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REVIEW

Phage Lysis: Three Steps, Three Choices, One Outcome

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The lysis of bacterial hosts by double-strand DNA bacteriophages, once thought to reflect merely the accumulation of sufficient lysozyme activity during the infection cycle, has been revealed to recently been revealed to be a carefully regulated and temporally scheduled process. For phages of Gram-negative hosts, there are three steps, corresponding to subversion of each of the three layers of the cell envelope: inner membrane, peptidoglycan, and outer membrane. The pathway is controlled at the level of the cytoplasmic membrane. In canonical lysis, a phage encoded protein, the holin, accumulates harmlessly in the cytoplasmic membrane until triggering at an allele-specific time to form micron-scale holes. This allows the soluble endolysin to escape from the cytoplasm to degrade the peptidoglycan. Recently a parallel pathway has been elucidated in which a different type of holin, the pinholin, which, instead of triggering to form large holes, triggers to form small, heptameric channels that serve to depolarize the membrane. Pinholins are associated with SAR endolysins, which accumulate in the periplasm as inactive, membrane-tethered enzymes. Pinholin triggering collapses the proton motive force, allowing the SAR endolysins to refold to an active form and attack the peptidoglycan. Surprisingly, a third step, the disruption of the outer membrane is also required. This is usually achieved by a spanin complex, consisting of a small outer membrane lipoprotein and an integral cytoplasmic membrane protein, designated as o-spanin and i-spanin, respectively. Without spanin function, lysis is blocked and progeny virions are trapped in dead spherical cells, suggesting that the outer membrane has considerable tensile strength. In addition to two-component spanins, there are some single-component spanins, or u-spanins, that have an N-terminal outer-membrane lipoprotein signal and a C-terminal transmembrane domain. A possible mechanism for spanin function to disrupt the outer membrane is to catalyze fusion of the inner and outer membranes.

***Keywords*:** lysis, holin, anti-holin, endolysin, SAR endolysin, i-spanin, o-spanin, and u-spanin

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Historical perspective

During the golden age of phage biology, the 1940s – early 1980s, research with paradigm systems like lambda and T4 was instrumental in establishing the fundamental rules of modern molecular biology (Cairns *et al.*, 2000). In addition, these and other phages of *E. coli*, *Salmonella* and *B. subtilis* became the best understood genetic entities in biology, mainly because of the relative simplicity of their genetic complement and the facile nature of their experimental systems. Nearly every aspect of gene expression and nucleic acid metabolism was the subject of profitable investigation in these phage systems. In addition, due to phage systems, viral self-assembly and structure became much more comprehensible, because no eukaryotic viral systems offered a comparable combination of genetic, structural, and biochemical approaches. Oddly, however, the terminal event in the infection cycle, **host lysis**, received relatively little attention. In the study of most of the paradigm phages, it was appreciated very early that most phage lysates contained a muralytic activity, which was invariably called "lysozyme", in honor of the first muralytic enzyme discovered in human mucus by Fleming (1922). Even into the early 1990s, the attitude of many phage biologists was exemplified by the following excerpt from the most popular textbook on molecular biology: "This enzyme [lysozyme] begins to be synthesized when the coat proteins appear and causes the rupture of the cell wall at about the time virus maturation is complete." (Watson *et al.*, 1987). This simple perspective turns out to be wrong at nearly every level (Young and Wang, 2006). First, for some simple phages, no lysozyme activity is produced at all. For the common double-stranded DNA phages, there is muralytic activity produced but the enzymes elaborated are diverse from phage to phage, with most of them unrelated to the classic lysozyme. (For a concise but comprehensive overview of endolysin diversity, see São-José *et al.* (2003)). Moreover, in some cases, like phage T7, the muralytic activity was expressed from the very start of infection, rather than concomitantly with structural proteins (Young and Wang, 2006). In addition, lysis has been shown to involve much more than just the "rupture" of the cell wall, as we document below. Finally, the temporal regulation of lysis is completely unrelated to any aspect of viral assembly. Instead, lysis is an independent, tightly regulated and temporally scheduled pathway, involving up to five proteins, that proceeds in parallel with the pathways of morphogenesis (Young, 2013). Moreover, the timing of lysis is the primary independent determinant of phage fecundity and is thus

the focus of extraordinary evolutionary pressure (Wang *et al.*, 1996; Bull *et al.*, 2004; Wang, 2006).

This review aims first to summarize the current state of understanding of the molecular basis of phage lysis as it has been defined for decades, i.e., a process required for destruction of the bacterial cell wall. Readers interested in more depth should also consider the recent review by Catalão *et al.* (2013). Much of the detailed description here will be directed towards a surprising new development indicating that *cell wall degradation is necessary but not sufficient* for phage lysis in Gram-negative hosts (Berry *et al.*, 2012).

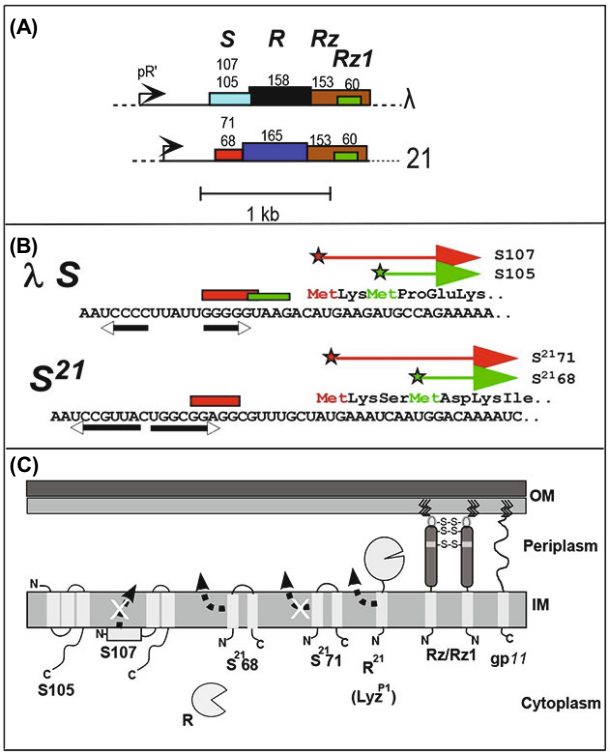

Fig. 1. The genes and proteins of lysis. (A) The lysis cassettes of phages lambda and lambdoid phage 21 are shown. In both of these lambdoid phages, the lysis genes are proximal to the late promoter, pR'. Lengths of primary translation products are shown above each gene; upper and lower numbers for *S* and S^{21} are for antiholin and holin, respectively. Gene color codes: light blue = canonical holing; red = pinholin; black = canonical endolysin (R transglycosylase); dark blue = SAR endolysin; brown = i-spanin; green = o-spanin. (B) Dual start motifs for lambda holin and phage 21 pinholin genes. Start codons for each product indicated by star; red = antiholin, green = holin (S105) or pinholin (S^{21}68). Red and green rectangles indicate Shine-Dalgarno sequences. Inverted arrows indicate RNA stem-loops that control choice of start codons. (C) Topological dynamics of lysis proteins. In the energized membrane the first transmembrane domain (TMD1) of the antiholin forms (S107 and S^{21}71) are inhibited from entering (S107) or exiting (S^{21}71) the bilayer, whereas TMD1 of the pinholin (S^{21}68) exits spontaneously during pinhole pathway. N-terminal SAR domain of R^{21} exits the membrane at a low rate spontaneously, or quantitatively upon membrane depolarization. The R transglycosylase is shown as fully active muralytic enzyme (oval with open "active site" cleft) in the periplasm, whereas the R^{21} SAR endolysin is shown in its inactive, membrane tethered form. The Rz-Rz1 complex is shown with the Rz i-spanin embedded in the IM with its N-terminal TMD and its periplasmic domain (elongated oval) disposed in the periplasm (dark grey = alpha-helical, coiled-coil domains; light gray = predicted hinge region). The Rz1 o-spanin is shown as a small oval attached to the inner leaflet of the OM by the three fatty acid chains of the lipoprotein motif. Intermolecular disulfide bonds are shown in approximate position. Finally, gp*11* is shown with its N-terminal OM lipoprotein motif already sorted to the OM by the Lol system, and its C-terminal TMD embedded in the IM. Modified from Young (2013).

