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Adapting T6SS Secretion Systems to Deliver Antibacterial Drugs to Eliminate *Pseudomonas aeruginosa*

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Abstract

Bacteria have evolved different mechanisms of communicating with their environment and with other prokaryotic and eukaryotic cells. A secretion system identified recently is the Type VI Secretion System (T6SS) found in approximately 25% of gram-negative bacteria. The T6SS secretion apparatus is associated with the opportunistic gram-negative pathogen, *Pseudomonas aeruginosa*, which is known to cause Cystic Fibrosis (CF) and other life-threatening bacterial infections such as Pneumonia and Septicemia. Studies have demonstrated that T6SS in *P. aeruginosa* can penetrate the peptidoglycan cell wall of prokaryotic bacteria and insert two key effectors proteins Tse1 and Tse3. The evolutionary adaption of T6SS secretion system gives the bacterium a unique survival advantage over other bacteria within the same niche. Although, the function, localization and mode of action of T6SS are being investigated, we do not fully understand the mechanism by which toxic proteins are delivered to neighboring prokaryotic cells.

The study will investigate T6SS apparatus in *P. aeruginosa* by exploring the structure and identifying the functions of the three effector proteins Tse1-3 delivered to neighboring cells. It will explore the hypothesis that T6SS secretion system can be manipulated, through adaption, to deliver antibacterial drugs advantageous to the host and potentially alter disease outcome. Our research provides a platform to divulge, elucidate and manipulate a truly remarkable mechanism that could deliver a new treatment model for treating bacterial infection.

Introduction

Bacteria communicate with their immediate environment. Several studies have identified secretion systems as a key mechanism employed by bacteria to interact with neighboring bacterial cells in the same niche. Recent research has identified seven secretion systems ranging from type I to type VII (T1SS to T7SS) that are utilized by a heterogeneous population of bacterial cells within the microbiome. It is now known that secretion systems are an integral biological arsenal used by bacteria to interact with competing prokaryotic and eukaryotic cells [1-3]. A specific type VI secretion system termed T6SS has received interest, particularly in bacterial species such as *Pseudomonas*, *Escherichia*, *Campylobacter*, *Agrobacterium*, *Aeromonas* and others [4].

The diverse functions performed by T6SS secretion systems are numerous and include nutrient acquisition, secretion of toxic effectors proteins outside of the cell and the deliberate targeting of eukaryotic cells causing disease [5,6]. As well as facilitating bacterial interactions, bacteria have evolved different mechanisms of communicating with their environment and with other prokaryotic and eukaryotic cells [7]. A secretion system identified recently is the Type VI Secretion System (T6SS) found in approximately 25% of gram-negative bacteria [5]. The T6SS apparatus is associated with the opportunistic gram-negative pathogen, *Pseudomonas aeruginosa*, which is known to cause Cystic Fibrosis (CF) and other life-threatening bacterial infections such as Pneumonia and Septicemia [5]. Studies have demonstrated that T6SS in *P. aeruginosa* can penetrate the peptidoglycan cell wall of prokaryotic bacteria and insert two key effectors proteins Tse1 and Tse3 [8,9]. The evolutionary adaption of T6SS secretion system gives the bacterium a unique survival advantage over other bacteria within the same niche [9]. Although, the function, localization and mode of action of T6SS are being investigated, we do not fully understand the mechanism by which toxic proteins are delivered to neighboring prokaryotic cells [6,7].

The type VI T6SS gene clusters demonstrates antagonistic or bactericidal activity towards neighboring bacteria [8]. It is known that secretion systems are used to deliver extracellular proteins or effectors by injection or secretion [10]. The same study (2014) identified that *P. aeruginosa* secret effector protein Tse1, Tse2 and Tse3 into the outer cell wall of competing bacteria to kill

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or inhibit adjacent prokaryotic cells. To prevent killing of own cells the bacterium encodes three immunity proteins Tsi1, Tsi2 and Tsi3 which are deployed to neutralize the bactericidal activity of the effector proteins [8,11].

The type VI is the newest secretion identified and was first mentioned in 2006 in two articles authored by that evidenced that the system was active in patients with Cystic Fibrosis (CF) [9], a disease caused by the *Bacterium Pseudomonas aeruginosa*, an opportunistic pathogen responsible for causing lung infection in CF patients [5]. *P. aeruginosa* bacterium is an excellent working model for the study of T6SS and has been shown to encode nearly all the secretion systems [5].

