Localization and Regulation of the T1 Unimolecular Spanin

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ABSTRACT Spanins are bacteriophage lysis proteins responsible for disruption of the outer membrane, the final step of Gram-negative host lysis. The absence of spanins results in a terminal phenotype of fragile spherical cells. The phage T1 employs a unimolecular spanin gp11 that has an N-terminal lipoylation signal and a C-terminal transmembrane domain. Upon maturation and localization, gp11 ends up as an outer membrane lipoprotein with a C-terminal transmembrane domain embedded in the inner membrane, thus connecting both membranes as a covalent polypeptide chain. Unlike the two-component spanins encoded by most of the other phages, including lambda, the unimolecular spanins have not been studied extensively. In this work, we show that the gp11 mutants lacking either membrane localization signal were nonfunctional and conferred a partially dominant phenotype. Translation from internal start sites within the gp11 coding sequence generated a shorter product which exhibited a negative regulatory effect on gp11 function. Fluorescence spectroscopy time-lapse videos of gp11-GFP expression showed gp11 accumulated in distinct punctate foci, suggesting localized clusters assembled within the peptidoglycan meshwork. In addition, gp11 was shown to mediate lysis in the absence of holin and endolysin function when peptidoglycan density was depleted by starvation for murein precursors. This result indicates that the peptidoglycan is a negative regulator of gp11 function. This supports a model in which gp11 acts by fusing the inner and outer membranes, a mode of action analogous to but mechanistically distinct from that proposed for the two-component spanin systems.

IMPORTANCE Spanins have been proposed to fuse the cytoplasmic and outer membranes during phage lysis. Recent work with the lambda spanins Rz-Rz1, which are similar to class I viral fusion proteins, has shed light on the functional domains and requirements for two-component spanin function. Here we report, for the first time, a genetic and biochemical approach to characterize unimolecular spanins, which are structurally and mechanistically different from two-component spanins. Considering similar predicted secondary structures within the ectodomains, unimolecular spanins can be regarded as a prokaryotic version of type II viral membrane fusion proteins. This study not only adds to our understanding of regulation of phage lysis at various levels but also provides a prokaryotic genetically tractable platform for interrogating class II-like membrane fusion proteins.

KEYWORDS *Escherichia coli*, bacteriophage lysis, membrane fusion, membrane proteins, spanins

It has been widely accepted that the *Caudovirales*, which dominate the microbial virome, use the holin-endolysin model for host lysis, in which the holin controls the timing of lysis by controlling access of the muralytic endolysin to the peptidoglycan (PG) (1). Only recently has it been shown that, in Gram-negative hosts, active disruption of the outer membrane (OM) is also required (2, 3). In most phages, OM disruption is

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FIG 1 (A) Different spanin genetic architectures from the phages λ (embedded), P2 (overlap), T4 (separate), and T1 (unimolecular). The boxes, color coded in pink, green, orange, cyan, and purple, represent holin, endolysin, i-spanin, o-spanin, and u-spanin, respectively, in each phage, while the dotted boxes represent genes of unrelated or unknown function. All genes are drawn to scale and labeled with appropriate gene names. The spanin genes are also labeled with their spanin type above the gene in each case. (B) Cartoon representation of the topology of two-component spanin prototype from phage λ and the u-spanin from phage T1. In λ, i-spanin Rz (orange) is embedded to the IM by an N-terminal TMD (yellow rectangle) and has a periplasmic domain that constitutes two alpha-helices (cylinders) connected by a linker, predicted to form coiled coils. The mature form of o-spanin Rz1 (cyan) is attached to the inner leaflet of the OM via three fatty acyl chains (dark blue lines) and has a periplasmic domain predicted to be unstructured. They interact through their C termini to form the spanin complex, linking the IM and OM through the PG meshwork. In T1, the u-spanin gp11 (purple) is attached to the inner leaflet of the OM by the three fatty acyl chains (dark blue lines) at the N terminus and to the inner membrane through the C-terminal TMD (red rectangle). The periplasmic domain of gp11, predicted to be mainly extended beta sheets (arrows), connects the IM and OM through the PG meshwork.

caused by the spanin complex, consisting of two subunits, an inner-membrane (IM) protein (i-spanin) with type II topology and an OM lipoprotein (o-spanin). In the well-studied phage lambda system, the i-spanin Rz and o-spanin Rz1 are encoded by nested genes in the lambda lysis cassette (Fig. 1A) (2). Both spanin subunits accumulate as covalent homodimers linked by disulfide bonds (4); these homodimers form a heterotetrameric protein bridge through the PG and across the periplasm by virtue of heterotypic C-terminal i-spanin/o-spanin interactions (Fig. 1B) (5, 6). Proposed structural models of these complexes rely heavily on detailed genetic and biochemical analysis (4–7), focused on the predominantly alpha-helical, coiled-coil structure of the i-spanin. Moreover, there is compelling evidence that the spanin complexes disrupt the OM by causing IM-OM fusion and that this topological function is blocked as long as the PG is intact (8).

Two-component spanins analogous to the Rz-Rz1 proteins can be identified in the genomes of most phages of Gram-negative hosts (9). However, some phages, including the paradigm phage T1, lack recognizable two-component spanins and instead have a single gene, like *gp11*, in the position immediately distal to the endolysin gene in the lysis cassette (Fig. 1A). Mature *gp11* is predicted to have an unprecedented localization. It has signals for localization to both membranes; an OM lipoprotein signal and a C-terminal transmembrane domain (TMD) (Fig. 1B and 2A). After posttranslational processing into a mature lipoprotein and subsequent sorting by the Lol (Localization of lipoproteins) system (Fig. 2B), *gp11* is connected to the OM via the N-terminal lipoylated end and anchored to the IM by the C-terminal TMD. This architecture, combined with the ability of *gp11* to complement the lysis defect of λ Rz_{am}Rz1_{am} (9), defined *gp11* as the prototype unimolecular spanin (u-spanin). Unlike the two-component spanins,

FIG 2 (A) Primary structure of T1gp11. The primary structure, charge distribution, and secondary structure of the T1 u-spanin gp11 is shown. Dark blue rectangle, N-terminal lipoylation signal sequence; boxed residues, lipobox; purple rectangle, alpha-helix; purple arrows, extended beta sheets; red rectangle, C-terminal TMD. Asterisks denote the alternative start sites, and carets (^) indicate potential SPaseI processing sites as predicted by LipoP 1.0. The C-terminal epitope where the gp11 antibody binds is highlighted by a hatched bar below. (B) Sorting of gp11 to OM by the Lol machinery. After getting processed into a mature lipoprotein, gp11 is connected to the IM from both the N-terminal and the C-terminal ends. Like any other OM lipoprotein, the N-terminal lipoylated end of gp11 is translocated to the OM in a stepwise manner by the Lol system, as indicated by the arrows. The N-terminal end interacts with the ABC transporter LolCDE complex (yellow) and is released from the IM to form a hydrophilic complex with the periplasmic transporter protein LolA (green). After crossing the periplasm, the N-terminal end is transferred to the OM receptor LolB (blue) and then incorporated into the OM. (C) Model for gp11 function. gp11 molecules accumulate in both IM and OM, stuck within the PG meshwork. Once the PG is degraded by the endolysin, the gp11 complexes can undergo higher-order oligomerization and/or conformational changes along the periplasmic domain (indicated by gray arrow), bringing both the membranes together and eventually resulting in fusion of the IM and OM.

gp11 has neither predicted helical structure nor any periplasmic cysteines for disulfide-linked dimerization. Instead, the periplasmic domain of gp11 is predicted to be dominated by beta strands (Fig. 2A). Nonetheless, the obvious analogy between the single polypeptide bridge between the OM and the IM supplied by the u-spanin and the noncovalent complexes spanning the periplasm supplied by Rz-Rz1 suggests that the u-spanin also functions by IM-OM fusion (Fig. 2C). The differences between the predicted secondary structure of the gp11 periplasmic domain and the dominant coiled-

coil structure of the Rz-Rz1 complex strongly suggest that the fusion pathways are dramatically different, yet functionally equivalent. Here, the results of genetic and molecular analysis of the subcellular localization, function, and regulation of T1gp11 are presented and discussed.

