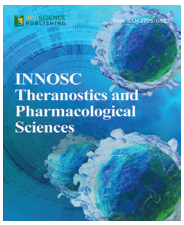
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RESEARCH ARTICLE

Horizontal Gene Transfer: Generating Antibiotic-resistant Bacteria

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Abstract:

This study aims to understand the gene mobility and its benefits. In this experiment, we transferred the significant gene horizontally under the appropriate condition and generated an antibiotic-resistant bacterial strain. The process of gene mobility is known as horizontal gene transfer, in which three different modes have been acknowledged that are transduction, transformation, and conjugation. Conjugation is a simple natural occurrence of cell-to-cell contact for transferring gene. The gene sequence that was analyzed in this study is responsible for tetracycline resistance in *Proteus mirabilis*; the gene was transferred horizontally to *Klebsiella pneumoniae*. Two methods were used for the conjugation, namely, plate- and broth-mating. The conjugation results were analyzed statistically, where events of probability across the time intervals at the highest spectrophotometric absorbance of 0.43 are tabulated. Broth mating was performed in 25 samples and the probability of an event is successful at $P = 0.88$. Further, broth mating was accredited to be better than plate-mating as claimed with a 95% confidence interval that yields the value of Z normal test of 4.49. This study suggests a feasible method for generating drug-resistant bacterial strains for use in medical research and industrial applications.

Keywords: Horizontal gene transfer, Antibiotic-resistant bacteria, *Proteus mirabilis*, *Klebsiella pneumoniae*, Tetracycline resistance gene

1. Introduction

Bacterial species are undeniably unique in nature due to their high survival rate in a wide range of environmental conditions [1-3]. Different species or strains that are growing under the same stress or unfavorable and stringent conditions possess genes that aid in the adaptation to survive in extreme habitats or environments [4,5]. The species or strains that do not have the specific genes have to rely on the bacterial species that possesses resistance gene. Thus, the species that cannot thrive well needs to overcome or survive through the stringent condition. In this context, horizontal gene transfer (HGT) that helps transfer genes for survival can transfer antibiotic-resistant genes from one bacterial strain to another strain that is susceptible to a particular antibiotic [6,7].

The antimicrobial-resistant bacteria have been long used in human vaccines; specifically, specific enzymes were extracted from the bacteria to counteract certain

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fatal infectious diseases [8,9]. However, the success of the extraction and application of the bacteria is bound for limited scope due to the constraints of methodology. It is due to the involvement of the same bacterial species. New evolution has changed the picture of the traditional gene transfer and proved that more benefits can be taken into account by mating different bacteria species [10].

Resistant bacteria possess several benefits to the society despite the known medical disadvantages [11-13]. However, the benefits of antimicrobial-resistant bacteria are largely ignored [14]. Moreover, many researchers lack the skills to identify the hidden features of resistant bacteria. Stubborn antibiotic-resistant bacteria continuously survive in the living system, irrespective of the consumed drugs [15]. Clearly, the bacteria impregnate fragment of the gene encoded for resistance against the harsh environment. Hence, this feature allows the bacteria to have a major advantage to overcome the current issues with most susceptible bacterial species for experimentation.

On the other hand, the susceptible bacteria are usually weak and easily destroyed by the drug. Recently, various studies proved that parts of gene fragment in weak bacteria, such as *Klebsiella pneumoniae*, have a characteristic of destroying pathogens in human oral mucosal system, thereby stimulating immune system to release specific enzymes that are capable to alter the binding site of many pathogenic agents to inactivate them [16]. The weak bacterial species, however, are incapable of surviving. The problem statement of this study is how to make the weak bacteria become stronger and capable to survive. In this context, HGT could be a possible solution. The traditional genetic tree of Darwinian theory only allows transfer of genes from the same species of bacteria by sexual or asexual reproduction [16]. The theory has stated that different species of bacteria are incapable of mating. However, it has been scientifically proven that different mating modes can be performed to successfully produce different bacterial species [17].

