Morphogenesis of Bacteriophage $\phi 29$ of *Bacillus subtilis*: Mapping and Functional Analysis of the Head Fiber Gene

BERNARD E. REILLY,* RODGER A. NELSON, AND DWIGHT L. ANDERSON

Department of Microbiology and School of Dentistry, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 18 May 1977

A set of mutants of *Bacillus subtilis* bacteriophage $\phi 29$ unable to synthesize the head fiber protein has been identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Infectious phage are produced during restrictive infection. We have focused on mutant *sus8.5*(900) because the mutation is suppressible by both the *su*⁺³ and *su*⁺⁴⁴ hosts, and it can be mapped by three- and four-factor crosses. After restrictive infection with mutant *sus8.5*(900), a fragment about 70% of the size of the normal fiber is produced as well as particles that are fast-sedimenting in sucrose gradients relative to $\phi 29$ ⁺. These particles have the buoyant density of particles with the fibers removed and have the absolute plating efficiency of $\phi 29$ ⁺. Fiber protein is absent from prohead as well as virion. A second set of mutants produces fiber protein with a slightly altered electrophoretic mobility. This type of fiber protein is either present or absent on both prohead and virion. A third class of mutants, typified by 914, produces a "normal" fiber, but a major head protein of altered electrophoretic mobility. After infection by this mutant, the fiber is absent from both prohead and virion, and the biological and physical properties of the 914⁻ particle are similar to those of particles produced after infection of the *su*⁻ host by *sus8.5*(900). These observations suggest that the head fiber is not an essential component of the prohead or virion and that the assembly process is efficient in the absence of fiber protein. Three- and four-factor genetic crosses have established the order *sus8*(769)-8(914)-*sus8.5*(900)-*sus9*(756) and indicate that cistrons 8 and 8.5 code for the major head protein and head fiber protein, respectively.

The virion of bacteriophage $\phi 29$ has protein fibers attached at both ends of its prolate head (2, 28). Fiberless particles produced by dimethyl sulfoxide treatment retain their infectivity (27). Three polypeptides have been assigned to the mature viral head (18), and there is general agreement that the major head protein and fiber are the major head constituents (10, 12, 22). Point mutants in cistron 8 were unable to synthesize head protein or fiber during restrictive infection (5, 9, 15), and tryptic peptide analysis of these proteins indicated that they were distinct proteins (27). Pulse-chase experiments demonstrated that fiber was a major component of the viral prohead, a particle that may be the first intermediate on the $\phi 29$ assembly pathway (20).

We have isolated a series of mutants that produce fiber protein with altered electrophoretic mobility or do not synthesize the fiber protein. After infection of the nonpermissive host, certain of these mutants produce both proheads and infectious particles that lack fibers. The absolute plating efficiency of the fi-

berless particles is the same as wild-type phage. Another mutant produces an altered major head protein and an apparently normal fiber, but the fiber is excluded from both prohead and virion. Genetic studies suggest that these mutations map between the mutants *sus8*(769) and *sus9*(756) of cistrons 8 and 9.

MATERIALS AND METHODS

The $\phi 29$ cistrons defined by quantitative complementation have been numbered sequentially from left to right (1 to 17), according to their relative map position. A protein product of cistron *n* has been referred to as *pn*. For example, the product of cistron 11, the neck lower collar protein, is now named *p11* (16). Although we have focused on the head fiber protein as a structural component, we have found the phenotypes of some of our new mutants to be quite complicated. For this reason we have referred to these mutants collectively as the "900 series," and particular mutants have a trivial designation, e.g., mutant 912, that does not imply that the 912 phenotype necessarily reflects a point mutation.

Phage and bacteria. Phage $\phi 29$ ⁺ and the mutant *sus14*(1241), a mutant having the delayed lysis phe-

notype, were employed (19, 24). The mutants used to construct the recombinants *sus*7(614)-*sus*8(769) and *sus*8(769)-*sus*9(756), and *sus*10(302), have been described (26).

The properties of *Bacillus subtilis* SpoA12 and *B. subtilis* L15, the nonpermissive (*su*⁻) and permissive bacterial hosts, respectively, have been described (26). To study suppression, we have employed *B. subtilis* MO-101-P *thr*⁻ [*met*⁻]⁺ *spoA*⁻ *su*⁺44 (17) and a prototroph derived from *B. subtilis* L5, strain L6. In the text these mutants will be referred to as *su*⁺44 and *su*⁺3, respectively.

Lysate production and bacteriophage assay. Our methods and media for lysate preparation have been described (3, 24), and standard phage techniques were used (1). The phage for genetic experiments were stored in Difco antibiotic medium 3. Viruses and lysates to be examined by electrophoresis and autoradiography were prepared in the minimal medium M40 (23) supplemented with 50 µg of tryptophan per ml (M40t medium), and input phage for these experiments were in TMS buffer (0.05 M Tris-hydrochloride [pH 7.8]-0.1 M NaCl-0.01 M MgCl₂).

Growth conditions, UV irradiation, and infection. Protocols described previously (12, 29) were generally followed. Bacteria were grown to 2×10^8 cells/ml in M40t medium, concentrated to 2×10^9 cells/ml by centrifugation, UV irradiated for 10 min (50 ergs/mm² per s) in M40t when indicated, infected with a multiplicity of infection (MOI) of 20 or 50, and then diluted to 2×10^8 cells/ml with prewarmed (37°C) M40t medium after adsorption for 2.5 or 5 min. The MOI as well as the time and temperature of adsorption is specified in the figure legends for each type of experiment. The M40t medium was sometimes supplemented with 200 µg of Casamino Acids (Difco) per ml (M40ta medium).

Radioactive labeling of proteins. UV-irradiated cells at 2×10^9 /ml in M40t medium at 37°C were infected with phage at an MOI of 50 (zero time). The culture was diluted to 2×10^8 cells/ml with prewarmed M40t medium and incubated with shaking at 37°C. ¹⁴C-labeled amino acids (5 µCi/ml) were added at 10 min after infection, and proteins were continuously labeled until 110 min after infection, at which time labeling was terminated (12). Cells were collected by centrifugation, and both the concentrated cells and the culture supernatant were processed for sodium dodecyl sulfate (SDS)-gel electrophoresis as described previously (12). Any modifications in the times of labeling, etc., are indicated in the appropriate figure legend.