RESULTS

Localization signal mutants. The primary structure of gp11, with its predicted N-terminal lipobox and OM-lipoprotein sorting signals and C-terminal TMD, suggests that it is localized to both membranes of the *Escherichia coli* envelope. While it is common for proteins to form complexes that span the cellular envelope, as in the cases of the type I secretion system (T1SS), T3SS, T4SS, and T6SS machineries (10), a single polypeptide chain covalently linking both the membranes has not been previously reported. To test whether the predicted localization signals were functional and required, we constructed a set of alleles with altered topological signals (Fig. 3A) and tested them for complementation of the lysis-defective λ Rz_{am}Rz1_{am} mutant. One of these, the gp11_{ΔTMD} allele, has the C-terminal TMD deleted and would thus be predicted to localize exclusively to the OM as a mature lipoprotein. Two other alleles encode products predicted to localize only in the IM: gp11_{C22S}, in which the Cys residue that is modified during lipoprotein processing has been replaced by a nonmodifiable Ser residue; and gp11_{IRS} (Inner membrane Retention Signal), an allele in which the residues immediately distal to the processed Cys residue are replaced by Asp residues. The latter mutation is predicted to abolish the OM sorting signal recognized by the Lol system (11), locking the N-terminal lipoylated end in the IM. All of these alleles exhibited absolute-defective lysis phenotypes, as judged by the inability to complement the lambda spanin lysis defect (Fig. 3B). Specific and either homotypic or heterotypic protein-protein interactions within TMD sequences constitute a hallmark of phage lysis proteins, especially the holins (12–15). To assess whether the C-terminal TMD in function of gp11 involved specific interactions, we constructed gp11_{art-TMD}, in which the entire TMD of gp11 is exchanged with an artificial TMD (VLLIIVVVVVVVILLI) (Fig. 3A). This TMD substitution allele retained full lysis function (Fig. 3B). Taken together, these results demonstrate that both the membrane localization signals of gp11 are necessary for lytic function, and the role of the C-terminal TMD is to anchor gp11 in the IM, rather than having a required interaction with itself or another integral membrane protein.

To compare the expression levels of these mutants to gp11_{WT}, we performed Western blotting on whole-cell samples from MC4100 λ Rz_{am}Rz1_{am} cultures expressing these constructs *in trans*. While gp11_{ΔTMD}, gp11_{C22S}, and gp11_{IRS} accumulated at levels similar to that for gp11_{WT}, we could not detect any gp11_{art-TMD} in the blot (Fig. 3C). In the case of the gp11_{art-TMD} construct, the TMD substitution is adjacent to the C-terminal cytoplasmic epitope, indicating that the lack of immunoblot signal is likely due to distortion of the epitope.

Interestingly, instead of the expected larger unprocessed gp11 species accumulating in the gp11_{C22S} sample, a smaller product is generated. Sequence analysis with SignalP (16) and LipoP (17) both indicate gp11 has potential signal peptidase I (SPaseI) cleavage sites at positions 26, 28, and 30, with position 28 having the highest probability of cleavage (Fig. 4A). Thus, we hypothesized that in the absence of signal peptidase II (SPaseII) processing, SPaseI can process the gp11 precursor to a periplasmic intermediate. To test this idea, the dependence of gp11 processing on SPaseII was examined by assessing the effect of treatment with globomycin (GI), a specific inhibitor of SPaseII (18), on gp11 maturation. We could not block complementation of the spanin lysis defect by gp11 in MC4100 λ Rz_{am}Rz1_{am} cultures even with the addition of GI at a concentration as high as 24 μ M (Fig. 4B). Cells treated with higher levels of GI show a loss of the cellular rod shape (19), thus making it difficult for us to differentiate between lysis and effect of GI on the integrity of the cellular envelope. However, Western blotting of whole-cell samples induced for gp11 expression with IPTG (isopropyl- β -D-thiogalactopyranoside; see Materials and Methods) indicated that the production of

FIG 3 (A) Cartoon representation of topological mutants of *gp11*. Upon posttranslational processing, wild-type *gp11* is translocated to both IM and OM. *gp11*_{ΔTMD} localizes to the OM only, whereas *gp11*_{C22S} and *gp11*_{IRS} localize to the IM only. *gp11*_{art-TMD} is translocated to both the membranes, but it has a different TMD (green rectangle) compared to the WT *gp11* (red rectangle). (B) Lysis phenotype of *gp11* topological mutants. Cultures of MC4100 λ Rz_{am}Rz1_{am} carrying pRE plasmids with different *gp11* alleles were grown at 30°C until the A_{550} was ~ 0.25 . The prophage and the plasmid carrying the spanin were induced by transferring to 42°C at $t = 0$ min and moved to 37°C at $t = 15$ min. The OD of the culture was monitored over time every 10 min. Open circles, no insert (pRE vector only control); closed circles, *gp11*; open square, *gp11*_{ΔTMD}; open triangles, *gp11*_{C22S}; crosses, *gp11*_{IRS}; closed squares, *gp11*_{art-TMD}. The slight decrease in the absorbance of the cultures which did not lyse is due to the change of cellular shape from rectangle to sphere. (C) Accumulation of *gp11* topological mutants. TCA precipitates were collected from the cultures described above at 40 min after induction and analyzed by Western blotting. The spliced images are taken from different regions of the same gel, and the samples in each lane are labeled at the top. The bands corresponding to the mature *gp11* and *gp11*^a, the smaller-molecular-weight product due to translation from an alternate start site, are indicated by arrows.

FIG 4 (A) Lipop prediction for gp11 processing. The graph shows the probability of cleavage of the gp11 signal sequence at different residues by SPasell (indicated by green bars) and SPasel (indicated by red bars), as predicted by Lipop 1.0. The x axis indicates the residue number, and the y axis indicates the log odds ratio of cleavage at that position. Residues 22 and 28 have the highest probability to be the processing sites for SPasell and SPasel, respectively. (B) Effect of SPasell processing on gp11 function. MC4100 λ Rz_{am}Rz1_{am} cells carrying pRE or pRzRz1 or pGp11 were grown at 30°C until reaching an OD of 0.2 and thermally induced for lysis. Different concentration of globomycin were added to the cultures, as indicated, at the point of induction, and the OD was monitored over time. (C) gp11 processing in the presence of GI. MG1655 *lacI*^q pQ cells carrying either pRE or pGp11 were induced for the expression with 1 mM IPTG in the presence or absence of 100 μ M GI. TCA samples were collected at 40 min after induction and analyzed by Western blotting. The spliced images are taken from different regions of the same gel, and the samples in each lane are labeled at the top. The bands corresponding to products cleaved by SPasell and SPasel, along with gp11^a, are indicated by arrows. The very faint band corresponding to the uncleaved product is also shown. (D) Lysis phenotype of gp11 internal start site mutants. Cultures of MC4100 λ Rz_{am}Rz1_{am} carrying pGp11 plasmids carrying different combinations of silent mutations at amino acid positions 63, 65, 81, and 83 were induced for lysis as described previously, and the optical density was monitored over time. The positions of the silent mutations in each of the constructs are indicated by symbols: closed triangles, Val63; closed squares, Val65; closed circles, Val63 and Val65; open triangles, Val81; open square, Val83; and open circles, Val81 and Val83. The onset of lysis is for the Val 63,65 mutant starts at 30 min after induction, compared to 40 min for all the other mutants. (E) Accumulation of gp11 internal start site mutants. MG1655 *lacI*^q pQ cells carrying either pRE or pGp11 plasmids with above-mentioned silent mutations were induced for expression with 1 mM IPTG. TCA samples were collected at 40 min after induction and analyzed by Western blotting. The bands corresponding to the mature gp11 product and gp11^a, the smaller-molecular-weight product due to translation from an alternate start site, are indicated by arrows. The gp11^a band disappears only when both V63 and V65 start sites are substituted with non-start codons.

mature gp11 was severely reduced in the presence of 100 μ M GI (Fig. 4C). Indeed, it also resulted in the accumulation of a smaller product, thus suggesting gp11 could potentially be a substrate for processing by SPasel.

