The Purification and Antibiotic Inhibition of L,D-transpeptidase in Enterobacter cloacae

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with Research Distinction* in the undergraduate colleges of The Ohio State University

by

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Abstract

β-lactam antibiotics such as penicillin have been particularly important in modern medicine through their ability to disrupt cross-linkages within bacterial cell walls. They work by binding to penicillin-binding proteins, or PBPs, which prevents cross-linkage between peptide strands of the cell wall from occurring, resulting in an overall weaker cell wall which subsequently kills bacterial cells. Many bacterial species, such as *Enterobacter cloacae,* also express several L,D-transpeptidases or Ldts, which can also cross-link peptidoglycan through an alternative pathway and are not as easily recognized by β-lactams. Based on these properties of the bacterial cell wall, it can be hypothesized that the presence of Ldts influence the effectiveness of β-lactam antibiotics. This project aims to quantify the activity of LdtE from *E. cloacae* in the presence of different β-lactam antibiotics. LdtE was over-expressed and purified as a recombinant protein from an *E. coli* expression system. Nitrocefin was used to quantify enzyme activity and inhibition in the presence of various β-lactam antibiotics. Through quantification of these interactions, it was determined that meropenem and nafcillin had the greatest inhibitory effect on LdtE. While a related carbapenem, imipenem, showed no inhibition, suggesting that the substituents on the carbapenem core play a critical role in enabling Ldt inhibition. Based on that data, we will also determine how the Ldts effect β-lactam susceptibility in *E. cloacae* by knocking out the genes and then performing assays to determine the minimum-inhibitory concentrations of antibiotic necessary to inhibit bacterial growth.

CHAPTER 1

INTRODUCTION

1.1 Overview

This thesis deals with antibiotic resistant *Enterobacter cloacae* and the role of L,D-transpeptidases in β-lactam antibiotic resistance, that typically work by inhibiting penicillin-binding proteins.

1.2 Penicillin discovery

The discovery of penicillin was a major shift in the ways in which humans responded to bacterial infections. Before the time of penicillin, the average lifespan of a human was a measly 47 years[1]. In 1928, scientist Alexander Fleming accidentally discovered that the fungus *Penicillium notatum* prevented bacterial growth in the area directly surrounding it[2]. Thus, the antibacterial chemical responsible for this phenomenon was named penicillin. However, Fleming was unable to purify penicillin[2]. Nearly 10 years later, Howard Florey and Ernst Chain would take Fleming's discovery and work to mass produce the fungus and purify penicillin, resulting in mice tests that would further prove the strength of penicillin as an antibacterial weapon[2]. By the mid-1940s, prescription penicillin was widely available, and an antibiotic revolution had started[1,2]. The family of β-lactams was then derived from penicillin, characterized by their conserved 4-member cyclic amide (**Fig. 1.1**).

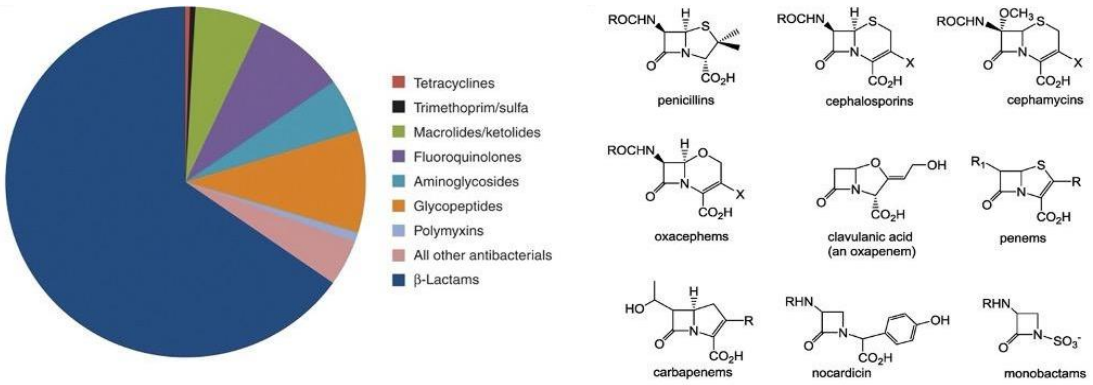


Figure 1.1 β-Lactam antibiotic prevalence. (A) The proportion of different classifications of antibiotics that make up prescriptions in the United States [2004-2014]. β-Lactam antibiotics comprise 65.24%[27]. (B) Structures of clinically relevant β-Lactams[29].

1.3 The bacterial cell wall and β-lactams

β-lactams specifically target the synthesis of the bacterial cell wall. The cell wall of both Gram-negative and Gram-positive bacteria have peptidoglycan strands, which are comprised of peptides attached to a rigid polysaccharide back-bone. In Gram-negative bacteria, a thin peptidoglycan layer is situated between an outer membrane and an inner cytoplasmic membranes[3]. This is different from Gram-positive bacteria where the peptidoglycan layer is thicker and there is no outer membrane present[3]. These peptidoglycan strands contribute to the

rigidity of the cell wall and provide the strength necessary to resist osmatic pressure[3]. This is due to the cross-linkage that occurs between the adjacent peptidoglycan strands catalyzed by D,D-transpeptidases, more commonly known as penicillin-binding proteins (PBPs). They specifically generate a peptide cross-linkage between the carboxyl group of the fourth position D-Alanine and an amino acid at the third position of the adjacent glycan strand (a 4 →3 cross-link)[4] (**Fig 1.2**). β-lactams covalently bind to the active-site serine of PBPs due to being structural analogs of the terminal D-Alanine-D-Alanine of peptidoglycan, functioning as an irreversible competitive inhibitor[5,6]. Ultimately this prevents cross-linkage and weakens the cell wall, resulting in bacteria that is susceptible to environmental pressures.

1.4 L,D transpeptidases

The 4→3 cross-linkage is not the only kind that occurs in the bacterial cell wall; a similar 3→3 linkage is also observed in many species[3]. D,D-carboxypeptidases function to cleave the terminal D-Ala of one of the peptide strands, which generates a tetrapeptide[3]. This serves as the substrate for L,D-transpeptidases (Ldt), which function similarly to PBPs and catalyze the linkage between two amino acids in the third positions of adjacent peptidoglycan strands (a 3 →3 linkage)[3]. Although they perform similar functions in the case of cross-linkage, a significant differentiating factor between Ldts and PBPs is the presence of a cysteine catalytic residue instead of the classical serine residue present in PBPs[7]. The Ldts and PBPs also show large differences in their sequences and protein structure. Evidence shows how meropenem, a carbapenem antibiotic, interacts differently with the catalytic site serine of a PBP and the cysteine of an Ldt in both binding and stabilization[8]. Noting these differences between Ldts and PBPs, it can be hypothesized that the presence of Ldts contributes to β-lactam antibiotic resistance. This issue becomes more prevalent based on the bacteria being studied, as some rely more heavily on Ldts than others. Although PBPs are the primary cross-linker in most cases, there are certain bacterial species such as *Mycobacterium tuberculosis*, where Ldts catalyze approximately 60% of the cross-linkage events in the cell[9].

