ORIGINAL ARTICLE

Determining the Efficacy of CRISPR-Cas9 Mechanisms on the *uppP* Gene of *Klebsiella pneumonia* using Lysozyme Assay

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ABSTRACT

Introduction: Multidrug resistance bacteria is alarming worldwide. A lot of research were done and are ongoing to search for the best, convenient and economically affordable ways to fight them. With the latest genome editing tool; Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, this research was performed to develop a novel strategy to genetically modify the genome and inhibit the growth of *Klebsiella pneumoniae* (UPM ESBLKP1), an Extended Spectrum Beta Lactamases (ESBL) organism. **Methods:** A CRISPR-Cas9 vector was constructed together with guide RNAs designed specifically for the targeted *uppP* gene, a gene responsible for bacterial cell growth and protection. **Results:** The growth and cell wall integrity of the modified *Klebsiella pneumoniae* (ΔUPM ESBLKP1) were significantly inhibited and reduced, respectively. Interestingly, wild type *Klebsiella pneumoniae* showed a normal growth curve while modified strains showed a faster doubling rate when supplemented with Luria-Bertani media. In contrast, slower growth rate of modified strain was observed in the M9 minimal media. This explained the higher doubling rate of mutants on nutrient rich medium earlier is being related to gene recovery. They grew slowly in the minimal media as they were adapting to a new environment while recovering the *uppP* gene and surviving, proving the success of its gene modification. **Conclusion:** The developed CRISPR-gRNA system was able to modify the targeted *Klebsiella pneumoniae* gene hence providing an opportunity to develop a new drug for *Klebsiella pneumoniae* infection as an alternative to antibiotics.

Keywords: CRISPR-Cas9, ESBL, *Klebsiella pneumoniae*, *uppP* gene, Cell growth and protection

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INTRODUCTION

The degree of resistance and the emergence of the Extended Spectrum Beta-Lactamases (ESBL) organisms have raised the world's attention towards the usage of antibiotics to treat these organisms (1). These organisms are known to be able to hydrolyse the beta-lactams ring of the beta lactam antibiotic hence inactivate them. According to Coque et al. (2008), ESBL organisms in the early 1990s were classified into TEM and SHV group beta lactamase producers only. But now, a new group had been identified (CTX-M). These three groups of beta lactamase enzyme producer are the most prevalence found in ESBL organisms (2). In 1980, Ambler has classified the ESBL into 4 classes, which are Class A, B, C and D with reference to the amino acid similarity rather the phenotypic expression. Meanwhile Bush-Jacoby-Medeiros classified the bacteria according to their functional similarity of the substrate and inhibitor profile, which contribute to the 2be and 2d grouping (3). In Ambler classification, TEM and SHV can be found in class A, C and D meanwhile in Bush-Jacoby-Medeiros classification, all of them are under the 2be class. Meanwhile CTX-M group with currently 50 allelic variants was placed in the class A ESBL organisms by Ambler group classification (4). As in the Bush-Jacoby-Medeiros classification, CTX-M member were also classified into the 2be group (5).

In a research conducted by a research team in an intensive care unit of a Brazilian hospital, 21 out of 25 (84%) *Klebsiella pneumoniae* samples were identified as ESBL organisms (6). In another report of *Klebsiella pneumoniae* isolated from blood, 52.9% out of 159 blood isolates were also identified as being ESBL organisms (7). According to the US Centre of Disease Control and Prevention (2018), a total of more than 18 different bacteria are already resistant toward drugs and antibiotics with different levels of threat: urgent, serious,

and concerning. Up to date, most of the third-generation cephalosporin antibiotics are no longer working on these organisms. Even the strongest antibiotic available on Earth, Colistin, has met it rival, *mcr* gene. This gene is able to silent Colistin and was first identified in China (1). This beta lactamases producer is common in Gram-negatives bacteria and was first discovered in *Klebsiella pneumonia* and transduced to *Escheria coli* as stated by (83).

