Assembly of *Bacillus subtilis* Phage Φ29

2. Mutants in the Cistrons Coding for the Non-structural Proteins

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The effect on phage morphogenesis of sus mutations in the cistrons coding for nonstructural proteins has been studied. Mutants in three cistrons analyzed that are involved in phage DNA synthesis, as well as in cistron 16 which codes for a late nonstructural protein, produce prolate capsids which are more rounded at the corners than complete phage heads and have an internal core; they contain the head proteins, the upper collar protein and protein p7, not present in mature phage particles. Mutants in cistron 7 do not produce capsids nor other phage-related structures; this result and the presence of p7 in phage capsids suggest an essential role in capsid assembly for this protein. The protein product of cistron 13 is probably needed for a stable DNA encapsulation since mutants in this cistron produce mainly DNA-free complete phage particles and only about 10% of uninfected DNA-containing complete phage. Cistron 15 codes for a late, partially dispensable, nonstructural protein which is present in the DNA-free capsids produced after infection with the delayed-lysis mutant sus14(1242), used as the wild-type control, or with mutants in cistrons 9, 11, 12 and 13. Proteins p15 and p16 are probably involved in the encapsulation of viral DNA in a prohead.

It is now well established that DNA encapsulation in several bacteriophages takes place on a preformed head, named prohead, containing some electron-dense internal material which is released when the DNA is encapsulated [1–6].

The results presented in the preceding paper suggest that in the *Bacillus subtilis* phage Φ29 a structure similar to a prohead is produced after infection with a delayed lytic mutant which behaves like wild-type phage, or with several mutants in cistrons coding for phage structural proteins. In this paper we present evidence on the accumulation of prohead-like structures after restrictive infection with sus mutants in several cistrons involved in phage DNA synthesis and in cistron 16, coding for a late, nonstructural protein (see Fig. 1 of the preceding paper).

Sus mutants in cistron 7, also coding for a late, nonstructural protein, p7, do not produce any kind of phage-related structure suggesting a role for this protein in head morphogenesis. Protein p7 is present in all prohead-like structures and is absent from all DNA-containing heads, suggesting that this protein is released from the phage heads prior to or simultaneously with the encapsulation of DNA. The effect on phage morphogenesis of mutations in cistrons coding for other nonstructural proteins is also described in this paper. A preliminary account of some of these results has already been given [7].

Taking into account the results presented in this and in the preceding paper, a preliminary morphogenetic route for phage Φ29 assembly is proposed.

MATERIALS AND METHODS

**Bacteria, Phage and Media**

The nonpermissive host, *B. subtilis* 110NA try"-spOA" su" and the permissive bacteria, *B. subtilis* 168 MO-99 spoA"[met"-thr"]" su"- su" were as described [8].

The new nomenclature adopted by Mellado et al. [9] for the Φ29 mutants has been used. Mutants sus2(513), sus3(91), sus13(342), sus13(53), sus15(212), sus16(241) and sus17(112) were from the collection of Moreno et al. [8]; mutants sus6(252) and sus7(81) were from the collection of Mellado et al. [10]; mutant sus14(1242), lacking the mutations in cistrons 6 and 17, was obtained as described [11]. In all cases, double mutants with sus14(1242), which produces delayed lysis and a phage burst higher than normal, were used [11].

Minimal medium [12] and phage buffer [13] were as described.

Bacteriophage Φ29 and empty heads, labeled with \(^3\)H labeled amino acids, used as markers, were prepared as indicated in the preceding paper [14].
Proteins and Particles Synthesized in Ultraviolet-Irradiated B. subtilis su− after Infection with sus Mutants

B. subtilis 110NA su− was grown at 42 °C in a low-sulphate defined medium adapted for the incorporation of [35S]sulphate, containing (NH₄)₂SO₄ and ZnSO₄ at concentrations of 0.1 mM and 0.001 mM, respectively, supplemented with 0.1 mM amino acids except methionine and cysteine, which were not added. The cells were grown to a concentration of 10⁸/ml and concentrated 2-fold by centrifugation and resuspension in the same medium lacking glucose and amino acids. The cells were subsequently irradiated for 7.5 min as described [12]. After irradiation, the bacteria were further concentrated 2.5-fold to give a final concentration of 5x10⁸ cells/ml by centrifugation and resuspension in low-sulphate medium. The bacteria were infected with the different sus mutant at a multiplicity of 20 and shaken at 42 °C. Immediately after infection, carrier-free [35S]sulphate (15 μCi/ml) was added. After 150 min of incubation the bacteria were lysed by addition of lysozyme (500 µg/ml) and incubated for 1–2 min at 42 °C. An aliquot of the different lysates was immediately centrifuged in analytical sucrose gradients to analyze for DNA-containing and for DNA-free particles as described below; another aliquot was precipitated by addition of 10 vol. of acetone and the sample was prepared for polyacrylamide gel electrophoresis as described below.