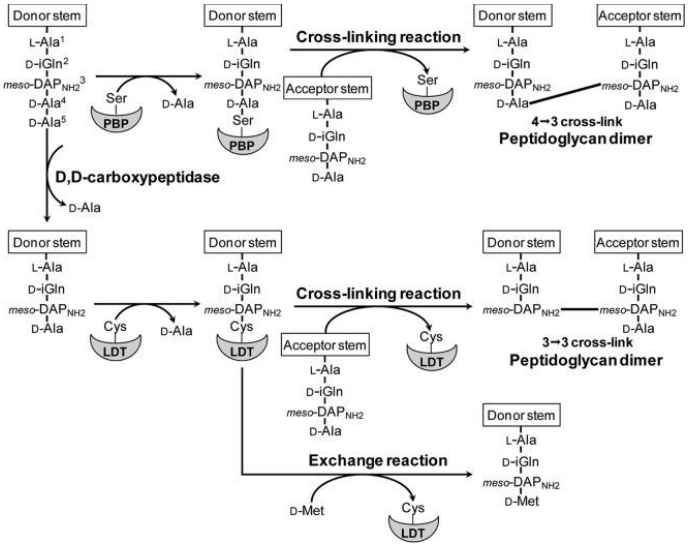


Figure 1.4 Diagram of the mechanism for cross-linkage in the bacterial cell wall. Penicillin-binding proteins (PBPs) facilitate the 4→3 cross-linkage of neighboring peptidoglycan strands via the cleavage of a terminal D-Ala. L,D-transpeptidase facilitates a similar 3→3 cross-linkage[5].

The function of each Ldt gene is still under investigation, with varying types of activity being discovered beyond their role in the formation of 3→3 cross-linkages[10]. Some Ldts have been found to function to strengthen the cell membrane in other ways. Studies have shown that some Ldts are responsible for the anchoring of Braun's lipoprotein to the outer membrane of Gram-negative bacteria[10]. The Braun's lipoprotein is an important structural protein that is present in many γ-proteobacteria, including *Enterobacteriaceae*[11]. LdtF in *E. coli* functions as an amidase

that cleaves the Braun's lipoprotein from the peptidoglycan layer in addition to being involved in biofilm production[7]. The Ldt_{Mt2} gene in *Mycobacterium tuberculosis* was shown to be associated with increased virulence[12]. This suggests that the Ldts also contribute to the success of a bacterial infection as well as antibiotic resistance. Overall, the workings of Ldts can be strongly linked to cell wall and membrane functionality that contribute to bacterial survival, even if each individual gene does not perform the exact same task.

There have been multiple studies focused on Ldts found in *Mycobacterium tuberculosis*, and several of these proteins have been crystallized and showed two domains, the N-terminus domain and the catalytic C-terminal domain[12,13]. The C-terminus consists of a ErfK/YbiS/YhnG-domain has a narrow tunnel or pocket with two entrances that seems to remain consistent among various Ldt proteins and bacterial species[12,14]. The N-terminal domain of the Ldts related to *Mycobacterium tuberculosis* contain immunoglobulin-related folds, which is not conserved amongst various bacterial species and greatly differentiates Ldt_{Mt} from other Ldts[12,15]. Despite the prevalence of Ldts in medicine, there is still a lack of structures present in literature[12]. This, along with the discovery of various Ldts whose functions are not always the same, leads to a lack of general knowledge into the structure, function, and means of combatting these enzymes that are seemingly linked to virulence and antibiotic resistance in bacteria.

1.5 β-lactamases

A major contributing factor to bacterial antibiotic resistance, especially in Gram-negative bacteria, is the presence of β-lactamases[16,17]. Having been a topic of research since the 1940s, β-lactamases entire purpose is to hydrolyze β-lactams, which inactivates them[16]. They do this through one of two mechanisms, either by acyl-enzyme formation with an active-site serine or by a hydrolytic reaction facilitated by zinc ions for metallo-β-lactamases[16,18]. The results is a β-lactam ring where the 4-membered ring containing the amide bond has been hydrolyzed and is thus inactive[18]. Naturally this has become a major concern for modern medicine as this function renders common β-lactams useless in fighting infection[19]. A way to combat this effect is with highly potent β-lactams that are more resistant to hydrolysis by β-lactamases, known as carbapenems[20,21]. Like penicillin, carbapenems have the 4-membered β-lactam ring, attached to a 5-membered ring[20]. They differ structurally by having carbon at position 1 instead of sulfur, and an unsaturated bond between carbon 2 and 3 in the thiazolidine ring[20]. Certain β-lactamases, as mentioned previously, have an active-site serine that functions like PBPs and reacts with the β-lactam ring in carbapenems[16,18]. The difference is that carbapenems also have a trans-1-hydroxyethyl substituent on the β-lactam ring which contributes to the resistance of hydrolysis by β-lactamases, resulting in the carbapenem[21] becoming irreversibly stuck to the β-lactamase.

Over time a new type of β-lactamases, known as carbapenemases, evolved, and has begun to decrease the efficacy of carbapenems in fighting bacterial infections[22,23]. Carbapenemases can hydrolyze every kind of β-lactam, including carbapenems[22]. The presence of carbapenemases is widespread with some being encoded in the chromosome while others are encoded in plasmids[24]. The class A *Klebsiella pneumoniae* carbapenemase (KPC) is encoded in plasmids and is important in *Enterobacteriaceae* bacteria[24].