Clustered, Regularly Interspaced Short Palindromic Repeat (CRISPR) is a system that works when guide RNAs (gRNAs) identify a sequence that is parallel to the Photospacer Adjacent Motif (PAM) sequence which the gRNA specifically carries. The gRNA then attaches to a protein called a Cas9 (CRISPR associated) where it creates a cleavage on the targeted gene at the 5'-NGG-3' of the protospacer adjacent motifs (PAM) downstream complementing the gRNA target sequence (9). Thus, this system is also known as CRISPR/Cas. The *uppP* gene, is responsible for the formation of the cell wall peptidoglycan of gram-negative bacteria. The cell wall has been identified as one of the reasons for bacterial resistance toward antibiotics (10-12). Hence, the objective of this study was to modify the *uppP* gene so as to disrupt the function of the *Klebsiella pneumonia* cell wall using the CRISPR-Cas9 system.

MATERIALS AND METHODS

Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed using antibiotic plates. Eleven antibiotics were used namely Tetracycline (10 µg/mL; Oxoid, UK), Nalidixic acid (30 µg/mL; Oxoid, UK), Trimethoprim-sulphamethoxazole (25 µg/mL; Oxoid, UK), Cefotaxime (30 µg/mL; Oxoid, UK), Ampicilin (100 µg/mL; Oxoid, UK), Polymyxin B (300 units; Oxoid, UK), Chloramphenicol (25 µg/mL in ethanol; Thermo Fisher Scientific, USA), Streptomycin (100 µg/mL; Thermo Fisher Scientific, USA), Erythromycin (150 µg/mL; Thermo Fisher Scientific, USA), Clindamycin (2 µg/mL; Thermo Fisher Scientific, USA), Kanamycin (50 µg/mL; Thermo Fisher Scientific, USA), Vancomaycin (5 µg/mL; Thermo Fisher Scientific, USA) and Spectinomycin (50 µg/mL; Thermo Fisher Scientific, USA).

A sterile cotton swab was used to spread the bacterial culture onto the nutrients agar (1.5%) (Thermo Scientific, USA). After overnight growth at 37°C, the number of colonies formed on the plate was counted and their susceptibilities determined.

Plasmid modification

Plasmid extraction

The pCasSA plasmids (Plasmid no: 98211) were purchased from Addgene.org on a stab agar. The plasmid contained both the gRNA insert site and the Cas9 coding region, and carried the kanamycin resistance gene. Thus, to select the right *E. coli* strain containing the pCasSA plasmid, kanamycin antibiotic was added to Luria Bertani agar used for the selection. E. coli culture that carried the plasmid was streaked on Luria-Bertani (LB) (MERCK, Canada) agar supplemented with the antibiotic kanamycin for selection and growth overnight at 30°C. Single colonies on the fourth quadrant were then picked and grown in LB broth (MERCK, Canada) (supplemented with kanamycin) overnight at 30°C. The culture was centrifuged at 11 200x*g* for 15 minutes to pellet down the bacterial culture. The pellet was then subjected to plasmid isolation using the innuPREP Plasmid Kit 2.0 by Analytik Jena (Germany), following the manufacturer's protocol. The isolated plasmid was observed on an agarose gel to confirm the size and was subjected to the double digestion method to remove the kanamycin resistance gene and to replace it with the chloramphenicol resistance gene.

Double digestion of pCasSA plasmid and Chloramphenicol resistance gene

The plasmids were double digested according to the method suggested by Chow (2005) with slight modifications. The plasmids concentration observed was 36 ng/µl. Then, 3.65 µl of plasmids were added to 2 µl of Tango buffer, followed by 1 µl each of the BgIII and NcoI restriction enzymes and 12.35 µl of ultrapure water totaling up to 20 µl reaction mixture. The mixture was then incubated for 1 hour at 30°C and terminated by heat at 60°C for 15 minutes. The same procedure was used for the chloramphenicol resistance gene. The chloramphenicol gene (having both BgIII and NcoI restriction sites at the 5' and the 3'- ends respectively) was purchased from Apical Scientific Sdn Bhd in powdered form and resuspended in sterile Milli-Q water (Biocel System, Europe). This gene was designed with the restriction sites of both restriction enzymes to enable the ligation of the plasmids with the new resistance gene effectively. The concentration of the gene was 32 ng/µl. Both digested samples were kept at -20°C until used.