Downstream alternative start site. A band of ~7 kDa (denoted by gp11^a in Fig. 3C) was always present in Western blots of membrane samples containing gp11. Since the epitope used for immunoblotting is at the extreme C terminus of gp11, we hypothesized that the smaller product was generated by translational initiation downstream of

the gene *11* start. There are four potential internal start sites in the *gp11* coding sequence, each a GTG (valine) codon with a reasonable Shine-Dalgarno sequence at an appropriate spacing: codons Val63, Val65, Val81, and Val83 (Fig. 2A). To ascertain where the translation of the smaller product started, we created silent mutations eliminating the GTG in each of the Val start codons. None of the individual mutations had any effect on the lytic function of *gp11* or the presence of the smaller species (Fig. 4D and E). However, the double-mutant allele with synonymous GTG-to-GTC codon changes at both Val63 and Val65 lacked the smaller species (Fig. 4E) and exhibited an altered lytic phenotype: earlier lysis in the complementation assay (Fig. 4D). Thus, the low-molecular-weight species was a result of translational initiations at Val63 and Val65, and the translational products of these downstream initiation events exert a dominant negative lysis delay (see Discussion).

Plasmid-borne *gp11* expression levels are comparable to T1 infections. In our experiments testing the ability of *gp11* to complement $\lambda R_{Z_{am}}R_{Z1_{am}}$ lysis defect, we noticed the timing of lysis onset changed from ~ 50 min in the case of *Rz-Rz1* to 40 min in the case of the *gp11* allele used in this study (pGp11). We considered that *gp11* might affect the cytoplasmic membrane integrity of the host cell and thus cause earlier lysis. Indeed, expression of *gp11* alone, in the absence of the prophage, reduced viability by ~ 4 orders of magnitude (Fig. 5a). This inducible lethality could also be seen during log-phase growth in liquid cultures, as cultures induced for *gp11* expression showed a decrease in optical density (OD) after ~ 50 min of induction (Fig. 5b). Both *gp11* $_{\Delta TMD}$ and *gp11* $_{C22S}$ did not show any decrease in OD in liquid cultures or inducer plates monitored over the same time period (Fig. 5a and b), indicating the toxic effect was dependent on both the membrane localization signals. Phase-contrast microscopy of samples from log-phase-growth cultures revealed that cells killed by *gp11* induction were transformed into rod-shaped ghosts (Fig. 5c), indicating that the cytoplasmic contents had leaked out even though the PG was intact. We wanted to test whether the expression levels of our *gp11* construct were physiologically relevant and comparable to the expression of the spanin in a T1 phage infection. Thus, we followed the lysis kinetics of MC4100 cells infected with T1 phage (Fig. 5d) and collected whole-cell samples at the onset of lysis. Western blotting revealed that *gp11* levels at the time of lysis in T1-infected cells were comparable to the level of expression from pGp11 induced in *trans* from a λ lysogen (Fig. 5e).

Dominance of localization signal mutants. Based on current models for the function of the *Rz-Rz1* two-component spanin system, it was considered likely that the u-spanin would also have to oligomerize to effect disruption of the OM (Fig. 1C). This suggested that some *gp11* lysis-defective missense alleles should show a dominant phenotype. To test the dominance or recessiveness of the *gp11* mutants, we constructed $\lambda::11$ hybrids (see Materials and Methods), where the entire *Rz* gene was replaced with one of the nonfunctional topological mutant alleles: *gp11* $_{\Delta TMD}$, *gp11* $_{C22S}$, or *gp11* $_{IRS}$. Lysogens carrying these hybrid prophages were transformed with pGp11 and induced for lysis. All of the topological mutants exhibited a significant retardation on the lysis mediated by *gp11* expressed in *trans* (Fig. 6A). This partial dominance was not observed when the loci of the alleles were exchanged, i.e., *gp11* expressed from the lysogen and the topological mutants were expressed from the plasmid, implying that the phenotype was dosage dependent. Indeed, Western blotting confirmed accumulation of *gp11* expressed from the prophage chromosome was significantly in excess compared to the expression from pGp11 (Fig. 6B).

Visualization of *gp11* localization *in vivo*. To observe the accumulation of *gp11* in the membranes, we created a chimeric allele, *gp11-gfp*, encoding a product in which the green fluorescent protein (GFP) domain is fused to the short, cytoplasmic C terminus of *gp11*. This chimera exhibited full complementation capacity when expressed in *trans* to an induced $\lambda R_{Z_{am}}R_{Z1_{am}}$ prophage and had a lysis profile almost indistinguishable from the parental allele in a shaker flask (Fig. 7A). As expected, introducing the C22S and IRS mutations into *gp11*-GFP rendered it nonfunctional.

FIG 5 (A) Toxicity of gp11 expression. Serial dilutions of overnight cultures of MG1655 *lacI^q* pQ cells carrying either pRE (○) or pGp11 (WT, ΔTMD, and C22S) alleles were made, and 5 μ l of each dilution was spotted onto LB agar plates with or without IPTG. (B) Expression profiles of gp11 and the topological mutants of gp11. MG1655 *lacI^q* pQ cells carrying either pRE (○) or pGp11 alleles (●, gp11; □, gp11_{ΔTMD}; and △, gp11_{C22S}) were induced for expression with 1 mM IPTG, and the OD was observed over time. (C) Micrograph of cells expressing pGp11 after induction with 1 mM IPTG for 60 min. The black and white arrows indicate an intact cell and a cell that has lost its cytoplasmic contents, respectively, presumably due to the fusion of IM and OM by gp11. (D) Lysis profiles of infection with phage T1. MC4100 cells at an OD of 550 of ~0.25 were infected with phage T1 at an MOI of 5 (●) and 10 (■), and the OD was observed over time. An uninfected culture (MOI = 0, ○) was used as a control. (E) TCA precipitates of MC4100 cells infected with phage T1 at different MOIs were collected at the onset of lysis and analyzed by Western blotting to compare to expression levels from the pGp11 plasmid. The spliced images are taken from different regions of the same gel, and the samples in each lane are labeled at the top. The band corresponding to the mature gp11 product is indicated by an asterisk (*).

Using the GFP fluorescence marker, we noticed that gp11 accumulated in punctate spots at poles and especially at the midcell, where, remarkably, it formed rings (Fig. 7B and C; see also Movie S1 in the supplemental material). The punctate distribution is dependent on both the membrane signals, as we found gp11_{C22S}-GFP and gp11_{IRS}-GFP both lost the accumulation phenotype and instead had uniform distribution all across the cells (Fig. 7B, D, and E; see also Movies S2 and S3 in the supplemental material). To test whether the punctate distribution of gp11-GFP was dependent on the context of the lambda prophage induction, the fusion allele was induced in the nonlysogenic context. Under these conditions, the punctate distribution of gp11 was still observed

FIG 6 (A) Dominance analysis of gp11 topological mutants. Cells carrying lambda lysogens with no spanin ($\lambda\Delta Rz$, red) or different topological alleles of gp11 ($\lambda gp11_{\Delta TMD}$, blue; $\lambda gp11_{C22S}$, green; and $\lambda gp11_{IRS}$, black) were transformed with pRE (●) or pGp11 (■) plasmids and thermally induced for lysis. All three topological alleles had a reproducible delaying effect on lysis by gp11. Each point indicates the average reading from three independent experiments and error bars indicate the standard deviations. (B) Expression levels of gp11 when expressed from the pGp11 plasmid and from the lambda chromosomal copy. The spliced images are taken from different regions of the same gel and the samples in each lane are labeled at the top. The band corresponding to the mature gp11 product is indicated by an asterisk (*).

but neither preferential accumulation at the midcell region nor ring formation was observed (Fig. 7F; see also Movie S4 in the supplemental material). Even in the nonlysogenic context, the punctate distribution is dependent on both membrane signals, since we found that gp11_{C22S}-GFP and gp11_{IRS}-GFP both showed uniform distribution throughout the cells (see Movies S5 and S6 in the supplemental material). Prolonged induction of gp11-GFP resulted in the production of ghosts, where the cytoplasmic contents had been leaked out.

