Phage single-gene lysis: Finding the weak spot in the bacterial cell wall

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In general, the last step in the vegetative cycle of bacterial viruses, or bacteriophages, is lysis of the host. dsDNA phages require multiple lysis proteins, including at least one enzyme that degrades the cell wall (peptidoglycan (PG)). In contrast, the lytic ssDNA and ssRNA phages have a single lysis protein that achieves cell lysis without enzymatically degrading the PG. Here, we review four “single-gene lysis” or Sgl proteins. Three of the Sgls block bacterial cell wall synthesis by binding to and inhibiting several enzymes in the PG precursor pathway. The target of the fourth Sgl, L from bacteriophage MS2, is still unknown, but we review evidence indicating that it is likely a protein involved in maintaining cell wall integrity. Although only a few phage genomes are available to date, the ssRNA Leviviridae are a rich source of novel Sgls, which may facilitate further unraveling of bacterial cell wall biosynthesis and discovery of new antibacterial agents.

Introduction to small lytic phages and “single-gene lysis”

By definition, the lytic bacteriophages encode proteins for disruption of the host envelope. The large dsDNA phages, the Caudovirales, have multiple lysis proteins, including holins, endolysins, and spanins, targeting the cytoplasmic or inner membrane (IM), peptidoglycan (PG), and outer membrane (OM), respectively, as well as multiple proteins that regulate the lysis process (1, 2). In contrast, the small lytic phages of Gram-negative hosts, comprising the ssDNA (Microviridae) and ssRNA (Leviviridae), achieve host lysis by a single gene, encoding a protein lacking any PG-degrading activity (3). This review exclusively focuses on these single-gene lysis (Sgl) proteins of small lytic phages.

Bacterial cell wall structure and biosynthesis

An exploration of Sgl mechanisms requires a brief review of the structure of the Gram-negative cell wall and its biosynthesis. The key to the structure and shape of the cell envelope is the PG layer, consisting of 2–3 layers of glycan strands made up of repeating disaccharide units of MurNAc-pentapeptide and GlcNAc, cross-linked by peptide bridges between pentapeptide side chains of MurNACs of adjacent strands (Fig. 1A) (4–6). The PG has considerable tensile strength (3–300 megapascals), allowing the cell to tolerate high internal osmotic pressures (3–10 atmospheres) while maintaining shape (7, 8). The entire PG of a cell can be isolated as a single complex polymer, the saccus, studies of which have revealed that the glycan chains run almost perpendicular to the long axis of the cell (9). In most Gram-negative bacteria, the PG layer is covalently linked through >10^5 peptide linkages to the C-terminal Lys residue of the major lipoprotein, Lpp; the PG-linked Lpp is anchored almost exclusively in the inner leaflet of OM (5, 10, 11).

Biosynthesis of the PG can be divided into cytoplasmic, membrane, and periplasmic stages. There are seven enzymatic steps in the cytoplasm, beginning with the transfer of an enolpyruvyl moiety from PEP to UDP-GlcNAc, catalyzed by MurA (Fig. S1) (12). After reduction of the enolpyruvyl moiety by MurB to create UDP-MurNAc, the next five enzymes are involved in adding amino acids that form the pentapeptide (L-Ala, D-Glu, m-Dap, D-Ala, and D-Ala) to the lactyl group, resulting in the final soluble intermediate UDP-MurNAc-pentapeptide (Fig. S1) (5, 13). The first membrane-linked step in PG synthesis begins with the transfer of this sugar nucleotide pentapeptide to the lipid carrier undecaprenyl phosphate (C55-P or UndP). This reaction is catalyzed on the cytoplasmic face of the IM by the integral membrane protein MraY to generate a monosaccharide–lipid compound, lipid I (Fig. 1A and Fig. S2A).

MurG then catalyzes the addition of a second sugar moiety (UDP-GlcNAc), resulting in the final precursor, lipid II (Fig. 1A). The last step of the membrane phase is the flipping of lipid II so that its disaccharide pentapeptide moiety is on the periplasmic face of the membrane. The enzyme “flippase” that effects this transfer has been controversial until very recently. Although FtsW was shown to flip lipid II in vitro (14, 15), several lines of evidence now support MurJ as being the essential lipid II flippase (16–21). The extracellular steps of PG biosynthesis utilize the energy stored in the phosphodiester–muramic acid bond of the flipped lipid II and in the D-Ala–D-Ala peptide bond to drive the glycosyltransferase and cross-linking reac-

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This article contains Table S1 and Figs. S1–S6.

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2 The abbreviations used are: IM, inner membrane; PG, peptidoglycan; OM, outer membrane; MurNAc, N-acetylmuramic acid; CTD, C-terminal domain; NTD, N-terminal domain; TMD, transmembrane domain; gRNA, guide RNA; SCAM, substituted-cysteine accessibility method; PDB, Protein Data Bank; mDAP, meso diaminopimelic acid.

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tions, respectively (13). These steps are carried out by mono- or bifunctional penicillin-binding proteins, and recently, RodA, a member of the SEDS superfamily, was shown to catalyze glycosyltransferase reactions (22–24).

**The first Sgl: Protein E from microvirus ϕX174**

*Famous phage, famous gene*

ϕX174 is the founding member and genetic paradigm of the *Microviridae*, which are nearly as widespread as the Caudovirales (25). It was the first gDNA to be completely sequenced and also to be synthesized *in vitro* (26, 27). The 10 genes include three that are embedded out-of-frame in essential cistrons (Fig. 1B). One of these embedded genes is E, encoding the Sgl protein in the +1 reading frame of the essential scaffolding gene D (Fig. 1B). E is famous not only for being the first embedded gene to be discovered but also the first gene to be subjected to site-directed mutagenesis (26, 28). More important here is the fact that it is the only DNA virus Sgl, and it was the first Sgl gene for which the lytic mechanism was firmly established. The methods for working out its functional pathway have been replicated for all of the other Sgl systems and thus will be reviewed here in some detail.