Gram-negative bacteria with their genes sequenced encode the gene cluster T6SS. The cluster of genes is known to comprise of a set of tightly clustered genes made up of 13 conserved genes which form the central core component [10]. The cluster has been found in the following gram-negative *bacteria*: *V. cholerae*, *P. aeruginosa* and *Acinetobacter* [5]. The central core component is made up of three groups. The first group makes up the membrane-associated proteins, also known as the integral membrane proteins TsO and TssM or TssJ, which is a lipoprotein. Collectively the group of protein forms the cell envelope. The second group constitutes proteins that form the components of the tail end of the bacteriophage. Proteins Hcp and VgrG make the syringe shape that form the tail structure of the bacteriophage [5,6,10]. The final groups include genes TssA, TssF, TssK and TssG although the function of these genes has not been elucidated at this time. Moreover, in silico analysis performed on bacterium *P. aeruginosa* showed that it was the only gram-negative bacteria that had three different T6SS gene clusters compared with nine different identified gram-negative bacteria [10,12].

An important finding by Mougous et al in 2006 found that one of the clusters that was named as Hcp 1 secretion island 1 (HSI-1), whose function was identified as a virulence locus of the bacterium *P. aeruginosa* which encoded a unique protein secretion system. The apparatus was identified as secreting a protein Hcp1 described as a hexameric protein with an internal diameter and a size of 40 Å. The study found that the HIS-encoded T6SS of *P. aeruginosa* (H1-T6SS) secreted three toxin proteins Tse1-3 to prokaryotic cells thus providing a survival advantage over other bacteria. The second protein whose function was associated with internalizing epithelial cells via activation of P13K-Ak host pathway [6]. The protein was identified as H2-T6SS. At present the structure and function of these proteins has not been solved and even less is known about the third protein HIS-111. Further phylogenetic analysis has shown that three T6SS gene clusters were present in different groups such that HSI-1 belonged to group A, HIS-II to group B and HIS-III to group D. It was suggested that each group had evolved differently and that the acquisition of genes and unique differences was associated with horizontal gene transfer [5]. Solving the structure and function of this remarkable system known as the HIS-1 has tremendous potential for the cure of many known diseases such as CF and other highly pathogenic infections. The HIS-1 apparatus has the potential to provide a platform as a possible therapeutic target. The idea of utilizing the apparatus to deliver drugs or to redirect the toxic effector proteins to specific bacterial targets is a promising idea and could facilitate controlling the dissemination of pathogenic bacteria in the human host [5,11].

One of the first effector proteins linked to *P. aeruginosa* K6SS is the VgrG protein [5]. Moreover, Hcp was shown to be secreted by gene cluster T6SS however; its function was identified as a chaperone and receptor of T65 effectors. Elucidating the ramifications of the T6SS system is to identify the functions of the three proteins (Tse1-3) the sophisticated mechanism by which the T6SS system can deliver these proteins into neighboring cells is integral for *P. aeruginosa* to acquire a dominant position within the microbiome [13]. The majestic mechanism provides the bacterium with a survival advantage therefore, understanding the function, localization and mode of action of the T6SS apparatus could alter the outcome of bacterial diseases in general [6].

The gene cluster HI-T6SS in *P. aeruginosa* was first identified by Hood in 2010, although, the exact function of these proteins has not been solved. The study suggested that Tse1 and Tse3 may be involved in targeting the peptidoglycan layer of the bacterial cell envelope thus causing the cells degradation. The structure of Tse1 has been solved using crystallography. The Tse1 protein comes from the super family of N1pC/P60 peptidases. The crystal structure identified six β strands (β1 to β6) and five helices (α1 to α5) which diffract at 1.5 Å resolutions [5,12].

The toxic element of the T6SS apparatus is protein Tse2 which has been suggested to target the bacterial cytoplasm. The protein was shown to inhibit bacterial growth of competing bacteria giving *P. aeruginosa* a unique advantage over other bacteria. The structure of Tse3 protein was also solved. The protein was shown to belong to the space group C121 and demonstrated diffraction at 1.5 Å resolutions [1].