Isolation of viral structures. For the isolation of fast-sedimenting mature phage, concentrated cells (2×10^9 cells/ml) in M40ta medium were infected at an MOI of 20. After a 5-min adsorption period at 23°C, the cells were diluted 10-fold with prewarmed (37°C) M40ta medium and incubated at 37°C with shaking. Proteins were continuously labeled with ¹⁴C-amino acids (10 µCi/ml) from 35 to 110 min after infection. Labeling was terminated (12), and infected cells were collected by centrifugation and suspended in a volume of TMS 1/50 that of the original culture; the concentrated cells were lysed and processed for sucrose gradient analysis as described pre-

viously (11, 29). Samples were centrifuged in gradients of 5 to 20% sucrose in TMS buffer in SW50.1 rotor at 35,000 rpm ($120,000 \times g$) for 25 min at 23°C. The gradients were formed and fractionated as described previously (29). For the isolation of proheads, UV-irradiated *B. subtilis* SpoA12 in M40t medium were infected and processed as described previously (20). Labeling in this case was from 6 to 100 min after infection (¹⁴C-labeled amino acids, 5 µCi/ml), and the lysate preparation was as described above. Conditions of centrifugation for the isolation of proheads are described in the appropriate figure legend.

Electron microscopy. Phage purified by density gradient centrifugation in cesium chloride and dialyzed against TMS buffer were fixed by adding 8% glutaraldehyde (Polysciences, Warrington, Pa.) to a final concentration of approximately 0.2%. Grids with carbon-coated Formvar films were floated on the sample drops for 5 to 10 min, and the specimens were then negatively stained with uranyl acetate.

To determine absolute plating efficiency, phage in a sample were counted by procedures described previously (4). Purified phage in water and 0.087-µm polystyrene latex particles (Dow Chemical Co., Midland, Mich.) were mixed and sprayed on carbon-coated Formvar films by use of an EFFA sprayer (E. F. Fullam, Inc., Schenectady, N.Y.). The specimens were shadowed with platinum-iridium at an angle of approximately 20°. Micrographs of droplet patterns containing phage and reference latex particles were made at an instrumental magnification of $\times 4,500$ and enlarged approximately three to four times for counting. Micrographs were taken with a Philips EM301 electron microscope, using an accelerating potential of 80 kV.

Electrophoresis. Proteins were separated by SDS-polyacrylamide gel electrophoresis as described previously (5, 6). Acrylamide concentrations are indicated in the appropriate figure legends. Gels were successively fixed in 45% methanol-10% acetic acid and 30% methanol-10% acetic acid prior to drying. The dried gels were processed for autoradiography as described previously (5).

Mutagenesis and genetic methods. A lysate of the mutant with the delayed lysis phenotype, *sus*14(1241), replicated in the presence of bromodeoxyuridine, was the source of mutants (25). Putative mutants were processed for examination by SDS-polyacrylamide gel electrophoresis and autoradiography, and the conditions for infection and isotopic labeling have been reported (5, 29). If the protein profile indicated that the major head protein or the head fiber were missing or exhibited an altered electrophoretic mobility, then the mutants were placed in the "900 series."

The details of two- and three-factor crosses as well as recombinant virus construction have been described (25, 26).

The methods employed to position a member of the 900 series were as follows. With each mutant of the 900 series, an attempt was made to construct three recombinants (see Table 3). For example, the recombinant *sus*9(756)-900 was isolated from the cross *sus*8(769)-*sus*9(756) $\times$ 900. These recombinants

were cloned three times and then examined for the presence of fibers by electron microscopy, and the observations were then confirmed by electrophoretic analysis. All recombinants used for genetic analysis had the wild-type allele of cistron 14 and did not exhibit delayed lysis.

In the three- and four-factor crosses, recombinants able to replicate in the su^- host were selected at random and cloned twice on the su^- host, and then lysates were produced. An analysis of the protein profile by electrophoresis and autoradiography enabled us to identify the allele of the cistron coding for the head protein or fiber protein that was present.

Chemicals and isotopes. The serylprotease inhibitor phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol to give a stock solution of 6 mg/ml and used at a final concentration of 300 μ g/ml. Egg-white lysozyme (A grade), DNase I (B grade), and RNase (bovine pancreatic) were from Calbiochem, La Jolla, Calif.

The 14 C-labeled amino acid mixture (New England Nuclear Corp., Boston, Mass.; NEC-445; 100 μ Ci/ml, about 200 mCi/mmol) was adjusted to neutrality by the addition of 0.1 volume of 1 N NaOH immediately before use. [3 H]thymidine was from New England Nuclear Corp. (NET-027Z; 1 mCi/ml, 50 Ci/mmol).

RESULTS

Isolation of mutants and preliminary characterization. A series of putative *sus* mutants were isolated after the replication of the delayed lysis mutant of $\phi 29$, *sus14*(1241), in the presence of bromodeoxyuridine (19, 25). The pattern of $\phi 29$ -specific protein synthesis after infection of the nonpermissive host (su^-) was examined by SDS-polyacrylamide gel electrophoresis and autoradiography (5). In 13 of 437 $\phi 29$ -specific protein profiles examined, the head fiber protein was missing or exhibited a slightly altered electrophoretic mobility (Fig. 1 and Table 1). For example, the fiber protein is missing after 907 or 908 su^- infection (Fig. 1h and l). A fragment apparently derived from the fiber protein and having about 70% of the mass of the fiber protein is present in the 900 $^-$ profile slightly below the position of the protein A2 (Fig. 1d, arrow). The profile representing the 909 su^- infection (Fig. 1p) illustrates increased relative mobility of the fiber protein as compared with its normal position when obtained from the purified virion (Fig. 1e and n) and shown in the *sus14*(1241) profile (Fig. 1a), whereas the profile of 902 su^- infection (Fig. 1g) shows decreased fiber mobility. Other mutant infections yielding fibers with slightly altered electrophoretic mobilities include 904, 906, 905, and 901, as illustrated in Fig. 1f, j, k, and o, respectively.

All mutants of the 900 series were able to

replicate in the su^- host (Table 1), which suggests that the fiber protein is nonessential.

To further characterize the mutants, lysates were prepared with either the permissive host su^{+3} or su^{+44} (Table 1 and Fig. 2). Of the mutants tested, apparently only 900 can be suppressed because both the fragment and a protein that migrates to the position of the fiber protein are visible in the electrophoretic profile (Fig. 2e).

