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Using a bacteriocin structure to engineer a phage lysin that targets *Yersinia pestis*

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Abstract

Purified phage lysins present an alternative to traditional antibiotics and work by hydrolyzing peptidoglycan. Phage lysins have been developed against Gram-positive pathogens such as *Bacillus anthracis* and *Streptococcus pneumoniae*, where the peptidoglycan layer is exposed on the cell surface. Addition of the lysin to a bacterial culture results in rapid death of the organism. Gram-negative bacteria are resistant to phage lysins because they contain an outer membrane that protects the peptidoglycan from degradation. We solved crystal structures of a *Yersinia pestis* outer membrane protein and the bacteriocin that targets it, which informed engineering of a bacterial-phage hybrid lysin that can be transported across the outer membrane to kill specific Gram-negative bacteria. This work provides a template for engineering phage lysins against a wide variety of bacterial pathogens.

Introduction

Bacteriophages are viruses that infect bacteria in order to proliferate. They were recognized as a potential treatment for bacterial infections over 100 years ago[1], but the development of small molecule antibiotics was more aggressively pursued because they are often smaller, cheaper to manufacture, and broadly efficacious. More recently the emergence of pan-resistant bacterial strains[2] has renewed interest in the use of phages as an alternative to traditional antibiotics[3]. Rather than using whole phages, purified phage lysins have been produced to target specific Gram-positive pathogens[4, 5], where the peptidoglycan (cell wall) is exposed on the cell surface. This approach, targeting specific pathogens rather than the entire gut flora, may reduce the development of multidrug resistant bacterial strains.

Until now, Gram-negative pathogens were resistant to treatment by phage lysins because the peptidoglycan layer is sandwiched between the inner and outer membranes. A lysin would need to be transported across the outer membrane in order to access and degrade the peptidoglycan. By targeting an outer membrane iron transporter and modifying a bacterial toxin that uses it for entry into the cell, we discovered a way to apply phage therapy to Gram-negative bacteria. Concurrent with our work, Braun, Zeth, and colleagues performed very similar experiments with the same outcome[6].

TonB dependent transporters and bacteriocins

TonB dependent transporters (TBDTs) normally transport ferric iron or cobalamin across the outer membrane[7]. In *Yersinia pestis*, a TBDT called FyuA is required for virulence in the early stages of a bubonic plague infection[8, 9]. The bacterium synthesizes the siderophore

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yersiniabactin[10], secretes it into the extracellular space where it binds Fe³⁺, and imports the Fe-yersiniabactin complex into the cell to meet its iron requirements. Transport across the outer membrane requires FyuA as the transporter and TonB-ExbB-ExbD plus proton motive force to energize the process. *Y. pestis* also makes a bacteriocin called pesticin[11] that requires FyuA for transport across the outer membrane. Pesticin is encoded on a plasmid[12] that also contains the genes for the pesticin immunity protein, and plasminogen activator, Pla. Pla is important for virulence so pesticin targets and kills *Yersinia* strains that express FyuA on the cell surface but have lost the bacteriocin-containing plasmid. Thus, the TBDT/bacteriocin couple maintains maximal virulence in the strain.

Crystals structures of FyuA and pesticin inform engineering of a Yersinia-targeting phage lysin

We solved the crystal structures of FyuA and pesticin to better understand what these proteins look like and how they function[13]. As mentioned above, FyuA is a TBDT that spans the outer membrane. We expressed FyuA in *E. coli* outer membranes, then extracted, purified, and crystallized it using various detergents. FyuA is a 71 kDa protein whose structure consists of two domains (mirroring all known TBDTs): a 22-stranded beta barrel spans the outer membrane and an N-terminal plug domain blocks the pore (Figure 1). From studies on FyuA and other TBDTs, we know that substrate is bound by loops of the plug domain and extracellular loops of the beta barrel. The plug domain contains a TonB box near its N-terminus that is required for import of either Fe-yersiniabactin or pesticin. In FyuA the TonB box consists of residues 8–14, STLVVTA, and this region is disordered in the structure.