DNA Synthesis in Φ29-Infected Bacteria in the Presence of 6-(p-Hydroxyphenylazo)-uracil

B. subtilis 110NA su− was grown at 42 °C in defined medium [12] until the cell concentration was 10⁸/ml. The cells were concentrated 2-fold by centrifugation and resuspension in the same medium except that the amino acids concentrations were 0.5 mM and that 6-(p-hydroxyphenylazo)-uracil (a gift from Dr N. C. Brown), to stop host DNA synthesis [15], was added. The cells were infected with the sus mutants at a multiplicity of 20 and labeled with [3H]thymidine (5 μCi/ml, 20 Ci/mmole) in the presence of uridine (200 μg/ml) [16]. At different times, aliquots were removed from the culture to assay for radioactivity insoluble in cold 5% trichloroacetic acid.

Labeling of Mutant Particles in the DNA and the Protein for Analytical Studies

B. subtilis 110NA su− was grown at 42 °C in defined medium, adapted for the incorporation of [35S]sulphate as described before. The cells were grown up to a concentration of 5x10⁷ bacteria/ml and concentrated 4-fold by centrifugation at room temperature and resuspension in the same medium containing 0.5 mM amino acids, except methionine and cysteine, in the presence of uridine (200 µg/ml). The cells were infected with the different mutants at a multiplicity of 20 and shaken at 42 °C. Immediately after infection, carrier-free [35S]sulphate (4 μCi/ml) was added and, at 15 min post-infection, [3H]thymidine (10 μCi/ml, 20 Ci/mmole) was added, both obtained from the Radiochemical Center, Amersham. 2 h after infection, a time at which phage development had reached a maximum, aliquots of 0.2 ml were removed from each culture, concentrated 2-fold by centrifugation at 42 °C and resuspension in a solution containing lysozyme (1 mg/ml in phage buffer) and the cells were lysed by incubation for 1 min at 42 °C. At different times after infection, aliquots were taken to follow phage development and the incorporation of [35S]sulphate and [3H]thymidine in cold 5% trichloroacetic acid. As a control, an uninfected culture was processed in the same way as the infected cultures.

Sucrose Gradient Centrifugation

Preparative Gradients. Linear 15–30% (w/v) sucrose gradients in phage buffer (7.5 ml of each solution) were prepared in 18-ml nitrocellulose tubes. 0.5–1 ml of the lysate was layered on the top of the gradient, and centrifugation was carried out at 4 °C and 88000 × g for 3 or 5 h depending on whether
DNA-containing or DNA-free particles were to be isolated. Fractions of about 0.5 ml were collected and the total radioactivity determined in an aliquot from each of them. The fractions containing the phage particles were either frozen for further analysis on polyacrylamide gel electrophoresis or dialyzed against phage buffer and stored at 4 °C. Electron microscopy of the particles was carried out both before and after dialysis.

Analytical Gradients. Linear 5–20% (w/v) sucrose gradients in phage buffer (2.5 ml of each solution) were prepared in 5-ml nitrocellulose tubes. About 0.2 ml of a solution containing the corresponding lysate and, as sedimentation markers, differently labeled phage Φ29 and/or empty heads purified from wild-type-infected cells, were layered on the top of the tube. Centrifugation was carried out at 4 °C either for 35 min at 114000 x g or for 55 min at 149000 x g depending on whether DNA-containing or DNA-free particles were being analyzed. Fractions of about 0.15 ml were collected in small vials and total radioactivity was determined in a scintillation counter after addition of 1 ml of a scintillation liquid composed of 2 vol. of a mixture containing 7 g of PPO and 0.4 g of dimethyl-POPOP per liter of toluene and 1 volume of Triton X-100.

RESULTS

Cistron 14

Mutant sus14(1242), which produces delayed lysis and a burst size higher than normal after artificial lysis [12] was used to prepare double mutants containing, in addition, the corresponding mutation to be analyzed in each case. The presence of the delayed lysis mutation is convenient to avoid spontaneous lysis. As already indicated in the preceding paper, the presence of the mutation sus14(1241) did not affect the results obtained with the different mutants. Mutant sus14(1242) differs from sus14(1241), used in the preceding paper, in that it lacks the two additional, functional mutations in cistrons 6 and 17, present in mutant sus14(1241) [12]. Since mutant sus14(1242) will represent the wild-type control the results obtained after infection with this mutant, under restrictive conditions, will be described first.