Partial characterization of the 900 particles. We have arbitrarily focused on the properties of the 900 particle from the su^- host because these properties are typical of the fiberless particle and are frequently distinctive when compared with $\phi 29^+$.

When 900 particles were examined by velocity sedimentation in 5 to 20% sucrose gradients, they were fast sedimenting relative to 14 $^-$ control phage (Fig. 3C and A, respectively). Electron microscopy and SDS-gel electrophoresis of samples of the fractions containing fast-sedimenting particles showed no visible fiber and no fragment of the head fiber protein (data not shown). When the fast-sedimenting particles were further purified by equilibrium density gradient centrifugation in CsCl and examined by electron microscopy, the 900 particles lacked visible fibers (compare Fig. 4A and B). When the proteins of the 900 particle were examined by electrophoresis and autoradiography, the head fiber protein was absent from the profile (Fig. 5c).

The absolute plating efficiency of the 900 particle on the su^- indicator strain (Table 2) is comparable to that of $\phi 29^+$.

When fibers are removed by treatment with dimethyl sulfoxide, the buoyant density of $\phi 29^+$ increases (27). The 900 particle has a buoyant density in CsCl approximately 0.03 g/cm 3 greater than mutant *sus14*(1241) phage (Fig. 6A). When head fibers were removed from the 14 $^-$ phage by treatment with 50% dimethyl sulfoxide, the particles had the buoyant density of the 900 particles (Fig. 6B).

Presence of fibers in the prohead versus the mature virion. An intermediate particle in the $\phi 29$ assembly pathway, the prohead, is composed of the fiber and the major head protein in addition to the upper collar p10 and an apparent scaffolding core protein p7 (14, 20).

Proheads formed during mutant 900 infection of UV-irradiated su^- cells were sedimented in sucrose gradients. The 900 proheads sediment slightly faster than 14 $^-$ proheads, as indicated by a shift in peak radioactivity from fraction 23 to fraction 21 (Fig. 7). The prohead of 900 lacks the fiber protein and contains only the major

FIG. 1. Autoradiographs of ^{14}C -labeled $\phi 29$ -specific proteins produced in mutant and $\phi 29^+$ infections of UV-irradiated *su⁻* *B. subtilis*. Cells were infected with a mutant of the 900 series or *sus14*(1241) (MOI of 50) and labeled with a mixture of ^{14}C -amino acids from 10 to 110 min after infection; $\phi 29^+$ -infected cells were labeled from 10 to 45 min. Cell and/or supernatant fractions were analyzed by SDS-gel electrophoresis (12 to 19% linear polyacrylamide gradient) followed by autoradiography as described in the text. Profiles are of lysates of cell pellets except for (h), which represents a supernatant. Proteins produced in fiber mutant infections are shown in profiles (d) 900; (f) 904; (g) 902; (h) 907; (j) 906; (k) 905; (l) 908; (m) 903; (o) 901; and (p) 909. Profile (c) is of mutant 914 (major head protein) infection as described in the text. Profile (b) represents an uninfected control culture. Profiles (a) and (i) are of *sus14*(1241) and $\phi 29^+$ infections, respectively. Profiles (e) and (n) are of purified $\phi 29^+$ virions. Symbols, names, and molecular weights (5, 12), respectively, of the $\phi 29$ -specific proteins are as follows: p12, 87,500; Ap, neck appendage, 75,300; p9, "tail," 62,300; Hd, major head protein, 45,000; p10, neck upper collar, 36,400; p16, 36,000; p11, neck lower collar, 35,200; F, head fiber, 28,500; p15, 25,100; A1, 22,400; A2, 21,800; LM2, 18,800. The apparent molecular weights of early proteins AF and BF (5) are slightly larger than that of the fiber protein in this electrophoresis system.

TABLE 1. *Partial phenotypic characterization of mutants of the 900 series*

Mutant	Relative electrophoretic mobility ^a	Phage yield ^b		Suppression ^c	
		<i>su</i> ⁺ ³	<i>su</i> ⁻	<i>su</i> ⁺ ³	<i>su</i> ⁺ ⁴⁴
900	0	0.85	1.2	+	+
901	f	0.21	0.25	0	
902	s	0.11	0.26	0	
903	s	1.9	1.9		
904	s	0.97	1.8	0	
905	s	0.63	2.2		
906	s	0.88	2.2		
907	o	1.1	1.4		0
908	o	2.1	1.5	0	0
909	f	2.7	3.9	0	
911	o	3.2	3.4		0
912	o	2.8	3.6		0
913	o	0.87	1.1		0
914	s	1.0	2.4		

^a Relative electrophoretic mobility of fiber protein (900 to 913) or head protein (914) in Fig. 1. s, Relatively slow migration; f, relatively fast migration; o, no protein is detected; and 0, a presumed fragment of the normal polypeptide is observed.

^b *su*⁺³ cells (2×10^8 /ml) were infected (MOI of 10), and $\phi 29$ antiserum was added 15 min later. After an additional 5 min, the infected cells were diluted 1,000-fold into Penassay broth, incubated for 2 h at 37°C, and lysed with lysozyme (10 μ g/ml). The data reflect the titer $\times 10^{-11}$.

^c Representative results reflecting suppression of mutation in the 900 series using *su*⁺⁴⁴ are presented in Fig. 2. Experiments employing *su*⁺³ were identical. 0, Lack of suppression.

head protein *p10* and the core protein *p7* (Fig. 8b).

Some fiber proteins with slightly altered electrophoretic mobilities appear to attach normally to phage heads. For example, a fiber protein with a slightly slower electrophoretic mobility is produced in the 903 mutant infection of the *su*⁻ host (Fig. 1m and Table 1). Figure 3B illustrates that 903 particles sediment normally as compared with the 14⁻ control phage, and electrophoretic analysis indicates that these particles contain the altered fiber protein (Fig. 5b). In this case the altered fibers are also present in the prohead (data not shown). The electrophoresis data of Fig. 9a through d illustrate the presence of normal, altered, missing, and altered F protein in the lysates of *su*14(1241)-, 901-, 908-, and 909-infected cells, respectively. The protein compositions of the corresponding proheads isolated from these lysates by sucrose gradient centrifugation are shown in Fig. 9h, g, f, and e, respectively. When the fiber is missing in the lysate, proheads are formed nevertheless. When an altered fiber is present, it may (e.g., 901) or may not (e.g., 909) appear in the

prohead. Note that all of these proheads contain the core protein *p7*.

