PLASMID TRANSFER IN PLANKTONICALLY-GROWN CULTURES AND

BIOFILMS

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Farhana Shamsad

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Bachelor of Science, University of Dhaka 2006

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Farhana Shamsad, Doctor of Philosophy, Molecular Science

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ABSTRACT

Conjugation of plasmids is widespread among bacteria and contributes to the spread of antibiotic resistance. In the natural environment, microorganisms predominantly exist in the form of biofilms or other bioaggregates, where they may be exposed to contaminants such as antibiotics, at subinhibitory concentrations. Bacterial cells in older biofilms have lower growth activity due to oxygen and nutrient limitation in the deeper layers of the biofilms. In batch culture, population growth eventually ceases during the stationary phase. Thus, the steady state of biofilms may resemble stationary growth phase cultures. Our objectives were to study (i) the effect of cell growth phases and (ii) subinhibitory and minimum inhibitory concentrations (MIC) of antibiotics on transconjugant formation in both batch cultures and biofilms. Additionally, (iii) the effect of variable nutrient concentrations on MIC was investigated and (iv) an optimization of RT-PCR method for the detection of *traA* gene (which encodes pilus biosynthesis) expression was carried out.

To study the effect of cell growth phases on transconjugant formation, plate matings were carried out utilizing planktonic cultures grown to exponential or stationary phase of donor and recipient strains. The results showed that transconjugant abundance was the highest (20± 0.08%) when both plasmid donor and recipient cells were grown to the stationary phase. However, the growth phase of the donor did not seem to play a role in biofilms. When donor cells were harvested from either the exponential or stationary phase of growth, and inoculated into 24 h old recipient

biofilms, there was no statistically significant difference between transconjugant abundance. A higher percentage of transconjugants was detected in plate matings when the donor was exposed to $0.5\times$ minimum inhibitory concentration of gentamicin and additionally challenged with gentamicin at MIC. In biofilms, transconjugant formation was not enhanced when the donor cells were grown with $0.5\times$ MIC gentamicin, and $0.5\times$ MIC gentamicin was added to the biofilms. A decrease in nutrient concentration was associated with a decrease in the MIC. *traA* expression, detected using RT-PCR in plasmid donor cells grown to early exponential and late exponential phases did not coincide with an increase in transconjugants.

ACKNOWLEDGEMENTS

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cDNA	Complementary deoxyribonucleic acid
CFU/mL	Colony forming units per milliliter
CLSM	Confocal laser scanning microscope
Da	Dalton
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
E. coli	*Escherichia coli*
EPS	Extracellular polymeric substance
GFP	Green fluorescent protein
HGT	Horizontal gene transfer
h	Hour
IncP-9	Incompatibility group P9
lac repressor	Lactose repressor
LB	Lysogeny broth
MIC	Minimum inhibitory concentration
min	Minutes
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
OD	Optical density
oriT	Origin of transfer
PBS	Phosphate buffered saline
P. putida	*Pseudomonas putida*
RT-PCR	Reverse transcriptase polymerase chain reaction

RNA	Ribonucleic acid
S. typhimurium	*Salmonella typhimurium*
spp.	Species
T4SS	Type IV secretion system
tra genes	Transfer genes

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Appendix A

1. Introduction

1.1. Motivation for the study

Horizontal transfer of mobile genetic elements is an important mechanism of microbial adaptation to a changing environment (Top and Springael, 2003). Previous studies in our lab investigated the transfer of catabolic plasmid pJP4 in bacterial biofilms in lab-scale continuous flow-through chambers. The results revealed that transconjugants were not detected microscopically after the addition of a plasmid donor strain (*Pseudomonas putida* SM1443) to mature (7 days-old) soil derived biofilms which were more than 60 μm thick (Shamsad, 2012). When the same donor strain was added to 24 h old *P. putida* ATCC12633 biofilms (15 μm thick), transconjugant cells were observed microscopically after 48 h of donor addition (Uddin, 2014). It is unclear whether more transconjugants were observed due to the potential increased penetration of donor cells into thinner biofilms, or if inhibition of donor penetration in older biofilms due to an increased production of extracellular polymeric substances (EPS) or cell growth stage–related parameters were involved. Limited information is available on how cell growth phases, of either donor or recipient cells, affect plasmid transfer in planktonic cell batch cultures and in biofilms (Muela et al., 1994).

Some studies revealed that antibiotics can act as signalling molecules. These studies showed that the presence of subinhibitory concentration of antibiotics altered the expression profiles for a variety of genes (Bagge et al., 2004, Hoffman et al., 2005). A study performed in our lab showed that a greater number of plasmid pJP4 transconjugant cells were detected when a soil-derived

mixed culture biofilm was exposed to sublethal concentration of gentamicin (100 µg/mL) in comparison to an experimental control (no exposure to gentamicin) (Postelnik, 2012).

Based on literature search and studies that were performed in our lab, the following questions arose:

1. How do cell growth phases affect plasmid transfer and transconjugant formation?

2. Is expression of *traA* (encodes pilus biosynthesis) linked with a specific growth phase and does it correspond to enhanced transconjugant formation?

3. How does an environmental factor such as nutrient availability affect susceptibility of bacterial cells to antibiotics?

4. How does an environmental stressor such as an antibiotic influence plasmid transfer and transconjugant formation?

We formulated the following hypotheses based on the research questions.

1. In the exponential growth phase, cells grow and increase in number. In the stationary phase cell growth eventually ceases due to the nutrient limitation and accumulation of waste products. We hypothesized that the abundance of transconjugants will be higher in the exponential growth phase than in the stationary growth phase of the plasmid donor and recipient cells in plate matings, as cell activity is greater in the exponential phase. We also hypothesized that transconjugant formation will be enhanced when plasmid donor cells grown to exponential phase will be added in the recipient biofilms.

2. The *traA* gene encodes pilus biosynthesis in plasmid donor cells. We hypothesized that the *traA* gene expression in donor cells will be linked to a higher percentage of transconjugants.

3. With decreased nutrient concentrations, bacterial cells will be less metabolically active and a lower minimum inhibitory concentration (MIC) of antibiotics will be needed to inhibit bacterial growth as fewer targets for the antibiotics will be available. We hypothesized that values of MIC of antibiotics will decrease with decreasing nutrient concentrations.

4. The number of transconjugants will be greater following antibiotic exposure due to a potential effect of antibiotics on gene expression.

To test the hypotheses we proposed the following objectives:

1a. To study the effect of cell growth phases (exponential and stationary) of plasmid donor and recipient cells on transconjugant formation using planktonic cell batch cultures.

1b. To study the effect of growth phases of plasmid donor cells on transconjugant formation in biofilms.

2. To develop and optimize an RT-PCR assay for the detection of *traA* expression.

3. To study the effect of varying nutrient concentrations on minimum inhibitory concentration of antibiotics.

4. To study the effect of exposure to subinhibitory and minimum inhibitory concentrations (MIC) of gentamicin on transconjugant formation using planktonically grown cells in plate matings and biofilms.

1.2. Horizontal gene transfer (HGT)

Horizontal gene transfer (HGT) is the acquisition of foreign genes by microorganisms and this is not dependent on cell division. There are three mechanisms of HGT: transformation, transduction and conjugation. Figure 1.1 summarizes the three different mechanisms of HGT. Among these three, transformation was the first mechanism discovered in prokaryotes (Frost et al., 2005). Transformation includes uptake, integration and expression of extracellular DNA by bacterial cells. In transformation, bacterial cells develop the ability to take up DNA which is referred to as competence (Thomas and Neilsen, 2005). Transduction is mediated by bacteriophages. Bacteriophages infect bacterial cells, inject DNA into the host cell and the DNA becomes incorporated into the host cellular chromosome (Frost et al., 2005). In conjugation, conjugative plasmids and transposons are transferred from donor to recipient cell via cell-cell contact (Frost et al., 2005).
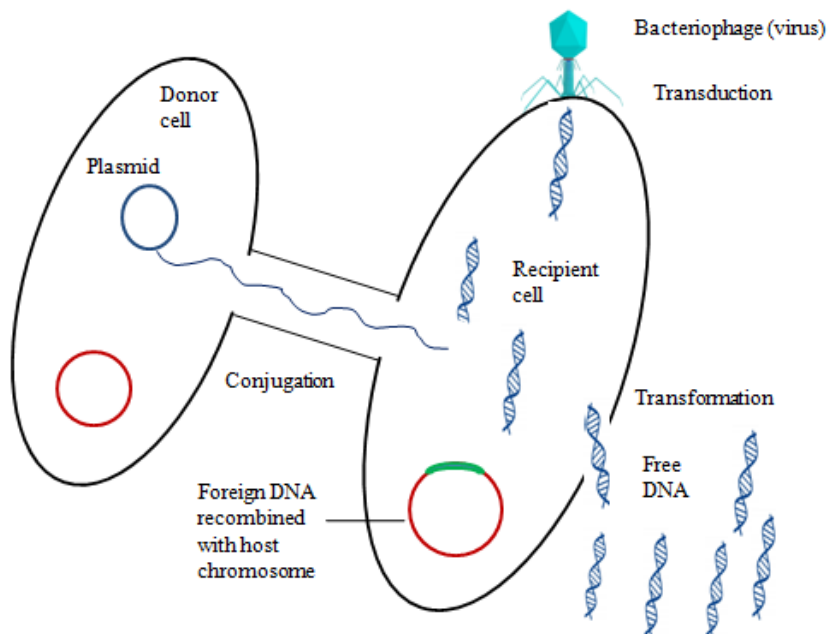
Figure 1.1. Mechanisms of horizontal gene transfer (HGT) (adapted from Stewart, 2013).

For our research project conjugation was the mechanism of interest to study plasmid transfer manifested by transconjugant formation in plate matings and biofilms.

