BACTERIOPHAGE GH15: DEVELOPING A NOVEL WEAPON AGAINST MRSA

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ABSTRACT

In recent years, numerous bacterial species have developed antibiotic resistance due to the overuse of antibiotics in the home, health care setting, and in agriculture. Alternative methods of treatment, including phage therapy (PT), have been proposed as solutions to this problem. PT is showing promise as an alternative method of treatment against the bacteria methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is a virulent and antibiotic resistant bacterium capable of causing infections of the skin, respiratory system, and various other body systems. In this research proposal, we propose investigating the use of the Staphylococcal bacteriophage (phage) GH15 as a therapeutic agent against MRSA infections due to its broad host range, its lack of bacterial virulence genes, and its strong ability to lyse various strains of MRSA. Specifically, we propose to evaluate the tail fibre genes of GH15 contributing to the phage's host range, in addition to the ability of the phage to induce antiphage humoral immune responses in human cells, in the interest of exploring GH15 as a therapeutic agent for use in PT, specifically against MRSA.

Ces dernières années, de nombreuses espèces bactériennes ont développé une résistance aux antibiotiques en raison de l'usage excessif des antibiotiques à la maison, dans les établissements de soins de santé et dans l'agriculture. Des méthodes alternatives de traitement, y compris la thérapie par phages (TP), ont été proposées comme solutions à ce problème. La PT est prometteuse en tant que méthode alternative de traitement contre la bactérie *Staphylococcus aureus* résistante à la méthicilline (SARM). Le SARM est une bactérie virulente et résistante aux antibiotiques capable de provoquer des infections de la peau, du système respiratoire et de divers autres systèmes du corps. Dans cette proposition de recherche, nous proposons d'étudier l'utilisation du bactériophage staphylococcique (phage) GH15 en tant qu'agent thérapeutique contre les infections à SARM en raison de sa large gamme d'hôtes, de son manque de gènes de virulence bactérienne et de sa forte capacité à lyser diverses souches de SARM. Plus précisément, nous proposons d'évaluer les gènes de la fibre caudale de GH15 contribuant à la gamme d'hôtes du phage, en plus de la capacité du phage à induire des réponses immunitaires humorales anti-phages dans les cellules humaines, dans l'intérêt d'explorer l'utilisation de GH15 comme agent thérapeutique en PT, précisément contre le SARM.

KEY WORDS

Bacteriophage; MRSA; phage therapy; phage GH15

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Introduction

Research Topic and Goals

Due to the appearance of antibiotic resistant bacterial strains, the scientific and medical communities have considered and experimented with the use of phages as a solution to the recent antibiotic crisis (Łusiak-Szelachowska et al., 2014; Pires et al., 2016; Xia et al., 2015). Phages are viruses that are only able to infect and lyse bacterial cells and subsequently have minimal to no impact on the surrounding human flora, making them ideal candidates for therapeutic use (Pires et al., 2016; Ross et al., 2016). Although phage therapy (PT), the use of phages to treat a bacterial infection (Pires et al., 2016; Ross et al., 2016), was controversial upon its discovery in the mid 1940s due to a lack of proper controls and inconsistent results reported by scientists at the time, new technology, and more controlled lab etiquette is allowing PT to be revisited and optimized to combat antibiotic resistant bacteria (Summers, 2012; Wittebole et al., 2013)

One of the most alarming and harmful antibiotic-resistant bacterial genera, methicillin-resistant *Staphylococcus aureus* (MRSA), has captured the attention of the scientific community due to its antibiotic resistance and its virulence (Wang et al., 2016; Xia et al., 2015; Zhang et al., 2016). MRSA is a pathogenic bacterium known to not only cause superficial skin infections, but also other infections including deep skin infections and respiratory infections (Wang et al., 2016; Xia et al., 2015). In 2011, the Centres for Disease Control and Prevention (CDC) estimated that 80,461 MRSA infections and 11,285 related deaths occurred in the United States. In 2013, the CDC placed MRSA under threat level "serious" (U.S. Department of Health and Human Services, 2013). Consequently, discovering phages that can be used to treat MRSA infections is of utmost importance as traditional antibiotic treatment methods are becoming ineffective (Verstappen et al., 2016; Wang et al., 2016).

The purpose of this research is to study the Staphylococcal phage GH15 and evaluate its potential as a therapeutic agent for the treatment of infections caused by MRSA. Previous studies have identified GH15 as a viable therapeutic agent due to its broad target range of *S. aureus* strains, and low host response in mammalian cell lines (Gu et al., 2012; Gu et al., 2013; Zhang et al., 2016). In this proposal, we present the idea of studying the tail fibre genes of GH15 responsible for cell attachment and host range in addition to monitoring the immune responses initiated by the human immune system against GH15 in order to determine the potential for GH15 as a therapeutic agent in humans. In this proposal, GH15 has been selected for study as its lack of bacterial virulence genes, its broad host range, and, additionally, its lytic ability make it an ideal candidate for the treatment of MRSA infections via PT (Gu et al., 2012; Gu et al., 2013).