We conclude that some altered fiber proteins can interact with the major head protein of the prohead and mature virion, whereas other altered fibers lose this capacity. In each mutant infection investigated, the altered fiber is either present in both the prohead and mature virion or absent in both structures, indicating conservation in the specificity of interaction of the two proteins during morphogenesis.

Characterization of mutant 914. The electrophoretic profile of $\phi 29$ -specific proteins after *su*⁻ infection by mutant 914 shows a major head protein of altered electrophoretic mobility and an apparently normal fiber protein (Fig. 1c). This mutant virus can replicate in the *su*⁻ host (Table 1), and the particles have a high efficiency of plating (Table 2). A fast-sedimenting

FIG. 2. Autoradiographs of ¹⁴C-labeled $\phi 29$ -specific proteins produced in 900 series mutant and $\phi 29^+$ infections of the UV-irradiated permissive host *B. subtilis su*⁺⁴⁴. Cells were grown, UV irradiated, infected, and labeled with ¹⁴C-amino acids as described in the text. Labeling was terminated at 45 min, and the cell pellet fractions were analyzed by SDS-gel electrophoresis (16 to 20% linear polyacrylamide gradient) followed by autoradiography as described in the text. (a) Purified $\phi 29$ virions; (b) 908; (c) 907; (d) $\phi 29^+$; (e) 900; and (f) uninfected control.

particle is produced (Fig. 3D) that lacks fibers by the criteria of electron microscopy and electrophoretic analysis (Fig. 4C and 5d). Although the fiber is in the lysate, no fiber can be detected in the prohead (Fig. 8c). From these results we conclude that, although an apparently normal fiber protein is present during infection, an alteration in the primary structure of the major head protein results in the complete exclusion of the fiber from any phage head structure we have been able to isolate.

Genetic studies. A point mutation in the $\phi 29$ genome can result in the loss of both the head and fiber proteins from the electrophoretic profile generated from *su*⁻ lysates. Other data obtained with the $\phi 29$ system suggest that this phenotype can result from polarity of translation (19, 25).

The 900 mutant was chosen for our initial genetic analysis because the presence of the fragment and suppression demonstrated by electrophoresis were typical for a *sus* mutation (Fig. 2e). Two- and three-factor crosses utilizing mutants of cistrons 7, 8, and 9 yielded the unusual results shown in Table 3. The recombination frequencies obtained by two-factor crosses do not give the additivity expected for the $\phi 29$ system (16, 26). In each cross in which the 900 marker was in a recombinant containing the *sus* mutant mapping to the "left," there was a significant reduction in recombination frequency. Table 4 shows that this phenomenon occurs with at least six mutants of the 900 series. The ratios shown in Table 4 emphasize the pronounced asymmetry characteristic of these three-factor crosses.

Because of these results we have completed the analysis of three- or four-factor crosses only when the recombination frequency obtained was similar to the control value, that value obtained when mutants of the 900 series were not employed. The recombination frequency and ratio of parental genotypes in the progeny of the *sus*8(769) $\times$ *sus*9(756)-900 cross support additional analysis (Table 5). Twenty recombinant clones chosen at random from the *su*⁻ indicator were cloned twice on the *su*⁻ host, and a lysate of each was prepared. The results of electrophoresis presented in Table 5 are consistent with the map order *cistron* 8-900-*cistron* 9.

The two-factor cross *sus*9(756) $\times$ *sus*10(302) and the three-factor cross *sus*9(756)-900 $\times$

FIG. 3. Sucrose gradient isolation of ^{14}C -labeled $\phi 29$ virions produced in *B. subtilis* SpoA12 infected with mutants (A) *sus*14(1241), (B) 903 (head fiber), (C) 900 (head fiber), and (D) 914 (major head protein). The preparation of lysates and determination of radioactivity are described in the text. Centrifugation was in an SW50.1 rotor at 35,000 rpm (120,000 $\times$ g) for 25 min at 23°C. Sedimentation is from right to left. Counts in fractions 1 through 26 of 36 fractions are plotted. Electron microscopy of phage purified by density gradient centrifugation in CsCl (Fig. 4) and SDS-polyacrylamide gel electrophoresis (Fig. 5) of mature phage in the peak fractions 7 (A and B) and 3 (C and D) show that virions produced in the *sus*14(1241) and 903 mutant infections (A and B) have head fibers, whereas the faster-sedimenting virions from the 900 and 914 mutant infections (C and D) lack fibers.

FIG. 4. Electron micrographs of virions produced in infections with $\phi 29^+$ (A), mutant 900 (B), or mutant 914 (C) and purified by cesium chloride density gradient centrifugation. Particles were fixed with glutaraldehyde and negatively stained for electron microscopy as described in the text. Fibers attached to the $\phi 29^+$ virions shown in (A) are clearly absent on the virions shown in panels (B) and (C). The magnification bar represents 100 nm.

sus10(302) gave comparable average ($n = 5$) recombination frequencies of 1.8 ± 0.21 and $1.6 \pm 0.26\%$, respectively (data not shown). The

results shown in Table 5 are consistent with the order 900-cistron 9-cistron 10.

Because the head protein of mutant 914 has a

FIG. 5. Autoradiograph of ^{14}C -labeled $\phi 29$ proteins comprising mature virions produced by *sus14*(1241) and 900 series mutant infections of *B. subtilis* SpoA12 and isolated by sucrose gradient centrifugation as shown in Fig. 3. Samples of the peak fractions 7 (A and B of Fig. 3) and 3 (C and D of Fig. 3) were analyzed by SDS-gel electrophoresis on a linear gradient of 16 to 20% polyacrylamide. (a) *sus14*(1241), (b) 903, (c) 900, (d) 914, and (e) *sus14*(1241) virions purified by cesium chloride density gradient centrifugation. The fiber (F) protein is present on virions from the *sus14*(1241) and 903 mutant infections, but not on virions from the 900 and 914 mutant infections.

distinctive electrophoretic mobility, a four-factor cross was possible. The recombinant *sus9*(756)-914 was constructed, and the phenotype was confirmed by electrophoresis. A series of *sus8*(769)-912 recombinants was constructed. One recombinant gave an average ($n = 5$) recombination frequency of $4.1 \pm 0.69\%$ when crossed with *sus9*(756) as compared with a control series average ($n = 4$) of $4.2 \pm 0.14\%$. These recombinants were used to perform the four-factor cross depicted in Table 6. Forty-eight recombinant clones chosen at random were processed as described above, and the results are consistent with the order *sus8*(769)-914-912-*sus9*(756).