*Genetics clarifies E function*

The E gene was cloned into a medium copy expression plasmid and shown to support lysis after induction (29, 30). Despite this early focus and the availability of the cloned E gene, the lysis mechanism of E remained controversial for 2 decades (3). Early transmission EM studies showed that cells infected with ϕX174 lysed as a result of septal blebs in dividing cells, generating a morphology that was remarkably similar to penicillin-mediated lysis (31, 32). Reproduction of this morphology after induction of the cloned E led to the general model that E interfered with PG biosynthesis (30, 33). However, based on physiological and scanning-EM studies, other groups proposed that E functioned either by the activation of unspecified autolytic functions (34–36) or by the formation of polymeric “transmembrane tunnels” that opened the cytoplasm directly to the medium (37). This profusion of models painted a confusing picture for the mechanism of E lysis, primarily because all lacked genetic evidence.

Mutational and deletion analysis of E revealed that the lytic function requires only the first 34 residues; lytic function was retained without the C-terminal 57 residues, a highly basic, Pro-rich segment, as long it was replaced by a stable cytoplasmic Pro-rich segment, as long it was replaced by a stable cytoplasmic Pro-rich segment. Purified SlyD was shown to accelerate folding of proteins limited by cis-trans peptidylprolyl isomerase (40). Indeed, ϕX174 infections of slyD knockout mutants proceed normally in every respect, except lysis never occurs and virions hyperaccumulate. Purified SlyD was shown to accelerate folding of proteins limited by cis-trans peptidylprolyl isomerase (40). Indeed, ϕX174 infections of slyD knockout mutants proceed normally in every respect, except lysis never occurs and virions hyperaccumulate. Purified SlyD was shown to accelerate folding of proteins limited by cis-trans peptidylprolyl isomerase (40). Indeed, ϕX174 infections of slyD knockout mutants proceed normally in every respect, except lysis never occurs and virions hyperaccumulate. Purified SlyD was shown to accelerate folding of proteins limited by cis-trans peptidylprolyl isomerase (40). Indeed, ϕX174 infections of slyD knockout mutants proceed normally in every respect, except lysis never occurs and virions hyperaccumulate.

To continue pursuit of the E target, bypass suppressor mutations were isolated as rare plaque formers on a slyD lawn (42). These Epos (plates on slyD) were found to be missense alleles at the N terminus of E, each of which resulted in a ~10-fold increase in the biosynthesis rate, thereby compensating for the
proteolytic instability of E. This proved to be a key technological advance. Spontaneous “Eps” host mutants (Epos sensitivity) that were resistant to induction of the plasmid-borne Epos allele were selected and cross-streaked for sensitivity to the phage. Of ~2000 survivors, all but three retained ϕX174 sensitivity and presumably were defective in E expression or plasmid copy number. Subsequent genetic mapping and sequencing revealed that the mutations mapped to residues (Pro-172 and Phe-288) in TMD5 and -9 of MraY (see Fig. 3). Labeling experiments with [3H]mDAP showed that E blocked cell wall synthesis ~20 min before lysis, and TLC chromatography revealed depletion of lipid-linked label and the accumulation of UDP-GlcNAc, confirming the inhibition of MraY (43). Based on the mutational data and membrane localization of both proteins, Bernhardt et al. (43, 44) proposed that E interacted with MraY through TMD–TMD interactions that were disrupted in the mutant alleles (Fig. S2B).

**In vitro analysis of E-mediated inhibition of MraY**

Initial attempts to overexpress full-length E failed due to its inherent lethality, but by doing inductions in the presence of the heterologous MraY from B. subtilis, a His-tagged full-length E protein was purified with a yield of 27 μg of E per liter of culture (45). Using this as an immunoblot standard, these workers determined that E was produced at ~500 molecules/cell at the time of lysis, in agreement with estimates from radiolabeling and E-LacZ enzyme assays (38, 46). An assay based on UDP-MurNAc-pentapeptide DNS, a fluorescent analogue of UDP-MurNAc-pentapeptide, and phytol-P, a 20-carbon analogue of C55-P, was developed to determine kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the apparent kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the apparent kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the apparent kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the apparent kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the apparent kinetic parameters in the presence and absence of E. For a 20-carbon analogue of UDP-MurNAc-pentapeptide, and phytol-P, the...
structures, leviviruses exploit retractable pili to initiate infection. By far the best studied leviviruses are MS2 and Qβ, both specific for the canonical F conjugation pilus; many other F-specific leviviruses that are related to these two paradigms have been studied (50–55). However, seven other distinct leviviruses are known, each targeting a different retractable pilus (Table S1) (56–61). The Sgl genes have been identified in eight of the nine distinct leviviruses by showing that, as for E, induction of a plasmid-borne clone is necessary and sufficient for lysis. Importantly, all cause disruption of the cell wall by accessing different cellular targets, suggesting that in each case, a new Sgl was evolved after radiation to a new retractable pilus, no doubt facilitated by the extremely high mutation rate of the RNA-dependent replicase (62). This means that even with the low total genomic database of less than 50,000 bases of unique Leviviridae genomes, there are multiple Sgl systems that might be exploited for probing the biosynthesis and dynamic homeostasis of the cell wall. In the following, the Sgl systems of Qβ, M, and MS2 will be reviewed. The order is not chronological but makes sense in that the targets of the first two Sgl proteins have been identified, whereas the MS2 Sgl system is an enduring mystery.