Hypothesis and Questions

Due to the need for an alternative to antibiotic treatments, the purpose of this proposal is to evaluate GH15 and its application to PT. Specifically, two objectives are proposed to determine the efficacy of GH15 in PT. Aim #1 proposes to identify which, of the seven genes encoding for tail fibre proteins of GH15, are responsible and have the largest impact on the host specificity of GH15. Aim #2 proposes to examine the safety and efficacy of GH15 in human cells by monitoring the anti-phage immune response by measuring cytokine production (ELISA) and cell death (cytotoxicity assays) in A549 lung epithelial cells.

Understanding how the human immune system responds and reacts to the presence of phages is key to evaluating GH15's potential as a therapeutic agent. We have chosen to monitor the levels of the cytokines IL-1, IL-6, TNFa, IL-4, IL-10 and the monocyte chemoattractant protein-1 (MCP-1 also known as CCL-2), which all play roles in inflammation, produced in human cells exposed to GH15 using ELISA assays. These cytokines

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were selected as they are all produced in response to a foreign (harmful) substance in the body – if the body recognizes the phages as harmful, an increase in the production of the abovementioned cytokines will be observed. Levels of all of these cytokines should be higher in response to a threat (Dinarello, 2000). ELISAs coupled with cytotoxicity assays will yield great insights into the immune response to the phage.

The information obtained through the following proposed experiments will prove to be useful in providing information about how GH15 can be used to treat MRSA infections through PT. PT has been proposed as an alternative to the use of antibiotics due to its ability to avoid collateral damage to the surrounding human tissues and its overall effectiveness in killing a target bacterial infection (Nobrega et al., 2015; Pires et al., 2016; Wang et al., 2016). In spite of the various benefits of PT, much still remains unknown about the full potential of using PT to treat bacterial infections.

Rationale

Due to the overuse of antibiotics, many bacterial genera are developing antibiotic resistance, indicating that a new method of treatment is required to combat bacterial infections (Zaczek et al., 2016). Specifically, MRSA is an alarming bacterial species resistant to methicillin and many other high-class antibiotics such as gentamicin, erythromycin, fluoroquinolones, and ofloxacin (Xia et al., 2015). MRSA is responsible for morbidity and mortality in a variety of lethal diseases including necrotizing pneumonia, overwhelming sepsis, pneumonia, endocarditis, and osteomyelitis (Wang et al., 2016; Xia et al., 2015). Due to its virulence and its antibiotic resistance, it is evident that a method of treatment be developed to fight MRSA infections. A number of solutions have been proposed in order to treat MRSA infections, including the use of phage lysins and PT (Verstappen et al., 2016; Xia et al., 2015; Zhang et al., 2016).

In this proposal, GH15 was selected for study. GH15 is a member of the Myoviridae phage family, of which it is among the largest phages. GH15 is a polyvalent phage; it is capable of infecting and lysing a wide range of *S. aureus* strains, including MRSA (Gu et al., 2012). GH15 lacks bacterial virulence genes and has a broad host range, making it an ideal candidate for use as a therapeutic agent against MRSA infections (Gu et al., 2012; Gu et al., 2013). Two studies in particular have been conducted on the use of phage lysins as therapeutic agents (Gu et al., 2011; Zhang et al., 2016). LysGH15, for example, is a lysin derived from the phage GH15. LysGH15 is an enzyme produced by GH15 that induces lysis of the host bacterial cell by targeting the peptidoglycan layer of the cell. When added externally, lysins typically access and cleave the cell wall peptidoglycans, making them useful as therapeutic agents (Zhang et al., 2016). A previous study (Gu et al., 2011) conducted on LysGH15 shows that LysGH15 has a strong ability to kill strains of *S. aureus*, especially MRSA, both in vitro and in vivo (in mice). The authors of this study demonstrate that a single injection of LysGH15 protected mice from fatal MRSA infection (Gu et al., 2011). Another study (Zhang et al., 2016) shows that the presence of LysGH15 did trigger the production of LysGH15-specific antibodies in mice, however, these antibodies did not block the lytic activity in vitro or the binding ability of LysGH15. The use of phage lysins to cure bacterial infections is one method under consideration as an alternative to antibiotics, however, these lysins are extremely host specific, suggesting that, unlike the phages used in PT, the host range of phage lysins cannot be altered as easily. This could be a major drawback to their use as therapeutic agents. PT, although still underdeveloped, allows the use of multiple phages (each specific to a particular bacterial species) in order to target multiple bacterial genera (mixtures consisting of multiple phages designed for therapeutic use are referred to as phage cocktails) (Pires et al., 2016).