1.6 Relevance of Enterobacter in modern medicine

It is hypothesized, due to the prevalence of Ldt genes in antibiotic resistance bacteria, that Ldts are often present in resistant infections in hospitals[25]. Looking specifically at *Enterobacteriaceae* family, they have consistently been ranked as containing most significant antibiotic-resistant pathogens in modern medicine[26]. The prevalence of β-lactam resistant *Enterobacteriaceae* is a dangerous development as β-lactams comprise a vast majority of prescription antibiotics, approximately 65% (**Fig 1.1**)[27]. The family of *Enterobacteriaceae* is considered a global threat based on the World Health Organization due these bacteria often expressing broad-spectrum β-lactamases[28]. Within the family of *Enterobacteriaceae* is the species *Enterobacter cloacae*, which is attributed to approximately 7% of intensive care unit (ICU) infections[29]. Rates of carbapenem-resistance have steadily increased from 1% to 9% from 2007-2019[30]. These multi-drug resistant bacterial infections are very difficult to treat, which makes their prevalence in hospitals concerning.

Goals: This project seeks to understand the biochemical role of a Ldt in *Enterobacter cloacae* and how it contributes to β-lactam resistance. Nitrocefin assays will be used to determine the ability of β-lactams to inhibit the activity of a Ldt. Genetic knockouts of Ldt genes will be performed, followed by determination of minimum-inhibitory concentrations (MIC) to determine how important different Ldts are in antibiotic resistance.

CHAPTER 2

PURIFICATION AND BIOCHEMICAL ASSAYS

2.1 Materials and Methods

2.1.1 Protein Expression

LdtE was expressed in fusion with Maltose-Binding Protein (MBP) at the N-terminus using a 3C protease cleavage sequence and a C-terminal hexahistadine tag in a pET29 with MBD-3C-LdtEC-sortase-6xHis vector. MBP-LdtE was overexpressed in a BL21 *E. coli* strain. 100 μM kanamycin was added to 5 mL of LB broth and inoculated with a glycerol stock of the BL21 *E. coli* transformed with the MBP-LdtE vector. This culture was incubated at 37ºC while spinning for ~16 hours. The next day, 500 mL LB broth with 100μM kanamycin was inoculated using the 5 mL culture. The 500 mL culture incubated while shaking at 225 rpm at 37ºC until the OD_{600} read at ~0.5. Once this optical density was reached, 300 mM IPTG was used to induce, and the culture was left to incubate for ~16 hours at 20ºC. On the third day, the 500 mL culture was centrifuged down at 4500 rpm for 15 minutes. The subsequent pellet was resuspended in 8 mL of lysis buffer (500 mM NaCl, 50 mM phosphate pH 7.2, 5 mM CHAPS, 2 Thermo Scientific Pierce Protease Inhibitor Mini Tablets). Then 1 mL aliquots of the bacterial resuspension were added to screw-cap centrifuge tubes containing 0.5 g of 0.1 mm beads. The bacteria were lysed using a Fisher Scientific Bead $Mill_4$ at 2,500 rpm for 2-minute cycles with 2 minutes of rest on ice in-between cycles: a total of 20 minutes. Afterwards the lysis tubes were centrifuged for 10 minutes at 14,000 rpm. The supernatant was extracted, and the lysis tubes were washed with 0.5 mL of lysis buffer and the pellet resuspended. The tubes were centrifuged again, and the supernatant extracted. The collection of supernatants was then filtered through a 0.2 μM Pall Corporation Supor filter.

2.1.2 Protein Purification

The filtered supernatant was then purified using fast-paced liquid chromatography, or FPLC (Bio-Rad NGC Chromatography System). The supernatant was loaded onto a nickel column using a Bio-Rad Bio-Scale Mini Ni-Charged 5 mL 40 x 12.6 mm column. The nickel chromatography was initiated with wash A (500 mM NaCl, 50 mM phosphate pH 7.2) and eluted using a linear gradient of wash B (500 mM NaCl, 50 mM phosphate pH 7.2, 200 mM imidazole). Collection tubes were analyzed using SDS-PAGE to determine the presence of LdtE. Those collection tubes were compiled together, concentrated to ~5 mL, and then buffer swapped into a 20 mM Tris pH 6.8 and 500 mM NaCl buffer. The concentrated protein solution was transferred to a 50 mL tube and 10% glycerol was added to the solution to a maximum volume of 5 mL. Between 250-500 μL of MBP-3C protease was added to cleave at the MBP-LdtE fusion sequence. The solution incubated at 4.5ºC overnight while stirring gently with a magnetic stir bar. The next day, the solution was run on SDS-PAGE to determine if MBP was properly cleaved and subsequently was buffer swapped back into wash A. The solution was run on a second nickel-column to further remove the bulk of the unwanted proteins. The collection tubes were run on SDS-PAGE to determine which had LdtE. The collection tubes that had the desired proteins were collected and buffer swapped into buffer MBP wash A (20 mM HEPES pH 7, 250

mM NaCl). The solution was then run on an MBP trap column using two connected Cytiva MBPTrap HP 1 mL columns. Initially run with MBP wash A and eluted using increasing concentrations of MBP wash B (20 mM HEPES pH 7, 250 mM NaCl, 10 mM maltose). Collection tubes were run on SDS-PAGE to determine which contained LdtE and those collection tubes were compiled together. The solution was run on a size-exclusion column with a Bio-Rad Enrich SEC70 10x300 mm column while using a buffer of 25mM HEPES and 150 mM NaCl. The resultant collection tubes were run on SDS-PAGE to determine the purity of the protein. Once the protein was determined pure, it was concentrated down to 500 μL.

The protein concentration was then quantified through bicinchoninic assay (BCA), specifically using the Thermo Scientific Pierce BCA Protein Assay Kit. Working reagent was created following the guidelines of the kit. The protein was diluted 5x using the 25 mM HEPES, 150 mM NaCl SEC70 buffer from the size-exclusion column. Bovine serum albumin (BSA) was used as a standard.

2.1.3 Nitrocefin Kinetics Assay

The β-lactams tested were meropenem and nafcillin at varying incubation times of 2 hours, 1 hour, and 30 minutes. Each reaction was performed in triplicate. A 96-well plate with a transparent bottom and lid was used in this procedure. 160 μL of buffer (20 mM TRIS pH 8.5, 150 mM NaCl), was added to row A. Another 80 μL of the same buffer was added to rows B-G. 100 μM stock of β-lactam was added to row A and a two-fold serial dilution using 80 μL down to row F was performed. This provided varying concentrations of 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, and 3.125 μM from row A-F. The enzyme was used at a 20 μM concentration and 10 μL was added into row A-G, providing a final concentration of 2 μM of enzyme per well. The plate was then covered with a lid and allowed to incubate at room temperature. After incubation, 100 μM of nitrocefin was added to each well and the plate was read using a BioTek Synergy H1 microplate reader. A reading was taken every minute for a total of 20 minutes. Absorbance was measured at 486 nm. A plot of time versus the absorbance was created to determine a linear regression. The slope, which is the velocity of the reactions, of this linear regression were converted to nM/min using the published extinction coefficient for nitrocefin. This velocity recorded in nM/min was then plotted against inhibitor concentration in μM. The non-linear regression of this new plot provided the IC_{50}, or the concentration of β-lactams necessary to inhibit the reactivity of LdtE by 50%.

