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Multifunctional Roles of a Bacteriophage ϕ 29 Morphogenetic Factor in Assembly and Infection

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SUMMARY

Low copy number proteins within macromolecular complexes, such as viruses, can be critical to biological function while comprising a minimal mass fraction of the complex. The *Bacillus subtilis* dsDNA bacteriophage ϕ 29 gene 13 product (gp13), previously undetected in the virion, was identified and localized to the distal tip of the tail knob. Western blots and immuno-electron microscopy detected a few copies of gp13 in ϕ 29, DNA-free particles, purified tails, and defective particles produced in suppressor-sensitive (*sus*) mutant *sus13(330)* infections. Particles assembled in the absence of intact gp13 (*sus13(342)* and *sus13(330)*) had the gross morphology of ϕ 29 but were noninfectious. gp13 has predicted structural homology and sequence similarity to the M23 metalloprotease LytM. Poised at the tip of the ϕ 29 tail knob, gp13 may serve as a plug to help restrain the highly pressurized packaged genome. Also, in this position, gp13 may be the first virion protein to contact the cell wall in infection, acting as a pilot protein to depolymerize the cell wall. gp13 may facilitate juxtaposing of the tail knob onto the cytoplasmic membrane and the triggering of genome injection.

Keywords

bacteriophage ϕ 29; morphogenetic factor; immuno-electron microscopy; tail protein; endopeptidase

INTRODUCTION

To understand in detail the events of morphogenesis and infection of viruses, such as the bacteriophage ϕ 29, all components of the virion involved in these processes must be identified. This is not trivial, as minor proteins present in only a few copies may be confused with contaminants or be obscured by major proteins. Here the ϕ 29 gene product 13 (gp13),

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previously identified as a morphogenetic factor in $\phi 29$ assembly,¹ is identified as a minor $\phi 29$ tail component that has escaped detection for more than three decades since identification of the major structural components of the virion.^{2, 3}

Phage $\phi 29$ morphogenesis begins with formation of the dodecameric head-tail connector (gene product 10, gp10), onto which the scaffolding (gp7), capsid (gp8) and head fiber (gp8.5) proteins assemble to form the 54×42 nm prolate prohead shell⁴⁻⁶ (Figure 1). Attachment of an oligomer of prohead RNA (pRNA) to the connector yields the mature prohead.^{7, 8} Multiple copies of the ATPase gp16 attach to the pRNA to constitute the packaging motor, and hydrolysis of ATP powers the translocation of the dsDNA genome with its covalently-bound gp3 (DNA-gp3)⁹ into the prohead with scaffold (gp7) exit.¹⁰⁻¹² Assembly of the lower collar (gp11) to the DNA-filled head¹³⁻¹⁵ triggers pRNA/gp16 release.¹⁶ Finally, assembly of the tail knob (gp9) and twelve appendages (gp12*, cleaved from the gp12 precursor)^{17, 18} yields the infectious virion.^{2, 4, 19, 20}

gp13 is required for $\phi 29$ assembly, and its activity as a morphogenetic factor, which occurs *in vivo* but not in extract complementation, co-purifies with the tail knob (gp9).¹ Using restrictive infections with suppressor-sensitive (*sus*) $\phi 29$ mutants, purified particles from a tail knob-defective lysate (*sus9*(756)) with functional gp13 ($9^- 13^+$) do not form infectious virions when mixed with an extract (*sus13*(342)) that lacks functional gp13 ($13^- 9^+$) and vice versa.¹ In contrast, complementation does occur with most virion proteins: filled heads isolated from a lower collar-defective lysate ($11^- 8^+$) form infectious phages when incubated with a capsid-defective extract ($8^- 11^+$) that provides gene products 11, 9, 13 and 12*, showing that gp8 activity in morphogenesis is not gp11 dependent nor vice versa.

gp13 was hypothesized to be a previously undetected tail component because the lower collar and tail tube of $\phi 29$ (Figure 1), reported to consist of gp11,²¹ appears to have more mass in cryo-electron microscopy reconstruction²² than can be accounted for by gp11 alone (compared to the dodecameric connector comprised of subunits of similar mass²³). Here, identification of gp13 as a $\phi 29$ structural component and its localization to the distal end of the tail knob was accomplished by the use of gp13-specific antibodies in Western blots and by immuno-electron microscopy of $\phi 29$, isolated tails, and defective particles produced in restrictive infection with *sus13*(342) and *sus13*(330) mutants. Previous investigations¹ found that assembly of an active tail knob requires both gp9 and gp13, and the present study demonstrates that the active knob contains both gp9 and gp13. Therefore the knob is a gp9/gp13 complex that is likely fixed *en bloc* onto the lower collar and tail tube before gp12* addition, defining a sub-assembly pathway for $\phi 29$. The use of specific antibody and electron microscopy to localize gp13 at the distal tip of the tail knob of $\phi 29$, a method pioneered to identify gene products on phage T4,²⁴ is applicable to the identification and localization of minor protein components with critical roles in assembly and function of other viruses and macromolecular complexes. Moreover, the method is applicable to detecting minor proteins in assembly intermediates and as a tool in unraveling assembly pathways.

Secondary structure predictions of gp13 suggest N-terminal α -helices and C-terminal β -strands. The C-terminal region shows sequence alignment to the zinc-metalloprotease LytM of *Staphylococcus aureus* (PDB: 1qwy) and a similarity in arrangement of β -strands. Biochemical identification of gp13 as an endopeptidase (Cohen et al., in preparation) provides direct evidence that gp13 has enzymatic activity analogous to LytM activity on the Staphylococcal peptidoglycan peptide cross-link that may be needed for the tail to penetrate the *Bacillus subtilis* cell wall during $\phi 29$ infection.

