REVIEW ARTICLE

Diversity in bacterial lysis systems: bacteriophages show the way

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Abstract

Bacteriophages have developed multiple host cell lysis strategies to promote release of descendant virions from infected bacteria. This review is focused on the lysis mechanisms employed by tailed double-stranded DNA bacteriophages, where new developments have recently emerged. These phages seem to use a least common denominator to induce lysis, the so-called holin-endolysin dyad. Endolysins are cell wall-degrading enzymes whereas holins form 'holes' in the cytoplasmic membrane at a precise scheduled time. The latter function was long viewed as essential to provide a pathway for endolysin escape to the cell wall. However, recent studies have shown that phages can also exploit the host cell secretion machinery to deliver endolysins to their target and subvert the bacterial autolytic arsenal to effectively accomplish lysis. In these systems the membrane-depolarizing holin function still seems to be essential to activate secreted endolysins. New lysis players have also been uncovered that promote degradation of particular bacterial cell envelopes, such as that of mycobacteria.

Introduction

Bacteriophages, the viruses that infect bacteria, are the most abundant biological entity on earth and probably the most diverse (Hendrix, 2002; Pedulla *et al.*, 2003). Phages can be fierce predators of bacteria and consequently they have a major impact on the ecological balance and dynamics of microbial life (Rodriguez-Valera *et al.*, 2009). However, they are at the same time key players in the evolution of bacteria by shaping their genome through horizontal gene transfer (Canchaya *et al.*, 2003; Brüssow *et al.*, 2004).

Like all viruses, phages must parasitize host cells, in this case bacteria, to replicate. In the extracellular environment they exist as supramolecular structures called virions. These are optimized to ensure viral propagation by protecting the phage genome and by promoting its efficient delivery to host bacteria. Once inside the host cell, the genetic information carried in the viral genome is responsible for its own replication and for the production of the components to make new virus particles. The virion descendence assembled within an infected cell then needs to be released to the environment, where host bacteria are potentially available for new infection cycles. As any other step underlying viral replication, the ones responsible for ending the multiplication process and for the subsequent passage of the virion progeny across the bacterial cell barriers have been shaped through evolution to maximize virus particle production and the chances of finding new hosts (Wang *et al.*, 2000; Wang, 2006).

With the exception of filamentous bacteriophages, whose virus particles are released from the host by a secretion-related mechanism that maintains bacterial cell integrity (Russel, 1995), phages typically induce lysis of infected bacteria to allow efficient escape of the virion progeny to the extracellular milieu (São-José *et al.*, 2003, 2007; Young & Wang, 2006). Phage-mediated lysis essentially proceeds by two basic mechanisms. Icosahedral phages with small, single-stranded genomes seem to employ a single lysis effector, whose function is to inhibit a given step of bacterial cell wall peptidoglycan biosynthesis. This strategy requires actively growing cells and lysis appears to be a consequence of rupture of the cell wall at the developing septum (Bernhardt *et al.*, 2001a, b, 2002). Double-stranded DNA (dsDNA) phages, which represent

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more than 95% of known bacterial viruses (Ackermann, 2009), employ at least two proteins whose coordinated action results in well timed and swift host cell lysis. These are a small hydrophobic protein called holin that oligomerizes in the cytoplasmic membrane until it induces formation of pores, and an endolysin, which is an enzyme that cleaves the cell wall peptidoglycan (Young & Wang, 2006; São-José *et al.*, 2007).

In this article we review the different lysis mechanisms described to date that are employed by tailed dsDNA bacteriophages. We discuss new lysis models that involve secreted endolysins endowed with canonical signal peptides or with signal-arrest-release sequences and the recent advances emerging from the studies of mycobacteriophage-induced host cell lysis.

Cell barriers to phage release

In the last step of phage infection the viral offspring must overcome the physical barriers mounted by the host cell envelope. Essentially, this complex multi-layered structure comprises a cytoplasmic membrane (CM), a cell wall and, in the case of Gram-negative bacteria and mycobacteria, an outer membrane (OM) (Fig. 1).

The bacterial CM is a hydrophobic phospholipid bilayer imbedded with proteins, which is common to all bacteria. It acts as a semi-permeable barrier preventing leakage of hydrophilic constituents from the cytoplasm and protecting this cell compartment from external insults. Importantly, the CM is impermeable to protons and other ions, allowing the cell to sustain an electrochemical gradient across the membrane and thus generating the so-called proton-motive force (pmf). This form of energy drives all reactions and physiologic processes that permit cells to live and propagate (Weiner & Rothery, 2007).

The major component of the cell wall is peptidoglycan (PG), a rigid structure that contributes to cell shape and confers mechanical resistance to osmotic pressure, preventing cells from lysing. The PG backbone consists of glycan chains with β-1,4 linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) units, which are cross-linked by peptide side chains attached to NAM via an amide bond. These side chains may be bridged by short peptides. The exact molecular makeup of these cross-links can be species-specific and accounts for more than 100 different peptidoglycans (Schleifer & Kandler, 1972; Vollmer *et al.*, 2008).

In Gram-positive bacteria the amount of cross-linking is higher and the wall is thicker than in Gram-negative cell walls. Interspersed in the PG are long anionic polymers, the wall teichoic and lipoteichoic acids, which are the major contributors to the envelope structure and function (Neuhaus & Baddiley, 2003; Bhavsar *et al.*, 2004) (Fig. 1a).

In Gram-negative bacteria the thinner layer of PG is covalently linked to an OM, an asymmetrical lipid bilayer consisting of phospholipids and lipopolysaccharides in

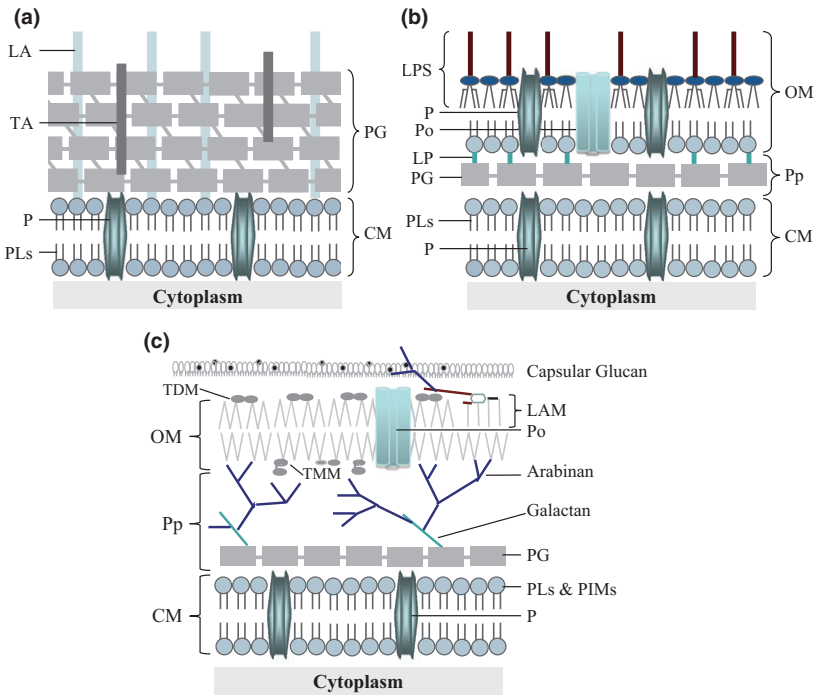

Fig. 1. Bacterial cell envelopes. (a) Gram-positive bacteria, (b) Gram-negative bacteria; (c) mycobacteria. CM, cytoplasmic membrane; LA, lipoteichoic acids; LAM, lipoarabinomannan; LP, lipoprotein; LPS, lipopolyssacharide; OM, outer membrane; P, protein; PG, peptidoglycan; PIMs, phosphatidylinositol mannosides; PLs, phospholipids; Po, porin; Pp, periplasm; TA, teichoic acids; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

the inner and outer leaflet, respectively. The OM, which serves as a protective barrier that controls the flux of solutes, also contains integral OM proteins and lipoproteins (Ruiz *et al.*, 2006) (Fig. 1b).

The structure of the mycobacteria cell envelope is much more complex; the CM which is structurally and functionally similar to other bacterial cytoplasmic membranes (Daffé *et al.*, 1989), is surrounded by a cell wall core that is composed of PG covalently attached to arabinogalactan (AG). This, in turn, is esterified to a mycolic acid layer forming the mycolyl arabinogalactan-peptidoglycan (mAGP) complex (Brennan, 2003). These covalently linked mycolic acids constitute all or part of the inner leaflet of a true outer membrane. The outermost leaflet is composed of various glycolipids, including trehalose mono- and dimycolate, phospholipids and species-specific lipids (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). Finally, outside of the outer membrane is a layer of proteins, polysaccharides and a small amount of lipids known as the capsule (Lemassu & Daffé, 1994; Lemassu *et al.*, 2006; Sani *et al.*, 2010) (Fig. 1c).

