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Bacteriophage Holins: Deadly Diversity

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Introduction

Bacteriophage biology has been the source of many of the fundamental paradigms of molecular genetics. The infective cycles of phages like λ, T4, and T7 have been characterized in great detail; the precise sequence of gene expression and many of the decisive regulatory events are known. Indeed, the only developmental step in biology that is sufficiently well understood to be quantitatively modelled at the molecular level is the choice between the lytic and lysogenic pathways in phage λ (McAdams and Shapiro, 1995). However, the terminating event of the infective cycle – lysis of the host cell – was generally viewed as the trivial and inevitable outcome of the accumulation of phage-encoded muralytic activity (Watson *et al*., 1987). It is only recently that it has become appreciated that the host lysis event is also exquisitely programmed. It will be argued here that, in fact, the *only* critical regulatory decision in the vegetative cycle is *when* to effect lysis of the host. The molecule which determines the timing of this event and also provides function essential to lysis is the *holin* (Wang *et al*., 2000). Holins are small membrane proteins that, as a functional group, have been defined for less than a decade (Young, 1992). Although considerable progress has been made in recent years in defining the scope of holin-mediated phenomena, it must be emphasized from the outset that we are still largely in the descriptive phase of the study of these remarkable proteins which constitute the most diverse group of functional homologs in nature (Wang *et al*., 2000). There is very little known about holins at the structural level or about how they function at the mechanistic level. In fact, the nature of the lethal lesion caused by holins in the process of lysis is still unknown; is it a defined pore in the membrane, a collection of stable pores of random dimensions, or an unstable, dynamic pore reflecting loose intermolecular interactions between holins, or irregular deformities caused by highly oligomeric clusters of holins? Nevertheless, holins have been revealed as such elegantly simple solutions to the problem of biological timing that they must attract increasing attention and study, even though their membrane localization and lethal phenotype pose daunting obstacles to biochemical investigation.

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The Genes of Phage Lysis

A Universal Strategy for dsDNA Phages

With the exception of filamentous phage, all phages resort to lysis of the host for release and dispersion of progeny (Young *et al*., 2000). Lysis requires compromising the structural integrity of the murein sacculus, which forms a tough, although dynamic, meshwork that surrounds the cell and confers on it its rod-like shape (Höltje and Glauner, 1990). Small phages with single-stranded nucleic acid genomes accomplish lysis by producing a protein that inhibits cell wall biosynthesis, at least in two cases (Bernhardt *et al*., 2000a; Bernhardt *et al*., 2000b; Bernhardt *et al*., 2001). However, apparently all phages with double-stranded nucleic acid (dsNA) genomes, both DNA and RNA, effect host lysis with a **holin-endolysin** system (Young, 1992; Young *et al*., 2000) named for the two proteins essential for programmed lysis. In most cases, the holin and endolysin genes are adjacent and often clustered with other genes providing ancillary lysis functions in a ''lysis cassette'' encoding up to five proteins (Figure 1). A brief description of the different proteins encoded by these cassettes is necessary to provide context for considering holin structure and function.

Endolysins: Phage Muralytic Enzymes

All dsNA phages encode at least one muralytic enzyme, the endolysin, essential for host lysis. The term endolysin is meant to distinguish these lysis proteins from a second muralytic activity often found associated with a protein component of the phage tail; this activity, always a glycosylase, is involved in facilitating the penetration of the murein during injection of the genome into the host and is not involved in programmed lysis (Caldentey *et al*., 1994; Moak and Molineux, 2000). At least four different muralytic activities are found in phage endolysins: transglycosidases and lysozymes that attack the glycosidic bond linking the amino-sugars in the cell wall; and amidases and endopeptidases that attack the amide and peptide bonds of the oligopeptide cross-linking chains (Young, 1992). Although the endolysins described previously, like the T4 E lysozyme, the T7 gp *3.5* amidase, and the λ R transglycosylase, at least shared the common feature of being small, globular proteins, recently endolysins have been identified that possess multiple muralytic activities and correspondingly greater molecular masses, up to 60kDa (Navarre *et al*., 1999).

With important exceptions noted below, endolysins in general have no intrinsic secretory signals and thus accumulate in the cytoplasm during the infective cycle.

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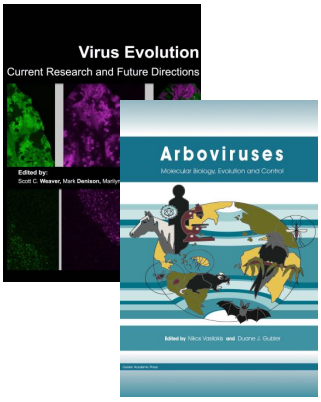

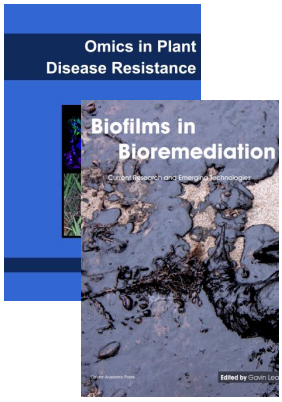

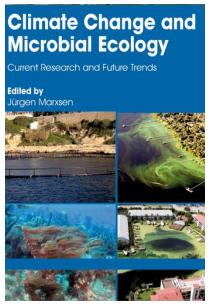

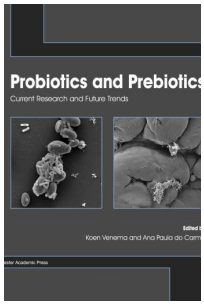

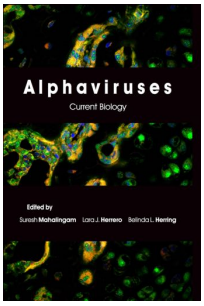

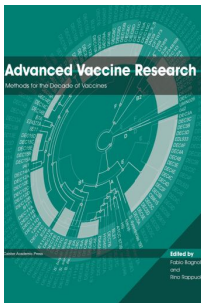

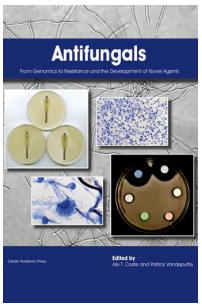

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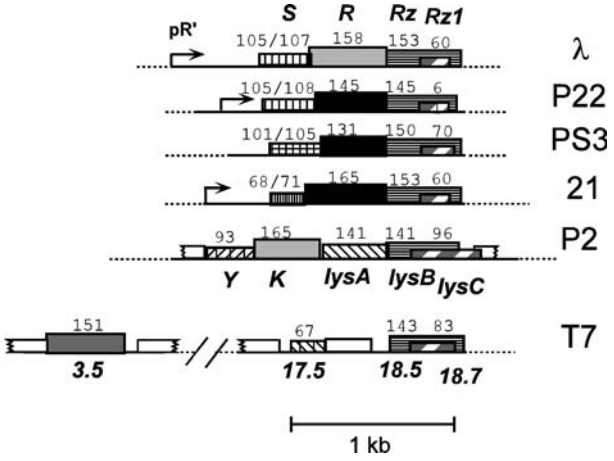

Figure 1. Lysis gene arrangements. The arrangement of the lysis genes of four lambdoid (λ, P22, PS3, 21) and two non-lambdoid (P2, T7) phages is depicted, with the size (in codons) shown above each gene. Each lysis gene set has at least a holin gene (all boxes decorated with any vertical lines), an endolysin (all solid boxes in gray or black), and an *Rz* (horizontally lined boxes) – *Rz1* (striped boxes) gene pair (Wang *et al.*, 2000). (*Rz* – *Rz1* genes in P2 and T7 are called *lysB*–*lysC* and *18.5*–*18.7*, respectively.) Sequence similarities are detectable only between genes of identical decoration. In addition, the putative antiholin gene, *lysA*, of P2 is shown (left-slanted lines) (Ziermann *et al.*, 1994). Among the endolysins, the enymatic activities are: black = muramidase (lysozyme); light gray = transglycosylase; dark gray = amidase. White boxes are other phage genes with no lysis function. In the case of the lambdoid holin genes with dual start motifs, the lengths of the holin and antiholin reading frames, in codons, are shown above each box. (Actual antiholin function has been demonstrated only for λ, P22, and 21.) The names of the λ lysis genes (*S, R, Rz,* and *Rz1*) are used for the lambdoid genes; the *Salmonella* phages P22 and PS3, the corresponding gene names would be *13, 19, 15* and *Rz1*, respectively. Note that in T7, the endolysin gene, *3.5*, is located in an early gene cluster, near the left end of the genome; its position in the lysis cassette is taken by an unrelated capsid structural gene.

Instead, in the absence of holin function, in general these enzymes exhibit no activity in vivo. For example, lysis is normally observed as a sudden event occurring throughout the induced culture 50 min after thermal induction of a λ lysogen. However, if the holin gene *S* is inactivated by a null mutation, culture mass and intracellular phage titers accumulate indefinitely. The activity of the endolysin, a transglycosylase encoded by the λ *R* gene, also accumulates indefinitely, without effect on cell growth or murein synthesis (Garrett *et al.*, 1981). If cells are concentrated, disrupted and fractionated, *R* activity is found in the soluble phase; moreover, addition of $CHCl_3$, freezing and thawing, or mechanical disruption causes very rapid lysis (Garrett *et al.*, 1981; Reader and Siminovitch, 1971a). Taken with the absence of secretion signals in the *R* sequence, these observations strongly suggest that *R* is retained in the cytoplasm in the λ*S* inductions. Moreover, this implies that holin function results in the release of endolysin across the membrane barrier, thus allowing the endolysin access to its murein substrate. Finally, to date, every endolysin gene tested, irrespective of its intrinsic muralytic activity and lack of sequence similarity to *R*, complements *R* null alleles (Wang *et al.*, 2000). This establishes the important principle that although a muralytic enzyme is required, it does not have to interact specifically with any other phage lysis protein, including the holin, the only other lysis protein that is essential for lysis.