RESULTS

gp13 is a previously unidentified structural component of ϕ 29

An abbreviated ϕ 29 assembly pathway is shown in Figure 1(a), and a cryo-electron microscopy three-dimensional reconstruction of ϕ 29 is shown in Figure 1(b), which illustrates the six visible protein components and the packaged genome: capsid (gp8), head fibers (gp8.5), head-tail connector (gp10), lower collar and tail tube (gp11), knob (gp9), appendages (gp12*) and DNA-gp3. gp13 was demonstrated to be a seventh structural component of ϕ 29 by the use of gp13-specific polyclonal antiserum (anti-gp13 serum) in Western blot analysis (Figure 2). Following LDS-PAGE, blots of various amounts of ϕ 29, proheads, and purified gp13 were incubated with anti-gp13 serum (Figure 2(a)) and developed with enzyme-linked secondary antibody (Methods). Mature ϕ 29 particles showed gp13-specific signal (lanes 1-3), while an equivalent number of proheads did not (lanes 4-6). Dilutions of purified gp13 served as a positive control (lanes 7-10). Re-probing with anti-connector (anti-gp10) serum served as a loading control. These results demonstrated that gp13 is present in ϕ 29 and is assembled after prohead formation and during tail assembly.

To demonstrate that gp13 is a tail component, a blot that included ϕ 29, proheads, particles emptied of DNA (ghosts), and isolated tails (gp10, 11, 12*, and 9) was probed with anti-gp13 serum (Figure 2(b) top). gp13 signal was observed in ϕ 29 (lanes 2-4 and 15), ghosts (lanes 7 and 8) and tails (lanes 9 and 10) but not in proheads (lanes 5 and 6). Signal with purified gp13 (lane 14) at approximately 41kDa marked the appropriate migration distance of gp13, in accordance with its sequence-predicted mass (Table 2). The purified proteins gp9, gp11 and gp13 (lanes 11, 13, 14) and purified connectors (gp10) (lane 12) served as controls both for loading and for cross-reactivity. Approximately equivalent numbers of ϕ 29, ghosts and tails were loaded as shown by gp9 signal with anti-gp9 serum (Figure 2(b) bottom). Additionally, anti-gp9 serum did not cross-react with gp13 (Figure 2(b) bottom), nor did anti-gp13 serum cross-react with gp9 (Figure 2(b) top). Tail preparations were confirmed by TEM and SDS-PAGE to lack proheads and phages (data not shown). The results demonstrated that gp13 is a structural component of the ϕ 29 tail, not previously recognized,^{1, 13} and led to experiments to localize gp13 in the ϕ 29 tail.

gp13 was localized in the ϕ 29 tail by immuno-electron microscopy

To localize gp13 in the tail, immuno-electron microscopy was utilized as described (Methods). Anti-gp13 IgGs were used to decorate mutant particles lacking the gp12* appendages, which serve as adsorption organelles (12⁻ particles; Figure 1), and this permitted access of the antibodies to the lower collar and tail tube. Particle/antibody complexes were observed by negative staining and transmission electron microscopy (TEM) (Figure 3 (a, c)). Appendageless 12⁻ particles formed antibody-dependent tail-bound aggregates when incubated with anti-gp13 IgG (Figure 3(a)), while 12⁻ particles incubated with pre-immune IgG (Figure 3(b)) and 12⁻ particles alone (data not shown) adsorbed randomly to the support film. Wild-type ϕ 29 particles behave similarly (Figure 7(b)). Additionally, the distal ends of the tail knobs were obscured in the presence of anti-gp13 IgG (Figure 3(a, i)), while particles treated with pre-immune IgG retained the sharp knob ends (Figure 3(b, i)). To confirm that antibodies caused the aggregation 12⁻ particles decorated with biotinylated antibodies were separated from excess antibody by HPLC (Methods), labeled with streptavidin-gold (6 nm), and observed by TEM. Electron dense gold spheres were observed only closely associated with ϕ 29 on the distal end of tails (Figure 3(c)). As a control, 12⁻ particles were decorated with biotinylated anti-capsid (anti-gp8) IgG and labeled with streptavidin-gold; no tail bound aggregates were observed, and particles aggregated by their heads were tagged with gold (data not shown). The results demonstrated that gp13 epitopes are present at the distal end of the ϕ 29 tail knob.

Prediction of gp13 secondary structure

The gp13 secondary structure was predicted by use of the Psipred and Profsec algorithms through the BioInfobank Metaserver (Methods) (Figure 4, α -helices ['H', red], β -strands ['E', blue]). A crystal structure database search using mGenTHREADER and 3D Jury yielded a significant homology hit by both methods. LytM (Figure 4, line 4) has 14% identity with gp13 and scored 0.764 (p -value = 3×10^{-8}) by mGenTHREADER analysis.

LytM is a Zn^{2+} metalloendopeptidase that has a His-x-His motif common to the M23 proteases.^{25, 26} gp13 has a His-Trp-His motif (Figure 4, circled), and alignment of this motif with the His-Val-His of LytM places all eight beta-strands of LytM in register with predicted beta-strands in gp13 (Figure 4, lines 2-4). This may suggest a role for gp13 as a Zn^{2+} -binding metalloendopeptidase. The Zn^{2+} ligands of active LytM have been demonstrated to involve three amino acids (His210, Asp214 and His293 in the His-x-His) and a fourth ligand that is a phosphate ion coordinated by three additional residues (His260, Asn286 and His291 in the His-x-His).²⁷ Three putative gp13 Zn-binding ligands were identified in gene 13 by sequence homology to LytM (His188, Asp195, His280 in the His-x-His) (Figure 4, squares). Homologous putative phosphate binding residues that enable formation of the fourth Zn^{2+} ligand were also observed in the gene 13 sequence (His247, Asn273 and His278 in the His-x-His) (Figure 4, triangles).