As already mentioned, dsDNA phages follow the most drastic strategy to overcome the host cell barriers and release their virion progeny, i.e. they induce cell lysis. As detailed below, lysis is accomplished through specialized and regulated functions that compromise the physical integrity of the different elements composing the bacterial cell envelope.

Phage-mediated bacterial lysis at a glance

The lysis mechanism of *Escherichia coli* phage λ (see detailed description below) is by far the best studied and still serves as a model for most dsDNA phages employing a holin-endolysin lysis strategy (Young & Wang, 2006; São-José *et al.*, 2007). According to this model, phage endolysins accumulate in the host cell cytoplasm during phage development, until holins suddenly induce formation of holes in the cytoplasmic membrane. These holes provide a pathway for endolysin release to the cell wall that is rapidly cleaved, leading to cell burst. Therefore, the holin function has the crucial role of defining the time of lysis, which is fundamental for phage fitness.

Confinement of endolysins in the host cell cytoplasm during phage replication was long considered a paramount and transversal feature of bacterial lysis mediated by dsDNA phages, simply because release of these lethal enzymes to the cell wall before full assembly of descendant virus particles would not make biological sense. However, as discussed further on, it is now known that several phages can engage the host cell secretion machinery, specifically the general secretion pathway (Sec system), to export their endolysins to the extracytoplasmatic medium way before completion of the viral reproductive cycle (Young, 2005; São-José *et al.*, 2007). These phages can either produce endolysins with secretion signals, that is, typical signal peptides (SP) or signal-arrest-release (SAR) sequences, or synthesize chaperone-like proteins that interact with endolysins and target them to the Sec translocase (São-José *et al.*, 2000; Xu *et al.*, 2004; Catalão *et al.*, 2010). It was shown that these lytic enzymes start to be exported to the cell wall compartment during phage development, without causing premature host cell lysis. This implies that endolysin action is somehow restrained until the proper moment for lysis to occur. Interestingly, phages producing secreted endolysins also produce a holin, although this is not involved in endolysin transport. It became rapidly clear that in these systems, holins still maintain the key role of setting up lysis timing, since their membrane pmf-dissipating action is directly or indirectly responsible for the abolishment of the mechanisms that restrain activity of the secreted endolysins. In some phages, like those infecting *Streptococcus pneumoniae*, the holin membrane-depolarizing function can even trigger the bacterial autolytic machinery as a means to accomplish optimal host cell lysis and virion progeny release (Frias *et al.*, 2009).

In addition to the basic holin and endolysin lysis players, dsDNA phages have evolved ancillary functions that are important for the effectiveness of bacterial lysis or for the fine-tuning of lysis timing (see below). A landmark example of the latter is the antiholin protein, which has the ability to tune the timing of the holin action in response to environmental cues. Supplementary functions that help to achieve proficient lysis include spanins that weaken the outer membrane barrier of Gram-negative hosts or lipases that are thought to compromise the mycolyl-arabinogalactan external layer of the mycobacterial cell envelope.

Basic features of the essential lysis players

Endolysin is a generic term used to describe dsDNA bacteriophage-encoded PG hydrolases that are released to or activated in the cell wall compartment at the end of the phage multiplication cycle. They are characterized by their ability to cleave different bonds of the PG meshwork and thus compromise the physical integrity of the bacterial cell wall (Loessner, 2005; Borysowski *et al.*, 2006). There are five major functional types of phage endolysins: *N*-acetylmuramidases (lysozymes), endo-β-*N*-acetylglucosaminidases, lytic transglycosylases, all cleaving the NAM-NAG moiety of the PG but with transglycosylases forming a cyclic 1,6-anhydro-*N*-acetylmuramic acid product, *N*-acetyl-muramoyl-L-alanine amidases, which hydrolyze

the amide bond between NAM and L-alanine residues in the oligopeptide cross-linking chains, and endopeptidases, which attack the peptide bonds in the same chains (Loessner, 2005).

With a few exceptions (Briers *et al.*, 2007; Walmagh *et al.*, 2012), phages infecting Gram-negative bacteria produce relatively small endolysins comprising a single domain responsible for the cleavage of a specific PG bond, while phages of Gram-positive bacteria or of mycobacteria encode endolysins with a typical modular architecture with at least two clearly separated functional regions: generally, an *N*-terminus carrying one or two catalytic domains, which is attached by a flexible linker to a *C*-terminus segment harbouring cell wall-binding domains (Loessner, 2005; Fischetti, 2008). It has been suggested that these cell wall-binding domains maintain endolysins attached to the cell wall debris of lysed bacterial cells, thus preventing collateral damage of potential new hosts (Fischetti, 2008). Some endolysins are also capable of degrading PG when applied externally to Gram-positive bacteria, resulting in a rapid lysis (Fischetti, 2005). This capacity has stimulated the research in bacteriophage endolysins with the purpose of using them as potential antibacterial agents. The exploitation of endolysins to kill pathogenic bacteria has been the subject of recent reviews (Borysowski *et al.*, 2006; O'Flaherty *et al.*, 2009; Fischetti, 2010).

Unlike endolysins, holins are much more diverse and frequently unique with respect to their amino acid sequence. Typically, they are small proteins (<150 aa) with at least one predicted transmembrane domain (TMD) and with a hydrophilic, highly charged *C*-terminus (Wang *et al.*, 2000; Young, 2002). Holins have been categorized in classes I, II and III, according to the predicted or experimentally determined number of TMDs – three, two or one, respectively (Wang *et al.*, 2000). The holins of *E. coli* phages λ, 21 and T4 are prototypes of these classes, respectively. Experimental data on a couple of phages strongly suggest that the 'holin functional unit' may actually be formed by a complex of at least two different polypeptides rather than by a single protein (see below).

Holins are late synthesized proteins that progressively accumulate in the bacterial cytoplasmic membrane and, as long as the pmf is maintained above a certain threshold, they assemble into oligomers and rafts of intrinsic stability (Young & Wang, 2006). Below this threshold, holins trigger to form pores that permeabilize the membrane. This event ends macromolecular synthesis and thus effectively terminates infection (Wang *et al.*, 2000; Young *et al.*, 2000). Holins can be prematurely triggered through the action of membrane-depolarizing agents such as cyanide and dinitrophenol (Gründling *et al.*, 2001; Young, 2005). The size of the holin-mediated hole can be significantly different among phages (see below).

Holins are subjected to opposing evolutionary forces. On the one hand, there is pressure to extend the vegetative cycle to allow continued accumulation of virions at a linear rate. On the other hand, there is pressure to trigger lysis earlier to release progeny phage particles so that they can infect new hosts and potentially yield exponential increases in phage numbers (Wang *et al.*, 2000; Young *et al.*, 2000; Wang, 2006).

The phage λ model of bacterial lysis

The lysis cassette of λ comprises four genes, *S*, *R*, *Rz* and *Rz1*, transcribed from the pR' late promoter, encoding a total of five proteins (Young, 1992). The *S* gene has a dual-start motif that allows for translation initiation events at two sites defined by codons 1 and 3, giving rise to two proteins with opposite functions, the antiholin (S_{107}) and the holin (S_{105}) (Bläsi *et al.*, 1989, 1990) The *R* gene encodes the endolysin, an 18-kDa soluble protein, with lytic transglycosylase activity (Bieńkowska-Szewczyk *et al.*, 1981). Rz and Rz1 are a class II CM protein and an OM lipoprotein, respectively, forming a complex (spanin) that potentially spans the entire periplasm. The Rz and Rz1 spanin (see below) is thought to promote fusion of the CM and OM, thus eliminating the last barrier that prevents efficient release of virion progeny from the infected cell (Berry *et al.*, 2008, 2010).

In the λ lysis system (Fig. 2a), the holin accumulates in the CM during late gene expression, without disturbing its integrity, whereas enzymatically active endolysin accumulates in the cytoplasm. At a genetically defined time, the holin triggers to disrupt the membrane, allowing endolysin to cross the membrane and attack the peptidoglycan, leading to cell lysis (Wang *et al.*, 2000; Gründling *et al.*, 2001).

The S holin, or S_{105}, is a 105-residue integral cytoplasmic membrane protein with three TMD and an N-out, C-in topology (Gründling *et al.*, 2000). S_{107} differs from S_{105} by two residues at the *N*-terminus, Met and Lys, which confers to S_{107} an extra positive charge. This basic residue is what confers to S_{107} its inhibitory effect, since it prevents insertion of the first TMD into the cytoplasmic membrane. S_{107} and S_{105} are produced at a ratio of 1 : 2 defined by two RNA structures in the 5′ end of the S gene. Modifications of this ratio alter the lysis time: the greater the amount of S_{107}, the more delayed lysis is (Bläsi *et al.*, 1989). The antiholin function is subverted when the membrane potential is dissipated, which results in the insertion of the S_{107} TMD_1 into the membrane, becoming a functional holin and thus increasing the amount of active holin.