The “protein antibiotic”: A2 from Qβ

Finding rat mutants

The Aβ protein has multiple functions during Qβ infection; it functions in virion assembly (63), is bound to and provides protection for the gRNA against RNase degradation (64), mediates interaction with the F-pilus, and is internalized into the host cytoplasm along with the genomic RNA (63–65). Remarkably, Aβ also functions as the Sgl protein. The lytic activity of Aβ was first demonstrated in 1983 by Winter and Gold (66), who showed that induction of Aβ cloned on a medium-copy plasmid is necessary and sufficient to cause lysis.

To identify the target of Aβ, the same method was used as for dX174 E, selecting for host mutants that survived induction of a plasmid-borne Aβ gene, followed by screening survivors by cross-streaking with Qβ phage, and the mutants that passed selection/screen were designated as rat (resistant to Aβ-two) mutants (67). Genetics and sequence analyses revealed a single missense change in murA, L138Q. As with E, the incorporation of [3H]mDAP into PG was blocked at least 20 min before the onset of lysis in cells induced for Aβ. Biochemical analysis of the sugar nucleotide pool from Aβ-inhibited cells revealed that UDP-GlcNAc was elevated, confirming MurA as the target. In vitro inhibition of MurA by purified Aβ could not be demonstrated, mainly because overexpressed Aβ was insoluble. However, in what seems to be the only instance of using virions for enzyme inhibition, it was shown that the catalytic activity of MurA, but not MurA<sup>L138Q</sup>, in crude extracts could be blocked by the addition of highly purified Qβ virions. Later experiments done with purified MurA and Qβ particles confirmed these results (68). Based on the turnover number of MurA and the number of purified virions needed to block its enzymatic activity, the MurA–Aβ<sub>2</sub> dissociation constant was determined to be ~10 nM (67).

The Aβ–MurA interaction

In addition to in vitro inhibition assays, direct protein–protein interaction between Aβ–MurA and Aβ–MurA<sup>L138Q</sup> was also demonstrated by yeast two-hybrid analysis, with the latter pair displaying a weaker signal, suggesting that the mutant allele weakens Aβ binding (68). To probe the interaction of MurA with Aβ in vitro, the well-characterized catalytic pathway of MurA was exploited. The MurA reaction is well-ordered, with UDP-GlcNAc binding in the catalytic cleft associated with a dramatic shift from open to closed conformation, which then allows PEP binding and catalysis. Interaction studies were done using a soluble MBP–Aβ<sub>2</sub> fusion protein and various forms of MurA, including the original rat allele, MurA<sup>L138Q</sup>, and MurA<sup>D305S</sup>, which is disabled for catalysis but not substrate binding, and with various combinations of the substrates and the suicide inhibitor fosfomycin. The results clearly showed that Aβ preferentially binds to the UDP-GlcNAc–liganded, closed form MurA, preventing PEP from binding. Details of the binding surface were obtained by site-directed substitutions of amino acids in the area around Leu-138, yielding a cluster of new rat alleles that blocked Qβ plaque formation and clearly defined an interaction surface surrounding the catalytic loop, including the catalytic domain, CTD, and the catalytic loop (Fig. S3).

Aβ differs significantly from the Mat proteins of MS2 and related Leviviridae, especially in the N terminus; deletion analysis confirmed that the lytic function is fully defined in the first 180 residues. To map the interaction domain, Aβ<sub>por</sub> (plates on rat) suppressor alleles were isolated and mapped to three positions (Leu-28, Asp-52, and Glu-125) in the N-terminal domain (69). However, none of the por alleles were lytic when cloned and induced in the rat1 host. Immunoblot analysis revealed that Aβ<sub>por</sub> mutant levels increased much more rapidly than the parental Aβ<sub>2</sub> during infection, resulting in early lysis and reduced yield of progeny virions in the WT host. Inspection of the sequence around the por sites confirmed that the mutations disrupt significant RNA structures that repress translational initiation in the viral RNA, thus bypassing the reduced Aβ<sub>2</sub>–MurA<sup>L138Q</sup> affinity by increasing the quantity of the phage protein.

The interaction interface was recently resolved in asymmetric cryo-EM structures of Qβ particles in complex with UDP-GlcNAc–liganded MurA or fosfomycin-liganded MurA (63) (Fig. 3, A and B). The cryo-EM structures validated the interaction interface on MurA inferred from the various rat alleles and also confirmed that the NTD of Aβ<sub>2</sub> is in contact with MurA (Fig. 3, C–E).

lys<sup>M</sup>: New target and settling a debate

The lysis gene (<i>lys<sup>M</sup></i>) of phage M has evolved completely embedded in the +1 reading frame of the rep gene, and it encodes a 37-amino acid protein with a single TMD (58). The functional Lys<sup>M</sup>–eGFP fusion suggests an N-out and C-in membrane topology (70). Early insights into the molecular mechanism of Lys<sup>M</sup> lethality came from the observations that lysis proceeded through septal catastrophes, like Aβ and E, suggesting that Lys<sup>M</sup> might be an inhibitor of cell wall biosynthesis (31,
proteins (radially colored) side. Both predictions were confirmed by an accumulation of lipid-linked PG precursors in the inner membrane. The SCAM labeling pattern is consistent with MurJ being the only active lipid II flippase in *E. coli*. However, the interpretation of the Lys\(^M\)-resistant *murJ* alleles and the interaction interface would greatly benefit from the structure of the MurJ–Lys\(^M\) complex.