Sequencing of gene 13 suppressor-sensitive mutants

To assess further the role of gp13 in ϕ 29 morphogenesis and infection, six suppressor-sensitive (*sus*) mutants that map to gene 13 were sequenced. Notably, 51 of 133 *sus* mutants reported²⁸ were in gene 13. Purified mutant genomes were sequenced by the use of four primers (Methods), permitting bidirectional double coverage through gene 13. The results (Table 2; Figure 4, top line) demonstrated that each mutant contained a C/T transition within the glutamine codon CAA, generating the nonsense codon TAA (ochre) and resulting in a truncated polypeptide. The *sus*13(55) and *sus*13(330) mutants were isolated separately but contained the same mutation. The mutants were predicted to produce protein fragments of variable length (ochre fragments) (Table 2), and mutants predicted to produce the longest (*sus*13(330)) and shortest (*sus*13(342)) ochre fragments were chosen for further study.

In comparison with the published gene 13 sequence,²⁹ two amino acid substitutions were detected in all eight gp13 genes sequenced for this study (two wt, from *sus*8.5(900)*sus*14(1241) and *sus*12(305)*sus*14(1241) and the six mutants of Figure 4). The aspartic acid at position 89 is asparagine, and the threonine at position 353 is isoleucine. The difference between the published sequence and our data may have arisen from lab strain differences or advancements in DNA sequencing fidelities during the last 20 years. These differences are present as italic letters in Figure 4 and do not change the secondary structure prediction (both the published and newly determined gene 13 sequence produced the same result [data not shown]).

*sus*13(330) mutant particles contain the long gp13 ochre fragment

To detect assembly of ochre fragments of gp13 into particles produced in restrictive *sus* mutant infections, DNA-filled particles were isolated by isopycnic CsCl density gradient centrifugation and their gp13 content assessed by Western blot analysis using anti-gp13 serum. gp13-specific signal was observed in the defective *sus*13(330) particles (Figure 5, lanes 4 and 5) above 37 kDa (see lane 1 marker), consistent with the predicted size of the ochre fragment (Table 2). In contrast, the defective *sus*13(342) particles did not show a gp13 signal, although the predicted 3.8kDa fragment (Table 2) might have run off the gel, passed through the membrane in blotting, or have fewer epitopes. Signal from the connector (gp10) on interaction with anti-gp10 serum served as a loading control (Figure 5, bottom).

***sus13(330)* mutant particles have a distinctive tail knob structure**

The phenotype of gp13-defective particles produced in restrictive infection with the mutants *sus13(342)* and *sus13(330)* is a DNA-filled particle with all of the structural proteins of earlier $\phi 29$ models,⁴ but is unstable, losing DNA and sometimes the tail tube-knob complex in sucrose density gradient centrifugation and in negative staining for TEM.^{1, 3} DNA-containing particles were preserved and purified for TEM, albeit with variable efficiency, by the use of isopycnic density gradient centrifugation in CsCl.^{19, 30} The defective particles of both mutants resembled $\phi 29$ (Figure 6). However, the *sus13(330)* particles differed in having “notched” tail knobs (Figure 6, dashed arrows). The mass distal to the notch has a cone-shaped structure in a cryo-EM 3D-reconstruction of the *sus13(330)* defective particle (Xiang et al., in preparation) that resembles the structure observed in chemically emptied (ghosted) wt $\phi 29$.²² Since the *sus13(330)* particle is aggregated by anti-gp9 IgG but not anti-gp13 IgG (Table 3, expt. 2 and 3), a possible explanation is that the cone-shaped structure represents a misassembly or rearrangement of a gp9 knob subunit that blocks access of antibody to the truncated gp13.

Tails of *sus13(342)* mutant particles contain the short ochre fragment of gp13

Because *sus13(342)* particles did not show gp13 signal by Western blot (Figure 5), attempts were made to detect gp13 by immuno-EM. *sus13(342)* particles were incubated with anti-gp13 IgG-biotin and observed by negative staining in TEM (Figure 7(a)). As a control, wt $\phi 29$ particles were incubated with anti-gp13 IgG-biotin (Figure 7(b)). An average of 96% of wild-type particles aggregated, while an average of only 20% of *sus13(342)* particles aggregated, a difference of greater than 4-fold (Table 3). In addition to the aggregation frequency, genome retention capacity was measured for each type of particle (Table 3). While 96% of wt $\phi 29$ retained the genome under EM staining conditions, gp13 defective particles variably lost the packaged genome (Table 3). In two EM preparations of *sus13(342)* particles, mounts of freshly prepared particles on support films (within two days of isolation) showed 81% DNA-retention (Table 3, expt. 2, 13(342)) while 24% of thirty day-old particles retained the packaged genome (Table 3, expt. 1, 13(342)). Freshly-mounted *sus13(342)* particles aggregated at 63% (Table 3, expt. 2), and thus the aggregation frequency was similar to the genome retention. The aggregation of the *sus13(342)* particles suggested that the short 3.8kDa fragment (Table 2) of gp13 from the N-terminus, although expected to display fewer epitopes, is sufficiently exposed in the assembled particle. However, of the *sus13(342)* particles observed, generally only those that contained DNA participated in aggregation; empty *sus13(342)* particles aggregated infrequently (Figure 7(a); Table 3). Thus, the 3.8 kDa *sus13(342)* ochre fragment may exit with the DNA. Taken together, these observations suggest that gp13 also serves as a plug that aids genome retention in $\phi 29$.