Holins: Saltatory Lethal Membrane Permeabilization

If endolysins require holin function to attack the peptidoglycan, what do holins do? In λ*R* inductions, lysis is not observed, due to the absence of endolysin activity, but nevertheless cell respiration stops. The membrane loses its ability to support respiration and a membrane potential and becomes permeable to ATP, other normally impermeant small molecules, and polyethylene glycol polymers of up to 10 kDa (Wilson, 1982; Adhya *et al.*, 1971; Reader and Siminovitch, 1971b; Garrett, 1982). These indirect assessments all point to a massive permeabilization of the membrane. The appropriately vague term ''hole formation'' has been used as short-hand for what would otherwise have to be termed ''apparent holin-dependent membrane permeabilization''. However, it should not be forgotten that nothing is known about the nature of the resultant membrane damage, or the structure of the holin-dependent lesions. Interestingly, even if induced in absence of all other λ genes, the *S* lesion is lethal, and the sharply-defined loss of viability precedes physiological death, as defined by cessation of culture growth and respiration, by more than 30 min (Garrett and Young, 1982). This suggests a lethal dose of *S* protein has been accumulated but does not cause permeabilization of the membrane until a defined time interval has elapsed. Below it will be argued that this delayed-action characteristic is the crucial feature which has defined the evolutionary success of holins as controllers of the host lysis phenomenon.

A recent report has claimed that holins have another distinct biological activity when synthesized at low levels in Gram-positive bacteria (Walker and Klaenhammer, 2001). The central observation in this report is that transcription of a single copy holin-endolysin gene cassette from the late gene region of a *L. lactis* prophage leads to quantitative release of some cytosolic proteins, including a 450kDa β-galactosidase produced from a cloned *lacZ* gene, but not others, all without affecting cell viability or membrane integrity. The notion that a holin-endolysin system can achieve semi-specific release is hard enough to credit, but the idea that it could do so without disrupting the membrane (much less the murein layer) or killing the cell is even more difficult to rationalize. An alternative interpretation of the data presented is that most of the culture is being gradually lysed and is then overrun with cells cured of the plasmid carrying the late gene activator and reporter gene. Moreover, the assay methods used to determine whether cytoplasmic enzymes are still internal are critical. Manipulation of cells carrying induced holin genes is very difficult; any physiological perturbation that can momentarily depress the proton motive force will trigger the accumulated holins. For example, Husson-Kao *et al.*

(2000) showed that the autolysis of *S. thermophilus* upon lactose depletion is due to triggering of sub-lethal amounts of holin proteins accumulated from a partially induced prophage. Thus, unless more convincing results can be obtained establishing that the kinetics and absolute levels of protein release are inconsistent with a partial lysis of the culture, it is premature to conclude that holins, at sub-lethal levels, can cause semi-specific release of large cytoplasmic proteins across the entire bacterial envelope.

As noted above, the crucial function of the holin in the phage vegetative cycle was not appreciated until relatively late, after the era when phage systems dominated molecular biology. Mostly this was due to the fact that, due to being small genes largely refractory to nonsense suppression, holin genes eluded detection in the systematic amber mutant hunts conducted early in the studies of the classic coliphage systems: λ, P22,T4, T5, T7, Mu, P1, and P2. Even in the modern era where new phages have been characterized primarily by cloning and sequencing rather than traditional mutant hunts, holin genes have been elusive, primarily because they are lethal and thus cannot be cloned in high copy plasmids. Nevertheless, there are about 150 holin genes now identified, defining more than 35 unrelated orthologous gene families. This diversity is too extensive to be reviewed here; instead, the reader is referred to a recent review (Wang *et al.*, 2000). In any case, holin genes are easily the most diverse group of genes with common function known in biology. Moreover, there are at least three different membrane topologies assumed by holins (Figure 2). Class I holins have three TMDs, with N-out, C-in, whereas class II holins have two, with N-in and C-in. Both class I and II holins are largely hydrophobic, with the helical TMDs defining two-thirds of the primary structure. However, recently, the T protein of phage T4 was shown to be a holin. T is highly hydrophilic, has only a single TMD, assumes the N-in, C-out disposition of a type II membrane protein and thus defines a third class of holins (Ramanculov and Young, 2000a). Despite these diverse forms, to date every holin gene tested, irrespective of class, complements lambda *S* nulls (Wang *et al.*, 2000). Coupled with the wide diversity of endolysin structures, activities, and molecular masses, the genetic interchangeability of holins strongly suggests that they do not interact with any other phage lysis protein. Moreover, the fact that qualitatively identical lysis can be obtained by induction of cloned lysis genes shows that no other phage proteins are directly involved in lysis either. Thus we are left with the picture that holins act on their own, without essential heterologous interactions, in accomplishing their required role in lysis.

Rz/Rz1: Two Lysis Genes Sharing the Same DNA

A holin and an endolysin constitute the essential minimum for programmed lysis in most cases, but to have a useful perspective on the lysis phenomenon, it is necessary to mention ancillary participants. A somewhat bizarre case is a pair of genes, *Rz* and *Rz1*, found in the lysis cassettes of many phages of Gram-negative bacteria (Figure 1). Mutations in either gene result in a lysis defect in the presence of millimolar concentrations of divalent cations; in these conditions, λ*Rz* or λ*Rz1* infections lead to the formation of spherical cell forms instead of frank lysis. What makes this bizarre is that the *Rz1* cistron is entirely embedded within *Rz* in a +1 reading frame. Nothing is known about *Rz/Rz1* function. The *Rz* protein has a secretory signal sequence and is predicted to be periplasmic, whereas the *Rz1* gene encodes a proline-rich lipoprotein. According to the rules established for signal-peptidase II-mediated lipoprotein sorting (Yokota *et al.*, 1999), *Rz1*, once processed to its mature 40 residue length, should be bound to the inner leaflet of the outer membrane by its N-terminal lipid moiety. At least two unrelated *Rz* gene families have been identified, all of which contain an embedded *Rz1* gene, recognizable by its signal peptidase II motif and its proline-rich amino acid sequence. It has been suggested that *Rz/Rz1* might account for an endopeptidase activity detected in λ lysates and originally ascribed to *R* (Taylor, 1971). In any case, because of the fact that millimolar levels of divalent cations stabilize the outer membrane in spheroplasts, it is thought that *Rz/Rz1* attacks the outer membrane or its covalent connections to the murein.

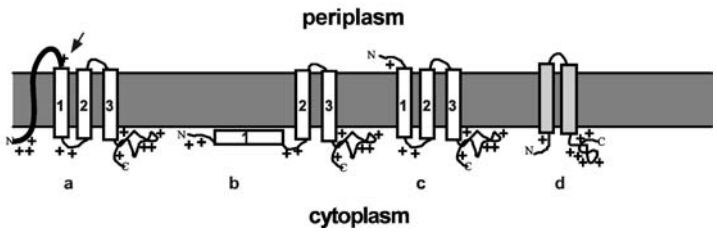

Figure 2. Membrane topologies of holins. The membrane topologies of λ S (items b, c) (Bläsi *et al.*, 1999; Gründling *et al.*, 2000b) and 21 S ((item d) (I.-N. Wang, M. Barenboim, and R. Young, unpublished) are depicted; each rectangle is a helical TMD. Item (a) shows the hybrid S protein bearing a secretory signal sequence constructed by Graschopf and Bläsi (1999), with the Lep cleavage site indicated by the arrow. Item (b) shows the putative topology of the antiholin S107, with its extra N-terminal positive charge; item (c) shows the topology of the holin, S105. The approximate positions of all residues with side chains predicted to be positively charged and located in the aqueous phase are shown. Modified from Wang *et al.* (2000).

Antiholins

In addition to the holin, endolysin, *Rz* and *Rz1* proteins, dsNA phages often encode a fifth lysis protein: the antiholin. The antiholins are specific negative regulators of holin function and will be considered in detail below in conjunction with the properties of their cognate holins. It is worth noting at this point, however, that, in parallel to the remarkable diversity of holins, at least five different types of antiholins are known, including, based on sequence analysis, three different types of membrane proteins, a cytoplasmic protein, and a periplasmic protein (see below). This is perhaps not surprising considering our premise that the host lysis event is the only important regulatory decision of the vegetative cycle, and that the regulation is purely temporal. It is intuitively attractive to think that it is easier to achieve fine-tuning of any molecular schedule if there are two opposing components to the clock. In fact, fine-tuning is apparently the role of antiholins in the lambdoid phages, where in the absence of the antiholin, the holin is still capable of supporting programmed lysis, albeit somewhat earlier than normal (Raab *et al*., 1988; Barenboim *et al*., 1999). Nevertheless, in at least one case, bacteriophage P1, it is clear that antiholin function is essential; without the antiholin, lysis occurs so catastrophically early that a productive yield of progeny virions is prevented (Walker and Walker, 1980).

Why Do Holins Exist?

