactivated by more than one mechanism and not all such damage is repairable by primary recombination. In a previous report, Rutberg and coworkers (268) demonstrated that, in ϕ105, both prophage and mature phage DNA show equal inactivation of single markers in competent cells, yet the above studies indicate that only transfection by mature DNA depends on primary recombination. These results imply that the mature DNA undergoes a very specific type of damage when entering a competent cell, different from the damage which can be measured by inactivation of single markers or unlinking of linked markers on the phage DNA. It has been suggested that these findings are consistent with a model in which mature ϕ105 DNA suffers exonucleolytic attack at one end, and recombination between two molecules with intact opposite ends restores a complete viral genome (79, 265). However, this model would appear to conflict with the studies of SP82G and H1, both of which show high DNA dose responses. In the latter it will be recalled, there is evidence that the transfecting DNA molecules undergo endonucleolytic damage which is repaired by primary recombination. The reasons for the differences between ϕ105 and SP82G and H1 are unknown.

One of the more puzzling aspects of the role of recombination is understanding why transfection can only be achieved using intact phage (or prophage) genomes as the source of DNA. Indeed, the loss of ability to serve as transfecting DNA is one of the most sensitive assays for double-stranded endonuclease activity. Apparently, at least one of the two DNA strands must be completely undamaged at the time DNA is added to the competent culture. The problem presumably does not reside in DNA uptake because fragmented DNA can be used in marker rescue. Havender and Trautner (121) studied the effect of partial DNA fragmentation in SP50 and showed that even when the phage DNA preparations contained a high concentration overlapping fragments, competent *B. subtilis* cells could not reconstruct an active phage genome.

One possible explanation is that fragmented DNA is more susceptible to cellular nucleases which quickly degrade it beyond the ability of the primary recombination system to restore a complete viral genome. Trautner and Spatz

(333) offer a different hypothesis. They note that recombination in *B. subtilis* transformation occurs via single-strand integration, while construction of an intact viral genome from half molecules of transfecting DNA requires double-strand exchange, a process which may not be within the capacity of the host enzymes thought to catalyze primary recombination. It would appear, however, that this is the type of repair needed to reactivate transfecting H1 DNA molecules subject to intracellular endonucleases (14), and if this explanation is correct, we shall require a better understanding of how transfecting DNA is fragmented.

Role of host enzymes in transfection. The phenomena of primary recombination and marker rescue suggest key roles for host enzymes in the transfection process, and several studies have been initiated to ascertain which host enzymes are involved. Dubnau and coworkers (66) have examined a collection of some 16 isogenic recombination-deficient strains isolated on the basis of sensitivity to methyl methane sulfonate or mitomycin C, and grouped them into five classes on the basis of various deficiencies in transformation and transduction. Other investigators have characterized some of these mutants with respect to their ability to support transfection; these studies are summarized in Table 5.

Absence of a normal *rec*A1 gene product (class I mutants) has multiple and somewhat confusing effects on the processing of infective DNA by competent cells. These bacteria are transformation-defective, but can be transduced by PBS1 lysates prepared in strain 168 (67). However, neither SP10 nor PBS1 lysates prepared on *B. subtilis* W23 can transduce this strain (67, 129, 130). Rutberg and Rutberg (269) noted that *rec*A1 mutants which are lysogenic for ϕ105 give a decreased yield of phage after induction with mitomycin C, but not after heat induction of a temperature-inducible prophage. Prophage ϕ105 DNA does not transfect strains lacking the *rec*A1 function, but these bacteria can be transfected with mature ϕ105 and with DNA from HMU-containing phage H1 (14, 269). Class I mutants do not form donor-recipient DNA complexes, the step in which transforming DNA becomes covalently sealed to the recipient genome.

B. subtilis 168 mutants deficient in the *rec*B2 function (class IIa) show a lowered frequency of transformation and SP10-mediated transduction, and a somewhat decreased efficiency of PBS1-mediated transduction (66, 130, 269). These bacteria remain susceptible to transfection by SP02 DNA (217) and by both prophage and mature ϕ105 DNA, but they show a 10-fold diminution in the frequency of marker rescue with DNA from the latter (269). On the other hand, the genomes of phages such as SPP1,

TABLE 5. *Transformation, transduction, and transfection of certain recombination-deficient mutants of B. subtilis 168*[a]

Mutant class	Examples	Transformation	PBS1 transduction with lysates prepared on		Formation of donor-recipient complex	Transfection with			Ability to support marker rescue
			W23	168		Phages showing high exponents of DNA concentration	ϕ105		
							Mature phage DNA	Prophage DNA	
I	*rec*A1 *rec*-8	−	−	+	Blocked	+	+	−	+
IIa	*rec*B2	−	−	−	Formed	−	+	+	−
IIb	*rec*-4 *rec*-3	−	−	−	Blocked	−	−	−	−
III	*rec*-5, 6, 7, 9, 10, 11, 12, 14, 15	+	+	+	Formed	NT	NT	NT	NT
IV	*rec*-13 *rec*-16	+	−	−	Formed	NT	NT	NT	NT

[a] After Dubnau et al. (66), Okubo and Romig (217), Rutberg et al. (268), Rutberg and Rutberg (269), Samojlenko et al. (271), and Spatz and Trautner (297). Key: (+) indicates that transformation, transduction, transfection, or marker rescue occurs at approximately the same level as in the wild type; (−) indicates the class of mutants are less susceptible to transformation, transduction, transfection, or marker rescue than the wild-type bacterium; and (NT) not tested.

SP50, and SP01, which require cooperation between several DNA molecules for transfection, have a reduced capacity to transfect *rec*B2 strains (217, 271). In transformation, class IIa mutants form apparently normal donor-recipient DNA complexes, but the latter fail to replicate and are diluted by replication of nontransformed recipient DNA.

Currently available evidence suggests that the product of the *rec*-4 gene is involved in the primary recombination event in transfection. Phages such as SPP1 and SP50 which show multipowered DNA responses cannot transfect mutants deficient in this function (class IIb), although DNAs from phages ϕ29 and SP02 which show linear dose responses are infective (297). As expected from the evidence discussed above that transfection by mature ϕ105 DNA also requires primary recombination, *rec*-4 mutants cannot be transfected by such DNA (79). The role of the *rec*-4 product is clouded somewhat, however, by the observation that transfection with ϕ105 prophage DNA, which is thought not to require primary recombination between two viral genomes, also cannot be carried out in IIb mutants (269). However, little is known about how transfecting prophage DNA is processed in competent cells, and it is possible than some recombination event, perhaps with the host genome, occurs before excision of the prophage from its associated host DNA. As noted in Table 5, *rec*-4 mutants cannot undergo transformation and transduction and do not form donor-recipient complexes.

Studies made in host mutants belonging to class I, IIa, and IIb indicate that none of these recombination-deficient strains depress normal phage growth nor is phage genetic recombination affected (269, 297). This finding has been interpreted to mean that phage-related enzymes mediate viral recombination. Marker rescue, on the other hand, apparently requires host enzymes; it does not take place in *rec*-4 mutants and is much reduced in strains deficient in the *rec*B2 function.

Transfection studies have not been carried out on the mutant host strains in classes III and IV. However, Samojlenko and co-workers (271) have made studies of transfection and transformation in mutants from four loci (*rec*B, *rec*D, *rec*E, and *rec*F) which specify recombination repair functions. Their *rec*B locus includes the *rec*B2 mutants of Dubnau and co-workers (66), while the latter three represent new linkage groups mapping to locations different from the previously mapped *rec*A and *rec*B loci (118). It is possible that mutants in the *rec*D, E, and/or F genes correspond to class III or class IV mutants, for all are defective in transformation. As noted above, *rec*B2 mutants show a reduced capacity to support transfection; similarly, *rec*F strains show very low levels of transfection. On the other hand, *rec*D and *rec*E mutants retain a high level of transfectability at least for some phage DNAs. These studies suggest that certain host functions are required for both transformation and transfection, but several appear to be unique to the former.

Yasbin and co-workers (357) have isolated mutants of *B. subtilis* 168 selected for reduced transforming ability. One of them, RUB 19, cannot be transfected or transformed, but is tranducible by both PBS1 and SP10 and yields recombinant phage genomes when multiply infected with strains of ϕ29. In addition, RUB 19 strains which are lysogenic for ϕ105 are not inducible at normal levels; however, SP02 induction in RUB 19 lysogens is normal.

Strains of *B. subtilis* 168 which are lysogenic for SP02 and/or ϕ105 show marked depressions in the level of transformation and superinfection marker rescue, although they remain susceptible to ϕ29 and SP01 transfection and PBS1- and SP10-mediated transduction (305, 358, 361). Lysogenic cells also retain the ability to produce genetically recombined phage progeny. Recent studies (92, 359, 360) indicate that low transformation efficiency results from the induction of lytic phage replication when these lysogenic bacteria become competent. Presumably the ability of *B. subtilis* 168 (SP02) and 168 (ϕ105) to be transfected indicates that transfection, unlike transformation, does not require continued cell viability. The finding that prophages are induced in the competent state leads to the question of whether the defective phages such as PBSX and PBSY might be induced under such conditions. However, Garro and Law (92) found no evidence of increased production of PBSX particles in competent *B. subtilis* 168 cultures. The possibility remains, nevertheless, that other strains of *B. subtilis* which do not undergo transformation may induce their respective defective prophages under competence conditions. Perhaps the ability to undergo transfection should be more commonly used as the criterion for detecting the ability to take up exogenous DNA.