Susceptible bacteria species includes *Klebsiella*, *Streptococcus*, and *Enterococcus*. Our research emphasized more on *Klebsiella* species because these species are often used in the laboratory due to its simplicity. *Klebsiella* species are Gram-negative, non-motile, encapsulated rod-shaped bacteria with swarming ability, and belong to the family of Enterobacteriaceae [10]. These bacteria yield lysine decarboxylase, cannot produce ornithine decarboxylase, and are generally positive in the Vogues Proskauer test [18]. On the other hand, one of the resistant bacteria commonly used for gene transfer is *Proteus* species. These bacteria are of Gram-negative, motile, and aerobic rod-shaped bacilli that belong to the family Enterobacteriaceae. Members of the Enterobacteriaceae family commonly have a range of size from 0.3 to 1.0 mm in width and 0.6–6.0 mm in length [19]. They are positive with urease test and having swarming motility on the solid media. Infection by this species can be injurious to the urinary tract, mucosal membrane, and lungs [18]. In this study, we applied *Proteus mirabilis* and *K. pneumoniae* for the process of HGT. *K. pneumoniae* (recipient) was made resistant to tetracycline by receiving the tetracycline resistance gene from *P. mirabilis* (donor). The primary objective of this investigation is to optimize the conditions for generating a wide spectrum of antibiotic-resistant strains so that they can be amenable to medical research and industrial applications.

2. Materials and methods

2.1. Bacterial strains and culturing

Two types of bacterial strains were provided by Asian Institute of Medical, Science, and Technology, namely, *P. mirabilis* and *K. pneumoniae*. These bacteria were first cultured on the Luria-Bertani (LB) agar plates (peptone from casein [10.0%], yeast extract [5.0%], and NaCl [5.0%] in distilled water). *P. mirabilis* harbors multiple antibiotic- and heavy metal-resistant plasmid; therefore, it was used as the donor. The recipient bacterium was determined to be *K. pneumoniae*, which is usually found in the natural habitats, such as soil.

2.2. Antimicrobial susceptibility test

The bacteria strains were tested for their antibiotic resistance characteristics. All bacteria were streaked on LB agar plates containing tetracycline on different plates. The plates were marked as PM for *P. mirabilis* and KP for *K. pneumoniae* and each bacterium was transferred to the respective plate using sterile inoculum loop. The bacteria were then

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allowed to grow at 37°C for 24 h. After 24 h of the incubation, the plates were inspected and observed. The presence of colony indicates that the bacterial species has survived and passed the susceptibility test on agar plate supplemented with tetracycline with a final concentration of 25 µg/ml.

2.3. Broth mating

The cultured cell density of donor and recipient bacteria in the conical flasks labeled as PM and KP, respectively, was adjusted to a density of 1.0 optical density (O.D.) measured at 600 nm using spectrophotometric method. A volume of 100 µl of donor culture as well as the recipient culture was aliquoted into a sterile centrifuge tube and 800 µl of sterile LB broth was added, and incubated (at 37°C) on a rotary shaker for 24 h.

2.4. Plate mating

Plate mating was carried out using the overnight cultures of donor and recipient. The density of the cells was made to a density of 1.0 O.D., measured at 600 nm using spectrophotometric method. About 100 µl of donor and recipient culture was spotted on LB agar plates. The plates were incubated (at 37°C) for 24 h. At the intervals of 4 h, the dried bacterial spots have been moistened with 0.1 M (pH 7) of sterile phosphate buffer and gently scraped using a sterile glass rod. The cell suspension was diluted serially with a factor of 3 and plated on LB agar with 25 µg/ml of tetracycline.

2.5. Statistical analysis

The event of gene transfer takes place in the conjugation experiment with the number of samples for broth mating that is given by n = 25 and the probability was calculated. The data from multiple experiments were considered and analyzed using Omni online software.

3. Results

In this study, two bacteria, namely, *P. mirabilis* and *K. pneumoniae,* were chosen as the donor and recipient to study HGT of tetracycline resistance gene. Before performing the study, the genome mapping of these two species, that is, *P. mirabilis* HI4320 genome and *K. pneumoniae* KCTT 2242 genome, were analyzed as shown in

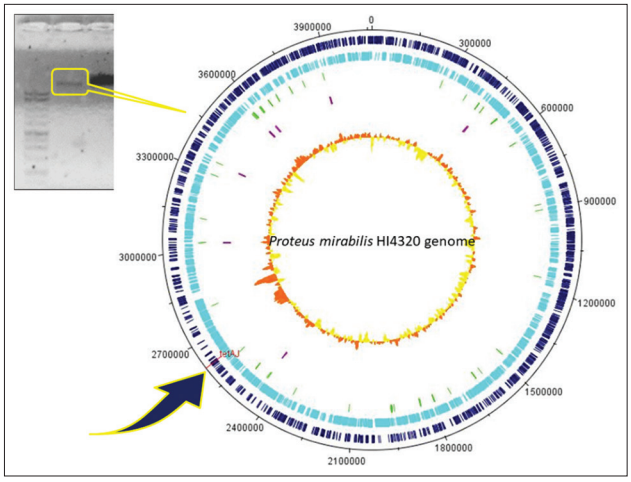


Figure 1. Circular mapping of *Proteus mirabilis* HI4320 genome. The region of tetracycline resistance gene is indicated by the bold arrow. Figure inset displays the electrophoresis results of genomic DNA from *P. mirabilis*. The figure displays the entire genome with the appropriate labeling on the regions. Arrow indicates the expected functional sequences. Figure inset is the agarose gel electrophoresis profile for the total genomic DNA. No smear indicates the intactness of the total gene.