As shown in Fig. 1B and 2B, infection of B. subtilis sus with mutant sus14(1242) produces infective, DNA-containing particles, and DNA-free particles in a proportion approximately 80% and 20%, respectively. Uninfected cells do not show a peak of radioactivity in any of the two regions of the gradient (Fig. 1A). The DNA-containing particles are infective and have the same sedimentation coefficient as purified phage Φ29 run in the same gradient as a marker.
Fig. 1. Sucrose gradient centrifugation of lysates of B. subtilis su* infected with sus mutants. B. subtilis 110NA su* was infected with different sus mutants, labeled with [3H]thymidine (O---O) and [35S]sulphate (●●●) and lysed as described in Materials and Methods. Centrifugation was carried out in 5–20% sucrose gradients for 35 min at 114000 × g. In this and in the following gradients the direction of sedimentation is from right to left. (A) Uninfected cells; (B – K) cells infected with mutants sus14(1242), sus2(513), sus3(91), sus6(252), sus7(81), sus13(342), sus13(53), sus15(212), sus16(241) and sus17(112). In all cases the double mutant with sus14(1242) was used (Fig.3A). However, as already shown for mutant sus14(1241) [14], the DNA-free particles produced after infection with mutant sus14(1242) sediment faster than purified empty heads (Fig.3A); in fact, a double peak of radioactivity could be seen (Fig.3B) when the centrifugation was carried out for a longer time to separate the peak corresponding to DNA-free capsids from peak R, also present in uninfected cells and which was shown to contain ribosomes (see Fig.2C of the preceding paper [14]). The sedimentation coefficients of the faster and slower sedimenting peaks were 147 S and 131 S, respectively, using a value of 120 S for the purified wild-type empty heads [13]. The DNA-containing particles and the two kinds of DNA-free particles were analyzed by electron microscopy and slab gel electrophoresis. Fig.4A shows that the DNA-containing particles are identical to the corresponding particles produced after infection
Fig. 2. DNA synthesis and phage development of B. subtilis sus" infected with sus mutants. (A) B. subtilis 1108A sus" was infected with mutants sus14(1242) (●), sus7(112)sus14(1242) (□-□), or sus3(91)sus14(1242) (○-○) and labeled with [3H]thymidine in the presence of 6-(p-hydroxyphenylazo)-uracil as described in Materials and Methods. (B) The cells were grown and infected as in (A) except that the drug and radioactive thymidine were not added. Infection with mutant sus15(212)sus14(1242) (□-□, □-□) was also carried out. At different times, samples were removed from the culture and the phage titer was determined after lysis with lysozyme.

Fig. 3. Sucrose gradient centrifugation of a lysate of B. subtilis sus" infected with mutant sus14(1242). (A) B. subtilis sus" was infected with mutant sus14(1242), labeled with [35S]sulfate as described in Materials and Methods and the lysate centrifuged in a 5-20% sucrose gradient for 30 min at 114,000 x g. Purified wild-type phage and empty capsids [3H]-labeled were run as a marker in the same gradient. (B) The cells infected with mutant sus14(1242) were labeled with [14C]-labeled protein hydrolysate. Purified [3H]-labeled wild-type empty heads were run as a marker in the same gradient. Centrifugation, in a 5-20% sucrose gradient, was carried out for 55 min at 149,000 x g. R indicates the ribosome peak. (●-●) [14C] or [35S] radioactivity; (○-○) [3H] radioactivity.

Fig. 5A-C shows the autoradiograph of the slab gel electrophoresis of the three types of particles; as a control, the proteins induced in ultraviolet-irradiated Q29-infected cells are shown. The DNA-containing particles (Fig. 5A) have protein NP1 (neck appendages), TP1 (tail), HP1 (main head protein), NP3 (lower collar), NP2 (upper collar) and HP3 (head fibers). In addition, protein HP0, a new protein resolved in this electrophoresis system and shown to be present both in purified phage and empty heads [11], is also seen. The faster sedimenting peak (Fig. 5B) resolved from the DNA-free capsids contained all the phage structural proteins, although proteins NP1, TP1 and NP3 were present in smaller amounts than in the complete phage particles; in addition, they contained the two late nonstructural proteins p7 and p15 in approximately 80 and 3 copies per particle, respectively, and a small amount of protein p16. The slower sedimenting peak (Fig. 5C) contained all the phage structural proteins and the nonstructural proteins p7 (35 copies per particle), p15 and p16; the amount of the two last proteins was too small to be measured.
Cistrons Involved in the Synthesis of Phage DNA

To study the role of the newly synthesized phage DNA in the formation of the capsid, the particles produced after infection of \textit{B. subtilis} sus with \textit{sus} mutants in cistrons 2, 3 and 6, involved in DNA synthesis \cite{11,22}, were analyzed. As shown in Fig. 1C and D, mutants \textit{sus}2(513)sus14(1242) and \textit{sus}3(91)sus14- (1242) produce a very small amount of DNA-free particles. Although mutant \textit{sus}6(252)sus14(1242) produces a larger amount of particles (Fig. 1E), it was not studied further since it also contains a mutation in cistron 15 (data not shown).