The unparalleled evolutionary diversity of holins suggests that they provide an advantage in fitness sufficiently powerful to cause multiple independent origins. Yet at first consideration, there is no obvious reason for holins to exist at all. The muralytic activity of endolysins must have access to its extracellular heteropolymeric target, the peptidoglycan, in order for lysis to occur. The simplest solution would seem to be, as defined for other proteins with required extracellular function, to equip the endolysin with a secretory signal sequence. The first problem, of course, is that endolysins are usually encoded on the same late gene transcripts as the virion structural and maturation proteins. Nevertheless, it would not seem difficult to reduce the translational efficiency of the endolysin cistron to achieve a delay in lysis appropriate for allowing the assembly of progeny virions. Indeed, Santos and colleagues recently reported the first discovery of an endolysin that achieve independent, *se* c-mediated externalization (Sao-Jose *et al*., 2000). The endolysin of fO_g44, a phage of the Gram-positive bacterium *Oenococcus oeni,* undergoes leader peptidase cleavage of its signal sequence and causes lysis of the host, independent of holin function, when expressed in *E. coli*. Preliminary evidence indicates the endolysin of phage P1 is also externalized by the *sec* system; nevertheless, even in this system, a holin is encoded (M. Xu and R. Young, unpublished). So why do holins exist?

The answer, at least in part, can be deduced from first principles. The first important lesson is that lysis is in general not the result of, or response to, a deterioration of the host biosynthetic capacity, as was originally proposed (Young, 1992). Indeed, some of the early work on the phage lytic cycle showed that, if the lysis system is inactivated, infected cells can continue to produce virions at a constant rate for a period greatly beyond the normal lysis time (Hutchison, III and Sinsheimer, 1966; Josslin, 1970; Reader and Siminovitch, 1971b). There have been several theoretical analyses of the contribution of lysis timing to overall fitness (Wang *et al*., 1996; Abedon, 1994; Abedon, 1989). Although these analyses differ in important quantitative details, nevertheless a common conclusion is that for any particular phage, host and defined physiological conditions, there is an ideal lysis time. The boundary conditions are the easiest to consider. Clearly, in conditions where the average host density is very low, there is an evolutionary pressure to postpone lysis and accumulate virions, linearly, as long as the biosynthetic capacity of the cell, in terms of the rate of virion assembly, is undamaged. In contrast, if the density of uninfected hosts is very high, there is pressure to escape the current host and infect as many as possible of the surrounding population of cells, thus pre-empting competitor phages and ensuring an exponential increase in phage titer. Thus evolution will tend to select for phages with lysis times optimized for the current average host density.

Again, all these considerations do is to establish that a defined lysis time is a fitness parameter. One can even argue that the timing of lysis is in fact the only molecular decision that must be made in the lytic cycle; all other processes, although temporally sequenced, presumably occur at a constitutive rate designed to maximize the rate of intracellular virion assembly. However, once the ideal lysis time has been reached, it would clearly be disadvantageous to be trapped in a dead host waiting for a limiting amount of muralytic activity to degrade the murein sufficiently. So one would expect that lysis should occur rapidly and completely, once begun. On the other hand, clearly it would be disadvantageous for the lysis system to have a deleterious effect on host physiology before the ideal lysis time; that is, anything that impairs the rate of production of intracellular progeny would be selected against. Thus the ideal lysis system is one that acts in a sudden or saltatory manner, at the ideal lysis time. This is one characteristic of holin-based lysis systems, which, except for the microviridae and leviviridae, with their tiny genomes, are used by all lytic phages. Moreover, the holin must be capable of rapid evolutionary adjustment, in terms of lysis times; this requirement for temporal plasticity is spectacularly met by the two holins that have been studied in detail, lambda S and T4 T. In both cases, single amino acid changes in the holin sequence have been shown to impose radically different temporal schedules on lysis, without affecting synthesis or accumulation of the holin protein (Gründling *et al*., 2000a; Ramanculov and Young, 2000b). It might be expected that not only should evolutionary adjustment be a hallmark of holins but also

there should be real-time regulation of holin action in response to environmental signals reflecting, for example, host cell density. Surprisingly, only in one case, the anti-holin of T4, has this been shown (see below); in all the other cases, although anti-holins are in fact made as negative regulators at the functional level, there has yet been no evidence that the activity or levels of the antiholins are regulated at all, much less in response to environmental stimuli. In any case, from a general consideration of the constraints of the phage lytic cycle, it appears that holins are the key to having a programmed, all–or-nothing lysis easily adjustable to changing environmental conditions. The next step is to achieve a mechanistic understanding of how holins achieve this critical funciton. Although the diversity in holin sequences is impressive and revealing, achieving an understanding of holin function at the molecular level requires the establishment and exploitation of a paradigm system.

Lambda S: The Holin Paradigm

S: One Reading Frame, Two Proteins of Opposing Function

The *S* gene spans 107 codons (Figure 3). There is an upstream element, called *sdi* (structure directed initiation) defining a stem-loop structure in the mRNA (Figure 4). The structure controls the partition of translational initiations between two start codons, codons Met_1 and Met_3 of the *S* reading frame; the products of initiation events at these two codons are designated S107 and S105, respectively. S105 is the holin and S107 is its specific antiholin. Both products are stable, integral membrane proteins. This unusual arrangement is not unusual among holin genes, many of which have two potential start codons early in the gene (Figure 4) (Wang *et al.*, 2000). In at least two of these, there is evidence that the proximal and distal start codons of these *dual start motifs* also encode an

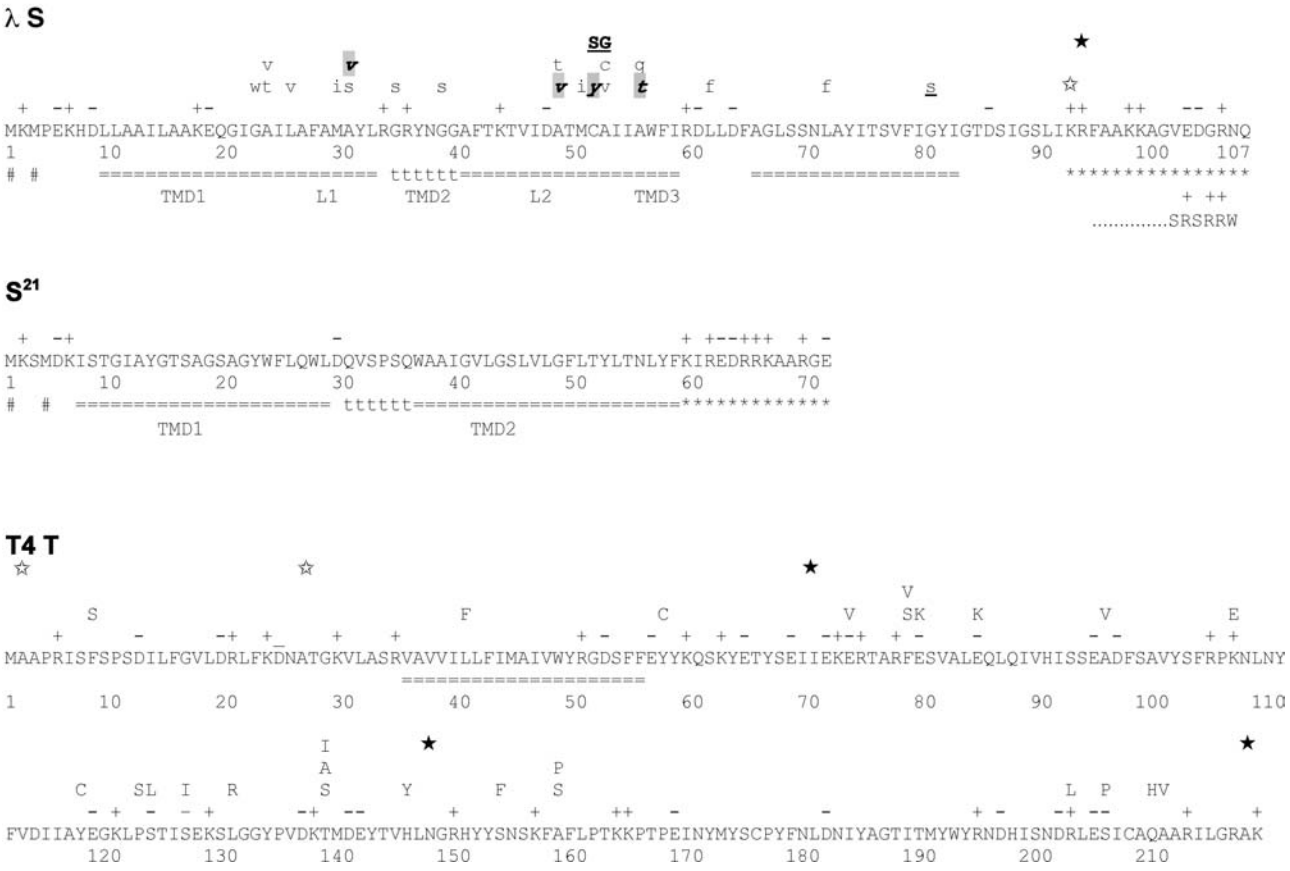

Figure 3. Holin proteins. The predicted primary structures of prototype holin gene products: λ *S* (class I), S^{21} (class II) and *T*. For *S*, lysis-defective missense mutants are shown as lower case substitutions above the sequence. For both *S* and S^{21}, the pound signs mark the dual start codons; in both cases, the holin sequence starts with the second Met codon, whereas the antiholin gene product starts with the first. Key: transmembrane domains (TM), connector loops (L), predicted turns (ttttttt), and hydrophilic C-terminal domains (*****). Above the *S* sequence, missense mutations leading to a block or delay in lysis (recessive alleles, lower case; co-dominant alleles, underlined lower case) or accelerated lysis (bold, underlined upper case) are shown. Shading indicates ''antidominant'' (formerly called ''early dominant'' (Raab *et al.*, 1988) alleles: lysis-defective alleles that accelerate lysis in trans to wt *S* (Raab *et al.*, 1988)). Clear star and filled star indicates positions of nonsense mutations leading to lysis-negative or early-lysis phenotypes, respectively. Below the sequence, the sequence of a C-terminal frameshift allele that suppresses the catastrophic early lysis defect of A52G(Johnson-Boaz *et al.*, 1994). For the *T* sequence, missense mutants that retard the onset of lysis but do not affect its efficiency or speed are indicated above the line; filled and empty stars indicate the positions of *phoA-lacZ*α sandwich fusion insertions that are $PhoA^+$ /βgal or $PhoA^-$ /$βgal^+$, respectively. Modified from (Young *et al.*, 2000).