A series of *sus8*(769)-900 recombinants was constructed. One recombinant gave an average ($n = 4$) recombination frequency of $5.2 \pm 0.76\%$

when crossed with *sus9*(756) as compared with a control series average ($n = 4$) of $4.6 \pm 0.57\%$. This recombinant was used to perform the four-factor cross depicted in Table 7. Forty-eight recombinant clones chosen at random were processed as described above, and the results of the electrophoresis are consistent with the order *sus8*(769)-914-900-*sus9*(756).

DISCUSSION

Our analysis of the role of the fiber protein in $\phi 29$ replication has been complicated by the fact that apparent mutation in the gene that codes for the head fiber protein does not seem to be conditionally lethal (Table 1) and because anomalous behavior in genetic crosses is characteristic of mutants of the 900 series (Tables 3 and 4). We have focused on the mutant *sus8.5*(900) because this mutant is suppressible by both the *su*⁺³ and *su*⁺⁴⁴ hosts (Fig. 2e and Table 1), and an apparent fragment of the fiber is produced. After backcrossing, *sus8.5*(900) could be used in three- and four-factor crosses (Tables 5 and 7). Our evidence suggests that the properties of the 900 particle are characteristic of particles lacking the fiber protein.

We were particularly interested in the properties of the 914 particles that form in the presence of an apparently normal fiber protein (Fig. 1c, 5d, and 8c and Table 1). In these protein profiles the protein p10 has an altered electrophoretic mobility. Backcrossing has restored the wild-type p10 in both virion and prohead (data not shown). Both 900 and 914 virions were fast sedimenting in sucrose gradients relative to $\phi 29^+$ (Fig. 3C and D). In contrast to $\phi 29^+$, 900 and 914 virions lacked fibers when examined by electron microscopy (Fig. 4B and C) or electrophoresis (Fig. 5c and d). All of these particles have approximately the same absolute efficiency of plating (Table 2). When $\phi 29^+$, 900, and 914 particles were heated at 70°C in TMS, the slopes of inactivation were indistinguishable

TABLE 2. Relationship between $\phi 29$ particles and PFU

Phage	Phage ^a /ml $\times 10^{-12}$	PFU ^b /ml $\times 10^{-12}$	PFU/phage
$\phi 29^+$	3.9 ± 0.8	2.9 ± 0.3	0.73
900	4.5 ± 1.3	4.8 ± 0.4	1.07
914	6.3 ± 1.1	5.3 ± 0.8	0.84

^a Based on five electron microscopic determinations of the ratio of phage to latex particles. Average counts of 179, 197, and 58 phage and 1,133, 909, and 397 latex particles were made for $\phi 29^+$, 900, and 914, respectively.

^b Average PFU determined on six plates of a single dilution series.

FIG. 6. Fiberless virions have a buoyant density greater than that of $\phi 29^+$. Phage were produced in *sus14*(1241)- and mutant 900-infected *B. subtilis* SpoA12 in the presence of ^{14}C - and ^3H -labeled amino acids, respectively, and were purified by density gradient centrifugation in cesium chloride as described previously (2). Some of the ^{14}C -labeled 14^- virions were treated with dimethylsulfoxide to remove the head fibers (27). ^{14}C -labeled 14^- particles with and without fibers were mixed separately with ^3H -labeled 900 particles, and cesium chloride was added to each mixture to give an average density of approximately 1.46 g/ml. The resulting mixtures were centrifuged in an SW50.1 rotor at 25,000 rpm ($60,000 \times g$) for 38 h at 25°C . Fourteen-drop fractions were collected from each centrifuge tube, and the ^{14}C and ^3H radioactivity in each fraction was determined by combusting a 20- μl sample in a Packard Tri-Carb oxidizer (model 306). To obtain the density of certain fractions, the refractive index was determined by use of an Abbe refractometer. The refractometer prism was maintained at $25 \pm 0.1^\circ\text{C}$ by use of a Lauda type K2-R recirculating water bath. Symbols: $\circ$, ^3H radioactivity in mutant 900 virions lacking fibers (A) and (B); $\bullet$, ^{14}C radioactivity in (A) 14^- phage with fibers and (B) dimethyl sulfoxide-treated 14^- phage lacking fibers; Δ , density at 25°C (A) and (B).

(data not shown). Survival of PFU in 1 and 2 M sodium perchlorate for 30 min at 37°C or after a rapid 200-fold dilution from 2 M NaCl or 20% sucrose into TMS gave comparable results for all three particles (data not shown). These results suggest that if the head of the 900 or 914 particle is less stable than $\phi 29^+$, it is masked by the relative instability of the neck-tail complex or the head-neck junction.

Apparently T4 phage can also be constructed without proteins normally found in the head of the virion. Infection with the T4 mutant IP 0 , defective for three internal head proteins, results in the production of active phage (7). Moreover, the *hoc* and *soc* proteins, which together with *p23** form the lattice of the T4 head shell and are distributed over the entire head surface, are nonessential for phage growth (13). The *hoc* and *soc* proteins form the six bridges and center unit, respectively, in the basic hexameric repeating unit. In addition, *p24**,

cleaved from *p24* during normal head assembly and thought to play a significant structural role in the T4 head, has recently been shown to be dispensable with a single compensating mutation in gene 23 known as bypass 24 (N. Sternberg, D. Tiemeier, and L. Enquist, Abstr. Fifth Biennial Conf. Bacteriophage Assembly, Snowbird, Utah, 1976; L. Black, personal communication). As another example, a coliphage λ vector constructed by Sternberg, Tiemeier, and Enquist to clone *EcoRI* fragments contains the *Dam15* mutation, yet plates on a *supO* host (Snowbird, Abstr. Fifth Biennial Conf. Bacteriophage Assembly). The λ gene D protein is a major external capsid protein.