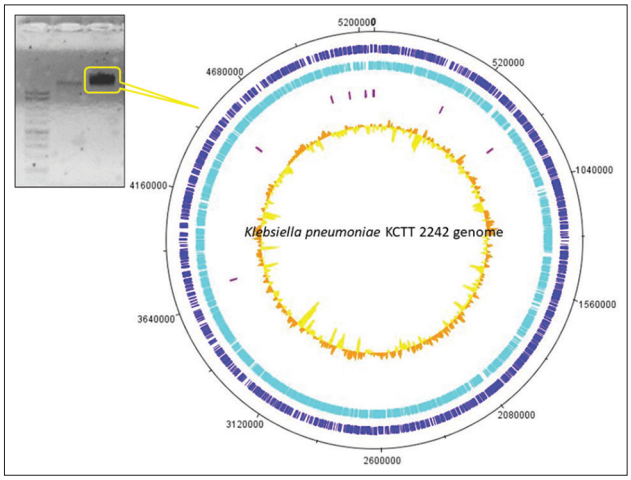


Figure 2. Circular mapping of *Klebsiella pneumoniae* KCTT 2242 genome. The figure displays the entire genome with the appropriate labeling on the regions. Figure inset displays the electrophoresis results of genomic DNA from *K. pneumoniae*. It was resolved agarose gel electrophoresis profile for the total genomic DNA. No smear indicates the intactness of the total gene.

Figures 1 and **2**. Furthermore, the appearance of genomic DNA from these species was observed on the gel electrophoresis as shown in the inset in **Figures 1** and **2**.

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Based on the circular genome mapping, the tetracycline resistance gene is positioned in the range between 2621559 and 2622755 (**Figure 3**). Twenty-six samples, in which 25 samples for broth mating and another one for plate mating technique, were used for further study. These samples were analyzed by antimicrobial susceptibility test (through bacterial culture) for plate mating and by spectrophotometry for broth mating. The samples of broth mating were replicated by serial dilution and samples were collected at the desired time intervals. **Figure 4** shows the morphology of colony on both bacteria culture plates (antimicrobial susceptibility test), in which initially only *P. mirabilis* (**Figure 3a**) could survive and grow on the tetracycline-supplemented media, whereas *K. pneumoniae* could grow well (**Figure 4b**).

3.1. Spectrophotometric analysis

Table 1 shows the results of statistical analysis on the transformation. The highest spectrometric value of broth mating conjugation is 0.44, in which sample BM-B5-S3 was tested at 960 min. Three inactive samples have no value and marked as "n/a" in which these samples gave the value of zero, which is equal to the absorbance value of standard blank solution. The samples were inactive, possibly because of transfer and death during the conjugation experiment.

3.2. Statistical verification on HGT

From the results tabulated (**Table 1**), the event of gene transfer takes place in the conjugation experiment that the number of samples for broth mating is given by $n = 25$ with the probability of a successful event, $P = 0.88$. Since the mean of is >5, the binomial probability requires Poisson approximation. Hence, using an approximation of $X \sim P0$ (19.36), the probability for gene transfer to take place at least in 8 events from all active samples is given by value of 0.0019; since the value is too small, the event is unlikely to take place. Meanwhile, after testing at least 10 events with the probability value of 0.9958, this can be interpreted as event that will certainly occur. From 10 samples out of 22 active samples, it can be approximated that about 55% or half of the samples exchange their genetic materials.