1.2.1. Mechanism of conjugation

Conjugation is found in Bacteria, Archaea, and Eukarya. In gram negative bacteria, conjugation can be divided into two stages. The first stage involves formation of a bridge between plasmid donor and recipient cells through extracellular conjugative pili. The second stage involves the transfer and processing of DNA (Yin and Stotzky, 1997). In gram negative bacteria, a conjugative pilus (mating pair formation apparatus) is formed in plasmid donor cell, which identifies plasmid recipient cell. Conjugative pilus retracts and brings the cells into close contact (Arutyunov and Frost, 2013). The type IV secretion system (T4SS) is needed for mating pair formation and gene transfer process. Type IV secretion systems are involved in protein secretions (Llosa et al., 2002). They contain channels through which protein- DNA complexes can be transferred between cells (Wallden et al., 2010). T4SS forms the transferosome which transports the DNA between cells. The relaxosome, a nucleoprotein complex at origin of transfer (*oriT*), contains the enzyme relaxase. In the F plasmid system, the relaxase is known as TraI. TraI makes a cut of DNA at the *oriT by* catalyzing the cleavage of specific phosphodiester and begins to separate a single DNA strand. The relaxosome is linked with the transferosome by the coupling protein (Frost and Koraimann, 2010). Coupling proteins are required in all conjugative systems. Some examples of coupling proteins are TrwB of IncW plasmid R388, TraD of IncF plasmids and TraG of IncP plasmids (Llosa et al., 2002). The DNA/relaxase complex is then recognized by the coupling protein and transferred to the secretion system (transferosome). The secretion system pumps the DNA/relaxase complex into the recipient cell. After entering the

recipient cell, single stranded DNA is replicated to become double stranded (Wong et al., 2012, Willey et al., 2011).

F-like plasmids carry two genes that encode proteins that reduce mating between similar donor cells. These two proteins are an inner membrane entry exclusion (Eex) protein, TraS, and an outer membrane surface exclusion (Sfx) protein, TraT. (Achtman et al., 1977, Arutyunov and Frost, 2013). Transfer of the F plasmid takes ~ 2 min at a rate of 45 kb/min (Arutyunov and Frost, 2013).

The majority of gram positive bacteria utilize T4SSs for conjugative passage of single stranded DNA (Goessweiner-Mohr et al., 2014). In gram positive enterococci, transfer apparatuses encoded by plasmids become activated in response to an appropriate recipient through production of pheromones. Recipient strains of gram positive *Enterococcus faecalis* produce and excrete diffusible, hydrophobic peptide molecules known as pheromones which act as chemical signals (Thomas and Neilsen, 2005, Writh, 1994). The donor strains of *E. faecalis* can sense the presence of the pheromones and produce aggregation substance which causes tight physical contact between donor and recipient cells until the transfer of the conjugative plasmids (Wirth, 1994). In gram positive bacteria, cell attachment occurs through the generation of surface factors or adhesins. PrgB is a protein encoded by plasmid pCF10, found in pheromone inducible conjugation in enterococci (Hirt et al., 2005). In gram positive *Streptomycetes*, the conjugative DNA transfer system depends on double stranded DNA translocation performed by a single protein TraB (Sepulveda et al., 2011). In *Streptomyces*, plasmid transfer occurs by the formation of "pock structures". Pocks are generated when donor spores germinate on a lawn of a plasmid free recipient. The pocks represent growth inhibition zones surrounding the plasmid donor where

sporulation of recipient is retarded. Pocks indicate the area where the recipient mycelium has acquired a plasmid by conjugation (Thoma and Muth, 2016).

There are at least 3 families of conjugative plasmids (pSK41, pWBG749 and pWBG4) found in staphylococci. Of these only 5-6 % of plasmids carry conjugation gene clusters required for independent conjugative transfer. The conjugative plasmid encodes all genes required for formation of the mating pore, the coupling protein, DNA relaxase and *oriT*. DNA is brought to the mating pore by the relaxase protein, which cleaves and attaches to the *oriT* sequence, forms a nucleoprotein complex, relaxosome. The relaxasome is brought to the mating-pore through coupling protein and transferred to the recipient cell through type IV secretion system (Ramsay et al., 2016).

1.3. Plasmids

Plasmids are mostly circular, double stranded, self replicating DNA molecules. Plasmid genes do not encode essential cellular functions. Plasmids have a major role within the pool of horizontally exchangeable genes as plasmids encode genes for stress response, their autonomous replication, also encode genes for their own transfer (Smets et al., 1993). Plasmids cannot co-exist in a cell together if they contain the same origin of replication. This is known as plasmid incompatibility (Velappan et al., 2007). Plasmids containing the same replication control are placed in the same incompatibility group. Plasmids are classified into different incompatibility groups e.g. IncC, IncN, IncP, IncQ, and IncW (Thomas and Smith, 1987).

Plasmids contain "backbone" genes that encode proteins involved in replication. Plasmids also contain accessory genes that encode different functions those are not encoded on the bacterial chromosome (Frost et al., 2005). The accessory region of plasmids contains the genes that

encode biofilm formation factors e.g. fimbriae that mediate cell-cell contact needed for cluster formation, cell surface adherence and promote biofilm formation (Madsen et al., 2012). Many bacterial species carry plasmids.

1.3.1. *Pseudomonas spp.*

Species within the genus *Pseudomonas* are gram negative*,* flagellated, rod shaped, aerobic bacteria (Anzai et al., 2000), belonging to the Gamma-proteobacterial class and are common inhabitants of soil and water (https://www.ncbi.nlm.nih.gov/genome, retrieved on Sep 27, 2017). *Pseudomonas* spp. contains chromosomally encoded efflux mechanisms, which help to increase the antibiotic resistance in *Pseudomonas* spp. (Devarajan et al., 2017). *Pseudomonas* spp. contains R factor plasmids which encode genes for resistance to various antibiotics, such as gentamicin, tobramycin, carbenicillin. Sex-factor plasmids are found in both *P. aeruginosa* and *P. putida.* Heavy metal (e.g. mercury) resistance plasmids are also found in *Pseudomonas* spp. (Chakrabarty, 1976). *P. aeruginosa* has been used as a model organism for biofilm formation (Sauer et al. 2002). *P. aeruginosa* is an opportunistic pathogen and isolated from plant, animals and humans. It can be isolated from respiratory therapy equipment, medicines, physiotherapy and hydrotherapy pools (Lister et al. 2009). *P. aeruginosa* is the cause of nosocomial pneumonia, health care associated pneumonia, urinary tract infection, surgical site infection (Lister et al., 2009, Kollef et al., 2009).

In contrast to *P. aeruginosa*, *P. putida* is not frequently associated with clinical infection studies but *P. putida* has been used in different plasmid transfer studies. *P. putida* is found in soil and aquatic environments, and it has been occasionally found to colonize human tissue (Molina et al., 2014). In another study Molina et al. (2016), performed genomic comparisons between the

environmental isolates and clinical isolates of *P. putida*. Genes detected in *P. putida* clinical isolates are related with survival under oxidative stress conditions, resistance against biocides, amino acid metabolism and toxin/antitoxin systems. These genes are absent in environmental *P. putida* isolates. These functions have influenced survival within human tissues since they help bacteria cells to avoid host immune response and developed enhanced stress resistance.

In this research project, we used *P. putida* strains to study pWWO transfer in biofilms and batch cultures. We investigated the effect of an environmental factor, i. e. nutrient availability, on susceptibility of bacterial cells to antibiotics and the effect of an environmental stressor, i. e. an antibiotic, on transconjugant formation in the biofilms. We used *P. putida* as it has been frequently used in different environmental studies, applications and also it is known for its capacity to form biofilms.

1.3.2. TOL plasmid

The TOL plasmid is a self-transmissible plasmid initially characterized in *P. putida (arvilla)* mt-2 strain (Chakrabarty, 1976).

For our research project, a TOL plasmid pWWO was used to study plasmid transfer in biofilms and batch culture systems. TOL plasmid pWWO isolated from *Pseudomonas putida* belongs to incompability group IncP-9 (Ikuma and Gunsch, 2012). Plasmid pWWO encodes genes for catabolism of aromatic compounds xylene, toluene, m-ethyltoluene and 1, 3, 4-trimethylbenzene (Abril et al., 1989). Transfer of plasmid pWWO by conjugation has been documented in *Pseudomonas* spp. and *Escherichia coli.* Christensen et al. (1998) studied conjugative transfer of TOL plasmid pWWO in biofilms composed of three different species *P. putida, Acinetobacter* and an unknown strain. Among these three only *P. putida* received the plasmid. In another study,

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Jussila et al. (2006) showed that pWWO was transferred from *Pseudomonas* to *Rhizobium.* Boon et al. (2005) studied transfer of pWWO from *P. putida* to *E. coli* DH5a. Their study revealed high nutrient condition (contained maximum amount of carbon and nitrogen source) and optimal temperature were necessary to obtain higher plasmid transfer frequency.

1.3.3. Plasmid replication process

Circular plasmids are replicated by three mechanisms: theta type, strand displacement and rolling circle mechanism.

The theta type replication has been well studied for plasmids of gram negative bacteria and also for some plasmids of gram positive bacteria (Solar et al., 1998, Lilly and Camps, 2015). DNA replication by the theta type mechanism includes melting of the parental strands, synthesis of a primer RNA (pRNA), and initiation of DNA synthesis by extension of the pRNA.

The theta type replication can be unidirectional or bidirectional and DNA synthesis can start from single or different origins. DNA synthesis is continuous in one strand (leading strand, polymerase clamped to DNA continuously) and discontinuous in other strand (lagging strand, synthesized as fragments) (Solar et al., 1998, Kelman and O'Donnell, 1995).