2.2 Results and Discussion
2.2.1 Purification of LdtE

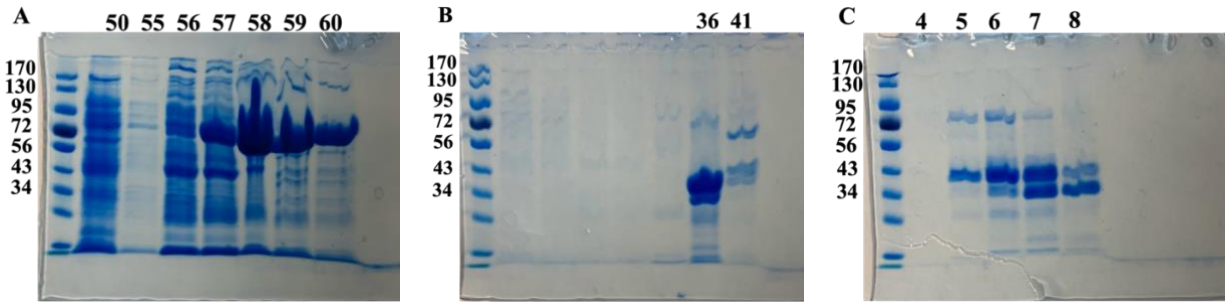


Figure 2.1.1. SDS-PAGE gels from the purification process of MBP-Ldt_{EC}. (A) Nickel-affinity column of MBP-Ldt_{EC} fusion (B) MBP-Trap column of Ldt_{EC} following overnight MBP cleavage (C) Size-exclusion column of Ldt_{EC}. Fraction tube numbers are included on the top of the gel images while molecular weight standards [kDa] are included on the left of the images.

The protein LdtE was expressed in fusion with Maltose Binding Protein (MBP) to maintain solubility of the protein, if the protein precipitates, then it cannot be properly purified. The size of the fusion complex was approximately 75 kDa. The MBP-LdtE construct was purified initially with nickel column chromatography, a purification step that utilized the His-tag on the C-terminus of the protein. The resulting protein extract was then incubated overnight with MBP-3C protease to cleave off the MBP and extract LdtE. This was followed by an MBPTrap column and then a size exclusion column. The initial nickel column step typically resulted in a large amount of protein being present (**Figure 2.1.1 A**). The MBP cleavage step that followed required adjustments. MBP-3C protease was added to the protein solution to cleave at the 3C protease recognition site. The initial process of cleavage involved just adding the MBP-3C protease and incubating overnight. Without the presence of any other buffer or solutes, the cleavage did not occur and any protein that was cleaved precipitated out of solution in the process (**Figure 2.1.2 A**) Another attempt included incubating

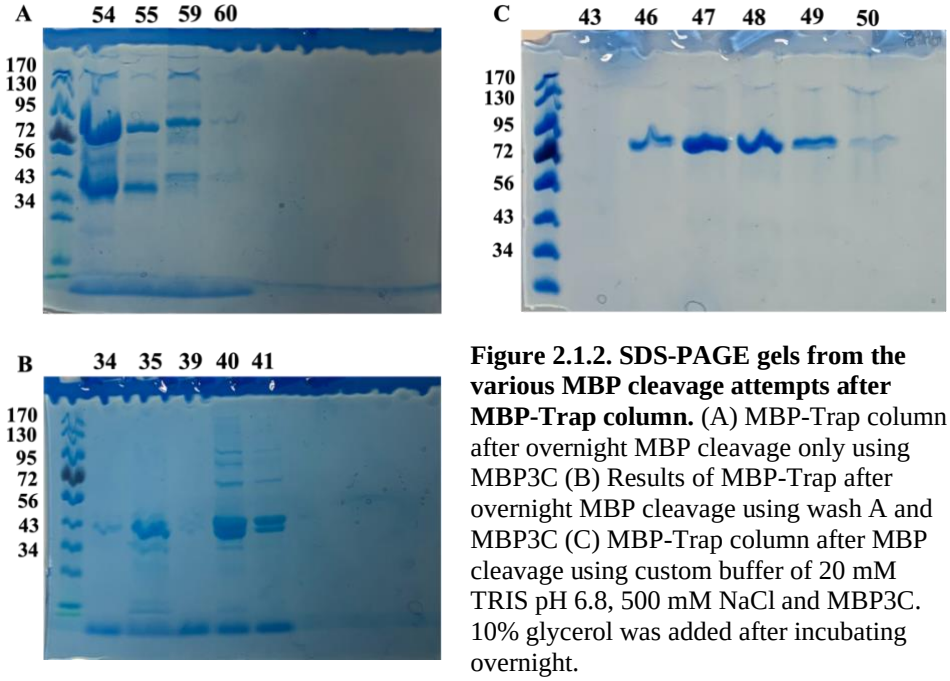
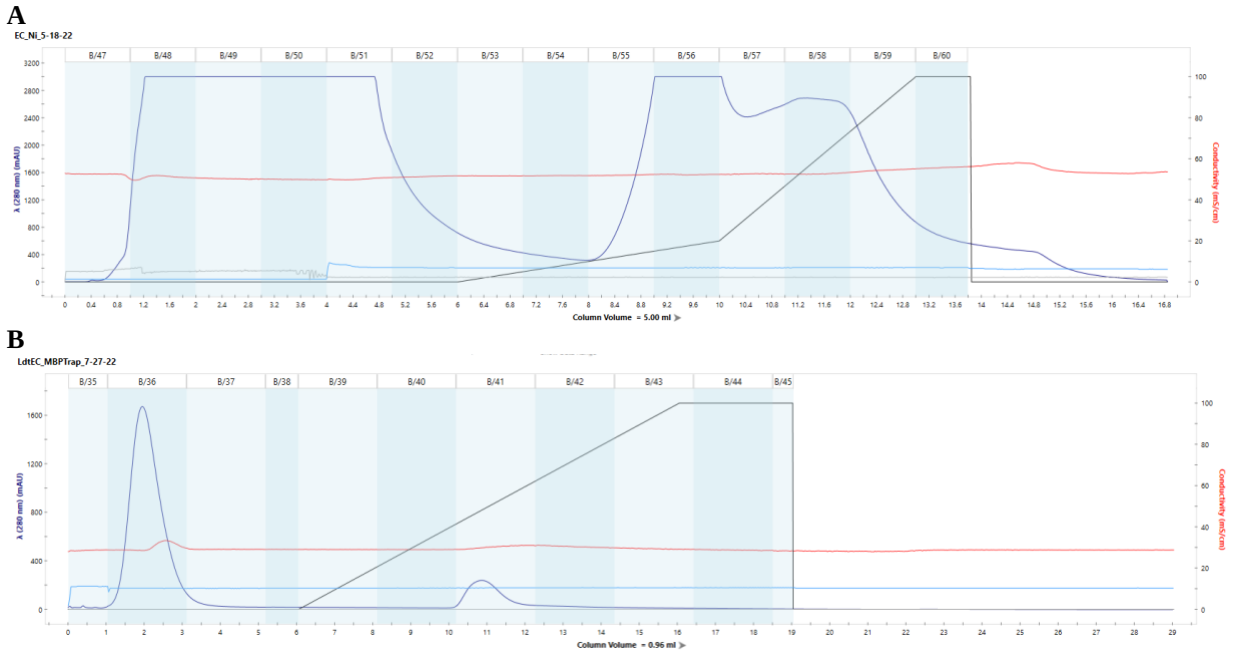