The holin-endolysin theory

In 1992, a general theory for phage lysis was explicitly formulated, featuring two phage proteins: the **holin** and the **endolysin** (Young, 1992). The latter term was promulgated for the phage muralytic activity required for lysis, since only a subset of these enzymes turn out to have the activity of classic lysozyme (i.e., hydrolysis of the MurNac-GlcNac glycosidic bond in the peptidoglycan) (São-José *et al.*, 2003). The term "holin" was invented to represent small membrane proteins that were proposed to control the timing of lysis (Young, 1992). The model was primarily based on genetic and molecular studies in phage lambda (Fig. 1). In overview, the idea was that, during the morphogenesis phase of the infection cycle, the holin, encoded by lambda *S*, accumulated in the membrane and the endolysin, encoded by lambda *R*, in the cytoplasm, while the pathways for virion assembly proceeded (Fig. 2A). At a time programmed into the primary structure of the holin (and completely independent of the progress of morphogenesis), the holin would form non-specific channels, or holes, in the cytoplasmic membrane allowing the endolysin to escape and attack the

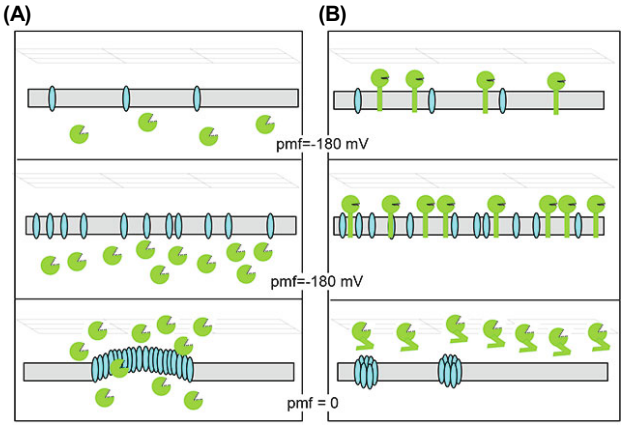

Fig. 2. Two pathways to murein degradation. Shown are cartoon views of the (A) canonical holin-endolysin and (B) pinholin-SAR endolysin pathways to murein degradation. Only the IM (grey rectangle) and PG (grid) are shown. The cartoon series begins early in late gene expression (morphogenesis period) and progresses downwards. Holins (blue ovals in A), pinholins (blue ovals in B), soluble endolysins (green ovals with open "active site cleft", and SAR endolysins (green ovals with N-terminal SAR domains depicted either in TMD conformation (green rectangle in top two panels under B) or extracted, refolded conformation (jack-knifed green rectangles in bottom panel), attached to the green globular (enzymatic and PG binding) domain. Holins accumulate in the IM (top two panels of A and B). Note that the prototype holin, S105, and pinholin S^{21}68, accumulate as homodimers or heterodimers with their cognate antiholins (Fig. 1; see text); however, holins are represented as single ovals here, for simplicity. The bottom panels represent the triggered cells, in which the canonical holins form a large ("micron-scale") hole (A) or the pinholins form many small heptameric pinholes (B) in the IM. Proton motive force (pmf) of IM is indicated for each stage.

peptidoglycan.

In the more than two decades since, the holin-endolysin model has been compellingly supported by many lines of evidence, using genetics, biochemistry, structural biology and, more recently, genomics. The interesting questions have been and remain: (a) what is the basis of the "lysis clock", i.e., what underlines the temporal scheduling effected by holins and (b) what is the nature of the hole formed by the holins. However, since its formulation more than 20 years ago, the holin-endolysin model has lost its universality, in that a second, fundamentally different pathway, the **pinholin-SAR endolysin** lysis, has been established (Fig. 2B). In both cases, the holin controls the timing and the endolysin effects the murein degradation, but at the molecular level, the mechanisms are radically different. Below, these two different strategies towards the same overall result are summarized, with the original holin-endolysin model now designated as **canonical**. As will be seen, in both of these schemes, a significant level of molecular and cell biological detail is now available for the functional characteristics of holins, although, as integral membrane proteins, rigorous definitions of structure and structure-function relationships are not yet available.

In case the reader finds the following somewhat "lambda-centric", a brief explanation is in order for why the primary experimental system for the study of lysis has been phage lambda. Mainly it is because the mutants defining the *S* and *R* cistrons were not only clustered but also had been identified with tight non-plaque-forming phenotypes, thus establishing both as essential genes. Moreover, because lambda is temperate and a thermo-sensitive allele existed for the lysogenic repressor (cI_{857}), one could perform inductions by a short period of heat-shock and have all the cells in a culture enter into the phage vegetative cycle simultaneously. This synchronicity allowed fine resolution of lysis physiology and timing with a minimum of culture manipulation that might affect the lysis phenotype. In fact, if standard conditions are rigorously imposed (i.e., host identity, culture volume, shaker speed, turbidity at time of induction, etc.), lysis of λcI_{857} occurs precisely 50 min after thermal induction, and mutant alleles with lysis timing phenotypes differing by only a few minutes can be easily discriminated (Raab *et al.*, 1986). With virulent phage, getting uniform infection in terms of number and time is impossible, given the reality of the Poisson distribution and the kinetics of adsorption. In addition, genetic and recombinant manipulation of the lambda lysis genes can be done on the resident prophage, so it is possible to create and maintain absolute lysis-defectives, which would also be impossible with virulent phages. As a result, nearly everything we know about lysis has its roots in the study of the lambda lysis genes or, in many cases, where the lysis genes of other phages have been substituted for *SR* in the lambda context.

Canonical holin-endolysin lysis

The current model for canonical holin-endolysin model is based on a wealth of physiology, genetics, and biochemistry, as well as on visible, fluorescence and cryo-electron microscopy (cryoEM). The model is most clearly described using the lambda system, where expression of all the late genes begins at ~8–10 min after the beginning of the infection cycle, when the late gene activator Q turns the single late promoter, pR' (Fig. 1). From that time on, the lambda holin protein, called S105, for its length in aa residues, accumulates as homodimers, mobile and uniformly distributed in the cytoplasmic or inner membrane (IM) (Fig. 2A) (Altman *et al.*, 1983). Meanwhile, the R endolysin, which has transglycosylase activity, accumulates in the cytoplasm as a monomeric, properly folded and fully active enzyme. This continues until suddenly, at 50 min, the holin "triggers" (Adhya *et al.*, 1971; Garrett and Young, 1982). At the culture level, triggering of a holin, in the absence of endolysin function, is detected as a sudden halt in culture growth and respiration, with a loss of cytoplasmic ions and small molecules to the medium and a collapse of the proton motive force (pmf). At the molecular level, GFP-fusion studies showed that triggering is correlated with a sudden redistribution of the holin molecules to relatively small number of discrete two-dimensional aggregates, designated as "rafts", constituted of hundreds of holin molecules (White *et al.*, 2011). At the ultrastructural level, cryoEM and tomography showed that in cells that have undergone S105 triggering, massive interruptions in the cytoplasmic membrane can be seen (Dewey *et al.*, 2010). The formation of these "holes", designated as "micron-scale" and ranging in scale up to > 1 μm, is dependent solely on the function of the S105 protein. It is presumed, but not yet demonstrated, that the holes form within the rafts of holin proteins. Little is known about the structure of the holes. Recently, cysteine-scanning accessibility studies have shown that nearly all of the S105 proteins participate in the holes and expose two of the three transmembrane domains (TMD1 and 3) to the aqueous environment of the lumen.

The timing of hole formation is allele-specific for S105 (Raab *et al.*, 1988; Johnson-Boaz *et al.*, 1994; Chang *et al.*, 1995; Pang *et al.*, 2010a). Single missense mutations in *S* can advance, retard, or abrogate triggering. It has been proposed that these changes affect the critical (two-dimensional)

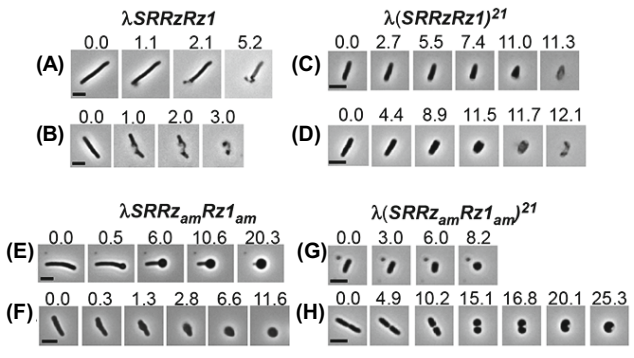

Fig. 3. Lysis morphologies. Shown are phase-contrast images of representative individual cells after induction of lambda lysogens with the indicated lysis genotype. Cultures were thermally induced and then, at just before the pre-determined time for triggering, a 10 μl sample was placed on a glass slide, covered with a coverslip, and imaged; all manipulations were done at 37°C. Numbers represent elapsed time in sec. For details see Berry *et al.* (2012), from which this was modified.

concentration for nucleation of the S105 rafts, after which rapid accretion of the S105 protein into the rafts culminates in a collapse of the membrane potential and formation of the micron-scale holes (White *et al.*, 2011). Overall, the number of S105 molecules at the time of triggering is ~1000–3000 for the wild-type allele (Zagotta and Wilson, 1990; Chang *et al.*, 1995); labelling data indicate that that the R endolysin is synthesized in comparable amounts (Altman *et al.*, 1983, 1985).

A key role, as yet not understood, is played by the polarization of the membrane. Premature triggering of the lambda holin can be imposed by any treatment that reduces the pmf by as little as 40%; this can be effected with energy poisons like the uncoupler dinitrophenol (DNP) or by sudden anaerobiosis (Gründling *et al.*, 2001). It has been proposed that the holin rafts compromise the electrochemical integrity of the membrane and thus generates a local depolarization that promotes hole-formation (White *et al.*, 2011).

Videomicroscopy of induced lambda lysogens reveal that lysis occurs as "local blowouts"; i.e., the rod-shaped cell suddenly develops a bulge at a particular site in the cell, wherein an envelope catastrophe occurs, leading to expulsion of the cytoplasmic contents (Fig. 3A and 3B) (Berry *et al.*, 2012). It is thought that the blow-out corresponds to a region opposite one of the micron-scale holes and is caused by the local exposure of the murein monolayer to the cytoplasmic endolysin escaping from those holes (Fig. 2A).

The characteristic features of the lambda pathway are thought to apply to all canonical holins. Recently, for example, it has been shown that two other canonical holins, P2 Y and T4 T, trigger to form the micron-scale holes observed for lambda (Savva *et al.*, 2014). The reader should be cautioned, however, that holin function has been assigned to many proteins, often based on little more than being small and having at least one TMD (Reddy and Saier, 2013). The most important holin-specific feature that should be used is DNP-inducible triggering; i.e., during holin expression, if a culture is treated with DNP or cyanide, viability should drop at least several orders of magnitude due to premature triggering and, if endolysins are also present, lysis should be observed (Garrett *et al.*, 1981; Garrett and Young, 1982).