Steiner & Bläsi (1993) have shown that the distribution of amino-terminal charged amino acids as well as the

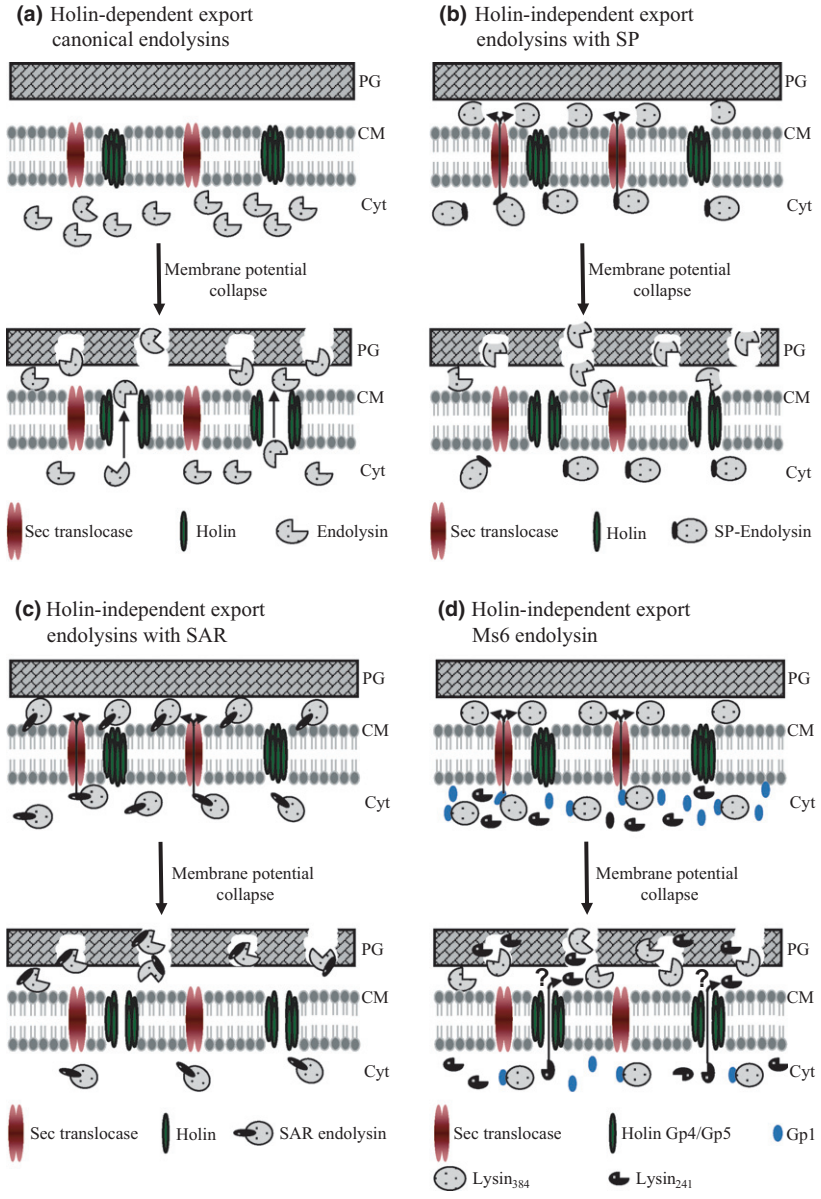

Fig. 2. Models for export and activation of phage endolysins. (a) In phages such as λ the export of the active endolysin to the cell wall is through the holin pores. Holin-independent, Sec-mediated export of endolysins is observed in: (b) phages producing endolysins with typical signal peptides (SP), such as oenophage fOg44; (c) in phages synthesizing signal-arrest-release (SAR) endolysins, as observed in coliphage P1; and (d) in mycobacteriophage Ms6, where export of the full-length endolysin (Lysin384) is assisted by the chaperone Gp1 (see text for details). When endolysins are exported through the Sec translocase, they are maintained in an inactive state in the cell wall compartment until holins dissipate the membrane pmf. The endolysin activation after pmf collapse is schematically represented by the change of the enzyme spherical configuration to a 'pacman' shape. Lysin241 in (d) is an *N*-terminally truncated version of the Ms6 endolysin produced by internal translation signals in the same reading frame. (?) indicates that export of this shorter version to the extracytoplasmatic environment is not known. PG and CM, as in Fig. 1; Cyt, cytoplasm.

total amino-terminal net charge of S_{105} and S_{107} influences their lethal potential. Functional assembly of the λ S holin requires periplasmic localization of its *N*-terminus (Graschopf & Bläsi, 1999). Recently, it has been demonstrated that the *N*-terminus of S_{105} retains its formylated Met residue, whereas that of S_{107} is fully deformylated (White *et al.*, 2010). Thus, compared with S_{105}, S_{107} actually has two positively charged extra residues, Lys_2 and the free *N*-terminal amino group. This supports the topological difference between S_{105} and S_{107}; the TMD_1 of S_{105} inserts into the membrane so rapidly that it escapes deformylation, whereas that of S_{107} is retained in the cytoplasm. The authors have shown that the antiholin character of S_{107} is due to the absence of TMD_1, since its deletion from S_{105} resulted in a lysis defect, with the mutant holin insensitive to membrane depolarization (White *et al.*, 2010).

Although the holin trigger is lethal to the cell, lysis requires the endolysin R. Thus, the lesion formed by the S holin is large enough to allow access of the endolysin to its PG substrate. Until recently, little was known about the nature of the λ S membrane lesions. Previously, it was stated that oligomerization of S_{105} is a condition to disrupt the membrane (Gründling *et al.*, 2000). Attempts to characterize the pore formed by S_{105} *in vitro* have shown that S_{105} oligomers were ring-shaped structures

that could further polymerize into head-to-tail filaments (Savva *et al.*, 2008). However, the observed dimensions of the rings were not in agreement with the previously described ability of S to release an endolysin-β-galactosidase chimera with a mass of 500 kDa (Wang *et al.*, 2003). More recently, cryo-electron microscopy analysis revealed that the scale of the holes is at least an order of magnitude greater than any previously described membrane channel, with an average diameter of 340 nm and some exceeding 1 μm. These large holes seem to support the notion that at the time of lethal triggering, the S_{105} holin exists in large aggregates, leading to one or a small number of holes rather than many smaller holes distributed throughout the membrane as initially thought (Dewey *et al.*, 2010; White *et al.*, 2011). By following S_{105} trigger in real time by fluorescence microscopy, White *et al.* (2011) proposed that λ induces host cell lysis when the holin reaches a critical concentration and nucleates to form rafts.

Holin-independent export of endolysins

In the following sections we discuss recent studies that provided evidence of phage lysis strategies that significantly deviate from the classical λ model. These studies have not only highlighted that dsDNA phages can adopt multiple strategies to accomplish lysis of their host cells, but have also given a new perspective on lysis regulation by the holin function.

Secreted endolysins in Gram-positive systems

The holin-dependent translocation of the endolysin to the cell wall compartment, observed in phage λ (Fig. 2a), was for long considered a hallmark of bacteriophage lysis (Young *et al.*, 2000; Wang *et al.*, 2003). However, studies on fOg44, a phage that infects the Gram-positive bacterium *Oenococcus oeni*, brought to light a new mechanism for endolysin targeting to the PG (Fig. 2b) (São-José *et al.*, 2000). This phage encodes a muramidase endolysin (Lys44) with a bona-fide, Sec-type SP sequence, which is processed by the leader peptidase during translocation of the enzyme to the cell wall compartment. Lys44 was shown to be exported by the Sec machinery from the moment of its synthesis, and its active SP-processed form is detected about halfway through the phage latent period. It was proposed that Lys44 is kept in an inactive state in the murein layer, perhaps by local conditions that directly inhibit the catalytic site of the enzyme, until the proper moment for lysis occurs (São-José *et al.*, 2000).

An intriguing observation arises, as all phages that synthesize secreted endolysins or are proposed to do so (São-José *et al.*, 2000, 2003, 2007), also appear to encode a holin-like protein. Indeed, fOg44 also seems to have a holin gene, *hol44* (Parreira *et al.*, 1999), whose expression under λ native regulatory signals can complement a nonsense mutation in the S holin gene (São-José *et al.*, 2004). These results suggested that even in these cases, the lysis clock is regulated by the holin-mediated dissipation of the pmf, which should activate the exported endolysins rather than allowing their exit. It was demonstrated that the bacterial membrane pmf somehow regulates the lytic activity of the secreted Lys44. Dissipation of the CM electrochemical gradient was shown to be required to sensitize *O. oeni* and *Lactococcus lactis* cells to the activity of Lys44, when this was added from the outside or coming from within cells, respectively. Strikingly, efficient sensitization of metabolically active cells to Lys44 action was only achieved with agents (e.g. nisin and chloroform) that form non-selective ion pores, i.e. agents that mimic the holin disruption of the CM electrical and chemical gradients (Nascimento *et al.*, 2008).