The production of a small amount of particles observed after infection with the DNA-negative mutants could be due either to the synthesis of a proportionally small amount of phage structural proteins or to a lack of protein assembly in the absence of DNA replication. To test these possibilities the structural proteins synthesized after infection of ultraviolet-irradiated sus bacteria were analyzed by polyacrylamide gel electrophoresis. In the same experiment the amount of particles produced was also analyzed by sucrose gradient centrifugation. Fig. 6D shows the autoradiograph of the electrophoresis of the proteins induced after infection of ultraviolet-irradiated bacte-
Fig. 5. Autoradiograph of the proteins, separated by slab gel electrophoresis, present in the particles produced after infection with mutant sus14(1242). The DNA-containing particles and the faster and slower sedimenting peaks from the DNA-free particles produced after infection with mutant sus14(1242) were purified by sucrose gradient centrifugation and subjected to slab electrophoresis in gels containing a 10-20% acrylamide gradient as described in Materials and Methods. (A) DNA-containing particles. (B) Faster sedimenting peak from the DNA-free particles. (C) Slower sedimenting peak from the DNA-free particles. (D) Proteins induced in ultraviolet-irradiated bacteria infected with wild-type phage and labeled with 14C-labeled protein hydrolysate in a 20-28-min pulse.

As can be seen, the amount of structural proteins produced after infection with mutants in cistrons 2, 3 and 6 is proportional to the amount of protein HP1 synthesized, in a ratio similar to the wild-type control, mutant sus14(1242), or to mutant sus16(241) sus14(1242).

Cistron 7

Cistron 7 codes for a nonstructural protein, p7, (molecular weight 9000), synthesized in large amounts late after infection [11,12]. As already described,
Particles and proteins synthesized in ultraviolet-irradiated B. subtilis $su^-$ after infection with sus mutants. B. subtilis $su^-$ was irradiated with ultraviolet light, labeled with $[^35]S$-sulfate and infected with mutants $sus16(241)sus14(1242)$ (A), $sus2(513)sus14(1242)$ (B) or $sus3(91)sus14(1242)$ (C) as described in Materials and Methods. An aliquot of each lysate was centrifuged in a 5–20% sucrose gradient for 55 min at 149,000 $xg$. Another aliquot was subjected to slab electrophoresis in 10% acrylamide gels (D) as described in Materials and Methods: (a) $sus16(241)sus14(1242)$; (b) $sus2(513)sus14(1242)$; (c) $sus3(91)sus14(1242)$.

although this protein is not present in DNA-containing phage particles, it is present in DNA-free capsids produced after infection with mutants $sus14(1241)$ and $sus14(1242)$ and in the DNA-free capsids produced after infection with mutants in different cistrons, in a number of copies between 10 and 90 [7] (see also below and preceding paper [14]).

Sus mutants in cistron 7 do not produce particles as analyzed by sucrose gradient sedimentation (see Fig.1F) and electron microscopy, either of thin sections (Fig.1B) or of crude lysates. However, the amount of protein HP1 synthesized, as well as that of the other phage structural proteins, is similar to that produced by the wild-type control mutant $sus14(1242)$ (Table1).

Cistron 13

The protein product of cistron 13 has not yet been identified. Infection with mutants $sus13(53)sus14(1242)$ or $sus13(342)sus14(1242)$ produces approximately 10% of uninfective DNA-containing particles, the rest are DNA-free particles (Fig.1G and H). As shown in Table1, the amount of particles produced after infection with either of the two mutants is proportional to the amount of protein HP1 synthesized in a ratio similar to that of the wild-type control, mutant $sus14(1242)$. To study whether the presence of the small amount of DNA-containing particles reflected the situation in vivo and was not an artifact of the isolation procedure, electron microscopy of thin
Table 1. Correlation between total amount of particles and of protein HP1 synthesized in ultraviolet-irradiated B. subtilis suinfected with different sus mutants
In all cases the double mutant with sus14(1242) was used. The amount of protein HP1 was determined by weighing the corresponding peak from the densitometry of the autoradiograph as described in Materials and Methods. The total amount of particles produced was calculated from the radioactivity in the DNA-containing and/or DNA-free peaks from the sucrose gradients. The ratio in the last column was calculated by dividing the ratio particles/HP1 obtained for mutant sus14(1242) by that for each of the sus mutants.