**MS2 lysis: To L and back**

*L: The first autolysin?*

The *L* gene was not recognized as a gene in MS2 until the isolation of a plaque-forming defective nonsense mutant that belonged to a complementation group distinct from *mat*, *coat*, and *rep* (72). Subsequent radioactive labeling experiments established *L* as the fourth gene of MS2, encoding a 75-amino acid polypeptide (73). As had been done with *E* and *A*\(_2\), a plasmid clone of *L* was shown to cause lysis after induction, and the *L* protein was shown to be associated with the membrane fraction (73, 74). Opposite to *E*, it is the 39-residue CTD of *L* that accounts for membrane localization and lytic function, with the N-terminal 36 highly basic residues shown to be dispensable for lysis (76). This clearly differentiates *L* action from the *E*, *A*\(_2\), and Lys\(^M\) Sgl proteins, all of which cause cessation of cell wall synthesis by interrupting the supply of lipid II to the PG machinery (43, 67, 70). To these workers, the most significant finding was that induced *L* lysis was severely compromised in acidic (pH 5.5) conditions, despite normal accumulation of *L*, raising a compelling analogy to penicillin-induced autolysis, which is also blocked under these conditions (77). This led to a general model in which *L* effect lysis by inducing autolysis, although the precise definition thereof was not provided. In immuno-EM experiments, *L* was shown to preferentially localize to apparent zones of adhesion between the IM and OM (78). This association with adhesion zones was emphasized by the fact that *L* lysis is also compromised in cells that lacked the periplasmic osmoprotectant membrane-derived oligosaccharide (79). These cells were shown to have many fewer adhesion zones and a much wider periplasm, and in this case, *L* appeared to be subject to degradation. Furthermore, a synthetic polypeptide corresponding to the C-terminal 25 amino acids of *L* was shown to permeabilize both liposomes and inverted membrane vesicles, leading the authors to invoke induction of autolysis after membrane permeabilization (80). However, these experiments lacked a negative polypeptide control, and the experiments were done at peptide-vesicle ratios in excess of 1000; moreover, permeabilization and depolarization does not result in rapid autolysis in *E. coli*, so the physiological relevance of these experiments is questionable.

**Back to L: Genetic and molecular analysis**

The consignment of MS2 *L* to the role of phage-encoded autolysin seemed to end further interest in its function, despite the likelihood that a critical component of cell wall homeostasis was targeted. However, over the next decades, a few new Levi-
viridae were characterized, many of which shared the same genetic architecture as MS2, despite no significant nucleotide sequence similarity (60, 61) (Table S1). This included not only new leviruses specific for the F pilus but also against the conjugational pilus of several R-factor plasmids and the polar pilus of Pseudomonas (Fig. S5) (57, 58, 60, 61). We noticed that the L proteins, although unrelated in terms of sequence, shared an apparent domain organization with L: domain 1, N-terminal, highly charged; domain 2, very hydrophobic and lacking charged residues; domain 3, a central Leu-Ser dipeptide; and domain 4, a variable CTD (81). The conserved architecture suggested that L-like Sgl systems were widespread among Gram-negative bacteria were all targeting the same host function.

Two genetics-based approaches were mounted, the first aimed at identifying host factors, using, as before, inducible plasmid-based clones of L (81, 82). To avoid mutations that reduced the copy number or L transcription, a blue/white reporter plasmid was constructed, and from hundreds of colonies surviving L induction from this construct, two blue colonies were identified and designated as ill (insensitive to L lysis) mutants (82). Surprisingly, the ill mutations mapped to dnaJ, which encodes a widely conserved chaperone involved in the heat shock response (83). Analysis revealed that, in both, a P330Q missense change in dnaJ accounted for the Ill phenotype and abolished MS2 plaque formation, with both phage and survival phenotypes recessive. The Pro-330 residue is the most conserved residue in the CTD of DnaJ, which is clearly a conserved segment, although its function is unclear. The P330Q change was found to preserve the heat shock function of DnaJ but abrogates the ability of DnaJ to form complexes with L. The L suppressors, designated as Lsodj (overcomes dnaJ) alleles, were isolated as mutants that allowed lysis in dnaJ\textsuperscript{P330Q} background; these proved to be deletions of the dispensable NTD of L. Isogenic inductions of the parental and Lodj alleles revealed that lysis was much earlier with the truncations. These results led to a model in which the NTD of L has a regulatory, lysis-delay function that blocks the interaction of L with its target; in this model, DnaJ is required for relief of this steric block (Fig. S6).

To identify key functional elements of L itself, a nearly saturated mutational analysis of L generated a collection of 103 alleles with single codon changes conferring absolute lysis defects (Fig. S4) (81). The mutational distribution validated the proposed four domain structure of the L Sgl proteins. Domain 1, comprising the dispensable, highly basic region, gave rise to only one nonlytic allele (Q33H). Domain 3 containing the LS dipeptide motif and the adjacent segments of domains 2 and 4 had the most missense changes conferring nonlytic character. All of the missense alleles tested were genetically recessive and generated membrane-associated products of parental size. In addition, several of the inactivating missense changes (i.e., L44V, F47L, F47Y, S49T, F51L, and L56F) were conservative, suggesting that the L protein makes specific heterotypic protein–protein contacts in the membrane.

Taken together, the isolation of dnaJ\textsuperscript{P330Q} and mutational analysis of L both suggest that L targets a host membrane protein; that the interaction is through the mutationally sensitive residues in domains 2, 3, and 4; and that, like SlyD and -E, a host chaperone is involved in regulating L function. Obviously, further investigation into the host factors involved in L lysis is needed to understand the mechanistic details of L function.