Ligation of pCasSA plasmid with Chloramphenicol resistance gene

The digested pCasSA and the chloramphenicol resistance gene were subjected to the ligation protocol. The reaction was set-up as follows: 2 µl of T4 DNA ligase buffer, 2 µl of T4 DNA ligase enzyme, 5 µl of chloramphenicol resistance gene, 3 µl of digested pCasSA plasmid and 8 µl of sterile-filtered ultrapure water. The ultrapure water was sterile-filtered using a PES filter with 0.22 µm pores (Bioflow, New Zealand). The reaction volume was 20 µl in total. The ligation was performed at 37°C for 4 hours, to optimize the reaction. After 4 hours, the reaction was terminated at 60°C for 15 minutes. The ligated samples were kept at -20°C until used.

Selection of plasmid

The ligated plasmids were transformed into *E. coli* DH5α (Yeastern Biotech) competent cells via high voltage

electroporation according to Binotto et al. (1991). The transformed *E. coli* were then plated on LB agar supplemented with the antibiotic chloramphenicol. The culture was then incubated at 30°C overnight. After the overnight incubation, a single colony was picked from the plate and then cultured into LB broth supplemented with chloramphenicol. It was subjected to incubation as mentioned above with shaking at 4x*g*. The plasmid were then termed as pCasSA+Cm^R for the downstream analysis.

gRNA design and insertion into pCasSA+Cm^R

The gRNAs of the *uppP* gene of *Klebsiella pneumoniae* were analysed using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/SCORE). Two selected gRNAs (gRNA1:5′-TCTAGAGGCCGCTGCCGCGACGATAAAGGGTCTAGA-3′ and gRNA 2: 5′-TCTAGAGGTCAGCCCCATCACCACCGCGGGTCTAGA-3′) were then being synthesized by Integrated DNA Technology Inc. (Malaysia). XbaI restriction sites were included at the 5′-and 3′-ends of both gRNAs sequences. Both plasmid and gRNAs were subjected to digestion with XbaI. The reaction set-up and the ligation reaction were performed as mentioned above.

Transformation of *Klebsiella pneumonia* with pCasSA+Cm^R

Klebsiella pneumoniae strain designated as UPM ESBLKP1 was obtained from Luria Bertani streaked agar plate of clinical sample of compromised patient submitted to the Lab of Microbiology, Faculty of Medicine and Health Sciences, UPM. Single colony was isolated and incubated into Luria Bertani broth with 4xg shaking overnight at 37°C. 10 tubes of stocks were prepared by finalizing the volume to 50% glycerol stock using 100% glycerol (R&M, UK). *Klebsiella pneumoniae* competent cells were then prepared following the method of Binotto et al. (1991) with bacterial culture of the colony-forming unit (CFU) of $10x10^8$ (14). The competent cells were prepared by inoculating fresh overnight culture into new fresh LB broth. The culture was then allowed to grow at 37°C with shaking at 4x*g* until the OD640 reached 0.75. Once the desired concentration was obtained, the cell culture was placed on an ice-water bath for 15 minutes to chill. The culture was then harvested by centrifugation at 4000x*g* for 10 minutes at 4°C. The pallet obtained was then washed two times with the original volume of culture with 10mM HEPES (Biowest, France) and one time with 100 µL of 10% glycerol. The culture was then centrifuged again at the above mentioned parameter. The competent cells were then resuspended with 1.5 mL of 10% glycerol and kept at -20°C until used.

The ligated pCasSA+Cm^R plasmid containing the gRNAs was then transformed into the *K. pneumoniae* competent cell by high voltage electroporation as suggested by Binotto et al., (1991). Three modifications namely uppP1 (treated with gRNA1), uppP2 (treated with gRNA2) and uppP3 (treated with a combination of gRNA1 and gRNA2) were made. Then, 1mL of SOC medium was added to the reaction immediately. The transformed cultures were allowed to enter the recovery phase by incubation at 37°C with shaking for one hour before being spread on LB + chloramphenicol agar with negative and positive controls. The positive control involved transformation of *Klebsiella pneumonia* strain UPM ESBLKP1 with pCasSA+Cm^R plasmid. The negative control only involved plating of *Klebsiella pneumonia* strain UPM ESBLKP1 without transformation with pCasSA+Cm^R plasmid. Plated cultures were then incubated at 37°C overnight.