Figure 2.1.2. SDS-PAGE gels from the various MBP cleavage attempts after MBP-Trap column. (A) MBP-Trap column after overnight MBP cleavage only using MBP3C (B) Results of MBP-Trap after overnight MBP cleavage using wash A and MBP3C (C) MBP-Trap column after MBP cleavage using custom buffer of 20 mM TRIS pH 6.8, 500 mM NaCl and MBP3C. 10% glycerol was added after incubating overnight.

the LdtE-MBP fusion overnight with MBP-3C protease in buffer Wash A. This specific attempt resulted in almost no cleavage at all. This is evident by SDS-page showing protein at a size of ~78 kDa which is the fusion size of MBP-LdtE (**Figure 2.1.2 B**) Another attempt before the finalized procedure used a unique buffer of 20 mM Tris pH 6.8, 500 mM NaCl, and MBP-3C protease. After incubating overnight at 4.5ºC, 10% glycerol was added. This resulted in very faint lines suggesting that most of the protein had crashed out of solution **(Figure 2.1.2 C)**. The buffer used that resulted in the best results was 20 mM TRIS pH 6.8, 500 mM NaCl, MBP-3C protease, and 10% glycerol that was added before the incubation instead of after. This unique solution allowed for proper cleavage of the protease site without resulting in the protein completely crashing out of solution; further adjustments will likely be needed to maximize the protein yield (**Figure 2.1.1 B**). Following successful MBP cleavage, the LdtE was run through an MBPTrap which would bind to the cleaved MBP while LdtE would be in the flow through (**Figure 2.1.1 B**). Typically, this step did not separate the MBP and LdtE entirely so another nickel column and MBPTrap would be run afterwards for extra purification. Due to the many modifications that needed to happen to properly cleave MBP from LdtE, there was typically very little protein left before running the size exclusion column (SEC). Once the process of MBP cleavage was adjusted to obtain adequate levels of protein, it was then run on a SEC70 column and further purified (**Figure 2.1.1 C**). The overall process of protein purification was successful after the adjustments to the cleavage. Most attempts at protein purification did not result in enough protein to proceed with the SEC column or BCA assay. Once adequate amount of pure protein was present the end of the purification process, the protein was then quantified using a BCA assay. The first buffer used for the MBP overnight cleavage, just wash A, resulted in a significant amount of protein falling out of solution. This resulted in the BCA assay results being a concentration of 1.6 mg/mL. With the adjustment of the buffer used for the MBP cleavage, the concentration of LdtE increased to ~2.0 mg/mL.



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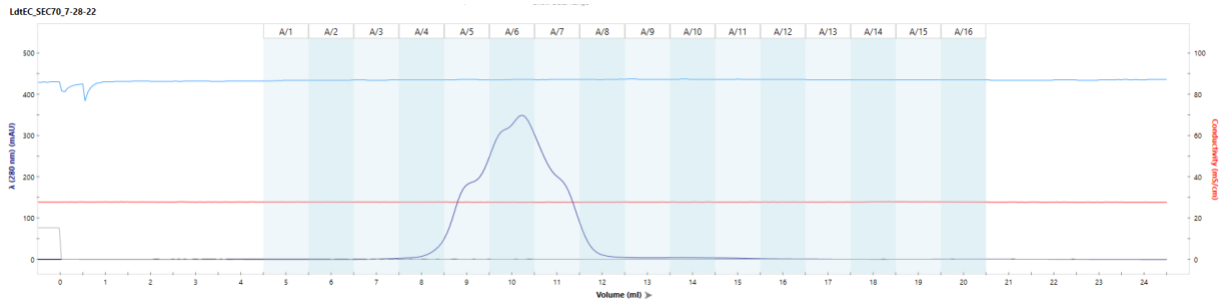
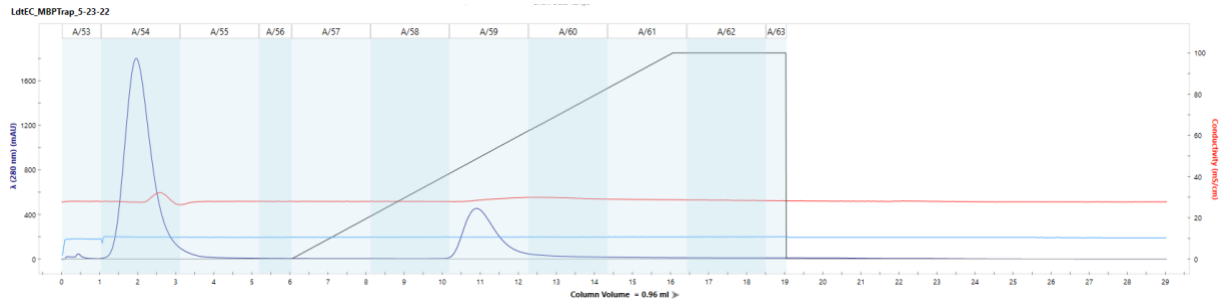


Figure 2.1.3. FPLC results corresponding to the gel images in figure 2.1.1. (A) Results corresponding to the Nickel-column gel in the mentioned figure. MBP-LdtE fusion is present in the second peak. (B) MBPTrap results corresponding to the mentioned figure. LdtE is present in the first peak at B36. (C) SEC70 column FPLC results. LdtE is present in the A5-8.