The PG meshwork acts as a negative inhibitor of gp11 function. Previously, we showed that PG depletion in the presence of physiological levels of Rz-Rz1 resulted in lysis, independent of holin or endolysin function (8). This implied the intact PG network acts a negative regulator of two-component spanin function, preventing IM-OM fusion until the lytic pathway is initiated by holin triggering (8). To test whether the u-spanin was subject to the same regulation, we repeated this PG depletion experiment with gp11 as reported by Rajaure et al., using a *dadX alr* host carrying a $\lambda S_{am}RRZ_{am}Rz1_{am}$ prophage which allows us to test gp11 function by depleting the PG in an endolysin-independent manner. The gene products of *dadX* and *alr* are alanine racemases essential for PG biosynthesis, responsible for conversion of L-alanine (Ala) to D-Ala (20). As with the *RzRz1* system, induced cultures of cells carrying the u-spanin gene in *trans* to the lysis-defective prophage (pGp11) resulted in a sharply defined lysis event in the absence of D-Ala at as early as 40 min (Fig. 8A). In comparison, both an isogenic control prototrophic for D-Ala and the auxotroph supplemented with D-Ala showed lysis beginning at ~55 min. Thus, PG depletion can activate the gp11 u-spanin to cause lysis without holin-endolysin function, in support of the membrane fusion model. Furthermore, we followed the accumulation of gp11-GFP over time in these lysogens auxotrophic for D-Ala in the presence or absence of supplemented D-Ala. In the presence of D-Ala, we observed that gp11-GFP showed a punctate distribution in the auxotrophic host similar to that seen in the wild-type host (Fig. 8B; see also Movie S7 in the supplemental material). However, in the absence of D-Ala, the cells lyse at an earlier time even before any gp11-GFP foci accumulate (Fig. 8C; see also Movie S8 in the supplemental material). These results suggest that lower levels of gp11 oligomers can

FIG 7 (A) Lysis phenotype of *gp11-gfp* alleles. Cultures of MC4100 λ Rz_{am}Rz1_{am} carrying pRE plasmids with different *gp11-gfp* alleles were induced for lysis, and the OD was monitored as described before. Open red circles, no insert (pRE vector only control); closed red circles, *gp11*; closed green circles, *gp11-gfp*; closed green triangles, *gp11_{C22S}-gfp*; closed green squares, *gp11_{IRS}-gfp*. (B) Histogram showing the distribution of *gp11*-GFP foci. Cultures of MC4100 λ Rz_{am}Rz1_{am} carrying pGp11-*gfp* or the topological mutant versions, pGp11_{C22S}-*gfp* and pGp11_{IRS}-*gfp*, were thermally induced for lysis in shaker flask cultures. After 15 min, cells were placed on an agarose pad containing MgCl₂, and the accumulation of *gp11*-GFP was monitored by using fluorescence microscopy. The numbers of cells showing GFP fluorescence (red bars), punctate distribution (yellow bars), midcell accumulation (green bars), and polar accumulation (blue bars) were counted for each population and plotted. (C) Representative images of accumulation of *gp11*-GFP in thermally induced MC4100 λ Rz_{am}Rz1_{am} cells over time. White and yellow arrows indicate the midcell and polar accumulation of *gp11*-GFP. The white bar in the first image is a scale bar (2 μ m). (D and E) Representative images of accumulation of *gp11_{C22S}-GFP* and *gp11_{IRS}-GFP* in thermally induced MC4100 λ Rz_{am}Rz1_{am} cells, respectively, over time. Due to the pressure of being under the agarose pad, the cells appear oval instead of the spherical phenotype characteristic of spanin-null mutants. The white bar in the first image is a scale bar (2 μ m). (F) Representative images of accumulation of *gp11*-GFP in MG1655 *lacI^q* pQ cells induced with 1 mM IPTG over time. The white bar in the first image is a scale bar (2 μ m).

effect lysis when the PG cross-linking is depleted, thus supporting our idea of PG acting as a inhibitor of *gp11* function.

DISCUSSION

Here, we report the first functional characterization of a u-spanin, the prototype *gp11* from phage T1. The key findings must be considered in the context of the current understanding of the biosynthetic and topological pathways of the Gram-negative envelope.

Posttranslational processing and sorting of *gp11*. *Gp11* has functional IM and OM localization signals; the loss of either leads to dosage-dependent-dominant, lysis-defective phenotypes. These findings raise questions regarding the processing of *gp11*. Of the ~1,000 membrane proteins encoded in *E. coli*, only 17 are predicted to have a single C-terminal TMD with the type I N-out, C-in topology like *Gp11* (see Table S1 in the supplemental material). Five of these have small N-terminal domains that would not require the *sec* system for export (21, 22). Of the remaining 11, only three have legitimate SPaseI or SPaseII signal sequences, suggesting the other 8 are erroneously predicted to have a single TMD or, in these cases, that the TMD is actually in N-in, C-out topology and thus belongs to the class of tail-associated membrane proteins that are

FIG 8 (a) Spanin function is negatively regulated by PG. (A) Cells carrying the $\lambda S_{am}RRz_{am}Rz1_{am}$ prophage and auxotrophic for D-Ala were transformed with pRE or pGp11, grown in LB medium supplemented with D-Ala until reaching an OD of ~ 0.2 , and thermally induced for lysis. An isogenic host which is not auxotrophic for D-Ala was used as a wild-type control. To observe the effect of D-Ala depletion, cells were collected by centrifugation, washed with fresh LB medium three times, and resuspended in LB medium without D-Ala to the same volume before thermal induction. Although the cultures expressing pRE (*dadX alr* pRE without D-Ala [Δ], *dadX alr* pRE with added D-Ala [$\blacktriangle$], and WT pRE [$\blacklozenge$]) continued to grow irrespective of the host type, cells expressing pGp11 showed a decrease in OD over time. The wild-type host ($\blacksquare$) and the auxotroph supplemented with D-Ala ($\bullet$) both followed similar growth kinetics, showing a decrease in OD at around 55 min. However, the auxotroph without any supplemental D-Ala showed a sharp change in the onset of lysis at about 40 min. (b and c) Representative images of the accumulation of gp11-GFP in thermally induced $\lambda S_{am}RRz_{am}Rz1_{am}$ lysogens auxotrophic for D-Ala in the presence (b) or absence (c) of D-Ala. In the presence of D-Ala, GFP foci can be observed similar to foci observed in Fig. 7C (wild-type host). However, in the absence of D-Ala, cells lyse even before GFP foci are accumulated. The white bar in the first image is a scale bar (2 μ m).

imbedded in the IM by a *sec*-independent, YidC-dependent process (23, 24). Only one protein, YhdV (NP_417733.1), has a legitimate lipobox and a predicted SPaseII cleavage site; even here, there is a predicted SPaseI cleavage site, so the localization is ambiguous. If SPaseII processed, YhdV is predicted to be an OM lipoprotein based on its Ala residue immediately following the presumptive Cys lipoylation residue. However, the mature length of processed YhdV would only be 59 residues, with only 36 N-proximal to the predicted C-terminal TMD; using 0.38 nm per residue for a maximally extended peptide, the periplasmic domain of YhdV would only extend ~ 14 nm, which would be insufficient to span the periplasm. This indicates that YhdV localization would depend on a competition between OM and IM localization of the processed lipoprotein form; more likely, YhdV may be primarily processed by SPaseI, resulting in a C-terminally tethered type I IM protein. Thus, in a T1-infected *E. coli* cell, Gp11 processing would be unique, in that two different localization pathways, the OM lipoprotein maturation and export pathway and the *sec*-dependent integration of the TMD into the IM, both occur, either simultaneously or in a kinetically determined sequence.