One other experimental observation indicates that a step common to transformation and transfection of those phages showing a multidose response may involve recombination and/or DNA synthesis. Wilson and Bott (352) showed that nalidixic acid affects both transformation and transfection by SP01 DNA at some step subsequent to attainment of DNase resistance. Since a number of lines of evidence suggest that nalidixic acid may act upon the mem-

brane-DNA replication complex (among other effects), it is possible that this site is involved in the reactivation of transfecting DNA molecules. As noted earlier, other evidence suggests that some of the endonucleases involved in damaging transfecting DNA may also be associated with the cell membrane (14).

Use of transfection to analyze phage development. Klotz (157) has shown that transfection combined with marker rescue is a useful method of gene titration which allows one to determine the relative proportions of various genetic markers during phage DNA synthesis, and he has used this technique in studies of the replication of the SPP1 chromosome. In these experiments, competent *B. subtilis* cells were allowed to incorporate SPP1 DNA, the cells were collected at various times in the latent period, and the DNA was isolated. This DNA was then used to transfect a population of competent bacteria which had been pre-infected with appropriate temperature-sensitive mutant helper phages. The number of wild-type recombinants for a given marker would then depend on the number of copies of the gene present at the time DNA was re-isolated from transfected cells.

Using this technique, it was found that SPP1 DNA replication is unidirectional, originating from a specific point on one end of the recombination map. It was also found that shearing the SPP1 genome into halves prior to transfection permitted only one round of replication of that half molecule which carries the origin. Marker rescue with DNA fragments resulting from hydrodynamic shear has also been used in making correlations between physical and genetic map distances in certain deletion mutants of SPP1 (158).

Another interesting use of transfection has been in studies of gene conversion. It will be recalled that the two strands of denatured SPP1 DNA can be separated on CsCl. Spatz and Trautner (296) isolated heavy and light strands obtained from wild-type and various mutant SPP1 phages and created heteroduplexes containing one strand of normal DNA and one strand of mutant DNA by mixing and reannealing various combinations of separated strands. Such heteroduplexes would have matched base pairs except at the locus of the mutation. These hybrid DNAs were then used in transfection, and the frequency of cells yielding mutants and wild-type, only wild-type and only mutant progeny were determined by analysis of single-cell bursts. Their data show that the majority of the transfected cells release but one kind of progeny, which implies there is some mechanism in *B. subtilis* which converts heterozygous DNA molecules to the homozygous condition before or during replication. These results might be interpreted to mean that one strand of DNA is destroyed during or after entry except that the frequencies of cells yielding only wild-type or only mutant progeny were unequal and the results were both strand and marker dependent (296, 334). Other lines of evidence indicate that gene conversion in transfection is not a product of nonspecific repair synthesis, but involves specific recognition of the heteroduplex region of the DNA molecule (278).

Finally, it should be noted that while many denatured phage DNAs demonstrate a bimodal density profile on CsCl gradients, it is not always clear whether the two fractions consist of complete, complementary, single-stranded halves of the original viral genomes, or, alternatively, are made up of fragments of single-stranded DNA with different buoyant densities. It will be recalled, however, that only intact double-stranded DNA can be used in transfection of competent *B. subtilis* cells. Thus, the finding that the heavy and light fractions of SPP1 can be reassociated to give transfecting DNA suggests that in this case, the two classes contained considerable amounts of intact strands which reformed complete double-stranded viral genomes. The transfection assay may be useful in other instances where it is not certain what is included in the heavy or light fractions.

SYNTHESIS OF MACROMOLECULES DURING THE DEVELOPMENT OF B. SUBTILIS PHAGES

Biochemical studies of the development of *B. subtilis* phages have concentrated on DNA replication and transcription; few investigations have been made of translation and nothing is known about assembly. Thus, most of the following discussion will deal with the synthesis of nucleic acids especially in HMU-containing phages and in ϕ29. Although details of macromolecular syntheses differ in each major group of phages, there are a few generalities. First, the infective process apparently does not bring about the dramatic immediate changes in cellular metabolism noted in many coliphage infections. For example, an efficient mechanism for superinfection exclusion is not set up after infection; both related and unrelated phages can adsorb and, as noted earlier, be expressed in some instances even as late as 15 min after addition of the first phage. Lysis from without has not been reported. Secondly, host-directed biochemical functions are not immediately suppressed, and while bacterial and viral replication are usually mutually exclusive, the shut-

off of host RNA and protein synthesis is slow compared to that in phage-infected *E. coli* (154); indeed, some species of bacterial RNA are often synthesized until lysis (88, 160, 171, 225, 272). Thirdly, phage-related transcription in most cases so far investigated involves temporally changing populations of RNA with new species of transcripts appearing as the latent period progresses; in some phages the synthesis of early RNAs is repressed as the infection progresses. Lastly, as in many other phages, temporal control over transcription is correlated with viral DNA replication; however, there is also some evidence that late proteins can be synthesized in the absence of phage DNA synthesis.

Development of HMU-Containing Phages

DNA replication. (i) Nucleotide metabolism. Production of viral DNA in cells infected with HMU-containing phages is dependent on the prior synthesis of the nucleotides of this unusual base, and the necessary enzymes for catalyzing appropriate reactions appear in the first minutes of the latent period. A new deoxycytidylate deaminase is detected within the first 5 to 10 min of the latent period of SP8 (191), 2C (225), ϕe (259), and presumably other phages of this group. This virus-related enzyme is unusual, compared to deoxycytidylate deaminases from other sources, in that it is not sensitive to feedback regulation by deoxythymidine nucleotides (206). The product of the deaminase-catalyzed reaction, deoxyuridylic acid, is converted directly to 5-hydroxymethyl-deoxyuridylic acid by a hydroxymethylase (2, 232, 259). The latter compound is then phosphorylated by 5-hydroxymethyldeoxyuridylic acid kinase to 5-hydroxymethyldeoxyuridine triphosphate, the presumed immediate precursor of HMU-containing DNA. The appearance of these new phage-directed enzymes after infection is accompanied by a decrease in the activity of the host thymidylate synthetase (the enzyme which converts deoxyuridylic acid to deoxythymidylic acid); the decreased activity may involve a protein inhibitor (259, 260).

Two phage-related enzymes involved in metabolizing thymine nucleotides, thymidine triphosphate nucleotidehydrolase (dTTPase; 257) and deoxythymidylate nucleotidehydrolase (dTMPase; 8) are also synthesized shortly after infection, but it is not clear whether these enzymes play an essential role in phage production (72, 232). Since host DNA replication stops 6 to 8 min after phage addition (123, 258), it was once thought that the two hydrolases might deplete the dTTP pool, thereby causing an inhibition in the synthesis of host DNA. However, it now appears that replication of the host genome is blocked by some other mechanism and the hydrolases may function to prevent incorporation of thymine into phage DNA. This conclusion is based on the following observations. When temperature-sensitive mutants of ϕe having a heat-labile dTTPase infect thymine auxotrophs of *B. subtilis* at 45 C, exogeneous thymine is incorporated. However, host DNA replication is blocked and the thymine is actually polymerized into phage DNA (189, 258). Although the phage DNA replication enzymes apparently cannot distinguish between thymine and HMU nucleotides, the native host enzymes can, as shown by the fact that HMU is not incorporated into the DNA of thymine-requiring auxotrophs of *B. subtilis* 168 (207). Curiously, ϕe phage which have as much as 20% of their HMU replaced by thymine are still viable (189).

(ii) DNA synthesis. The fate of viral DNA and its mode of replication in infections of HMU-containing phages have been studied primarily in SP01. As noted earlier, the genome of this phage is a linear molecule of 110×10^6 to 130×10^6 molecular weight, injection is unidirectional, requiring 2 to 4 min (depending on the temperature), and the DNA-encoding early functions enter the cell first (193). The fate of the viral DNA after penetration has been investigated (172, 174) by reisolating DNA from infected cells and determining its ability to transfect other cells. An eclipse in transfecting activity, correlated with the appearance of single-strand breaks in the DNA, was found a few minutes after infection. DNA extracted at later times, after viral DNA replication, was repaired to some extent and had regained transfecting activity (173).

SP01 DNA synthesis begins at the end of the first quarter of the latent period—i.e., at 9 min in a latent period of 33 min. Shortly after the onset of viral replication, the viral genome can be found in a complex with RNA and protein called "VF" (174). This intermediate contains more than one phage equivalent of DNA and sediments 4 to 6 times faster than mature SP01 DNA. Presumably, VF is a membrane-bound intermediate, although it may not be of the type detected in coliphage infections (73), in SP82G-infected *B. subtilis* (161) and in other *B. subtilis* phage infections (160). About 10 min after SP01 DNA replication begins, a new intermediate called "F" appears, which consists of concatomers of many phage equivalents. Later some free mature viral DNA can be detected

and infectious particles can be isolated from the cell.