Colony morphology and Growth Observations

The colonies which appeared on the transformed plates were counted and their morphologies were noted. A single colony was then picked out from the plate and subjected to growth observation by growing them in Luria-Bertani broth (MERCK, Canada) and M9 minimal medium (15). These cultures were observed using a spectrometer at OD600 every hour for 16 hours (16). The results were then plotted as a single graph for the respective media.

Sequencing

A single colony each of the wild type and the modified strains were picked and subjected to the colony PCR technique. The PCR amplicons were then submitted for sequencing to 1st Base Asia (https://order.base-asia.com/). The PCR was performed by initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 1 minute, elongation at 56°C for 1 minute, extension at 72°C for 1 minute. After completing 35 cycles, the PCR samples were subjected to final extension at 72°C for 3 minutes and held at 4°C until used.

Cell wall integrity

Lysozyme assay was performed to determine the cell wall integrity by following the method suggested by Kim et al. (2013). Lysozyme buffer was prepared by combining 20 mMTris·Cl (pH 8.0) (R&M, UK), 2 mM sodium EDTA (Sigma-Aldrich; MERCK, Germany), and 1.2% Triton® X-100 (Sigma-Aldrich; MERCK, Germany). Then, 100 µL Lysozyme (10 mg/ml) (Vivantis, Malaysia) was added to the solution immediately before use.

Statistical analysis

Two-way ANOVA was performed using the software SAS 9.4 M (SAS Institute Inc.) to study the significance of the findings.

RESULTS

Antibiotic Susceptibility Test

Result shows the *Klebsiella pneumonia* strain was sensitived to Polymyxin B and chloramphenicol while resistance to other nine antibiotics tested for screening with abundant growth on the plate. This result is in

agreement with previous study by Sood (2016) which mentioned that chloramphenicol is still potent towards multidrug resistance bacteria including the ESBL organisms. Hence, chloramphenicol was selected to perform the downstream analysis.

Plasmid Modification

After overnight incubation, the chloramphenicol plate which was spread with *E. coli* containing pCasSA plasmids showed no growth. Meanwhile, the chloramphenicol plate that was spread with the modified pCasSA plasmids namely pCasSA+Cm^R showed positive growth. This result indicated the positive insertion of the chloramphenicol resistance gene replacing the kanamycin gene in the pCasSA plasmids.

The expected size of the *Cm^R* gene insert was 660 base pairs (bp) and it was successfully amplified (Figure 1). Hence, the replacement of the kanamycin resistance gene with the chloramphenicol resistance gene was achieved.

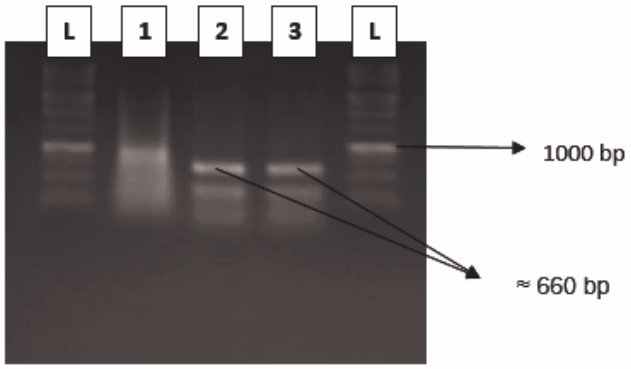

Figure 1: Agarose gel detection of insert gene (CmR) at 1% agarose concentration. Lane L: 1 kb RTU DNA ladder (ABM, Canada); Lane 1: control of CmR gene; Lane 2: amplified CmR gene from pCasSA+CmR plasmid.

Transformation of pCasSA+Cm^R plasmid in *Klebsiella pneumoniae*

The ligated pCasSA+Cm^R plasmid with gRNAs was transformed into *K. pneumoniae* using high voltage electroporation (13). pCasSA+Cm^R plasmid without gRNA was used as control, transformed into *K. pneumoniae* and named as $_{contr}$*K. pneumoniae*. Transformed *K. pneumoniae* growth was then observed after 16 hours' incubation. The results of the transformation are shown in Figure 2. It was observed that the growth of the modified strains was much slower compared to the $_{contr}$*K. pneumoniae* The number of colonies which appeared on the plates for the modified strains were lower compared to the $_{contr}$*K. pneumoniae*. However, only plates C and D were countable.