<table>
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<th>Particles</th>
<th>Particles/HP1</th>
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<td>29720</td>
<td>320</td>
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sections of bacteria infected with mutants in cistron 13 was carried out. Fig. 10C shows the results obtained after infection with mutant sus13(342)sus14(1242); although some of the particles produced contained DNA, most of them were DNA-free (about 80%). The same results were obtained after infection with mutant sus13(53)sus14(1242). As a control, Fig. 10A shows an electron micrograph of thin sections of cells infected with mutant sus14(1242). About 80% of the particles contained DNA, a proportion similar to that obtained when the lysates are analyzed by sucrose gradient centrifugation.

To study the nature of the particles produced after infection with mutants in cistron 13, the DNA-free particles produced after infection with mutant sus13(53)sus14(1242) were purified by sucrose gradient centrifugation. As shown in Fig. 7B, they sediment as a broad peak, moving faster than purified wild-type empty heads, with a sedimentation coefficient of about 135 S. Fig. 8B shows an electron micrograph of a lysate of sus13(53)sus14(1242)-infected cells; most of the particles consist of empty phage particles, although some do not have tails.

Slab gel electrophoresis of the DNA-free particles isolated after infection with mutant sus13(53)-sus14(1242) showed that the faster sedimenting fractions contain all the phage structural proteins, including HP0, and, in addition, the nonstructural proteins p7, p15 and p16 in 6, 4 and 1 copies per particle, respectively. The slower sedimenting fractions also contain all the structural proteins and the nonstructural proteins p7 and p15 in 7 and 1 copies per particle, respectively (Fig. 9C). The amount of proteins NP1, TP1 and NP3 was higher in the last fractions than in the first ones, although in both cases the amount of protein TP1 was low.

Cistron 15

Cistron 15 codes for a late, nonstructural protein, of molecular weight 26,000, synthesized in large amounts [11, 12, 23]. This protein, although not present in the final phage particles, is present in the DNA-free particles produced after infection with the delayed lysis mutant sus14(241), with mutants in the cistrons coding for the structural proteins TP1, NP3 and NP1 (see preceding paper) and also in those pro-
duced after infection with mutants in cistron 13, as well as in the more rapidly sedimenting peak of DNA-free particles produced after infection with the delayed lysis mutant sus14(1242).

Protein p15 is partially dispensable under certain conditions since the burst size produced after infection with mutant sus15(212)sus14(1242) in defined medium is only approximately twice as low as that produced after infection with mutant sus14(1242) (see Fig. 2B), although no protein p15 was synthesized (data not shown). However, under different growth conditions, like those used for complementation experiments, protein p15 is essential for phage development [8].

Infection of B. subtilis su with mutant sus15(212)-sus14(1242) produces approximately 70% and 30% of infective DNA-containing particles and DNA-free heads, respectively, when analyzed by sucrose gradient centrifugation (Fig. 1I). When thin sections of cells infected with the same mutant are analyzed with the electron microscope, essentially the same result is obtained (60% and 40% of DNA-containing and DNA-free particles, respectively) (Fig. 10D). As in other cases, the DNA-free particles produced after

![Electron micrographs of the particles produced after infection with mutants sus3(91)sus14(1242), sus13(53)sus14(1242) and sus16(241)sus14(1242). (A) Purified particles from sus3(91)sus14(1242)-infected bacteria. (B) Lysate from sus13(53)sus14(1242)-infected bacteria. (C) Purified particles from sus16(241)sus14(1242)-infected bacteria. The scale line represents 0.1 μm.](image-url)
infection with mutant sus3(91)sus14(1242) sediment faster than purified empty heads. Electron microscopy of lysates of sus15(212)sus14(1242)-infected cells showed the presence of normal phage particles and a smaller proportion of prolate heads, more rounded at the corners than complete phage particles, many of which contained an internal core. As shown in Table 1, the amount of particles produced is proportional to the amount of protein HP1 synthesized in a ratio slightly higher than that of the wild-type control mutant sus14(1242). By slab gel electrophoresis the DNA-containing and DNA-free particles produced by mutant sus15(212)sus14(1242) were shown to contain the same proteins as the particles produced after infection with mutant sus14(1242), except that protein p15 was not present (data not shown).