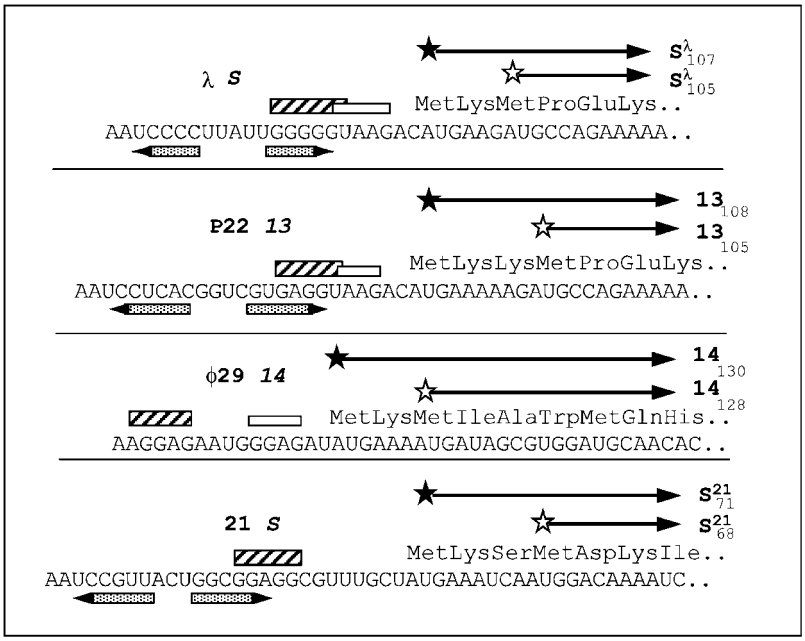

Figure 4. Dual start motifs. The dual start motifs of two orthologous (λ *S* and P22 *13*) and one non-orthologous (*ϕ*29 *14*) class I genes and a class II holin gene are shown, with the anti-holin and holin translational start indicated by filled and empty stars, respectively (Bläsi *et al*., 1989; Bläsi *et al*., 1990; Nam *et al*., 1990; Steiner *et al*., 1993; Barenboim *et al*., 1999). The Shine-Dalgarno sequences serving the anti-holin and holin start codons are indicated by striped and clear rectangles, respectively, except for S^{21}, where both starts are served by the same sequence. Inverted repeat sequences that, in the mRNA context, control the partition of translational initiations between the two start codons are indicated by the pairs of divergent arrows. The length of each gene product is indicated to the right.

antiholin and holin, respectively (Tedin *et al*., 1995; Barenboim *et al*., 1999). Thus it is crucial to consider the properties of the holin and the antiholin to understand holin function in general. Here, the properties of the holin, S105, will be considered first.

S105: Molecular Details for a Function Holin

S has been quantitatively localized to the inner membrane (Altman *et al*., 1985). Various TMD-prediction algorithms predict two or three TMDs for the S105 protein. This ambiguity arises from the fact that each of the TMDs has substantial hydrophilic character, either in terms of charged residues (TMD1, TMD2) or hydroxylated residues (TM3) (Figure 3). Gene fusion analysis of *S* indicated that the C-terminus is cytoplasmic (Bläsi *et al*., 1999) and the loop between TMD2 and TMD3 is periplasmic (I.-N. Wang and R. Young, unpublished). Gründling *et al.* (2000) used a combination of cysteine-scanning and chemical modification to demonstrate unequivocally that, in the membrane, S105 has three TMDs, with N-out and C-in.

Quantitation of *S* indicates only a very small fraction of the cell membrane surface can be occupied by the holin molecules. For the wt S^+ allele, the total amount of *S* at the time of lysis has been estimated by quantitative immunoblotting, at about 1000–3000 molecules, of which about two-thirds is S105. It is not known to what degree the holins cluster in the membrane. Immunoblot analysis of membranes treated with the cross-linking reagent DSP results in covalently-linked *S* oligomers up to at least the 6-mer state (Johnson-Boaz *et al*., 1994; Gründling *et al*., 2000a), so it seems clear that *S* oligomerizes during hole formation, as would be expected from the fact that it has only 3 TMDs.

Allelic Variability: Recessiveness, Dominance, and Anti-Dominance

S is an ideal genetic subject because of small size and lethal action. Moreover, one can easily select both gain and loss of function mutants. For example, if one infects *E. coli* with mutagenized λ, allows the infection to proceed through the lysis of the bulk culture, isolates unlysed cells by filtration or centrifugation of the lysate, and then treats these recovered cells with chloroform, all of the phage released will be *S* mutants. Alternatively, one can enrich for early lysis phenotypes by isolating phage from the filtrate of samples taken long before the normal lysis time. Moreover, it is not even necessary to use the phage context. Plasmid clones of the *SR* genes are directly released to the medium after induction (Kloos *et al*., 1994) (H. Fang, E. Ramanculov and R. Young, unpublished), where they can be recovered and retransformed into new hosts. Thus positive and negative selections can be applied to *S*, and indeed any holin gene, which makes the relatively undeveloped state of holin genetics all the more surprising.

Many *S* alleles have been isolated, mostly by selecting for loss of lethality in inducible plasmid-borne *S* genes (Raab *et al*., 1988; Raab *et al*., 1986). When these alleles were recombined back onto λ, many of them exhibited a delayed-lysis phenotype instead of a total lysis defect, presumably because expression is somewhat higher in the context of λ late gene expression than from an induced *lac* promoter on

pBR322. To correlate the lysis defects with defects at the molecular level, representative *S* mutants were analyzed by the cross-linking and immunoblot strategy described above (Gründling *et al*., 2000a). Some alleles were found to have defects in dimer formation, while many showed defects in oligomer formation, and a few had a normal pattern of oligomerization despite the loss of lytic function. The simplest interpretation is that these alleles define the pathway for hole formation: formation of dimers, followed by higher order oligomerization and then a concerted conformational change leading to hole-formation (Figure 5).

Given its oligomeric character, it was anticipated that mapping dominant and recessive alleles, it might be possible to discern domains of *S* needed for intermolecular interaction. It is crucial to consider the nature of *S* function when analyzing lysis phenotypes for dominance and recessiveness. The simplest system is a double lysogen, where one prophage carries a null allele, *Sam* 7, or the wt allele S^+, and the other carries the allele being tested. The boundary conditions of this analysis are defined by comparing lysis kinetics of the (S^+, S^+) pair with that of the (*Sam*7, S^+), which undergoes lysis about 10 min later. This null phenotype defines the recessive phenotype shared with a number of lysis-defective alleles. As predicted, some lysis-defective alleles cause a substantial delay in lysis and are thus judged to have dominant (strictly, co-dominant) character (Figure 3). Unexpectedly, however, a number of lysis-defective alleles test out as S^+ in the double-lysogen test, and a few, when paired with S^+ cause lysis even earlier than the double wild-type! This unprecedented phenotypic inversion is designated ''anti-dominance'' (originally, ''early dominance'' (Raab *et al*., 1988)) and constrains models for holin timing and hole-formation.

Features of the Lysis Clock

Not all *S* mutants are lysis-defective. Two adjacent missense mutants, C51S and A52G, have early-lysis phenotypes that have been crucial to the current understanding of holin function. A52G is especially noteworthy; lysis occurs at 20 min, only 12 min after the onset of late protein synthesis. Because this is about the time that the first infectious virion appears in the cytoplasm (the end of the eclipse phase), S_{a52g} confers a plaque-forming defect (Johnson-Boaz *et al*., 1994). This is formal genetic proof that *S* has two essential functions: it must provide the lethal membrane lesion required for lysis and it also must do so at the proper time, certainly no earlier than the time when a burst size of progeny sufficient to support plaque formation is available. Thus *S*, and by extension all holins, constitute biological clocks. Moreover, the early lysis supported by S_{a52g} is not due to increased accumulation of the holin (Johnson-Boaz *et al*., 1994). Thus a small change, precisely 14 daltons in side chain mass in the middle of TMD2, has no effect on the efficiency of lysis but a catastrophic effect on its timing. By comparative immunoblot, S_{a52g} triggers lysis when it has reached about 10% of the level of S^+, or 100-300 molecules. This puts significant constraints on models for *S* function. Finally, the plaque- forming defect of S_{a52g} has been used to isolate intragenic suppressors restoring the lysis delay. One such suppressor is a frame-shift that scrambles the sequence of the N-terminus and results in a net gain of four positive charges (Figure 3). Thus the role of the C-terminal sequence of *S*, beyond TMD3, has no essential role in hole-formation but instead provides a charge-sensitive timing feature.