Plate A is the control plate containing pCasSA+Cm^R ($_{contr}$*K. pneumonia*). Plate B and C are transformed *K. pneumoniae* with gRNA 1 and gRNA 2 respectively. Plate D is the transformed *K. pneumoniae* with both gRNA 1 and gRNA 2. The mutants obtained from plates

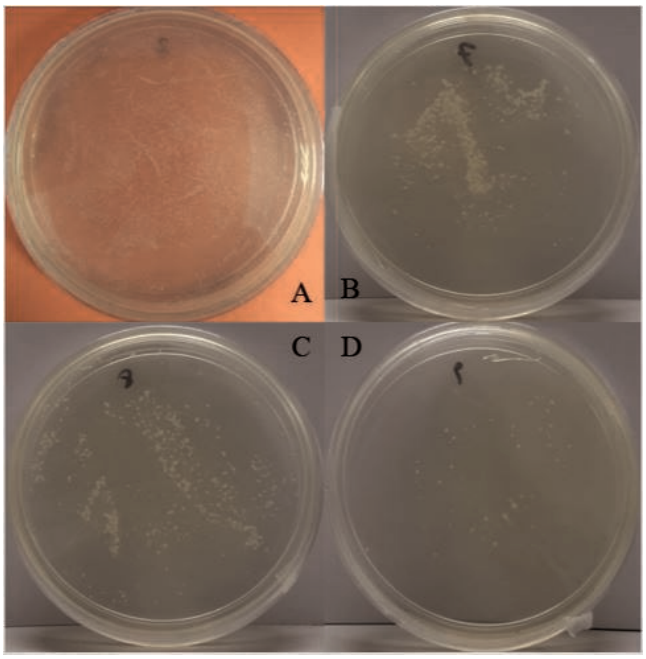

Figure 2: Transformed K. pneumoniae grown on plate with chloramphenicol (1.0mg/ml) antibiotic after overnight incubation. A: Transformed K. pneumoniae with pCasSA+CmR without gRNA as control. B: Transformed K. pneumoniae with pCasSA+CmR with gRNA1. C: Transformed K. pneumoniae with pCasSA+CmR with gRNA2. D: Transformed K. pneumoniae with pCasSA+CmR with gRNA1 and gRNA2.

B, C and D were termed as Mutant uppP1, Mutant uppP2 and Mutant uppP3 respectively. The results showed reduction of the growth of the modified strains compared to the control. The colony counts are shown in Table I and it was noted the mutant strains had less growth compared to the control.

Colony Morphology and Growth Observations

Colonies which appeared on the plates were observed and recorded based on their colours and shaped. Colonies which appeared on plate A were larger in sizes with yellowish colour as expected from *K. pneumoniae*. Meanwhile, the modified strains' colonies in plates B, C and D appeared to be lighter in colour; being whitish colonies. However, the shapes of the colonies observed were all the same. They were circular, raised-convex shaped.

A single colony inoculated in 100 mL LB broth (MERCK, Germany) was observed for its growth curve (Figure 3A). The growth of the control underwent a longer lag

Table I: Number of colony count per plate of wild type against the modified strains.