PT is one of the most promising solutions to the antibiotic crisis (Górski and Weber-Dąbrowska, 2004; Nobrega et al., 2015; Pires et al., 2016). Unlike other methods of treatment, such as the use of antibiotics, PT is highly specific to its target bacterial species, however, unlike phage lysins, it can be altered to target many bacterial species. PT involves the use of phages in order to combat bacterial infections. Despite the many benefits of PT, including the many simple, rapid, and low-cost phage production processes available, the ability of this treatment to avoid collateral damage to host tissue, and the overall effectiveness of this treatment, there are still many drawbacks to the use of phages as therapeutic agents (Nobrega et al., 2015; Pires et al., 2016).

The host specificity of PT is one such drawback as the tail fibre proteins (proteins on the tails of phages) of phages can only recognize a specific host receptor (displayed on the surface of the host bacterial cell), allowing the phage to attach to that cell (Pires et al., 2016; Takeuchi et al., 2016). An exact diagnostic of the bacteria resulting in infection is required before phages can be administered as one phage specific to a particular set of bacterial strain(s) will not work in the treatment of disease caused by other bacterial genera (Pires et al., 2016). Currently, the primary solution to this issue is the use of phage cocktails (they consist of a mixture of various phages all specific to individual bacterial genera) (Nobrega et al., 2015). Phage cocktails, however, are underdeveloped and more research is required before their clinical usage.

Additionally, phages can induce anti-phage immune responses when administered to humans (Daniel-Paul et al., 2011; Górski et al., 2012; Łusiak-Szelachowska et al., 2014). These immune responses do not always lead to the inactivation of phages, however, this has to be determined for each phage individually (Górski et al., 2012; Łusiak-Szelachowska et al., 2014). In order for the full potential of PT to be uncovered, more research is required on phages and their applications to PT.

In order to evaluate the potential of phage GH15 as a therapeutic agent, two aims have been proposed. The first aim is to determine which, of the seven identified tail fibre proteins in GH15, have the biggest impact on cell attachment and host range of GH15. The second aim is to simply observe the ability of GH15 to induce cytokine production in the human cell line A549. Through conducting experiments to study each of these aims we hope to add to the growing scientific knowledge of the phage GH15 and further its potential as a therapeutic agent in PT, specifically against MRSA.

Methodology and Materials

Specific Aim #1: Determine how the seven tail fibre proteins of GH15 affect the host specificity of GH15

In order to evaluate which genes are responsible for the tail fibre proteins in phage GH15, we analyzed the genome sequence of GH15 on the NCBI. The seven potential tail fibre protein genes were identified. These genes encode for putative proteins: the exact impact of each individual gene on the host range of GH15 is unknown. To determine this, we propose the deletion of each gene individually, producing seven different mutants of GH15 (each mutant has the deletion of one of the putative proteins). Homologous recombination would be used to create each of the different mutants.

Homologous recombination allows scientists to alter the host specificity of phages through the "swapping" of the tail fibre genes (Mahichi et al., 2009; Pires et al., 2016). The desired genes of one phage are usually introduced into a bacterial cell via a plasmid, whereby they can be packed into progeny phages upon infection of the bacterial cell by a different phage (Pires et al., 2016). The progeny phage produced will contain the tail fibre genes of another phage, resulting in an altered host-range from that of the parental phage. Homologous recombination can also be used to delete genes in phages. In a previous

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study (Daniel-Paul et al., 2011), homologous recombination was used to create a mutant of the phage P954 where the endolysin gene of the phage was inactivated through insertional inactivation. In this study, we want to follow the procedure outlined by Daniel-Paul et al., 2011 as a method to inactivate the genes encoding the tail fibre proteins in phage GH15. These mutants of GH15 produced through homologous recombination would then be exposed to MRSA strain USA300 in addition to strains of *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Escherichia coli* in order to observe the host specificity of the mutant phages. Their ability to infect and lyse MRSA strain USA300 as well as the other bacterial strains will be determined by plaque assays.

Specific Aim #2: Examine cytokine production in response to GH15

To evaluate the levels of the cytokines IL-1, IL-6, TNFa, IL-4, IL-10 and MCP-1 produced by cells A549 in the presence of GH15, and the mutants that have a desired response to USA300 created in Aim 1, an indirect enzyme-linked immunosorbent assay (ELISA) will be conducted. Monoclonal antibodies to the biomarker molecules will be monitored by using a secondary antibody conjugated to Horseradish peroxidase (HRP) for colorimetric detection. Additionally, a cytotoxicity assay will be conducted to detect whether GH15 induces human cell lysis. GH15 and mutants will be exposed to cell line A549 and the release of lactate dehydrogenase will be monitored to determine cell lysis. As a negative control, the phage T4 will be used as it is known not to induce high production levels of cytokines or induce human cell lysis (Miernikiewicz et al. 2013).