Several lines of evidence, in addition to temporal considerations, suggest that VF is a true intermediate in the synthesis of F. Pulse-chase experiments are consistent with this sequence, treatment of VF with pronase changes its sedimentation properties to those of F and exposure of cells to chloramphenicol after the appearance of VF prevents conversion to F. In vivo the conversion of VF to F is rapid and requires at least one late viral protein. Suppressible mutants of SP01 which are blocked in this conversion have been isolated (174). The production of mature DNA from F probably requires the action of a phage coded nuclease and repair enzymes; numerous single-strand breaks can be detected in the viral DNA at all stages prior to packaging into the infectious particles. One point of interest in this sequence is that maturation of SP01 DNA does not seem to be coupled to head assembly as in the coliphages. Mature SP01 DNA is produced via the above maturation sequence in normal amounts and at normal rates by a suppressible SP01 mutant (F-1) which cannot produce phage heads. Moreover, 50% of the mature SP01 DNA is not packaged at the end of the latent period in infections with wild-type SP01 (174); unpackaged DNA was also found concomitantly with free mature phage particles in infections with ϕ25 (176).

Yehle and Ganesan (366, 367) have examined DNA polymerase activity after SP01 infection of wild-type and a polymerase-deficient (pol I^-) mutant of *B. subtilis*. They found that the endogenous polymerase activity measured in extracts of wild-type cells declined to 10 to 15% of normal 6 to 9 min after addition of the phage, and then increased again as viral DNA replication was initiated. In the *pol* I^- host, there was little detectable polymerase activity early in the latent period and a large increase 10 to 12 min after infection. Thus, the DNA polymerase I activity which apparently declines in wild-type and is very low in the mutant is replaced by a new activity detectable by the same assay. The phage-related enzyme has now been purified 1,000-fold (367) and shown to consist of single polypeptide chain of approximately 122,000 molecular weight. In vitro, the SP01 DNA polymerase requires single-stranded DNA or bihelical DNA which is partially denatured. A variety of evidence suggests the phage enzyme is not derived from host polymerases I, II, or III, and is, therefore, likely encoded in the SP01 genome (367). In keeping with results obtained from studies of ϕe mutants with temperature-sensitive dTTPase (189), the SP01 DNA polymerase isolated by Yehle and Ganesan can use dTTP as a substrate in vitro.

Although host DNA polymerase I is apparently not required for SP01 phage development, there is evidence that the bacterial DNA polymerase III is necessary for replication of related phage ϕe. Thus, mutants of *B. subtilis* with temperature-sensitive lesions in the *pol* C gene which directs the synthesis of host polymerase III cannot be productively infected with ϕe at the nonpermissive temperature (167). The role of host polymerase II in either host or phage development has not yet been elucidated.

In many *B. subtilis* phage infections, bacterial DNA synthesis can be inhibited specifically by addition of 6-(*p*-hydroxyphenylazo)-2,4-dihydroxypyrimidine (HPUra) (38), which reversibly inhibits semiconservative DNA synthesis in gram-positive bacteria by blocking the action of DNA polymerase III (21, 187, 205); it has no effect on repair reactions (39). To date, HPUra has been used to investigate phage replication in *B. subtilis* infected with ϕe (166), SP02 (266), ϕ29 (142, 276), and PBS2 (234), and with the exception of ϕ105 (267), the development of all these viruses is resistant to HPUra. This result might reasonably be interpreted to mean that host polymerase III is not required for the development of most *B. subtilis* phages, and it appears to contradict the just-mentioned studies of Lavi and co-workers that indicate this enzyme is essential for replication of ϕe DNA, even though development of this phage is resistant to HPUra. The conflict between these findings remains to be resolved although it has been suggested that resistance to the drug, at least in the case of ϕe, may be associated with a phage-directed modification of DNA polymerase III which is responsible for a shift to exclusive synthesis of viral DNA and a cessation of host genome replication (166, 167).

DNA synthesis of HMU-containing phage 2C does not occur in a *B. subtilis* mutant unable to concentrate K^+ from the medium (348). Although the primary effect of this mutation is to inhibit protein synthesis, there is apparently an independent K^+ requirement for the synthesis of phage DNA. Interestingly, bacterial DNA synthesis was not shut off in 2C-infected cells when K^+ was absent, thus suggesting that the inhibition of this host function may require virus-induced protein synthesis.

(iii) Arrest of host DNA synthesis. As stated above, consideration was first given to the possibility that suppression of host DNA replication by HMU-containing phages was due to depletion of the dTTP pool after infection.

Two types of phage-directed proteins were thought to contribute to this depletion, an inhibitor of thymidylate synthetase (119), and hydrolases capable of degrading dTTP (257). This idea has been abandoned because suppression of host DNA replication occurs in cells infected with dTTPase-less phage mutants (258). A second hypothesis is that virus-related nucleases might degrade the host genome in a manner similar to that seen in coliphage infections. Increases in DNase activity capable of degrading host DNA have been observed after infection with SP3 (this enzyme is active against denatured DNA; 338) and SP10 (29) but the function of these DNases is not clear. In most *B. subtilis* phage infections and especially those with HMU-containing phages, the *B. subtilis* chromosome probably is not degraded at all or is degraded only late in infection (174). This conclusion is based on careful studies of transforming activities (366) and molecular weight determinations of host DNA isolated from infected cells (258). The techniques used were sensitive enough to detect any DNA breakdown except a few single-strand nicks (258). Moreover, additional experiments (166, 189) showed that arrest of host DNA synthesis is not caused by the production of repairable single-strand breaks in the host DNA, detachment of host DNA from the membrane or the presence of dHMU derivatives.

Physiological studies (166, 189) with intact and toluenized *B. subtilis* infected with wild-type and mutant ϕe suggest instead that a specific protein is produced after phage infection which represses host DNA replication. This conclusion is based on the following evidence. (i) Addition of chloramphenicol up to 13 min after infection prevents the shut-off of bacterial DNA synthesis. (ii) A mutant of ϕe has been isolated which does not block host DNA synthesis. (iii) A substance has been detected that leaks out of toluenized cells infected with a suppressible mutant of ϕe which has a defect in DNA replication; this substance inhibits DNA synthesis in toluenized uninfected cells. The experiments mentioned above, employing a K^+-deficient mutant of *B. subtilis* infected with HMU-containing phage 2C, are also compatible with the synthesis of a phage-related protein which inhibits host DNA synthesis (348). Moreover, addition of rifampin at different times early in the SP01 infection (89) indicates that synthesis of an RNA necessary for the shut-off of host replication occurs at approximately 4 min after infection.

RNA synthesis. (i) Program of transcription. Investigations of RNA synthesis in *B. subtilis* cells infected with HMU-containing phages has centered on SP01 and SP82G. The program of viral transcription in both (Fig. 11) involves six classes or populations of RNA which can be distinguished on the basis of their times of first appearance and cessation of synthesis (88, 89, 102, 299). Slight differences in the

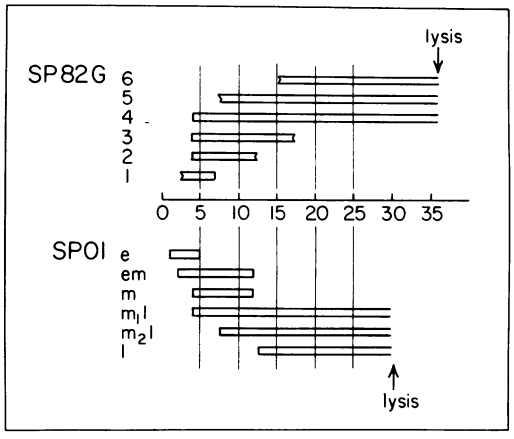

FIG. 11. *Comparison of transcription programs of SP82G and SP01. The classes of RNA produced during the infection by SP82G are described by Spiegelman and Whiteley (299). The transcription program for SP01 was taken from the data of Gage and Geiduschek (88) and the classes are labeled according to the nomenclature of these authors. The time of onset or cessation of synthesis is indicated by the position of the verticle line closing the bar; notched ends, placed at the most probable time of onset or cessation of synthesis, are used to show that the exact time is not known. Figure taken from Spiegelman and Whiteley (299).*

TABLE 6. *Strand specificity of RNAs synthesized during SP82G infection of B. subtilis*

^{3}H-labeled RNA from cells labeled at	H strand[a]		L strand[a]		Self annealing[c]
	counts/min	%[b]	counts/min	%[b]	
−2 to 3 MAI[d]	1,669	47	1,862	53	1.2
0 to 4 MAI	3,110	73	1,174	27	0.7
0 to 5 MAI	4,242	80	1,071	20	0.5
0 to 7 MAI	23,198	94	1,607	6	0.9
5 to 7 MAI	19,569	95	936	5	2.3
9 to 13 MAI	24,439	95	1,188	5	1.7
12 to 15 MAI	10,178	97	320	3	3.5
17 to 20 MAI	20,133	97	522	3	4.8
21 to 25 MAI	26,468	98	498	2	1.7