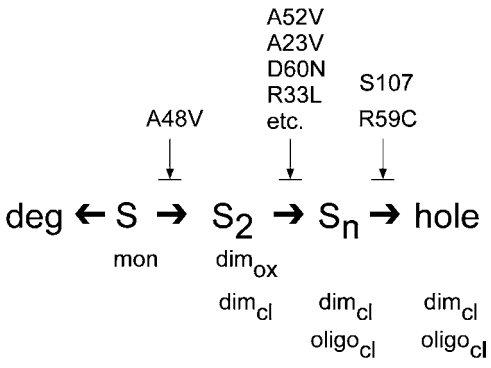

Figure 5. Genetically-defined pathway to hole-formation. Results of Cu^{++}-phenanthroline-mediated site-specific disulfide cross-linking and DSP-mediated random cross-linking studies on isolated membranes reveal that different lysis-defective *S* alleles are blocked at different stages in the pathway to hole-formation. Modified from Gründling *et al.* (2000a). The A48V protein is partially defective in dimerization and is present at lower levels under standard induction conditions; consequently, a proteolytic step is proposed for monomers that fail to dimerize.

Phenotypic Assignment with the Dual Start Motif

As mentioned above, genetic analysis of *S* is facile; indeed, it was the analysis of dominant mutants with single-base changes in the putative RNA structure upstream of the *S* reading frame that led to the discovery of the S107 antiholin. There is precedent for a protein and its negative regulator to be produced from the same reading frame, by virtue of a downstream transcriptional or translational start (e.g., Tn*5* transposase and its inhibitor, encoded on IS*50*; (Krebs and Reznikoff, 1986) but only in the case of *S* is the full-length gene product the negative regulator. It is important to note that the existence of the dual start motif should complicate interpretation of the lysis phenotypes of the collection of *S* mutants, most of which originated from the full-length parental S^+ reading frame. In principle, for example, a delay in lysis might mean either reduced lytic function for the S105 protein, or increased inhibitor function for the S107 protein, or both. Fortunately, one can inactivate either start codon of *S* without affecting the initiation rate at the other in vivo (Chang *et al*., 1995), so in effect an inhibitor-free *S* gene, designated *S105*, can be created by converting the AUG of codon 1 to CUG. When representative alleles derived from *S* have been converted to *S105* alleles in this way, there has been no significant change in the phenotype (Gründling *et al*., 2000a; Gründling *et al*., 2000b). This suggests that in the initial selection for loss of lethality, it was not

easy to get alleles in which the antiholin gains function at the same time that the holin loses it. In any case, clearly genetic studies with other dual-start holin genes should be done with this potential complication in mind. Unfortunately, it is not always true that the dual starts can be manipulated independently. Inactivation of the start codon for the antiholin of S^{21} causes a reduction in initiation frequency at the Met_4 start codon for the holin reading frame (Barenboim *et al.*, 1999), which complicates interpretation of lysis phenotypes.

S107: Dynamic Topology and Function

The S107 protein differs from S105 only its N-terminal dipeptide sequence, Met_1 -Lys_2. Normally, the S105:S107 ratio is about 2.5:1. Mutations in *sdi* and the Shine-Dalgarno sequences identified for these two start codons change the proportion and total amount of *S* protein synthesized. The triggering time of lysis is inversely related to the excess of S105 over S107. When S107 is made in excess of S105, programmed, saltatory lysis does not occur in a physiologically meaningful time frame (Raab *et al.*, 1988; Bläsi *et al.*, 1990; Chang *et al.*, 1995), suggesting that the antiholin acts by titrating out the holin. Recently, support for this notion was provided by the finding that S107 preferentially forms dimers with S105 in the membrane (Gründling *et al.*, 2000c). The operative distinction between S107 and S105 is the positively-charged residue Lys_2; moreover, the antiholin loses its ability to inhibit the holin if the membrane is de-energized by an energy poison or uncoupler and, in fact, seems to be converted into an active holin form (Bläsi *et al.*, 1990). These findings led to the model that the N-terminal TMD1 of S107 is blocked from flipping up through the cytoplasmic membrane by the energized membrane, as found for the N-terminal TMD of Lep and ProW (Cao and Dalbey, 1994; Dalbey *et al.*, 1995; Schuenemann *et al.*, 1999) (Figure 2). Graschopf and Bläsi (1999) provided evidence supporting this by showing that attaching an N-terminal signal sequence suppressed the holin-antiholin distinction, and correlating signal sequence cleavage with lysis function (Figure 2). Recently, unambiguous evidence for a differential topology of the S107 antiholin and the S105 holin was obtained: the N-terminus of S105 is >95% blocked, presumably because of N-formylation, whereas >95% of the S107 protein is unblocked, presumably because of deformylation by cytoplasmic deformylase (J. Deaton and R. Young, unpublished). It should be noted that, because of the exemption from cytoplasmic processing, S105 has no charge on its N-terminal residue, whereas S107 has two positive charges: the protonated amino groups of the N-terminal methionine and the Lys_2 residue.

It is not known how the cytoplasmic localization of the N-terminal domain of S107 causes an inhibition of S105 function. Presumably the S105-S107 heterodimers somehow poison the process of hole formation. Recently, a *S* deletion allele missing all of TMD1 has been shown to have a strong dominant-negative phenotype, suggesting it is the lack of TMD1 rather than the inappropriate presence of the N-terminal domain in the cytoplasm that is the inhibitory feature (I.-N. Wang and R. Young, unpublished). A reasonable model for antiholin function at the operational level has emerged (Figure 6). Each molecule of S107 titrates out a molecule of the holin, S105, by forming a heterodimer with it; assuming a 2:1 synthesis ration, this reduces the number of S105-S105 holin homodimers by 50%. At some point triggering occurs, defined at least in part by the accumulation of the holin homodimers; thus the first hole forms, the membrane depolarizes, allowing the N-terminal TMD1 of S107 in each S105-S107 heterodimer to flip up across the membrane. This has the effect that the ''clock'', defined by the accumulation rate of S105, actually runs slower because much of the S105 protein is removed from participation by S107. However, once the programmed triggering time is reached, the inactive heterodimers are converted into active hole-forming subunits, thus instantly tripling the amount of active holin protein involved in compromising the membrane. Clearly this feature confers on the holin lysis system an ''all or nothing'' character, which, above, we have argued is one of the features that confer a compelling evolutionary advantage on holin-mediated lysis.

Purification and Characterization of Holins

Ultimately, mechanistic understanding of holin function will require reconstitution in a purified in vitro system. The first major obstacle is inherent in the nature of holins. The genes for ordinary membrane proteins are notoriously toxic when cloned on multicopy plasmids, and holin genes are even more difficult to clone because they encode membrane proteins designed to kill bacterial cells. Fortunately, however, *S* protein accumulates to about 100-fold over the normal triggering levels for lethal hole formation if tightly-repressed but inducible multicopy T7 expression systems are used, even though the inductions only last a few minutes before the culture dies. Moreover, the protein accumulates in the membrane in detergent extractable-form. The basis for the ability of cells to accumulate these quantities of holin is unknown; the levels vary inversely with the lethality of the *S* allele, with the A52V variant accumulating to 500,000 copies per cell, the wt to 100,000 copies per cell and the A52G early lysis allele to 10,000 copies per cell (Smith *et al.*, 1998) (J. Deaton and R. Young, unpublished). Presumably, the normal lethal levels are exceeded because there is a minimum time needed to form the lesion, irrespective of the rate of synthesis of *S*. The apparent lethality of the A52V protein may actually reflect the available membrane space of the cell, when one considers that the total surface area of a 3 μm cell is about 8 μm^2, and 500,000 S_{A52V} molecules with three TMDs would be expected to occupy about 2 μm^2.

The identical approach has been used on the class II holin from phage 21 and the *T* holin of phage T4. Purification of holins has been greatly facilitated by incorporation of an oligohistidine tag, but caution must

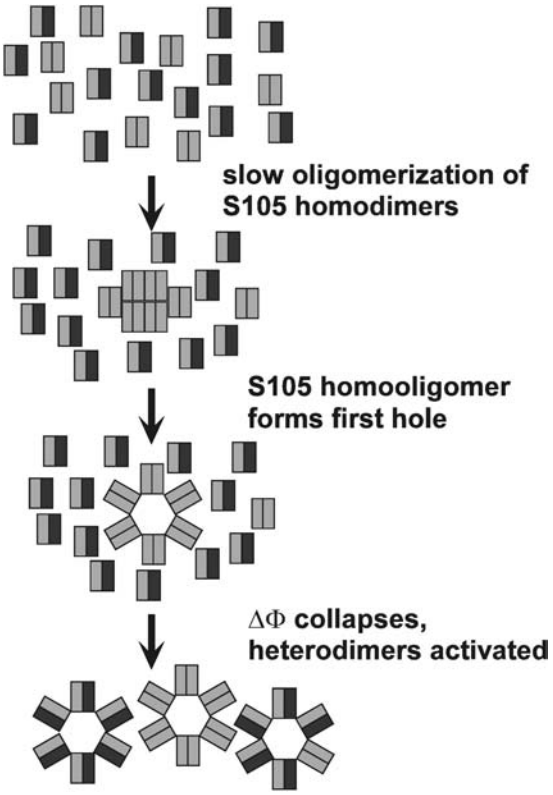

Figure 6. The dual-start motif of holin genes increases hole-forming potential without accelerating the approach to lysis triggering. The model shows the holin (gray) accumulating as homodimers or in heterodimers with the antiholin (black). Only holin-holin homodimers contribute to the formation of the first oligomeric pre-hole. When the pre-hole spontaneously triggers into a hole, the membrane is de-energized, resulting in conformational changes in the anti-holins (e.g., insertion of TMD1 through the membrane, in the case of S107). Thus the heterodimers are now fully capable of oligomerization into holes. At a holin-antiholin ratio of 2:1, as observed for S105 and S107, the effect is that the functional holes are tripled at the instant of triggering. Modified from Gründling *et al.* (2000c).

be used in this approach. In light of the complex phenotypes deriving from single residue changes throughout the *S* sequence, it is perhaps not surprising that it took 13 attempts to find a position within the protein where the oligohistidine sequence was tolerated without gross effects on the timing or lysis functions (Smith and Young, 1998). With S, as with all membrane proteins, the choice of detergent is a source of essentially arbitrary variability. To facilitate in vitro manipulation, the use of detergents with a CMC sufficiently high to permit efficient dialysis is essential. For example, S was found to be stably soluble even at micromolar concentrations in the zwitterionic detergent Empigen BB, whereas it precipitated from octyl glucoside solutions with a time constant of about 24 hours. Moreover, CD analysis of the S protein in the former detergent yielded alpha-helical content exactly consistent with the presence of three helical TMDs, whereas in octyl glucoside, the ellipticity suggested that only two TMDs persisted (J. Deaton and R. Young, unpublished). Similar results were obtained with the S^{21} holin and the two detergents.