DISCUSSION

A sensitive method with general applicability for identifying and localizing a minor protein structural constituent of a virus or other macromolecular complex is described. Western blot analyses and immuno-electron microscopy were used to identify gp13 as a previously undetected structural component of the phage $\phi 29$ tail, in addition to its role as a morphogenetic factor in assembly of the tail knob.¹ gp13 was identified in $\phi 29$, ghosts, isolated tails and defective *sus* mutant particles by the use of gp13-specific antibodies, but not in proheads, becoming the eighth virion protein in the linear $\phi 29$ assembly pathway first proposed by Mendez et al.² Mutant studies confirmed that gp13 is in the tail, as the predicted long ochre fragment of gp13 produced in restrictive infection with the mutant *sus13(330)* was identified in defective particles by Western blot analysis. Previous studies of gp13^{1, 3, 15, 31} likely did not identify gp13 in virion or knob preparations because in some PAGE conditions tested for this study, purified gp13 co-migrated with gp10 and gp11 despite a 5-7kDa predicted difference in size (data not shown). A similar co-migration artifact has been reported for T4 gp11 and

gp26³² and T7 proteins 6.7 and 13³³ whose predicted mass differences are also ~6kDa. The use of gp13-specific antibodies provided the sensitivity required for gp13 detection. Historical literature review demonstrated an unidentified band between the connector (gp10) and lower collar (gp11) proteins in an autoradiogram of purified ϕ 29 that may, in hindsight, have been gp13.¹³

The *in vitro* assembly activity of gp13 copurifies with gp9, yet the ϕ 29 tail knob (gp9) assembles in cells infected with the mutant *sus13*(342), which produces a small 3.8kDa ochre fragment of gp13, to yield noninfectious particles that resemble ϕ 29¹ (Figs. 4, 6 and 7; Table 1). [Similarly, the T7 gene product 6.7 has been suggested to be essential for infectivity but not for formation of morphologically normal particles.³³] To produce infectious ϕ 29, it was found that gp9 must interact with gp13 *in vivo*, suggesting that gp13 is a morphogenetic assembly factor.¹ The λ tail protein gpM appears as a morphogenetic factor by the same rationale: gpM⁻ lysates (gpG⁺ gpH⁺) cannot complement gpG⁻ (gpM⁺ gpH⁺) nor gpH⁻ (gpM⁺ gpG⁺) extracts from λ .³⁴ While gpG is not a virion component, the presence or absence of gpM in the λ virion remains unclear³⁵ and may be studied with antibody localization.

The present work shows that gp13 is a virion protein that likely co-assembles with gp9 to produce the tail knob. The gp13/gp9 knob complex then attaches *en bloc* to the tail tube during ϕ 29 morphogenesis. Attempted sequential addition of tail components gp9 and gp13 to assembly intermediates has utilized expression of ϕ 29 genes in *E. coli* from restriction fragments that contain flanking genes.^{36, 37} The authors have argued for the ordering of gp9 activity prior to gp13; however, because partially assembled complexes were not purified away from residual proteins in the lysates, unreacted gp9 or gp13 may have remained available.^{36, 37} In previous reports involving gp13 defective lysates^{1, 3} the defective gp13 protein may have acted as a dominant-negative inactivating functional gp9 from subsequent reaction with wt gp13 and vice versa. The purported purified component experiments of Lee et al.^{36, 37} did not test if defective gp13 blocks gp9 action or vice versa.

Incorporation of gp13 into ϕ 29 occurs before appendage (gp12*) attachment, because 12⁻ particles aggregate with anti-gp13 IgG (Figure 3), consistent with prior reports.¹⁵ With the gp13/gp9 knob attached, the twelve appendages that function in adsorption assemble to yield the infectious virion. Since purified ϕ 29 proteins such as head fibers³⁸ and appendages¹⁸ are readily assembled to intermediates having DNA-filled heads *in vitro*, the requirement for *in vivo* assembly of the gp13/gp9 knob appears to define a distinct multi-protein tail sub-assembly pathway in a podophage. A model for this gp13/gp9 accessory or sub-assembly pathway is shown in Figure 8. The myo- and siphoviridae (T4-like and λ -like, respectively) have tail assembly pathways independent of prohead/capsid assembly. In contrast, tails of the podophage T3 are assembled only on the head.³⁹ However, assembly of the lambdoid podophage phage P22 gp9 into tail spikes in P22 virions⁴⁰ can be considered a tail sub-assembly pathway,⁴¹ and similarly, oligomers of the ϕ 29 appendages (gp12*) are preassembled.¹ The ϕ 29 gp13/gp9 sub-assembly proposed here involves multiple copies of two polypeptides, suggesting that ϕ 29 uses a mixture of traditional podophage and myo/siphoviridae assembly pathway processes.

Secondary structure prediction and alignment to known crystal structures shows similarity between the gp13 C-terminal domain and LytM. LytM is a Zn²⁺-containing metalloprotease autolysin from *Staphylococcus aureus* that cleaves the pentaglycine cross-links of the staphylococcal cell wall.^{25, 27} The HxH Zn²⁺-binding motif of the M23 family (e.g. LytM [*Staphylococcus aureus*], Lysostaphin [*S. simulans*]) is present in ϕ 29 gp13 and in the ϕ 29 relatives GA-1 and B103.²⁰ Other phage proteins, such as T4 gp12, contain the HxH motif and have been shown to bind Zn²⁺ in a fashion that aids oligomerization.⁴²⁻⁴⁴ Additionally, the tape measure proteins in λ and mycobacteriophages⁴⁵ as well as the λ Ur short-tail fiber

(*stf* gene product) contain HxH motifs (data not shown). The metal-chelating role for these HxH motifs that aids protein oligomerization or enzymatic activity has yet to be demonstrated.