SAR-endolysins and pinholins: a different molecular tactic fulfilling the same overall strategy

In 2000, it was reported that the endolysin of fOg44, a phage of *Oenococcus*, had a signal sequence and could be exported by the *E. coli sec* system (São-José *et al.*, 2000). This led to a general appreciation that many endolysins had export signals. Two such endolysins, Lyz (gp*17*) of the coliphage P1 and R^{21} of the lambdoid phage 21, have been the subject of detailed biochemical, genetic and crystallographic studies and are now considered prototypes of a new class of enzymes, the **SAR endolysins** (Xu *et al.*, 2004, 2005; Sun *et al.*, 2009). These enzymes are "true lysozymes", homologs of the T4 endolysin, that hydrolyze the MurNac-GlcNac glycosidic bond using a catalytic triad motif (E-x5-(C/D)-x3-T) (Matthews *et al.*, 1981). However, unlike T4 E, these enzymes have an N-terminal SAR (for signal-anchor-release) domain that engages the host *sec* system (Xu *et al.*, 2004, 2005). As a result, the SAR endolysin is exported to the periplasm in a holin-independent manner. Unlike other secretory enzymes, however, SAR endolysins are not processed by signal peptidase and released into the periplasm but instead accumulate in a membrane-tethered form. Moreover, the tethered enzymes are enzymatically inactive, thus avoiding a premature muralytic catastrophe for the phage. Activation occurs when the enzyme is released from the membrane and refolds into the catalytically active form (Xu *et al.*, 2005; Sun *et al.*, 2009). This can occur at an enzyme-specific, slow spontaneous rate or rapidly and quantitatively if the membrane is depolarized. Thus the SAR domain is, in effect, an unstable TMD, dependent on the pmf to be retained in the bilayer. In addition, the SAR domain, once extracted from the bilayer, plays an important role in the refolding of the enzyme, providing either covalent (disulfide bond) or non-covalent interactions with the main body of the enzyme (Xu *et al.*, 2005; Sun *et al.*, 2009; Kuty *et al.*, 2010).

The discovery of SAR endolysins raised the possibility that a phage could conduct a lytic pathway without the necessity for a holin. Indeed, in phage P1, *lydA*, encoding the holin, is non-essential (Walker and Walker, 1975; Schmidt *et al.*, 1996), because during the morphogenesis period, eventually enough Lyz spontaneously escapes from the bilayer and refolds to its active form to cause lysis of the cell (and thus enable plaque-formation). Nevertheless, phages with SAR endolysins encode holins and the latter play a key role, as best illustrated in studies with lambda phage constructs carrying the phage 21 lysis genes, including S^{21}, encoding S^{21}68, a 68 residue holin with 2 TMDs (N-in, C-in topology) (Fig. 1B) (Bonovich and Young, 1991; Park *et al.*, 2006, 2007; Pang *et al.*, 2010a, 2010b). The link is the sensitivity of the SAR endolysin to the pmf. When the S^{21} holin triggers, the pmf collapses, thus causing the SAR endolysin to release from the bilayer and become activated. Thus, in SAR endolysin phages, the holin is purely a timer for endolysin activation and plays no role in its export (Fig. 2B). In fact, soon after its discovery, the S^{21} holin was shown to be incompetent for release of canonical endolysins (Park *et al.*, 2007). The molecular basis for this made clear when ultrastructural, biochemical and modeling studies showed, instead of the micron-scale holes formed by canonical holins, S^{21} formed regular heptameric channels lined by TMD2, with a lumen estimated at ~2 nm (Pang *et al.*, 2009). Instead of a few (1–3) micron-scale holes observed after triggering of the canonical holins, the S^{21} holin triggered to form ~10^3 heptameric channels (Fig. 2B). To distinguish S^{21} from the canonical holins, it was designated as a "pinholin" and its lesions, the "pinholes".

Although both pathways result in lysis, the pinholin-SAR endolysin pathway can be discerned from canonical holin-endolysin pathway by videomicroscopy. As noted above, cells lysing by the latter pathway are generally seen to undergo a localized envelope catastrophe ("local blow-out"), presumably because of the sudden exposure of a region of the peptidoglycan (PG) to the endolysins escaping from the micron-scale holes (Fig. 3A and 3B). However, as far as we know, the SAR endolysins are uniformly distributed in

the IM before pinholin triggering, so after triggering, there should be isotropic activation of the SAR endolysins and degradation of the PG. What is visualized is the gradual shortening of the cells, culminating in lysis (Fig. 3C and 3D).

To date, S^{21} remains the only pinholin characterized in detail. Other than homology with S^{21}, there is no clear way to discern whether or not holin is a canonical holin or a pinholin. Recent results indicated that the presence of a SAR endolysin gene, i.e., an endolysin with a predicted N-terminal TMD, is necessary but not sufficient to indict an associated holin as a pinholin. The holin LydA of phage P1, which encodes the SAR endolysin that was first characterized at the functional and structural level, turns out to be a canonical holin. It should also be noted that other variations of secreted endolysins must exist; indeed, in the oenococcal phage fOg44, the secreted endolysin has a cleavable signal sequence instead of a SAR domain (São-José *et al.*, 2000), so it is unclear how the fOg44 holin controls lysis.

Regulation of lysis

The overall take-home-lesson from the above is that the control of lysis is vested in the holin protein (Catalão *et al.*, 2013; Young, 2013). Lysis starts when the holin triggers, either spontaneously at a time programmed into the holin, or prematurely, as a result of a sudden reduction in the pmf. Thus the determinant of the timing of lysis is the primary structure of the holin protein. In several different holin systems, it has been shown that timing can be advanced or retarded radically by relatively conservative missense mutations, especially in the TMDs of the holins (Raab *et al.*, 1988; Johnson-Boaz *et al.*, 1994; Gründling *et al.*, 2000a; Ramanculov and Young, 2001c; Rydman and Bamford, 2003; Pang *et al.*, 2010b). It is thought that these changes exert their influence by changing the critical concentration for holin triggering (White *et al.*, 2011; Young, 2013); it follows that altering the level of transcription or translation of the holin gene will also alter the timing of lysis (Chang *et al.*, 1995). However, in general once turned on by anti-termination or a transcription factor, late gene transcription in phage infection cycles is constitutive and varies only by gene dosage. Translational control has been well-documented for lambda *S*, which encodes a second product, S107, by virtue of a start two codons upstream of the start codon of the holin S105 (Fig. 1B) (Raab *et al.*, 1988; Bläsi *et al.*, 1989, 1990; Chang *et al.*, 1993; Steiner and Bläsi, 1993). S107 has been shown to be a specific inhibitor, or **antiholin**, of the holin S105, acting by heterodimerizing with it (Gründling *et al.*, 2000b). The ability of S107 to block holin function has to do with the dynamic membrane topology of TMD1, although the details of this "dual-start" regulation are not relevant to the subject here (Bläsi *et al.*, 1990; Graschopf and Bläsi, 1999; White *et al.*, 2010). It should be noted that the ratio of S105 to S107, normally ~2:1, defines the normal triggering time and does not change during the infection cycle (Chang *et al.*, 1993; Chang *et al.*, 1995). The ratio can be altered by mutation but whether or not there are changes dependent on cell physiology is not known. Dual start regulation has been demonstrated for other holins, including the pinholin, $S^{21}68$, for which the cognate antiholin is $S^{21}71$, generated by initiation events three codons upstream (Fig. 1B) (Bonovich and Young, 1991; Barenboim *et al.*, 1999). Real-time regulation of lysis has, to date, been demonstrated only in one system, phage T4. In T4, the holin T has a single TMD and a large periplasmic globular domain, which is the binding target of periplasmic protein RI, the antiholin (Ramanculov and Young, 2001a , 2001b, 2001c; Tran *et al.*, 2005, 2007). Normally, the T holin triggers at ~25 min after infection, but if the RI antiholin is activated, it binds to the periplasmic domain of T and blocks hole formation. The mechanism of activation is beyond the scope of this review; suffice it to say that conditions where there is a lack of possible hosts for the progeny virions cause RI activation (Tran *et al.*, 2007; Moussa *et al.*, 2012).

New player: the discovery of lambda Rz

Irrespective of which of the two established pathways, i.e., canonical holin-endolysin or pinholin-SAR endolysin, the underlying premise was the same: lysis was effected by the action of the muralytic endolysin and controlled by the holin. Implicit in this is the notion that the peptidoglycan layer is **necessary** to prevent cell lysis and that its destruction is **sufficient** to ensure lysis on a time scale useful to bacteriophages. A corollary is that the outer membrane (OM) of Gram-negative hosts is not a significant barrier to the release of the progeny virions. The rest of this review is devoted to documenting that this perspective was wrong. In fact, for most phages of Gram-negative hosts, both the PG and the OM must be actively disrupted to achieve lysis, which is achieved by a third functional class of lysis proteins, the **spanins** (Berry *et al.*, 2012). Moreover, at least two entirely different types of spanins are found and both conduct processes of protein and membrane dynamics unprecedented in prokaryotic systems (Summer *et al.*, 2007). Before describing these unique molecules and considering their mechanisms, it is useful to recount the time-line of how they were discovered.