Lysis systems involving secreted endolysins uncovered a role of the CM pmf in the control of the activity of these lytic enzymes, and suggested a more general function for holins, i.e. to activate the endolysins, regardless of its enzymatic activity or subcellular localization. This fact led to the speculation that, at least in Gram-positive systems, the holin-mediated pmf dissipation could also activate the bacterial host autolytic machinery, since this is also known to be turned on upon CM injury (São-José *et al.*, 2000, 2003, 2007). This prediction recently gained experimental support when it was demonstrated that CM permeabilization induced by the holin of *S. pneumoniae* phage SV1 activates the ubiquitous host autolysin LytA, which contributes to optimal host cell lysis and subsequent virion escape (Frias *et al.*, 2009). Interestingly, it was also proposed that the lipid-containing dsDNA phage PM2 also relies on host cell (*Pseudoalteromonas* sp.) factors for digestion of the PG (Krupovič *et al.*, 2007).

Secreted endolysins in Gram-negative systems

Recently, a new class of endolysins has come to light that, similarly to Lys44, can also engage the Sec system to target them to the PG. Particularly notable are the cases of the endolysins of *E. coli* phages P1 (Lyz^{P1}) and 21 (R^{21}), which differ significantly from the fOg44 secretory endolysin in not having a typical cleavable SP sequence, although they were found to cause lysis of the host without a holin (Xu *et al.*, 2004). The *N*-terminus of Lyz^{P1} and R^{21} is unusual in that it contains a signal-arrest-release (SAR) sequence, a TMD that is characterized by the presence of weakly hydrophobic amino acids, flanked by charged residues. This domain allows the export of the

endolysins through the host Sec system, but in this case the SAR sequence retains them in the periplasm as inactive forms tethered to the CM (Xu *et al.*, 2004, 2005). In these cases holin function is confined to imposing the timing of lysis.

Lyz^{P1} and R^{21} are homologous to the T4 canonical lysozyme. Sequence comparison with other phage or prophage encoded homologues revealed that many have *N*-terminal domains that resemble the SAR sequences defined in Lyz^{P1} and R^{21} (Xu *et al.*, 2004). Occurrence of SAR endolysins outside lambdoid phages was meanwhile demonstrated (Stojković & Rothman-Denes, 2007; Briers *et al.*, 2011).

A model for triggering of lysis with SAR endolysins was proposed (Fig. 2c) where the SAR endolysin is initially tethered in an inactive form to the energized membrane, whereas the holin protein accumulates without affecting the CM pmf. At the programmed lysis time, the holin triggers, disrupting the membrane sufficiently to abolish the pmf, and depolarization accelerates the endolysin release from the membrane, which results in its activation, leading to rapid lysis (Xu *et al.*, 2004). This functional regulation is essential to avoid premature lysis of the infected host.

The molecular details of the activation event were further dissected. When the P1 lysozyme is secreted to the periplasm and accumulates in a membrane-tethered form, a disulfide bond is formed between cysteine residue Cys_{44} and the active site residue Cys_{51}, keeping the enzyme inactive. Upon release of the SAR sequence from the bilayer, Lyz^{P1} is activated by an intramolecular thiol-disulphide isomerization, which requires the Cys_{13} residue of the SAR domain: the thiol group of Cys_{13} attacks and reduces the Cys_{44}-Cys_{51} disulphide bond, resulting in the unblocking of the enzyme active site. Once the protein is released from the membrane, its Cys_{13} residue is involved in the isomerization reaction that relieves topological, covalent, and conformational constraints from Lyz^{P1}. This allows the protein to assume an active conformation mediated by DsbA (the periplasmic primary oxidant involved in disulphide bond formation), and possibly facilitated by periplasmic foldases and chaperones (Xu *et al.*, 2004, 2005). Crystal structures confirmed that in the active form of Lyz^{P1} the disulphide linkage is formed between Cys_{13} and Cys_{44}, whereas in the inactive form it occurs between Cys_{44} and Cys_{51}, and revealed dramatic conformational differences in the catalytic domain (Xu *et al.*, 2005).

The model of Lyz^{P1}activation is not common to all SAR endolysins. The R^{21} does not have a cysteine residue within the SAR domain and it is, therefore, differently activated. Recently, Sun *et al.* (2009) reported that a dynamic membrane topology regulates activation of R^{21}. When membrane depolarization occurs upon holin triggering, the released SAR domain refolds into the body of the enzyme, effecting muralytic activation by repositioning one residue of the canonical lysozyme catalytic triad. Besides controlling the protein topology, the SAR domain in R^{21} plays a specific and more integral role in the catalytic activity of the enzyme.

More recently, the endolysin Lyz^{103} of the *Erwinia amylovora* phage ERA103 was shown to exemplify a third distinct mode of SAR endolysins regulation. The nascent Lyz^{103} is maintained in an inactive state as the result of the inhibitory Cys_{42}-Cys_{45} disulphide bond flanking the catalytic Glu_{43}, caging the enzyme active site. Release of the SAR sequence from the membrane provides a Cys_{12} thiol group that disrupts the Cys_{42}-Cys_{45} disulphide bond, resulting in the activation of Lyz^{103} (Kuty *et al.*, 2010). This is the first enzyme whose enzymatic activity is regulated by disulphide bond caging of its active site.

Pinholins: formers of small-sized pores

An unusual and remarkable different class of holin-endolysin systems has arisen in recent years (Park *et al.*, 2006, 2007). This class, represented by the lambdoid bacteriophage 21, employs endolysins having *N*-terminal SAR domains and pinholins. For phages encoding SAR endolysins it has been shown that the holin protein needs only to produce lesions large enough to allow the passage of ions and depolarize the CM in order to fulfil its role in controlling the timing of lysis. Indeed, unlike holes formed by the λ holin, lesions formed by the phage 21 holin (S^{21}) do not allow the passage of the λ endolysin (Park *et al.*, 2007). The term 'pinholin' was thus proposed to differentiate the small-hole (pinhole)-forming character of S^{21} from the canonical holins that form large holes (Park *et al.*, 2007). Like the λ *S* gene, the *S*^{21} gene encodes two proteins, $S^{21}71$ and $S^{21}68$, due to alternative translational starts: the shorter product ($S^{21}68$) is required for lysis and the longer polypeptide ($S^{21}71$), like λ S_{107}, has an inhibitory character on $S^{21}68$, and thus is an antiholin (Barenboim *et al.*, 1999). Despite its small size (68 residues), $S^{21}68$ has two functional domains corresponding to the two predicted TMDs. TMD_1 is not only dispensable for hole-formation and lysis, but is in fact a SAR domain that must exit the bilayer so that TMD_2 is competent for hole formation. The departure of TMD_1 from the bilayer coincides with the lethal triggering of the holin and is accelerated by membrane depolarization (Park *et al.*, 2007). In addition, TMD_1 acts in *trans* as an inhibitor of the TMD_2 lethal function (Pang *et al.*, 2010). The structure of the prototype pinholin S^{21} was examined by negative-stain transmission electron-microscopy, cysteine-accessibility, and chemical cross-linking, as well as by

computational approaches. Together, the results suggested that the pinholin forms symmetric heptameric structures with the hydrophilic surface of one TMD lining the surface of a central channel of ~15 Å in diameter (Pang *et al.*, 2009). It was suggested that the S^{21}/R^{21} gene pair, encoding a pinholin and a SAR endolysin, might represent an intermediate stage in the evolution of holin-endolysin systems.

For both canonical holins and pinholins, hole formation occurs suddenly after a period of harmless accumulation in the membrane, during which membrane energization, macromolecular synthesis and virion assembly continues undisturbed. Triggering determines the timing of lysis, although the molecular strategies are completely different for both (Pang *et al.*, 2009).