Bacteriophage tail-associated enzymes exemplify highly evolved virus entry machines. Biochemical studies of the T7-like phage K1-E,⁴⁶ the P22 endorhamnosidase tail spike (gp9)^{47, 48} and the ϕ 29 relative GA-1 appendages (gp12*)⁴⁹ have demonstrated the critical role of oligomeric adsorption/cell wall degrading complexes for infectivity. Low copy number enzymes can also facilitate cell wall puncture. The tail-associated lysozyme gp5 of T4⁵⁰ is present in only three copies in each virion and is critically located on the tail axis near the bottom of the tail tube.⁵¹ T4 does not appear to have the radially oriented enzymatic complexes that are present in phages K1-E, P22 and the appendages of GA-1/ ϕ 29.⁴⁹ Conversely, the phage tails of P22 and K1-E do not appear to have axially positioned enzymes like T4^{50, 51} or ϕ 29 (this study). Thus ϕ 29 emerges as hybrid of structures and functions with both axially and radially positioned enzymes required for virus puncture of the cell wall.

The ϕ 29 tail protein gp13 closely resembles a metalloendopeptidase, and low copy number virion proteins are likely present in other viruses. In seeking a gp13 counterpart in T4, gp26 of T4⁵² may be a candidate. gp26, which has been recovered from isolated T4 baseplates and studied with gp26-specific antibodies,³² is likely present in only a few copies and has been suggested to be attached to the distal gp5 trimer at the tip of the T4 tail tube.⁵³

Immuno-EM of ϕ 29 and *sus*13(342) particles localized gp13 to the distal tip of the tail knob (Figs. 3 and 7). Further, aggregation of *sus*13(342) defective particles with anti-gp13 IgG (Table 3) demonstrated that gp13 epitopes are accessible, suggesting that the short 3.8 kDa fragment, the gp13 N-terminus, is exposed at the tip of the knob. In this position, full length gp13 likely serves as a plug to help restrain the highly pressurized packaged genome. The defective particles produced in the restrictive gene 13 *sus* mutant infections were unstable and readily lost their DNA (Figure 7, Table 3). Also, prior work attributed ϕ 29 genome stability to gp13,^{1, 3} in a similar fashion to gp4 and gp10 of phage P22.⁵⁴ In the absence of these proteins, ϕ 29 and P22 particles lose their DNA and are rendered non-infectious. Non-functional gpZ in λ results in particles defective for DNA injection;⁵⁵ this defect is because the DNA does not descend into the tail in preparation for injection.⁵⁶ Immuno-EM may aid the localization of gpZ in a λ pretail assembly intermediate.

In addition to its function of stabilizing the particle, gp13 is likely the first viral protein to contact the *B. subtilis* cell wall. Both purified gp13 and ϕ 29 show enzymatic activity on the *Bacillus subtilis* peptidoglycan (Cohen et al., in preparation). The mass observed distal to the “notch” in the tail knob of the defective *sus*13(330) particle by negative stain TEM (Figure 6 (b)) likely corresponds to a cone-shaped protrusion in a cryo-EM reconstruction of the particle (Xiang et al., in preparation). The cone is similar to the knob protrusion in a cryo-EM reconstruction of ϕ 29 ghosts (particles emptied of DNA by chemical treatment).²² This structure might form a channel for passage of DNA through the cell membrane. The dimensions of the cone suggest that it would have a mass approximately equivalent to one copy of gp9 (67 kDa) or two copies of gp13 (2×41 kDa). Whereas the immuno-EM data suggest that the cone of the *sus*13(330) particle is gp9 (Table 3), a crystal structure of gp13 may fit into the cryo-EM density (Xiang et al., in preparation). Atomic structures of distal knobs with and without gp13 will be needed to determine the molecular boundaries of gp9 and gp13 in this important infection organelle. The degradation of peptidoglycan by gp13 (Cohen et al., in preparation) likely enables tail knob penetration of the wall, access of the tail appendages (gp12*, Figure 1) to the outer cell wall to stabilize adsorption, and juxtaposing of the tail and cell membrane that triggers genome ejection.

METHODS

Phage Particle Preparation

Stocks of *sus* mutants were prepared by infection of the permissive host *Bacillus subtilis* *su*⁺⁴⁴ (*spoA*⁻ *met*⁻ *thr*⁻ *sup*⁻).⁵⁷ ϕ 29 with and without head fibers were produced in infections of the nonpermissive host *Bacillus subtilis* strain SpoOA12 (*trpC2*, *spoOA12*, *sup*⁻)⁵⁸ with the suppressor-sensitive mutants *sus14*(1241),⁵⁹ which confers the delayed lysis phenotype and assembles wild-type (wt) ϕ 29 progeny, and *sus8.5*(900)*sus14*(1241) (fiberless ϕ 29).²⁸ Wild-type and fiberless particles were purified as described.³ DNA-free particles (ghosts) and tails were produced from chemically treated particles and separated by sucrose density gradient centrifugation (S. Grimes, personal communication).

gp13-defective particles were produced in infection of SpoOA12 with the mutants *sus13*(342) *sus14*(1242)^{1, 59} and *sus13*(330)*sus14*(1241).³ Briefly, 2×10^9 SpoOA12 cells per ml of 416 medium [2% Bactotryptone, 1% yeast extract, 170 mM NaCl] were infected at a multiplicity of infection (MOI) of 15, diluted ten-fold in fresh 416 and concentrated 20-fold by centrifugation 5-10 min prior to lysis. The lysates were treated with DNase I (50 μ g/ml) and clarified by centrifugation. DNA containing particles were isolated by two successive isopycnic centrifugations in 60% (w/v) CsCl gradients, first in 416 medium and then in TMS buffer [50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂ and 100 mM NaCl] at 60,000 g for 30-40 hr at 4°C in the SW55 rotor. DNA-filled particles were stored at 4°C in CsCl and dialyzed on 0.025 μ m filters (Millipore) against TMS buffer prior to use.