Some of the T4 particles assembled without the above proteins differ in certain respects from the normal particles. For example, phage lacking *p24* (*am24 byp24*) have apparently normal morphology and heat and pH stability, but are osmotic shock resistant. Also, the *am24*

FIG. 7. Sedimentation properties of control 14^- and mutant 900 proheads. UV-irradiated *B. subtilis* SpoA12 (12) was infected with *sus14*(1241) or mutant 900 phage (MOI of 20), and after a 5-min adsorption period at 23°C the cultures were incubated at 37°C with shaking. The infected cultures were labeled continuously with ^{14}C -amino acids (5 $\mu\text{Ci/ml}$) from 6 to 100 min after infection. Labeling was terminated and lysates were prepared as described previously (29). Proheads were isolated by centrifugation of the lysates in 5 to 20% sucrose gradients, and representative gradient profiles are shown in (A) and (B). Centrifugation was in an SW50.1 rotor at 35,000 rpm ($120,000 \times g$) for 30 min at 23°C. Sedimentation is from right to left, and the radioactivities in fractions 1 to 30 of 42 fractions, determined as described previously (29), are plotted. Proheads are found in the major peak of each gradient profile (20). (A) *sus14*(1241)-infected cells; (B) mutant 900-infected cells.

byp24 phage are formed more slowly than wild-type phage, and protein cleavage occurs at a greatly reduced rate. *hoc* $^-$ phage are more resistant to osmotic shock than wild-type T4, but *soc* $^-$ *hoc* $^-$ phage are much more sensitive to osmotic shock than is T4 $^+$. We have not detected differences between $\phi 29^+$ and the fiberless particles in sensitivity to heat or osmotic shock.

The prohead of $\phi 29$ contains the head, fiber, p10, and p7 proteins. Assuming that the number of copies of head protein is comparable in the prohead and virion, at least 50 copies of the fiber protein are present in the virion (11, 30) and at least 30 copies are present in the prohead (20). The proheads of 900 and 914 lack the fiber protein (Fig. 8). With other mutants, if an altered fiber protein can be detected on the virion, it is present in the prohead. In contrast, if the altered fiber is absent from the virion, it cannot be detected in the prohead. This indicates a very specific interaction in a primitive organizing structure, the prohead.

We have observed bromodeoxyuridine-generated mutants that contained more than one point mutation (25; this study, data not shown). Nevertheless, the anomalous behavior of the recombinants containing mutations of the 900 series (Tables 3 and 4) was unexpected. In addition, one of four reciprocal three-factor

FIG. 8. Autoradiograph of ^{14}C -labeled proteins comprising proheads isolated by sucrose gradient centrifugation. Samples from the peak fractions 23 (Fig. 7A) and 21 (Fig. 7B) containing *sus14*(1241) and mutant 900 proheads, respectively, were examined for viral protein composition along with proheads from mutant 904- and 914-infected cells obtained similarly (gradient profiles not shown). SDS-gel electrophoresis was in 12% polyacrylamide. (a) Purified $\phi 29^+$ virions; (b) 900 proheads; (c) 914 proheads; (d) 904 proheads; (e) *sus14*(1241) proheads.

crosses that gave anomalous recombination frequencies (Table 3) gave inconsistent results when the recombinants were examined by electrophoresis (data not shown). For this reason we focused on recombinants that were "typical" by the criterion of recombination frequency. The four multiple-factor crosses employing electrophoretic analysis (Tables 5, 6, and 7) are consistent with the order *sus8*(769)-914-912-*sus9*(756) or *sus8*(769)-914-900-*sus9*(756). Melhado et al. (15a) have isolated and mapped the mutation *sus8*(22) (19). We have confirmed the position of *sus8*(22) by four-factor crosses (data not shown). During restrictive infection with *sus8*(22), neither head protein nor fiber is pro-

duced; however, an apparent fragment with a molecular weight of 25,000 can be detected. Both head protein and fiber are produced by revertants. The tryptic peptides of the fragment are consistent with a mutation in the cistron coding for the head protein, and the absence of fiber may be the result of polarity of translation. Taken together, these results are consistent with a scheme in which cistron 8 codes for the major head protein and cistron 8.5 codes for the fiber. Therefore, the mutation 900 could be named *sus8.5*(900).

There are two views as to the composition of the $\phi 29$ head and hence the structure of $\phi 29$. Mendez et al. (18) report seven polypeptides in $\phi 29$, with three being present in the head. The results of Pène et al. (22) are in agreement. The structure proposed by Viñuela et al. (30) contains five subunits of the minor head protein HP2 per phage. Carrascosa et al. (9) now detect a fourth head protein, HO0, in the virion.

Our view is that the virus head consists of the major head protein (HP1), the fiber (HP3), and the upper collar protein p10 (NP2) and that the fiber is nonessential. We have not observed HP0 or HP2 in any lysate, defective particle, intermediate particle, or virus, employing slab discontinuous SDS-gel systems with single concentrations of polyacrylamide (e.g., 10 and 12%) or with linear gradients of polyacrylamide ranging from 16 to 20% to 12 to 19% (5, 6). HP2 has never been detected when cistron 8 mutants have been grown in the *su*⁻ host (5, 9, 15). If HP2 exists, then its absence must reflect the polarity of the cistron 8 mutation [e.g., *sus8*(769)] as does the absence of fiber protein.

FIG. 9. Autoradiograph of ^{14}C -labeled proteins present in lysates and proheads from *sus14*(1241)-, 901-, 908-, and 909-infected *B. subtilis* SpoA12. Phage infection, radioactive labeling, and prohead isolation were as described in the Fig. 7 legend. Preparation of cell lysates, SDS-gel electrophoresis (12 to 19% linear gradient of polyacrylamide), and autoradiography were as described in the text. (a) *sus14*(1241) lysate; (b) 901 lysate; (c) 908 lysate; (d) 909 lysate; (e) 909 proheads; (f) 908 proheads; (g) 901 proheads; (h) *sus14*(1241) proheads. (i) represents an uninfected control culture, and (j) is a profile of purified $\phi 29^+$ virions. Symbols, names, and molecular weights of the $\phi 29$ structural proteins are as described in the legend to Fig. 1. Other viral proteins indicated here are the low-molecular-weight proteins LM3 and LM6 having molecular weights of 16,700 and 13,000, respectively, and the prohead "scaffolding" protein p7 having a molecular weight of 14,200 (5).