Analysis of the periplasmic domain. Confirmation of the requirements for integration into both membranes indicates that the 85-residue segment between Cys22 and the C-terminal TMD crosses the periplasm. Secondary structure predictions indicate

14% of the residues in the periplasmic domain adopt an alpha-helical conformation, while 31% of them arrange into beta strands (Fig. 2A). At 0.15 nm per residue, the predicted alpha-helix would extend only ~2 nm, leaving 73 residues to account for the rest of the distance between the membranes. Given the ~25 nm width of the periplasm (6, 25), the rest of gp11 periplasmic domain would need to be in a fully extended conformation, spanning ~0.38 nm per residue, to account for the remaining ~23 nm. After degradation of the PG, lateral oligomerization of gp11 molecules and subsequent collapsing conformational changes into beta sheets or beta barrels would shorten the length dramatically, thus facilitating membrane fusion.

BLAST analysis of gp11 detected predicted u-spanin homologs from other phages but no other proteins. Since no insights could be gained from sequence similarity searches, we looked for proteins with secondary structure patterns similar to that predicted for gp11. One such protein is YuaF, a member of the NfeD-like (nodule formation efficiency D) clan with a potential role in maintaining membrane integrity in *Bacillus subtilis* (26). The C-terminal fragment of YuaF, residues 97 to 174, is predicted to have an alpha-helix followed by five beta sheets; this secondary structure was confirmed using high resolution NMR (27). According to Walker et al., this soluble fragment of YuaF (sYuaF) adopts an oligonucleotide/oligosaccharide-binding-protein (OB)-fold topology, wherein the five beta sheets arrange themselves into a closed beta barrel structure. While gp11 and sYuaF share no significant sequence similarity, it is possible the periplasmic domain of gp11 assumes a similar topology as that of sYuaF, given the secondary structure pattern. Change from an initial stretched/extended conformation to a collapsed conformation, such as the OB-fold, would serve the purpose of bringing the IM and OM together, eventually resulting in membrane fusion. OB-folds are known to frequently oligomerize or are found in large multicomponent assemblies (28), which makes the idea of gp11 adopting such a conformation plausible. Moreover, the PG interaction feature of YuaF suggests that Gp11 may interact directly with the PG or with PG fragments derived from endolysin activity, raising the attractive possibility that the coupling of the u-spanin function with the endolysin may be more specific than liberation from the constraints of the PG cage.

Punctate localization of gp11-GFP indicates clustered assembly of the u-spanin.

Gp11 depends on the host Lol system for transport of its N-terminal lipoprotein domain to the OM (Fig. 2B). Once the periplasmic transporter LolA transfers the lipoylated N terminus from the IM to OM, mature gp11 molecules are trapped in the PG lacuna within which they are threaded. Periplasmic interactions between the beta-sheet structures of adjacent gp11 molecules within the same mesh-hole of the PG network could lead to gp11 oligomers and thus the punctate pattern observed for the GFP fusions. The oligomerization depends on the extended conformation of gp11, since gp11_{IRS} did not show any foci. Another interesting finding is the midcell ring accumulation phenotype observed when gp11 was expressed in the background of an induced λ prophage. Lambda induction results in the blockage of septation via a direct inhibition of FtsZ ring formation by the lambda Kil protein (29). The simplest notion is that PG in the region where there is aborted septation may have less cross-linking, possibly increasing the room for u-spanins to accumulate. Thus, although ectopic expression of the u-spanin alone results in foci dispersed throughout the envelope, the effects of other phage-mediated processes may play a role in localizing gp11 more specifically. If T1 hybrids carrying gp11-GFP can be constructed, it will be interesting to determine whether the foci show more asymmetric subcellular distribution in the envelope of the T1-infected cell. Irrespective of the context, the punctate foci indicating high local concentrations of gp11 would likely prime the u-spanin for further oligomerization and faster OM disruption after the PG is removed.

Oligomerization in gp11 function. The punctate distribution displayed by gp11-GFP suggests that it oligomerizes in the envelope. This is unlikely to be an artifact caused by the GFP domain, especially considering the near-identity of lysis kinetics with the parental gp11 and the gp11-GFP hybrid (Fig. 6A). Moreover, since the fusion is nearly 500 bp from the ribosome binding site of gene 11, the level of translation of the

hybrid is unlikely to be elevated at all, much less to nonphysiological levels prone to supporting artifactual aggregation of the GFP domains. Assuming the punctate foci for the GFP fusion construct reflects oligomerization by gp11 itself, any level of oligomerization before PG degradation is somewhat surprising. Any gp11 molecule newly inserted in the OM and IM is immediately trapped in a single lacuna of the PG. Moreover, there is genetic evidence that gp11 oligomerizes at some point in the lytic pathway, in that, defects in both IM and OM localization signals lead to dosage-dependent dominant negative phenotypes. We interpret this dosage-dependent dominant negative phenotype to be a result of hetero-oligomerization of mature gp11 with mutant gp11 molecules localized to only one membrane. It is possible that there is nonspecific aggregate formation due to excess of the localization mutant proteins and also that the gp11 maturation rate may be affected by competition for posttranslational processing and transportation systems. We consider this unlikely given the moderate level of expression (low thousands per infected cell) observed for phage lysis proteins (15, 30, 31). This raises the general issue about how gp11 is localized. If the transfer to the OM occurs after integration of the C-terminal TMD, then "inchworm" forms of gp11, with both lipoylated N terminus and C-terminal TMD inserted in the IM, may accumulate and multimerize via two-dimensional diffusion in the IM. Specific interactions that could support homo-oligomerization would have to be in the periplasmic domain, in view of the ability of the synthetic TMD to replace the parental TMD. Once the oligomers are formed, the Lol system could export the lipoylated N termini to the OM one molecule at a time, allowing punctate foci of periplasm-spanning gp11 molecules to form. In contrast, if gp11 is exported to the OM before the TMD escapes laterally into the IM from the translocon, each molecule would presumably be trapped in a single lacuna of the PG network, which would make oligomerization into foci conceptually difficult. Homo-oligomerization has already been demonstrated for the Rz-Rz1 two-component spanin system, where the presence of coiled-coil domains provides a ready mode for protein-protein interactions. In contrast, the gp11 periplasmic domain is dominated by predicted beta-strand segments, implying the interactions between the subunits might involve mixed strand beta-sheet structures.

Gp11, like the Rz-Rz1 complex, can effect lysis without holin-endolysin function if PG is depleted. The findings with the D-Ala auxotroph indicate u-spanins can effect "holin-endolysin"-independent lysis upon partial PG degradation. This suggests that the step of OM disruption by gp11 is not coupled to the preceding lytic steps dealing with the IM and the PG. Leakage of cell contents upon expression of gp11 alone also supports this notion. These results are in accordance with our observations of Rz-Rz1, the two-component spanins from lambda (8). It is important to note that, in these situations, the cells retain their rod-shaped morphology. The simplest interpretation is that both types of spanins use the topological solution of IM-OM fusion to subvert the OM. This also supports the notion of PG acting as an inhibitor of spanin function until the ideal time for host lysis, which is dictated by the release of endolysin by the holin.

From this perspective, the importance of regulating gp11 function is paramount, since premature membrane fusion would terminate the phage infection cycle. Thus, the physical trapping of spanin complexes by PG could be considered a damping mechanism against premature IM-OM fusion. Even though intrinsically different from the λ two-component spanins Rz-Rz1 in terms of secondary structure, gp11 seems to achieve the same result and to be regulated in a similar fashion by the PG. The parallels also extend to eukaryotic viral fusion systems (32). The two-component systems can be considered analogous to the class I viral membrane fusion proteins, which are dependent on alpha-helical coiled coils for their function. With the predicted beta-sheet secondary structures, gp11 and other u-spanins would be comparable to class II viral membrane fusion proteins, which consist of globular domains made up of beta sheets. Similar to the oligomerization and series of conformational changes in the SFV E1 ectodomain induced by low pH (33), the trigger in the case of u-spanins could be the breakdown of PG.