The plasmid rolling circle mechanism depends on the cleavage at the nick site of the double-strand origin of one of the parental DNA strands by an initiator Rep protein. This cleavage generates a 3-hydroxyl end that allows DNA polymerases to initiate the leading strand replication. Elongation of the leading strand takes place as the parental double helix is unwound by host DNA helicase. The $3'$ end is lengthened and the growing point forms a circular template. The $5'$ end of the strand is displaced to form an ever lengthening tail (Figure 1.2). A trans-esterification occurs that joins the $5'$ end to the $3'$ end, generated in the termination

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cleavage, and releases the displaced parental strand as a single-stranded DNA (ssDNA). This ssDNA serves as the template for the lagging strand. The ssDNA can be converted in double stranded DNA form by synthesis of a complementary strand (Ruiz-Maso et al., 2005, Willey et al., 2011).
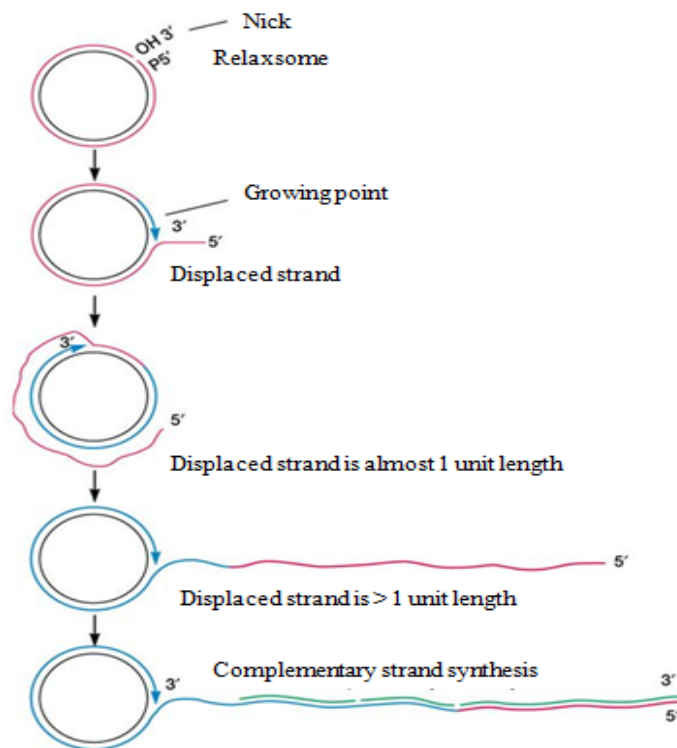
Figure 1.2. Rolling circle replication mechanism (adapted from Willey et al., 2011).

1.3.4. Role of plasmid donor and recipient in conjugation

In conjugation, the formation of contacts between two gram negative bacteria (plasmid donor and recipient) is mediated by conjugative pili. Conjugative pili are cylindrical filaments with an outside diameter of 8 nm and a 2 nm central, hydrophilic lumen, extending from the surface of

donor cells (Silverman, 1997). According to Novotny and Fives-Taylor (1973), pili disappear as a result of retraction and some environmental factors, e.g. temperature, have an effect on pili retraction. Their study suggests that in *Escherichia coli* cells, retraction of pili occurs at a slower rate at 25° C, but retraction is very slow or completely inhibited at temperatures below 24° C. According to Seoane et al. (2011), recipient cells receive plasmids more frequently at the advanced stage of the growth cycle when cells are elongated. Further details are discussed in chapter 2.

1.3.5. Genes and proteins involved in conjugation

The products of three F genes, *traA, traQ,* and *traX* within the *tra* region are involved in synthesis of F-pilin subunits (Firth et al., 1996). In addition to the product of these three genes, TraB, TraC, TraE, TraF, TraG, TraH, TraK, TraL, TraU, TraV, TraW and TrbC are involed in pilus assembly (Frost et al., 1994). According to Anthony et al. (1999) TraL, TraE, TraK, TraC, TraG were involved in pilus tip formation. TraB, TraF, TraH, TraW, TraV were involved in pilus outgrowth and TrbI was involved in pilus retraction. F-like pilins have four domains, two hydrophobic domains inserted in the inner membrane, the N- and C-termini exposed in the periplasm, and a loop containing basic amino acids oriented towards the cytoplasm (Silverman 1997).

TraN, which is involved in mating pair stabilization, interacts with outer membrane protein and lipopolysacharide. TraG was the second stabilization protein and involved in entry exclusion. The functions encoded by the transfer region genes are summarized in Table 1.1.

Table 1.1. Transfer region genes involved in plasmid transfer during conjugation (adapted from Firth et al., 1996)

Gene	Functional group	Product location	References
traA	Pilus biogenesis (encodes 121 aa precursor of the pilus subunit, propilin)	Inner membrane and extracellularly	Frost et al., 1984
traB	Pilus biogenesis	Inner membrane	Moore et al., 1987
traC	Pilus biogenesis	Cytoplasm/ Inner membrane	Schandel et al., 1990
traD	DNA metabolism (Purified TraD is an integral inner membrane protein that it is capable of binding to DNA. It has been suggested that it forms or is part of a non-specific pore for DNA export, or that it directly energizes DNA transport. TraD redirects energy utilization towards DNA transport. It could be also involved in signalling that the relaxosome should move to a competent conjugation pore.)	Inner membrane	Panicker and Minkley 1992, Jalajakumari and Manning 1989, Arutyunov and Frost 2013
traI	DNA metabolism	Cytoplasm	Abdel-Monem et al., 1983
traM	DNA metabolism	Cytoplasm	Di Laurenzio et al.,1992
traN	Aggregate stabilization	Outer membrane	Maneewannakul et al., 1993
traQ	Pilus biogenesis (propilin maturation)	Inner membrane	Maneewannakul et al., 1993
traX	Pilus biogenesis (propilin maturation)	Inner membrane	Cram et al.,1991
traY	DNA metabolism	Cytoplasm	Fowler et al., 1983

Figure 1.3. shows the genetic map of plasmid pWWO. Nineteen genes on the plasmid backbone are involved in transfer of pWWO. For plasmid pWWO, origin of transfer includes TraA, TraB, TraC and TraD and two hypothetical proteins (Orf176 and Orf177) (Greated et al., 2002). TraD is the DNA binding protein that facilitates relaxosome formation. Plasmid pWWO *oriT* sequences contain low G+C content and *nic* sequence. *mpf*A-J genes encode the products involved in synthesis of conjugative pilus during mating pair formation.

Plasmid pWWO carries the *xyl* genes for toluenes and xylenes degradation. This plasmid also carries *mer* operon which contains mercury resistance genes (Greated et al., 2002).
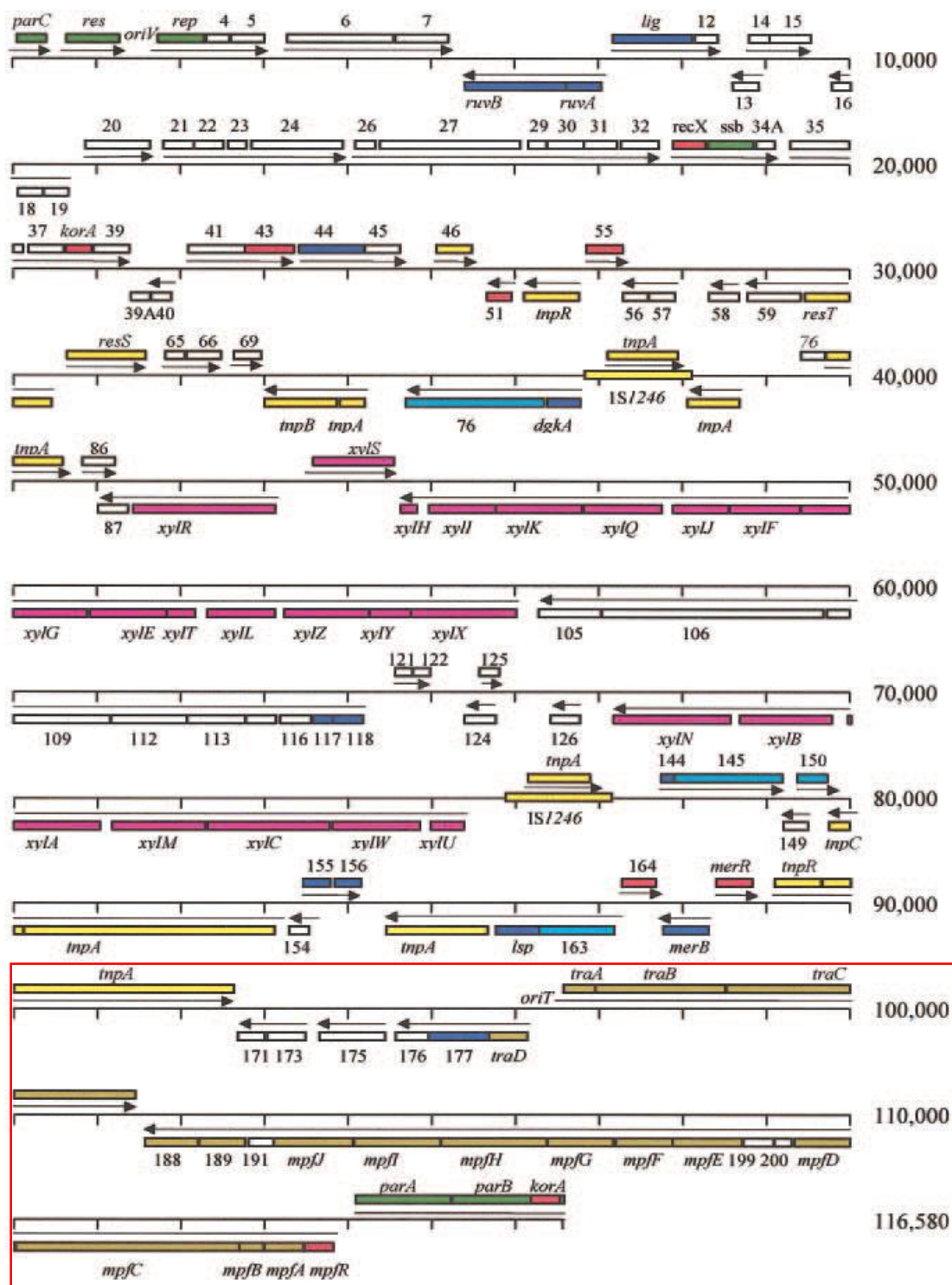
Figure 1.3. Genetic map of plasmid pWWO. Light green colour (in red box) represents proteins involved in conjugative transfer (From Greated et al., 2002).

1.3.6. Regulatory processes influencing conjugation and expression of genes in transconjugants

Sorek et al. (2007) reported that the ability of bacteria to take up DNA by HGT depends on the Guanine-Cytosine (G-C) content of the genome. Their analysis suggests that G-C rich genomes contain fewer untransferable genes. Popa et al. (2011) found that with a high percentage of the horizontal gene transfer occurred between donor–recipient pairs having ≤5% difference in G-C content. Their results suggest that the barrier for gene acquisition from donors of dissimilar genomic G-C content creates a biological barrier and HGT occurs more frequently among closely related species, having similar genomes.