The first published clue that the holin-endolysin model was inaccurate, or at least incomplete, came from a 1979 study of Tn*903* insertions in phage lambda (Young *et al.*, 1979). Two of the insertions conferred a plating defect, which was unexpected since the Tn*903* insertions occurred in the prophage and virions had been produced by subsequent induction. Complementation analysis for plaque formation indicated that the insertions defined a new gene, designated *Rz* (Zhang and Young, 1999). The DNA analysis tools available at that time, heteroduplex and restriction fragment mapping, both indicated that these insertions were at two different sites immediately downstream of the *SR* lysis genes. Analysis showed that both mutants had unusual conditional lysis defects. Induced cultures of the λ*Rz*::Tn*903* lysogens lysed normally, in terms of the classical sudden A_{550} decrease at 50 min after induction, but cultures infected with the same phage stopped growing at the normal lysis time and exhibited only a 20% reduction in A_{550} with no release of virions. Microscopic observation of the infected cells revealed that they had been transformed from rod shape to

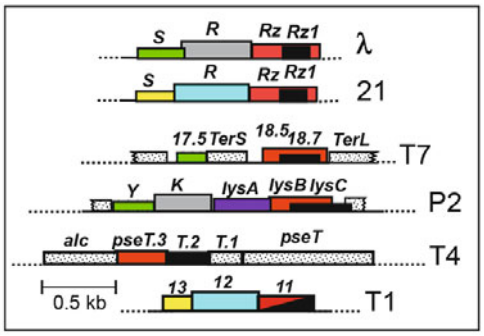

Fig. 4. Lysis gene clusters in paradigm phages. Genes are color-coded as follows: green = canonical holin; yellow = pinholin; grey = canonical endolysin; light blue = SAR endolysin; red = i-spanin; black = o-spanin; red/black hatch = u-spanin. Other genes are unrelated to lysis function and are marked by dots, except P2 *lysA*, thought to encode an antiholin (purple). No significant sequence homology exists between any genes except ~96% identity between the i-spanin/o-spanin genes of lambda and 21 (Bonovich and Young, 1991) and 57% identity between the endolysins R of lambda and K of P2 (Ziermann *et al.*, 1994).

spherical shape, thus accounting for the partial reduction in A_{550}. The difference between the induction and infection lysis phenotypes was found to be the use in the infection experiments of 10 mM $MgCl_2$, required for lambda adsorption. Thus the *Rz* was assigned a conditional lysis phenotype dependent on the presence of millimolar concentrations of Mg^{2+}, with the defective lysis process ending in the formation of spherical cells. When the *Rz* gene and its lysis-defective Tn*903* insertion were sequenced, it was found that Rz encoded a novel 153 aa protein (Taylor *et al.*, 1983).

A possible role for Rz that would still be consistent with the general precept of holin-endolysin lysis was available because A. Taylor and her colleagues had identified two different bacteriolytic activities in lambda lysates: an endopeptidase that cleaved the cross-linking peptide (Taylor, 1971) and a transglycosylase (Taylor and Gorazdowska, 1974; Bienkowska-Szewczyk and Taylor, 1980). When this group purified the R enzyme to homogeneity, it was discovered that it had the latter activity (Bienkowska-Szewczyk *et al.*, 1981). Thus, in lambda infections, the cell wall is degraded by R without generation of reducing ends. This led to the proposal that Rz was the missing endopeptidase activity, a notion supported by the finding that purified R was inhibited by Mg^{2+}. In this scenario, conditions with a high divalent cation concentration would inhibit R function during lysis and thus make lysis dependent on Rz. However, inspection of the Rz sequence revealed that it had an N-terminal hydrophobic domain variously predicted as a signal sequence or a TMD. Immuno-blotting using an antibody raised against an Rz oligopeptide sequence, Hanych and collaborators found that Rz did not appear to be processed but instead was quantitatively associated with the membrane fraction as a full-length protein, suggesting that the N-terminus was a TMD (Hanych *et al.*, 1993a). In 1993, Casjens *et al.* (1989) determined the DNA sequence of the lysis gene region of the *Salmonella* phage P22 and defined a new gene, designated as *15*, just downstream of the holin (*13*; *S* homolog) and endolysin *(14*; homolog of T4 *e*). Deletions of *15* exhibited the same cation-dependent lysis phenotype as the lambda Rz insertion mutants; in fact, the cation sensitivity could be generalized to other alkaline earth divalent cations (Mn^{2+}, Sr^{2+}, Ca^{2+} and Ba^{2+}), which do not facilitate lambda adsorption. Moreover, P22 hybrids were constructed in which gene *18.5* of phage T7, not detectably related to *Rz*, was shown to complement the *15* lysis defect (see Fig. 4 for the T7 lysis gene map).

Rz1: genes inside of genes

These findings showed that *Rz*-like genes might have a general role in lysis not predicted by the holin-endolysin model, leading the Taylor group to attempt to clone the

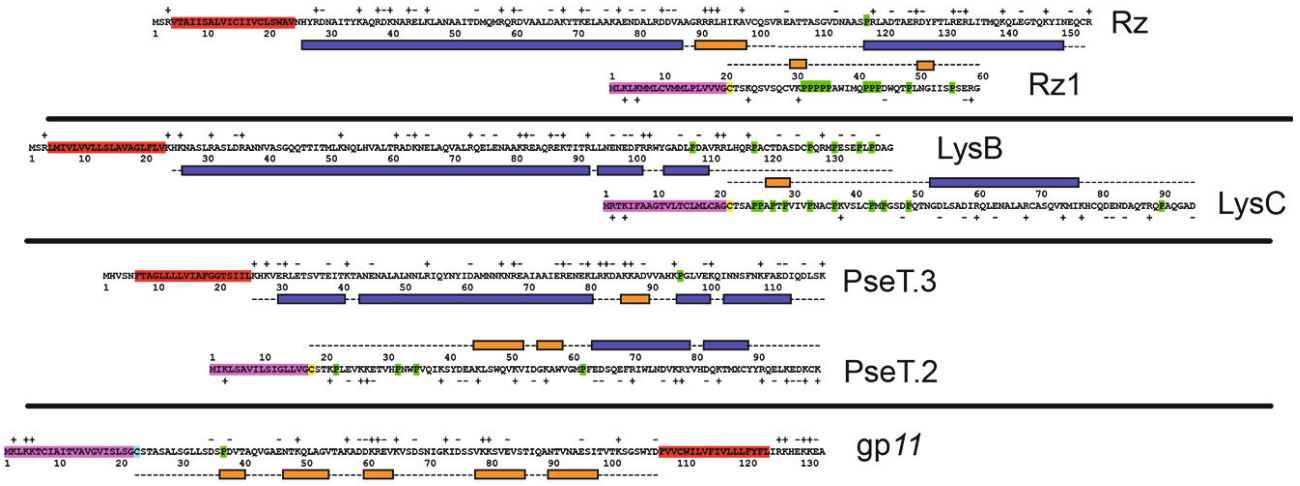

Fig. 5. Spanin sequences. Primary structures of i-spanin/o-spanin pairs from prototype **embedded** (lambda *Rz-Rz1*), **overlapped** (P2 *lysB-lysC*) and **separated** (T4 *pseT.3-pseT.2*) gene architectures, as well as the product of the prototype u-spanin gene, T1*11* (Summer *et al.*, 2007). The primary structures for the embedded and overlapped o-spanins are shown aligned to the i-spanin sequence as they are aligned in the gene structure.

gene and over-express the protein (Hanych *et al.*, 1993b). Adventitious cloning events generated constructs which expressed a 6.4 kDa species detectable by radioactive labeling. Sequence analysis revealed that a small deletion had inactivated the *Rz* gene and that the 6.4 kDa species originated from an open reading frame (orf) embedded in the +1 register from *Rz* (Fig. 5). This orf, designated as *Rz1*, had a strong Shine-Dalgarno sequence serving it and encompassed 60 codons. The predicted Rz1 sequence had a strong signal peptidase II cleavage motif, indicating that the precursor Rz1 protein would be processed and modified as a lipoprotein. Moreover, the residues in the +1 and +2 positions beyond the modified Cys were Thr and Ser, which would be predicted to result in the mature Rz1 lipoprotein to be sorted by the Lol system to the OM. Subsequently, labelling experiments showed the 6.4 kDa species was exclusively localized to the OM and was labeled with palmitate, confirming its lipoprotein character.

The bizarre nested genetic architecture of *Rz* and *Rz1* was also found to be conserved in the other *Rz*-like genes mentioned above (Fig. 4). However, this was not the first time that nested cistrons had been identified in prokaryotic biology. There were several other instances, two of which involved phage lysis functions. In the first two genomes ever completely determined, the ssRNA phage MS2 and the ssDNA phage ϕX174, the lysis genes *E* and *L*, respectively, were found to be embedded in different reading frames of other genes, unrelated to lysis function (Sanger *et al.*, 1977; Atkins *et al.*, 1979). Whether or not *Rz1* was involved in lysis was not determined until Zhang *et al.* (1999) performed complementation analysis, using nonsense mutations in *Rz* that were silent in *Rz1*, and vice versa. The results clearly showed that mutations in either *Rz* or *Rz1* generated the identical cation-dependent lysis defective phenotype. This was unique in biology: two proteins in the same DNA but different reading frames required for the same biological function! Moreover, the fact that Rz1 had its own lipoprotein processing and OM-sorting signal strongly indicated that its subcellular localization was holin-independent, as was already indicated for Rz.

The first evidence that Rz and Rz1-like proteins might actually interact came as a little-noted result in a landmark work in which the complete genome of phage T7 was subjected to yeast two-hybrid analysis (Bartel *et al.*, 1996). In this work, which first demonstrated the potential of this technology for determining a genome-wide "interactome", Fields *et al.* found, among many other interactions, a number of fragments of proteins gp*18.5* and pg*18.7* that interacted in both bait and prey frameworks. The smallest positive interactions were between the last 50 residues of the gp*18.5* and the last10 residues of gp*18.7*, the Rz and Rz1 equivalents, respectively indicating these proteins interacted by their extreme C-termini. Taken together, all these data made it more difficult to accommodate Rz and Rz1 lytic function to the classical holin-endolysin perspective.