A



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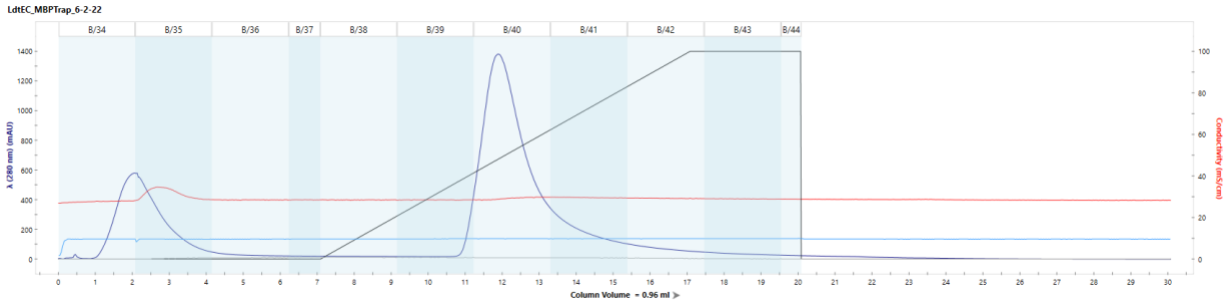

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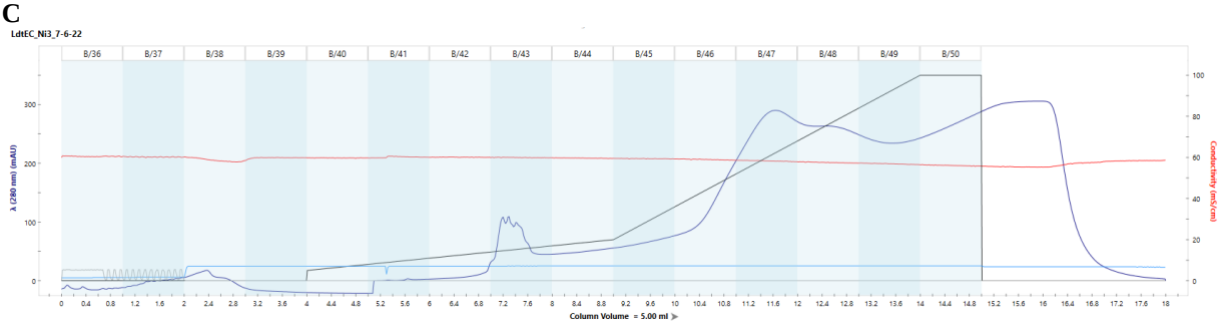

Figure 2.1.4. FPLC results corresponding to failed MBP cleavage attempts in figure 2.1.2. (A) MBP-Trap following the MBP3C protease cleavage. Corresponds to the [A] gel picture in the mentioned figure. Incubated with only the MBP3C in water. (B) MBP-Trap corresponding to image [B] in the mentioned figure. This followed overnight cleavage in wash A. (C) Nickel-column to further purify after an MBP-Trap. Corresponds to image [C] in the mentioned figure. Overnight cleavage in 20 mM TRIS pH 6.8, 500 mM NaCl, and 10% glycerol.

2.2.2 Kinetics Assay Results

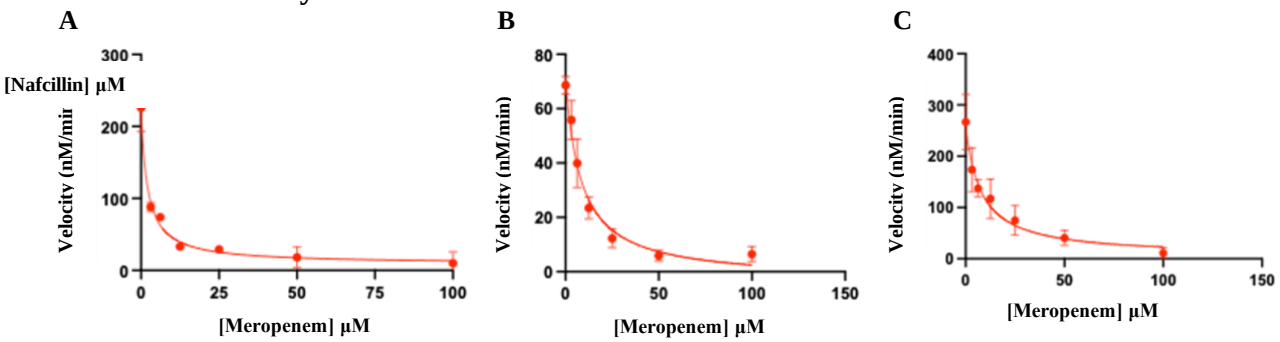

Figure 2.2.1. Inhibition of LdtE by meropenem. Velocity (nM/min) is plotted against concentration of meropenem (µM). (A) Reaction rate after an incubation period of 2-hours. IC_{50} = 2.05 ± 0.41 µM. (B) Reaction rate after incubation period of 1-hour. IC_{50} = 8.34 ± 1.86 µM. (C) Reaction rate after 30-minutes incubation. IC_{50} = 7.72 ± 2.41 µM.

Nitrocefin kinetics assays were performed to determine the rate of reaction between different β-lactams with L,d Transpeptidase in *Enterobacter cloacae* in vitro. Kinetics assays were performed using the MBP-LdtE fusion to determine how the protein might function against varying β-lactams *in vitro*. Using data from a panel of β-lactams studied previously, it was determined that meropenem and nafcillin had the highest rate of inhibition against LdtE. The two antibiotics were then tested *in vitro* with LdtE at varying incubation periods before adding nitrocefin and reading absorption. Following the plate reading, the IC_{50} was determined. The 2-hour incubation between LdtE and meropenem resulted in an IC_{50} of 2.05 ± 0.41 µM (**Figure 2.2.1 A**). The 1-hour and 30-minute incubation times had IC_{50} results of 8.34 ± 1.86 µM and 7.72 ± 2.41 µM respectively. Comparing these results, the difference in IC_{50} between the 2-hour and 1-hour incubation times is statistically significant. The difference between the 1-hour and 30-minute incubation however was not significant based on the standard deviation. This suggests a significant change in the effectiveness of meropenem inhibiting LdtE between 2-hour and 1-hour incubations. This is not replicated between the 1-hour and 30-minute incubation. Further testing with other incubation times would need to be done to

