Classification of Elizabethkingia anophelis Resistance Mechanisms and Characterization of Novel β-Lactamase Genes

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Abstract:

Due to the cause of recent outbreaks within regions of the United States and other parts of the world, the study of *Elizabethkingia anophelis* has been of great interest due to high mortality and presence of multiple antibiotic resistance mechanisms. Of the many different resistance mechanisms that *E. anophelis* can employ, the presence of multiple β-lactamase enzymes has been of great interest for studies on the pathogenicity of this bacterium. Closely related to many of the other *Elizabethkingia* species, understanding the novel genes in both genomes and identifying the novel β-lactamase genes is crucial in developing better diagnostic and treatment methods for *Elizabethkingia* infections.

Introduction:

Elizabethkingia Species and Characteristics:

Elizabetkingia is a genus of bacteria with six characterized species. Discovered primarily as a branch from *Flavobacteriaceae* family branching from the *Chryseobacterium* genus, *Elizabethkinga* was first discovered in 2005 (9). Characterized as gram-negative, non-motile, non-spore forming rods, this new genus of bacteria, the use of 16s rRNA sequencing, whole genome sequencing, DNA-DNA Hybridization, phylogenetic studies and phenotypic tests have differentiated the *Elizabethkingia* genus from *Chryseobacterium* (2, 9, 11). The six species currently identified are *E. menigoseptica, E. miricola, E. anophelis, E. bruuniana, E. ursingii, and E. occulta* (5, 11). *E. menigoseptica* being one of the first two *Elizabethkinga* species characterized within the genus has shown association with meningitis and septicemia (9). First isolated from a case of neonatal meningitis, E. meningoseptica still remains a cause of outbreaks and a bacterium of interest for comparative studies with other species within the genus. The

second species identified it the *Elizabethkingia* genus is *E. miricola*, isolated from condensed water obtained from the Russian Mir Space Station (9). The next species identified is *E. anophelis*. Isolated in 2011, this bacterium was obtained from the midgut of mosquitos *(Anopheles gambiae*) (10). Prior to characterization of three new novel species, the *Elizabethkingia* species were characterized by 16s rRNA sequencing and DNA-DNA hybridization (11). Upon further analysis, other species within the *Elizabethkingia* genus were identified. Distinct from the *E. miricola* branch, *E. bruuniana* and *E. ursingii* were discovered using average nucleotide identity BLASTIN, DNA-DNA hybridization, and phylogenetic analysis (11). *E. occulta* was originally proposed as a strain of *E. ursingii*, but upon utilization of the average nucleotide identity BLASTIN and determination of the genome-to-genome distance, was correctly identified as a novel species (11). Further comparison of the strain first identified as species *occulta* with other strains from the CDC strain collection have lead to the proposal of two strains within the *E. occulta* species (11). The name thus implies that the *occulta* species was hidden in plain sight (11). All three of the novel species have nearly identical phenotypic characteristics that are shared among the entire *Elizabethkingia*, therefore phenotypic testing cannot be used to differentiate the different species within this genus (11). While most species share common characteristics, *E. meingoseptica* and *E. anophelis* have presented as opportunistic pathogens (1). In addition to the specific locations of isolation, many of the *Elizabethkinga* species have been isolated from the soil, water, air, and nosocomial environments, showing the abundance of this genus (1, 9, 10, 11). Most genomic discrimination methods involve 16s rRNA sequencing, but whole-genome sequencing has shown a potential for another novel species within the *Elizabethkingia* genus, but the new potential species has yet to be classified (5). Having shown significant prominence due to outbreak cases and increased resistance,

understanding the resistance mechanisms exhibited by species within this genus can give a better understanding of how to treat infections caused by *Elizabethkingia* bacteria, as well as apply to other resistance mechanisms exhibited by similar types of bacteria. Understanding the pathogenicity and differences in related species has given insight in to the most recent outbreaks of *E. anophelis*. For example, discriminating between *E. anophelis* and *E. menigoseptica* is proving very difficult be means of shared 16s rRNA sequence similarity approximately at 99.3% (5) and many resistance genes shared between the two species (1).