Proheads were isolated from lysates of SpoOA12 cells infected with the mutant *sus14*(1241) *sus16*(300) as described.⁶⁰ Particles without appendages (gp12*-minus, 12⁻ particles), were prepared after infection of SpoOA12 cells with the mutant *sus12*(305)*sus14*(1241),¹⁸ and the DNA-containing particles were isolated by centrifugation in 10-30% (w/v) sucrose density gradients at 45,000 rpm for 60 min at 4°C in the SW55 rotor.

Expression and purification of gp9, gp10, gp11 and gp13

ϕ 29 gene product 10 (gp10) was expressed in *Escherichia coli* (pPLc28D1) and purified as described.⁶¹ gp9, gp11 and gp13 were expressed from cloned genes. Briefly, purified PCR products of gp13 amplified from purified ϕ 29 DNA were cloned into pTYB1 (New England Biolabs, NEB, Ipswich, MA, NEB) using the NdeI-SapI sites, which generated a C-terminal intein fusion protein (NEB). The recombinant proteins were expressed in *E. coli* BL21(DE3) CodonPlus-RP cells (Stratagene, La Jolla, CA) and purified to homogeneity using chitin-affinity chromatography (NEB) per manufacture guidelines. Self-cleavage activity of the intein tag was induced by 50mM DTT. Free gp13 released from the chitin beads were further purified with a Superdex 200 column (GE Healthcare, Piscataway, NJ) and then concentrated to ~20 mg/ml. Similarly, purified PCR products of gp9 and gp11 amplified from purified ϕ 29 DNA were cloned into pET30b (Novagen, EMD, Madison, WI) using the NcoI-XbaI and the NdeI-XbaI sites, respectively, which generated C-terminal hexa-histidine fusion proteins. The recombinant proteins of gp9 and gp11 were expressed in *E. coli* BL21(DE3) CodonPlus-RP cells and purified to homogeneity using NTA-Cobalt resin (BD) and a Superdex 200 column.

Antibody preparation and IgG Purification

Polyclonal antisera against purified ϕ 29 proteins (gp9, gp11, gp13) were produced in rabbits by Rockland Immunochemicals Inc. (Gilbertsville, PA). Terminal bleed serum was delipidated by centrifugation at 10,000 g for 15 min; immunoglobulins were precipitated with 40% ammonium sulfate⁶² and resolubilized in PBS buffer, pH 8.0. The IgG fraction from each antiserum (gp9, gp11, gp13 and pre-immune) was isolated on separate protein A columns (Pierce, Rockford, IL). Polyclonal antiserum specific for gp8 was raised in rabbits against

fiberless (gp8.5⁻), connector-free (gp10⁻) isometric particles produced in restrictive infection with the mutant *sus8.5(900)sus10(302)*. Polyclonal antiserum prepared against purified connectors (gp10) has been described.^{61, 63} Biotinylation of purified IgG was performed using EZ Link NHS-LC-LC-Biotin (Pierce) following the product recommendations. Biotin-IgG was purified from free biotin-NHS using a PD10 column (Pharmacia, Piscataway, NJ), eluted with PBS and stored at 4°C.

Electrophoresis and Western blotting

Polyacrylamide gel electrophoresis (PAGE) utilized 12% acrylamide precast Invitrogen Novex gels in MOPS buffer with samples prepared in lithium dodecylsulfate (LDS) buffer (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines, with 10 mM dithiothreitol. Transfer of proteins to nitrocellulose membranes (Biotrace NT, Pall Corp, NY) was performed by standard procedures,⁶⁴ and membranes were blocked in TS buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl] with 1% w/v BSA (TS-B) and 0.1% v/v Tween-20 (TS-BT) at 4°C for at least two hours. Primary antisera raised to recombinant proteins (described above) was diluted 1:10,000 or 1:20,000 (final serum protein concentration 4-8 µg/ml) in TS-B and 0.05% Tween-20 and used to probe the blot for 2-3 hr at 4°C or 1 hr at 23°C. Antisera against isometric particles (anti-gp8) or connectors (anti-gp10) were diluted 1:100,000 in TS-B and 0.05% Tween-20 as above. Blots were washed free of serum with TS-BT and probed with goat anti-rabbit-horseradish peroxidase secondary antibody diluted 1:20,000 in TS-BT for 1 hr at 4°C or 23°C, washed with TS-T and imaged in Super-Signal West Pico-enhanced chemiluminescent (ECL) reagent. CCD imaging of 10 sec delayed frames was performed using the Chemidoc system (Biorad, Hercules, CA), and unsaturated images were used for all analyses. Primary antibodies to purified proteins were from Rockland Immuno Chemicals (Gilbertsville, PA), and secondary antibody and ECL reagents from Pierce (Rockland, IL).

