Antibiotics are the molecules of choice to treat bacterial infections. However, because of the rapid emergence of drug-resistant bacteria, alternative modes of combating infections are being envisaged. Bacteriophages, which infect and lyse bacterial cells, may function as effective antimicrobial agents. Most bacteriophages produce their own peptidoglycan hydrolase called endolysin or lysin, which breaks down the cell wall of bacteria and aids in the release of newly assembled virions. Here, we discuss several findings that help us in understanding how endolysins are regulated. We observe that there is no common mechanism that is followed in all cases. Many different modes of activity regulation have been observed in endolysins, including regulation of protein expression, translocation across the cell membrane and post-translational modifications. These processes not only demonstrate how endolysins are made dependent on other accessory proteins and non-protein factors for their synthesis, translocation across the cytoplasmic membrane and activity, but also show how autoregulation helps in maintaining the enzyme in an inactive form. Various regulatory mechanisms that are discussed are particularly applicable to endolysins. Nevertheless, a detailed study of these methods opens new avenues of investigation in the area of protein translocation systems and the novel ways of enzyme activation and regulation in bacteria.

INTRODUCTION

Bacteriophages, since their discovery, have always fascinated microbiologists, biochemists, biophysicists and geneticists for the peculiar characteristics that they display (Duckworth, 1976; Wittebole et al., 2014). Bacteriophages are able to infect and lyse bacterial cells, and it is this feature that makes them important antimicrobial agents (Sulakvelidze et al., 2001; Hermoso et al., 2007), especially because of the rapid emergence of multidrug-resistant bacteria (Loeffler et al., 2001; Schuch et al., 2002; O’Flaherty et al., 2009; Fischetti, 2010). Bacteriophages use two independent mechanisms to lyse host cells. Whereas some ssDNA and RNA viruses use a peptidoglycan synthesis inhibitor (Young et al., 2000; Zheng et al., 2009; Tanaka & Clemons, 2012), all of the dsDNA viruses follow a lysin-mediated breakdown of peptidoglycan (Young et al., 2000; Young, 2014; Lood et al., 2015). Endolysin is a peptidoglycan-hydrolysing enzyme that carries out enzymic digestion of the cell wall peptidoglycan at the end of the phage infection cycle, which ensures the release of newly packed phage particles.

Endolysins produced by bacteriophages infecting Gram-positive and Gram-negative bacteria differ from each other. Phages infecting Gram-negative bacteria (G− phages) produce endolysins that are mostly uni-domain in nature, with a few exceptions (Zhang & Studier, 2004; Lukacik et al., 2012; Walmagh et al., 2012). However, endolysins from phages infecting Gram-positive bacteria (G+ phage) are modular in nature (Sudiarta et al., 2010). The G+ phage endolysin consists of one or two domains at the N terminus and a C-terminal cell-wall-binding domain (CBD) (Loessner, 2005). Depending on the requirement of the CBD for enzyme activity, endolysins can be further classified as CBD-dependent and CBD-independent (Porter et al., 2007; Low et al., 2011). Endolysin is produced in the cytoplasm and is translocated to the periplasmic space where it acts upon the cell wall peptidoglycan. In this review, we summarize several findings that have demonstrated how the activity of endolysin is regulated in various systems. The processes by which endolysin activity is regulated include endolysin synthesis, export, activation and substrate specificity, and are depicted in Fig. 1.

REGULATION OF ENDOLYSIN EXPRESSION

The bacteriophage endolysin is required at the final stage of the phage infection cycle. Therefore, it is expected that expression of endolysin occurs only towards the end of the infection cycle. Interestingly, several studies have shown that transcription of the endolysin gene occurs at
The early stage of phage infection. A Northern blotting experiment with temperate *Streptococcus thermophilus* phage Sfi21 suggested that the transcription of both holin and endolysin occurs at the early stage of the lytic cycle. Additionally, the amount of transcript increases progressively, and it is maintained until the last stage of the cycle (Ventura *et al.*, 2002). In one of the most characterized lytic phages of *Escherichia coli*, the T4 phage, DNA microarray data showed that the transcription of lytic genes starts at the early stage, but the proteins are synthesized only at the late stage (Luke *et al.*, 2002); suppression of mRNA translation is achieved by means of a stable hairpin structure adopted by endolysin mRNA that inhibits translation (McPheeters *et al.*, 1986). It is, however, not clear why translation of mRNA is suppressed as the endolysin, even if synthesized early, will accumulate in the cytoplasm and will remain physically separated from the substrate (peptidoglycan).