The Importance Behind *E. anophelis* study:

Elizabethkingia anophelis has been a bacterium of interest in studying resistance mechanisms and viability in many different environments. Having many different strains, several genes for adaptation to environments, as well as, antibiotic resistance have been characterized with genomic studies. As mentioned, *E. anophelis* was isolated from the mid gut of mosquitos (*Anopheles gambiae*) (1, 5, 10). Living in the gut of *A. gambiae*, *E. anophelis* elicit a symbiotic relationship for the ability to use multiple metabolite sources, hemolysis activity, and resistance to harsh environments (1). Due to the diet of mosquitos, *E. anophelis* must be able to survive on a variety of sugar sources. To compensate for the variety of metabolite sources, *E. anophelis* have genes encoding for a variety of glycoside hydrolase enzymes, which are responsible for metabolizing a variety of polysaccharides (1).

E. anophelis has shown observable resistance to several antibiotics, and studying the resistance genes has shown consistency with those observations (1). Within the genome, resistance genes that code for multi-drug efflux pumps have been identified from the resistance-nodulation-division, major facilitator multidrug, and multidrug and toxic compound extrusion

transporter families (1). In addition to the multi-drug efflux pumps encoded in the genome, transferase enzymes used to inactivate antibiotics are also coded for, specifically chloramphenicol acetyltransferase (3, 7) and aminoglycoside 6-adenyltansferase (3). Another major component to the resistance exhibited by *E. anophelis* are genes encoding for all classes of β-lactamase enzymes, which degrade antibiotic, rendering the antibiotic ineffective (1). Acquisition of the β-lactamase enzymes have shown to be conserved among the species, but also transferred across species through plasmids.

E. anophelis has been of great interest in recent years due to outbreaks across the United States in Wisconsin, Illinois, and Michigan (3). Extending beyond the United States, there have also been a reported *E. anophelis* outbreaks in Singapore, Hong Kong, and parts of the Central African Republic (5, 6, 7). As previously mentioned, the pathogenic nature of *E. anopheli*s has required further genomic classification to better identify the different species associated with the *Elizabethkinga* genus, but the actual functionality of the resistance genes within the genome of the various *Elizabethkinga* species has yet to be confirmed. The mechanism for transmission is not well understood and requires further insight for means of prevention in clinical settings.

Modes of Antibiotic Resistance:

E. anophelis resistance genes code for several different modes of antibiotic resistance (1, 7). Within the genome there are genes encoding for several classes of multidrug efflux-pumps, antibiotic transferase enzymes, and novel β-lactamase enzymes (1, 3, 7). Due to the diversity in resistance mechanisms, *E. anophelis* can evade many defenses used to ward of infection via *E. anophelis* and survive in environments where antibiotics may be prominent. Antibiotic widespread use has contributed to the progression of these resistance mechanisms, as well as, the utilization of multiple resistance mechanisms in order to ward off as many modes of attack as

possible. Here, efflux pumps and transferase enzymes are discussed, while beta-lactamase enzymes are discussed in the next section. Within the *E. anophelis* genome, multi-drug efflux pumps are coded fore in the resistance-nodulation-division (RND), major facilitator multidrug (MFS), and multidrug and toxic compound extrusion (MATE) families of efflux pumps (1). Most of the efflux pumps that are coded for are apart of the RND and MFS families though (1). RND pumps are responsible for pumping substrates such as heavy metals and toxic chemicals in order to maintain intracellular conditions (1). *E. meningoseptica* in particular has 5 putative novel operons for RND efflux pumps (19). The presence of putative novel RND efflux pump operons within *E. meningoseptica* implies that the presence of potential novel RND efflux pump operons in *E. anophelis* as well. Either through branching species, conjugation, or mutations, the novel efflux pumps may lead to increased resistance due to lack of target specificity. MFS pumps facilitate transport of an even broader range of substrates, including proteins, sugars, metabolites, and various drugs (1, 14). *E. anophelis* has shown resistance to fluroquinolones and heavy metals, which may attributable to the efflux pumps (2, 4). Another mode of antibiotic resistance conferred by transferase enzymes, specifically chloramphenicol acetyltransferase and aminoglycoside 6-adenyltransferase (3, 7). By attaching a functional group to an antibiotic, the antibiotic is unable to bind to the active site of the target, rendering the drug ineffective. Continued study of the E. anophelis genome for antibiotic resistance covalent modification genes, as well as antibiotic resistance efflux pumps, but further characterization of the novel putative genes coding for resistance mechanisms is required.