**Cistron 16**

Cistron 16 codes for a late, nonstructural protein, p16, with molecular weight of about 35,000 [11, 16, 23]. Infection of B. subtilis su- with mutant sus16(241)-sus14(1242) produces 100% DNA-free heads as analyzed by sucrose gradient centrifugation (Fig. 1J) and electron microscopy of thin sections of infected cells (Fig. 10E). The sedimentation rate of the 16-particles is 135 S, higher than that of purified empty heads (Fig. 7C). When these particles were examined with the electron microscope they were shown to consist of prolate heads, more rounded at the corners than complete phage heads, with an internal core (Fig. 8C). Analysis by slab gel electrophoresis showed that they contained proteins HP0, HP1, NP2 and HP3 and also the nonstructural protein p7 (Fig. 9D). The number of copies of protein p7 per particle is approximately 90.

**Cistron 17**

Infection of B. subtilis su- with mutant sus17(112) does not give place to the synthesis of protein p17, which is an early protein essential for Φ29 replication [11]. However, when the double mutant sus17(112)-sus14(1242), used in the present work, infected B. subtilis su-, although no protein p17 was synthesized (data not shown), some phage DNA synthesis took place (Fig. 2A) in accordance with the production of a phage burst of 15% relative to that produced after infection with mutant sus14(1242) (see Fig. 2B). As a control, the DNA-synthesis and phage burst produced after infection with mutants sus14(1242) and sus3(91)sus14(1242) is shown (Fig. 2A and B). One possible interpretation of these results is that protein p17 is partially dispensable and, when the lysis of the bacteria is inhibited, an unidentified bacterial function replaces, to some extent, the function of protein p17.

The proportion of DNA-containing and DNA-free particles produced after infection with mutant sus17(112)sus14(1242) was 40% and 60%, respectively, when analyzed by sucrose gradient centrifugation.
Fig. 10. Electron micrographs of thin sections of B. subtilis sus− infected with sus mutants. Thin sections of B. subtilis sus− infected with different sus mutants were prepared as described in Materials and Methods. The scale line represents 0.1 μm. (A) Infection with mutant sus14(1242), (B) sus7(81)sus14(1242), (C) sus13(342)sus14(1242), (D) sus15(212)sus14(1242), (E) sus16(241)sus14(1242), (F) sus17(112)sus14(1242). Between 500 and 1000 particles in about 20 different cells were counted in each case to measure the DNA-containing and DNA-free particles.

(Fig. 1 K) and 25% and 75% when thin sections of infected cells were analyzed with the electron microscope (Fig. 10 F). As shown in Table 1, the amount of particles produced is proportional to the amount of protein HP1 synthesized in a ratio similar to that of the wild-type control, mutant sus14(1242). The DNA-containing and DNA-free particles produced after infection with mutant sus17(112)sus14(1242) were similar to those produced after infection with mutant sus14(1242), when analyzed by polyacrylamide gel electrophoresis (data not shown).

DISCUSSION

In the studies reported in this paper mutant sus14(1242) was considered as a wild-type control.
since it behaves like wild-type phage except that, when it infects *B. subtilis* su', the lysis is delayed. About 80% of the total amount of particles produced after infection with this mutant contain DNA and the rest of the particles lack DNA. By sucrose gradient centrifugation the DNA-free particles are resolved into two components with sedimentation coefficients 147 S and 131 S, both of them larger than that of purified wild-type empty heads. The 147-S peak consists mainly of particles which appear more rounded at the corners than complete phage capsids and contain an electron-dense internal core. The structural proteins present in these particles are the head proteins HP0, HP1 and HP3 and the upper collar protein NP2, the rest of the structural proteins being present in a small amount; these particles contain in addition proteins p7 and p15, not present in complete phage particles, in approximately 80 and 3 copies, respectively, and a small amount of protein p16. As already discussed in the preceding paper, p7 probably forms the internal core seen with the electron microscope and these particles might represent a prohead-type of structure, ready for DNA encapsulation, similar to that described in other phages [1–6]. The 131-S peak resembles more the composition of complete phage particles, having a larger amount of proteins NP1, TP1 and NP3 and smaller amounts of proteins p7, p15 and p16, relative to the 147-S peak. Under the electron microscope, these particles appear mainly as empty phage and heads, and they are probably derived from phage which has lost its DNA.

Mutants in cistron 7, coding for a late protein [11], not present in the final phage particle, do not produce phage heads or tail-neck complexes, even though all the phage structural proteins are synthesized in normal amounts. These results suggest that protein p7 plays an essential role in head assembly. In accordance with this is the fact that protein p7 is present in the DNA-free particles accumulated after infection with several mutants, as well as in the prohead type of structures produced after infection with mutants sus14(1241) or sus14(1242), which behave like wild-type phage. Protein p7 is not present in any DNA-containing heads, suggesting that it is degraded or released from the phage heads prior to or simultaneously with the encapsulation of the DNA. Protein p7 resembles protein p8 from phage P22 in several ways: it is required for the assembly of the coat protein into heads; it is transiently incorporated in phage heads, being released or degraded, probably upon DNA encapsulation; its location in the genetic map is contiguous to the gene coding for the major head protein [3, 24, 25, 9].