Even upon successful purification, the nature of holin function makes an in vitro assay difficult. Some success has been achieved with a liposome permeabilization assay used for the characterization of soluble cytolytic toxins. In these assays, liposomes are formed pre-loaded with a self-quenching fluorescent dye, and permeabilization is followed by the increase in fluorescence as the toxins bind, oligomerize and isomerize into the transmembrane pore (Smith, 1998) (J. Deaton and R. Young, unpublished). Such systems are exquisitely sensitive to trace detergents, so it has been necessary to dialyze the holin into a chaotrope first and then dilute the detergent-free holin into the liposome suspension. Only a small fraction of the holin protein actually binds to the liposomes, with the rest forming insoluble aggregates, but the fraction that does causes dye release with kinetics on the time scales of in vivo holin function. Moreover, the allelic state of the added holin protein faithfully reflects the in vivo phenotype: the A52V variant is compromised in dye release at 37°C, whereas the A55T variant (the product of the only existing *Sts* allele), causes dye release at the permissive temperature (30°C) but not the non-permissive temperature (42°C) (J. Deaton and R. Young, unpublished). These controls for non-specific damage to the membrane strongly suggest that the permeabilization observed is closely related, if not identical, to the hole-formation event in vivo. However, although this in vitro system offers the best chance for dissecting holin function at the molecular level, further progress towards mechanistic understanding requires that a number of problems be addressed, expecially more efficient delivery of the holin to the liposomes, and deconvolution of the insertion, diffusion, oligomerization steps that presumably precede hole formation. Moreover, the critical function of the polarized state of the membrane in vivo suggests that liposomes must be energized, perhaps through incorporation of the light-activated bacteriorhodopsin proton pump. Nevertheless, at the least these experiments offer compelling biochemical evidence to support the thesis that S, and by extension, any holin, is necessary and sufficient for formation of a permeabilizing lesion in the membrane.

New Lessons from T4 T: A Holin Regulated in Real Time

T4 t: A New Kind of Holin

Bacteriophage T4 was the first phage to be subjected to physiological and genetic analysis, and, as noted in the Introduction, its mode of host lysis was one of the first phenomena to be analyzed in detail. It has been more than 30 years since the *t* and *e* genes were defined as the two essential lysis genes, and in fact E is the canonical lysozyme and was one of the earliest proteins for which crystal and solution structures were determined (Gutter *et al.*, 1983). The phenotypes of *t* null mutants were identical to those of *S* amber mutants: an absolute lysis-defect concomitant with an prolonged increase in

intracellular virion and lysozyme content (Josslin, 1970; Josslin, 1971). Unexpectedly, however, the predicted T sequence was found to be unlike any other holin sequence, not only in its size, which, at 218 residues, is about twice that of S, but also because it is a highly hydrophilic sequence and possessed not even a single consensus TMD (Montag *et al.*, 1987)(Figure 3). However, induction of multicopy plasmid clones of *t* caused host lysis in the presence of either lambda or T4 endolysin, and a hybrid lambda phage with *S* replaced by *t* exhibited a sharply defined lytic profile that could be triggered early by energy poisons and was qualitatively identical to that of the parental lambda phage, albeit occuring much earlier, at 20 min rather than 50 min (Ramanculov and Young, 2000a). Thus T, although non-canonical in terms of its primary structure, is clearly a holin.

The membrane topology of T has been probed by fusion gene analysis with the *pho-lac* cassette (Alexeyev and Winkler, 1999); this ''sandwich'' cassette, consisting of a fusion of *phoA* and *lac* Zα, generates a Pho^+ signal if the insertion point is located in the periplasm and a Lac^+ signal if the insertion point is located in the cytoplasm. Given the small size of holins, the use of a sandwich reporter is crucial, because traditional *phoA* and *lacZ* fusions are truncations of the subject gene and thus may lack crucial C-terminal topology determinants. Analysis of the *t* sequence revealed that there is only a single TMD, spanning approximately between residue 35 and 55 (Figure 3), with the N-terminus in (cytoplasmic) and the C-terminus out (periplasmic). The large periplasmic domain should make *T* dependent on the *sec* system for membrane integration, but this has not yet been tested.

The early lysis phenotype supported by λS::*t* resulted in a severely reduced burst size and thus a pronounced plating defect; by the simple stratagem of selecting large plaque revertants, it was possible to isolate a large number of *t* mutants with delayed lysis times (Ramanculov and Young, 2000b). A number of these alleles supports very rapid but late-onset lysis; these alleles, designated as ''clock'' mutants, map almost exclusively to the hydrophilic periplasmic domain. Immunoblot analysis shows that the T proteins from these alleles accumulate at a normal rate, indicating that the timing differences are not due to a reduced synthesis or stability but instead reflect a change in the intrinsic lysis timing of T. Moreover, deletions of the large periplasmic domain retained significant holin function, albeit with radically altered timing. The other delayed-lysis mutants exhibited not only late onset but also gradual lysis, and these alleles mostly mapped to the TMD. Taken together, these results suggest that the lethal holin function is primarily defined by the amino-terminal region of T, including the TMD, whereas the bulk of the T protein is a periplasmic regulatory domain.

Lysis-Inhibition Linked to Holin Function

The finding that T4 effected lysis by a holin-endolysin system opened the way to molecular analysis of the most venerable biological phenomenon of molecular genetics: the lysis-inhibition (LIN) system of T-even phage (Doermann, 1948). Considering its antiquity in the era of modern biology, this phenomenon deserves a brief review. If T4-infected cells are subjected to a secondary infection, the normal schedule for lysis and termination of the infective cycle is over-ridden. In this LIN state, lysis is blocked and the vegetative cycle is extended, resulting in virions continuning to accumulate intracellularlly at a constant rate; LIN can be maintained for hours if continued secondary infection events are experienced. Moreover, LIN can be instantly subverted by the addition of the same energy poisons that trigger holins, a fact that strongly indicated that LIN involved inhibition of holin function, rather than a negative effect on holin accumulation or stability (Young, 1992).

The first heritable phenotype established for bacteriophage was the plaque-morphology phenotype of LIN^- mutants. LIN^- mutants make large clear plaques, whereas the parental LIN^+ phage make small, fuzzy-edged plaques, reflecting the continuous LIN state experienced by the infected cells immobilized in the growing plaque. These plaque-morphology mutants, described more than 50 years ago, were given the designation ''*r* '' mutants, for rapid-lysis (i.e., not subject to lysis delay) (Hershey, 1946). A number of genetic loci were associated with the LIN^-, *r*-plaque phenotype; however, Paddison *et al.* (Paddison *et al.*, 1998) have proposed that only three *r* genes are required for LIN: *rI*, which is required in all hosts; *rIII*, which is required for stability of the LIN state; and *r* V, which was recently found to be allelic to *t* (Dressman and Drake, 1999). Sequence analysis indicates that *rI* and *rIII* encodes a periplasmic protein, which should be 8.7 kDa after proteolytic removal of the signal sequence, and a 9.2 kDa cytoplasmic protein, respectively. The prediction that RI is located in the periplasm is consistent with what little is known about the LIN signal, which is carried by T-even phages but not empty phage ghosts or u.v.-irradiated phage. It is thought that the genome of the secondary phage is blocked from entering the host cytoplasm by a small membrane protein, Imm, synthesized as an early gene product by the primary phage. The DNA is diverted to the periplasm and degraded by host endonucleases. Thus the LIN signal could be the DNA or perhaps internal capsid polypeptides that are also injected (Paddison *et al.*, 1998). Despite the detailed genetics available more than 50 years ago, however, little or no progress on dissecting the LIN phenomenon was made, mostly because T4 abolishes all host gene expression and destroys endogenous DNA and is thus largely intractable to the methods of recombinant DNA and cloning.