Results and Conclusions

Observing the Impact of tail fibre gene deletion on the host range of GH15

Since the seven putative tail fibre protein genes will be deleted individually in each of the mutants, we expect to see a decrease in the ability of the mutants to infect USA300. We expect that the deletion of some of the putative tail fibre genes will have a greater impact on the host specificity of GH15 than the deletion of others. It is predicted that some of the mutants will still be able to infect and lyse MRSA strain USA300, though with a limited ability, and others will not be able to infect USA300. Through the use of homologous recombination, it is also possible that one of the genes deleted could code for an essential protein in GH15. In the case of a deleted essential protein, the mutants would die before infection of the bacterial cells begins, suggesting that the genes encoding the essential protein cannot be removed from the phage. The identified genes could then be modified to change host specificity.

Studying the host specificity of GH15 is key in order to evaluate its potential as a therapeutic agent, as knowing which genes are responsible for GH15's host range allows for their alteration. Changing the host range of a phage would allow this phage to be used to treat multiple bacterial infections as opposed to just one infection, promoting its use as a therapeutic agent.
By observing the impact of tail fibre gene deletion on the host range of GH15, we hope to identify which genes are directly related to and have the largest impact on the host specificity of GH15, allowing further studies to be conducted to alter the host range of GH15. Altering the host range of GH15 would allow GH15 to not only be used to treat infections caused by all strains of MRSA, but also infections caused by other bacterial genera.

Monitoring levels of Cytokines Produced in Response to GH15

Through the ELISA and the cytotoxicity assays we will be able to determine the levels of cytokines produced by cells A549 in the presence of GH15 in addition to observing whether GH15 is able to induce lysis of the A549 cells. Upon conduction of the ELISA, two possible outcomes could occur. In each of the scenarios GH15 will induce the production of cytokines. Either medium-high levels of cytokines will be produced or low levels of cytokines will be produced. In the case of medium-high cytokine production, we will have to further test whether these levels of cytokines

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will impact the ability of GH15 to infect MRSA. If so, GH15 will have to be modified (the protein coat of the phage may have to be altered) in order to avoid inducing immune responses. In the case of low cytokine production, GH15 will not be affected by the immune responses, which would further promote GH15 as a therapeutic agent. Additionally, we expect the cytotoxicity assays to prove that GH15 does not induce lysis of cells A549.

Before a phage can be used as a therapeutic agent, its impact on the human immune system, and its ability to induce an antiphage immune response must be evaluated. A phage associated with high levels of cytokine and antibody production will not necessarily work as well in PT as a phage that induces low levels of cytokines and antibodies. Through ELISA and cytotoxicity assays we will evaluate the cytokine production in the human cell line A549 in response to the presence of phage GH15. Determining the amount of cytokines produced in response to GH15 is an important factor to consider before GH15 can be used as a therapeutic agent.

It must also be noted that method of phage administration does have an impact on the degree of antiphage activity (Górski et al. 2012; Hwang et al. 2015; Łusiak-Szelachowska et al. 2015). Previous studies have monitored levels of antiphage antibodies in the serum of patients treated with phages (Hong et al. 2016; Miernikiewicz et al. 2013; Zaczek et al. 2016). They concluded that, in addition to phage type, the route of phage administration also has an impact on the levels of antiphage antibodies. Our studies do not involve the use of human sera and so our results are a basis for studying the cytokine production in response to GH15. Further studies will have to be conducted to determine the exact immune responses initiated against GH15 in vivo.

Final Discussion and Comments

The Staphylococcal phage GH15 has high potential as a therapeutic agent against MRSA infections. A therapeutic phage must have the ability to lyse its host cell with minimal impact on the human flora and the presence of a broad host range further impacts the decision for a phage to be used as a therapeutic agent. Furthermore, the phage must lack bacterial virulence genes so as to avoid making a bacterial infection worse. Phage GH15 not only lacks virulence genes but has a broad host range and a strong ability to lyse MRSA strains in vivo. Through determining the impact of the tail fibre genes on the host range of GH15 and the monitoring of cytokine production in human cells in response to GH15, we hope to further understand the therapeutic potential of GH15.

Abbreviations

Abbreviation	Full Form
MRSA	Methicillin-resistant *Staphylococcus aureus*
PT	Phage therapy
Phage	Bacteriophage
ELISA	Enzyme-linked immunosorbent assay
NCBI	National Center for Biotechnology Information

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