The mutants negative in DNA synthesis studied, corresponding to three different cistrons, produce an amount of particles which is related to the amount of phage structural proteins synthesized. These results discard the possibility that *de novo* synthesis of phage DNA is needed for the assembly of phage proheads. These particles, like those produced after infection with mutant sus16(241)sus14(1242), contain an electron-dense internal core and they are formed by the structural proteins HP0, HP1, HP3 and NP2 and the nonstructural protein p7; the number of copies of p7 is approximately 20 in the case of mutant sus3(91)-sus14(1242), 30 for mutant sus2(513)sus14(1242) (data not shown) and 90 for mutant sus16(241)sus14(1242). As seen in Fig. 7A, the sedimentation rate of the heads produced after infection with mutant sus3(91)sus14(1242), as with mutant sus2(513)sus14(1242) (data not shown), is slightly smaller than that of the heads formed after infection with mutant sus16(241)sus14(1242), suggesting that the higher sedimentation coefficient of the heads having an internal core relative to the purified empty heads could be related to the existence of a larger amount of protein p7. The reason for the smaller amount of protein p7 in the particles produced after infection with the DNA-negative mutants relative to that present in the particles formed after infection with mutant sus16(241)sus14(1242) is unknown. The formation of DNA-free heads in the latter case could be due to the possibility that protein p16 has a role either in the maturation of phage DNA or in the encapsulation process.

It is interesting to note that the particles produced after infection either with the DNA-negative mutants or with mutants in cistron 16, do not contain proteins p15 and p16, unlike the particles produced after infection with mutants in cistrons 14 (wild-type infection) or with mutants in cistrons 9, 10 (in the case of the prolate heads produced), 11, 12 and 13 (see also preceding paper [14]). These results suggest that proteins p15 and p16 are assembled in phage heads already containing protein p7 in a step prior to or concomitant with DNA encapsulation. However, we cannot say at present whether or not p7 is released from the head at the time of the assembly of p15 and p16 or later on, when the DNA is encapsulated.

Thin sections of mutant sus16(241)sus14(1242)-infected cells show that the heads produced are found throughout the cytoplasm (see Fig. 10E), in a way similar to the proheads of phage P22 [26] and different from that which happens in the case of the Tau particles of phage T4, which are found attached to the cell membrane [27].

Infection with sus mutants in cistron 13 produces about 10% of uninfected DNA-containing particles, the rest being DNA-free phage particles with or without tails. To study whether the small proportion of DNA-containing particles was due to instability of the phage DNA during the isolation procedure, thin sections of cells infected with sus mutants in cistron 13 were analyzed with the electron microscope. In agreement with the above results, approx-
imate 20% of the total particles contained DNA, which means that if the particles are empty because of instability of the DNA, the exit of the DNA from the phage particle might take place within the cell or during the preparation of the thin sections. This resembles the case of mutants in cistrons 10 and 26 from phage P22 in which empty particles are accumulated due to instability of the DNA in the particle within the cell [26]. The analysis by polyacrylamide gel electrophoresis of the DNA-free particles produced after infection with sus mutants in cistron 13 showed that the faster sedimenting fractions of the peak contained mainly the phage structural proteins HP0, HP1, NP2 and HP3 and a small amount of proteins NP1, TP1 and NP3. The amount of protein p7 in this peak was very low, 6 subunits per particle compared with 80 subunits present in the 147-S peak produced after infection with mutant sus14(1242). Proteins p15 and p16 were present in about 4 and 1 copies per particle, respectively. The slower sedimenting fractions resembled the 131-S peak produced after sus14(1242)-infection and contained a larger amount of proteins NP1, TP1 and NP3 than the faster sedimenting fractions; a small amount of proteins p7 and p15 were also present (7 and 1 copies, respectively). If the exit of protein p7 from the phage heads takes place when DNA is encapsulated, the small amount of this protein present in the particles suggests that many of them have lost the encapsulated DNA. On the other hand, the amount of protein TP1 in these particles is smaller than expected from the proportion of phages with tails seen with the electron microscope in lysates from cells infected with sus mutants in this cistron. These results suggest that the tail protein is unstable in those particles, being lost to a certain extent in the purification process. In the case of mutants in cistrons 10 and 26 from phage P22, the particles produced after infection with these mutants are rescued by complementation in vitro; this means that the particles with DNA, though unstable, are true intermediates in phage morphogenesis [24]. Similar complementation experiments in vitro will have to be carried out with mutants in cistron 13 to determine whether or not the particles produced are intermediates in phage φ29 morphogenesis.