Plate	Colony counts
A	Full plate (TNTC)
B	863
C	415
D	103

Plate A, B, C, and D are shown in Fig. 2
*TNTC = Too Numerous to Count

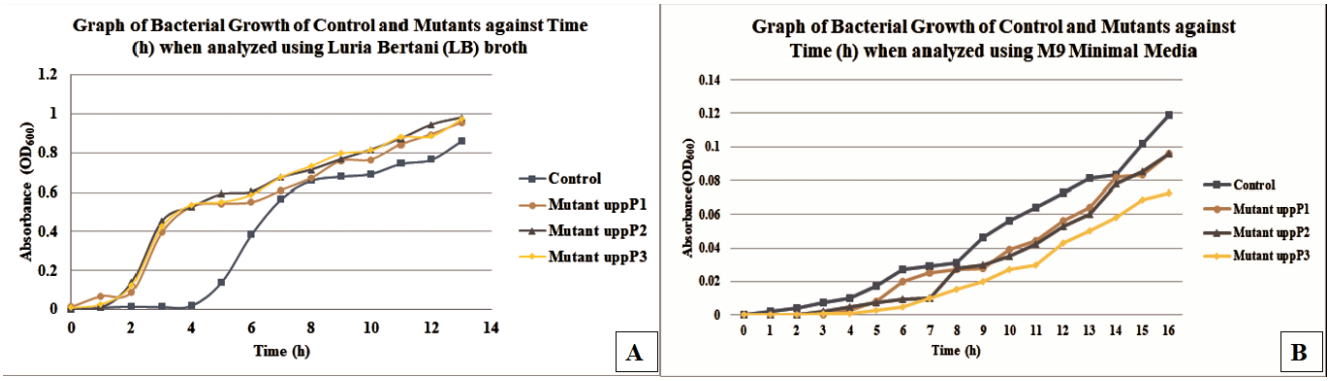

Figure 3: Graphs representing the Growth Curve of Control against Mutants. A: Graph representing the growth curve of the Control against the Mutants in Luria Bertani (LB) broth. B: Graph representing the growth curve of the Control and the Mutants in M9 minimal media.

phase before entering the log phase. On the other hand, all the modified strains required two hours to enter the log phase from the lag phase. Since the modified strains entered the lag phase earlier than the control, thus, they entered the stationary phase earlier. The graph shows the modified strains entering the stationary phase after three hours compared to the control which required eight hours of growth.

Mutant bacteria grew at a faster rate and doubled very fast due to the modifications on their genome (17). They also showed that the bacterial growth rate depended on both intrinsic and extrinsic conditions faced by the bacteria. In this study, the mutant bacteria needed to grow faster to recover the *uppP* gene sequence. As could be seen in the graph, all mutant strains were still growing after entering the lag phase at the 6-hour mark even though they entered the lag phase at the 3-hour mark. This indicated that even in the lag phase the bacteria were still trying to survive the condition. However, the control strain grew steadily and entered the lag phase after 8 hours. Since the media were supplemented for the bacteria at high volumes, there were still nutrients left for them to uptake and grow. Thus, the bacteria kept growing even though they entered the lag phase. Even though there were slight increments of readings at hours 11 and 12, the graph for the control strain was as expected. The strains were then grown in M9 minimal media (15) to confirm the finding. Interestingly, the mutants' growth in M9 media was lower compared to the control strain (Figure 3B). Perhaps, this was due to the control strain not needing to recover the *uppP* gene unlike the mutants. Thus, they grew faster than the mutant strains. Hence, this explained the higher doubling rate of the mutants from the previous growth analysis on LB medium being related to gene recovery. In contrast, they grew slowly in the minimal media as they needed to adapt to a new environment while recovering the *uppP* gene at the same time.

The control strain started to double at 4 hours culturing time, but the mutant strains started to double at 6 hours culturing time. This delay indicated that the mutant strains underwent DNA repair mechanisms of the damaged gene. The growth curve showed that the *uppP* gene might be disrupted and the mutant strains were *uppP* deficient.

Sequencing

The modified strain sequences were compared with the control strain at the region where the gRNAs were designated. From the sequencing result, it was noted there was no obvious deletion found in all the mutants except for Mutant upp1 (Table II). This might be the result of the DNA repair mechanisms during the cell growth.

Table II: Arrangement of the nucleotide sequences of wild type against modified strains. Mutant uppP1: transformed with pCasSA+Cm^R carrying gRNA1. Mutant uppP2: transformed with pCasSA+Cm^R carrying gRNA2. Mutant uppP3: transformed with pCasSA+Cm^R carrying gRNA1+gRNA2. There are no changes in sequences of all transformed strains except for Mutant uppP1.

Sample	Sequence	
	gRNA1	gRNA2
Mutant uppP1	CCGCTGCCGCGACGA-TAAAG	TCAGCCCCATCACCAC-CGCG
Mutant uppP2	-CGCTGCCGCGACGA-TAAAG	TCAGCCCCATCACCAC-CGCG
Mutant uppP3	CCGCTGCCGCGACGA-TAAAG	TCAGCCCCATCACCAC-CGCG

Cell wall integrity

The lysozyme assay was used to determine the cell wall integrity of the modified strains. From the graph (Figure 4), all modified strains showed weaker cell walls compared to the control. The modified strains showed lower readings compared to the control for the first 20 minutes although they were having the same concentration (0.77 at OD_{600}).