The T7 phage, another well-studied bacteriophage of *E. coli*, shows an early transcription of its endolysin gene coding for T7 lysozyme and holin (an integral membrane protein required for membrane perforation and passive diffusion of endolysin into the periplasmic space; discussed later). In T7, the mRNA for both lysozyme and holin could be observed just after 3 min of infection (Nguyen & Kang, 2014). DNA microarray studies carried out with the Gram-positive *Streptococcus thermophilus* phages cos-type DT1 and pac-type 2972 also revealed that their endolysin genes show a gradual increase in transcript levels from early stage and reach a maximum at late stage (Duplessis *et al.*, 2005). Similarly, RNA sequencing studies of the mycobacteriophage Giles demonstrated that the transcription of lytic genes started at an early stage of infection, although it reached its highest levels at the late stage of infection (Dedrick *et al.*, 2013). Early transcription clearly suggests that endolysin can be transcribed by the host RNA polymerase, preferably using the housekeeping sigma factor. Indeed, in order to identify the promoter for the endolysin gene of mycobacteriophage Ms6, the desired region was fused to the *lacZ* reporter. It was then demonstrated that *lacZ* was expressed from two $\sigma^{70}$-like promoters, providing evidence that transcription of endolysin genes can be carried out by host transcription factors (Garcia *et al.*, 2002). It would be of immense interest to explore the involvement of various transcription factors in the expression of endolysin genes. For example, in *Thermus thermophilus* HB8, it has been shown that gp39 and gp76 of bacteriophage P23-45 can recognize host RNA polymerase and regulate phage gene transcription (Berdygulova *et al.*, 2011).

**REGULATION DURING TRANSLOCATION**

The site of action of endolysin is the peptidoglycan layer of the bacterial cell wall. In order to reach the target, endolysin must travel from the cytoplasm to the periplasm. To accomplish this, endolysin uses different methods. One of the most common and well-studied mechanisms involves holin, and forms what is known as the holin–endolysin cell lysis system. Holin is a small transmembrane protein that possesses one to three transmembrane regions (Bläsi & Young, 1996; Young, 2002; Reddy & Saier, 2013; Savva *et al.*, 2014). Upon oligomerization at a specific time, holin forms holes in the cytoplasmic membrane of bacteria and allows the passive diffusion of endolysin from the cytoplasm to the periplasm, thus triggering the cell lysis event (Park...
However, the discovery of holin-independent export of endolysin in many bacteriophages suggested that the holin–endolysin system may not be the only method of choice. In several bacteriophages, the endolysin is produced with an N-terminally located signal-arrest-release (SAR) sequence that is responsible for the transport of endolysin. Therefore, in these bacteriophages, endolysin is secreted with the help of the SAR sequence. The first secretory endolysin Lys44 was reported from Oenococcus oeni phage fOg44 (São-Jose et al., 2000). Upon expression in E. coli cells, Lys44 showed two bands on SDS-PAGE with apparent molecular masses of 51 and 43 kDa, belonging to the primary and the mature forms, respectively. Upon expression, endolysin crosses the cytoplasmic membrane and, with the help of the proteolytic activity of periplasmic protease LepB, is released into the periplasm (São-Jose et al., 2000). Furthermore, the work also showed that processing of endolysin is necessary for its lytic activity. Although the processing of endolysin was observed in many other bacteriophages, there are some exceptions wherein the SAR sequence is not cleaved by the periplasmic protease. E. coli bacteriophage P1 lysin, for example, showed the presence of N-terminal SAR sequence when its periplasmic and cytoplasmic fractions were tested on SDS-PAGE, suggesting that the SAR sequence remains attached to the endolysin even after export (Xu et al., 2004). Similar observations were made for Erwinia amylovora phage ERA103 and other bacteriophages (Kuty et al., 2010; Briers et al., 2011).

Recently, two reports showed very different modes of translocation of endolysin to the periplasmic space. The pneumococcal phage SV1 lysin Svl was found to contain no signal sequence at its N terminus, could be transported to the periplasmic space in a holin-deficient phage and carried out holin-independent killing (Frias et al., 2013). Interestingly, the choline-containing teichoic acid was found to be responsible for endolysin transport. Svl was not translocated to the periplasmic space when the culture was grown in ethanolamine-containing medium, which is the blocking agent for the loading of teichoic acid on the choline precursor in the cytoplasm.