[a] H, Heavy; L, light.
[b] Percentage of total bound radioactivity.
[c] Ribonuclease-resistant radioactivity after hybridization in the absence of DNA.
[d] [^{3}H]uridine (1 μCi/ml) (36.4 Ci/mmol, New England Nuclear) was added at different intervals expressed as minutes after infection (MAI) with SP82G.

timing of the RNA classes are noted between SP01 and SP82G infections, but this might be attributable to the differences in the length of the respective latent periods (30 to 35 min for SP01 and 35 to 40 min for SP82G in the same medium at 37 C). Table 6 compares the extent of hybridization of SP82G RNAs synthesized at different intervals during infection to isolated heavy and light fractions of SP82G DNA prepared by CsCl density centrifugation of denatured phage DNA (168). The results indicate that sequences present in both heavy and light fractions are transcribed early in infection, whereas transcription of regions of the DNA separated in the heavy fraction predominates in the middle and latter part of the latent period. A similar pattern of hybridization to heavy and light fractions has been reported for SP01 RNAs (88). An analysis of RNA classes synthesized from strands of SP82G DNA which can be separated into heavy and light fractions (J. M. Lawrie, G. B. Spiegelman, and H. R. Whiteley, unpublished observations) is summarized in Fig. 12.

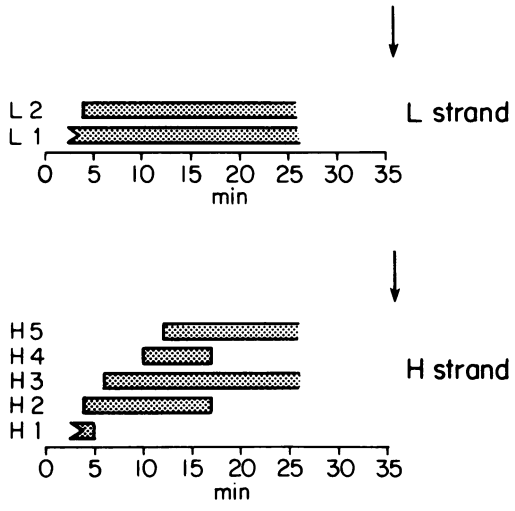

FIG. 12. *Time of synthesis of classes of RNA transcribed from the heavy (H) and light (L) strands of phage DNA during the infection of B. subtilis by SP82G. Classes were detected by competition-hybridization analysis as described earlier (299) with isolated H and L fractions of denatured DNA (168). Notched ends indicate that the time of onset of synthesis is not known; open ends indicate the latest interval of RNA synthesis analyzed in these experiments.*

Fujita et al. (84) have described a number of suppressible mutants of SP01 with alterations in the program of transcription. Mutants *sus*F-4 and *sus*F-14, located, respectively, in cistrons 33 and 34 (Fig. 10) which are proximal to genes controlling DNA replication (221), can synthesize viral DNA but fail to make the m_2l and l classes of RNA shown in Fig. 11. These two complementation groups are presumed to have regulatory functions. Suppressible mutant F-21 (cistron 28), which is located among genes involved in DNA synthesis, can transcribe only the *e* and *em* classes in a nonpermissive host, and phage-directed RNA synthesis essentially stops 10 min after infection. Finally, mutant F-30 in cistron 22 is unable to make the *l* class of RNAs (84, 88, 89) and is DNA negative (86, 221). Similar mutants have not yet been identified in SP82G.

Synthesis of the two early classes of RNA (*e*, *em* in SP01; 1, 2 in SP82G) is terminated as the infection progresses. In the presence of chloramphenicol, however, late transcripts are not made and production of early RNAs continues indefinitely (87, 88). Consequently, it has been postulated that transcription of the genes corresponding to the four late classes of RNA depends on protein synthesis, and that the synthesis of *e* and *em* RNAs is blocked by a phage-directed repressor at late times in the infection. A protein called "TF-1" has been purified from SP01-infected cells and this protein specifically inhibits transcription of HMU-containing DNA in vitro (144, 346, 350). However, the actual role of TF-1 in controlling RNA synthesis is not clear. It does not degrade the DNA template nor any component of the reaction, but, on the other hand, in vitro it binds to many sites on SP82G DNA and inhibits the initiation of all classes of RNA transcripts rather than just classes *e* and *em* (144, 291). It is possible, of course, that in vitro assays do not contain the same proportions of components that are present in vivo or that prior interaction of TF-1 with some other controlling element is required for greater specificity. A protein similar to TF-1 has been isolated from SP82G-infected cells (J. M. Lawrie and H. R. Whiteley, unpublished observations) and it also completely inhibits in vitro synthesis by host polymerase and by the SP82G-modified polymerase described below. An additional, as yet unidentified, mechanism(s) must exist in SP01 and SP82G-infected cells for the shut-off of one of the middle classes of RNA (*m* and class 3, respectively).

There is conflicting evidence concerning the question of whether DNA replication is a prerequisite for the synthesis of late RNA and certain proteins. Pene and Marmur (226) reported that a late protein, lysozyme, does not appear in 2C-infected cells which were treated with mitomycin C, and they interpreted this observation as indicating that DNA replication was required for the transcription of late functions as in coliphages (28, 44). Moreover, as

already noted, infection of *B. subtilis* with DNA-negative mutant F-30 of SP01 (88, 89) yields all classes of SP01 RNA except the *l* class. However, investigations of SP82G by Stewart and co-workers (304) indicate that late proteins (lysozyme- and serum-blocking power) are produced when *B. subtilis* is infected with a double mutant having defects in each of two cistrons required for DNA replication, and this observation suggests that late transcription is not directly linked to DNA synthesis. Evidence is also presented that the apparent contradiction between the studies with 2C and those with SP82G may result from a heretofore unrecognized effect of mitomycin C on transcription of viral DNA. Stewart et al. (304, 306) then further propose that some change in the physical state of SP82G DNA is required for both replication and synthesis of late RNAs as proposed for T_4-infected *E. coli* (40, 250, 293), and that the mutants which they studied may be blocked in some step of DNA replication unrelated to or subsequent to this alteration. On the other hand, the F30 mutant of SP01 which is both DNA negative and unable to transcribe the *l* class of late RNAs may be defective in a function prior to this change. Replication-uncoupled late gene function has been reported for T_4 mutants which lack DNA polymerase (22), as well as for mutants which permit the accumulation of single-strand breaks in parental phage DNA when replication is blocked (49, 250). The synthesis of late proteins independently of phage replication has also been demonstrated for coliphages T_5 (126, 229) and T_7 (reviewed by Studier [307]) and for *B. subtilis* phage ϕ29 (7, 122, 142).

(ii) Regulation of transcription. The chloramphenicol experiments cited above indicate that, as in coliphage infections (reviewed by Calendar [44] and Mathews [192]), transcription beyond the early genes requires phage-directed protein synthesis. A great many investigations have been made concerning the role of regulatory proteins in the synthesis of RNA during infection of *E. coli* by its phages, especially by T_4 (reviewed by Bautz [19]; and Chamberlin [50]) and concerning the structure of RNA polymerase (reviewed by Burgess [42], by Losick [178], and by Chamberlin [50]). A detailed discussion of transcription and the mechanisms regulating transcription is beyond the scope of this review but a brief statement of the current status of the problem will be made before discussing the results obtained to date with the *B. subtilis* phages.

Several nonexclusive mechanisms have been proposed to account for the changes in transcription which occur in coliphage infections: (i) that a new or modified RNA polymerase is produced after infection and that this enzyme is capable of recognizing new initiation sites on the phage genome; (ii) that an antiterminator protein is synthesized (279) which prevents *rho*-mediated termination (253), thus allowing transcription of new cistrons by a read-through mechanism; and (iii) that some classes of RNA subsequent to early classes are synthesized by read-through without the participations of any phage-directed proteins (25).

Since the sigma subunit of *E. coli* polymerse functions in initiation (337) and also provides specificity (309), Travers (335, 336) first proposed that a new or modified sigma subunit may be synthesized during T_4 infection, thereby altering the specificity of the enzyme. However, conclusive evidence supporting this idea has not been obtained, although it was found that the enzyme undergoes substantial modification after infection. The enzyme extracted from T_4-infected cells lacks the sigma subunit (20, 344) and has relatively low activity in vitro. In addition, the α subunit (98, 99, 283) and perhaps the $\beta'\beta$ subunits (283) are modified and the enzyme contains small polypeptides synthesized after infection (134, 239, 302, 303).

Production of a completely new polymerase has been thoroughly documented for *E. coli* infected with T_7 (51); a new enzyme is also produced in infections with T_3 (188). In these infections, the host enzyme transcribes early genes, one of which codes for the new polymerase; the latter then transcribes the remainder of the genome. The new polymerase consists of a single type of subunit and is resistant to rifampin, an inhibitor specific for the β' subunit of the host enzyme.