TABLE 3. *Anomalous behavior of mutant 900 in three-factor crosses*

Parental genotypes			No. of tests	Recombination frequencies	Ratio ^a
7(614)	+	+	23	0.90 ± 0.24	1.00
+	8(769)	+			
7(614)	+	+	5	0.68 ± 0.05	0.76
+	8(769)	900			
7(614)	+	900	6	0.32 ± 0.08	0.36
+	8(769)	+			
8(769)	+	+	33	5.3 ± 0.95	1.00
+	+	9(756)			
8(769)	+	+	6	3.8 ± 0.34	0.72
+	900	9(756)			
8(769)	900	+	10	1.3 ± 0.16	0.25
+	+	9(756)			
7(614)	+	+	24	4.5 ± 0.95	1.00
+	+	9(756)			
7(614)	+	+	8	3.3 ± 0.88	0.73
+	900	9(756)			
7(614)	900	+	8	0.85 ± 0.20	0.19
+	+	9(756)			

^a The ratio reflects the comparison of the recombination frequency obtained in a three-factor cross by that obtained with the corresponding control two-factor cross.

TABLE 4. *Anomalous behavior of mutants of the 900 series in three-factor crosses*

Parental genotype			Recombination frequency ^a of fiber mutant X						Ratio ^b
			900	907	908	909	911	912	
7(614)	+	+	4.2	5.0	4.6	5.4	4.9	3.8	1.00
+	+	9(756)							
7(614)	+	+	3.8	5.0	3.5	6.6	8.3	3.7	1.07 $\pm$ 0.27
+	(X)	9(756)							
7(614)	(X)	+	0.85	1.1	0.81	1.0	1.2	1.0	0.22 $\pm$ 0.09
+	+	9(756)							
8(769)	+	+	5.0	6.4	5.3	5.7	5.5	4.2	1.00
+	+	9(756)							
8(769)	+	+	3.8	7.1	3.9	7.7	6.2	4.7	1.04 $\pm$ 0.14
+	(X)	9(756)							
8(769)	(X)	+	1.3	0.90	1.2	1.9	2.4	1.0	0.27 $\pm$ 0.06
+	+	9(756)							

^a Each cross was made in quadruplicate, and the average recombination frequency is listed.

^b This ratio reflects the recombination frequency obtained by a given three-factor cross compared with the control two-factor cross. The ratio listed reflects the average of the six values obtained.

TABLE 5. *Results of three-factor crosses to position the 900 fiber mutation*

Parental genotypes			Recombination frequency	Progeny phage		
				Parental types	Fiber in recombinants ^a	
					+	-
8(769)	+	+	6.7	49	11	9
+	900	9(756)		51		
900	9(756)	+	2.0	50	18	2
+	+	10(302)		50		

^a Twenty recombinants were studied by SDS-polyacrylamide gel electrophoresis. Recombinants are able (like $\phi 29^+$) or unable (like 900) to produce normal fibers in *su*⁻ infections. The gels contained 12% polyacrylamide.

TABLE 6. Results of a four-factor cross to orient the cistrons coding for the major head protein and the fiber

Parental genotype				Progeny genotype ^a				No. of recombinants
8(769)	+	912	+ ^b	+	+	912	+	17
+	914	+	9(756)	+	914	+	+	27
				+	914	912	+	4
				+	+	+	+	0

^a The *sus*8(769)-912 parent constituted 53% of the progeny phage. The recombination frequency was 6.7%. The phenotype of 48 recombinants was studied by SDS-gel electrophoresis in 12% polyacrylamide.

^b The 912 mutant is unable to synthesize the fiber protein during restrictive infection. The 914 mutant is characterized by a major head protein of altered electrophoretic mobility, and the phenotype is assumed to reflect mutation in the cistron coding for the head protein.

TABLE 7. Results of a four-factor cross to orient the cistrons coding for the major head protein and the fiber

Parental genotype				Progeny genotype ^a				No. of recombinants
8(769)	+	900	+ ^b	+	+	900	+	16
+	914	+	9(756)	+	914	+	+	18
				+	914	900	+	13
				+	+	+	+	1

^a The *sus*8(769)-*sus*8.5(900) parent constituted 50% of the progeny phage. The recombination frequency was 4.2%. The phenotype of 48 recombinants was studied by SDS-gel electrophoresis in 12% polyacrylamide.

^b The 900 mutant is unable to synthesize the fiber protein although an apparent fragment of the fiber is produced during restrictive infection. The 914 mutant is characterized by a major head protein of altered electrophoretic mobility, and the phenotype is assumed to reflect mutation in the cistron coding for the head protein.

The position of the major head protein after 914 infection would be expected to permit the visualization of HP2. It does not. The putative protein HP0 bands at the approximate position of the head protein of 914. We suspect that both HP0 and HP2 could be generated by a low level of misreading and/or proteolytic cleavage and for this reason could appear at random in any particular lysate.

We believe that the genes coding for structural proteins of ϕ 29 have been identified. The results of Yanofsky et al. (31) suggest that the DNA "linker" protein(s) described by Ortin et al. (21) is the product of cistron 3 and that the remaining structural proteins are coded for by cistrons 8, 8.5, 9, 10, 11, and 12 (5, 9, 15, 16). We have found no evidence for any function of the fiber protein except adornment under the artificial laboratory conditions conventionally used.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Richard Rau, Charlene Peterson, and Viola M. Zeece.

This work was aided by grant GB-29393 from the National Science Foundation and by Public Health Service grants DE-3606 and GM-19743 from the National Institute of Dental Research and the National Institute of General Medical Sciences, respectively.