Not only does gp11 seem to be capable of effecting lysis by itself, but also alter the timing of lysis in the lambda prophage context. An optimal lysis time is vital for phage propagation, and changes can affect burst size and phage fitness (34, 35). Holins accumulate in the IM harmlessly until reaching a threshold concentration, after which “triggering” occurs, forming lesions in the IM (34). This timing of triggering is encoded into the primary structure of holin and single missense mutations in the holin sequence result in a varied spectrum of lysis times, ranging from very early to very late/blocked lysis (31, 36, 37). Moreover, lysis proteins are thought to function independently of each other; i.e., the presence or absence of endolysin and spanins does not affect holin triggering time. However, triggering can be induced prematurely by the addition of energy poisons, like potassium cyanide, which collapse the proton-motive force across the IM (30, 38). Thus, the simplest interpretation is that accumulation of gp11 has an impact on the energization and membrane integrity of the IM, thus indirectly influencing holin triggering.

Downstream translational starts generate “anti-spanin” gene products. The product of an internal translational start gp11^a (Fig. 3) has a negative dominant effect on gp11 function, suggesting the shorter and mislocalized gene product serves as an intrinsic “anti-spanin,” providing another level of regulation for u-spanin function. This is reminiscent of the “dual-start” holin genes, lambda *S* and phi21 *S21*, in which separate translational starts are used to generate anti-holin products (14, 39, 40). However, in these cases the anti-holins are expressed from upstream translational starts, in contrast to the downstream start as in the case of gp11. Inhibition of the SPaseII processing using GI revealed that gp11 could act as a substrate for SPaseII processing, resulting in a nonlipoylated product. Given the negative dominant effect of the topological mutants of gp11, such as the gp11C22S, it is possible the mature product of gp11 cleaved by SPaseII is physiologically relevant. The competitive processing of gp11 between the SPases could thus act as another step of negative regulation of u-spanin function. Thus, there may be three different levels of regulation of u-spanin function: the physical barrier provided by the PG, the negative regulation from the nonlipoylated gp11 produced by SPaseII cleavage, and the downstream start anti-spanin gp11^a product. The smaller u-spanin products potentially interact with the extended periplasmic domain of full-length gp11, essentially poisoning the functional gp11 oligomers. This perspective suggests that trans inhibitors, based on the periplasmic domain of gp11, could be designed to block the disruption of OM.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and growth conditions. The bacterial strains, bacteriophages, and plasmids used in this study are described in Table 1. Bacterial cultures were grown in standard Luria-Bertani (LB) medium, supplemented with the antibiotics ampicillin (Amp; 100 µg/ml) and kanamycin (Kan; 40 µg/ml) as appropriate. Expression of the pRE plasmid is governed by the lambda late promoter pR'. Thus, to activate pR', the antiterminator Q is supplied in *trans* by either the induced prophage or the pQ plasmid. For experiments to test complementation of the λ RzRz1 lysis defect, MC4100 (λ 900Rz_{am}Rz1_{am}) cells were transformed with the pRE plasmid carrying the concerned allele and transformants selected on Amp-Kan plates at 30°C were used to inoculate overnight cultures. The overnight culture was diluted 1:250 into 25 ml of fresh LB medium and grown to an OD of ~0.25 at 30°C. Lysis was thermally induced by a shift from 30°C to 42°C for 15 min, followed by continued growth at 37°C. MgCl₂ was added to a final concentration of 10 mM at the time of induction to stabilize the outer membrane. Growth and lysis of cultures over time was monitored by determining the A₅₅₀ using a Gilford Stasar III spectrophotometer. For the expression of gp11 in nonlysogenic context, MG1655 Δ tonA *lacI*^{q1} pQ cells were transformed with the pRE plasmid carrying the concerned allele, and transformants selected on Amp-Kan plates at 37°C were used to inoculate overnight cultures. The overnight culture was diluted 1:250 into 25 ml of fresh LB medium and grown to an OD of ~0.2 before inducing by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM for plasmid induction. For the globomycin experiments, GI was added at mentioned concentrations to cultures at the point of induction of gp11 expression. Lysis and growth curves were plotted using a Kaleidagraph.

DNA manipulation and construction of λ ::11 hybrids. A list of primers used for site-directed mutagenesis used in this study can be provided on request. All primers were ordered from Integrated DNA technologies (Coralville, IA). Plasmid isolation was performed using the Qiagen spin miniprep kit, and mutations were confirmed by sequencing results from Eton Biosciences (San Diego, CA).

TABLE 1 Phages, strains, and plasmids used in this study

Phage, strain, or plasmid	Description ^a	Source or reference
Phages		
λ900	λΔ(<i>stf tfa</i> :: <i>cat</i> c1857 <i>bor</i> :: <i>kan</i> ; carries Cam ^r and Kan ^r	Laboratory stock
λ900Rz _{am} Rz1 _{am}	λ900 carrying the Rz _{Q100am} Rz1 _{W38am} nonsense alleles of the spanin genes	5
λ901	λ900 carrying the S _{am7} nonsense allele of the S holin gene	Laboratory stock
λ901Rz _{am} Rz1 _{am}	λ901 carrying the Rz _{Q100am} Rz1 _{W38am} nonsense alleles of the spanin genes	5
λΔSR	<i>stf</i> :: <i>cat</i> :: <i>tfa</i> c1857 Δ(<i>SR</i>); carries Cam ^r	2
λ900 ΔRz	λ900 carrying a deletion of the Rz gene	This study
λ900 Rz::gp11	λ900 with the Rz gene deleted and substituted with gp11	This study
E. coli strains		
MC4100 tonA::Tn10	<i>Escherichia coli</i> K-12 F araD139 Δ(<i>argF-lac</i>)U169 rpsL15 relA1 flbB3501 deo pstF25 rbsR tonA	Laboratory stock
MC4100 (λ900Rz _{am} Rz1 _{am})	MC4100 tonA::Tn10 carrying λ900 prophage with Rz _{Q100am} Rz1 _{W38am} nonsense alleles	5
MC4100 (λΔSR)	MC4100 tonA::Tn10 carrying the λΔSR prophage	2
MC4100 (λ900ΔRz)	MC4100 tonA::Tn10 lysogen carrying λ900 prophage with Rz gene deleted	This study
MC4100 (λ900 Rz::gp11)	MC4100 tonA::Tn10 lysogen carrying λ900 prophage with gp11 substituted for the Rz gene	This study
MC1000	araD139 Δ(<i>ara-leu</i>)7679 galU galK Δ <i>lac</i>	20
MB2159	MC1000 dadX _{EC} :: <i>frt</i> <i>alr</i> _{EC} :: <i>frt</i>	20
MC1000 (λ901)	MC1000 lysogen carrying λ901 prophage	8
MC1000 (λ901Rz _{am} Rz1 _{am})	MC1000 lysogen carrying λ901 prophage with Rz _{Q100am} Rz1 _{W38am} nonsense alleles	8
MB2159 (λ901)	MB2159 lysogen carrying λ901 prophage	8
MB2159 (λ901Rz _{am} Rz1 _{am})	MB2159 lysogen carrying λ901 prophage with Rz _{Q100am} Rz1 _{W38am} nonsense alleles	8
MG1655 ΔtonA lacI ^{q1} pQ	MG1655 ΔtonA lacI ^{q1} carrying the pQ plasmid	42
Plasmids		
pRE	Medium copy vector with the λ late promoter pR' that is transcriptionally activated by λQ; Amp ^r	42
pRzRz1	pRE plasmid carrying the embedded Rz Rz1 genes	5
pGp11	pRE plasmid carrying the T1gp11 gene	9
pGp11 _{ΔTMD}	pRE plasmid carrying the T1gp11 allele in which the C-terminal TMD from residues 108 to 124 is deleted	This study
pGp11 _{C22S}	pRE plasmid carrying the T1gp11 allele in which the lipoylation Cys22 residue is substituted with Ser	This study
pGp11 _{IRS}	pRE plasmid carrying the T1gp11 allele in which the Ser23 and Thr24 residues are substituted with Asp	This study
pGp11 _{art-TMD}	pRE plasmid carrying the T1gp11 allele in which the C-terminal TMD from residues 108 to 124 is replaced with the residues VLLIIIVVVVVVILLI	This study
pGp11-gfp	pRE plasmid carrying the T1gp11 allele with a translational GFP fusion at the C terminus	This study
pQ	λ Q gene cloned under P _{lac/ara-1} promoter in a low-copy-number plasmid pZS-24; Kan ^r	38
pER157	pBR322 Δ <i>tet</i> (<i>SRRzRz1 bor</i> :: <i>kan</i>); The insert is flanked by regions with homology to the phage chromosome	2
pRK1	pBR322 Δ <i>tet</i> (<i>SRRzRz bor</i> :: <i>kan</i>)	This study
pRK2	pBR322 Δ <i>tet</i> (<i>SRgp11 bor</i> :: <i>kan</i>)	This study