Anti-*rho* proteins have not been isolated but the read-through mechanism receives support from studies of the effect of *rho* on the in vitro synthesis of T_4-specified enzymes by a coupled transcription-translation system. However, this mechanism cannot account for the synthesis of RNAs coding for some quasi-late T_4 enzymes (56, 143, 212), thus implicating the participation of some other kind of a phage-coded control element. It should be recalled also that synthesis of late RNA classes usually depends on concomitant DNA synthesis.

Much less is known about the mechanisms regulating transcription in phage-infected *B. subtilis*. As in coliphage infections, RNA polymerase isolated from uninfected host cells transcribes only early phage genes in vitro (94, 124, 299). Geiduschek and Sklar (95) demonstrated that at least one of the subunits of the host polymerase (the β' subunit) functions throughout SP01 phage infection. They found that addi-

tion of rifampin, which directly affects β', at any time during the latent period inhibited RNA synthesis and that if rifampin-resistant mutants of *B. subtilis* were used as the host cells, then phage-specific RNA synthesis was also resistant to rifampin. Similar observations have been made in β22 (125).

More recently, RNA polymerase has been isolated from SP82G-infected *B. subtilis* (298, 299), from SP01-infected *B. subtilis* (69, 81), and from ϕ29-, β22-, and SP02c$_1$-infected *Bacilli* (132; H. R. Whiteley, H. E. Hemphill and G. B. Spiegelman, unpublished observations). A comparison of polymerases from uninfected and SP82G-infected cells has shown that the enzymes differ in subunit composition and in the classes of RNA transcribed from SP82G DNA in vitro; no significant differences were found in sedimentation constants, sensitivity to rifampin or streptolydigin, or in template specificities. In addition to the core and σ subunits (16, 17), the host enzyme contains small molecular weight polypeptides (69, 228; G. B. Spiegleman and H. R. Whiteley, unpublished observations). The RNA polymerase purified from SP82G-infected *B. subtilis* contains the core subunits ($\beta'\beta$ and α) but is lacking or has a greatly decreased quantity of the σ subunit. It also contains at least six small polypeptides having molecular weights ranging from 12,000 to 32,000. Three of these small polypeptides were found in association with the core subunits by the method of enzyme purification reported earlier (298); three additional polypeptides were detected when the enzyme was analyzed by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels (G. B. Spiegelman and H. R. Whiteley, unpublished observations). Two forms of the polymerase from SP82G-infected cells, having different complements of small polypeptides, can be separated by chromatography on DNA cellulose. The subunit composition of these forms is similar to that reported for two forms of the SP01-modified polymerase (228). It is not known if all of the small polypeptides contribute to the transcriptional specificity of the modified polymerases. No changes have been found in the electrophoretic properties of the $\beta'\beta$ and α subunits. If chloramphenicol is added to *B. subtilis* cells together with SP82G no modification of the host enzyme is found (G. B. Spiegelman and H. R. Whiteley, unpublished observations).

Analysis of the classes of RNA produced in vitro by polymerases isolated from uninfected and SP82G-infected *B. subtilis* (299), showed that as expected the host enzyme produced only early RNA species. Competition-hybridization experiments with RNA synthesized in vitro by the enzyme from phage-infected bacteria, however, disclosed the presence of both early and middle classes of RNA. RNAs synthesized in vitro were also examined by hybridization to isolated heavy and light fractions of SP82G DNA. The results of these studies (168) showed that RNA produced in vitro by the host polymerase resembled the transcripts made early in infection and those made in the presence of chloramphenicol. In contrast, RNA produced by the SP82G enzyme resembled that found in the middle and late stages of infection.

Experiments with RNA polymerase isolated from SP01-infected *B. subtilis* (68, 69, 81; G. B. Spiegelman and H. R. Whiteley, unpublished observations) showed that this enzyme contained several small polypeptides and a decreased content of the σ subunit. Competition-hybridization experiments (69, 228) demonstrated that the modified enzyme is capable of producing asymmetric middle classes of RNA in vitro. The enzyme isolated by Duffy and Geiduschek (69) contained core subunits, two phage-coded polypeptides (molecular weights of 28,000 and 13,000), and two components (11,000 and 9,500) which were also found in polymerase isolated from uninfected cells. Recent studies (69a) indicate the transcriptional specificity of the modified enzyme involves the 28,000-molecular-weight polypeptide. Similar asymmetric transcription of middle classes in vitro was reported by Pero et al. (228) for polymerase from SP01-infected cells (isolated by a different procedure) containing core subunits, three phage-induced polypeptides (26,000, 24,000, and 13,500), and two host polypeptides (21,500 and 11,000 molecular weight). Evidence was presented that the 21,500-molecular-weight polypeptide plays a role in determining the asymmetry of transcription.

A slightly different complement of small polypeptides associated with core was found when extracts of SP01-infected cells were treated with antibodies to host core subunits. This method (81) showed five polypeptides (molecular weights of 85,000, 40,000, 28,000, 25,000, and 23,000) in association with the core. This same technique was also used to examine the subunit composition of polymerase synthesized by the *sus* mutants described earlier (84). Fox and Pero (81) found that polymerase isolated from cells infected with *sus* F21, the mutant which is blocked early in transcription, lacks the 85,000-, 25,000-, and 23,000-molecular-weight polypeptides; polymerase purified from nonpermissive bacteria infected with *sus* F4 and *sus* F14 mutants, which are blocked late in transcription, lack the 23,000-molecular-weight polypeptide. These interesting results suggest

specific functions for each of the small polypeptides.

(iii) Synthesis of host RNA. As already noted, infection with HMU-containing phages brings about a decline but not a complete cessation of host RNA synthesis (89, 225). Although there have not been many studies of the mechanisms responsible for decreased transcription of the host genome in SP01-infected *B. subtilis*, the problem has been examined in β22 and 2C infections (124, 225, 226). Any mechanism which would result in degradation of the host DNA could affect host transcription but, as discussed earlier, there is no evidence that such degradation occurs early in infection. Another possible mechanism would be synthesis of a specific protein capable of interfering with host transcription. In *E. coli* apparently two mechanisms operate to shut off host RNA synthesis, a multiplicity-dependent process which does not involve protein synthesis, and another which requires protein synthesis (155, 209, 211). Extensive studies have not been made in phage-infected *B. subtilis* but Pene (225) reported that host messenger RNA (mRNA) synthesis is shut off in 2C-infected cells in the presence of chloramphenicol.

A particularly interesting aspect of infections with HMU-containing phages is that there is an apparent differential effect on messenger versus ribosomal RNA synthesis. RNA-DNA hybridization experiments indicate there is a reasonably rapid inhibition of the former in infections with SP01 (89, 102), SP82G (161) and 2C (225, 226, 348), but studies of the incorporation of labeled uridine demonstrate that host ribosomal RNA continues to be made through most, if not all, of the latent period. In addition, synthesis of host proteins including polymerase core subunits (H. R. Whiteley et al., unpublished observations) continues, albeit at a diminished rate, throughout at least the first half of the SP82G latent period. Possibly, the phage-modified polymerase is able to transcribe stable RNA cistrons from the host DNA. It appears more likely, however, that the production of these RNAs may be ascribed to a small amount of host polymerase (present initially or subsequently synthesized) which escapes modification after phage infection.

Protein synthesis. (i) In vivo synthesis. Investigations of protein synthesis in SP01-infected cells were first made by D. A. Shub (Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1966) and by Levinthal and co-workers (171) with the aim of correlating the time of RNA synthesis and the appearance of phage proteins. Gel electrophoresis patterns of pulse-labeled proteins isolated from SP01-infected cells demonstrated that phage proteins, like RNAs, can be separated into three general groups: populations whose synthesis starts immediately after infection and stops a short time later, a group whose production starts several minutes into the latent period and stops just before DNA replication begins, and proteins which appear late and are made until lysis (171). Measurements of the rate of incorporation of labeled amino acids into various SP01 proteins after treatment with actinomycin D indicated that the half-lives of phage mRNA's were about the same as those of *B. subtilis* mRNA's. Very little is known about the protein-synthesizing machinery involved in the production of the phage proteins except that host lysyl- and tryptophanyl-transfer RNA synthetases are required for the production of several HMU-containing phages (236).

Relatively few specific proteins have been investigated for their time of appearance during infection by HMU-containing viruses. Enzymes required for the synthesis of phage DNA precursors are, of course, produced prior to DNA replication. dTTPase and dUMP hydroxymethylase appear at about 5 to 7 min and 10 min, respectively, after infection with φe (71, 257); dCMP deaminase is also detected at about 10 min in cells infected with SP01 (D. A. Shub, Ph.D thesis, Massachusetts Institute of Technology, Cambridge, 1966; [349]), SP8 (206) and 2C (2, 226). The modified RNA polymerase produced in SP82G infections is also detected prior to 10 min (G. B. Spiegelman and H. R. Whiteley, unpublished observations); a similar early modification of the SP01 polymerase has been reported (81). In contrast, studies with SP01-infected cells show that DNA polymerase (366) and serum-blocking power (349) appear at approximately 15 min after infection and increase markedly starting at the 18th minute; a similar time course has been reported for lysozyme in cells infected with 2C (226) and SP82G (304).