Mutations in genes encoding for antibiotic resistance can alter the binding affinity of certain drugs to antibiotic resistance enzymes, thus conferring antibiotic resistance (18). Alterations in gene expression and can make designing a drug to bind to a particular target very

difficult due to the rapid nature that bacterial DNA can change. Beyond enzymes that code for antibiotic resistance, targets for antibiotics can also mutate, such as the gene coding for particular rRNA (18). Additionally, transformational and recombinatorial processes can form "mosaic" genes which can modify a potential target for antibiotics (18). In particular to *E. anophelis* outbreak strains, mutations in the mut genes were found to lead to inactivation of certain adenine glycosylase enzymes responsible for repairing DNA (3). This led to a hypermutative state, meaning the genome constantly mutates and leads to an alteration in the function of many different proteins coded for in the *E. anophelis* genome. Further investigation is required to identify mutational events associated with antibiotic resistances.

In addition to the coded mechanisms of antibiotic resistance, gram-negative cells confer a mode resistance by nature of gram negative cells. Due to the presence of the cell wall, gram negative cells are naturally less permeable due to the presence of the outer membrane (18). Having the double membrane as mentioned makes antibiotic targeting difficult, but gram-negative cells can also regulate the entrance of certain materials into the periplasmic space by regulating the expression of porins (18). Because many antibiotics enter through porins, down-regulation of porins or expression of more selective porins can be detrimental to effectively attacking gram-negative cells (18).

β-Lactamase Enzymes:

Gram-negative bacteria can produce five types of β-lactamase enzymes, all serving the function to inactivate antibiotic molecules. The general function that are shared among all β-lactamase enzymes is the ability to cleave the amid bond on the β-lactam ring in order to render the antibiotic ineffective (13). Utilized by many different bacteria, β-lactamase enzymes share

common characteristics, but also have distinctions that allow segregation into four distinct classes and one class that is less characterized (13). With a wide range of antibiotics, different classes act on different types of antibiotics, contributing to the diversity of the β-lactamase enzymes. In particular, each class has a specific type of antibiotic that they target, while the extended spectrum β-lactamase enzymes can target several types of antibiotics (12).

Based on Ambler's classification guide, the four classes of β-lactamase enzymes are classified on their phenotypic characteristics. Class A β-lactamase enzymes are part of the serine beta-lactamase enzymes and exhibit penicillinase activity (15). Class A β-lactamase enzymes can be coded chromosomally or transferred via horizontal gene transfer between bacteria (15). Class B β-lactamase differ from all of the other classes of β-lactamase enzymes by means of identity at the active site (15). Class B enzymes are classified as the metallo-β-lactamses enzymes and are distinctive in their carapenenemase activity. Requiring zinc molecules for coordination at the active site, the enzymatic mechanism is completely different from that of the serine classes of β-lactamases (8). Class B β-lactamases are largely chromosomally encoded (15). Class C enzymes are apart of the serine class of β-lactamase enzymes and are characterized by their cephalosporinase activity (15). Generally, class C β-lactamase enzymes are chromosomally encoded (15). Lastly, there is the class D β-lactamase enzymes, which are apart of the serine class of β-lactamase enzymes and are characterized by their oxacillin-hydrolase activity (15). Class D β-lactamases can be both chromosomally encoded and plasmid acquired (15). The last classification of β-lactamase enzymes is less well-defined. Extended spectrum β-lactamase enzymes are responsible for resistance to multiple classes of antibiotics, specifically several classes of penicillins, third generation cephalosporins, and aztreonam (12). Encompassing a

broader range of β-lactamase enzymes, the extended-spectrum β-lactamase enzymes are not as well characterized as the other classes.

Because many β-lactamase enzymes are acquired via horizontal gene transfer among bacteria, many of the associated resistance mechanisms can also be associated with acquisition of other resistance genes, whether that be resistance acquired by plasmids or other resistance mechanisms that could be utilized for further drug resistance and survival. By identifying the novel β-lactamase enzymes and by obtaining a better understanding of the resistance mechanisms exhibited by the different species within the *Elizabethkingia* genus, better diagnostic methods can be developed along with better treatment methods for approaching outbreaks. Because *E. meningoseptica* and *E. anophelis* share many characteristics and share many genes, developing better discrimination methods is crucial for identifying the source of infection. Having been discovered first, many of the *E. meningoseptica* class B β-lactamase enzymes have previously been classified, but novel β-lactamase genes still exist in many of the *Elizabethkingia* genomes, and classification of the novel genes and further study is required for future classification and for future targeting.