We solved the structure of pesticin (40 KDa) after expressing it in *E. coli*, purifying and crystallizing it using standard techniques. The pesticin structure consists of two domains joined by a short linker (Figure 2). The N-terminal domain is a mixed alpha-beta structure while the C-terminal domain is primarily alpha helical. The N-terminal domain contains a TonB box motif at the N-terminus, DTMVV, which is required for import of pesticin by FyuA[11]. This region is disordered in our structure. Although pesticin had no structural or sequence similarity to any known colicin structure, we discovered that it has significant structural similarity to phage T4 lysozyme, even conserving 2 of the 3 active site residues[14]. Both T4 lysozyme and pesticin degrade peptidoglycan[15, 16].

Structure determination of pesticin revealed how similar its C-terminal domain is to T4 lysozyme, so we undertook engineering of a bacterial-phage hybrid protein to create a phage lysin that targets Gram-negative bacteria. The hybrid protein consists of the N-terminal domain of pesticin, which we determined to be essential for binding to FyuA, fused to T4 lysozyme in place of the C-terminal pesticin (muramidase/killing) domain. Determination of the hybrid structure showed that the domain architecture is preserved and the two proteins (pesticin and hybrid) are similar in size and shape (Figure 2).

Pesticin and the hybrid lysin specifically target cells expressing FyuA

We tested the activities of pesticin and the hybrid lysin in *E. coli* by expressing FyuA in the outer membrane[13]. When FyuA is present, both pesticin and the hybrid lysin are transported across the outer membrane, where they attack the peptidoglycan layer to kill the cell. *E. coli* cells that did not express FyuA were unaffected by both toxins. The pesticin immunity protein, Pim, confers protection by residing in the periplasm where it inactivates pesticin that has been transported across the outer membrane[11]. When we expressed FyuA in the outer membrane and Pim in the periplasm of *E. coli*, cells were no longer killed by pesticin but they were still killed by the hybrid lysin. We visualized these events using

electron microscopy on whole, frozen *E. coli* cells and learned that the two toxins appear to kill differently: pesticin made discreet holes in the outer membrane which eventually resulted in cell lysis, while the hybrid lysin caused massive vesiculation of inner and outer membranes, resulting in total destruction of the cell. Although it appears to degrade peptidoglycan more aggressively than pesticin, the hybrid lysin may be imported less efficiently because the overall efficacy of pesticin was higher than the hybrid lysin. Nonetheless, since Pim does not inactivate the hybrid lysin, it can target bacterial strains expressing pesticin and Pim, extending its potential use to any organism expressing FyuA on the cell surface.

The efficacy of pesticin and the hybrid lysin against *Yersinia* and UPEC strains

We evaluated killing by pesticin and the hybrid lysin in two strains of *Y. pestis* and also in *Y. pseudotuberculosis*[13]. *Y. pestis* KIM6+ expresses pesticin and Pim, and is killed by the hybrid lysin but not by pesticin. *Y. pestis* KIM10+ does not make pesticin or Pim, so both toxins can kill this strain. Similarly, *Y. pseudotuberculosis* does not make pesticin or Pim and is killed by both toxins. FyuA is also expressed in certain pathogenic *E. coli* strains that cause urinary tract and kidney infections (UPEC)[17]. We obtained 18 clinical isolates and evaluated our toxins in these strains too. In every case, if FyuA is expressed on the cell surface, pesticin and the hybrid lysin were able to kill the cells (none of these strains had the plasmid expressing pesticin and Pim). Therefore, both pesticin and the hybrid lysin can kill clinically relevant bacterial cultures in broth and plate assays, and the next experiment to undertake is to determine efficacy in an animal model of disease.