Mycobacteriophage-mediated lysis: a new model of endolysin export

Mycobacteriophages are viruses that specifically infect mycobacterial hosts, bacteria that have a complex cell envelope (see above). At the time of this writing, more than 2500 mycobacteriophages have already been isolated, most of them having *Mycobacterium smegmatis* as host, with a total of 223 genome sequences available in GenBank (http://www.phagesdb.org). Based on gross nucleotide sequence similarity, these phages have been grouped into 36 clusters and subclusters (A–O) and eight singletons that have no close relatives (Hatfull, 2012a, b). To date, only mycobacteriophages with a double-stranded DNA genome have been described (Hatfull, 2010, 2012a; Hatfull *et al.*, 2010) and, like all dsDNA phages, they have to face the host cell barriers to release progeny virions at the end of a lytic cycle. Until the recent studies on phage Ms6 (Gil *et al.*, 2008; Catalão *et al.*, 2010), little was known about the mechanisms underlying mycobacteriophage-induced lysis of mycobacteria. The work of Garcia *et al.* (2002) reported for the first time the genetic organization of the lysis module of mycobacteriophage Ms6. The lysis cassette of Ms6 is composed of five genes (Fig. 3) that, in addition to encode a specialized function related to the particular nature of its host cell envelope, displays peculiar features that have not been described for other phages, thus deserving a detailed description.

In Ms6 lysis strategy, the access of the Ms6 endolysin (LysA) to the PG is also independent of the holin function (Catalão *et al.*, 2010). However, the Ms6 LysA is not endowed with a predicted Sec-type SP or SAR sequence, as happens with the secreted endolysins described above. Catalão *et al.* (2010) showed that the export of the Ms6 endolysin, encoded by gene *gp2* (*lysA*), is assisted by a chaperone-like protein encoded by the first gene (*gp1*) of the Ms6 lysis cassette. Several physical and predicted structural characteristics of Gp1 are consistent with those of chaperones. As described for chaperones, Ms6 Gp1 interacts with its effector, LysA, an interaction that was shown to encompass the *N*-terminal region of the chaperone and the first 60 amino acids of the Ms6 endolysin (Catalão *et al.*, 2010, 2011b). The requirement of Gp1 for LysA export is supported by experiments performed in *M. smegmatis*, where alkaline phosphatase activity of a PhoA-LysA hybrid protein decreased in the absence of Gp1 (Catalão *et al.*, 2010). The role of Gp1 in lysis was clearly demonstrated by Catalão *et al.* (2010). A deletion of *gp1* from the Ms6 genome revealed that, although not essential for plaque formation, Gp1 is necessary to achieve an efficient lysis, since its absence resulted in a decrease of ~70% in the burst size.

In common with the secreted endolysins described above, experiments in *E. coli* showed that the Sec system is involved in Gp1-assisted export of LysA to the cell wall (Catalão *et al.*, 2010). The authors have shown that lysis is prevented in *E. coli* cells expressing Gp1 and LysA where the Sec system is defective, either by the use of a mutant strain displaying pleiotropic defects in protein export or by addition of the SecA inhibitor sodium azide to cells carrying the wild-type *secA* locus. Thus, as suggested for the other secreted endolysins, holin function also seems to be confined to the regulation of lysis timing through endolysin activation. Indeed, the potential role of the Ms6 holin function in LysA activation was supported by the results of an experiment using nisin, a permeabilizing compound that triggers cytoplasmic membrane depolarization (Catalão *et al.*, 2010). In contrast to what happened with *M. smegmatis* cells expressing only Gp1 or LysA, addition of nisin to the cells expressing both proteins resulted in complete lysis. This result indicates that the endolysin was already positioned next to its target, the peptidoglycan, at the onset of lysis, since the pore diameter produced by nisin (2 nm) should not allow the passage of a protein as large as Ms6 endolysin (Catalão *et al.*, 2010). However, how Ms6 endolysin is kept inactive until holin triggering occurs is a question that remains to be elucidated.

Gp1 homologues have been identified in other mycobacteriophage genomes (Fig. 3), particularly in the lysis cassette of phages that belong to subcluster A1 and subcluster F1 (Hatfull *et al.*, 2010; Henry *et al.*, 2010a) and of phage Charlie, a member of the recently created subcluster N (Hatfull, 2012b; http://www.phagesdb.org/). One exception is *gp90* of phage TM4, from subcluster K1, which is positioned outside the lysis cassette (Hatfull, 2012a). Gp1 homologues seem to be missing in several mycobacteriophage genomes, suggesting that, in these phages, endolysin export occurs in a different way.

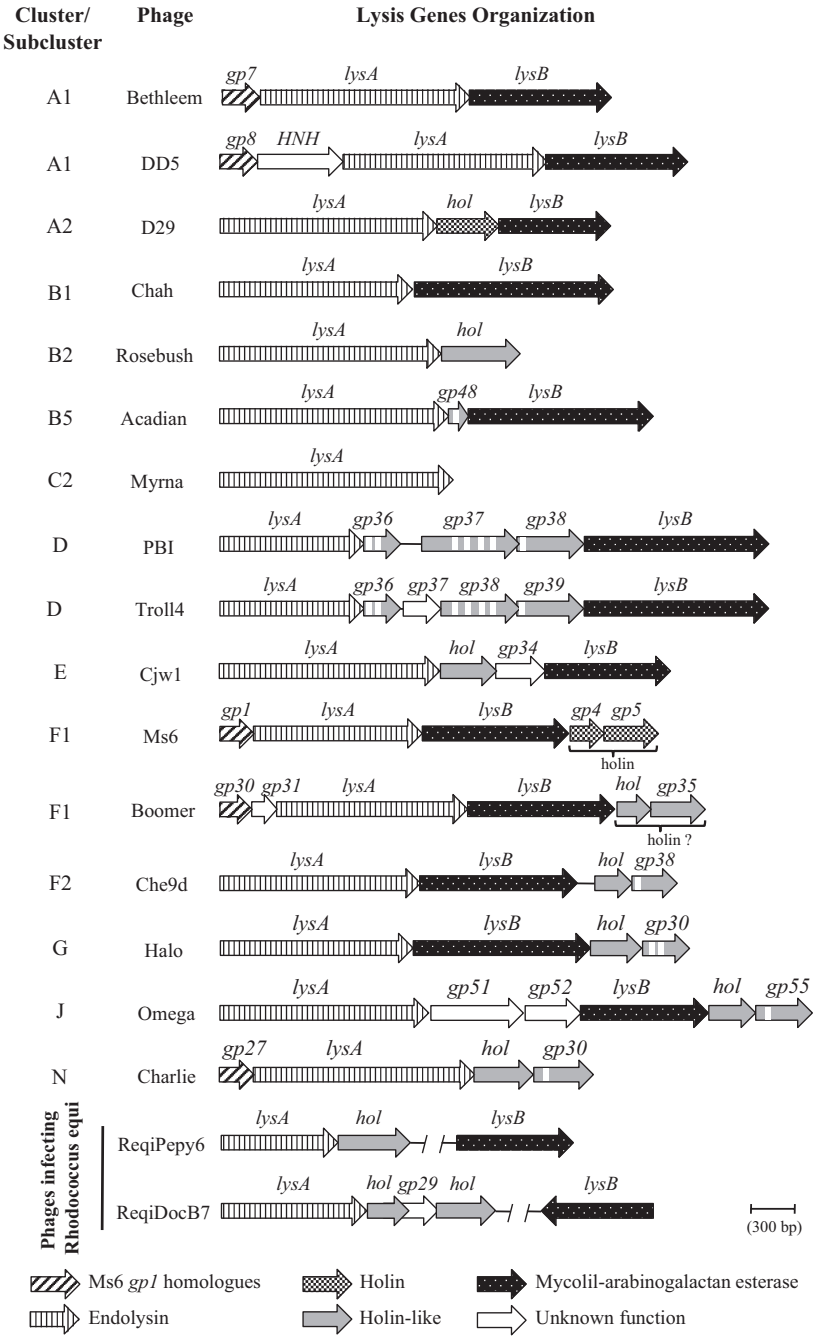

Fig. 3. Diversity of lysis cassettes from phages infecting members of the *Corynebacteriaceae* family. Illustrated are representatives of mycobacteriophages and phages infecting *Rhodococcus equi* with diverse genome organization. Holin-like genes not previously assigned, display white bars that represent the number and location of putative TMD coding sequences.

The Ms6 endolysin is an enzyme with *N*-acetylmuramoyl-L-alanine amidase activity, holding a central peptidoglycan recognition protein (PGRP) conserved domain, localized between amino acid residues 168 and 312 (Catalão *et al.*, 2011c). Interestingly, the *lysA* gene generates two products, designated $Lysin_{384}$ and $Lysin_{241}$ according to the size of the polypeptides produced. $Lysin_{241}$ is produced from a second translation initiation codon in the same reading frame of $Lysin_{384}$. Ms6 deleted of *lysA* is not viable, suggesting that this mutant completely fails to induce host cell lysis. However, Ms6 mutants producing only one of the forms of LysA were shown to be viable, albeit defective in the normal timing, progression and completion of host cell lysis. Lack of $Lysin_{384}$ resulted in a lysis delay of 30 min and in a reduction in the number of phage particles released, whereas in the absence of $Lysin_{241}$, lysis starts 90 min later with no significant effect on the number of phage particles released (Catalão *et al.*, 2011c). These results indicate that both proteins are necessary for complete and efficient lysis of *M. smegmatis*. Of

note is the fact that although $Lysin_{241}$ keeps the enzyme catalytic domain, the *N*-terminal region that interacts with Gp1 is absent. This indicates that translocation of this shorter version across the CM is not assisted by Gp1. At this time it is not known how $Lysin_{241}$ accesses the PG. One may speculate that in *M. smegmatis*, $Lysin_{241}$ may gain access to its target once the CM is disrupted by the Ms6 holin. A proposed model for *M. smegmatis* lysis achieved by Ms6 is represented in Fig. 2d.