LITERATURE CITED

- Adams, M. 1969. Bacteriophages. John Wiley & Sons, Inc., New York.
- Anderson, D. L., D. D. Hickman, and B. E. Reilly. 1966. Structure of *Bacillus subtilis* bacteriophage ϕ 29 and the length of ϕ 29 deoxyribonucleic acid. *J. Bacteriol.* 91:2081-2089.
- Anderson, D. L., and E. T. Mosharrafa. 1968. Physical and biological properties of phage ϕ 29 deoxyribonucleic acid. *J. Virol.* 2:1185-1190.
- Anderson, D. L., M. E. Pollock, and L. F. Brower. 1965. Morphology of *Mycoplasma laidlawii* type A. I. Comparison of electron microscopic counts with colony-forming units. *J. Bacteriol.* 90:1764-1767.
- Anderson, D. L., and B. E. Reilly. 1974. Analysis of bacteriophage ϕ 29 gene function: protein synthesis in suppressor-sensitive mutant infection of *Bacillus subtilis*. *J. Virol.* 13:211-221.
- Anderson, D. L., and B. E. Reilly. 1976. Analysis of gene function in *Bacillus subtilis* bacteriophage ϕ 29, p. 254-274. In D. Schlessinger (ed.), *Microbiology—1976*. American Society for Microbiology, Washington, D.C.
- Black, L. W. 1974. Bacteriophage T4 internal protein mutants: isolation and properties. *Virology* 60:166-179.
- Camacho, A., E. Moreno, J. L. Carrascosa, E. Viñuela, and M. Salas. 1974. A suppressor of nonsense mutations in *Bacillus subtilis*. *Eur. J. Biochem.* 47:199-205.
- Carrascosa, J. L., A. Camacho, F. Moreno, F. Jimenez, R. P. Mellado, E. Viñuela, and M. Salas. 1976. *Bacillus subtilis* phage ϕ 29—characterization of gene products and functions. *Eur. J. Biochem.* 66:229-241.
- Carrascosa, J. L., E. Viñuela, and M. Salas. 1973.

- Proteins induced in *Bacillus subtilis* infected with bacteriophage ϕ 29. *Virology* 56:291-299.
11. Hagen, E. W., B. E. Reilly, M. E. Tosi, and D. L. Anderson. 1976. Analysis of gene function of bacteriophage ϕ 29 of *Bacillus subtilis*: identification of cistrons essential for viral assembly. *J. Virol.* 19:501-517.
 12. Hawley, L. A., B. E. Reilly, E. W. Hagen, and D. L. Anderson. 1973. Viral protein synthesis in bacteriophage ϕ 29-infected *Bacillus subtilis*. *J. Virol.* 12:1149-1159.
 13. Ishi, T., and M. Yanagida. 1975. Molecular organization of the shell of the T_{even} bacteriophage head. *J. Mol. Biol.* 97:655-660.
 14. Jimenez, F., A. Camacho, J. De La Torre, E. Viñuela, and M. Salas. 1977. Assembly of *Bacillus subtilis* phage ϕ 29. 2. Mutants in the cistrons coding for the non-structural proteins. *Eur. J. Biochem.* 73:57-72.
 15. McGuire, J. C., J. J. Pène, and J. Barrow-Carraway. 1974. Gene expression during the development of bacteriophage ϕ 29. III. Analysis of viral-specific protein synthesis with suppressible mutants. *J. Virol.* 13:690-698.
 - 15a. Mellado, R. P., E. Méndez, E. Viñuela, and M. Salas. 1977. Order of the two major head protein genes of bacteriophage ϕ 29 of *Bacillus subtilis*. *J. Virol.* 24:378-382.
 16. Mellado, R. P., F. Moreno, E. Viñuela, M. Salas, B. E. Reilly, and D. L. Anderson. 1976. Genetic analysis of bacteriophage ϕ 29 of *Bacillus subtilis*: integration and mapping of reference mutants of two collections. *J. Virol.* 19:495-500.
 17. Mellado, R. P., E. Viñuela, and M. Salas. 1976. Isolation of a strong suppressor of nonsense mutations in *Bacillus subtilis*. *Eur. J. Biochem.* 65:213-223.
 18. Méndez, E., G. Ramirez, M. Salas, and E. Viñuela. 1971. Structural proteins of bacteriophage ϕ 29. *Virology* 45:567-576.
 19. Moreno, R., A. Camacho, E. Viñuela, and M. Salas. 1974. Suppressor-sensitive mutants and genetic map of *Bacillus subtilis* bacteriophage ϕ 29. *Virology* 62:1-16.
 20. Nelson, R. A., B. E. Reilly, and D. L. Anderson. 1976. Morphogenesis of bacteriophage ϕ 29 of *Bacillus subtilis*: preliminary isolation and characterization of intermediate particles of the assembly pathway. *J. Virol.* 19:518-532.
 21. Ortin, J., C. Vazquez, E. Viñuela, and M. Salas. 1971. DNA-protein complex in circular DNA from phage ϕ 29. *Nature (London) New Biol.* 234:275-277.
 22. Pène, J. J., P. C. Murr, and J. Barrow-Carraway. 1973. Synthesis of bacteriophage ϕ 29 proteins in *Bacillus subtilis*. *J. Virol.* 12:61-67.
 23. Polsinelli, M., and M. Beretta. 1966. Genetic recombination in crosses between *Streptomyces aureofaciens* and *Streptomyces rimosus*. *J. Bacteriol.* 91:63-68.
 24. Reilly, B. E., and J. Spizizen. 1965. Bacteriophage deoxyribonucleate infection of competent *Bacillus subtilis*. *J. Bacteriol.* 89:782-790.
 25. Reilly, B. E., M. E. Tosi, and D. L. Anderson. 1975. Genetic analysis of bacteriophage ϕ 29 of *Bacillus subtilis*: mapping of the cistrons coding for structural proteins. *J. Virol.* 16:1010-1016.
 26. Reilly, B. E., V. M. Zeece, and D. L. Anderson. 1973. A genetic study of suppressor-sensitive mutants of the *Bacillus subtilis* bacteriophage ϕ 29. *J. Virol.* 11:756-760.
 27. Salas, M., C. Vazquez, E. Méndez, and E. Viñuela. 1972. Head fibers of bacteriophage ϕ 29. *Virology* 50:180-188.
 28. Tosi, M., and D. L. Anderson. 1973. Antigenic properties of bacteriophage ϕ 29 structural proteins. *J. Virol.* 12:1548-1559.
 29. Tosi, M. E., B. E. Reilly, and D. L. Anderson. 1975. Morphogenesis of bacteriophage ϕ 29 of *Bacillus subtilis*: cleavage and assembly of the neck appendage protein. *J. Virol.* 16:1282-1295.
 30. Viñuela, E., A. Camacho, F. Jiménez, J. L. Carras-cosa, G. Ramirez, and M. Salas. 1976. Structure and assembly of phage ϕ 29. *Philos. Trans. R. Soc. London Ser. B* 276:29-35.
 31. Yanofsky, S., F. Kawamura, and J. Ito. 1976. Thermolabile transfecting DNA from temperature-sensitive mutants of phage ϕ 29. *Nature (London)* 259:60-63.