Spanins revealed and defined

Despite the unprecedented genetic architecture, *Rz* and *Rz1* remained as obscure genes of uncertain generality for phage biology in general until, *ca* 2000, the pace of phage genome sequencing began to accelerate. Homologs of the lambda or T7 Rz-equivalents began to accumulate and, lacking any alternative, were usually annotated as "auxiliary lysis protein" or "endopeptidase". Finally, Summer *et al.* (2007) conducted a comprehensive survey of all the available genomes from phages of Gram-negative hosts and found that the vast majority of these genomes encoded two genes that had architectures and internal structures reminiscent of *Rz* and *Rz1*. Besides providing strong evidence that these proteins fulfilled an important function, despite the weak and unexplained lysis phenotype, this work revealed astounding diversity of Rz-like and Rz1-like proteins. In addition to lambda and T7, 43 other phages had embedded gene pairs like *Rz-Rz1* and *18.5-18.7*, grouped into 8 distinct sequence families. In all cases, the larger gene encoded a protein of ~150 aa with an N-terminal TMD, and the embedded gene encodes a short OM lipoprotein. In addition to this architecture, designated as the *embedded* class, two other gene pair architectures encoding proteins with these same features were found (Figs. 4 and 5): the *overlapped* class, comprised of 51 gene pairs in 23 different families, in which the OM lipoprotein gene began within the Rz-like gene but extended beyond its 3′ end; and, most surprisingly, the *separated* class, where the Rz1-like gene was entirely separated from the Rz-like gene but immediately downstream, comprised of 18 gene pairs in 6 unrelated sequence families. Included in the latter class were two genes, *pseT.3* and *pseT.2*, in the paradigm phage T4. Although these genes had escaped identification by T4 genetics, Summer *et al.* were able to show that they complemented the *Rz-Rz1* lysis defect in phage lambda.

These findings raised intriguing questions about the evolution of *Rz-Rz1* like genes, especially since, if Occam's Razor is applied, the simplest notion is that these genes started out as separated cistrons and developed the ability to interact and function in lysis, and then gradually evolved into the overlapped and embedded class. Given the previous finding that the putative T7 gp*18.5*/*18.7* proteins interacted by their C-termini, it was speculated that the evolutionary pressure driving the movement of the *Rz1*-like gene into the *Rz* gene was derived from minimizing the recombinational segregation of the interacting domains. Thus in the embedded architecture, the DNA encoding the interacting domains could hardly be closer, occupying the same nucleotide sequence in different reading frames. The concept of recombinational segregation is especially noteworthy for phages, which tend to induce "hyper-recombinational" states during infection; note that in phage T4, a single bp represents 0.01 map units (0.01% recombination) (Benzer *et al.*, 1966). Likely, this underlies the fact that in several instances (e.g., *nu1*/*A*, *O*/*P*), adjacent lambda genes encode proteins that interact via the C-terminus of the upstream gene product and N-terminus of the downstream gene product.

Tracking down all these *Rz* and *Rz1*-like genes was, and still is, complicated. The features of Rz, i.e., a type II integral membrane protein with a predicted periplasmic domain, are, unfortunately, not uncommon in phage genomes. Moreover, as with holins, the bewildering sequence diversity often precludes identification by homology. Most importantly, virtually none of the *Rz1*-like genes in the embedded and

overlapped classes were annotated in the GenBank records. In general, gene-calling software is simply not equipped to handle genes within genes. Thus often it was necessary to examine every possible open reading frame in the genome for the potential of encoding an OM lipoprotein. Fortunately, there is a widely-conserved logo, the "lipobox", that defines signal peptidase II processing sites: (L/V-X-G/A/S-C), where X is not a charged or helix-breaking residue (Okuda and Tokuda, 2011). A software suite using this logo, LipoP can be useful for finding lipoprotein candidates, although even this software relies on this motif being near the designated N-terminus (Juncker *et al.*, 2003). Unfortunately, *Rz1* genes very often have multiple possible start codons served by likely Shine-Dalgarno sequences (Summer *et al.*, 2007). While this presumably reflects something about the strange evolutionary history of these genes within genes, it also unfortunately means that *Rz1*-like genes often had to be located manually.

In addition to this wealth of information applicable to the consideration of the evolution of the Rz/Rz1-like proteins, the Summer *et al.* survey also provided a striking clue about their function. This came about when the genome of phage T1 was examined and found to have a lysis cassette consisting of three genes, the first two (genes *13* and *12*) encoding a putative pinholin and SAR endolysin, very similar in arrangement, if not sequence, to the paradigm S^{21}-R^{21} pinholin-SAR endolysin genes (Fig. 4). However, the phage 21 cassette and other lysis cassettes encoding pinholin-SAR endolysins all have immediately distal embedded *Rz-Rz1* like genes. As shown in Fig. 5, in T1, the distal gene of the lysis cassette, gene *11*, encodes a 131 aa polypeptide with elements of both Rz-like and Rz1-like proteins: an N-terminal lipobox and a C-terminal TMD. Moreover, the fact that the Cys residue destined for lipoylation is followed by Ser and Thr residues mandates that the N-terminus of this protein would be sorted to the OM by the Lol system. Thus gp*11* is predicted to be embedded in both the OM, via the three fatty acyl groups that modify the processed Cys residue, and the IM, via the TMD (Fig. 1C). To confirm the functional equivalence, gp*11* was shown to complement the *RzRz1* lysis defect of phage lambda. In addition, proteins with similar features were found to be encoded by six other phages, all lacking "normal" Rz-Rz1 like genes. In all, the 7 proteins could be grouped into three unrelated sequence families.

Although the bioinformatic story that had been elaborated for the *Rz-Rz1* like genes in this survey was already decidedly strange, the unexpected discovery of the gp*11* class had a profound effect on thinking about the whole problem. First, the unambiguous topology of the gp*11* class, **spanning** the entire periplasm, led Summer *et al.* to propose a unifying name for this functional class of proteins: the "spanins". Thus gp*11* is a unimolecular spanin (**u-spanin**), and Rz and Rz1 would constitute a two-component spanin, drawing on the previous results with yeast two hybrid analysis that indicated the Rz and Rz1 equivalents of T7 interacted by their C-termini. To avoid confusion between these topologically distinct spanins, Rz-like proteins and Rz1-like proteins were designated as inner membrane spanin subunits (**i-spanins**), and outer-membrane spanin subunits, or **o-spanins**, respectively. A corollary of these definitions was that the lytic function of these unimolecular or two-component spanins must in some way involve the linkage of the IM and OM.

Rz and Rz1 are located in the IM and OM and form complexes that span the periplasm

In retrospect, and especially in view of the existence and unambiguous topology of gp*11*, the subcellular localization of Rz and Rz1 to the IM and OM should not have been a conceptual challenge. Rz clearly has an N-terminal TMD and the charge distribution flanking that TMD clearly signals that it has type II topology (N-in, C-out) (Fig. 5). Rz1 has a consensus lipobox and, with Thr-Ser in the +1 and +2 positions in the putative mature lipoprotein, would certainly be sorted to the inner leaflet of the OM by the Lol system. Nevertheless, several attempts to actually localize the two proteins were made without unambiguous outcomes before the definitive work of Berry *et al.* (2008). These authors showed that, when expressed separately under native conditions (i.e., during a lysogenic induction) Rz and Rz1 were localized to the IM and OM, respectively, as predicted. They went on to show that in the S_{am} $Rz^{+}Rz1^{+}$ induction, the Rz protein co-fractionated with Rz1 in the OM fractions. This was shown to be due to a complex formed between Rz and Rz1, as judged by co-immunoprecipitation. This spanin complex connecting the IM and OM was necessary for Rz-Rz1 function, as judged by two findings. First, redirecting the Rz1 protein to the IM by converting the first two residues of the mature Rz1 sequence to Asp residues blocked Rz-Rz1 function. Second, replacing the N-terminal TMD of Rz with an efficient cleavable signal sequence created a negative-dominant *Rz* allele, which formed complexes with Rz1 but did not support lytic function and masked Rz1 from binding native IM-localized Rz. Moreover, the spanin complexes were detectable throughout the late gene expression period, which means that the proteins must interact by threading through the lacunae formed by the cross-linking of the PG. This suggested the simple model that spanin function, whatever it was at the molecular level, was negatively regulated by the intact PG, thus conceptually providing a direct mechanism for coupling with endolysin function. Of course, this same rationale would apply to the u-spanins, given that Lol-mediated sorting of the N-terminal lipoylated Cys residue to the OM occurs during the morphogenesis period.

Spanin function is essential for lysis of the Gram-negative host

Counting holins, anti-holins and endolysins, spanins constitute a fourth functional class of phage lysis proteins. From the outset, the unique and diverse primary structures, unprecedented sub-cellular disposition, bizarre genetic organization and genomic ubiquity was strikingly mismatched with a putative "auxiliary" lysis function. Recently, this mismatch was resolved when Berry *et al.* (2012) conducted systematic video-microscopic studies of the lytic morphologies of induced lambda lysogens in the presence or absence spanin function. For $Rz_{am}Rz1_{am}$ lysogens, cells were observed to undergo a sudden local deformation after the triggering

time but instead of proceeding immediately to the lytic lesion, the bulged region was seen to expand and progress throughout the rod-shaped length of the cell, resulting within ~10–20 sec in the final spherical cell morphology without loss of cellular contents (as judged by phase contrast) (Fig. 3E and 3F). The conclusion was that the lytic event was blocked at a step after destruction of the PG. Moreover, the spherical cells persisted indefinitely, despite the absence of supplementary divalent cations in the medium.