A recent bioinformatic analysis of mycobacteriophages endolysins shows that they are highly diverse and modular in nature. Most contain three domains with a novel *N*-terminal predicted peptidase, a central catalytic domain that may specify an amidase, muramidase or transglycosylase activity, and a *C*-terminal region harbouring putative cell wall-binding motifs (Payne & Hatfull, 2012). Some endolysins contain glycosidase and amidase domains, such as Che9d Gp35 and Wildcat Gp49 (Payne & Hatfull, 2012). Peptidoglycan hydrolase activity has already been demonstrated for the endolysins of Ms6, TM4 and D29 (Payne *et al.*, 2009; Catalão *et al.*, 2010; Henry *et al.*, 2010b).

Ms6 regulation of mycobacteria lysis timing seems also to display peculiar features. Achievement of the correct lysis timing was shown to be dependent on the interaction and concerted action of two membrane proteins with holin-like features (Catalão *et al.*, 2011a), even though the presence of both is not absolutely required for *M. smegmatis* lysis. The gene product encoded by *gp4* was identified as the Ms6 holin, as it shares characteristics of class II holins and can complement an S^{λ}-defective mutant (Garcia *et al.*, 2002). A more recent characterization of Ms6 Gp4 function suggested that Gp4 might function as a pinholin, since the first TMD has characteristics of a SAR domain with a high percentage of weakly hydrophobic and uncharged polar residues (Catalão *et al.*, 2011a). Co-expression of Ms6 LysA and Gp4 in *E. coli* did not result in bacterial lysis (Garcia *et al.*, 2002). However, when the Ms6 *gp4* was changed by the mycobacteriophage D29 holin gene, the co-expression resulted in *E. coli* lysis. This is consistent with the idea that, in contrast to the D29 holin, Ms6 Gp4 forms pores too small to allow passage of the 43-kDa Ms6 endolysin. Noteworthy is the fact that *gp4* deletion from the Ms6 genome results in a viable phage but with an earlier timing of lysis, which is more consistent with antiholin function for Gp4 rather than holin (Catalão *et al.*, 2011a).

Although in the majority of mycobacteriophages sequenced so far, no holin function could be experimentally assigned to any gene (Hatfull, 2012a), in Ms6 an additional lysis gene (*gp5*), encoding a holin-like protein with one predicted TMD, was found to be positioned immediately downstream of the *gp4* gene (Garcia *et al.*, 2002; Catalão *et al.*, 2011a). Deletion of *gp5* from the Ms6 genome resulted in a viable phage showing a delayed time of lysis, confirming that Gp5 has a regulatory role in the timing of lysis. Surprisingly, and contrary to Gp4, Gp5 did not complement an S^{λ}-defective mutant. Taking these results as a whole it was suggested that the combined action of Gp4 and Gp5 could play the functional role of a holin (Catalão *et al.*, 2011a), similarly to what was proposed for *xhlA* and *xhlB* gene products of *Bacillus subtilis* prophage PBSX (see below). Expression of both proteins is necessary to effect a host cell lysis at the correct timing. It was proposed that interaction of Gp5 with Gp4 contributes to the precise adjustment of the timing of hole formation (Catalão *et al.*, 2011a).

Homologues of Ms6 Gp5 were found in other mycobacteriophages, with the highest identity within subcluster F1 (Fig. 3). It is known that holin proteins are highly diverse (Wang *et al.*, 2000) and do not share high sequence identity, and thus it is not easy to identify holin genes in phage genomes unless they are in the close vicinity of endolysin genes. Among the 223 annotated mycobacteriophage genomes, few have annotated holin genes; however, many genes encoding putative proteins with predicted TMDs can be identified close to endolysin genes (Fig. 3), suggesting that they may function as holins. Whenever a holin gene has been assigned, including in Ms6, it is closely linked and downstream to *lysA*.

It is now obvious that mycobacteriophage lysis cassettes are also highly diverse and the few representatives indicated in Fig. 3 clearly show it. Of note is the fact that the lysis module of Ms6, and of the majority of mycobacteriophages already sequenced, presents a gene, *lysB*, which is exclusive of phages infecting bacteria with similar cell envelopes, as in the case of the mycolata group, which includes mycobacteria and rhodococci. The product of *lysB* was shown to target the mycobacteria outer membrane, the last barrier to bacteriophage release (see below). With this exciting finding in mind it is becoming clear that to overcome the disadvantage that a complex cell envelope may represent for a successful phage infection cycle, mycobacteriophages have evolved new lytic systems through the acquisition of specific genes that likely confer a substantial selective advantage contributing to an optimal lysis.

The last barrier to virion release: targeting of the outer membrane by accessory lysis proteins

Phage holins and endolysins have been regarded for long as the only essential functions to accomplish lysis of bacterial host cells. Although this might be true for most laboratory conditions, the fact is that, through evolution,

phages seem to have preserved accessory lysis functions that probably have a more pronounced role in the environmental conditions found in nature. Recent studies have not only reinforced the importance of known auxiliary lytic functions but have also uncovered new lysis players in phages that need to cope with complex bacterial cell envelopes such as that of mycobacteria. A detailed description of examples of these ancillary lysis functions is provided next.

Spanins

Rz and *Rz1* are the two last genes of the phage λ lysis cassette. *Rz* encodes a type II integral membrane protein, with an *N*-terminal TDM and a periplasmic *C*-terminus (Summer *et al.*, 2007; Berry *et al.*, 2008). The Rz1 coding region is embedded entirely within *Rz* in the +1 reading frame and encodes a small proline-rich outer membrane lipoprotein having a signal peptidase II cleavage site (Hanych *et al.*, 1993; Kedzierska *et al.*, 1996; Summer *et al.*, 2007).

For many years the function of λ Rz and Rz1 proteins was unclear. The involvement of Rz/Rz1 in host lysis was restricted to the observation that, under laboratory conditions, in the presence of millimolar concentrations of divalent cations, both proteins are necessary to achieve lysis. Nonsense mutations in either Rz or Rz1 block lysis, leading to metastable spherical cell forms (Young *et al.*, 1979; Zhang & Young, 1999). The precise role of these proteins in host cell lysis was recently elucidated. Berry *et al.* (2008) demonstrated that, at physiological levels of expression, Rz and Rz1 are localized in the inner (IM) and outer membrane (OM), respectively, with their *C*-terminal domains predicted to lie in the periplasm. Recently, it was demonstrated that these two proteins interact and form a complex (Berry *et al.*, 2008, 2010) which was proposed to be involved in a third and final step of host lysis. The first step is the temporally programmed permeabilization of the cytoplasmic membrane by the holin, resulting in the release of a cytoplasmic endolysin to the periplasm or in the activation of a SAR endolysin already positioned in this cell compartment. The second stage is the degradation of the cell wall PG by the endolysin. These two stages are followed by a third stage involving the fusion of the IM and OM mediated by the Rz/Rz1 complexes. These three steps will eliminate all conceivable barriers to the release of the progeny virions (Berry *et al.*, 2008). It was suggested that to facilitate the fusion of the two membranes, Rz/Rz1 complexes may undergo conformational changes (Jahn & Scheller, 2006).

A bioinformatic search identified *Rz/Rz1* equivalents in nearly all genomes from phages infecting Gram-negative bacteria (Summer *et al.*, 2007). The variety of *Rz/Rz1* equivalents was enormous, with 37 unrelated gene families grouped in accordance with gene arrangement. *Rz/Rz1* gene pairs of the λ group share the embedded gene architecture (eight families); the P2 group includes 23 families in which *Rz1* extends beyond *Rz* (overlapped structure); the third group includes six families with Rz/Rz1 homologues encoded in non-overlapping genes, as exemplified by the T4 *pseT.3* and *pseT.2* genes, which encode proteins unrelated to all other Rz/Rz1 equivalents but possess the *N*-terminal TMD signature and the signal sequence of outer membrane lipoproteins, respectively (Summer *et al.*, 2007). In this study, functional homologues of Rz/Rz1 encoded by a single gene were also identified in seven phages, as exemplified by the *gp11* of coliphage T1. This new class of proteins, named spanins, are predicted outer membrane lipoproteins that also possess a predicted *C*-terminal TMD and thus will span the entire periplasm, providing a physical link between the inner and outer membranes.