Cistron 15 codes for a protein, p15, which is partially dispensable under certain growth conditions, since the burst size produced after infection with mutant sus15(212)sus14(1242) is only approximately twice as low as that produced after infection with the wild-type control mutant sus14(1242). Protein p15, although not present in mature phage particles, is present in the prohead-like particles produced after infection with mutant sus14(1242) as well as in the heads accumulated after infection with mutants in cistrons 9, 10 (in the prolate heads produced), 11, 12 and 13. Since protein p15 has affinity either for native or denatured DNA (unpublished results), a possible role for this protein is in the process of DNA encapsulation.

**Morphogenetic Route for the Assembly of Phage φ29**

The results presented in this and in the previous paper [14] suggest a preliminary morphogenetic route for the assembly of phage φ29, shown in Fig. 11. Protein p7 and the major head protein, HP1, interact in order to get head assembly. The fiber protein HP3 does not seem to be needed for head assembly since mutants lacking this protein produce fiber-less infective phage particles (Anderson and Reilly, personal communication). In order to get a prolate head, the upper collar protein NP2 must be functional since mutants in cistron 10, coding for this protein, produce isometric heads; these heads have an electron-dense internal core and they contain, apart from protein HP0 and the modified proteins HP1 and HP3, named HP1* and HP3*, the nonstructural protein p7, suggesting that this protein forms the internal core. In the absence of DNA synthesis or of protein p16 a prolate head more rounded at the corners than complete phage particles and with an internal core, is accumulated. It contains the head proteins, the upper collar protein and the nonstructural protein p7, again suggesting that this protein forms the internal core present in the particles. We do not know presently whether these particles are normal intermediates in phage morphogenesis or abortive structures.

In order to get DNA encapsulation, the tail protein TP1 must also be functional since in either sus or ts mutants in cistron 9 a prolate head, more rounded at the corners than complete phage heads, with an internal core is produced containing proteins HP0, HP1*, HP3*, NP2, p7, p15 and p16.

Another prohead-like structure with an internal core has been observed; it contains the head proteins, the upper collar protein and the nonstructural proteins p7, p15 and p16. These heads are obtained after infection with sus mutants in cistron 14 (wild-type infection) or with mutants in cistrons 11 and 12. They could represent heads in the process of encapsulation of the DNA which lost it during the isolation process of the particles; proteins p15 and p16 might enter into the heads together with the DNA and, at the same time, protein p7 could be released. Once the DNA has been completely encapsulated, the release of proteins p15 and p16 could take place. We do not know presently whether proteins p7, p15 and p16 are recycled as in the case of protein p8 from phage P22 [25] or if they are degraded as happens with protein p22 from phage T4 [28].

Mutants in cistron 11, coding for the lower collagen protein NP3, produce DNA-containing heads formed by the head proteins and the upper collar protein.
Fig. 11. Morphogenetic route for the assembly of phage Φ29. The particles between the heavy lines represent intermediates in phage assembly. The continuous lines with the names of the different proteins represent the moment of action of the protein. The dashed lines represent the particles accumulated after infection with the different mutants. The parenthesis in protein p15 indicates that it may be dispensable. The particles between brackets represent a possible intermediate in the process of DNA encapsulation. The dotted lines indicate the moment of exit of proteins p7, p15 and p16 from the phage heads. The proteins present in the different particles are indicated below each drawing. The points within the particles represent the internal core and the circles represent DNA.

No tail protein can be detected in these particles. We do not know at present whether this is due to instability of the assembled tail protein in the absence of the lower collar protein or that the tail protein is assembled at the same time or after the lower collar protein.

Either for a stable DNA encapsulation or for a stable tail assembly, the protein product of cistron 13 must be functional since mutants in this cistron produce 10% of uninfected DNA-containing particles, the rest of the structures accumulated being mainly DNA-free particles, with and without tails; many of these particles come from phage which have lost the DNA since the amount of protein p7 present in them is very low and no internal core is seen with the electron microscope.

Finally, mutants in the cistron coding for the precursor of the neck appendages, P-NP1, produce normal amounts of DNA-containing particles having all the phage structural proteins except the neck appendages, indicating that this protein is the last structural component assembled into the phage particles. A similar conclusion has been reached by complementation in vitro [29] (and unpublished results).

The results presented suggest the existence of a single morphogenetic route for the assembly of phage Φ29.

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