Another interesting mode of transport of endolysin was shown in mycobacteriophage Ms6 lysin. The deletion of holin in mycobacteriophage Ms6 did not result in the loss of viability of the phage. This suggested that Ms6 lysin could translocate in a holin-independent manner (Catalão et al., 2010). Expression of Ms6 lysin along with Gp1 showed a lysis phenotype. Interestingly, Gp1 does not belong to the holin class of proteins. Instead, biophysical analysis of Gp1 suggested that it belongs to the chaperones of the type III secretion system. It was suggested that the N-terminal region of Gp1 physically interacts with lysin and transports it using the SecA-dependent pathway (Catalão et al., 2011b). Mycobacteriophages L5, D29 and Kostya have also shown holin-independent killing of mycobacteria (Payne & Hatfull, 2012; Pohane et al., 2014). However, the mechanism of transport of endolysin in these cases is yet to be elucidated. Nevertheless, these studies suggest that, apart from the canonical mode of transport through holin, bacteriophages also use other strategies for the translocation of endolysins.

**REGULATION BY INACTIVATION**

It is interesting to note that although endolysin in several bacteriophages can be translocated in a holin-independent manner, many of these phages do produce holin during their infection cycle. So, why is holin required, if endolysin can be translocated without it? Several studies have shown that endolysin remains in an inactive form after localization to the periplasmic space. Various processes then either activate the inactive endolysin or enhance its catalytic activity for the rapid lysis of the host cells. One of the most common modes of activation of endolysin in the periplasmic space is the depolarization of the cytoplasmic membrane that occurs by the activity of holin or pinholin (Young, 2002). Pinholins, like holins, form holes in the cytoplasmic membrane of the host. However, unlike holins, the holes formed by pinholins are not of sufficient size to allow for the diffusion of endolysin, and yet are large enough to cause leakage of protons leading to the disruption of proton motive force, thus resulting in the depolarization of the membrane (Park et al., 2007; Pang et al., 2010, 2013). As mentioned earlier, the O. oeni phage endolysin Lys44 is transported by a cleavable SAR signal sequence (São-Jose et al., 2000). When the purified endolysin from phage fOg44 was added externally to O. oeni culture, the protein was unable to carry out lysis of the bacterial cells. However, addition of a membrane-disrupting agent such as nisin along with the endolysin resulted in a rapid decline in the culture optical density (Nascimento et al., 2008). Furthermore, when endolysin was expressed within the cells, lysis occurred only when nisin was supplied externally (Nascimento et al., 2008). Although the study verifies the requirement of the disruption of proton motive force in endolysin activation, a direct involvement of fOg44 holin in the phage’s endolysin activation has currently not been examined. In another report, the expression of Pseudomonas aeruginosa phage φKMV endolysin resulted in the lysis of E. coli 60–90 min after induction, whereas the co-expression of endolysin and holin led to bacterial cell lysis within 15 min of induction (Briers et al., 2011). So far, we have noted that phage-encoded endolysin, upon activation due to the disruption of the proton motive force, causes cell wall peptidoglycan degradation resulting in phage release. However, interestingly, bacteriophages have also been shown to manipulate bacteria-encoded autolysins with the help of holin for their own benefit. Autolysins are the bacteria-encoded peptidoglycan hydrolases that are required for cellular processes such as cell growth, cell division and pathogenesis, amongst others (Shockman et al., 1996; Smith et al., 1996). In the case of phage SV1 of Streptococcus pneumoniae, the autolysin LytA was able to lyse the
host cells after induction with holin and, consequently, SV1 phage was shown to form plaques in the absence of its endolysin. This observation underlines the importance of LytA for efficient release of phage particles (Frias et al., 2009). A similar property of autolysin induction by phage was previously reported in the case of Micrococcus lysodeikticus infected with phage N1 (Goeppert & Naylor, 1967). When the holin of pneumococcal bacteriophage EJ-1 was expressed in E. coli, it also activated LytA autolysin and caused cell death (Diaz et al., 1996). These experiments suggest that bacterial cell death after phage infection, occurring due to interference in cell wall biosynthesis or cell wall disruption, requires depolarization of the cell membrane (Bayles, 2000, 2003; Martinez-Cuesta et al., 2000; Clementi et al., 2012; Sadykov & Bayles, 2012).