Figure 4. Lys\textsuperscript{M} resistance mutations map to TMD2 and TMD7 of MurJ. The amino acid changes in E. coli MurJ (MurJ\textsubscript{EC}) resulting in Lys\textsuperscript{M} resistance were mapped onto the structure of MurJ from Thermosipho africanus (MurJ\textsubscript{TA}) (PDB code 5T77). A, cytoplasmic-open conformation. B, model of the periplasmic-open conformation. Left, lateral view; right, periplasmic view. The changes in MurJ\textsubscript{EC} and homologous amino acids in MurJ\textsubscript{TA} are shown on the right. This research was originally published in Nature Microbiology. Chamakura, K. R., Sham, L. T., Davis, R. M., Min, L., Cho, H., Ruiz, N., Bernhardt, T. G., and Young, R. A viral protein antibiotic inhibits lipid II flippase activity. Nat. Microbiol. 2017; 2:1480–1484. © Nature Research.
What’s next?

The premise of this review was that the study of the Sgl systems of small lytic phages would be interesting and lead to a better understanding of the bacterial cell wall biosynthesis and homeostasis. To summarize what has been discussed, four Sgl systems have been studied in depth, one from the microvirus φX174 and three from the Levirviridae. In three cases, the Sgl proteins turned out to be “protein antibiotics,” specific inhibitors of different enzymes of the highly conserved PG biosynthesis pathway (3, 70, 84). Consideration of the available genetic and biochemical data has already increased our understanding of how these important enzymes function and, in the case of the LysM, settled the identification of the flippase that exports lipid II to the periplasm. The next level of understanding will come from detailed structural information about the Sgl–enzyme inhibition complexes. The fourth case, the L protein of MS2, has not yet been fully characterized but appears to be the prototype of an Sgl type that has evolved multiple times in the Levirviridae, infecting a wide range of Gram-negative bacteria (81).

Genetic and biochemical evidence was cited showing that L does not inhibit any of the steps that lead to externalized lipid II and its incorporation into existing PG and suggests that it targets a host protein. There is no conceivable answer to the L target mystery that would not be important, possibly identifying conserved proteins that are essential for proper coordination or localization of cell wall synthesis machineries or are involved in the control of powerful autolytic enzymes.

Even with the L story still incomplete, this seems like a pretty good haul of information from the study of four small genes, starting with very “low-tech,” old-fashioned and simple genetic selections. The shocking thing is that this wealth of molecular information is derived from the study of only nine distinct Levirviridae (Table S1), comprising a total of ~50 kb of total genomic information. Despite the low number, these nine phages segregate into five different phage types based on where the Sgl evolved. Listed from 5’ to 3’ of the gRNA, they are as follows: AP205 (5’ of mat), Qβ (mat = A2), MS2 (overlapping end of coat and beginning of rep), phiCb5 (middle of rep), and M (near 3’ end of rep) (Fig. 2B). The diverse location of the Sgl genes suggests that they have evolved more than once and probably as a late addition to the genome after speciation to different pili or hosts (58, 61). (This includes all of the L-like genes; none of the 6 L-type Sgls has any detectable sequence identity other than the LS dipeptide sequence.) Given the diversity of Sgl systems and the existence of multiple protein targets in PG biosynthesis and maintenance, it is not difficult to imagine the existence of Sgl inhibitors for every known step in PG biosynthesis and, if L is any indicator, possibly uncharacterized components critical for dynamic cell wall homeostasis.

Taken together, it seems obvious that it would be useful to identify new Sgl genes, and the old-fashioned “phage hunt” is a reliable approach. RNA phage hunts have so far been done for five conjugational pili, resulting in two protein antibiotic Sgls (A2 and LysM) and four unrelated L-type Sgls (LMS2, LHGAL, LCI, and LPRR). Only three nonconjugational pili (Caulobacter, Acinetobacter, and Pseudomonas) have been targeted, resulting in two L-type Sgls and one, Lys of Caulobacter phage phiCBS, that does not have an L-type domain structure but does have a single N-terminal TMD, resembling both E and LysM. Considering the existence of many more retractable pili systems, there is a clear rationale to conduct RNA phage hunts in many other systems with retractable pili, especially in pathogenic bacteria.

Metagenomics is also having an impact. A recent survey of publicly available RNA-inclusive metagenomes and RNA virome studies of invertebrate species led to the identification of ~200 new ssRNA phage genomes (85, 86). Although most of the new levirival genomes are partial, ~80 are either complete or nearly so, with all three core genes annotated. Only one (AVE017) of these ~200 genomes had an annotated Sgl gene, being a close relative (38% sequence identity) of MS2 L (85). Given their small size, predilection for being embedded in the core genes, and extreme sequence diversity at the protein level, finding Sgl candidates in these genomes poses unique challenges to the traditional gene annotation tools. Moreover, currently, there is no direct way to sort out these levirival genomes to a particular bacterial host or, more specifically, to a particular retractable pilus. Nevertheless, the promise of more intriguing Sgl proteins targeting novel components of the bacterial cell wall machinery surely makes our current effort, which involves identifying potential Sgl ORFs and characterizing them one by one for the ability to support lysis after induction of synthetic clones, worth doing.