β-Lactam Antibiotics Mode of Action:

Mechanistically, all of the β-lactam class antibiotics function in identical ways. For the Penicillin class of antibiotics, the target is the cell wall. Penicillin antibiotics are responsible for inhibiting the synthesis of the peptidoglycan within in the cell wall, ultimately causing cell lysis due to a compromised cell wall and the effects of osmotic pressure on the cell wall (16). This is done via targeting and binding penicillin to the transpeptidase enzymes responsible for cross-linking the peptidoglycan layer within the cell wall (16). Residing in the class of β-lactam

antibiotics, both carbepenems and cephalosporins have identical mechanisms, but with different efficiencies from the penicillin classes of antibiotics. The major differences between the different classes of β-lactam antibiotics is the shape of the molecule itself, making the different classes of β-lactamase enzymes specific for the specific molecule shape.

Methods and Materials:

In order to understand the functional nature of one of the novel β-lactamase genes in the *E. anophelis* genome, cloning of the TLA-1 Homolog gene (Bla3533) was required, where future protein purification and functionality tests will continue after optimal cloning has been performed. Using plasmid DNA with the insert of bla3533, the plasmid DNA was grown overnight at room temperature. The next day a plasmid miniprep was done in order to clean up and isolate the plasmid DNA. Using the plasmid DNA, polymerase chain reaction trials set up according to table one were conducted in order to find optimal conditions for PCR trials. After every run, the PCR product was checked on a 1% agarose gel for identification of proper band size. Following the isolation of 7-8 successful PCR trials, the PCR product was cleaned up using the Enoch PCR clean-up kit following the given procedures within the clean-up kit. With isolated PCR product, restriction digests using BAMH1 and SAC1 restriction enzymes. Restriction digests were done on both the purified PCR product and pSKB3 vector, which were ligated together following the heat-killed restriction digests. After ligation of the target gene into the digested pSKB3 vector, the plasmid is then transformed into DH5α competent cells. The

transformed cells were grown on Kanamycin plates and observed for colony growth.

Reagent	Tube 1	Tube 2	Tube 3	Tube 4
Water	32	30.5	30.5	29
5X phusion buffer	1	1	1	1
10mM dNTPs	1	1	1	1
Template	1	1	1	1
10uM for Primer	2.5	2.5	2.5	2.5
10 uM Rev Primer	2.5	2.5	2.5	2.5
DMSO	0	1.5	0	1.5
50mM $MgCl_2$	0	0	1.5	1.5
Taq	0.5	0.5	0.5	0.5

Table 1: PCR protocol. All of the values listed in the table are in in μL

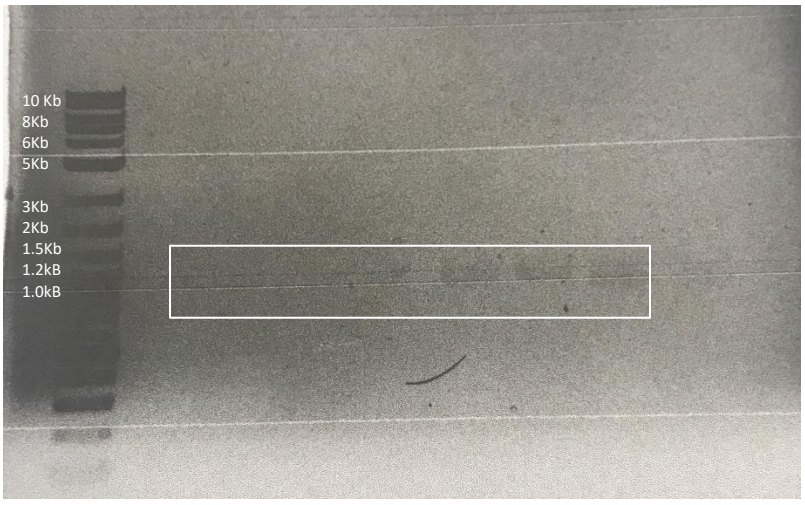

Figure 1: PCR trial confirmation on 1% agarose gel.