Considerations for further development

In order to develop modified bacteriocins for clinical use, we need to improve both efficiency of transport across the outer membrane and enzymatic activity in the organism. Our electron microscopy observation that pesticin disrupts cell membranes at discreet locations while the hybrid lysin causes massive destruction, and yet pesticin still kills better[13], suggests that the hybrid lysin is transported across the outer membrane less efficiently than pesticin. Because both pesticin and the hybrid lysin appear to be too large to be transported by FyuA without some degree of unfolding[13], we made a thermostable hybrid lysin containing two disulfide bonds[18] that should prevent unfolding of the lysozyme domain. While the thermostable hybrid lysin was still able to kill cells, it was 10-fold less active than hybrid lysin containing the wild-type T4 lysozyme domain. Additional data comes from Braun, Zeth, and colleagues where single disulfides conferred a complete loss of activity[6]. These results indicate that by increasing rigidity of the lysozyme domain, we have reduced the efficacy of the hybrid lysin. There is still much to be learned about how the killing domains of bacteriocins are imported and unanswered questions are the following: [1] the precise pathway(s) remains unknown, [2] whether energy is required at this stage is unclear, [3] whether electrostatics or interactions between specific residues play a role, and [4] whether size, shape, and flexibility (or ability to unfold) are important. These are questions relevant not only to pesticin and the hybrid lysin, but for the bacteriocin field as a whole. Once we better understand the mechanism(s) by which killing domains are imported, we will be better able to engineer effective phage therapy reagents.

In addition to improving the transport efficiency, it may be possible to increase and/or change the enzymatic activity of the killing domain. To increase *in vivo* activity of the pesticin-T4 lysozyme hybrid lysin, we could change the type of lysozyme used for killing. This might be beneficial because *E. coli* makes a periplasmic protein that inhibits vertebrate lysozymes called Ivy, and homologues are also found in *Shigella*, *Yersinia*, *Klebsiella*,

Pseudomonas, and other genera. Ivy preferentially inhibits vertebrate lysozymes such as hen egg white lysozyme, but also inhibits the activity of phage T4 lysozyme by 40% [19]. In contrast some lysozymes are resistant to Ivy, with (bacteriophage) lambda lysozyme, cauliflower lysozyme, and mutanolysin from *Streptomyces globisporus* showing no reduction in activity. By substituting the T4 lysozyme domain in our hybrid lysin with an Ivy-resistant lysozyme, we might increase activity by 40% or more.

Another way to increase activity might be to change it. Rather than attacking peptidoglycan using a lysozyme-type activity, we could take advantage of the fact that bacteriocins are modular protein toxins with killing domains that include RNA and DNA nucleases, ion channels, and domains that target peptidoglycan [20]. By substituting a nuclease or ion channel killing domain for T4 lysozyme, we could completely change the mode of killing by the hybrid toxin while retaining specificity for FyuA. Since FyuA is required for virulence [8, 9] and primarily expressed in virulent strains [8, 17, 21–27], this strategy targets specific pathogens rather than causing widespread bacterial eradication. Conversely, we could retain T4 lysozyme and substitute the FyuA-binding domain with a receptor-binding domain specific for a TBDT in a different organism. This strategy should allow us to target other bacterial strains, extending the phage therapy approach beyond *Yersinia* and *E. coli*.

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Figure 1. Ribbon diagram of the FyuA crystal structure. A) The 22-stranded β -barrel of FyuA is shown in gray while the inner plug domain is shown in red. The TonB box is indicated by a rectangle near the N-terminus of FyuA. This region is disordered in the electron density map and shown as a dashed line. B) Surface representation of FyuA viewed from the top of the barrel and down the barrel axis. The barrel is colored gray and rendered transparent while the plug is red. The purpose of this panel is to show that the plug completely fills the barrel pore.

Figure 2.

Ribbon diagrams of pesticin and the hybrid lysin. A) The N-terminal binding domain of pesticin is shown in light green while the C-terminal killing domain is shown in dark green. B) The N-terminal binding domain of the hybrid lysin is shown in yellow while the C-terminal killing domain is shown in orange. C) Superposition of the binding and killing domains of pesticin and the hybrid toxin. The domains were separated and aligned individually. Each domain is colored as in panels A and B.