Ikuma and Gunsch (2012) investigated the effect of additional carbon sources, different pH values, different nutrient sources on toluene degradation ability of TOL pWWO plasmid containing transconjugants. Their results showed that glucose addition increased the toluene degradation ability in transconjugants. Different pH and nitrogen sources did not have a significant effect on the toluene degradation rate by the transconjugants.

A higher conjugation rate is linked to either the formation of bacterial aggregates under certain inoculum conditions or the recipient cell's genetic makeup (Pei and Gunsch, 2009). The highest amount of conjugation was seen for the treatment condition with the highest recipient cell ratio of 20:1 (activated sludge to *P. putida* BBC443). This result suggests that conjugation rate is associated with the availability of the recipient cells. Dahlberg et al. (1998) reported a similar trend in sea water where the highest plasmid transfer frequencies occurred with the lowest number of donor cells.

In conclusion, conjugation rate has been shown to depend on the G-C content of the genome, genome similarity and the ratio of donor to recipient cells.

1.4. Antibiotics

Antibiotics are low molecular weight (<3,000 Da) molecules produced by some fungi and bacteria (Laureti et al., 2013). Among bacteria, the genera *Streptomyces*, *Bacillus*, *Pseudomonas* are well known for their antibiotic-producing properties. Antibiotics might target cellular structures or enzymes. Common mode of action of antibiotics are the inhibition of the bacterial cell wall biosynthesis (e.g. β-lactam), the inhibition of protein (e.g. macrolides), RNA (e.g. ansamycins) or DNA synthesis (e.g. quinolones), the damage of cell membranes (e.g. polymyxins) and inhibition of essential metabolites (e.g. folate) synthesis (e.g. trimethoprim, class sulfonamides) (Laureti et al., 2013, Rasamiravaka and Jaziri, 2016). Antibiotics either kill bacteria directly (bactericidal mode) or inhibit bacterial growth (bacteriostatic mode) (Rasamiravaka and Jaziri, 2016).

The concentrations of these molecules required to achieve an antimicrobial effect is likely extremely high compared to the concentrations in which these molecules can be found in natural environments (Romero et al., 2011). The soluble concentrations of some antibiotics (e.g. tetracycline, ciprofloxacin, erythromycin) were found to be ≤1.3 µg/L in the wastewater facilities (Karthikeyan and Meyer, 2005). Antibiotics are used to treat human infections, they are also used in veterinary medicine and agriculture (Hirsch et al., 1999). Antibiotics are neither well metabolized by humans and animals nor are they fully biodegradable. These active forms of antibiotics pollute the soil and aquatic environments (Laureti et al., 2013). Subinhibitory concentrations (lower than the minimum inhibitory concentration) of antibiotics are found in the environment.

1.4.1. Subinhibitory concentrations of antibiotics act as signalling molecules, mechanisms of signalling molecules

Some studies have revealed that exposure to subinhibitory concentrations of antibiotics induces changes in the expression profile of a wide range of genes in different bacterial species (Davies et al., 2006, Bagge et al., 2004, Hoffman et al., 2005).

Bagge et al. (2004) studied the influence of subinhibitory concentration of imipenem (β-lactam antibiotic) on the structure of *P. aeruginosa* PAO1 biofilm. The authors compared nontreated (not exposed to imipenem) biofilms to biofilms exposed to imipenem for 18 or 37 h. Biofilms exposed to imipenem developed more biomass per substratum area and an increased average thickness. Control (not exposed to imipenem) biofilms formed numerous towers and pillars of microcolonies separated by areas devoid of cells. Their study demonstrated that among the genes that responded to imipenem exposure were the alginate biosynthesis genes (*algD* to *algA*). The exposure to subinhibitory concentrations of imipenem (a β-lactam antibiotic) caused an increased biofilm volume due to the production of alginate (exopolysaccharide) in the *P. aeruginosa* strain PAO1 biofilm. The authors concluded that increased production of alginate decreases the susceptibilities of bacteria growing in biofilms to antibiotics. The penetration of antibiotics through the biofilm may be reduced due to binding to alginate.

According to Goh et al. (2002) in the presence of low concentrations of erythromycin and rifampicin, global bacterial transcription patterns were altered by the stimulation or inhibition of a variety of promoter–*lux* reporter constructs in a library of *Salmonella typhimurium*. At subinhibitory concentrations these two antibiotics stimulated or inhibited many different promoters in *S. typhimurium*. Genes encoded different functions e.g. transport, virulence and DNA repair were activated by subinhibitory concentrations of antibiotics. The authors concluded

that in the presence of subinhibitory concentrations of antibiotics "the transcription machinery must have the means to sense these subtle conformational or stoichiometric changes and respond by specific up or down regulation." They also concluded that transcriptional modulation by antibiotics could lead to negative effects during the treatment of bacterial infections in human hosts.

1.4.2. Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), Minimum biofilm eradication concentration (MBEC)

Antimicrobial susceptibility testing is important to detect susceptibility or resistance in bacteria to different antimicrobials (Jorgensen and Ferraro, 2009). Minimum inhibitory concentrations (MIC) are the lowest concentrations of antimicrobials that inhibit visible growth of bacteria after overnight incubation. MIC is the standard technique for determination of susceptibility of microorganisms to antimicrobials. Minimum bactericidal concentrations (MBC) are the lowest concentrations of an antimicrobial agent that prevent the growth of bacteria after sub culturing onto antibiotic free media. The minimal biofilm eradication concentration (MBEC) is the minimal concentration of antimicrobial agents that can kill bacteria existing in a biofilm (Takei et al., 2013).

Biofilm cells are more resistant to antibiotics than the planktonic cells. Some factors provide antibiotic tolerance for biofilms e.g. (1) slow growth (2) presence of exopolysaccharides that prevent antibiotic diffusion and bind with the antibiotics (3) presence of persister cells (4) expression of resistance genes (Spoering and Lewis, 2001, Lewis, 2001). According to Giwercman et al. (1991) in the presence of β-lactam antibiotics, imipenem and piperacillin, β-lactamase production in *P. aeruginosa* biofilms was induced. The authors concluded that the

production of β-lactamase was the reason for persistence of *P. aeruginosa* in chronic infections. The β-lactamase enzymes provide resistance to β-lactam antibiotics by disrupting the beta lactam ring (http://medical-dictionary.thefreedictionary.com/Beta+lactamase, retrieved Jan 08, 2017).

For our research project MIC, MBC, and MBEC were measured for gentamicin (an aminoglycoside), kanamycin (an aminoglycoside), tetracycline (tetracyclines) and rifampicin (rifamycin) for *P. putida* strains at different nutrient concentrations.

1.4.3. Aminoglycoside antibiotics: usage, toxicity

Aminoglycosides produced by *Streptomyces* and *Micromonospora* species (Farouk et al., 2015), are broad spectrum antibiotics that are used to treat infections caused by gram positive and gram negative bacteria, such as *Staphylococci*, *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter* spp (Hanberger et al., 2013). Examples of aminoglycoside antibiotics are kanamycin, gentamicin, neomycin, tobramycin (Farouk et al., 2015). Aminoglycoside antibiotics are administered as ointments, ear drops, nasal drops or by injections. These antibiotics are also used to promote growth and prevent infections in animals (Farouk et al., 2015). Aminoglycosides kill bacteria by binding to the bacterial ribosome and inhibiting protein synthesis. These antibiotics bind to the highly conserved A-site (transfer RNA acceptor site) of bacterial 16S ribosomal RNA of the 30S ribosomal subunit (Magnet and Blanchard, 2005). The toxicity of aminoglycoside antibiotics includes ototoxicity (e.g. streptomycin, gentamicin) and nephrotoxicity (e.g. neomycin). Aminoglycosides generate free radicals within the inner ear which may cause permanent damage to sensory cells and neurons, resulting in permanent hearing loss (Hanberger et al., 2013, Selimoglu, 2007). In this research project the effect of subinhibitory concentration

and minimum inhibitory concentration of an aminoglycoside antibiotic, gentamicin was studied on transconjugant formation in batch cultures and biofilms.

1.5. Scope of work and thesis outline

Existing literature only sparsely addresses questions such as how cell growth phases affect plasmid transfer and transconjugant formation in batch cultures and biofilms, and how subinhibitory and minimum inhibitory concentrations of a selected antibiotic (gentamicin) affect plasmid transfer and transconjugant formation in batch cultures and biofilms. The research carried out within the frame of this thesis consisted of four projects to satisfy the four objectives listed in section 1.1.

The projects are listed below:

1. Effect of cell growth phases on transconjugant formation in plate matings and biofilms.

2. Optimization of RT-PCR to detect expression of the *traA* gene in plasmid donor cells.

3. Effect of nutrient concentrations on the determination of minimum inhibitory concentration of antibiotics.

4. Effect of subinhibitory and minimum inhibitory concentration of gentamicin on transconjugant formation in plate matings and biofilms.

The background information of the projects is described in chapter 2. The materials and methods of the experiments are described in chapter 3. The results obtained from the experiments are described in chapter 4. The discussion of experimental data is found in chapter 5.

Chapter 6 outlines the conclusions and suggests recommendations as a follow up for this project. The appendices contain the supporting data for "Effect of nutrient concentrations on the

determination of minimum inhibitory concentration of antibiotics". References are listed at the end of the thesis.

2. Background

2.1. Project 1: Effect of cell growth phases on transconjugant formation in plate matings and in biofilms

Horizontal transfer of mobile genetic elements is an important mechanism of microbial adaptation to a changing environment and bacterial cells evolve rapidly by transfer of DNA (Top and Springael, 2003, Thomas and Neilsen, 2005). Conjugation of plasmids, the spread of extrachromosomal DNA between cells mediated by cell to cell contact, is an important mechanism of HGT. Plasmid transfer has been studied in both batch cultures and in biofilms (e.g. Bathe et al., 2004, 2005, 2009).

In the natural environment, microorganisms predominantly exist enclosed in a self-secreted matrix of extracellular polymeric substances (EPS) in the form of biofilms or other bioaggregates, rather than as individual cells, and usually exist in the stationary growth phase (Gefen et al., 2014). The steps involved in biofilm formation are (i) reversible attachment: initial attachment of the bacteria cells to the substratum, (ii) irreversible attachment: cells are cemented to the substratum and form cell clusters, (iii) cell clusters are matured and cells are embedded in the EPS, (iv) dispersion: cells are detached from the cell cluster (Sauer et al., 2002). Bester et al. (2005) showed that bacterial cells are not detached only from the established biofilms but also from the younger biofilms. The bacterial cells in the deeper layer of the biofilms have decreased growth rate due to spatial and substrate limitation.