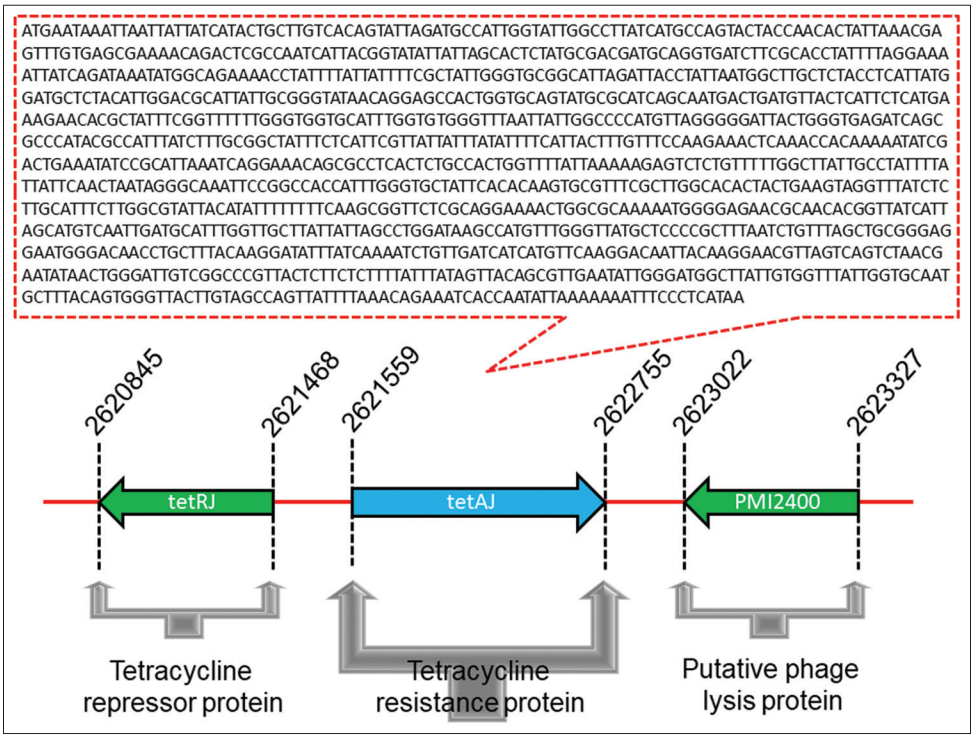


Figure 3. Illustration depicting the location of tetracycline resistance gene of *Proteus mirabilis*. The directions are indicated by the arrows. The region between 2620845 and 2621468 encodes for tetracycline repressor protein. The region between 2621559 and 2622755 encodes for tetracycline resistance protein. The complete sequences for this region are given. The region between 2623022 and 2623327 encodes for putative phage lysis protein.

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Table 1. Statistical analysis on the transformation

Statistical value	Statistical test
Number of active broth samples**:** n=22	a. Binomial Distribution: $X \sim B$ (22,0.88) b. Poisson Approximation: $X \sim P_0$ (19.36)
Mean of active samples: $\bar{x} = \frac{9.0892}{22}$ $\bar{x} = 0.41315$	a. Exactly eight events: $P(X=8)=1.9133\times10^{-3}$ b. More than 10 events: $P(X>10)=0.9958$
Standard deviation of data: σ=0.01373 Variance of data: σ^2=0.00019	a. Hypothesis testing α=0.001: $Z_{test} = 4.4923 > Z_{\alpha}$=3.091

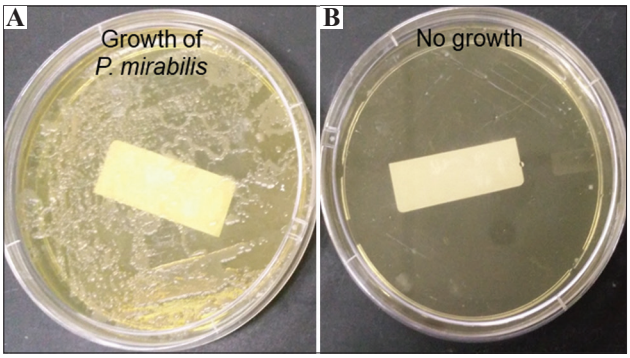


Figure 4. Plate mating macroscopy before conjugation experiment. (A) *Proteus mirabilis* exhibits growth in scattered colonies. (B) *Klebsiella pneumoniae* exhibits no growth in the presence of tetracycline.

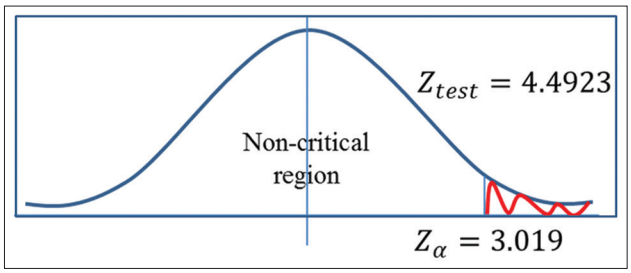


Figure 5. The normal distribution curve of data dispersions with the shaded region is a critical region for rejection.