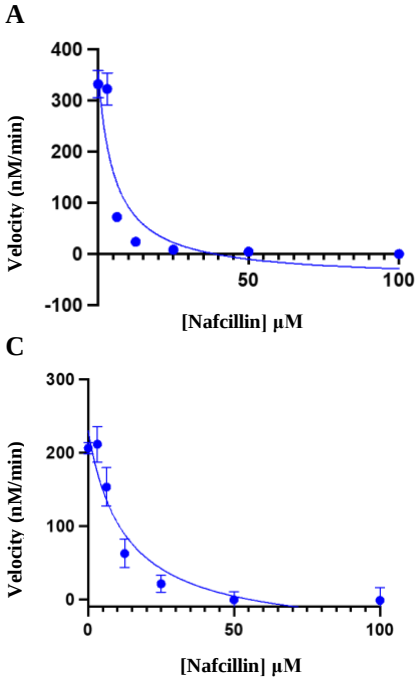

Figure 2.2.2. Inhibition of LdtE by nafcillin. Velocity (nM/min) is plotted against concentration of nafcillin (µM). (A) Reaction rate after an incubation period of 2-hours. IC_{50} = 5.04 ± 4.49 µM. (B) Reaction rate after incubation period of 1-hour. IC_{50} = 12.52 ± 8.15 µM. (C) Reaction rate after 30-minutes incubation. IC_{50} = 5.99 ± 5.45 µM.

better understand the effect of time on activity between meropenem and LdtE. Nitrocefin tests were inconclusive when done with nafcillin. This can be concluded based on the standard deviations for the IC_{50} results. When looking at the 2-hour incubation, the IC_{50} is 5.04 with a standard deviation of 4.49 μM **(Figure 2.2.2 A)**. This essentially means that the calculated logarithmic line does not fit the data points very closely. The data for the 1-hour incubation also has an incredibly high standard deviation with the IC_{50} being 12.52 ± 8.15 μM **(Figure 2.2.2 B)**. The IC_{50} for the 1-hour incubation increased significantly however with such a high deviation, it is unreliable. This is further shown by the sudden decrease in IC_{50} for the 30-minute incubation back down into the 5 μM range. The 30-minute incubation had an IC_{50} of 5.99 ± 5.45 μM **(Figure 2.2.2 C)**. The data for nafcillin was inconsistent between the different incubation times, ultimately preventing any conclusion from being drawn. The nafcillin nitrocefin assays will need to be performed again to support accuracy of the data already produced. The results provide a general direction on what antibiotics to use when performing minimum inhibitory concentration assays (MICs) in vivo.

CHAPTER 3

GENETIC KNOCKOUTS

3.1 Materials and Methods

3.1.1 Plasmid Preparation

A LdtE Kanamycin Knock-Out in pUC-GW-Amp plasmid (4034 bp) and a LdtD Tetracycline Knock-Out in pUC-GW-AMP plasmid (4435 bp) were used. The plasmids stocks were diluted to 200 ng/μL using Tris-EDTA (TE) Buffer (10 mM TRIS pH 8.0, 1 mM EDTA pH 8.0). The plasmids were then transformed into *E. coli* using chemically competent BioLabs inc. NEB5-alpha *E. coli* cells, a derivative of DH5α cells. 1 μL of the 200ng/μL plasmid stocks were added to an Eppendorf tube containing 50 μL of NEB5-alpha *E. coli* cells and incubated on ice for 30 minutes. The cell and plasmid mixtures were then heat shocked at 42ºC for 20 seconds. The solutions were incubated on ice again for 2 minutes, afterwards 1 mL of SOC medium was added to both. The cells were then incubated for 1 hour at 37ºC while shaking at 225 rpm. After the incubation, the cells were plated on LB carbenicillin plates and left to incubate at 37ºC for 48 hours. The cell colonies were used to inoculate a 5 mL of LB with 100 μg/mL carbenicillin and incubated while spinning at 37ºC overnight. 500 μL of the overnight cultures and 500 μL of 50% glycerol was used to create glycerol stocks for both plasmids. The rest of the overnight cultures were miniprepped using a Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit. The procedure used was provided with the kit.

3.1.2 Knockout Gene Isolation

In the LdtE KAN KO in pUC-GW-Amp plasmid, a kanamycin gene with LdtE homologous ends were present. In the LdtD TET KO in pUC-GW-AMP plasmid, a tetracycline gene with LdtD homologous ends was present. These genes were cut out using restriction enzymes in an overnight double digest. 10 μL of the miniprepped plasmids were added to separate Eppendorf tubes with 10 μL BioLabs inc. Cutsmart 10X buffer and 2 μL each of restriction enzymes BamHI and XhoI. MQ water was added for an overall volume of 100 μL in both. The double digests were incubated overnight at 37ºC. The entire double digest produced were then run on an agarose DNA gel (100 mL TBE Buffer, 1 g agarose, 5 μL ethidium bromide). The desired DNA fragments were then extracted using a Thermo Fisher Scientific Silica Bead DNA Gel Extraction Kit. The procedure followed was an industry procedure provided the kit. Afterwards, a portion of the extracted DNA was then run again on an agarose DNA gel to ensure proper extraction occurred.

3.1.3 Electrocompetence

ATCC strain *Enterobacter cloacae* were plated on Lennox Broth (LB) plates and incubated overnight at 30ºC. The resultant colonies were then used to inoculate a liquid 5 mL LB culture and incubated while rotating overnight at 30ºC. 500 μL of the overnight culture and 500 μL of 10% glycerol was used to make a glycerol stock while the remaining cell culture was used to induce a 500 mL flask of LB. The flask was shaken at 32ºC until it reached it reached an OD_{600} of ~0.5. The culture was taken out and centrifuged down for 20 minutes at 4500 rpm. The supernatant was discarded, and the rest of this process was done on ice. The cell pellet was gently resuspended with 50 mL of ice cold 10% glycerol and centrifuged again for 20 minutes.

The supernatant was discarded, and the cell pellet was gently resuspended again with 25 mL of cold 10% glycerol. It was centrifuged again for 20 minutes, and the supernatant discarded. The cell culture was again gently resuspended in 5 mL of cold 10% glycerol and centrifuged for 20 minutes. The supernatant was discarded, and the cell pellet was resuspended with 500 μL of 10% glycerol and aliquoted. The electrocompetent cells were stored at -80ºC.