Biofilms provide favourable conditions for HGT due to the close contact between microbial cells (Madsen et al., 2012). A study done by Cook et al. (2011) showed that the copy number of plasmid pCF10 was increased 1.5 – 2 times in gram positive *Enterococcus faecalis* biofilms than

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that of the planktonic cells. The increased number of pCF10 altered the responses to cCF10 which is a sex pheromone excreted by the recipient cell. Donor cells with more copy numbers of pCF10 responded to cCF10 more efficiently than the cells with low copy number of plasmid.

Further, plasmid carriage promotes biofilm formation through the expression of conjugative F pili which stimulate cell to cell contact between plasmid donor and recipient cells (Ghigo, 2001, Reisner et al., 2006, D'Alvise et al., 2010).

During conjugation, the filamentous tube like F-pilus (or conjugative pilus) is needed to form a mating pair between the plasmid donor and recipient cell to facilitate the transfer of DNA from donor to recipient cell. The DNA is transferred from the donor to the recipient cell as single stranded and becomes double stranded in the recipient cell by the formation of a complementary strand. F-pilus which is 1-2 μm in length, 8.5-9 nm in diameter attached to the surface of plasmid donor cells (Firth et al., 1996). *traA*, is the first gene in the *tra* operon. (Babic et al., 2008, Kalkum et al., 2002, Greated et al., 2002). *traA* encodes 121 amino acid precursors of the pilus subunit (Frost et al.,1984).

Bacterial cells in the deeper layers of established biofilms exhibit lower growth activity due to oxygen and nutrient limitation, similar to a stationary phase in a batch culture system where population growth eventually ceases due to nutrient limitation and waste product accumulation. Thus, the steady state of biofilm development may resemble stationary growth phase cultures (Collet et al., 2008). Cell size changes during the different phases of growth have been documented, showing that cell size decreases as cells near the end of the exponential growth phase and enter the stationary phase (Akerlund et al., 1995). Variations in cell size may influence conjugation rates. Seoane et al. (2011) have shown that cell elongation likely facilitates conjugation. In addition to cell size, plasmid conjugation rates can be affected by many biotic

and abiotic factors such as bacterial cell density (Normander et al., 1998), nutrient availability (Smets et al., 1993, Fox et al., 2008), or spatial architecture of the bacterial community (Molin and Tolker-Nielsen, 2003, Licht et al., 1999). In the exponential growth phase, microorganisms grow and divide at the maximum rate. Contrary, in the stationary growth phase, population growth slows down due to nutrient and oxygen limitation (Willey et al., 2011). In the long term stationary phase, expression of stress response genes (e.g. *rpoS*) is needed for the survival of bacterial cells (Finkel, 2006).

Limited information is available on how cell growth phases (and associated changes in cell size) affect plasmid transfer in planktonic cell batch cultures and in biofilms. Our objectives for this project were to investigate the effect of cell growth phases (exponential and stationary) of plasmid donor and recipient cells on transconjugant formation using planktonic cells in plate mating experiments and to study the effect of different growth phases of the plasmid donor on transconjugant formation in biofilms. To determine if conjugative pilus biosynthesis is linked to a specific growth phase of donor cells and enhanced transconjugant formation, we developed an RT-PCR assay to determine the expression of *traA* gene. For our study, we used the TOL plasmid pWWO which is a 117 kb plasmid and belongs to the IncP-9 group (Greated et al., 2002). Previously, pWWO was used to study plasmid transfer in batch cultures and biofilms (Pei and Gunch, 2009, Seoane et al., 2011, Boon et al., 2002, Christensen et al., 1993). In the exponential growth phase, cells grow and increase in number.

In the exponential growth phase, cells grow and increase in number. In the stationary phase cell growth eventually ceases due to the nutrient limitation and accumulation of waste products. We hypothesized that the abundance of transconjugants will be higher in the exponential growth phase than in the stationary growth phase of the plasmid donor and recipient cells in plate

matings, as cell activity is greater in the exponential phase. We also hypothesized that transconjugant formation will be enhanced when plasmid donor cells grown to exponential phase will be added in the recipient biofilms. The *traA* gene encodes pilus biosynthesis in plasmid donor cells. We hypothesized that the *traA* gene expression in donor cells will be linked to a higher percentage of transconjugants.

2.2. Project 2: Optimization of RT-PCR to detect expression of *traA* in plasmid donor cells

The polymerase chain reaction (PCR) is an accurate and rapid method for the detection of target DNA. As DNA is stable outside of cells, PCR provides positive results for both live and dead bacterial cells (Molaee et al., 2015). Quantitative PCR (qPCR) is the quantitative measurement of amplified DNA. Reverse transcriptase polymerase chain reaction (RT-PCR) is a suitable method for detecting the presence of living cells as well as gene expression, as this method targets RNA and in contrast to DNA, RNA is not stable once released from cells. RT-PCR is used to detect and quantify gene expression through the creation and amplification of complementary DNA (cDNA) reverse-transcribed from mRNA. Reverse transcription quantitative PCR (RT-qPCR) has been established as a method of choice for the quantification of mRNA transcripts of a gene of interest in biological samples (Volland et al., 2017). To determine relative changes in gene expression, a relative quantification can be used based on the relative expression of a target gene versus a reference gene (Pfaffl, 2001). An absolute quantification can be done using a calibration curve.

Bustin et al. (2009) summarized the essential information that should be included in publications in "The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR

Experiments". According to their guidelines, the authors recommended to include information about experimental set up, sample preparation and storage, nucleic acid extraction, reverse transcription, qPCR target information, qPCR protocol, qPCR oligonucleotides, qPCR validation, data analysis in the manuscripts. In this research project RT-PCR was used to detect the expression of the *traA* gene which encodes pilus synthesis in *Pseudomonas putida* BBC443 plasmid donor cells. This section outlines steps undertaken to optimize the RT-PCR technique (Figure 2.1) for the detection of *traA* gene expression. It highlights the importance of the use of appropriate controls to eliminate false-positive results.
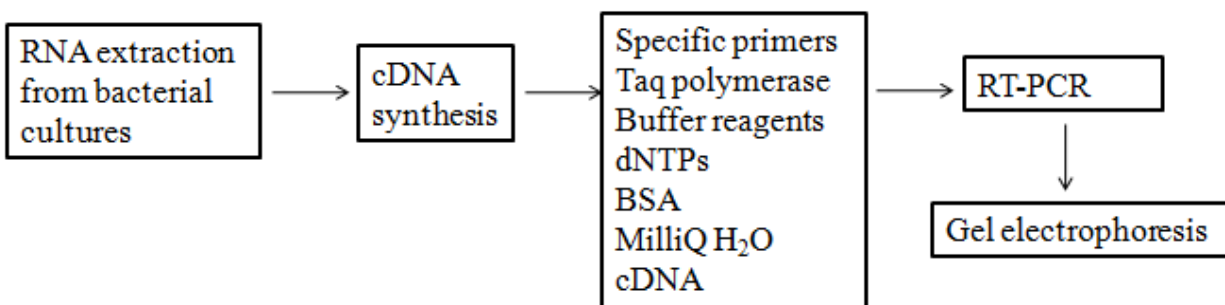
Figure 2.1. Steps involved in detection of gene expression using RT-PCR

2.3. Project 3: Effect of nutrient concentrations on the determination of minimum inhibitory concentration of antibiotics

In the environment, antibiotics are present at subinhibitory concentrations due to their extensive use in the treatment of human infections, veterinary medicine and agriculture (Laureti et al., 2013, Romero et al., 2011).

Spread of antibiotic resistance has become a problem for medical practice as resistant bacteria are causing increased number of infections which cannot be treated with existing antibiotics (Laureti et al., 2013, Bruchmann et al., 2013). Nutrient availability influences the physiological status of bacterial cells and this, in turn, may affect bacterial response to antibiotics (Smith and Prairie, 2004). In this study, we investigated the effect of nutrient concentration on MIC of antibiotic determination. MIC is the lowest concentrations of an antimicrobial that inhibits visible growth of bacteria after overnight incubation (Andrews, 2001). Nutrient concentration varies in both environmental and clinical conditions and microorganisms may experience nutrient limiting conditions in such environments. In our study, we used the strains *Pseudomonas putida* BBC443 and *P. putida* ATCC12633. These two strains were used in our previous studies (Shamsad and Hausner, submitted) and also in project 4 (refer to page 29). *P. putida* BBC443 carries plasmid pWWO which contains a kanamycin resistance gene (Pei and Gunsh, 2009, Christensen et al., 1993). Kanamycin is an aminoglycoside antibiotic (Mingeot-Leclercq et al., 1999). For our study, we investigated the effect of another aminoglycoside antibiotic gentamicin for which resistance was not encoded on the plasmid or the chromosome of either one of the two *Pseudomonas* strains. Neither strain is known to possess resistance against gentamicin.

A large group of aminoglycoside antibiotics are bactericidal although they have different ribosomal binding sites (Mingeot-Leclercq et al., 1999). Aminoglycoside antibiotics are bactericidal (Willey et al., 2011). Gentamicin binds with the 30S ribosomal subunit and inhibits protein synthesis (Kadurugamuwa et al., 1993). Gentamicin is an aminoglycoside antibiotic and inhibitor of protein synthesis by disturbing the elongation of peptide chain (Hahn and Sarre, 1969, Kadurugamuwa et al., 1993, Molina et al., 2014). Aminoglycoside modifying enzymes e.g. aminoglycoside 6'-N acetyltransferase, aminoglycoside 2"-adenylyltransferase, and

aminoglycoside acetyltransferases provide resistance to gentamicin (Zembower et al., 1998). A functional group of these enzymes binds with the antibiotic and inhibits the activity of the antibiotic (Azucena and Mobashery, 2001). Transport of aminoglycosides depends on electron transport and can be inhibited by low pH, hyperosmolarity and anaerobic conditions (Mingeot-Leclercq et al., 1999). A decreased uptake of the antibiotics due to membrane impermeability can also cause resistance for all aminoglycosides (Mingeot-Leclercq et al., 1999).