The fact that genes for Rz/Rz1 equivalents are found in nearly all phages of Gram-negative hosts indicates that in the osmotic and ionic conditions found in nature, the OM is a significant barrier to virion progeny release, even after holin–endolysin mediated destruction of the cell wall. Therefore, in nature they should confer an advantage that is not apparent under laboratory conditions (Summer *et al.*, 2007). Rz and Rz1 equivalents were also identified in the tailless, membrane-containing dsDNA virus PRD1 (*Tectiviridae*) that infects a variety of Gram-negative hosts. Possibly Rz and Rz1 interact not only with each other but also as a complex with the holin, transmitting the mechanical stress of the holin-mediated lesion in the cytoplasmic membrane to the outer membrane, which results in its disintegration (Krupovič *et al.*, 2008).

While the three steps of phage lysis mediated by holins, endolysins and Rz/Rz1 complexes form a sequential pathway in which holin function is required for endolysin function, which in turn is required for Rz/Rz1 function, they are mechanistically independent (i.e. do not require heterotypic interactions with each other). This likely accounts for the remarkable diversity and mosaicism found in phage lysis cassettes, which are composed of many unrelated families of holins and Rz/Rz1 proteins, and at least three types of endolysins (Wang *et al.*, 2000; Young, 2002; Young & Wang, 2006; Summer *et al.*, 2007).

Mycobacteriophage LysB

As already mentioned, the Ms6 lysis cassette contains a particular gene, designated *lysB*, encoding a protein whose function is related to the particular composition of the mycobacteria cell envelope. The Ms6 LysB amino acid

sequence contains the pentapeptide G-X-S-X-G characteristic of lipolytic enzymes. Biochemical studies have shown that Ms6 LysB has activity on both esterase and lipase substrates, showing significant preference for long chain substrates (Gil *et al.*, 2008), a characteristic that is in agreement with the high lipid content of the mycobacteria cell envelope. The crystal structure of D29 LysB revealed structural similarities to members of the cutinase family of lipolytic enzymes (Payne *et al.*, 2009). The major target for LysB activity was identified as being the mycolyl-arabinogalactan-peptidoglycan complex (mAGP), hydrolyzing the ester bonds between the mycolic acids and the arabinogalactan (Payne *et al.*, 2009; Gil *et al.*, 2010). In addition to a mycolyl-arabinogalactan esterase activity, Ms6 LysB was also shown to hydrolyse other mycobacterial lipid components of the cell envelope, particularly trehalose dimycolate (TDM, a glycolipid involved in the virulence of pathogenic species) of *M. smegmatis*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Ra (Gil *et al.*, 2010). Nevertheless, due to the importance of the mAGP-complex for the stability of the mycobacteria cell envelope it seems reasonable to consider that the cleavage of the mycobacterial outer membrane from the peptidoglycan-arabinogalactan layer is the primary role of LysB.

Deletion of *lysB* from the Ms6 (Gil, 2012) and Giles (Payne *et al.*, 2009) genomes have shown that LysB is not essential for lysis; however, in Giles this results in a 30-min lysis delay and in a reduction of plaque size (Payne *et al.*, 2009).

Mycobacteria are considered to be Gram-positive bacteria; however, the existence of the mycobacterial outer membrane composed of mycolic acids and free lipids presents a barrier analogous to the OM of Gram-negative bacteria (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). Analogies can be made between the function of LysB against the mycobacterial OM and the function of the λ Rz/Rz1 homologues or spanins in Gram-negative hosts. Despite their different mode of action, where spanins promote the fusion of the inner and outer membranes and LysB breaks the linkage between the OM and the cell wall, they both mediate the final step in host lysis by eliminating the last barrier to the release of progeny virions.

Until recently the LysB-like proteins were found exclusively in phages infecting mycobacteria, but Summer *et al.* (2011) identified LysB homologues in phages infecting *Rhodococcus equi*. One of the features that *Rhodococcus* shares with mycobacteria is the presence of an outer layer composed of mycolic acids covalently linked to the PG (Sutcliffe, 1998). This layer presents an additional challenge to phages infecting the mycolata group and thus it can be anticipated that to overcome this barrier, phages infecting other members of the *Corynebacterineae* suborder, including *Rhodococcus*, would also encode LysB equivalents (Summer *et al.*, 2011). The importance of LysB is illustrated by its presence in almost all mycobacteriophage genomes sequenced so far (Hatfull *et al.*, 2010; Henry *et al.*, 2010a), indicating that the mycolyl arabinogalactan layer is a significant barrier to virion release even after the endolysin-mediated destruction of the cell wall. Thus, it is proposed that LysB activity, together with LysA acting on the PG results in a more efficient breakage of the cell barriers, allowing a robust dissemination of the new phage particles (Gil *et al.*, 2010). Exceptions are Che12, Subcluster B2 phages, and the C2 phage Myrna (Fig. 3) that lack a LysB-like protein (Hatfull, 2012a, b). A hypothesis suggested is that these phages have evolved a mechanism for utilizing a host-encoded cutinase-like enzyme for this function (Hatfull, 2010). Interestingly, in mycobacteriophages the *lysB* genes are positioned close to the *lysA* genes, whereas in *Rhodococcus equi* phages ReqiPepy6 and ReqiDocB7 they are not linked to the lysis genes. Moreover, in the latter, *lysB* gene is transcribed in opposite direction (Summer *et al.*, 2011) (Fig. 3).

At this time it is unknown how Ms6 LysB reaches its substrate, since no signal sequences allowing its transport across the cell barriers have been identified (Gil *et al.*, 2008). One can hypothesize that LysB might reach the OM simply by diffusion after PG breakdown by LysA.

Genome organization and regulation of lysis players

The vast majority of dsDNA phages carry the lysis genes clustered in the viral genome and frequently contiguous holin and endolysin genes are co-transcribed as part of the late expressed genes (São-José *et al.*, 2003). In fact, when an *orf* adjacent to a readily identifiable endolysin gene codes for a small (<150 aa) putative product with the potential to form at least one TMD, it is frequently assigned as the holin gene (Wang *et al.*, 2000). As described above, in lambdoid phages such as λ, 21 and P22, a dual-start motif enables holin and antiholin functions to be encoded in a single gene lying immediately upstream of that of the endolysin (Young & Wang, 2006). In these phages, the ancillary lysis genes *Rz* and *Rz1* encoding the spanin components also cluster with the antiholin/holin-endolysin genes, being located immediately downstream of the endolysin (Summer *et al.*, 2007).

Several examples of subtle or clear deviations from this spatial and temporal gene organization are found in both Gram-negative and Gram-positive systems (reviewed in São-José *et al.*, 2003). Differences can involve rearrangements of the gene synteny described for lambdoid phages, with some cases of divergently transcribed genes, the non-clustering of lysis genes and even different timings of their expression.