This might indicate that the cell walls of the modified strains were disturbed and had low associations of lysozyme compared to the wild type. Mutant uppP3 showed the lowest absorbance reading hence it had the weakest cell wall property. Two-way ANOVA analysis showed significant differences between the control and the mutant strains (Figure 5).

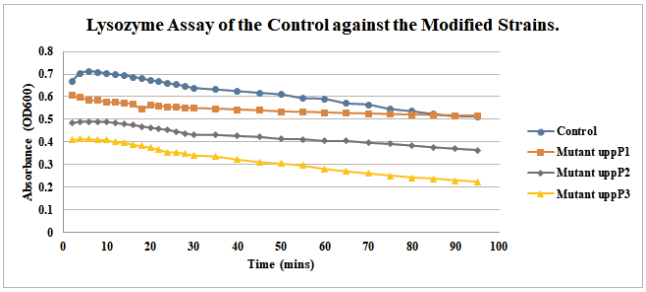

Figure 4: Lysozyme Assay of the Control against the Modified Strains

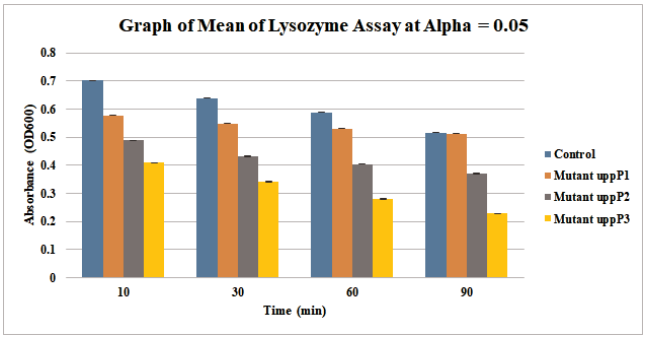

Figure 5: Graph bar of the mean at Confident Interval (CI) of 95% (Alpha = 0.05)

DISCUSSION

ESBL *K. pneumoniae* is known to be resistant to many antibiotics (19). Thus, normal or even the third-generation antibiotic cephalosporin cannot be used on it. Our antimicrobial study showed UPM ESBLKP1 to be resistant to Tetracycline, Nalidixic acid, Trimethoprim-sulphamethoxazole, Cefotaxime, Ampicilin, Polymyxin B, Streptomycin, Erythromycin, Clindamycin, Kanamycin, Vancomaycin and Spectinomycin and sensitive towards Chloramphenicol even at 1.0 mg/ml concentration. These antibiotic resistance and susceptibility were consistent with the claim made by Sood (2016), in that most of the ESBL organisms are still sensitive towards chloramphenicol. Morosini et al., (2016), performed an experiment in which ESBL Enterobacteriaceae was tested with a wide range of classes of antibiotics and they found that chloramphenicol was still effective against ESBL organisms. Due to this finding, we constructed and modified the original pCasSA plasmid which originally carried the kanamycin/neomycin resistance gene to carry the chloramphenicol resistance gene (Cm^R).

In our study, we observed the colony formations of the control and the mutant strains of ESBL *Klebsiella pneumoniae* and proved that disturbing the *uppP* gene could restrict the growth of the bacteria due to cell wall damage. It was also noted that the mutant strains might have weaker cell wall compositions compared to the control when treated with the same concentration of lysozyme as shown in the graph readings. This agreed with the results of the study performed by Kim et al., (2013) who concluded that by having a mutation in the *uppP* gene, the *Bulkholderia* cell wall formation was disturbed. Thus, they could not establish symbiotic interaction with the host and was unable to survive. We observed that colonies of the modified strains were lower in numbers and possessed whitish colonies compared to the control strain which expressed the functional *uppP* gene. This might be due to the modified strains needing to recover the gene by obtaining a fully functional gene from other colonies that were not modified throughout the process so that they were able to survive. Growth performance analysis using LB and M9 minimal media showed different growth patterns between the control and the mutant strains. The control strain exhibited a normal growth curve while the mutant strains had slower growth rates. Lei and Schmidt (2018) stated that when a bacterium lost one of its main genes for growth, it would start to grow faster than at the usual rate in order to recover the removed or modified gene. Thus, when analysed using the LB medium, the doubling rate of all mutant strains were higher than that of the control. Growth analysis using both LB and M9 media proved that the CRISPR modification on the *uppP* gene was successfully performed.