When cells lyse, DNA is released, and cells may also actively secrete DNA. Binding of DNA with aminoglycosides can contribute to inhibition of uptake and increases resistance (Chiang et al., 2013). Negatively charged cell components may also bind aminoglycosides and affect MIC. Bacterial metabolism may lead to changes in pH and influence aminoglycoside uptake. Limited information is available on the effect of nutrient concentration, inactivated biomass, extracellular DNA and pH on MIC of antibiotics. The objective of this project was to determine the MIC of selected antibiotics under varying nutrient concentrations and to investigate potential effects of biomass and extracellular DNA availability on the MIC of gentamicin. We tested the effect of cell numbers (as inoculum), extracellular DNA and biomass on MIC of gentamicin to determine if theses provide protective effect from gentamicin. We measured pH when different cell numbers were added as inoculum to detect if pH changes after incubation.

With decreased nutrient concentrations, bacterial cells will be less metabolically active and a lower MIC of antibiotics will be needed to inhibit bacterial growth as fewer targets for the antibiotics will be available. We hypothesized that values of MIC of antibiotics will decrease with decreasing nutrient concentrations.

We chose gentamicin as a representative of the aminoglycoside antibiotics and also because gentamicin was used in project 4 where we investigated the effect of MIC and subinhibitory

concentration of gentamicin on plasmid transfer in batch cultures and biofilms (Shamsad and Hausner, in preparation).

2.4. Project 4: The effects of subinhibitory and minimum inhibitory concentrations of gentamicin on transconjugant formation in plate matings and biofilms

Antibiotics are chemotherapeutic agents, which are a powerful tool to treat bacterial diseases (Sengupta et al., 2013). These molecules are produced by fungi and bacteria. Antibiotics are used in medicine and agriculture. Antibiotics are not fully metabolized by humans and are not biodegradable. Depending on the class of antibiotics, a large amount (20-80%) of the administered antibiotics are excreted by humans and animals as unchanged chemicals and released in the environment (Anderson and Hughes, 2014). As a result, an active form of antibiotics remains in the environment at subinhibitory concentrations (Laureti et al., 2013). Antibiotics also have been shown to act as signalling molecules that induce changes in gene expression in microbial populations and influence physiological functions such as motility and production of metabolites (Romero et al., 2011, Laureti et al., 2013, Baharoglu and Mazel, 2011).

Some studies have demonstrated that subinhibitory concentrations of antibiotics can upregulate expression of genes (e.g. in *Pseudomonas aeruginosa*, an aminoglycoside response regulator, *arr* gene) and enhance biofilm formation (Hoffman et al., 2005, Bagge et al., 2004). Ma and Bryers (2012) demonstrated that plasmid transfer was enhanced in biofilms exposed to subinhibitory concentration of antibiotics for which resistance was encoded on the plasmid.

Biofilms present considerable challenges to the successful eradication of bacterial infection since they act to protect bacteria from the effects of both the host immune system and antibacterial

drugs (Davies, 2003). Biofilm bacteria are less responsive to antibiotic stressors than planktonic organisms of the same species as extracellular polymeric substances (EPS) of biofilms provide protection (Stewart, 2002). EPS contain exopolysaccharides, proteins and extracellular DNA. Binding of antibiotics with these components reduces the penetration of antibiotics through the biofilms (Ciofu and Tolker-Nielsen, 2011, Hoiby et al., 2010). Other reasons of antibiotic resistance of biofilms include slow growth of biofilm cells, presence of persister cells (spore like state) and adaptive stress response of biofilm cells (Stewart, 2002). Walters III et al. (2003) analysed the roles of antimicrobial penetration, oxygen limitation, and low metabolic activity in the tolerance of *P. aeruginosa* biofilms to ciprofloxacin and tobramycin. Biofilms were treated with ciprofloxacin (1.0 µg/mL) for 12 h and tobramycin (10 µg/mL) for 36 h. The minimum inhibitory concentrations (MICs) for ciprofloxacin and tobramycin were 0.1 µg/mL and 0.01 µg/mL respectively. The authors found that both antibiotics were able to kill only metabolically active bacteria located in zones with high oxygen concentration, suggesting that low metabolic activity is more relevant to biofilm antibiotic tolerance.

Subinhibitory concentrations of aminoglycoside antibiotics (which are widely used to treat chronic bacterial infections of heart, lung and urinary tract e.g. kanamycin, gentamicin) induced biofilm formation in *P. aeruginosa* and *E. coli* (Hoffman et al., 2005). Ma and Bryers (2012) investigated the effect of subinhibitory concentration of kanamycin and imipenem on the transfer of the TOL plasmid pWWO in *Pseudomonas putida* donor and *Pseudomonas putida* recipient cultures. Their results demonstrated enhanced transfer of pWWO (which carried a kanamycin resistance gene) in biofilms exposed to subinhibitory concentration of kanamycin (2.5 µg/mL), up to tenfold. Also, the biofilms exposed to subinhibitory concentrations of kanamycin had higher thickness and cell density than biofilms which were not exposed to antibiotics. However,

after imipenem (for which resistance was not encoded on the plasmid) exposure, biofilm cell population and overall biofilm amount were also enhanced compared to the controls (no antibiotic), but transconjugant populations were not increased. The authors concluded that "biofilm bacteria might sense antibiotics to which they are resistant and subsequently enhance the spread of that resistance". According to Salcedo et al. (2015), the highest biomass and number of transconjugants were obtained for *E. coli* (plasmid donor) and *P. aeruginosa* (plasmid recipient) mixed culture biofilms at the 100-1000 ppb levels (approximately corresponding to values near or below MIC for e.g. gentamicin) of antibiotics. In summary, subinhibitory concentrations of antibiotics may enhance both horizontal gene transfer and biofilm development.

In this study, we investigated the effect of subinhibitory concentration and minimum inhibitory concentration of gentamicin on transconjugant formation in planktonic cell batch cultures. We also investigated the effect of subinhibitory concentration of gentamicin in biofilms, grown in 24 well plates. Gentamicin is an aminoglycoside antibiotic and effective in the treatment of gram-negative infections (Hathorn et al., 2014). We hypothesized that the number of transconjugant will be greater with the exposure to gentamicin from that of the control (without gentamicin) due to the action of antibiotics as signalling molecules which may change gene expression due to the alteration of transcription patterns. For this study, we used plasmid pWWO donor strain *Pseudomonas putida* BBC443. A gene for kanamycin resistance is encoded on the plasmid. Like kanamycin, gentamicin is also an aminoglycoside antibiotic, but the test strain does not carry genetically-encoded resistance against gentamicin. The objective of this study was to test the effect of gentamicin on transconjugant formation in plate matings and biofilms.

Chapter 3

3. Materials and methods

3.1. Effect of cell growth phases on transconjugant formation using planktonic cultures and in biofilms

3.1.1. Strains and media

Modified *Pseudomonas putida* BBC443 strain (Christensen et al., 1998, Pei and Gunsch, 2009) carrying a green fluorescent protein tagged derivative of TOL plasmid pWWO, which also carries a kanamycin resistance gene was used as a plasmid donor strain (kindly provided by Dr. C. Gunsch, Duke University, with permission of Dr. S. Molin, Technical University of Denmark). *Pseudomonas putida* ATCC12633 was used as a plasmid recipient strain. *P. putida* BBC443 was derived by inserting a mini-Tn5-LacIq-cassette into the *P. putida* KT2442 chromosome and by modifying the TOL plasmid inserting a mini-Tn5-PA$_{1-/04/03}$::gfpmut3b cassette (Christensen et al., 1998).

P. putida BBC443 has chromosomally encoded rifampicin resistance and contains the *lacI^q* repressor gene on its chromosome. The product of this gene represses the expression of the green fluorescent protein which is under the control of an *Escherichia coli* lac-derived promoter. The recipient strain *P. putida* ATCC12633 did not carry *lacI^q* repressor gene, therefore, green fluorescence was expressed in transconjugant cells after the transfer of plasmid from donor to recipient cell. Lysogeny broth (LB) (Bioshop Inc., ON, Canada) was used to grow the plasmid donor and recipient strains. For donor strain cultivation, kanamycin (Bioshop Inc., ON, Canada) was added for a final concentration of 50 µg/mL. Cultures were incubated at room temperature on a table shaker (390 rpm).

3.1.2. Growth curve

To study the effect of growth phases on plasmid transfer in planktonic cell batch cultures, growth curves of *P. putida* BBC443 and *P. putida* ATCC12633 were generated. Separate flasks with LB broth medium were inoculated with overnight cultures of each strain and incubated at room temperature with shaking at 390 rpm. Optical density readings were measured by spectrophotometer (Biophotometer, Eppendorf) and plate counts on LB plates were done to calculate CFU/mL and generate the growth curves for each strain.

3.1.3. Plate mating experiments

Recipient and donor strains were grown for 6.5 to 7.0 h to reach early exponential growth phase or to 144 h to reach late stationary growth phase prior to the setup of mating experiments. A concentration of 10^8 CFU/mL of donor and recipient strains was used for all mating experiments. Cells were washed twice with 0.9% saline. Cell pellets were resuspended with 1 mL 0.9% saline. Donor and recipient cell solutions (200 µL of each) were transferred into a 1.5 mL vial and mixed well by vortexing. Cell suspensions (20 µL) of the mixture of the donor and recipient cells were spot-plated onto 3 different LB plates. Plates were incubated at room temperature for 72 h. On day 3 the entire mating patch was harvested and resuspended in 0.9% saline solution, mixed well and centrifuged at 7000× g for 8 minutes, stained and analyzed as described below.

3.1.4. Detection and quantification of cells and EPS

To estimate transconjugants expressed as a percentage of all cells and the amount of EPS produced in plate mating experiments, cell pellets from mating patches were stained with the nucleic acid binding stain Syto 62 (1:1000 dilution) (Invitrogen, MA, USA) and a fluorescently

labelled lectin (Con A – TRITC; Invitrogen, MA, USA) (0.1 mg/mL) (Johnsen et al., 2000) to visualize all cells and polysaccharides within EPS, respectively. Staining solutions (250 µL) were added to cell pellets in 1.5 mL vials and the vials were incubated for 20 minutes in the dark. Next, each pellet was washed with 1 mL of 0.9% saline. Five µL of the mating patch solutions were examined microscopically. Transconjugants were detected on the basis of their GFP mediated fluorescence. Twenty to twenty five images were collected using a confocal laser scanning microscope (CLSM) (LSM510, Carl Zeiss, Jena, Germany) using the 488 nm and 633 nm laser lines to detect transconjugants and to estimate total cells, respectively. The 543 nm laser line was used to visualize ConA binding to EPS. Images obtained with the CLSM were analyzed using NIH ImageJ (http.//rsb.info.nih.gov/ij/). Images were manually thresholded and the proportion of transconjugants as a percentage of all cells was determined using the "area fraction" command within the "analyze" menu of ImageJ.