Similar results were obtained with induced lysogens of lambda hybrids carrying the phage 21 pinholin-SAR endolysin lysis cassette (Fig. 3G and 3H). As with the parental, induced cells were seen to undergo a generalized shortening and rounding up after holin triggering, but instead of terminating with lysis at some point, the shortening/rounding process continued until spherical cells were formed, which again persisted indefinitely even in the absence of supplementary divalent cations. Thus in both canonical and pinholin-SAR endolysin pathways, degradation of the PG in the absence of spanin function leaves the OM intact and, apparently, capable of withstanding the internal osmotic pressure of the cell and resisting lysis.

These results were clearly contradictory to the long-established conditional phenotype for Rz-Rz1 mutants, where in shaker batch cultures, *RzRz1* mutants are lysis proficient unless the medium is supplemented with millimolar concentrations of divalent cations. It was proposed that the lysis observed with these mutants in shaker flask conditions was actually an artifact imposed by the shearing forces attendant to aerobic growth. This was confirmed by tracking A_{550} in induced cultures in which shaking was halted at various times after induction. It is still unclear why the *RzRz1* mutants, although having a reduced and variable plaque size, do not have a clear plating defect without the presence of divalent cations. Possibly the osmotic conditions in soft agar are deleterious to the fragile spherical cells, thus allowing sufficient phage to be released for plaque formation.

Molecular properties of the lambda spanins

These results, taken together with the ubiquity and diversity of spanin genes discovered through genomic analysis, elevated the spanins to the status of holins and endolysins as essential lysis proteins. Consequently, the spanins have become attractive targets for study at the molecular level. Although crystal structures are not yet available, several intriguing features of the lambda spanins have been recently revealed, in addition to one older study on the properties of the Rz1 o-spanin protein.

Conformational and covalent dynamics of the lambda spanins

Both Rz and Rz1 are membrane proteins and as such are unattractive targets for standard over-expression systems. Since there is no particular function assigned to either the N-terminal fatty acids of Rz1 or the N-terminal TMD of Rz other than membrane attachment, Berry *et al.* (2010) made over-expression constructs of the soluble periplasmic domains of both Rz and Rz1 and purified both proteins, designated as sRz and sRz1, to homogeneity. The sRz protein was a homodimer in solution, with significant alpha-helical character, as determined by CD spectroscopy. The sRz1 protein purified as a monomer and was unstructured in terms of CD analysis, as predicted from its proline-rich primary structure (25% proline in 40 total residues; see Fig. 5). When sRz and sRz1 were mixed, a complex was formed with equimolar stoichiometry, accompanied by a major increase in

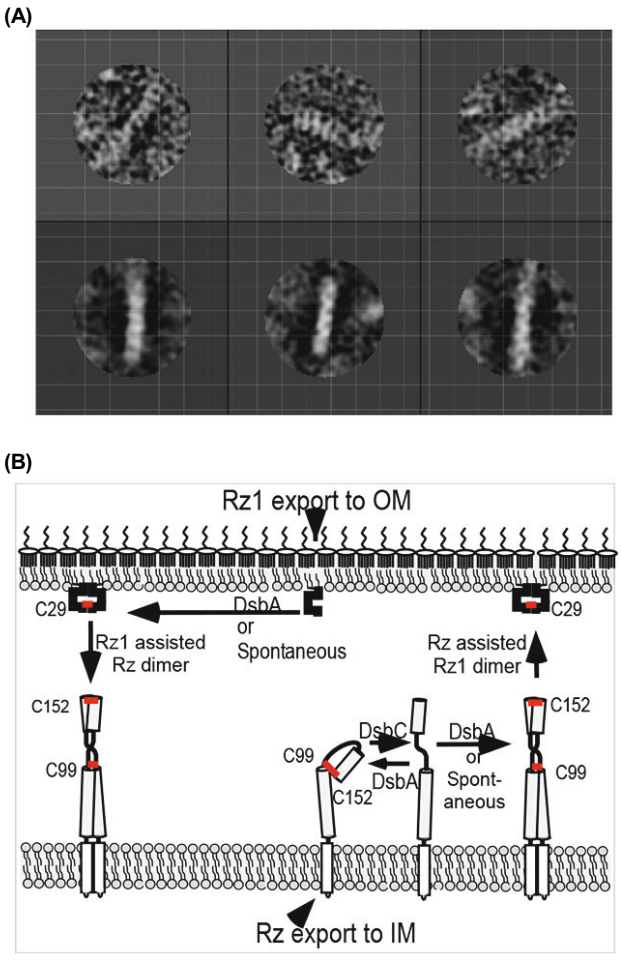

Fig. 6. Spanin complex structure and formation. (A) Single particle analysis of sRz-sRz1 complexes imaged by transmission electron microscopy with negative stain. Upper 3 panels are representative single particles and lower 3 panels are class averages. For details, see Fig. 6 of Berry *et al.* (2010), from which this was modified with permission. (B) Model for intermolecular disulfide bond formation in the spanin complex. Red bars indicate disulfide bonds. Normally, DsbA catalyzes the formation of a non-productive intramolecular disulfide between the two Cys residues, Cys99 and Cys152, of Rz. DsbC reduces this bond, allowing DsbA to catalyze the formation of intermolecular disulfide bonds in the covalent Rz homodimer. In the presence of Rz1, the disulfide linkages of Rz–Rz covalent homodimer can form spontaneously. For Rz1, DsbA catalyzes the covalent dimerization of Rz1 using its single Cys, Cys29. In the absence of DsbA, the homodimer disulfide linkage in each subunit can form as long as the cognate subunit is present, presumably as a template. For details see Berry *et al.* (2013).

alpha-helical content. When examined by negative-stain electron microscopy, the sRz-sRz1 complexes were observed as rod-like bundles, ~5 nm wide and ~24 nm in length; single particle analysis revealed that several of the class average images displayed coiled-coil structures, with a repeat length of ~4 nm (Fig. 6). The length corresponds well to the average width of the lateral periplasm of *E. coli*, which was observed to be 25.5 nm ± 1 nm in cryoEM images.

As noted above, it had been shown that Rz-Rz1 spanin complexes formed while the PG was intact (Berry *et al.*, 2008). This led to the model that after localization of the i-spanin and o-spanin subunits to the IM and OM respectively, complex formation would occur by interactions between the C-termini of both proteins. However, because the complexes would be constrained to 2-dimensional diffusion and each spanin complex would be isolated within a lacuna of the PG network, oligomerization would be prevented. In this perspective, destruction of the PG by the endolysin would liberate the spanins to undergo lateral oligomerization and thus provide the free energy for tertiary and quaternary changes necessary to disrupt the OM (Berry *et al.*, 2008, 2010, 2012).

Covalent dimerization of the lambda spanin

Further insight into the molecular function of the lambda spanins was obtained when Rz was analyzed for the state of its two Cys residues, at positions 99 and 152. Most Cys residues in periplasmic proteins occur in pairs and are oxidized to intramolecular disulfide bonds by the periplasmic Dsb system during *sec*-mediated secretion (Ito and Inaba, 2008). However, covalent linkage between positions 99 and 152 of Rz would certainly block oligomerization into rod-like bundles of extended coiled-coil alpha helices. Moreover, Berry *et al.* (2010) had shown that *Rz* alleles with either or both Cys residues converted to Ala retained lytic function. To address the state of the two Rz thiols, whole cell samples were analyzed by SDS-PAGE with or without reducing agent, revealing that the parental Rz protein existed as a mercaptoethanol-sensitive dimer (Berry *et al.*, 2013). This was also observed for Rz_{C99A} and Rz_{C152A}, but not for $Rz_{C99A,152A}$. The conclusion was that Rz accumulates in the envelope as a covalent dimer, linked by two intermolecular disulfide bonds. The same result was obtained with Rz1, which has a single Cys residue at position 29, also previously shown to be non-essential to spanin function. Coupled with previous results using co-immunoprecipitation methods, these findings indicated that the spanin complexes accumulate in the envelope as heterodimers of covalently-linked homodimers (Fig. 1C).

This finding was particularly surprising in light of the biochemical properties of sRz and sRz1, which had been converted to their Cys-free alleles to avoid the possibility of oxidative damage during purification. The sRz protein was shown to exist as a dimer in solution, demonstrating that the disulfide links were not required for homodimerization of the i-spanin. However, sRz1 purified as a monomer, indicating it did require the disulfide bond for dimerization, despite the fact that $Rz1_{C29A}$ was lytically functional. This led the authors to test all possible combinations of Cys→Ala substitution alleles in *RzRz1* for lytic function. The results demonstrated a synthetic lethality between $Rz1_{C29A}$ and Rz_{C152A}; that is, the spanin complex was functional if either Rz or Rz1 forms a covalent homodimer, with the requirement that in Rz, the disulfide link must be at the distal Cys.

Intermolecular disulfide bonds have rarely been identified in prokaryotes, so it was of interest to see if the well-characterized Dsb system was involved in the spanin homodimerization. Experiments with *dsbA* and *dsbC* mutants showed that neither mutation exerted an absolute block on spanin function but in both cases, lysis was delayed. The kinetic defect was much more severe in the *dsbC* background, nearly 15 min, compared to 10–15 sec with *dsbA* or the double mutant *dsbAdsbC*. This asymmetric dependence could be attributed to efficient DsbA-mediated formation of an intramolecular disulfide bond in Rz, rendering it incompetent for covalent homodimerization unless DsbC was present to provide remedial reduction. Moreover, Dsb-independent covalent homodimerization of either spanin subunit was found to occur but in both cases, the presence of the cognate subunit as a template was required (Fig. 6). These findings added yet another unprecedented feature to the Rz-Rz1 system, as the first cases where specific intermolecular disulfide bond formation between specific thiols can be attributed to Dsb function.