Another significant component of the apparatus is the ability of T6SS to avoid killing own cells. The mechanism through which *P. aeruginosa* can prevent harm and damage to self-cell provides a unique and clever mechanism which ensures the bacterium's survival. An example is the T6SS encoded three immunity proteins Tsi1-3 these proteins function to neutralize the inhibitory action of Tse1-3. It has been suggested that the HI loop from Tsi1 blocks the activity of the protein by inserting into the Y-shaped grooved located on the surface of Tse1. The mechanism stops the binding of the protein to the peptidoglycan of the bacterial cell envelope and prevents cell degradation. This example demonstrates the unique ability of T6SS to regulate the function of the apparatus for its own advantage and to prevent its own death. This capability of T6SS in killing and paralyzing neighboring cells while preventing bactericidal activity towards self-cells ensures that *P. aeruginosais* king in the bacterial niche of the microbiome. The T6SS apparatus affords a survival advantage and bacteria that lack a similar secretion system are simply not able to compete with *P. aeruginosa*.

Aims

Our long-term goal is to develop and adapt the T6SS apparatus to deliver antibacterial proteins into *P. aeruginosa* microorganisms. Thus, the objectives of the study are 1) to understand the function of Tse1-3 proteins and that targeted delivery of these proteins to competing bacteria cause bactericidal activity. Objective 2) to determine whether adapting T6SS to deliver antibacterial proteins can eliminate *P. aeruginosa* and to develop a new treatment modality to fight pathogenic bacterial infection. Our rationale is that T6SS apparatus is a critical component that affords *P. aeruginosa* a survival advantage, therefore targeting the secretion system is an important part in finding new ways and models that will be successful in eliminating bacterial infection in the human host. Our central

hypothesis is that by elucidating the function of proteins that form the T6SS apparatus will lead to the elimination of the bacterium and is a key jigsaw piece in finding the next cure for diseases such as CF and others that cause death in the human host. We propose the following aims:

Specific aim 1: To study the function of Tse1-3 proteins and determine the role in the formation of the T6SS secretion system. Our working hypothesis is that it will lead to adapting the system to eliminate *P. aeruginosa* from the human host. We will monitor the function of proteins by performing mutagenesis on key effector proteins and monitor the effect on T6SS using Immunoblotting and Pull-Down Assays and Western Blotting analysis. To elucidate the effect of these proteins on T6SS system.

Specific aim 2: To understand the mechanism of immunity proteins Tsi1-3 in neutralizing the bactericidal activity of T6SS and preventing self-cell death. Our working hypothesis is it will lead to developing antibacterial drugs that function like immunity enzymes. Delivering these drugs to eukaryotic cells to neutralize the activity of toxic effector proteins by *P. aeruginosa* bacterium. We will monitor the function by performing Cell Invasion Assays and visualize with Time-lapse microscopy.

At the completion of our study it is hoped that we will have developed a therapeutic tool facilitated by T6SS to deliver antibacterial proteins targeted to pathogenic bacteria. We hope that our research will lead to a better understanding and will address the gap in current knowledge of the function of the Tse1-3 proteins in forming the T6SS bacterial secretion systems in *P. aeruginosa*. Our aim is that our research will save lives by developing new ways to deliver therapeutic drugs to eliminate CF, pneumonia and septicemia linked to the *P. aeruginosa* bacterium.

Method

The study will involve using *P. aeruginosa* strains which are derived from PAO1 in order to test the function of Tse1 to Tse3 proteins. Isolated *E. coli* peptidoglycan sacculi will be incubated with purified enzymes and separated by HPLC and the resulting peptidoglycan and soluble fragments identified using Mass Spectrometry (MS).

Pseudomonas aeruginosa cells using a RetS strain which has an active H1-T6SS (T6SS-active) secretion system and a mutant cell with a deletion of the HI-T6SS gene cluster (T6SS-inactive) strains will be grown overnight on LBA at 37°C.

Competition Assays will be set up by using *P. putida* as prey. A constitutively active strain that is T6SS active, PAKΔretS was used to perform the competition assay. Competition assays will be performed in the presence of different genes such as vgrG1b and with mutant genes to test the efficiency of killing by T6SS.

Other experimental methods carried out will be gene deletions, assays for T6SS secretion, Western blotting analysis, assays for T6SS killing and imaging will be performed with an Ibidi 35 mm μ Dish. Images will be observed with an Axio Observer Z1 fluorescence microscope.

Conclusion

The project is estimated to take three years to complete with all experimental procedures completed within three years. A total of six months will be enough to collate the results and analyze the data with the written dissertation completed within 6 months of performing all experiments. The outcome of the research will be a significant contribution to science. The T6SS secretion system warrants further investigations as it has therapeutic potential in the management of bacterial infections. It could be used to replace current failures in existing treatment modalities.

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