3.1.5. Transconjugant formation in biofilms

To grow the recipient biofilm, sterile microscopic slides were submerged in a sterile 50 mL tube with 24.5 mL of LB medium and 0.5 mL of overnight culture of recipient *P. putida* ATCC12633, and incubated for 24 h at room temperature on a shaker at 100 rpm, similar to a procedure described by Krol et al. (2011). Microscopic slides with 24 h old biofilms were washed by dipping in 50 mL 0.9% saline to remove non-attached cells and then the slides were transferred to 25 mL of donor cultures grown to early exponential phase or late stationary phase and incubated for 12-14 h at room temperature on a shaker at 100 rpm. After 12-14 h unattached cells were removed by dipping the slides in 50 mL 0.9% saline and then transferred to fresh LB medium. The slides were transferred daily to tubes with 25 mL fresh LB medium. After 72 h, the

biofilms on slides were washed with 0.9% saline and stained with Syto 62 for 20 min in the dark. After staining the slides were washed by dipping in 50 mL 0.9% saline. To quantify transconjugant formation in biofilms, biofilms were stained with Syto 62 (1:1000 dilution) directly on slides and images were collected by CLSM as described above. Eight images per treatment were analyzed using NIH ImageJ. Cells in microscopic images were analyzed by measuring the percent area covered by transconjugant cells and all cells to yield percentages of green fluorescing cells (transconjugants) within the total cells.

3.1.6. Cell size measurement at different growth phases

P. putida BBC443 and *P. putida* ATCC12633 were grown to early exponential (6.5 to 7.5 h) and late stationary (144 h) growth phases. Cells were spun down at 7000 g for 5 min, the supernatants were discarded, and the cell pellets were stained with Syto 62 for 20 min in dark. Cells were washed twice with 0.9% saline. Ten to fifteen images were collected using CLSM. Fifty cells of each the donor and recipient strain were measured by NIH ImageJ to obtain the cell size at early exponential and late stationary growth phases. Using these values, the average cell size at different growth phases of donor and recipient strains was calculated.

3.1.7. DNA extraction, primers and polymerase chain reactions (PCR)

DNA extraction from cell cultures of plasmid donor strain grown to early exponential (6.5 h) and late stationary growth phases (144 h) was performed using a GeneElute Bacterial Genomic DNA kit (Sigma-Aldrich) following the manufacturer's instructions. PCR was used to amplify portions (200 bp) of the 16S rRNA and *tra*A genes.

The primer sequences used to amplify 16S rRNA and *tra*A genes (Greated et al., 2002) were F-AGGGTGGTGGAATTTCCTGT, R-AGCTGCGCCACTAAAATCTC and F-ACAGTACCCATCCGGTTCAA, R-GCTTGTTCCTGGGTGATGTT (designed in this work), respectively. The 50 µL PCR reaction mixture contained 6 µL of template DNA, MilliQ H_2O, 1 µL of both the forward and reverse primer (primers working concentration to amplify was 25 µM), 0.6875 µL BSA (20×) (New England BioLabs, MA, USA), 200 µM of each dNTP (Bio Basics, ON, Canada), 2.5 units of Taq polymerase (New England BioLabs, MA, USA) in 10× Taq buffer (100 mM Tris hCl pH 9.0, 500 mM KCl, 15 mM MgCl2) (New England BioLabs, MA, USA).

PCR was performed according to the following program: denaturing at 96º C for 5 min, thermocycling at 94º C for 1 min, annealing temperature was 55º C for 1 min and decreased by 1º C in every cycle for 10 cycles, followed by 3 min elongation time at 72º C. Additional 19 cycles were performed without decreasing the annealing temperature.

PCR products were loaded into a 1% agarose gel with Red safe DNA gel stain (FroggaBio, ON, Canada) with a 100-bp ladder (Froggabio, ON, Canada), ran for 25 minutes at 100 V, followed by visualization using Molecular Imager ChemiDoc^{TM} XRS (Bio-Rad) to determine the presence of the PCR products.

3.1.8. RNA isolation and cDNA synthesis

Total RNA was extracted from the plasmid donor strain grown to early exponential phase (6.5 h), 48 h and late stationary phase (144 h) using the Fast prep system (MP Biomedicals, CA, USA) following the manufacturer's instructions. Twenty µg RNA was treated with DNase 1

(Promega, Madison, USA). iScript cDNA synthesis kit (Bio-Rad,CA,USA) was used to synthesize cDNA from 1 µg RNA.

3.1.9. RT-PCR amplification

RT-PCR was used to amplify segments (200 bp) of the 16S rRNA gene and to detect the expression of *tra*A genes. The 50 µL PCR reaction mixture contained 0.2 µg of template cDNA. Primer sequences, reagents and the program used to amplify 16S rRNA gene are mentioned above.

PCR reactions for *traA* was performed according to the following program:

96°C for 5 min (one cycle) followed by 94ºC for 1 min, 55.3 º C for 1 min, 72ºC for 3 min. 30 cycles were performed. The PCR products were sent to Applied Genomics at SickKids in Toronto to perform gel electrophoresis. To confirm cDNA was synthesized and RNA samples were free from genomic DNA, no RT control (reverse transcriptase enzyme was not added during the cDNA synthesis), was used for all samples.

3.1.10. Statistical analysis

For batch culture experiments, one-way ANOVA with post hoc Tukey HSD test was used to determine the differences between different conditions. For biofilm experiments, t-test was used to determine the difference in transconjugant percentages in all cells. Data sets were considered to be significantly different at $p < 0.05$.

3.2. Optimization of RT-PCR to detect expression of *traA* in plasmid donor cells

3.2.1. Bacterial culture and growth condition

Pseudomonas putida BBC443 carrying TOL plasmid pWWO (Christensen et al., 1998, Pei and Gunsch, 2009) was used as plasmid donor strain in this research project. 100% LB medium amended with 50 µg/mL kanamycin was used to grow the donor cells at room temperature on a shaker at 390 rpm.

3.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from *Pseudomonas putida* BBC443 donor cells grown to different growth phases: early exponential (6.5 h), mid exponential (24 h), late exponential (48 h), mid stationary (72 h) and late stationary phase (144 h or day 6) using the illustra RNA spin mini kit from GE healthcare life sciences (ON, Canada) or FastPrep system (MP Biomedicals, CA, USA) following the manufacturer's instructions by using TRIzol (Thermo Scientific, ON, Canada). For the Fast prep system, twenty µg RNA was treated with DNase 1 (Promega, Madison, USA).

iScript cDNA synthesis kit (Bio-Rad, CA, USA) was used to synthesize cDNA from 1 µg RNA. cDNA was quantified by NanoDrop (Implen, Munchen, Germany).

3.2.3. RT-PCR amplification

RT-PCR was used to amplify segments (200 bp) of the 16S rRNA and to detect the expression of *tra*A genes. Primer sequences are mentioned in section 3.1.7. To optimize the reaction, 50 µL PCR reaction mixture containing 0.1, 0.2, 0.4, 0.8, or 1.5 µg of template cDNA was used. Other reagents are mentioned in section 3.1.7.

PCR for 16S rRNA was performed according to the following program: denaturing at 96º C for 5 min, thermocycling at 94º C for 1 min, annealing temperature was 55º C for 1 min and decreased by 1º C in every cycle for 10 cycles, followed by 3 min elongation time at 72º C. Additional 19 cycles were performed without decreasing the annealing temperature.

PCR reactions for *traA* gene was performed according to the above program that used to amplify 16S rRNA gene. A PCR program used to amplify *traA* is described in section 3.1.9.

RT-PCR (10 µL) products were loaded into a 1% agarose gel with ethidium bromide stain (FroggaBio, ON, Canada) with a 100 bp ladder (Froggabio, ON, Canada), ran for 25 minutes at 100 V, followed by visualization using Molecular Imager ChemiDoc™ XRS (Bio-Rad, CA, USA) to determine the presence of the RT-PCR products.

3.3. Effect of nutrient concentrations on the determination of minimum inhibitory concentration of antibiotics

3.3.1. Bacterial strains, growth media and inoculum preparation

Pseudomonas putida ATCC12633 and *P. putida* BBC443 (carrying TOL plasmid pWWO which contains a kanamycin resistance gene) (Christensen et al., 1998, Pei and Gunsch, 2009) were used for this study. (Details mentioned in section 3.1.1). To prepare 100%, 50% and 10% Lysogeny Broth (LB), 20 g, 10 g, and 2 g LB broth powder (Bio Basics, ON, Canada) was added to 1 L deionized water, respectively.

To obtain inocula of *P. putida* BBC443, a volume (40 mL) of 100% or 50% LB broth was amended with 50 µg/mL kanamycin and added to 50 mL Falcon tubes. LB broth (10%) was not amended with kanamycin as the growth of *P. putida* BBC443 was not detected after overnight incubation when amended with kanamycin. The three different concentrations of LB broth were

inoculated with *P. putida* BBC443. Tubes were incubated on a shaker for 16-20 h at 320 rpm at room temperature until the optical density (OD_{600}) reached 0.4. These cultures were used to obtain 10^5 CFU/mL as inoculum for MIC experiments. Kanamycin was used to grow *P. putida* BBC443 because resistance is encoded on the plasmid pWWO. *P. putida*ATCC12633 cultures were prepared as described for *P. putida* BBC443, without the addition of kanamycin.