A very interesting mechanism of regulation of endolysin was identified in bacteriophage P1. The P1 endolysin is transported by the SAR domain sequence present at the N terminus (Xu et al., 2005). The active site of P1 endolysin consists of Glu42, Thr57 and Cys51, and the regulation of the activity is achieved by the involvement of two other cysteines, Cys44 and Cys13. By carrying out chemical cleavage using cyanlated cysteines and ammonium hydroxide, P1 endolysin was found to have two isoforms: one in which Cys13 had a free thiol group and the other in which Cys51 had a reduced thiol group (Cys-SH). Based on cell lysis and X-ray crystallography data, it was suggested that the formation of a disulphide bond between Cys44 and Cys51 results in the inactivation of endolysin, whereas a disulphide bond between Cys13 and Cys44 activates the protein (Xu et al., 2005). A similar mechanism of regulation was observed in the case of Erwinia phage ERA103 endolysin (Kutty et al., 2010). Here, the N-terminal catalytic domain of endolysin contains three Cys residues, i.e. Cys12, Cys42 and Cys45. Cys12 is present in the SAR domain of ERA103 endolysin. A Cys12Ser mutation results in a catalytically inactive protein. Genetic and biochemical studies suggested that a disulphide bond between Cys42 and Cys45 forms a cage that traps the catalytic Glu43 residue and thus blocks the enzyme activity. Shifting of the disulphide bond from Cys42–Cys45 to Cys42–Cys12 leads to removal of the block, thus making the enzyme catalytically active.

Such endolysin activity regulation by disulphide bond isomerization to trap a catalytic residue is an interesting way of inhibiting enzyme activity in SAR endolysins. However, not all endolysins contain cysteine residues in their SAR domain. How do these endolysins block their active site? In the case of coliphage 21 endolysin (R₁), although a SAR domain is present at the N terminus of endolysin, it lacks a Cys residue (Xu et al., 2004). When the SAR–truncated derivative of R₁ was prepared, it showed no activity in vitro. Although the addition of a cleavable secretory signal sequence from PelB (an Erwinia carotovora pectate lyase) resulted in the transport of endolysin, the protein remained catalytically inactive. These experiments demonstrated that SAR has an important role in active-site regulation. The structural studies suggested that the conformation of the SAR domain in the inactive form of endolysin differs from that in the active form of protein. Whilst the SAR domain conformation in the active form allows the formation of the catalytic triad, the SAR domain remains associated with the membrane in the inactive form (Sun et al., 2009). However, the mechanism by which the SAR domain is released from the membrane to activate endolysin needs to be elucidated.

Lysin activity regulation by trapping the active-site residue in a loop has been reported in Gp5 of T4 bacteriophage. Gp5 is a tail protein that is required for phage infection (Arisaka et al., 2003). In Gp5, the enzyme inactivation occurs by the formation of an inactivation loop that spans from Pro346 to Ser360. The enzyme is activated by an autoproteolytic cleavage of the protein between Ser351 and Ala352 within the loop (Kanamaru et al., 2005).

The activity of endolysin has also been shown to be regulated by means of oligomerization. One of the best-studied examples includes PlyC, an endolysin produced by streptococcal C₄ phage. PlyC oligomer is formed by the association of one molecule of PlyCA, which bears the catalytic domains, with eight molecules of PlyCB, which has the cell-wall-binding ability. Interestingly, PlyCA shows no lytic activity in the absence of PlyCB, suggesting that an oligomeric assembly is required for the functioning of the protein (McGowan et al., 2012). The CTP1L endolysin of CTP1 phage that infects Clostridium tyrobutyricum is known to form dimers. Biochemical and biophysical examination of CTP1L suggested that the side-by-side dimerization of the protein leads to an autocleavage in the molecule that enhances endolysin activity (Dunne et al., 2014). Dimerization-based endolysin activity regulation has been reported in the case of Cpl-1 endolysin produced by Cp-1 bacteriophage that infects S. pneumoniae. Cpl-1 endolysin shows enhanced activity upon dimerization that is a result of the interaction between its CBD and the bacterial cell wall component choline (Sanz et al., 1992; Buey et al., 2007; Monterroso et al., 2008). It is worth adding here that introduction of a disulphide bond leading to the stabilization of the dimeric form of Cpl-1 resulted in higher activity of the endolysin and reduced plasma clearance (Resch et al., 2011). This report thus also demonstrated that the bioengineering of endolysins for therapeutic purposes can be carried out. Although in some cases, as noted here, endolysin activity has been shown to be affected by multimerization, it is not clear how phage utilizes the oligomerization property of endolysin to regulate the protein’s activity during host cell lysis.