An Ancient Player Unmasked: RI is an Antiholin

Recently, the molecular basis of LIN has been studied using the hybrid λS::*t* phage (Ramanculov and Young, 2000c). LIN has been reconstituted in inductions of the λS::*t* hybrid phage, but not an isogenic λ S^+ induction, by secondary infection with T4 at 20 min, just before lysis of the induced

lysogen. Moreover, stable LIN was not achieved if the infecting T4 phage carried null alleles in *rI* or, most significantly, *t*. Moreover, secondary infections with either T4*rV* (a lysis-proficient, LIN-defective *t* allele) or T4*rIII*, LIN was briefly established but collapses within 20 min. In these induction-infection experiments, the pre-existing pool of T protein synthesized from the induced λ genome, at 20 min already about to trigger lysis, is susceptible to LIN only if the new T protein synthesized from the infecting T4 phage is fully LIN-proficient. This suggests that once T enters into a conformation or state that is committed to hole-formation, it is no longer susceptible to the LIN signal. Surprisingly, when the *rI* gene was finally cloned and expressed in trans to a *λS*::*t* induction, it was found to impose LIN, even without a secondary T4 infection to supply the LIN signal. The LIN achieved in these conditions was only partial but, fortuitously, a complete and stable LIN state was obtained under the same conditions with a modified *rI* allele carrying a C-terminal *c-myc* tag ($rI^{c\text{-}myc}$), constructed to facilitate identification and subcellular localization of RI. These results suggest that despite the complex genetics and physiology involved, LIN might be simple in molecular terms. A model was proposed that RI, as a periplasmic protein, is rapidly degraded until the LIN signal somehow achieves its stabilization; a simple version of this scheme might have the diverted DNA of the secondary phage stabilizing the protein by direct interaction or by inhibiting the periplasmic proteases. In any case, the RI protein consequently accumulates to a higher level, a level sufficient to bind a significant amount of the newly synthesized T protein. The T-RI complex would poison the oligomeric pre-hole assembly and thus impose LIN. Direct evidence for this interaction has been difficult to obtain, because to date RI has not been independently visualized even with the highly immuno-reactive *c-myc* epitope. Nevertheless, formaldehyde cross-linking reveals a molecular species immunoreactive to both anti-T and anti-cmyc antibodies, at an apparent mass corresponding to a single T and a single RI-cmyc polypeptide; this species is not detected when an *rV* allele of *t* is used. These results demonstrate that RI acts as an antiholin, in the sense that, like S107, it binds to its cognate holin and blocks hole-formation, and that this blockage can be subverted by de-energization of the membrane. Similar experiments with a cloned *rIII* gene showed no LIN, suggesting that RIII plays a role in stabilizing the RI-T interaction but cannot replace RI.

Presumably, RI, as a periplasmic protein, binds to the large, polyionic C-terminal domain of T, as suggested by the fact that the C-terminal deletion alleles of *t* with residual lysis activity are all LIN^-. Interestingly, LIN can be instituted without RI, if Ni^{++} or Zn^{++} salts are added at millimolar concentrations to inductions of *λS*::*t* carrying a *t* allele encoding a T protein tagged at the C-terminus with a hexahistidine sequence. This suggests that liganding of *T* on the periplasmic domain is all that is required to impose LIN. There is some evidence that the C-terminus of *T* undergoes a dramatic conformational change as it proceeds through the pathway to the formation of the lethal hole. As T_{His6} accumulates in the membrane, it can be detected first in monomers and SDS-resistant dimers but later in higher order, SDS-resistant oligomers; remarkably, the monomers and dimers are detected by immunoblot with anti-T polyclonal antibodies, but anti-His_6 antibodies react only with the higher order species. It seems likely that this conformational change and LIN resulting from the cation or RI-mediated liganding are related.

Current Perspectives and Conundrums

LIN Finally has its Day

Even if the existence of T as a holin makes any hope of a common structural motif for holins even more unlikely, the emerging outlines of the molecular basis of the LIN phenomenon help in the construction of a new perspective of holin and antiholin function. Clearly, T, like λS, has an ideal lysis time and the plasticity to assume a different ideal lysis time by single missense changes. Thus T is well equipped to optimize T4 in its competitive environment. It is equally clear that T has also evolved to respond to an environmental signal: the presence of excess T4 virions in the environment. The occurrence of secondary infections means that the surrounding medium is depleted of free hosts. In these conditions, it is obviously advantageous to postpone host lysis and continue the linear intracellular accumulation of virions, and thus the evolutionarily-selected ideal lysis time of T must be over-ridden. The *r* system defines an elegant signal transduction mechanism and deserves further attention. The nature of the signal is of primary interest. The idea that it is the presence of the bulk DNA in the periplasm, diverted by the injection-blocking function of the Imm protein, is attractive. This would provide specificity, since Imm blocks only T-even injection (Lu and Henning, 1994). Moreover, the diverted DNA is degraded by host periplasmic nucleases, which could explain why the LIN signal seems to deteriorate with an intrinsic time constant (Bode, 1967). Direct examination of secondarily-infected cells using DNA-specific dyes and fluorescent microscopy should make it possible to confirm the establishment and subsequent deterioration of the periplasmic DNA pool. How the signal causes activation of RI is also an important question. Host mutants with defects in periplasmic nucleases and proteases should be examined for the duration of the LIN phenotype, both with the plasmid-based *rI* overexpression system (signal-independent LIN) and the canonical T4 infection system (signal-dependent LIN). The role of RIII can be addressed by supplying it from a compatible plasmid; the simplest model predicts that *rIII* -expression should improve the partial LIN derived from the cloned *rI* wild-type allele. Moreover, the subcellular localization of RI and RIII needs to be directly confirmed. If indeed RI and RIII are demon-

strated to be in the periplasm and cytoplasm, respectively, then RIII would have to bind to the small cytoplasmic domain of T to exert a direct molecular effect. Alternatively, RIII may act indirectly by facilitating the synthesis or export of RI, or reducing its periplasmic degradation. The RI protein is apparently stabilized by the extension of its C-terminus with the c-myc epitope, to the extent that *t*-mediated lysis is completely blocked even in the absence of a LIN signal. This tight lysis-defective phenotype opens the door to exploration of what is important in the RI sequence and what is required in the *T* sequence for imposition and maintenance of LIN. Moreover, the current perspective predicts that the C-terminal periplasmic domain of *T*, if synthesized with a cleavable signal sequence, should titrate out periplasmic RI and thus block LIN establishment. All of these questions can be approached with the powerful positive and negative genetic selections available for studying lysis phenomena. Thus the potential for productive mutation-suppression analysis facilitating a genetic exploration of specific and indirect interactions is great.

Holins and Antiholins: How Many Ways are there to Adjust the Clock?

The *r*- mediated LIN system and the dual start motif of λ S are two elegant and utterly different schemes to regulate holin function. Obviously many questions remain about the *r* system, as outlined above, but although the S107 antiholin is somewhat better defined at the molecular level, its fundamental rationale is still elusive. We have suggested that the dual start motif in lambdoid holin genes provides a mechanism to increase the abruptness of lysis, by, in effect, concealing two-thirds of the potential hole-forming proteins in inactive holin-anti-holin heterodimers. Moreover, altering the production of anti-holin by site-directed mutagenesis can lead to dramatic changes in lysis timing (Chang *et al*., 1995). However, although it is clear that the λ lysis time can be adjusted over a very wide range by changing the proportion of S105 and S107 produced, it has not been shown that this proportion is actually changed in any physiological situation. There is some evidence for a host factor that binds specifically to the RNA structural motif that controls the partition of translational initiations between the two start codons of *S* (Nam, 1991), suggesting that conditions may exist that alter S105-S107 production. The simplest notion is that expression of the anti-holin should be favored when a delay in lysis is favorable, presumably in poor growth conditions. Given the relationship between host density and the ideal lysis time, it is very tempting to think that the dual start motif will respond in some way to quorum sensing systems, but to date no systematic exploration of the relationship between the length of the vegetative cycle and host density has been conducted. Thus, with the exception of the *r* system in T4, the biological role of anti-holins in the regulation of timing is not yet apparent.

Many of the class II holin genes have putative dual start motifs, and an anti-holin/holin relationship function has been demonstrated for S^{21} 71 and S^{21} 68 gene products of S^{21} (Barenboim *et al*., 1999). Despite the apparent similarity of the S^{λ} and S^{21} dual start motifs, with a positively charged residue defining the anti-holin character of the longer gene product, the inhibitory mechanism must be completely different for a number of reasons. First, the N-terminus of the class II antiholin is permanently cytoplasmic and thus cannot be expected to change its membrane topology upon membrane depolarization; in fact, unlike S107, S^{21} 71 is not converted into a functional holin conformation by addition of an energy posion to the medium (Barenboim *et al*., 1999). Thus even dual start motifs, once thought to be a conserved and defining signature of holin genes, have at least two different modes of exerting an inhibitory effect on lysis timing. Considering that that dual starts are found in a number of unrelated holin gene families of both class I and class II, suggesting multiple independent evolutionary origins, it is perhaps not surprising that these systems are mechanistically divergent.

Only the λ, 21 and T4 anti-holin systems have been studied. At least two more completely different types of anti-holins are known. In phage P1, the antiholin, LydB, is essential to prevent catastrophically early lysis mediated by its class I holin, LydA, and the gene *17* endolysin, a T4 E-like lysozyme (Yarmolinsky and Sternberg, 1988; Sandmeier *et al*., 1991; Schmidt *et al*., 1996). Sequence analysis indicates that LydB is a hydrophilic, cytoplasmic protein and thus apparently defines yet another mode for anti-holin function, given that T4 RI is predicted to be periplasmic and the two dual start anti-holins are integral membrane proteins. Another difference is that gp*17* has an N-terminal extension with significant hydrophobic character (Guidolin *et al*., 1989); preliminary results indicate that this sequence is indeed a secretory signal that serves to engage the *sec* translocon, either as a cleavable signal sequence or signal-arrest domain (M. Xu and R. Young, unpublished). Thus P1 has two essential lysis proteins: the endolysin and the anti-holin! Apparently, P1 lysis does not require holin function for lysis but instead only to make lysis occur at a defined time and with an abrupt character. Moreover, these data suggest that the P1 anti-holin inhibits both the holin and the endolysin.