(ii) In vitro synthesis. RNA isolated from SP01-infected cells can direct the production of SP01 proteins in vitro when incubated with an *E. coli* protein-synthesizing system (290). Such RNA preparations gain and lose the capacity to direct the synthesis of individual polypeptides, paralleling similar changes taking place in protein synthesis in vivo. The technique is sensitive enough to quantitate specific mRNA's and has been used to show that the mRNA for dCMP deaminase first appears 5 min after infection, reaches a maximum at 10 min and then declines. These results have been interpreted to mean the dCMP deaminase mRNA is produced in the middle of the infection, perhaps as part of the *m* class of transcripts (Fig. 11). Experi-

ments with rifampin support this conclusion and indicate that new initiations, rather than read-through, may be involved in the synthesis of this mRNA in vivo.

The translation system has also been coupled to a transcription system with *E. coli* RNA polymerase, and this permits a rigorous examination of the fidelity of RNA synthesis in vitro. Such experiments (290, 291) confirm results obtained earlier—i.e., that *E. coli* RNA polymerase makes predominantly early RNA from SP01 DNA (94). However, small amounts of dCMP deaminase are produced in the coupled system with DNA from SP82G (280) or SP01 (290); it has been suggested that there may be some read-through of middle classes of RNA in vitro. RNA synthesized in vitro by *B. subtilis* polymerase or by the SP82G-modified enzyme can also be translated by an *E. coli* protein-synthesizing system. Such experiments show that the dCMP deaminase gene is transcribed by the modified enzyme, but that very little or no dCMP deaminase RNA is produced from SP82G DNA by the unmodified host polymerase (W. Hiatt and H. R. Whiteley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S123, p. 234).

Development of φ29

φ29 infection has little effect on host protein, RNA, or DNA syntheses which continue until the end of the latent period (272, 275), thereby complicating the analysis of φ29-related syntheses. Bacterial functions can, however, be reduced by irradiating host cells with UV prior to infection or by performing the infection at higher temperature; host DNA synthesis can also be specifically inhibited by arylhydrazineopyrimidines (HPUra). These methods, together with nucleic acid hybridization and gel electrophoresis, have enabled investigators to take advantage of the potential of this small but morphologically complex phage.

DNA replication. The incorporation of [^{3}H]thymidine in the presence of HPUra has been used to follow the time course of φ29 replication in *B. subtilis* (276) and in *B. amyloliquefaciens* (142). φ29 DNA synthesis begins at approximately 10 to 12 min after infection, increases until about 30 to 35 min and then levels off until lysis (40 min after infection). As in other *B. subtilis* phage infections (160, 161, 174), viral replication involves a rapidly sedimenting component containing host DNA, phospholipid, protein, and some pulse-labeled RNA (142). Experiments with φ29 having ^{32}P-labeled DNA show that the parental genome remains free in the cytoplasm for approximately 10 min and then forms a complex with the membrane. Newly synthesized φ29 DNA is also first detected in this membrane-bound complex; then it is found as free DNA and finally appears in mature phage. A significant amount of the progeny DNA remains with the complex even at late times, sugesting that there are a considerable number of phage DNA binding sites on the membrane. Replicating φ29 DNA does not appear to be in large concatamers as in replicative intermediates detected in other phage infections (142, 276).

Temperature-sensitive and suppressible mutants in cistron I of McGuire and co-workers (195) lack the ability to attach the φ29 chromosome to the host membrane under nonpermissive conditions (142), suggesting the product of this gene, which is a 67,000-molecular-weight protein, may be required to form the viral DNA-membrane complex. Involvement of a φ29-directed function is also indicated by the finding that actinomycin and rifampin prevent association of infecting DNA with the membrane if added at 5 min but not if added 10 min after infection; chloramphenicol and puromycin also inhibit formation of the complex (142). Interestingly, *ts* mutants lacking the cistron I activity can transcribe the entire φ29 genome at the restrictive temperature, indicating that neither the association of the DNA with membrane nor viral genome replication is a necessary prerequisite for late transcription.

RNA synthesis. Competition-hybridization experiments indicate there are two temporally separable classes of φ29 RNA: early RNAs (representing approximately 40% of the genome) which are produced before the onset of φ29 DNA replication, and late RNAs (representing the remaining 60% of the genome) whose synthesis begins at approximately the same time as viral DNA replication (181, 182, 272, 276). Temperature shift-up experiments with *ts* mutants also suggest two major populations of viral transcripts (276, 317, 318), although some evidence indicates that the product of one cistron required for DNA replication appears in the middle of the latent period (274).

The work of Schachtele et al. (272, 273) and Loskutoff et al. (181, 182) has disclosed several interesting points about the program of transcription. First, very little phage-specific RNA is made until approximately 6 to 8 min after infection, and the RNA made before the onset of viral DNA replication (early RNA) is transcribed only from the light strand of DNA (200, 272, 273). Secondly, at least some light strand transcripts identical to those synthesized early are made throughout the latent period, and early RNA molecules may also accumulate because of the slow turnover of φ29-specific RNA

(182). Finally, a new class of mRNAs made exclusively from the heavy strand of ϕ29 DNA is detected at approximately the same time as phage DNA replication is initiated, although this late transcription is apparently not dependent upon DNA synthesis (182, 273).

Loskutoff et al. (181, 182) have used gel electrophoresis and sedimentation in sucrose gradients to analyze the size distribution of RNA molecules synthesized at early and late times during ϕ29 infection. Early RNA was found to consist of six components having the following molecular weights: 750,000, 440,000, 370,000, 250,000, 90,000, and 40,000. These six molecules accounted for 35% of the coding capacity of the ϕ29 genome, a value which agrees quite well with the estimates of transcription derived from hybridization experiments. All but the 440,000-molecular-weight transcript are produced in the presence of chloramphenicol; late RNAs are not made under these conditions. Gel electrophoresis indicates that synthesis of the 750,000-molecular-weight component stops at the same time as viral DNA replication begins but is apparently independent of replication since cessation was also found in a *ts* mutant unable to replicate DNA. On the other hand, the synthesis of the remaining early components is apparently not repressed at late times in the infection, and in addition to the continued production of these five RNA molecules, three new transcripts (molecular weights of 1,750,000, 930,000, and 70,000) are made after DNA replication is initiated. Loskutoff and Pene (181) suggest that the two very large late RNAs could be multi-cistronic mRNA's coding for the seven structural proteins of the phage.

The mechanisms controlling transcription are not known. In vitro transcription of ϕ29 DNA by host polymerase yields only early RNA (H. R. Whiteley, unpublished observations). Studies of rifampin sensitivity during the lytic cycle indicate that at least the β' subunit of the host enzyme is required for both early and late functions. Since late RNAs and one of the early RNA components cannot be made in the presence of chloramphenicol, it seems likely that a phage-directed protein is required for late transcription, some or all of which is from the heavy strand (182, 273). Investigations of RNA polymerase isolated from uninfected and ϕ29-infected *B. amyloliquefaciens* (132) showed that approximately 10 to 15% of the host enzyme is modified within 10 min after infection. The modified enzyme differed from the host polymerase with regard to template specificities, sedimentation constants, and subunit composition. As in the SP82G- and SP01-modified enzymes, the ϕ29-modified polymerase had a decreased content of the sigma subunit and contained an additional polypeptide (molecular weight of 30,000) which copurified with the enzyme. The classes of RNA produced by this polymerase from ϕ29 DNA in vitro have not been investigated.

Protein synthesis. Approximately 60% of the genetic information of ϕ29 is apparently used to code for the six to seven structural proteins of the virion, and most of these polypeptides are synthesized after, or concomitant with, phage DNA replication (3, 47, 122, 196). The numbers of the nonstructural proteins have not been established with certainty, and little is known about their functions.

Studies of phage-directed protein synthesis have been carried out by infecting UV-irradiated *B. subtilis* with wild-type or mutant ϕ29 in the presence of radioactive amino acids, and subsequently separating the isolated proteins on polyacrylamide gels. Using this technique Carrascosa et al. (47) detected 12 nonstructural proteins in addition to seven structural components; Pene and co-workers (227) found seven structural proteins and as many as 10 early and four late nonstructural polypeptides. Moreover, this latter group found that synthesis of six of the early nonstructural proteins ceased late in infection, and they suggest these proteins may be related to the early 750,000-molecular-weight RNA transcript which is shut off at the time DNA synthesis is initiated. A total of 23 viral proteins were found by Hawley et al. (122), although some of these may be precursors of other polypeptides found in the same gels.

The finding that some suppressor-sensitive ϕ29 mutants cannot produce two or more polypeptides under nonpermissive conditions has led to the suggestion that at least some ϕ29 proteins are made by cleavage of higher-molecular-weight precursor proteins (7). Carrascosa and co-workers (48) have shown by comparison of tryptic peptide digests that the neck appendage protein (molecular weight 80,000) is apparently processed from a precursor with a molecular weight of about 90,000.