**REGULATION BY SPECIFICITY**

Bacteriophages infecting Gram-positive bacteria harbour endolysins that are modular in nature, and contain at least one catalytic and one CBD (Fischetti, 2005, 2010; Sudiarta et al., 2010). However, endolysins of phages infecting Gram-negative bacteria contain only a catalytic domain (Cheng et al., 1994). It was found that the CBD of endolysin binds specifically to the host cell wall.
Interestingly, however, most of the bacterial autolysins do not display peptidoglycan-binding specificity (Steen et al., 2003; Nelson et al., 2006; Fischetti, 2008). The G+ phage endolysins have a very narrow range of substrate specificity, i.e. their ability to degrade the cell wall is confined to specific host species or strains (Fischetti, 2008). For example, the Dp-1 bacteriophage lytic enzyme Pal shows lytic activity specifically on S. pneumoniae, but it has either very low or no activity on other species of streptococci (Loeffler et al., 2001). Similar observations were made for C1 phage endolysin of streptococci, where the enzyme showed very specific lytic activity on group A Streptococcus, but not on B, D, F, G, L and other species of streptococci (Nelson et al., 2001). This is an intriguing property of G+ phage endolysin that makes the protein very useful not only for the diagnostic purposes, but also for the targeted elimination of pathogenic Gram-positive bacteria.

Many studies have shown that the species-specific cell lysis property of G+ phage endolysins is attributed to the CBD (Low et al., 2005) and it has also been studied in the case of Mycobacterium phage D29 lysin A (Pohane et al., 2014). Lysin A of phage D29 contains two N-terminal catalytic domains and a C-terminal CBD (Payne & Hatfull, 2012; Pohane et al., 2014). Whilst the full-length lysin A was toxic to mycobacterial cells, it failed to kill E. coli. Interestingly, however, the individual catalytic domains could kill E. coli cells. It has been proposed that the ability of D29 lysin A to kill M. smegmatis, but not E. coli, is a result of interaction between the catalytic domains and the CBDs. Furthermore, the CBD domain of D29 lysin A binds only to the mycobacterial cell wall (Pohane et al., 2014, 2015). The data thus suggest that the endolysin stays in a ‘lock’ conformation in the non-host species, owing to an interaction between the catalytic domains and the CBDs. In the canonical host, this interaction is abrogated by the interference from the cell wall, thus allowing the catalytic domain to act on the peptidoglycan. The interaction of the CBD with the cell wall has been studied in the Listeria monocytogenes phage endolysins Ply118 and Ply500 (Loessner et al., 2002), and Bacillus anthracis bacteriophage endolysins PlyL and PlyG (Mo et al., 2012). In these examples, the affinity of the CBDs with the cell wall components was found to be in nanomolar range (Pohane et al., 2014). It was hypothesized that the CBD of endolysin helps in avoiding the killing of uninfected bacterial cells by perturbing the free diffusion of endolysin, thus resulting in the availability of more host cells to be infected by the phages (Loessner et al., 2002; Fischetti, 2008). Additionally, the interaction between the catalytic domains and the CBDs of endolysin may not allow the enzyme to lyse other cells, in a case where an endolysin does get released from an infected cell. Together, these regulatory mechanisms in the bacteriophage endolysins will help to maintain balance in microbial ecosystems.

CONCLUSIONS AND FUTURE PROSPECTS

Endolysins are regulated not only at the transcription level, but also post-translationally. Many of them rely on either holin-mediated transport or a signal sequence to reach the periplasmic space. However, the involvement of teichoic acids and chaperones in the transportation of endolysins opens new avenues for exploring the protein transport systems in bacteria. Upon reaching the periplasmic space, in some studied cases, endolysin stays in an inactive form and the activation requires significant changes in the protein conformation. However, it is unclear how these intramolecular conformational changes occur. Furthermore, the host specificity of phages infecting Gram-positive bacteria is an interesting observation in the field of bacteriophage research. The interaction between the catalytic and the CBDs will allow us to further explore it in the light of enzyme autoregulation. However, this may not be the only mechanism; another important aspect such as substrate choice also needs exploration. Moreover, the mechanism employed for the disruption of the interaction between the catalytic domains and the CBDs is yet to be studied in more detail. The species-specific substrate selection in the G+ phage endolysin will allow for the development of novel therapeutics to control the diseases caused by pathogenic Gram-positive bacteria. Further research in this area will certainly help in combating bacterial infections.

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