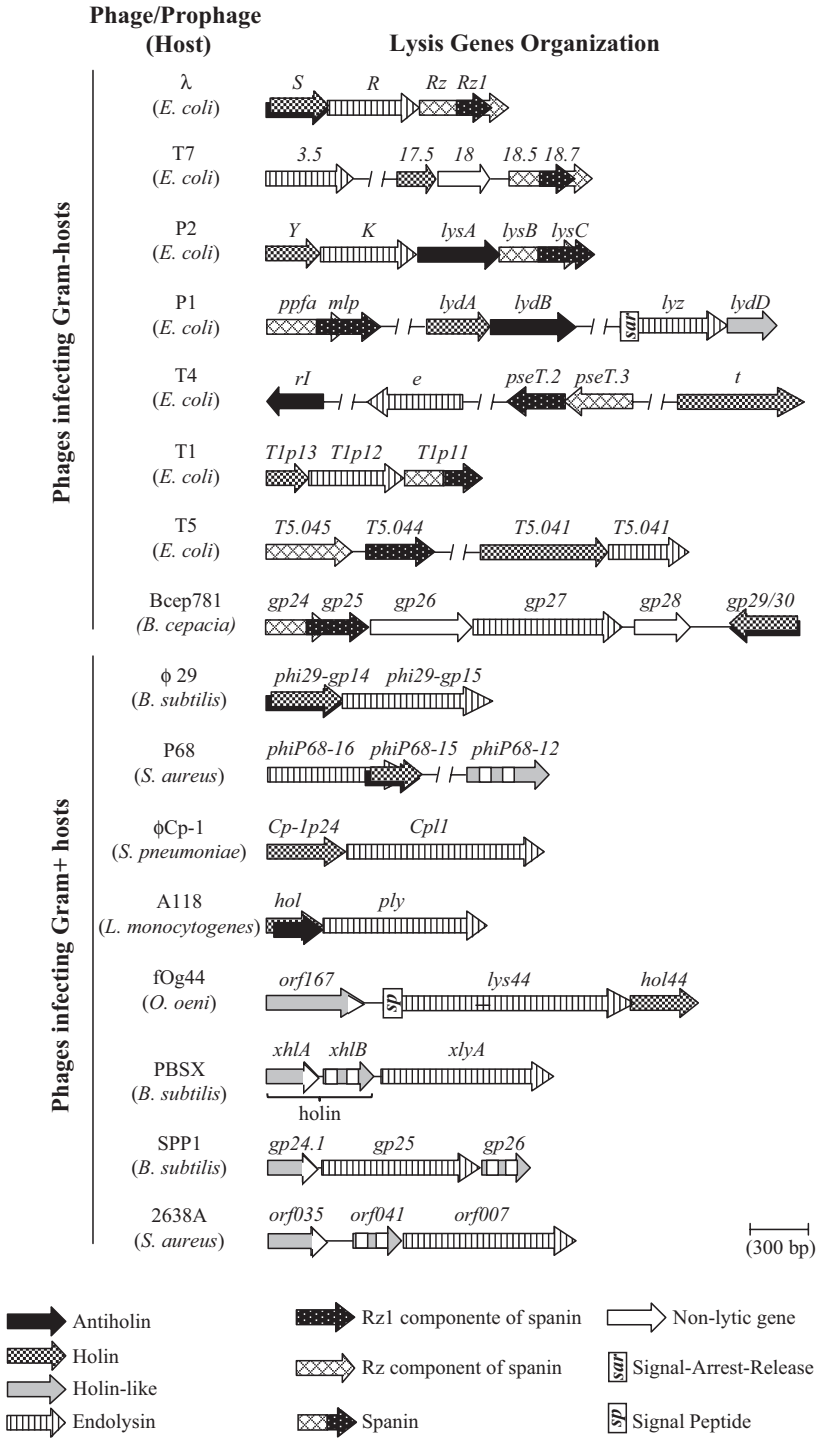

Fig. 4. Diversity of lysis genes and their genome arrangement in selected examples of phages infecting Gram-negative and Gram-positive bacteria. The white segments in holin-like genes of phages from Gram-positive indicate the number and position of putative TMD coding sequences.

Complete enumeration and description of such deviations is beyond the scope of this review but Fig. 4 provides a few illustrative examples that include well known phages or lysis mechanisms, except the case of phage 2638A, which is shown just to illustrate a lysis gene arrangement commonly found in *Staphylococcus aureus* phages (our analysis).

Frequently, and particularly in Gram-positive systems, two putative genes encoding products with holin features are found in the close vicinity of the endolysin gene (holin-like genes in Fig. 4). Inspired by the lambdoid lysis mechanism, some authors have speculated that these genes might correspond to the antiholin and holin func-

tions (Sheehan *et al.*, 1999). There are, however, some examples where such a pair of holin-like proteins seems in fact to form the actual holin 'functional unit', as mentioned above for Gp4 and Gp5 of mycobacteriophage Ms6 and XhlA and XhlB of *B. subtilis* phage PBSX (Figs 3 and 4). Krogh *et al.* (1998) studied the role of these PBSX holin-like products in host cell lysis by expressing them in *B. subtilis*, in different combinations with the PBSX endolysin XlyA, under the control of native transcription signals. In contrast to what happens with phage λ, the study revealed that neither XhlA nor XhlB in pairwise combinations with the endolysin could trigger host cell lysis efficiently. This could only be achieved, in a typical saltatory fashion, when XhlA and XhlB were co-expressed with XlyA. The authors proposed that XhlA and XhlB would associate in the membrane to form the functional holin that allows endolysin release.

From the examples of lysis mechanisms discussed in the previous sections we can easily anticipate that temporal and efficiency regulation of phage-mediated bacterial lysis can occur at the transcription, translation and post-translation levels. In most phages, lytic functions start to be expressed late in the phage development cycle, since lysis genes lay within late transcribed operons, which generally also encompass genes involved in virus particle morphogenesis (Young, 1992; Parreira *et al.*, 1999; Altermann & Henrich, 2003; Ventura & Brüssow, 2004; Duplessis *et al.*, 2005). Tight control of lysis genes transcription may depend on the synthesis of phage-encoded activators of late promoters and/or of anti-termination functions (Roberts, 1975; Luk & Szybalski, 1983; Young, 1992; Alonso *et al.*, 1997; Garcia *et al.*, 2002; Pedersen *et al.*, 2006; Datta *et al.*, 2007). A stem-loop structure overlapping the Shine-Dalgarno sequence in the mRNA of the phage λ S gene reduces the frequency of translation initiation at the S_{107} start codon. As described above, this example of translation control imposes that S_{107} and S_{105} are synthesized in an approximate molar ratio of 1 : 2 (Bläsi & Young, 1996; Wang *et al.*, 2000).

The paradigm of post-translation regulation in phage-induced host cell lysis is provided by the antiholin function that controls holin activity. In fact, it has been argued that all phages employing a holin-endolysin system would also need an antiholin function to ensure the control of holin activity in response to physiological cues (Young & Bläsi, 1995). This is clearly patent in the classic lysis inhibition (LIN) phenomenon of phage T4 (Doermann, 1948), where the T4 antiholin function has been shown to impose lysis postponing in response to superinfecting phages, by blocking holin-mediated lysis (Ramanculov & Young, 2001a). The biological significance of this is that, by delaying lysis, T4 'chooses' to maximize virion production within the infected cell, since the incoming of superinfecting DNA is a signal indicating a reduced number of host cells available in the medium for subsequent rounds of infection. The T4 holin T (Lu & Henning, 1992; Dressman & Drake, 1999) has a particular membrane topology, since it features a large *C*-terminal periplasmic domain (CTPD) that is responsible for its relatively large size when compared with other holins (Ramanculov & Young, 2001b). The T4 antiholin, encoded by the *rI* gene, exerts its function by also featuring a CTPD that interacts with that of T (Ramanculov & Young, 2001a; Tran *et al.*, 2005). The molecular details of RI and T interaction have recently been elucidated (Moussa *et al.*, 2012).

Another level of post-translation control is common to all known lysis systems employing secreted endolysins (see above). In these systems the endolysin is targeted to the extracytoplasmatic cell compartment, via the Sec system, during intracellular phage development. Therefore, a mechanism restraining endolysin activity must be operating to prevent premature host cell lysis. To date, this mechanism has only been clearly elucidated for some SAR endolysins (see above). Indirect experimental data strongly suggest that activation of secreted endolysins with a typical, cleavable SP sequence or of those whose secretion is chaperone-assisted, such as the enzymes Lys44 and LysA of phages fOg44 and Ms6, respectively (see above), is also dependent on holin trigger. In this case, however, the mechanism restraining endolysin activity is unknown, although it seems to be intimately linked to the cytoplasmic membrane pmf (Nascimento *et al.*, 2008; Catalão *et al.*, 2010). It has been speculated that these secreted endolysins are maintained inactive in the cell wall, until the holin trigger, by the same mechanisms that regulate the activity of bacterial host autolysins (São-José *et al.*, 2000; Nascimento *et al.*, 2008). In fact, by collapsing the pmf, the holins may not only activate the cell wall-positioned endolysins but also the host autolysins, as observed in pneumococcal systems (Frias *et al.*, 2009).

Concluding remarks

During the past years it has become evident that during evolution, bacteriophages have developed a variety of lysis strategies beyond the most well known prototype λ holin-endolysin lysis system. The recently discovered phage lysis models include secretory endolysins endowed with Sec-type SP or SAR sequences and a new class of holins, the pinholins, encoded by phages with SAR endolysins, whose function is confined to membrane depolarization which triggers endolysin activation. A particularly remarkable example of such diversity is given by mycobacteriophages, which specifically target mycobacterial hosts possessing an

extremely complex cell envelope. These viruses have evolved specific lytic functions by acquiring additional and specific lysis genes that confer a selective advantage by allowing efficient degradation of all cell barriers that mycobacteriophages have to face, thus contributing to a successful lysis. This genetic diversity provides bacteriophages with an obvious evolutionary benefit, allowing adaptation to different environmental conditions and to a particular host, and opens new perspectives to the exploitation of undiscovered bacterial lysis pathways. This could bring new tools for the battle against bacterial diseases such as the production of recombinant antibacterial peptides based on the knowledge of key genomic regions such as the lytic cassette, as well as revealing interesting biological features of bacterial hosts.

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Authors' contribution

The authors M.P. and C.S.-J. have contributed equally to this manuscript.

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