Nucleic Acid Metabolism in Other B. subtilis Phage Infections

No other *Bacillus* phages have been as extensively studied at the molecular level as have the HMU-containing phages and ϕ29. However, preliminary investigations have been made of several other phages, and in a few instances either the findings or the viruses themselves are unique.

PBS1 and PBS2. Evidence that SP82G, ϕe, and SP01 direct the production of a number of enzymes specifically involved in replicating

HMU-containing DNA is bound to elicit interest in how phages containing uracil instead of thymine carry out DNA synthesis. Studies with transducing phage PBS1 and its clear-plaque derivative, PBS2, indicate that the pattern is similar to that observed in other phages having base substitutions. PBS2-induced dUMP kinase and dTMP phosphatase (F. M. Kahan, Fed. Proc. 22:406, 1963) as well as deoxycytidine triphosphate (dCTP) deaminase (328) have been reported. The properties of the dTMPase have been investigated in some detail (233); it hydrolyzes many deoxyribonucleotides including dUMP but is most active against dTMP. Price and Fogt (233) suggest that the enzymes induced by PBS2 infection would tend to exclude thymine from PBS2 DNA; the dUMP kinase and dTMPase activities, respectively, would remove the substrate and end products of the reaction catalyzed by dTMP synthetase. In addition a dTTPase may also be induced after infection (232).

It is likely that PBS2 directs the synthesis of a new DNA replication enzyme(s) during the latent period, as the specific activity of DNA polymerase increases 5- to 10-fold after PBS1 infection of wild-type cells and 50- to 100-fold in infections of a mutant of *B. subtilis* deficient in DNA polymerase I (231). The presumed new enzyme utilizes deoxyuridine triphosphate dUTP or dTTP equally well, while the host polymerase uses dUTP only 60% as efficiently as dTTP. The incorporating activity has not yet been purified sufficiently to determine its properties and template specificities. HPUra, which as already noted, blocks *B. subtilis* DNA replication, does not inhibit PBS2 DNA synthesis (234).

Investigations of RNA synthesis in PBS2-infected cells led to the intriguing discovery (235, 247) that addition of rifampin does not block RNA synthesis and that PBS2 can develop in cells which have been pretreated with this antibiotic. One possible explanation for the latter observation is that PBS2, like *Pseudomonas* phage PM2 (59), carries its own complement of RNA polymerase and this enzyme is resistant to rifampin. Clark et al. (55) have isolated the RNA polymerase from PBS2-infected *B. subtilis*; the enzyme is rifampin resistant, has a higher activity with PBS2 DNA than with other templates, and has a completely different subunit composition. The PBS2 polymerase consists of approximately equimolar amounts of four major polypeptides having molecular weights of 80,000, 76,000, 58,000, and 48,000, plus several other polypeptides in smaller amounts. The possibility that all or some of these polypeptides are present in the mature phage is currently under investigation.

It has recently been found that *B. subtilis* cells contain a DNase which specifically hydrolyzes uracil-containing DNA (329, 330); this endonuclease has no activity against thymine- or HMU-containing DNA. The normal role of the enzyme in *B. subtilis* is unknown, but its activity is blocked soon after PBS1 infection by a phage-induced inhibitor which binds specifically to the DNase molecules.

SPP1. The ease with which denatured SPP1 DNA can be separated into heavy and light fractions on CsCl density gradients makes this phage potentially useful in determining how DNA is replicated and whether there are changes in strand specificity during transcription. Polsinelli et al. (230) have used very short periods of pulse labeling (2 to 15 s) of SPP1-infected cells to determine whether fragments of nascent DNA similar to those reported by Okazaki et al. (215) for replicating *E. coli* are produced in the SPP1 infection and whether they are complementary to one or both strands of SPP1 DNA. These studies indicated that the short nucleotide sequences synthesized during the pulse periods are complementary to both strands of SPP1 DNA without any asymmetry in the proportions of fragments binding to either strand. Thus, these results support the discontinuous models of DNA replication.

Pulse labeling of SPP1-infected cells (78) shows that phage-specific RNA synthesis increases rapidly in the first few minutes after infection, then remains constant until about 15 min after infection and finally decreases slightly; host synthesis is shut off very gradually (197). Transcription in vivo is predominantly from regions of the SPP1 genome which separate with the heavy fraction of denatured DNA on CsCl density gradients; only a short period of transcription from genes on the light fraction is detected (198, 249). The latter occurs at approximately the midpoint of the infection (20 min) when as much as 50% of the phage-specific RNA hybridizes to light DNA.

At the present time, there is no evidence for and, indeed, there may be no reason to invoke a modification of the host DNA-directed RNA polymerase to explain transcription of all SPP1-related RNAs. Conservation of at least the β' subunit of the host polymerase is indicated, because phage production is sensitive to rifampin and streptolydigin at all times during the latent period (197). Moreover, Milanesi and Melgara (198) have shown the RNA polymerase from uninfected *B. subtilis* is capable of transcribing the entire SPP1 genome in vitro. The transcription by the host enzyme is predominantly from regions of the genome associated

with the heavy fraction of denatured DNA, but as in vivo, a small amount of RNA is read from light-fraction DNA. These results do not exclude control mechanisms affecting SPP1 transcription, but suggest they may not be mediated by modification of RNA polymerase.

β22. Bacteriophage β22 is a large virus similar in size to HMU-containing phages but with no known base substitutions. Production of progeny β22 remains sensitive to rifampin throughout the eclipse period, which indicates that, as in most other *B. subtilis* phage infections, at least a portion of the host polymerase functions in transcription (125). Host nucleic acid syntheses are suppressed in β22 infection, but not immediately. Bacterial DNA replication ceases just as phage DNA synthesis is initiated and unlike the situation in HMU-phage infections, eventually both the transcription of host messenger and stable RNA species are suppressed.

Like the HMU-containing phages, β22 demonstrates a very complicated pattern of transcription with several classes of late RNA appearing as the infection progresses; synthesis of some early RNAs is turned off by the midpoint of the latent period (124). Production of late RNAs, some of which appear at the beginning of DNA replication, is inhibited by chloramphenicol. As in many other instances, RNA polymerase isolated from uninfected *B. subtilis* transcribes only early RNAs, suggesting that a modification of the RNA polymerase may be necessary for late transcription. Preliminary experiments with RNA polymerase isolated from β22-infected cells provide evidence for structural changes in the enzyme (H. R. Whiteley, H. E. Hemphill, and G. B. Spiegelman, unpublished observation). As noted earlier (section on major groups of *B. subtilis* phages), Yehle and Doi (365) reported that β22 can productively infect sporulating cultures of *B. subtilis*. Since changes have been reported in RNA polymerase isolated from late log-phase cells (133) and from sporulating cells (177), it would be of interest to determine what changes, if any, polymerase undergoes if cells are infected with β22 at these points of the growth curve.

SP02 and φ105. Studies of nucleic acid synthesis in SP02 infection have usually been done with SP02c$_1$, a clear-plaque mutant of this temperate phage, or on lysogenic cells induced with mitomycin C. As in the case of other *B. subtilis* phages, SP02c$_1$ infection does not have a dramatic effect on host metabolism and bacterial mRNA synthesis is only suppressed gradually, while ribosomal RNA apparently continues to be made until lysis (160). Early and late phage RNA classes have been detected (160), and preliminary studies of the subunit composition of RNA polymerase extracted from infected cells (H. R. Whiteley, unpublished observations) indicate the enzyme undergoes modification as a consequence of phage infection.

Studies of cells infected with SP02c$_1$ containing ^{32}P-labeled DNA indicate the viral genome becomes associated with the cytoplasmic membrane very early in the latent period and remains bound to the membrane until the time infectious particles first appear in the cell. Host DNA synthesis continues in the infected cell for about 10 min then ceases coincidentally with the beginning of viral DNA replication. It has also been found that the bacterial DNA becomes detached from the membrane after SP02c$_1$ infection and this could mean it is displaced from a membrane-associated replication complex which in turn would stop host DNA replication (160). Evidence has been presented that a new DNA polymerase is synthesized in cells infected with SP02 but not in cells infected with φ105 (266). The enzyme induced in SP02-infected cells is resistant to HPUra but can incorporate deoxynucleotide precursors in the presence of either *B. subtilis* or SP02 DNA in vitro.

Studies of the processes of lysogenization by SP02 and φ105 are not far advanced. As noted earlier, mutants in the presumed structural gene (*c* gene) for a repressor have been isolated. Chow and Davidson (54) have presented indirect evidence which suggests that insertion of prophage φ105 or SP02 DNA occurs by a process similar to the Campbell model (46). In their study, electron microscopy was used to visualize hybrids formed between denatured DNA from lysogens of SP02 or φ105 and DNA extracted from mature virions. In the case of SP02, such hybrids tend to form circles and this has been interpreted to mean the prophage is a circular permutation of the mature phage DNA. This, in turn, suggests the lysogenizing SP02 genome is injected linearly, circularized, and then undergoes reciprocal recombination with the host genome at a site on the phage DNA (the presumed *att* region) different from the point of circularization. By using deletion mutants of SP02, the hybridization-mapping technique has been extended to demonstrate that the *att* region is located at a site which is 61.2% from one end of the linear phage genome. In contrast, φ105 prophage is not circularly permuted and its *att* region is presumed to be very close to or to overlap the ends for the mature phage genome. As noted earlier, colinearity of marker order in φ105 vegetative phage and prophage is also indicated by genetic studies (9). Attempts to find sequence homology

between SP02 and the genome of *B. subtilis* have been unsuccessful (364).