^aCam^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

Construction of lambda hybrids with modified lysis cassettes was performed as described previously (2). First, pRK1 was constructed from pER157 (which carries the lambda lysis cassette followed by the Δ*bor*::*kan* insertion) by deleting the Rz gene via PCR slicing using the λ ΔRz primers. The gp11 gene was amplified using the λ ΔRz::gp11 primers on the pGp11 plasmid, and the amplicon was purified using a Qiagen PCR purification kit. The purified amplicon was then used as a “mega-primer” to insert the gp11 gene into pRK1, resulting in pRK2. These plasmids were then used to recombine the modified lambda lysis cassette with either no spanin or the u-spanin gp11 into the prophage chromosome. Briefly, the plasmids carrying the modified lambda lysis cassette were transformed into the strain MC4100 (λΔSR) and induced for lysis. The phage lysate was used to lysogenize MC4100, and lysogens that were Kan resistant and Amp sensitive were tested for S⁺R⁺. Single lysogens from the above-mentioned positives were identified by screening lysogens using colony PCR (41), and the changes in the lysis cassette were confirmed by sequencing. The pRK2 plasmid was used as a template to create lysis cassette versions carrying topological mutant alleles of gp11 and then recombined to constructs λ::11 hybrids carrying the respective signal sequence mutations.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed as described previously (36). Trichloroacetic acid (TCA) pellets of whole-cell samples were acetone washed and resuspended in 1× SDS-PAGE buffer with 100 mM β-mercaptoethanol. Protein samples were loaded onto 10% resolving Tris-tricine polyacrylamide gels after loading volumes were normalized according to an A₅₅₀ at the time of collection of TCA precipitates. Proteins were transferred to polyvinylidene difluoride membrane (Pall Life Sciences) using a Hoefer TE unit at 0.5 mA for 2 h. Antibodies (GenScript) were generated in rabbits against a synthetic peptide (CIRKHEKKEA) corresponding to the C-terminal end of gp11. The primary antibodies were used at a dilution of 1:1,000, while the secondary antibody,

goat-anti-rabbit-HRP (Thermo Scientific), was used at a dilution of 1:5,000. Chemiluminescence was detected using the Bio-Rad XR Gel Doc system. SeeBlue Plus2 (Invitrogen) prestained standard served as a molecular mass standard.

Fluorescence microscopy. MC4100 ($\lambda 900Rz_{am}Rz1_{am}$) cells carrying the different topological mutant alleles of pGp11-*gfp* were grown to an OD of ~ 0.25 at 30°C. Lysis was thermally induced by a shift to 42°C for 15 min, and then 1 μ l of sample was placed onto a coverslip (24 $\times$ 50 mm; thickness, 0.16 mm). After the sample was gently covered with an agarose pad (ca. 5 by 5 mm; 1% agarose [wt/vol] in LB medium plus 10 mM MgCl₂), the cells continued to grow at 37°C, while imaging was performed simultaneously on an inverted microscope (Ti-E; Nikon, Tokyo, Japan) with a cage incubator (InVivo Scientific, St. Louis, MO) set at 37°C. Images were taken using 100 $\times$ objective (Plan Fluo, NA 1.40, oil immersion) with standard filter sets and a cooled EMCCD camera (iXon 3 897; Andor, Belfast, United Kingdom). Images were taken every 1 min for the specified times through the phase-contrast and GFP channels (100-ms exposure) over 8 to 10 different coordinates. Image analysis and processing was done using NIS-Elements imaging software. For the midcell, polar, and punctate accumulation statistics, each image from different coordinates was analyzed, and cells exhibiting the desired phenotype were manually counted and plotted using the Kaleidagraph. The final movies showing the phase, GFP, and merged channels together were created using a VSDC video editor. To visualize the accumulation of gp11 in a nonlysogenic background, MG1655 *lacI^q* pQ cells carrying pGp11-*gfp* alleles were grown at 37°C to an OD of ~ 0.25 and induced with 1 mM IPTG. After 15 min of induction, 1 μ l of culture was placed under an agarose pad with IPTG (1% [wt/vol] in LB medium plus 1 mM IPTG) and visualized as described above.

PG depletion experiments. The D-Ala depletion experiments were performed as described previously (8). Briefly, MC1000 ($\lambda 901Rz_{am}Rz1_{am}$) and MB2159 ($\lambda 901Rz_{am}Rz1_{am}$) were used as the isogenic wild-type and racemase mutant (*dadX alr*) hosts. Competent cells of these strains were transformed with pRE or pGp11 and selected on Amp-Kan plates supplemented with 150 μ M D-Ala. Overnight cultures of these transformants were diluted 1:300 into fresh LB medium and 150 μ M D-Ala and grown at 30°C until they reached an OD of ~ 0.25 before thermal induction. For the depleting D-Ala condition, the culture was harvested by centrifugation at 6,500 $\times g$ for 5 min at room temperature using a Thermo Scientific F15S-8 $\times$ 50cy rotor, washed three times with LB medium to remove the remaining D-Ala in the medium, and resuspended in fresh medium before thermal induction. Microscopy experiments to follow the accumulation of gp11-GFP in these strains were performed as described above. The agarose pad was supplemented with 150 μ M D-Ala to provide D-Ala while imaging cells.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00380-18>.

SUPPLEMENTAL FILE 1, AVI file, 12.2 MB.

SUPPLEMENTAL FILE 2, AVI file, 18.2 MB.

SUPPLEMENTAL FILE 3, AVI file, 17.2 MB.

SUPPLEMENTAL FILE 4, AVI file, 8.3 MB.

SUPPLEMENTAL FILE 5, AVI file, 6.0 MB.

SUPPLEMENTAL FILE 6, AVI file, 7.2 MB.

SUPPLEMENTAL FILE 7, AVI file, 6.4 MB.

SUPPLEMENTAL FILE 8, AVI file, 5.6 MB.

SUPPLEMENTAL FILE 9, PDF file, 0.1 MB.