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References

1. Young, R. (2013) Phage lysis: do we have the whole story yet? Curr. Opin. Microbiol. 16, 790–797 CrossRef Medline
2. Young, R. (2014) Phage lysis: three steps, three choices, one outcome. J. Microbiol. 52, 243–258 CrossRef Medline
3. Bernhardt, T. G., Wang, I. N., Struck, D. K., and Young, R. (2002) Breaking free: “protein antibiotics” and phage lysis. Res. Microbiol. 153, 493–501 CrossRef Medline
4. Vollmer, W., Blanot, D., and de Pedro, M. A. (2008) Peptidoglycan structure and architecture. FEMS Microbiol. Rev. 32, 149–167 CrossRef Medline
5. Tippas, A., Banzhaf, M., Gross, C. A., and Vollmer, W. (2011) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat. Rev. Microbiol. 10, 123–136 CrossRef Medline
6. Silhavy, T. I., Kahne, D., and Walker, S. (2010) The bacterial cell envelope. Cold Spring Harb. Perspect. Biol. 2, a004144 Medline
7. Thwaites, J. L., and Mendelson, N. H. (1989) Mechanical properties of peptidoglycan as determined from bacterial thread. Int. J. Biol. Macromol. 11, 201–206 CrossRef Medline
8. Stock, J. B., Rauch, B., and Roseman, S. (1977) Periplasmic space in Salmonella typhimurium and Escherichia coli. J. Biol. Chem. 252, 7850–7861 Medline
9. Gan, L., Chen, S., and Jensen, G. J. (2008) Molecular organization of Gram-negative peptidoglycan. Proc. Natl. Acad. Sci. U.S.A. 105, 18953–18957 CrossRef Medline
10. Braun, V. (1975) Covalent lipoprotein from the outer membrane of Escherichia coli. Biochim. Biophys. Acta 415, 335–377 CrossRef Medline

3356 J. Biol. Chem. (2019) 294(10) 3350–3358
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11. Cowles, C. E., Li, Y., Semmelhack, M. F., Cristea, I. M., and Silhavy, T. J. (2011) The free and bound forms of Lpp occupy distinct subcellular locations in Escherichia coli. Mol. Microbiol. 79, 1168–1181 Medline

12. Brown, E. D., Vivas, E. I., Walsh, C. T., and Kolter, R. (1995) MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in Escherichia coli. J. Bacteriol. 177, 4194–4197 Medline

13. Lovering, A. L., Safadi, S. S., and Strynadka, N. C. (2012) Structural perspective of peptidoglycan biosynthesis and assembly. Annu. Rev. Biochem. 81, 451–478 CrossRef Medline

14. Mohammadi, T., van Dam, V., Sibbrandi, R., Vernet, T., Zapun, A., Bouhs, A., Diepeveen-de Bruin, M., Nguyen-Distèche, M., de Krijff, B., and Breukink, E. (2011) Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. EMBO J. 30, 1425–1432 CrossRef Medline

15. Mohammadi, T., Sibbrandi, R., Lutters, M., Verheul, J., Martin, N. I., den Blauwten, T., de Krijff, B., and Breukink, E. (2014) Specificity of the transport of lipid II by FtsW in Escherichia coli. J. Biol. Chem. 289, 14707–14718 Medline

16. Ruiz, N. (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flipase in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 105, 15553–15557 CrossRef Medline

17. Sham, L. T., Butler, E. K., Lebar, M. D., Kahne, D., Bernhardt, T. G., and Ruiz, N. (2014) Bacterial cell wall. MurJ is the flipase of lipid-linked precursors for peptidoglycan biogenesis. Science 345, 220–222 CrossRef Medline

18. Meeske, A. J., Sham, L. T., Kimsey, H., Koo, B. M., Gross, C. A., Bernhardt, T. G., and Rudner, D. Z. (2015) MurJ and a novel lipid II flipase are required for cell wall biogenesis in Bacillus subtilis. Proc. Natl. Acad. Sci. U.S.A. 112, 6437–6442 CrossRef Medline

19. Kuk, A. C., Marshalisid, E. H., and Lee, S. Y. (2017) Crystal structure of the OMP flipase MurJ in an inward-facing conformation. Nat. Struct. Mol. Biol. 24, 171–176 CrossRef Medline

20. Bolla, J. R., Sauer, J. B., Wu, D., Mehmood, S., Allison, T. M., and Robinson, C. V. (2018) Direct observation of the influence of cardiolipin and antibiotics on lipid II binding to MurJ. Nat. Chem. 10, 363–371 CrossRef Medline

21. Zheng, Y., Struck, D. K., and Young, R. (2009) Purification and functional analysis of the lipid II flippase MurJ from Escherichia coli by dX174 gene E product. In Microbial Cell Wall Synthesis and Autolysis (Nombele, C., ed) pp. 213–218, Elsevier Science Publishers, New York

22. Lutz, W., Halfmann, G., and Lutz, W. (1984) Induction of autolysis of Escherichia coli by dX174 gene E product. In Microbial Cell Wall Synthesis and Autolysis (Nombele, C., ed) pp. 213–218, Elsevier Science Publishers, New York

23. Lutz, W., Halfmann, G., and Lutz, W. (1984) Lysis of Escherichia coli after infection with dX174 depends on the regulation of the cellular autolytic system. J. Gen. Microbiol. 130, 1079–1087 Medline

24. Witte, A., Wanner, G., Blasi, U., Halfmann, G., Szostak, M., and Lutz, W. (1990) Endogenous transmembrane tunnel formation mediated by dX174 lysis protein E. J. Bacteriol. 172, 4109–4114 CrossRef Medline

25. Maratea, D., Young, K., and Young, R. (1985) Deletion and fusion analysis of the dX174 lysis gene E. Gene 40, 39–46 CrossRef Medline

26. Buckley, K. J., and Hayashi, M. (1986) Lytic activity localized to membrane-spanning region of dX174 E protein. Mol. Gen. Genet. 204, 120–125 CrossRef Medline

27. Roof, W. D., Horne, S. M., Young, K. D., and Young, R. (1994) slyD, a host gene required for dX174 lysis, is related to the FK506-binding protein family of peptidyl-prolyl cis-trans-isomerases. J. Biol. Chem. 269, 2902–2910 Medline