Fusogenic properties of Rz1

There is only one published example of biochemical experiments with purified native spanins. Bryl *et al.* (2000) reported the over-expression of Rz1 from inductions of a T7-overexpression plasmid construct and subsequent purification of Rz1 by extracting bands from SDS-PAGE gels. In over-expression experiments, Rz1 had been shown to accumulate in membrane fractions of intermediate density (see below), leading to the hypothesis that Rz1 was fusogenic. To test this notion, the gel-extracted Rz1 protein was reconstituted into liposomes and then assessing lipid and lumenal content mixing using these Rz1 proteoliposomes and target liposomes of varying composition. The results showed that the presence of the Rz1 protein facilitated mixing of both the contents and lipids with target liposomes made from both neutral and anionic lipids. Moreover, these apparent fusion events occurred without the embedding of the single Trp residue of Rz1 in the hydrophobic environment of the liposome membrane. Unfortunately, these reports did not document the amount or purity of the Rz1 protein used; moreover, no control in terms of a different protein with matched pI, solubility and lipid modification was used to see if any of these effects were specific to Rz1 or were simply derived from the presence of protein in one of the liposome preparations. Nevertheless these results present a tantalizing possibility that membrane fusion might be involved in spanin function.

Mechanism of spanin function

The results summarized so far have established that for phage lambda and probably in general for phages of Gram-negative hosts, the spanins are essential for lysis. It follows that the two-step holin-endolysin model involving cytoplasmic membrane permeabilization followed by murein degradation should be augmented to include a third step, OM disruption. The focus now is on how spanins accomplish OM disruption. At the operational level, there have been three distinctly different modes proposed for how spanins function to disrupt the OM: enzymatic degradation (of either PG cross-links or peptide links to OM; (Young *et al.*, 1979; Bienkowska-Szewczyk *et al.*, 1981), pore formation (Krupovič *et al.*, 2008), and IM-OM fusion (Berry *et al.*, 2010, 2012). In addition, for Gram-negative bacteria, controversial cellular structures, the Bayer patches, or zones of adhesion between the IM and OM, have been invoked as sites where Rz1 could promote OM disruption (Bryl *et al.*, 2000). While no definitive resolution has yet been achieved favoring any of these models, the author favors membrane fusion, at least in part because the other possibilities have too many problems, as we consider below.

Spanins as enzymes

In all likelihood, despite the still-widespread annotation of Rz-like proteins as endopeptidases, a model that spanins operate by any kind of enzymatic action can be ruled out, especially if the simplifying assumption is made that both u-spanins and two-component spanins function in the same basic way. No hint in the way of homology to a proteolytic or lipolytic domain has shown up in any of the many spanin genes discovered to date. If the u-spanin were an enzyme, it would face a severe contortional challenge in terms of accessing the OM, with its C-terminus embedded in the IM. Secondary structure analysis of the i-spanins and o-spanins (see below) indicate the former is nearly completely coiled-coil alpha-helix, while the latter is unstructured and dominated by proline residues, neither one of which would lend itself to a globular enzymatic domain. Moreover, the predicted length of the Rz periplasmic domain, assuming it is mostly alpha-helix, matches rather closely with the measured width of the periplasm, again suggesting there is little scope for a globular enzymatic domain.

Spanins as pore-formers

The simplest scenario for a pore formation strategy would be that after destruction of the PG, the spanins would oligomerize laterally in the envelope to form pores, basically mimicking the action of canonical holins but in the context of the OM. Just such a model was proposed by Krupovič *et al.* (2008) when they identified and characterized the i-spanin and o-spanin genes, *XXXV* and *XXXVI* respectively, of the cystovirus PRD1. As in lambda, transposon insertions inactivating these genes resulted in a divalent cation-dependent lysis defect, with a spherical cell form as the terminal phenotype in the presence of 10 mM $MgCl_2$. These authors proposed a model in which the spanin complex was coupled through the TMD of the i-spanin to the holin. Thus, when the holin triggered, the rapid dispersion of the holins into the hole walls would force "co-movement" of the i-spanin, which, through its complex with the o-spanin, would subject the OM to mechanical stress leading to local disruption and hole formation over the IM lesion. In this scenario, then, the pore-formation in the OM is mechanically linked to holin-mediated hole-formation in the IM. This model was tested by Berry *et al.* (2008). These authors prepared plasmid constructs that expressed the phage P1 SAR endolysin, Lyz, with or without Rz-Rz1. As noted above, SAR endolysins can cause lysis in the absence of holin function because each one has a characteristic rate at which it spontaneously escapes from its tethered state and refolds to full enzymatic activity. Using an allele of P1 *lyz* that had relatively early spontaneous lysis, Berry *et al.* showed that the complete *RzRz1* mutant phenotype (i.e., lysis defect with a cation-dependent spherical cell terminal phenotype) could be recapitulated, irrespective of holin function. Thus spanin function does not depend on holin function and, instead, simply requires degradation of the PG. Moreover, such a direct coupling of holin and spanin function is even more unlikely if one considers that pinholin-SAR endolysin lysis is just as dependent on spanin function as is canonical holin-endolysin lysis, despite the fact that the pinholins do not form micron-scale holes in the IM.

Do phages exploit the zones of adhesion?

Perhaps the biggest conceptual problem with the pore-forming idea is that it is difficult to imagine how the walls of the holes could be established, given that the only spanin moiety that is actually embedded in the OM are the fatty acid and diacylglycerol groups of the modified N-terminal Cys residue of the o-spanin or u-spanin. In contrast, the fact that two completely different functional classes of spanins both connect the IM and OM suggests that some sort of IM-OM interaction is involved in the lytic function. Moreover, there have been reports that the two membranes became intimately associated as a result of late gene expression in both the T4 and lambda infection cycles. The earliest report from C. Earhart's group found that both standard techniques for separating IM and OM components, i.e., isopycnic density gradient centrifugation and selective detergent solubilization, became ineffective as a result of late gene expression (Fletcher *et al.*, 1974). In practical terms, what this meant was that instead of getting well-separated peaks of low density (IM) and high density (OM) in isopycnic sucrose gradients of membranes, most of the membrane material was clustered in a peak of intermediate density. Similarly, A. Taylor's group found that IM and OM proteins moved into the intermediate density peak, in lambda inductions, in a manner dependent on the late activator gene *Q* (Kucharczyk *et al.*, 1991). In this case, the intermediate density peak was designated as the "A" peak because it was reputedly enriched in the so-called zones of adhesion (Bayer, 1968). Accordingly, Kucharczyk *et al.* suggested that the lambda *S* holin was

somehow expanding and subverting the zones of adhesion to effect the efficient release of the *R* endolysin to the murein. Later, the same group had several findings that suggested that both spanins were located to the intermediate density material. Indeed, as cited above, when Hanych *et al.* (1993a) had achieved the first subcellular localization of the Rz protein to the membrane fraction, they also noted that most of the Rz protein synthesized after hyper-expression from a T7-promoter vector plasmid was localized in membranes of intermediate density. Similarly, when Kedzierska *et al.* (1996) achieved over-expression of the *Rz1* gene, the Rz1 product was found to accumulate in both the OM and A fractions, with the majority in the latter.

Many of these conflicting results were rationalized in the work cited above by Berry *et al.* (2008) that showed Rz and Rz1 formed complexes and gave rise to the spanin concept. In addition to these important results, these authors also showed that it was essential to use an *S* nonsense allele in all inductions. This is critical because expression of the S105 holin protein prevented the formation of spheroplasts, presumably because of the micron-scale holes in the IM. If spheroplast formation is prevented, the standard Osborn technique (Osborn and Munson, 1974) for separating IM and OM was shown to fail, with the bulk of the membrane material was found in membranes of intermediate density. Over-expression of any membrane protein is also likely to be toxic for spheroplasts. Thus, the prior suggestions that zones of adhesions were expanded and presumably subverted by phage lysis proteins as a strategy for delivery of the muralytic activity were likely based on artifacts caused by interference with spheroplast formation during the membrane preparation protocol. Moreover, the existence of adhesion zones between the IM and OM is widely disputed. Originally described by M. Bayer and colleagues in EM thin sections of plasmolyzed, fixed *E. coli* cells, the adhesion zones were proposed to have a number of important functions, including providing a transit point for lipid and protein translocation between the membranes (Bayer, 1968). However, it has also been argued that the contact points are artifacts of fixation and plasmolysis (Kellenberger, 1990). Moreover, recent careful measurements of lateral cell envelope structure by cryo-EM of plunge-frozen, cells not subjected to plasmolysis or fixation revealed a uniform periplasmic space with no visible zones of adhesion (Berry *et al.*, 2008). In summary, there is no compelling evidence that phages exploit pre-existing zones of adhesion for lysis, even if such zones exist.

The membrane fusion model for spanin function

The foregoing has documented the difficulties with various models that have been advanced for how the spanins achieve disruption of the OM, although none except the enzymatic model can be ruled out definitively. In the view of this author, however, the simple model that the spanins mediate randomly-sited fusion between the OM and IM is much less problematic. The first attraction is its simplicity. Presumably, the reason that the OM has sufficient tensile strength to maintain the spherical cell forms that persist after PG degradation in the absence of spanin function is based on the chemical properties of the asymmetric bilayer, with the inner phospholipid layer supporting the outer LPS leaflet. Operationally, even if only local fusion occurred between the OM and IM, the consequence would surely be catastrophic for the integrity of the OM, especially since the load of the cellular osmotic pressure is no longer supported by the PG layer and would be exerted on one side of the fusion joint. Likely, instantaneous and violent bursting would occur as soon as the membranes began to fuse.