3.1.4 Transformation

Bio-Rad 0.2 cm Electrode Gene Pulser Cuvettes and Eppendorf tubes were incubated on ice until cold. The electrocompetent *E. cloacae* cells were thawed on ice and 40 μL of the cell culture was transferred to each of the cold Eppendorf tubes. 1 μL of the LdtD-TET double digested plasmid was transferred to one of the cold Eppendorf tubes with the cells, and 1 μL of the LdtE-KAN double digested plasmid was transferred to the other cold Eppendorf tube. The cell mixtures were incubated on ice for 1 minute. The cell mixtures were then transferred to their own respective cold gene pulser cuvettes. Using a Bio-Rad MicroPulser set to Bacteria E2, the cell mixtures were pulsed and then immediately suspended in 1 mL of LB. The transformed cells were transferred to their own new Eppendorf tubes and incubated at 37ºC while shaking at 225 rpm for 1 hour. Following incubation, 200 μL of the 1 mL transformed cells were plated on antibiotic selection plates. The LdtD TET knockout cells were plated on LB agar plates supplemented with 10 μg/mL of Tetracycline. The LdtE KAN knockout cells were plated on LB agar plates that had 100 μg/mL of Kanamycin. The plates were incubated at 30ºC for 48 hours.

3.1.3 Colony PCR and Sequencing

The plates that managed to grow colonies suggested a successful transformation process. The individual colonies were then used in a colony PCR. 20 μL of 20 mM NaOH was transferred to a PCR tube. Using a pipette tip, the colony was scraped up and mixed into the NaOH PCR tube via pipetting. The tube was then incubated for 8 minutes at 100ºC in a Thermo Fisher Scientific MiniAmp Thermal Cycler. After incubation, the tube was vortexed for 15 seconds and the contents were transferred to an Eppendorf tube and centrifuged for 1 minute. The supernatant was used as the template DNA. The primers used were provided by Integrated DNA Technologies. The sequences for the LdtE KAN KO primers were CCACAGCTCCGCGTGCC CCAT and TTAGTTCAAACGCACCGGCA. The sequences for the LdtD TET KO primers were ATGGCCTGCTTCTCGCCGAA and TTCGGAATCTTGCACGCCCTC. PCR conditions were an annealing temperature of 60ºC at 35 cycles with a 5-minute extension time. After the PCR, the products were purified using a Thermo Fisher Scientific GeneJET PCR Purification Kit. The procedure used was the standard procedure provided with the kit.

The cleaned-up colony PCR products were sent to sequencing to determine if the homologous recombination knockouts were successful. The products were out into a tube with the proper primers and outsourced to be sequenced. The sequencing primers for LdtE KAN KO were AATGCAACCGGCGCAGGAAC and CCAGGTATTAGAAGAAT. The sequencing primers for LdtD TET were TTCGGAATCTTGCACGCCCTC and ATGGCCTGCTTCTCGCC.

3.2 Results and Discussion

3.2.1 Double Digest and Transformations

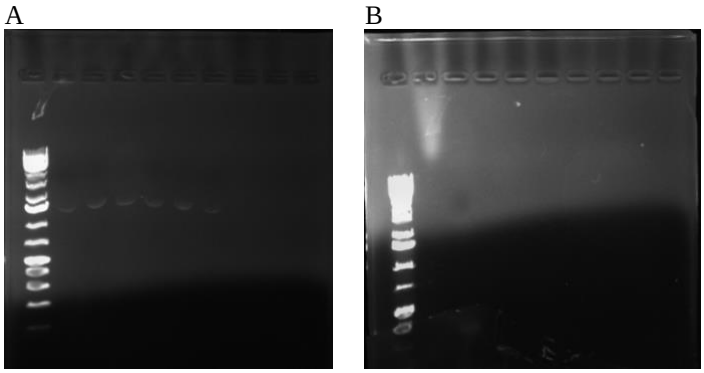

Figure 3.2.1. Results of the double digest gel extraction. (A) Confirmation gel for the double digest gel extraction of LdtE KAN. Bands visible at ~1500 kDa confirm the presence of KAN gene. (B) Confirmation gel for the double digest of LdtD TET. There was no DNA present.

The double digest and subsequent agarose gel extraction was successful in obtaining large amounts of DNA to then perform transformation. The LdtE-KAN knockouts and LdtD-TET double digest gene knockouts were run on agarose gels to confirm that the products were of correct length. The gel for the LdtE KAN gene showed faint bands at around the 1500 kDa mark which is precisely the size of the desired LdtE KAN gene (**Figure 3.2.1 A**). This provided the proper confirmation to use this DNA for transformations in *E. cloacae*. The gel showing the double digest gel extraction for LdtE TET **(Figure 3.2.1 B)** did not show any DNA present at all. This contradicts the nanodrop reading for the double digest, which calculated one digest at 2.9 ng/μL and the other at 7.0 ng/μL. With this nanodrop calculation, the LdtD TET gene was transformed regardless of the gel results. Both LdtE KAN and LdtD TET were transformed into *E. cloacae*. This was done to knockout the gene responsible for the affiliated Ldt gene, LdtE for the LdtE KAN and LdtD for the LdtD TET gene. The knockouts were plated on their appropriate antibiotic plates and incubated.

Upon the incubation of these plates, the LdtE KAN *E. cloacae* knockouts had 5 colonies grow on the plates while the LdtD TET *E. cloacae* knockouts grew 14 colonies. The 5 LdtE KAN colonies that grew were dense and large. The 14 LdtD TET colonies in comparison were small and took longer to grow, requiring a 72-hour incubation instead of 48 hours. The results of the sequencing for the LdtE KAN gene were inconclusive initially as it was discovered that the wrong primers were used. Both knockouts will be sent to sequencing in the future to determine if homologous recombination occurred in the correct section of the bacterial chromosome.

4.1 Conclusion

Nitrocefin assays showed that the incubation of meropenem with LdtE is important to the overall effectiveness of meropenem inhibiting LdtE, especially with the difference between 1- and 2-hour incubation periods. The longer incubation period incubation periods correlated to a lower IC_{50}. For this part of the project, it would be necessary to redo the nitrocefin assays for nafcillin as the assays performed previously were inconclusive. The genetic knockouts have so far been inconclusive. The gene was present in the bacterial chromosome however, without the sequencing results it is unknown if the gene has performed homologous recombination in the correct spot. Once it is determined that the knockouts were successful, the next steps for this project would be to perform minimum inhibitory concentration assays (MIC) do determine how the bacteria would perform against antibiotics with their Ldt genes knocked out.

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