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REFERENCES

1. Young R. 1992. Bacteriophage lysis: mechanism and regulation. *Microbiol Rev* 56:430–481.
2. Zhang N, Young R. 1999. Complementation and characterization of the nested Rz and Rz1 reading frames in the genome of bacteriophage lambda. *Mol Gen Genet* 262:659–667. <https://doi.org/10.1007/s004380051128>.
3. Berry J, Rajaure M, Pang T, Young R. 2012. The spanin complex is essential for lambda lysis. *J Bacteriol* 194:5667–5674. <https://doi.org/10.1128/JB.01245-12>.
4. Berry JD, Rajaure M, Young R. 2013. Spanin function requires subunit homodimerization through intermolecular disulfide bonds. *Mol Microbiol* 88:35–47. <https://doi.org/10.1111/mmi.12167>.
5. Berry J, Summer EJ, Struck DK, Young R. 2008. The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. *Mol Microbiol* 70:341–351. <https://doi.org/10.1111/j.1365-2958.2008.06408.x>.
6. Berry J, Savva C, Holzenburg A, Young R. 2010. The lambda spanin components Rz and Rz1 undergo tertiary and quaternary rearrangements upon complex formation. *Protein Sci* 19:1967–1977. <https://doi.org/10.1002/pro.485>.
7. Cahill J, Rajaure M, O'Leary C, Sloan J, Marrufo A, Holt A, Kulkarni A, Hernandez O, Young R. 2017. Genetic analysis of the lambda spanins Rz and Rz1: identification of functional domains. *G3* 7:741–753. <https://doi.org/10.1534/g3.116.037192>.
8. Rajaure M, Berry J, Kongari R, Cahill J, Young R. 2015. Membrane fusion during phage lysis. *Proc Natl Acad Sci U S A* 112:5497–5502. <https://doi.org/10.1073/pnas.1420588112>.
9. Summer EJ, Berry J, Tran TAT, Niu L, Struck DK, Young R. 2007. Rz/Rz1 lysis gene equivalents in phages of Gram-negative hosts. *J Mol Biol* 373:1098–1112. <https://doi.org/10.1016/j.jmb.2007.08.045>.
10. Green ER, Mecsas J. 2016. Bacterial secretion systems: an overview. *Microbiol Spectr* 4. <https://doi.org/10.1128/microbiolspec.VMBF-0012-2015>.
11. Seydel A, Gounon P, Pugsley AP. 1999. Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol Microbiol* 34:810–821. <https://doi.org/10.1046/j.1365-2958.1999.01647.x>.
12. Gründling A, Smith DL, Bläsi U, Young R. 2000. Dimerization between the holin and holin inhibitor of phage λ. *J Bacteriol* 182:6075–6081. <https://doi.org/10.1128/JB.182.21.6075-6081.2000>.
13. Dewey JS, Savva CG, White RL, Vitha S, Holzenburg A, Young R. 2010. Micron-scale holes terminate the phage infection cycle. *Proc Natl Acad Sci U S A* 107:2219–2223. <https://doi.org/10.1073/pnas.0914030107>.
14. Park T, Struck DK, Dankenbring CA, Young R. 2007. The pinholin of lambdaoid phage 21: control of lysis by membrane depolarization. *J Bacteriol* 189:9135–9139. <https://doi.org/10.1128/JB.00847-07>.
15. Pang T, Savva CG, Fleming KG, Struck DK, Young R. 2009. Structure of the lethal phage pinhole. *Proc Natl Acad Sci U S A* 106:18966–18971. <https://doi.org/10.1073/pnas.0907941106>.
16. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Meth* 8:785–786. <https://doi.org/10.1038/nmeth.1701>.
17. Juncker AS, Willenbrock H, von Heijne G, Brunak S, Nielsen H, Krogh. 2003. A prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 12:1652–1662. <https://doi.org/10.1110/ps.0303703>.
18. Hussain M, Ichihara S, Mizushima S. 1980. Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. *J Biol Chem* 255:3707–3712.
19. Inukai M, Takeuchi M, Shimizu K. 1984. Effects of globomycin on the morphology of bacteria and the isolation of resistant mutants. *Agric Biol Chem* 48:513–518. <https://doi.org/10.1080/00021369.1984.10866153>.
20. Strych U, Penland RL, Jimenez M, Krause KL, Benedik MJ. 2001. Characterization of the alanine racemases from two mycobacteria. *FEMS Microbiol Lett* 196:93–98. <https://doi.org/10.1111/j.1574-6968.2001.tb10547.x>.
21. Andersson H, von Heijne G. 1993. Sec dependent and sec independent assembly of *Escherichia coli* inner membrane proteins: the topological rules depend on chain length. *EMBO J* 12:683–691.
22. Andersson H, von Heijne G. 1994. Membrane protein topology: effects of delta mu H⁺ on the translocation of charged residues explain the 'positive inside' rule. *EMBO J* 13:2267–2272.
23. Aschtgen M-S, Zoued A, Llobès R, Journé L, Cascales E. 2012. The C-tail anchored TssL subunit, an essential protein of the enteroaggregative *Escherichia coli* Sci-1 type VI secretion system, is inserted by YidC. *Microbiologyopen* 1:71–82. <https://doi.org/10.1002/mbo3.9>.
24. Borgese N, Righi M. 2010. Remote origins of tail-anchored proteins. *Traffic* 11:877–885. <https://doi.org/10.1111/j.1600-0854.2010.01068.x>.
25. Matias VRF, Al-Amoudi A, Dubochet J, Beveridge TJ. 2003. Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. *J Bacteriol* 185:6112–6118. <https://doi.org/10.1128/JB.185.20.6112-6118.2003>.
26. Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer ELL, Studholme DJ, Yeats C, Eddy SR. 2004. The Pfam protein families database. *Nucleic Acids Res* 32(Database Issue):D138–D141.
27. Walker CA, Hinderhofer M, Witte DJ, Boos W, Möller HM. 2008. Solution structure of the soluble domain of the NfeD protein YuaF from *Bacillus subtilis*. *Biomol J NMR* 42:69–76. <https://doi.org/10.1007/s10858-008-9261-3>.
28. Theobald DL, Mitton-Fry RM, Wuttke DS. 2003. Nucleic acid recognition by OB-fold proteins. *Annu Rev Biophys Biomol Struct* 32:115–133. <https://doi.org/10.1146/annurev.biophys.32.110601.142506>.
29. Haeusser DP, Hoashi M, Weaver A, Brown N, Pan J, Sawitzke JA, Thomason LC, Court DL, Margolin W. 2014. The Kil peptide of bacteriophage λ blocks *Escherichia coli* cytokinesis via ZipA-dependent inhibition of FtsZ assembly. *PLoS Genet* 10:e1004217. <https://doi.org/10.1371/journal.pgen.1004217>.
30. White R, Chiba S, Pang T, Dewey JS, Savva CG, Holzenburg A, Pogliano K, Young R. 2011. Holin triggering in real time. *Proc Natl Acad Sci U S A* 108:798–803. <https://doi.org/10.1073/pnas.1011921108>.
31. Chang CY, Nam K, Young R. 1995. S gene expression and the timing of lysis by bacteriophage lambda. *J Bacteriol* 177:3283–3294. <https://doi.org/10.1128/jb.177.11.3283-3294.1995>.
32. Kielian M, Rey FA. 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nat Rev Microbiol* 4:67–76. <https://doi.org/10.1038/nrmicro1326>.
33. Kielian M, Klimjack MR, Ghosh S, Duffus WA. 1996. Mechanisms of mutations inhibiting fusion and infection by Semliki Forest virus. *J Cell Biol* 134:863. <https://doi.org/10.1083/jcb.134.4.863>.
34. Wang I-N, Smith DL, Young R. 2000. Holins: the protein clocks of bacteriophage infections. *Annu Rev Microbiol* 54:799–825. <https://doi.org/10.1146/annurev.micro.54.1.799>.
35. Wang I-N. 2006. Lysis timing and bacteriophage fitness. *Genetics* 172:17–26. <https://doi.org/10.1534/genetics.105.052811>.
36. Gründling A, Bläsi U, Young R. 2000. Genetic and biochemical analysis of dimer and oligomer interactions of the λ S holin. *J Bacteriol* 182:6082–6090. <https://doi.org/10.1128/JB.182.21.6082-6090.2000>.
37. To KH, Dewey J, Weaver J, Park T, Young R. 2013. Functional analysis of a class I holin, P2 Y. *J Bacteriol* 195:1346–1355. <https://doi.org/10.1128/JB.01986-12>.
38. Gründling A, Manson MD, Young R. 2001. Holins kill without warning. *Proc Natl Acad Sci U S A* 98:9348–9352. <https://doi.org/10.1073/pnas.151247598>.
39. Bonovich MT, Young R. 1991. Dual start motif in two lambdaoid S genes unrelated to lambda S. *J Bacteriol* 173:2897–2905. <https://doi.org/10.1128/jb.173.9.2897-2905.1991>.
40. Graschopf A, Bläsi U. 1999. Molecular function of the dual-start motif in the λ S holin. *Mol Microbiol* 33:569–582. <https://doi.org/10.1046/j.1365-2958.1999.01501.x>.
41. Powell BS, Rivas MP, Court DL, Nakamura Y, Turnbough CL. 1994. Rapid confirmation of single copy lambda prophage integration by PCR. *Nucleic Acids Res* 22:5765–5766. <https://doi.org/10.1093/nar/22.25.5765>.
42. Park T, Struck DK, Deaton JF, Young R. 2006. Topological dynamics of holins in programmed bacterial lysis. *Proc Natl Acad Sci U S A* 103:19713–19718. <https://doi.org/10.1073/pnas.0600943103>.