Electron microscopy

Electron microscopy of specimens mounted on thin carbon films over fenestrated supports or 0.15% polyvinyl formal (Formvar) films on 400-mesh copper grids (Fullam Inc., Clifton Park, NY) was performed at 80 keV using a Philips EM301 instrument.

Immuno-electron microscopy was performed with purified immunoglobulin type G (IgG) specific to gp13, gp9 and gp8 diluted in TMS or pre-immune non-specific IgG from the same animal used for gp13 specific preparation, as adapted from prior work.²⁴ Immuno-gold labeling of 2×10^{10} phage particles was conducted with 4 µg biotin-conjugated IgG and streptavidin-6 nm gold (Aurion Inc., Costerwegs, The Netherlands), adapted from prior studies.⁶⁵ Phage particle/antibody complexes were incubated on support films and stained with phosphotungstate (PTA), pH 7. Streptavidin-gold labeled complexes were prepared from particle-IgG complexes separated from free immunoglobulins by size exclusion chromatography using HPLC, System Gold (Beckman, Fullerton, CA).

Aggregation frequency

The frequency of aggregation was determined either from electron micrographs or by direct grid visualization. Tail aggregation was defined as two or more virus particles that had coincident tails on the surface where the knobs were within a knob-width from each other. Particles 1) with deformed or otherwise abnormal tail morphologies, 2) lacking sufficient staining to visualize the entire virion or 3) with tails incompletely visible in the micrograph or grid space were excluded from measurements.

Secondary structure prediction modeling

Secondary structure predictions of gp13 (GenBank accession no. **P15132**) and crystal structure searches were performed via the Bioinfobank metaserver with Profsec and the 3D Jury approach (<<http://bioinfo.pl/meta>>)⁶⁶ and with Psipred (<<http://bioinf.cs.ucl.ac.uk/psipred/>>)⁶⁷ and mGenTHREADER.⁶⁸

Phage genome isolation and sequencing

Phages containing *sus* mutations were purified by isopycnic CsCl gradient centrifugation^{19, 30} and used for genome extraction. 10¹⁰ particles were heated at 65°C in TE buffer [50 mM Tris, pH 7.8, 10 mM EDTA] and 10 mg/ml proteinase K to release the genome as described.⁶⁹ Nucleic acid was extracted twice with phenol:isoamyl alcohol:Tris-HCl (pH 7.8), EtOH-precipitated and EtOH washed, lyophilized and resuspended in water. Gene 13 sequencing utilized 2 direct (5' AGACGGCGATAACTTTATT3', 5' AATTAGCACAAATCCCAA3') and 2 complementary (5' TGGGAATTGTGCTAATTG3', 5' ACTGTATCAACAACCATACAA3') primers that bind relative to the gene 13 start site at -99-81, +499-516, +498-515 and +1196-1216 bp, respectively.

Mutants *sus13*(44), *sus13*(53) and *sus13*(336) were sequenced from single mutants, while the *sus13*(330) and *sus13*(55) mutants were sequenced from mutants that contained the additional *sus14*(1241) mutation that provides delayed lysis.⁵⁹ The *sus13*(342) mutant was sequenced both from the single mutant background and the double mutant background with *sus14*(1242) which also provides the delayed lysis phenotype.⁵⁹ The mutant *sus13*(55) was a triple mutant (*sus12-sus13-sus14*) and was not used further. Sequencing was performed at the University of Minnesota Biomedical Genomics core facility.

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Abbreviations used

PAGE	polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
cryo-EM	cryo-electron microscopy
gp	gene product

Figure 1.

$\phi 29$ assembly pathway and cryo-EM 3D reconstruction. (a) Assembly begins with the formation of the head-tail connector (gp10) onto which the scaffolding (gp7) (hatched particle), major capsid (gp8) and head fiber (gp8.5) proteins and prohead RNA (pRNA) assemble to form the prohead. The DNA packaging motor is complete on assembly of the ATPase gp16 to pRNA, and on hydrolysis of ATP, drives translocation of DNA-gp3 into the prohead concurrent with scaffold extrusion (un-hatched particle). Lower collar and tail tube (gp11) assembly triggers release of gp16/pRNA after complete packaging (filled grey particle). The tail knob (gp9), pre-assembled with gp13 (this study), is fixed onto the tail tube. Lastly, the appendages

(gp12*), which serve as adsorption organelles, are attached at the connector-lower collar junction to form the infectious virion. (b) ϕ 29 structures revealed in a cryo-EM 3D reconstruction.²²

Figure 2.

Western blot analysis of $\phi 29$ particles and tails using gp13-specific antiserum. gp13 is demonstrated in $\phi 29$ but not the prohead (a, top). Particle number is controlled and shown with connector-specific (anti-gp10) serum (a, bottom). gp13 is shown to be present in $\phi 29$ (b, top, lanes 2-4), ghosts (DNA-free particles) (lanes 7-8) and isolated tails (gp10, gp11, gp9, gp12*) (lanes 9-10) but not in proheads (lanes 5-6). gp9 does not react with anti-gp13 serum (b, top, lane 11), and gp13 does not react with anti-gp9 serum (b, bottom, lane 14). Anti-gp9 serum was used as a loading control, and $\phi 29$, ghosts and tails show equivalent signal (b, bottom, lanes 2-4 and 7-10).

Figure 3.