The most suggestive elements for a membrane fusion model, besides the possible fusogenic character of purified Rz1 reported previously (Bryl *et al.*, 2000), are the primary structure of the Rz periplasmic domain and the conformational dynamics attendant to spanin complex formation. The Rz soluble domain is nearly completely dominated by strongly predicted alpha-helical character, except for a single putative helix-breaking "hinge" region (Fig. 5). Moreover, the alpha-helical domain is strongly predicted to favor a coiled-coil structure. Both helical and coiled-coil predictions are borne out in both CD and EM studies of the complex formation (Berry *et al.*, 2010), which suggests that the binding of the Rz1 protein to the Rz homodimer leads to a substantial increase of alpha helix as well as formation of coiled-coil oligomers. This evokes the coiled-coil dynamics of the SNARE system that is integral to trans-Golgi vesicle fusion (Jahn and Scheller, 2006; Risselada and Grubmuller, 2012). SNARE proteins undergo coiled-coil oligomerization to bring the vesicle membrane and cell membranes into contact. In the current model for spanin function, the spanin hetero-tetramers (e.g., Rz_2:$Rz1_2$), once liberated from the PG network, oligomerize by lateral diffusion and form coiled-coil struc-

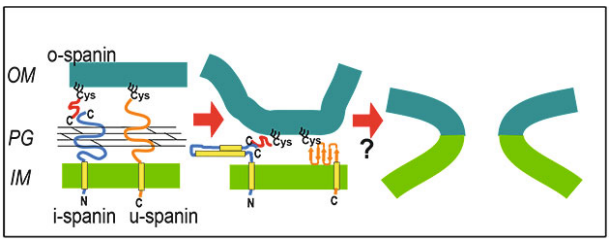

Fig. 7. Model for spanin-mediated disruption of the OM by membrane fusion. Both two-component spanin complexes and u-spanins accumulate in the envelope during the morphogenesis period, trapped within lacuna of the PG network. For simplicity, the two-component spanin complex is shown as monomer-monomer complexes interacting by C-terminal domains, although in fact, both the i-spanin and the o-spanin are covalent homodimers with intermolecular disulfide linkages in the lambda complex (See cartoon in Fig. 1C). After removal of the PG by the endolysin or SAR endolysin, the spanins undergo conformational changes that bring the two membranes together. Again, a simplified model is shown where the two coiled-coil domains of the Rz i-spanin interact in the collapsed conformation of the complex. Not shown is the two-dimensional oligomerization of these complexes (see Fig. 6) (Berry *et al.*, 2010). With the u-spanin, which lacks predicted alpha-helical or coiled-coil structures, the cartoon depicts formation of a beta-sheet from the predicted beta-strand elements. Whether the u-spanin also oligomerizes after removal of the PG layer is not known. In both cases, some feature of the collapsed conformation (middle cartoon) acts to destabilize either the inner leaflet of the OM or the outer leaflet of the IM, or both. The Rz1 periplasmic domain may be a good candidate for this activity (Bryl *et al.*, 2000). From Young (2013).

tures (Fig. 7). It is proposed that either these coiled-coil bundles have affinity for one of the bilayers, or perhaps both, so that bundle formation would lead to binding to one or both membrane surfaces. Alternatively, under the conditions of the periplasm with both ends of the spanin complex embedded in a membrane, formation of the coiled-coil structure leads to a collapsing conformational change, possibly involving the hinge region of the i-spanin.

Bringing two membranes together is necessary but not sufficient to initiate fusion. In the SNARE fusion, the model is that some feature of the clustered N-terminal TMDs of the oligomerized SNARE proteins destabilize the two apposed membrane leaflets. In other fusion events, like viral entry or synaptic vesicle fusion, specific proteins are involved. For the putative spanin fusion, the results of Bryl *et al.* (2000) suggest that the fusogenic agent might be the o-spanin subunit. The simplest overall idea is thus that the i-spanin subunit uses its coiled-coil propensity to bring about juxtaposition of the membranes, bringing the o-spanin into position to catalyze the actual leaflet fusion event.

Obviously, most aspects of this scenario are inappropriate for the u-spanin, in which the periplasmic domain has almost no predicted alpha-helical content but instead is dominated by several stretches of predicted beta-sheet structure (Fig. 5). Once again using Occam's Razor, the simplest idea is that gp*11* is regulated in the same way as the Rz-Rz1 complex; i.e., it is restrained from tertiary and quaternary rearrangements as long as the PG is intact. Nothing has been published about the biochemical properties of the periplasmic domain but some of the same considerations apply in terms of models for u-spanin function. Since the N-terminus of gp*11* is attached to the OM only by its N-terminal lipid moieties, it is difficult to imagine that tertiary and quaternary rearrangement could lead to formation of either a lipolytic or proteolytic domain in the periplasm or formation of a large pore in the OM. Thus membrane fusion is the preferred model for u-spanin function too, possibly involving intramolecular or intermolecular interactions of the predicted beta-strand domains.

Overview and Conclusions

Three steps in lock-step

In overview, lysis of Gram-negative hosts must now be regarded as a three-step pathway that attacks each component of the cellular envelope in a temporally-regulated, extremely efficient, and saltatory manner. Saltatory in this context is meant to emphasize the all-or-nothing character of the lytic event. Instead of lysis being basically a reflection of the accumulation of a single muralytic enzyme, multiple phage-encoded protein factors are involved: holin, anti-holin, endolysin, and spanin. The broad outlines of how these proteins operate to achieve the lytic event are now established, and some mechanistic understanding is now available. Two aspects of the process deserve re-emphasis. First, throughout the late-gene or morphogenesis period of the infection cycle, all the participant proteins accumulate in parallel without a detectable deleterious effect on the physiology of the infected cell. It seems apparent that the lysis pathway has evolved to avoid affecting the fecundity of the infection cycle, based on two underlying factors. First, the membrane components, which include the holin, anti-holin, SAR endolysin, and spanin proteins, are not synthesized in levels that are problematic for the integrity of the IM. So generalized "membrane toxicity" does not play a role in regularly-scheduled phage lysis (although it might be in situations where the normal pathway has been mutationally disrupted; see discussion of phage T4 lysis inhibition in Young (1992). Moreover, for each component, a physical or biophysical barrier prevents premature lytic function:

(a) the holin and pinholin are inhibited from hole formation by the pmf;

(b) the canonical endolysin is inhibited from degrading the PG by being sequestered in the cytoplasm;

(c) the SAR endolysin is inhibited from degrading the PG by being tethered to the IM and by having its catalytic domain locked in an inactive conformation either covalently or non-covalently;

(d) the spanin proteins are inhibited from disrupting the OM by being trapped within the meshwork of the intact PG.

Thus, the entire pathway is designed to be blocked at every level until holin triggering occurs, after which lysis ensues as rapidly as possible. It seems clear that, once the decision is made to terminate the infection cycle, every second wasted in the physiologically dead cell is a fitness loss.

Three steps, three choices

From an evolutionary perspective, it should be noted that there are at least two choices at each of the three steps of the lysis pathway. For the first step targeting the IM, the holin can either be a canonical holin or a pinholin. PG degradation can be achieved by either canonical soluble endolysins, which exist in at least four different enzymatic varieties, or secreted SAR endolysins. OM disruption can be achieved by either two-component spanins or u-spanins. Of the eight possible combinatorial permutations of these three choices, two are intrinsically impossible, because pinholins require SAR endolysins. However, of the other six possibilities, five have been observed in nature or shown to function in the laboratory, as documented in the preceding. Undoubtedly, one reason that the lysis pathway exhibits such combinatorial facility is that the components that subvert each layer of the envelope do not physically interact. It is also possible that in some bacterial hosts or some ecological niches, different selections from the "lysis menu" have a substantially different fitness values. Absent the necessity of keeping the pathway genes together, the well-documented modular character of phage evolution would be expected to generate every possible combination. Indeed, the extraordinary architecture of the embedded i-spanin/o-spanin gene class, which places the code for the interacting domains in the *same* DNA, can be regarded as further evidence for the high level of recombinational re-assortment that dominates phage evolution.

The outer membrane as a structural feature

Outside of phage biology, perhaps the most jarring aspect of this new understanding is the notion that the OM can withstand the osmotic pressure of the cell in the absence of

the PG layer, at least on time scales that are intolerable for a phage infection cycle. Thus the very existence of spanins and their requirement in lysis says that our understanding of the structural character of the bacterial envelope is at least incomplete. Unfortunately, currently it is not possible to create artificial OM samples in vesicles or planar bilayers, so the actual tensile strength of the OM is unknown. In fact, using SAR endolysins, it should be possible to create spherical cell populations that can be used to address the mechanical properties of the OM using atomic force microscopy and other nano-scale methods. In passing, it should be noted that preliminary analysis of a much larger set of curated phage genomes indicates that in a significant fraction of phages no lipoprotein with characteristics of either an o-spanin or a u-spanin is encoded, even though holin and endolysin candidates can be identified. Most of these are phages of poorly characterized Gram-negative hosts and many are from marine environments. Although some of this may be due to annotation errors, it is possible that in some Gram-negative bacteria and in some environments, either the OM does not need to be disrupted to effect efficient virion release. Alternatively, at least one other strategy may exist for the disruption of the OM, which, considering the options already identified, would not be surprising. Finally, it must be noted that all of the foregoing is directed towards the strategies of host lysis by phages of Gram-negative hosts. There are hints that similar complexity may be in place for phages of Gram-positive hosts and of the mycolata. Considering the value of understanding the vulnerabilities of bacterial pathogens and the time that phages have had to explore and exploit these vulnerabilities, there seems to be abundant motivation for continuing to focus on the mechanisms by which phages lyse their hosts.

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