Studies of the process of induction of ϕ105 and SP02 prophages have concentrated on the time of prophage excision. Although, as noted above, HPUra does not block SP02 DNA replication, it does induce SP02 and ϕ105 prophages in appropriate lysogenic cells (12). Using this chemical both to inhibit host replication enzymes and to induce the prophage, Arwert and Rutberg (12) have studied the process of induction of wild-type and DNA-negative mutants of SP02, and have concluded that the prophage can excise from the bacterial chromosome without viral DNA synthesis (13).

In contrast to the findings with SP02 and the results of other investigators with temperate *E. coli* phages, Armentrout and Rutberg (10), using a temperature-inducible mutant of ϕ105, found that excision of this prophage from the host genome does not occur early in induction. Rather the phage genome is replicated in situ through much of the latent period, with mature phage DNA first appearing 30 min after induction. Just how this occurs is not known, but it appears that induction leads to the formation of a new DNA replication initiation site at the prophage and this is followed by preferential replication of the phage genome along with a number of adjacent bacterial chromosome markers. This phage-induced bacterial DNA replication is bidirectional (264). The advantage of this extensive in situ DNA replication is hard to understand unless it is required for excision. It is presumably not required for ϕ105 replication, since clear-plaque mutants are thought to develop without integrating into the host genome. It should also be noted that ϕ105 replication, unlike that of SP02 or any other known *B. subtilis* phage, is sensitive to HPUra (267).

SUMMARY

It is apparent from the survey presented here that the various groups of *B. subtilis* phages, like the much better studied coliphages, differ in size and morphology and that some have unusual bases in their DNA. However, the genetic information of all *B. subtilis* phages described to date is contained in double-stranded DNA and, in almost all, ϕ29, ϕ105, and SP02 being possible exceptions, the DNA is linear and nonpermuted. There have not been any reports of single-stranded DNA, or RNA phages capable of infecting *B. subtilis*, although in any such negative findings it can be argued that the search has been inadequate. One can only speculate as to why the morphological and biochemical diversity of coliphages is apparently greater than that of the *Bacillaceae*. One factor, of course, may be that the complexity of the envelope of gram-negative bacteria provides a greater variety of phage receptor sites and permits the evolution of viruses adapted to different modes of infection. Also, the life cycle of RNA and filamentous DNA phages is apparently attuned to the presence of various sex factors in *E. coli*, and the latter are not known to exist in *Bacillus*. Possibly, the absence of *B. subtilis* phages containing single-stranded genomes is related to the presence of nucleases specific for single-stranded DNA which are known to occur in this species (76, 323).

The catalog of viruses capable of infecting *B. subtilis* encompasses the entire spectrum of virulence: lytic, temperate, pseudotemperate, and defective phages. Several of these phages have been used to study unique aspects of the metabolism of the host such as transformation, sporulation, and thermophily. In some instances, for example spore entrapment, these investigations have contributed toward an understanding of host processes and have also led to interesting observations pertaining to phage development. On the whole, however, the level of knowledge concerning almost all aspects of the biochemistry of phage infection in *B. subtilis* is very significantly less than that accumulated during the past 30 years of intensive investigation of the coliphages. Nevertheless, some comparisons can be made between *B. subtilis* phages and coliphages which, it is hoped, will point out the value of continued investigations of the former. These comparisons will focus mainly on the large (130×10^6 molecular weight DNA) HMU-containing *B. subtilis* phages (SP01, SP82G, and ϕe) and T_4, the large (130×10^6 molecular weight DNA) HMC-containing coliphage; some comparisons between the smaller double-stranded DNA viruses represented by the *B. subtilis* phage ϕ29 (11×10^6 molecular weight DNA) and T_7 (24×10^6 molecular weight DNA) can also be made.

One of the interesting differences between the T-even coliphages and the HMU-containing phages of *B. subtilis* is that superinfection exclusion does not occur in infections of the latter, and related or unrelated phages may sequentially infect *B. subtilis* although the yields of progeny are often reduced compared to single infections. It is likely that advantage will eventually be taken of the ease with which superinfection can be achieved in this bacterium to study such phenomena as the nature and number of viral DNA replication sites and factors limiting the maximum number of phage genomes which may be expressed in a single cell.

A second difference between coliphages and those of *B. subtilis* concerns the shut-off of host functions. In the latter, the synthesis of host macromolecules decreases gradually after infection with large phages, and host DNA is apparently not broken down. In T-even phage infections of *E. coli*, on the other hand, a phage-coded function mediates a fairly rapid shut-off of host synthesis; degradation of host DNA begins soon after infection. Infection of either *B. subtilis* or *E. coli* by small phages (ϕ29 and T_7, respectively) has little or no effect on the synthesis of host macromolecules. From the limited studies made to date, it seems reasonable to conclude that the DNAs of both coliphages and *B. subtilis* phages become bound to the membrane before they undergo replication. It may be noted that the enzymes participating in replication of the latter phages have received relatively little attention whereas replication of T_4 and T_7 have been investigated in considerable detail.

As in coliphage infections, the early genes of large *B. subtilis* phages are transcribed by the host RNA polymerase and transcription of middle and late genes requires modification of the polymerase. The modified enzyme retains the core subunits but not the sigma subunit of the host polymerase, and additional small polypeptides are co-purified with the core. A similar modification occurs in T_4 infection of *E. coli*. In contrast to the latter, however, the modified polymerase produced in at least two subtilis-phage infections (SP01 and SP82G) retains its specificity in vitro and produces classes of RNA which are different from the transcripts made by the host enzyme. Late RNA synthesis in both *B. subtilis* and *E. coli* infected with these large phages is temporally correlated with replication of the viral genome, and this may reflect some requisite change in DNA structure for both DNA synthesis and late transcription. The possibility that all or part of the PBS2 polymerase may be packaged in the phage head and injected with the phage suggests an unusual alternative mechanism for controlling the transcription of this extremely large phage.

The synthesis of at least six classes of RNA can be detected by competition-hybridization during the infection of *B. subtilis* by SP01 and SP82G. The ease of detecting these classes depends, at least in part, on the fact that the synthesis of some of the classes is shut off during the early and middle stages of transcription, possibly through the action of phage-encoded "transcription factors." Such factors have not been found in *E. coli* infected by T_4 and only three or four classes of T_4 RNA can be distinguished by competition-hybridization. The program of sequential transcription in coliphage is in turn reflected by the sequential synthesis of viral proteins. The complexity of the pattern of proteins synthesized after SP01 infection is similar to that observed during T_4 infection except that the processing of precursor proteins, as described for the latter, has not yet been documented for SP01 or SP82G proteins.

Synthesis of a new polymerase, as in T_7 infections, has not been found in *B. subtilis* infected with ϕ29, but has been reported, as noted earlier, for PBS2. There is some evidence suggesting modification of approximately 15% of the total host enzyme after infection with ϕ29; however, synthesis of early classes of RNA, presumably catalyzed by the host polymerase, continues throughout infection. Two very large RNAs are produced by transcription of late genes; and it has been proposed that these RNAs are multicistronic. Transcription of late genes does not require DNA synthesis (7, 122, 142). Processing of large ϕ29 proteins into smaller functional polypeptides has also been suggested. Two additional points concerning the synthesis of ϕ29 proteins deserve comment. Indirect evidence has been presented indicating that one or more cistrons code for positive regulatory proteins. The isolation of mutants having defects in these cistrons will have considerable value in studies of regulation of development of ϕ29. Secondly, a great number of small-molecular-weight ϕ29 proteins have been detected (7); studies of mutants having defects in the synthesis of these polypeptides will be of interest. Anderson and Reilly (7) have proposed that these polypeptides may regulate the synthesis of host macromolecules, a process which continues throughout ϕ29 infection, and this idea also merits investigation.

Phage assembly has not been investigated in *B. subtilis* infections although there are indications that some of the structures seen in mutants of T_4 are also produced in mutants of SP82G blocked in DNA synthesis. One very obvious difficulty which has prevented investigations of assembly and other stages of development of *B. subtilis* phages is the paucity of information concerning the genetics of most *B. subtilis* phages. For example, only approximately 20% of the genome of SP82G has been mapped and even less is known about the genetics of other large phages such as β22. One exception to this lack of genetic information is ϕ29, which is currently under investigation in at least three laboratories. It can be expected that the genetic map, the functions of phage-encoded proteins, information concerning the

control of ϕ29 development and, possibly, investigations on the assembly of this small but complex phage will be forthcoming in the near future. It will be of great interest to see what parallels can be drawn in the production of ϕ29 and T_7, both of which are small, double-stranded lytic DNA phages.

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