28. Kay, J. E. (1996) Structure-function relationships in the FK506-binding protein (FKBP) family of peptidyl-prolyl cis-trans isomerases. Biochem. J. 314, 361–385 CrossRef Medline

29. Bernhardt, T. G., Roof, W. D., and Young, R. (2002) The Escherichia coli FKBP-type PPlase SlyD is required for the stabilization of the E lysis protein of bacteriophage dX174. Mol. Microbiol. 45, 99–108 CrossRef Medline

30. Bernhardt, T. G., Struck, D. K., and Young, R. (2001) The lysis protein E of dX174 is a specific inhibitor of the MrAY-catalyzed step in peptidoglycan synthesis. J. Biol. Chem. 276, 6093–6097 CrossRef Medline

31. Bernhardt, T. G., Roof, W. D., and Young, R. (2000) Genetic evidence that the bacteriophage dX174 lysis protein inhibits cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. 97, 4297–4302 CrossRef Medline

32. Zheng, Y., Struck, D. K., and Young, R. (2009) Purification and functional characterization of dX174 lysis protein E. Biochemistry 48, 4999–5006 CrossRef Medline

33. Pollock, T. J., Tessman, E. S., and Tessman, I. (2017) Identification of lysis protein E of bacteriophage dX174. J. Virol. 88, 408–410 Medline

34. Zheng, Y., Struck, D. K., Bernhardt, T. G., and Young, R. (2008) Genetic analysis of MrAY inhibition by the dX174 protein E. Genetics 180, 1459–1466 CrossRef Medline

35. Chung, B. C., Zhao, J., Gillespie, R. A., Kwon, D. Y., Guan, Z., Hong, J., Zhou, P., and Lee, S. Y. (2013) Crystal structure of MrAY, an essential membrane enzyme for bacterial cell wall synthesis. Science 341, 1012–1016 CrossRef Medline

36. Tanaka, S., and Clemons, W. M., Jr. (2012) Minimal requirements for inhibition of MrAY by lysis protein E from bacteriophage dX174. Mol. Microbiol. 85, 975–985 CrossRef Medline

37. Inokuchi, Y., Jacobson, A. B., Hirose, T., Inayama, S., and Hirashima, A. (1988) Analysis of the complete nucleotide sequence of the group IV RNA coliphage SP. Nucleic Acids Res. 16, 6205–6221 CrossRef Medline
51. Inokuchi, Y., Takahashi, R., Hirose, T., Inayama, S., Jacobson, A. B., and Hirashima, A. (1986) The complete nucleotide sequence of the group II RNA coliphage GA. J. Bacteriol. 99, 1169–1180 CrossRef Medline

52. Inokuchi, Y., Hirashima, A., and Watanabe, I. (1982) Comparison of the nucleotide sequences at the 3′-terminal region of RNAs from RNA coliphages. J. Mol. Biol. 158, 711–730 CrossRef Medline

53. Stewart, J. R., Vinjé, J., Oudejans, J. S., Scott, G. L., and Sobsey, M. D. (2006) Sequence variation among group III F-specific RNA coliphages from water samples and swine lagoons. Appl. Environ. Microbiol. 72, 1226–1230 CrossRef Medline

54. Friedman, S. D., Cooper, E. M., Casanova, L., Sobsey, M. D., and Gentner, F. J. (2009) A reverse transcription-PCR assay to distinguish the four genotypes of male-specific (F′) RNA coliphages. J. Virol. Methods 157, 49–52 CrossRef Medline

55. Adhin, M. R., Hirashima, A., and van Duin, J. (1989) Nucleotide sequence of the ssRNA bacteriophage JP34 resolves the discrepancy between serological and biophysical classification. Virology 170, 238–242 CrossRef Medline

56. Kazaks, A., Voronkova, T., Rumnieks, J., Dishlers, A., and Tars, K. (2011) Embo J. 158, 263–275 CrossRef Medline

57. Ruokoranta, T. M., Grahn, A. M., Ravantti, J. J., Poranen, M. M., and Tammela, T. (2007) Nucleotide sequence and characterization of two R-plasmid-dependent ssRNA coliphage GA. J. Mol. Biol. 371, 1067–1077 CrossRef Medline

58. Rummiekis, J., and Tars, K. (2012) Diversity of pili-specific bacteriophages: genome sequence of IncM plasmid-dependent RNA phage M. BMC Microbiol. 12, 277 CrossRef Medline

59. Klovins, J., Overbeek, G. P., van den Worm, S. H., Ackermann, H. W., and van Duin, J. (2002) Nucleotide sequence of a ssRNA phage from Acinetobacter: kinship to coliphages. J. Gen. Virol. 83, 1523–1533 CrossRef Medline

60. Oshthoorn, R. C., Garde, G., Dayhuff, T., Atkins, J. F., and van Duin, J. (1995) Nucleotide sequence of a single-stranded RNA phage from Pseudomonas aeruginosa: kinship to coliphages and conservation of regulatory RNA structures. Virology 206, 611–625 CrossRef Medline

61. Kannoly, S., Shao, Y., and Wang, I. N. (2012) Rethinking the evolution of single-stranded RNA (ssRNA) bacteriophages based on genomic sequences and characterizations of two R-plasmid-dependent ssRNA phages, C-1 and Hgal. J. Bacteriol. 194, 5073–5079 CrossRef Medline

62. Domingo, E., and Holland, J. J. (1997) RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51, 151–178 CrossRef Medline