According to Ghachi et al. (2005), the synthesis of peptidoglycan was performed by multiple genes including the *uppP*, *ybjG* and *pgpB* genes. They mentioned that all three genes needed to be deleted in order to abolish the formation of peptidoglycan. However, the disruption of the *bacA* (*uppP*) gene caused a significant depletion but a non-lethal loss to the formation of peptidoglycan. According to Bickford and Nick (2015), the *bacA* (*uppP*) gene accounted for 75% of the undecaprenyl pyrophosphate phosphatase activity in bacteria.

On top of bacterial peptidoglycan synthesis, the *uppP* gene also synthesized the LPS (Lipopolysaccharides) unit of the cell wall and a few other cell membranes related compounds. A few papers had reported that the *uppP* gene also known as *bacA* gene is responsible for the antibiotic resistance of bacitracin (15, 23-25). Thus, deletion or knockout of this sequence of the bacteria would inhibit both antibiotic resistance and cell wall formation. In this study, the results tabulated in Table II show the control against the modified strain *uppP* gene's sequences respectively at the targeted sites of gRNA. Even though only one modified strain had indel deletion, it showed that the modification using the CRISPR/Cas system was working. The modification performed in this research did not include the introduction of a new gene fragment. It only involved a targeted gene for modification. Thus, a deletion of a single nucleotide that was found in the Mutant uppP1 was expected as a non-homologous end-joining modification. After treatment with the CRISPR mechanisms, the DNA repair depended solely on the host cell. There are various types of DNA repair mechanisms; direct repair, template-dependent DNA repair, mismatches repair and others

(26-28). Two possible repairs that may be involved in this study are direct repair and template-dependent DNA repair. Rao and Prasad (2016) mentioned that direct repair involves chemical reversal that does not damage the phosphodiester backbone of the DNA. Meanwhile, template-dependent DNA repair utilizes the antiparallel sequence of the DNA so that the sequences are conserved on both strands (28). Bhaya et al. (2011) in their review stated that CRIPSR/Cas was not only responsible as a defence mechanism but also played a role in the host cell to maintain the genetic material. Due to the fact that our *K. pneumoniae* survived and grew on the plate might indicate that they had recovered the sequence to the fullest and managed to synthesise the peptidoglycan needed for cell wall formation. Thus, there were no differences in the sequences of the living, successful transformants. The sequences analysed were the sequences from the established colonies in the plate, thus it was expected that the sequence for the *uppP* gene was present. Since both gRNAs targeted either the upstream or the downstream sequences, thus the sequence could be repaired by copying the sequence from the non-modified bacteria present in the cultures of all the mutants. This was shown in the growth of Mutant uppP3 as the *uppP* gene was targeted for removal; it grew at the lowest rate when compared to Mutant uppP1 and Mutant uppP2, as it needed to recover the whole gene.

The lysozyme assay was used in this research because of the ability of lysozyme itself to denature the cell wall of bacteria. Thus, by measuring the concentration of bacteria during cell wall denaturation, the cell wall stability could be observed. Wild type bacteria usually had a higher reading of the absorbance at OD600 whereas a modified strain usually had a lower reading. The results obtained from this research were consistent with the analysis performed by Kim et al., (2013) who found that bacteria with wild type *uppP* gene had a higher reading compared to modified *uppP* deficient strains. But when a modified strain (*uppP* deficient) was introduced with a fragment of the full sequence of the *uppP* gene, the reading of the absorbance was as high as that of the wild type. From the lysozyme assay, we concluded that cell wall integrity depended on the *uppP* gene. When the gene was disrupted, the absorbance representing cell wall stability became lower from what it was supposed to be. From the results of this research, we also observed that the modified strains had lower readings than the wild type. Moreover, the strain which had flanking gRNA targeted sites had the lowest absorbance reading. Hence, we suggest that to efficiently edit bacteria genomic materials using the CRISPR/Cas system, a flanking set of gRNA is required to increase the efficiency of the gRNA.

CONCLUSION

This study showed the successful modification of the *uppP* gene using the CRISPR/Cas9 mechanism. The modified strains showed lower rates of growth as observed in the growth curves plotted but their growths were faster than that of the control strain. It was also noted that the cell wall integrity of the mutants had been reduced and weakened.

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