Immuno-electron microscopy localization of gp13 in $\phi 29$ using anti-gp13 serum. Particles without appendages (12^-) form antibody-dependent tail-bound aggregates in the presence of anti-gp13 serum (a) while 12^- particles incubated with pre-immune serum (b) do not. Insets (a, i-iii) show magnified aggregates and isolated particles, demonstrating that tail knobs are obscured in the presence of anti-gp13 serum but not in the presence of non-specific IgG (b, i). Immuno-gold localization targets the tail in the presence of anti-gp13 IgG (c), as the only phage-associated gold is tail bound. Negative staining with phosphotungstate. Magnification bars = 100 nm.

Figure 4.

Secondary structure prediction of the gp13 primary amino acid sequence (line 1), showing mutant glutamine (Q) residues, ochre mutations (arrows) and a His-x-His motif (circled green). Two secondary structure prediction models (psipred, line 2, and profsec, line 3) show predicted N-terminal α -helices ('H', red) and C-terminal β -sheets ('E', blue). Three putative Zn^{2+} ligands align with LytM- Zn^{2+} ligands (squares) following the canonical motif structure H-x(3,6)-D and H-x-H, and three residues provide putative anchoring for the fourth Zn^{2+} ligand as either a phosphate or water molecule in LytM (triangles).²⁷ Zn^{2+} -ligand residues in LytM are numbered below each residue. Amino acids N89 and I353 are italicized to denote differences compared to the published gp13 sequence.²⁹

Figure 5.

Western blot analysis of gp13-defective particles produced in restrictive infection with the mutants *sus13(330)* and *sus13(342)*. *sus13(330)* particles contain a long ochre fragment of gp13 (top panel, arrow with box down-shifted). *sus13(342)* particles contain no detectible gp13 (top panel). Reprobing of the blot with anti-connector (anti-gp10) serum serves as a loading control (bottom panel). Molecular weight standards are with visible light illumination.

Figure 6.

Transmission electron microscopy of gp13-defective mutant particles. Particles produced in restrictive infection with the mutants *sus13(342)* (left panel) and *sus13(330)* (right panel) look grossly normal. However, on close inspection, *sus13(330)* particles have notched tail knobs (right panel, dashed arrows). For reference, $\phi 29$ is shown (insets). Negative staining with phosphotungstate. $\phi 29$ inset was stained with uranyl acetate. Magnification bar = 100 nm.

Figure 7. Immuno-electron microscopy detects the short *sus13*(342) ochre fragment of gp13 in DNA-filled but not empty particles. (A) Representative micrographs of DNA-containing and empty *sus13*(342) mutant particles incubated with anti-gp13 IgG. (B) ϕ 29 aggregated with anti-gp13 IgG. Negative staining with phosphotungstate. Magnification bar = 100 nm.

Figure 8.

The $\phi 29$ Accessory Tail Assembly Pathway. gp13 and gp9 pre-assemble to form the tail knob (step A), and this complex is then attached *en bloc* onto the lower collar and tail tube (gp11) of parallel assembling filled heads (step B) as the penultimate morphogenesis step (C) that precedes attachment of the adsorption organelle appendages (gp12*) (step D) to form the infectious virion.

Table 1 ϕ 29 phages used in this study

Phages	Source
<i>sus</i> 8.5(900) <i>sus</i> 14(1241)	38
<i>sus</i> 12(305) <i>sus</i> 14(1241)	70
<i>sus</i> 12(305) <i>sus</i> 13(55) <i>sus</i> 14(1241)	Reilly et al., unpublished
<i>sus</i> 13(336)	28
<i>sus</i> 13(44)	28
<i>sus</i> 13(53)	28
<i>sus</i> 13(330) <i>sus</i> 14(1241)	3, 28
<i>sus</i> 13(342)	1, 59
<i>sus</i> 13(342) <i>sus</i> 14(1242)	1
<i>sus</i> 14(1241) ^a	59
<i>sus</i> 14(1241) <i>sus</i> 16(300)	10

^aDelayed lysis phenotype results in wild-type particles

Table 2
Sequence analysis of gene 13 suppressor-sensitive mutants

Mutant	Position ^a	Fragment (aa length)	Fragment (Da mass) ^b
<i>sus13</i> (342)	Q33*	32	3777.3
<i>sus13</i> (44)	Q43*	42	4796.6
<i>sus13</i> (336)	Q83*	82	9189.3
<i>sus13</i> (53)	Q167*	166	18883.2
<i>sus13</i> (55)	Q332*	331	37314.9
<i>sus13</i> (330)	Q332*	331	37314.9
wild-type	n/a	365	40924.7

^a Ochre nonsense mutation amino acid change.

^b Masses (Daltons) predicted using ExPASy ProtParam tool (<http://www.expasy.ch/tools/protparam.html>).⁷¹

Table 3
Aggregation of ϕ 29 and gp13-defective mutants with antibodies to tail components

Expt.	Particle ^a	Antibody	Total Particles	DNA Filled (%)	Particles Aggregated (%)
1	WT (1241)	gp13	574	549 (96)	552 (96)
	13- (342)	gp13	272	64 (24)	53 (20)
2	13- (330)	gp13	299	201 (67)	6 (2)
	13- (342)	gp13	300	243 (81)	189 (63)
3	13- (330)	gp13	355	257 (72)	5 (1)
	13- (330)	gp9	501	444 (89)	322 (64)
	13- (330)	p.i. ^b	305	215 (71)	9 (3)

Aggregation of ϕ 29 and gp13-defective mutants with specific immunoglobulins was determined by immuno-electron microscopy. Background aggregation is less than 5% as determined by pre-immune (p.i.) IgG incubation with 13(330) particles.

^aNumbers in parentheses indicate the phage mutant used.

^bp.i. indicates pre-immune immunoglobulins isolated from the same animal used to produce the anti-gp13 IgG.