63. Cui, Z., Gorzelniak, K. V., Chang, J. Y., Langlais, C., Jakana, J., Young, R., and Zhang, J. (2017) Structures of Q β virions, virus-like particles, and the Qβ-MurA complex reveal internal coat proteins and the mechanism of lysis. J. Virol. 91, 3331–3336 CrossRef Medline

64. Weber, K., and Konigsberg, W. (1975) Proteins of the RNA phages. In RNA Phages (Zinder, N. D., ed) pp. 51–84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

65. Kozak, M., and Nathans, D. (1971) Fate of maturation protein during infection by coliphage MS2. Nat. New Biol. 234, 209–211 CrossRef Medline

66. Winter, R. B., and Gold, L. (1983) Overproduction of bacteriophage Qβ maturation (A2) protein leads to cell lysis. Cell 33, 877–885 CrossRef Medline

67. Bernhardt, T. G., Wang, I. N., Struck, D. K., and Young, R. (2001) A protein antibiotic in the phage Qβ virion: diversity in lysis targets. Science 292, 2326–2329 CrossRef Medline

68. Reed, C. A., Langlais, C., Kuznetsov, V., and Young, R. (2012) Inhibitory mechanism of the Qβ lysin protein A2. Mol. Microbiol. 86, 836–844 CrossRef Medline

69. Reed, C. A., Langlais, C., Wang, I. N., and Young, R. (2013) A2 expression and assembly regulates lysis in Qβ infections. Microbiology 159, 507–514 CrossRef Medline

70. Chamakura, K. R., Sham, L. T., Davis, R. M., Min, L., Cho, H., Ruiz, N., Bernhardt, T. G., and Young, R. (2017) A viral protein antibiotic inhibits lipid II flipase activity. Nat. Microbiol. 2, 1480–1484 CrossRef Medline

71. Butler, E. K., Davis, R. M., Bari, V., Nicholson, P. A., and Ruiz, N. (2013) Structure-function analysis of MurJ reveals a solvent-exposed cavity containing residues essential for peptidoglycan biosynthesis in Escherichia coli. J. Bacteriol. 195, 4639–4649 Medline

72. Model, P., Webster, R. E., and Zinder, N. D. (1979) Characterization of Op3, a lysis-defective mutant of bacteriophage f2. Cell 18, 235–246 CrossRef Medline

73. Beremand, M. N., and Blumenthal, T. (1979) Overlapping genes in RNA phage: a new protein implicated in lysis. Cell 18, 257–266 CrossRef Medline

74. Coleman, J., Inouye, M., and Atkins, J. (1983) Bacteriophage MS2 lysin protein does not require coat protein to mediate cell lysis. J. Bacteriol. 153, 1098–1100 Medline

75. Berkhourt, B., de Smit, M. H., Spanjaard, R. A., Blom, T., and van Duin, J. (1985) The amino terminal half of the MS2-coded lysis protein is dispensable for function: implications for our understanding of coding region overlaps. EMBO J. 4, 3315–3320 CrossRef Medline

76. Holtje, J. V., and van Duin, J. (1984) MS2 phage induced lysis of E. coli depends upon the activity of the bacterial autolysins. In Microbial Cell Wall Synthesis and Autolysis (Nombela, C., ed) pp. 195–199, Elsevier Science Publishers, New York

77. Walderich, B., Ursinus-Wössner, A., van Duin, J., and Holtje, J. V. (1988) Induction of the autolytic system of Escherichia coli by specific insertion of bacteriophage MS2 lysis protein into the bacterial cell envelope. J. Bacteriol. 170, 5027–5033 CrossRef Medline

78. Walderich, B., and Holtje, J. V. (1989) Specific localization of the lysis protein of bacteriophage MS2 in membrane adhesion sites of Escherichia coli. J. Bacteriol. 171, 3331–3336 CrossRef Medline

79. Holtje, J. V., Fiedler, W., Rotering, H., Walderich, B., and van Duin, J. (1988) Lysis induction of Escherichia coli by the cloned lysis protein of the phage MS2 depends on the presence of osmoregulatory membrane-derived oligosaccharides. J. Biol. Chem. 263, 3539–3541 Medline

80. Goessens, W. H. F., Driessen, A. J. M., Wilschut, J., and van Duin, J. (1988) A synthetic peptide corresponding to the C-terminal 25 residues of phage MS2-coded lysis protein dissipates the proton-motive force in Escherichia coli membrane vesicles by generating hydrophobic pores. EMBO J. 7, 867–873 CrossRef Medline

81. Chamakura, K. R., Sham, L. T., Davis, R. M., Min, L., Cho, H., Ruiz, N., Bernhardt, T. G., and Young, R. (2013) A2 expression and assembly regulates lysis in Qβ infections. Microbiology 159, 507–514 CrossRef Medline

82. Chamakura, K. R., Sham, L. T., Davis, R. M., Min, L., Cho, H., Ruiz, N., Bernhardt, T. G., and Young, R. (2017) A viral protein antibiotic inhibits lipid II flipase activity. Nat. Microbiol. 2, 1480–1484 CrossRef Medline

83. Butler, E. K., Davis, R. M., Bari, V., Nicholson, P. A., and Ruiz, N. (2013) Structure-function analysis of MurJ reveals a solvent-exposed cavity containing residues essential for peptidoglycan biosynthesis in Escherichia coli. J. Bacteriol. 195, 4639–4649 Medline

84. Model, P., Webster, R. E., and Zinder, N. D. (1979) Characterization of Op3, a lysis-defective mutant of bacteriophage f2. Cell 18, 235–246 CrossRef Medline

85. Beremand, M. N., and Blumenthal, T. (1979) Overlapping genes in RNA phage: a new protein implicated in lysis. Cell 18, 257–266 CrossRef Medline