Yet another anti-holin mode is suggested by the structure of the lysis cassette of phage P2, which encodes a class I holin, gp*Y*, an endolysin, gp*K*, orthologous to λ R, and functional homologs of the Rz and Rz1 proteins (LysB and LysC). In this case, there is some evidence that the gene *lysA* distal to the endolysin encodes an antiholin, because a *lysA* null has an advanced lysis timing phenotype (Ziermann *et al*., 1994). Although the ability of *lysA* to retard Y-mediated lysis in trans has not yet been demonstrated, if LysA is indeed an anti-holin, it is a new type, because it is clearly an integral membrane protein with four predicted TMDs. Interestingly, it has a positively charged N-terminal domain preceding the first TMD, like the class I and class II anti-holins, although, unlike the two prototype membrane anti-holins, LysA has no

detectable sequence relationship to the holin it would inhibit.

There is clearly great diversity in the anti-holin schemes evolved by bacteriophages. Presumably this reflects the evolutionary impact of achieving lysis ideally timed and consummated to suit every variation of the growth environment. Possibly it also reflects the ease by which holin timing can be altered; perhaps any protein-mediated interaction with the holins can distort the fragile balance between maintenance of the pre-hole state and the formation of the catastrophic lytic lesion.

What Defines the Clock: Two Apparently Contradictory Experiments

Considerations of anti-holin function aside, the clock of lysis is ultimately defined by the rate of holin accumulation in the membrane, at least within reasonable limits (Chang *et al*., 1995; Smith *et al*., 1998). The simplest model is that the holin has to reach a critical concentration in the membrane; in this view, as holins populate higher oligomeric states, some N-mer is formed with an intrinsic ability to form the hole as a consequence of a spontaneous, highly co-operative tertiary or quaternary conformational change. Once the first hole is formed, the membrane will be de-energized, and all the other holin molecules will be recruited into holes, just as in the case of lysis prematurely triggered by addition of energy poisons. Anti-holins could work by slowing the accumulation of N-mers fit for the spontaneous isomerization or by inhibiting the isomerization itself. A prediction of this model is that if two different holins are accumulating in the same cell, the lysis time will be defined by whichever holin reaches its critical concentration first. That is, assuming the two holins do not interact and thus form separate mass-action pools, the presence of another holin should have no effect on the timing of a particular holin. This hypothesis has been tested and falsified using the *S* and *t* genes of λ and T4 (Ramanculov and Young, 2000a). Expression of *t* and *S* together causes lysis significantly earlier than identical experiments with either holin gene (Figure 7). Thus either the two holins participate in the same mass-action pool, which seems highly unlikely, or both holins impose an additive deleterious effect on the host cell, and the deleterious effect is what eventually determines the triggering time.

The simplest notion for such a common deleterious effect is that both holins, and by extension all holins, cause a deterioration of the energized state of the membrane during the accumulation phase. According to this scheme, some holin N-mer intermediate along the hole-formation pathway causes a leakage of protons; when the proton-motive-force (pmf) is reduced to a certain level, the holins are triggered into the formation of the lethal hole. This model predicts that there should be a detectable reduction in the pmf before lysis; it too has been falsified. Recently, in cells induced for expression of a cloned λ lysis cassette, the pmf has been monitored in vivo by measuring the rotation speed of motile cells tethered to a glass slide (Gründling, Manson and Young, 2001). In this system, rotation speed, a linear function of the pmf, was found to be unaffected throughout the induction period up to seconds before lysis. Interestingly, using the same system and graded concentrations of an uncoupler, the authors were able to show that S is triggered when the pmf is reduced only marginally, to about 70% of normal, which highlights the delicate poise of the holin timing system. The author's first experience with a plasmid clone of *S* illustrated this point vividly; when aerating several induced, late-logarithmic cultures of cells carrying *S* under a *lac* promoter, the shaker bath was inadvertently left off for a minute before samples were taken to assess the optical density. The cultures all cleared,

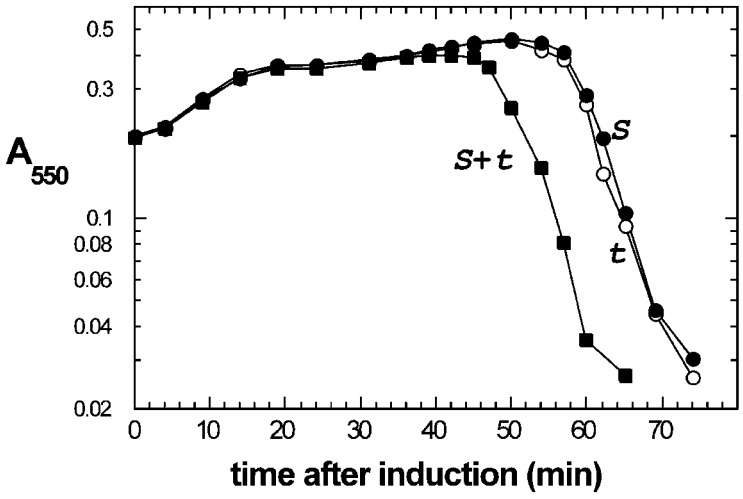

Figure 7. The accumulation of two unrelated holins with radically different topologies causes accelerated lysis timing, compared to either holin alone. In this system, cells carry an inducible λ prophage and a plasmid with the λ late promoter serving the lysis gene cluster. After induction, late gene expression is activated from both the phage late gene transcriptional unit and the plasmid. The lysis phenotype of cultures with an *S* allele in the plasmid (filled squares and filled circles), T4 *t* substituted for *S* in the prophage (black squares, open circles), or null alleles on the plasmid (open circles) or prophage (filled circles). Modified from Ramanculov and Young, (2000a).

irrespective of the interval since induction, as a result of the sudden drop in oxygen tension in the medium and consequent momentary and partial membrane depolarization in the cells (R. Young, unpublished).

In any case, the pmf across the cytoplasmic membrane is unaffected by the accumulation of the S holin. This is satisfyingly consistent with expectations that holin-mediated timing should not compromise the biosynthetic capacity of the host; that is, after all, one of the principles that we have invoked as defining the role of holin in the first place. Nevertheless, it leaves us with less attractive alternatives to explain the clear implication of the two-holin experiment, that holins exert a common deleterious effect. One possibility is that there is a common physical target for holins. It is unlikely that the common target is a host protein. First of all, holins from a very wide range of phages from many species of Gram-negative and Gram-positive bacteria all seem to be fully functional on *E. coli*. Second, S has been shown to be lethal even in yeast cells. Finally, purified holin protein is able to form holes in artificial membranes, and mutations which block hole-formation in vivo also block this in vitro hole formation. What remains for a possible common target is some undefined physical property of the bacterial envelope somehow not reflected by the undisturbed pmf. In this view, holins accumulate and form oligomeric foci that are potential zones for generalized membrane disruption but do not increase membrane permeability to protons or other ions. The spontaneous conformational change in one such local oligomeric zone leads to generalized disruption of the membrane in this area; immediately, the entire membrane is de-energized and all the other holin foci reorganize into disruption zones too. The additive results of the two holin experiment can be rationalized if the nearby presence of heterologous foci affect the physical nature of the local membrane environment in a way to facilitate the disruption event. At present, it is unclear how such effects could be propagated. However, there is growing evidence that non-bilayer structures, and non-bilayer forming phospholipids, are involved in membrane transport events (Dowhan, 1997). It will be interesting to see if holin aggregates influence the bilayer-forming tendency of neighboring phospholipids in artificial membranes.

What is to be Done?

This review has posed more questions than it has answered. Holins are much less well characterized than most other lethal, cytolytic toxins and integral membrane channel-forming proteins. Holins are interesting both from the nature of the hole, by which is meant the unknown, catastrophic damage done to the membrane, and the nature of the clock that times the imposition of this damage. Holins are small, lethal, and have a macroscopically accessible phenotype, and are thus highly tractable to genetic analysis. Nevertheless, holin genetics are distinctly underdeveloped; except for the two instances of λ *S* and T4 *t*, no mutational study has been conducted on any holin. Fortunately, it appears that phage λ is suitable test-bed for the study of holins from phages of all eubacteria (Ramanculov and Young, 2000a; Vukov *et al*., 2000), so the lack of suitable cloning vectors and suppressor backgrounds in most bacteria need not hinder genetic analysis of any holin. At the least, genetic analysis should be extended to at least one more class I holin, to see if the phenotypic patterns observed in *S* are common to other holins with the same membrane topology. The class I holins from phages P1 and P2 are excellent candidates; simple questions of the specificity of holin-holin and holin-antiholin interactions can be addressed by exchanging the components of these lysis systems at the level first of intact proteins and then at the level of TMDs and cytoplasmic and periplasmic domains. Saturation mutagenesis and phenotypic analysis has been initiated with S^{21} and should be mirrored with at least one unrelated class II holin, for the same reasons.

Ultimately, however, two kinds of structural information are necessary. The most distressing void is our lack of knowledge of the nature of the hole. The development of the in vitro hole-formation assay offers an opportunity to visualize the membrane lesion in a purified system. Although the possibility of artifact has to be kept in mind, nevertheless the parallels between holin-permeabilization capacity of wild type and mutant alleles in vitro and the lysis competence of these alleles in vivo suggest that the membrane lesions in the two systems are at least related, if not identical. Thus the next step must be to see the holes. Finally, even when the nature of the permeabilizing lesion has been established, mechanistic understanding of how holins effect the permeabilization and do so with such precise timing and capacity for regulation will depend on getting structural information at the molecular level. Despite its lethal character, S105 has been purified in stable, detergent-solubilized concentrations exceeding 30 μM (J. Deaton and R. Young, unpub.), suggesting that crystal or solution structure may not be an unreasonable goal for at least one holin.

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