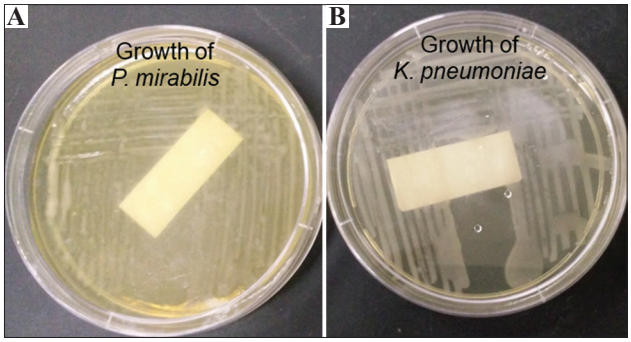


Figure 6. Visible colony is seen on the surface of the agar plate. (A) *Proteus mirabilis* exhibits growth in scattered form. (B) *Klebsiella pneumoniae* exhibits growth in mucoid colony.

After performing conjugation experiment, a claim that broth mating, rather than plate mating, has a higher affinity for conjugation of the gene to take place, in which the average of the transfer is 0.4132 and dispersed with a standard deviation of 0.0137 with a sample size of 22 different conjugations, can be made. The claim was tested at a mean of at least 0.4000 with a value of α =0.001. From the normal curve, $Ztest > Z\alpha$, the calculated statistical value falls in the rejection region with value of 4.4923, as shown in **Figure 5**. Therefore, there is a enough evidence to reject the claim that broth mating has higher affinity for conjugation of gene to take place than plate mating. After the mating experiment successfully carried out, both cultures were tested on the tetracycline agar plates. The cultured plates show that both bacteria survived and exhibited growth (**Figure 6**).

4. Discussion

Bacteria are ubiquitous and can thrive well in a wide range of conditions. To ensure survival, bacteria secrete compounds that utilize or degrade the surrounding materials. In this context, some bacterial species naturally have the ability to detoxify the harmful agents surrounding them, whereas others need to rely on other classes of bacteria species to obtain such capability. The achievement with the approach described in this article is the production of antibiotic-resistant strains, which were originally susceptible to particular drug(s). The importance of bacterial genes has been widely investigated

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in omics studies and functional analyzes [20-26]. The present investigation provides the insight into understanding gene mobility and its benefits.

This study aimed to transfer the gene of interest horizontally under the condition made for generating antibiotic-resistant bacterial strain. As stated, the process of HGT encompasses transduction, transformation, and conjugation. In this study, the gene responsible for tetracycline resistance was transferred horizontally from *P. mirabilis* to *K. pneumoniae*. Two different methods, that is, plate mating and broth mating, were utilized for conjugation. The results from broth mating have a probability value estimated at $P = 0.88$ with the significance of greater than 5. In addition, the efficacy of broth mating was found to be better than that of plate mating, as implied by the 95% confidence interval. This study presented a way for generating antibiotic-resistant bacterial strains for use in medical research and industrial applications. This result shows that the resistance gene from *P. mirabilis* was transferred to *K. pneumoniae* through cell-to-cell contact. The present study provides an insight into generating antibiotic-resistant bacterial strains that were previously susceptible for therapeutic or pharmacology-based applications.

5. Conclusion

This study was performed to assess the gene transfer between different progeny of bacteria using a mating technique that applies the principle of conjugation. Samples were collected in accordance with time intervals, where conjugation rate of gene transfer was assessed based on time. This indicates that the more time is spent, the less likely for a gene to transfer due to the environmental or other factors, such as plasmid shelf-life and compatibility of bacteria when mating is in process. The susceptibility test was performed to identify either *P. mirabilis* or *K. pneumoniae* has the resistance characteristic toward tetracycline. The results show that *P. mirabilis* is resistant toward tetracycline. After conjugation, both *P. mirabilis* and *K. pneumoniae* were assessed in susceptibility test, which also proved that both bacteria could survive in antibiotic-supplemented media. In a nutshell, the events of conjugation could take place through mating between bacteria of different progenies. This study confirms that novel bacterial strains can be applied for HGT to generate and expand different strains for varied purposes. Moreover, these strains can be screened for their potentials before being used in medical research and industrial applications. However, it is important to note that the success of gene transfer relies on the optimum condition; therefore, the conditions need to be optimized for generating different strains.

Conflict of interest

No conflict of interest declared.

Author contributions

Mohamad Amin Awang Teh@Ismail was responsible for data curation, formal analysis, investigation, resources, validation, writing, reviewing, and editing the paper. Thangavel Lakshmipriya completed the methodology, data curation, validation, writing, reviewing, and editing. Subash C.B. Gopinatah made the conceptualization, data curation, project administration, resources, supervision, visualization, validation, writing, reviewing, and editing. Suresh V. Chinni have conducted data curation, visualization, writing, reviewing, and editing.

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