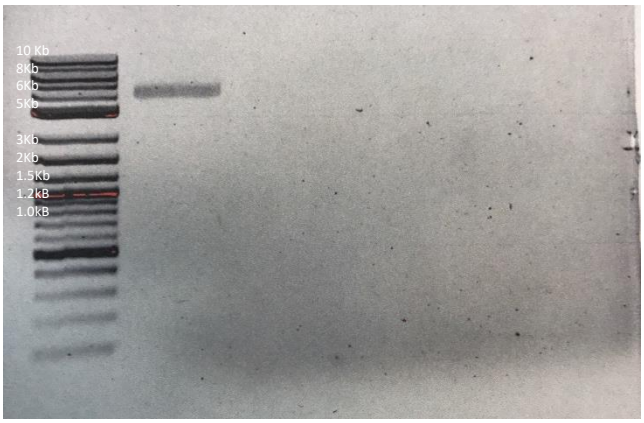

Figure 2: DNA ligation step. The PCR product (approximately 1000 bp) was ligated into the pSKB3 vector

Results:

While the cloning project progressed passed the isolation of the targeted DNA sequence via polymerase chain reaction, successful clones were not able to be isolated passed the transformation step. Figure 1 shows the result of the PCR trials, with faint bands around 900 base pairs, the size of the gene. Past the PCR trials, the restriction digested vector and PCR products were successfully ligated (Figure 2). Upon the transformation step into competent cells, 0 colonies were isolated from any of the incubated plates with Kanamycin. This implies that the Kanamycin plates may have been to concentrated with Kanamycin, meaning all the cell growth was susceptible to the antibiotic. We could not effectively identify if the plasmid transformed due to the lack of growth on the plates, again, attributable to potential error in the Kanamycin plate concentrations.

Discussion:

Due to the presence of various *Elizabethkinga* outbreaks around the world, further genomic classification of the antibiotic resistance mechanisms and varying *Elizabethkinga* species is required for better targeting in clinical settings. Due to the abundance of this genus

throughout a range of environments, fully understanding the pathogenicity of species within this genus is important for understanding treatments. With many of the *Elizabethkinga* species sharing up to 99.8% homology among genomic sequences, misidentification of species and improper targeting have made finding a treatment very difficult (5). Specifically, *E. anophelis* has been a bacterium of interest due to the large Wisconsin outbreak in 2015 and 2016, the outbreaks in Central Africa and the outbreaks in Singapore (5, 6, 7). Understanding novel resistance mechanisms and understanding the natural resistance that this bacterium utilizes is crucial. Housing many novel β-lactamase genes, further classification of the β-lactamase genes is important for expansion of antibiotic development and multi-drug antibiotic treatments.

While cloning the Ag1 3533 gene proved unsuccessful in this particular experiment, further cloning projects can identify the genes that code for β-lactamase enzymes as well as classify and identify proper functionality. Because of the abundance of novel genes that exist within the *Elizabethkingia* genus, cloning of the putative β-lactamase genes and protein studies can help understanding the pathogenicity associated with *E. meningoseptica* and *E. anophelis.*

Future Directions:

In the future, the project must be repeated from the plasmid prep step of the plasmid DNA with the isolated gene of interested. The plasmid prep step should be re-isolated and the cloning project must be redone prior to analyzing the functionality of the beta-lactamase enzyme. Following all of the standard steps required for cloning, a positive clone must be isolated and sequenced to ensure isolation of the correct gene. Following a successful sequence step, transformations of the plasmid into DH5α cells and additionally BL21 cells will allow more variety for future functional testing and protein work. Small and large Kirby-Bauer assays can

then be done in order to test antibiotic susceptibility to different types of antibiotics. Following the standard protocol for the Kirby-Bauer assays will allow analysis of the different types of antibiotics that this specific beta lactamase enzyme is susceptible to and allow measurement of how susceptible the enzyme is to particular antibiotics. Moving past Kirby-Bauer assays, minimum inhibitory concentration procedures can be done in order to determine the minimum concentration of antibiotic used to inhibit the growth of *E. anophelies*. This is another test that can be used to determine the susceptibility of this particular bacterium to various concentrations of antibiotic and different types of antibiotic. After testing various reactions to antibiotics, functionality of the beta-lactamase enzyme can be further analyzed. Protein purification must be conducted in order to isolate the protein for functional tests. No protocols have been developed, so optimizing a protocol for the protein purification step is required for further investigation. Following protein purification, various enzyme assays can be used to determine some of the functional features associated with the Ag1 3533 gene expression of the subsequent β-lactamase enzyme. Protocols for the enzyme assays have not been developed and require the development and optimization in order to be used. In addition to this gene, there are still many novel genes within the *E. menigoseptica* and *E. anophelis* genome that need further identification.

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