3.3.2. Antibiotics

MICs of kanamycin, gentamicin, rifampicin and tetracycline were measured for the two *Pseudomonas* strains at different nutrient concentrations. Kanamycin is an aminoglycoside antibiotic for which resistance was encoded on plasmid pWWO, carried by *P. putida* BBC443. Gentamicin is an antibiotic in the same class as kanamycin, but the donor strain does not carry gentamicin resistance determinants. Rifampicin resistance is encoded on the *P. putida* BBC443 chromosome. Tetracycline resistance was neither plasmid- nor chromosomally encoded, but this antibiotic is widely used in human and veterinary medicines and distributed in the environment (Auerbach et al., 2006). Antibiotic stock solutions were made according to Andrews (2001) using powder of the antibiotics purchased from Bio Basics, ON, Canada. To prepare a stock solution, a weighed amount of the antibiotic powder was mixed with a volume of autoclaved milliQ H_2O. The prepared antibiotics were transferred into sterile 1.5 mL microcentrifuge tubes and stored at -20 °C (Andrews, 2001) until further use.

3.3.3. Determination of the minimal inhibitory concentration (MIC)

Overnight culture (10^5 CFU/mL), a specific volume of 100% LB broth, a specific volume of antibiotic stock solution, and a specific volume of autoclaved deionized water (for 10% and 50%

LB) were added in a 13 mL test tube to obtain total volume of 5 mL. Each treatment was carried out in triplicate. The test tubes were incubated on a shaker for 18-24 h at 320 rpm at room temperature. At the end of the incubation period, the OD (at 600 nm) of the trials was measured. The lowest concentration of antibiotic at which bacterial growth was inhibited was determined as the minimal inhibitory concentration of that antibiotic. The experiment was carried out in 10%, 50%, and 100% LB broth concentrations to determine the MIC for each of the antibiotics.

The positive (no antibiotic) control for the experimental trials consisted of the appropriate amounts of 100% LB broth, deionized water (only for 10% and 50% LB conc.), and an overnight culture to obtain 5 mL total volume. The negative control (no overnight culture) contained appropriate amounts of 100% LB broth, deionized water (only for 10% and 50% LB concentrations) and an antibiotic to obtain 5 mL total volume.

3.3.4. Determination of minimum bactericidal concentration (MBC)

To determine minimum inhibitory concentration (MBC) of the antibiotics 100% LB was used. The experimental test tubes that were used for MIC experiments were also used to determine the MBC. After incubating for 24 h at room temperature, the OD was measured for all tubes and the test tubes that had an OD value of zero were plated on 100% LB agar plates using the spot plate technique. After 3-4 days, the plates were observed for colony appearance. The lowest concentration of antibiotic at which no colony was observed was determined as the minimum bactericidal concentration of that antibiotic (Andrews, 2001).

3.3.5. Determination of minimum biofilm eradication concentration (MBEC)

To determine the minimum biofilm eradication concentration for *P. putida* ATCC12633, the biofilms were grown for 48 h in 24 well plates at room temperature. To grow the biofilms 490 µL 100% LB broth and 10 µL overnight culture (OD was ~ 0.4) were added into the wells. Every 24 h, 250 µL medium was removed and fresh LB was added. After 48 h, 250 µL of the LB medium was removed and fresh LB was added to the wells to adjust the volume. Each concentration of antibiotics was added to four different wells. Positive (LB broth, overnight culture) and negative controls (LB broth, lowest concentration of antibiotic) were also set up for each antibiotic. After addition of the antibiotic, biofilms were grown for an additional 18-20 h. After 18-20 h, almost all the liquid was removed from the wells. The wells were washed twice with 100 µL of 0.9% saline to remove planktonic cells. Another 50 µL of 0.9% saline was added in the wells, mixed thoroughly by pipetting and 5 µL of the solution from each well was spot plated three times on LB agar plates. After incubation for 3-4 days at room temperature, LB plates were visually observed for bacterial growth. The lowest concentration of antibiotic at which no colony was observed on the plates was determined as the minimum biofilm eradication concentration of that antibiotic.

3.3.6. Effect of cell number on MIC

To detect the effect of cell number on MIC with varying nutrient concentrations, 10^3, 10^5 or 10^7 CFU/mL of overnight culture, a specific volume of 100% LB broth, a specific volume of antibiotic stock solution, and a specific volume of autoclaved deionized water (for 10% and 50% LB) were added in a 13 mL test tube to obtain total volume of 5 mL.

3.3.7. Effect of pH

P. putida BBC443 cells were grown overnight with 100%, 50% and 10% LB. To determine the effect of pH on MIC of gentamicin, $10^3/10^5/10^7$ CFU/mL overnight culture, antibiotic (gentamicin), a specific volume of autoclaved deionized water (for 50% and 10% LB) and LB broth were added to get final volume 5 mL. Cultures were incubated for 18 h on 320 rpm shaker at room temperature. pH (Mettler Toledo, ON, Canada) was measured before and after 18 h incubation.

3.3.8. Effect of DNA on MIC

DNA was extracted (Sigma Aldrich, MO, USA) from 10^7 CFU/mL *P. putida* BBC443 grown with 100% LB. To determine the effect of DNA on MIC of gentamicin, 10^3 and 10^5 CFU/mL with 10% LB were tested.

10^3 or 10^5 CFU/mL, antibiotic concentrations (0.2, 0.4, 0.8, 1.5 µg/mL), dH_2O (to dilute LB to 10%), LB and extracted DNA (5 or 10 µg/mL) were combined to a final volume of 5 mL in 13 ml tubes. The tubes were incubated for 18 h on 320 rpm shaker at room temperature. Turbidity was measured after 18 h.

3.3.9. Effect of biomass on MIC

To detect the effect of cell biomass, a culture grown to 10^7 CFU/mL was boiled to inactivate cells for 25 min. 10^3 or 10^5 CFU/mL, antibiotic concentrations (0.2, 0.4, 0.8, 1.5 µg/mL), dH_2O (to dilute LB to 10%), LB and biomass were combined to a final volume of 5 mL in 13 mL tubes. The tubes were incubated for 18 h on 320 rpm shaker at room temperature. Turbidity was measured after 18 h incubation.

3.4. The effects of subinhibitory and minimum inhibitory concentrations of gentamicin on transconjugant formation in biofilms and plate matings

3.4.1. Bacteria strains and growth conditions

A modified *Pseudomonas putida* BBC443 strain (Christensen et al., 1998, Pei and Gunsch, 2009) carrying a green fluorescent protein tagged derivative of TOL plasmid pWWO, which also carries a kanamycin resistance gene was used as a plasmid donor strain.

P. putida ATCC12633 was used as plasmid recipient strain. Details of these strains are mentioned in section 3.1.1.

Lysogeny broth (LB) (Bioshop Inc., ON, Canada) was used to grow the plasmid donor and recipient strains. For donor strain cultivation, kanamycin (Bioshop Inc., ON, Canada) was added to a final concentration of 50 µg/mL. Cultures were grown at room temperature (RT) with shaking at 390 rpm for 48 h.

3.4.2. Plasmid transfer in plate mating experiments in the presence of gentamicin

Plasmid donor *P. putida* BBC443 and recipient *P. putida* ATCC12633 were grown for 48 h prior to the set up of plate mating experiments with LB broth at room temperature with shaking (390 rpm). Donor cells were grown with 50 µg/mL kanamycin and also with combined 50 µg/mL kanamycin and 0.5× MIC concentration of antibiotic gentamicin for 48 h. Also, donor cells grown with kanamycin and 0.5× MIC gentamicin, were additionally challenged with MIC gentamicin. Donor and recipient cell solutions were collected into a 1.5 mL vials and washed twice with 0.9% saline. Mixtures of the donor and recipient cells were spot-plated on LB plates (triplicate), LB + 0.5× MIC gentamicin (Bioshop, ON, Canada) and LB + MIC gentamicin plates

(triplicate). The plates were incubated at room temperature. MICs of gentamicin for the plasmid donor and recipient were 1.5 µg/mL and 3.6 µg/mL, respectively.

After 72 h, entire mating patches were collected by scraping, resuspended in 0.9% saline, washed two times with 0.9% saline, and stained with Syto 62 (Invitrogen, MA, USA) for 20 min in the dark. Mating patch solutions were examined by CLSM. Images were analyzed using Image J. The abundance of transconjugants was expressed as a percentage of total cells.

3.4.3. Plasmid transfer in biofilms in the presence of gentamicin (in 24 well plates)

Plasmid recipient *Pseudomonas putida* ATCC12633 cells were grown overnight in LB medium to optical density (OD) of 0.4. To grow the biofilms, 10^5 CFU/mL of this culture was added together with 490 µL LB to 24 well plates. Recipient biofilms were grown at RT for 48 h in 24 well plates in a stationary mode. Donor *P. putida* BBC443 cells were grown for 48 h with 50 µg/mL kanamycin and also with combined 50 µg/mL kanamycin and 0.5× MIC concentration of antibiotic gentamicin.

Donor cells grown with only kanamycin (10^9 CFU/mL) (after washing with 0.9% saline) or kanamycin and 0.5× MIC concentration of gentamicin were added to the 48 h old recipient biofilms (in 3 different wells of 24 well plate). After addition of donor cells, 0.5× MIC gentamicin was added in the biofilms. Gentamicin was not added in the control biofilms. To check that donor cells alone do not fluoresce green, donor cells were added to 3 wells with LB and also with and without gentamicin (control).

After donor addition, 500 µL of liquid from the wells was removed and fresh LB medium was added to the wells every 24 h. Three days (72 h) after donor cell addition, 850 µL liquid was removed from the wells in order to remove planktonic cells. Next, the biofilms were washed

once with 100 µL 0.9% saline. Then the biofilms were scraped and transferred to 1.5 mL vials containing 0.9% saline. The biofilms were stained, and microscopic images were obtained. Details of staining and microscopy are mentioned below.

3.4.4. Staining and microscopy

To quantify transconjugant formation in biofilms, biofilms were stained with Syto 62 (1:1000 dilution) for 20 min in the dark. Images were collected by CLSM using 488 nm and 633 nm laser lines to detect GFP and Syto 62, respectively, and analyzed using NIH ImageJ. The percent area covered by transconjugant cells and the total cells were determined to yield green fluorescing cells (transconjugants) as a percentage of total cells.

3.4.5. Statistical analysis

For batch culture and biofilm experiments, one way ANOVA with post hoc Tukey HSD test was used to determine the differences between different conditions. Data sets were considered to be significantly different at $p < 0.05$.

Chapter 4

4. Results

4.1. Effect of cell growth phases on transconjugant formation using planktonic cultures and in biofilms

4.1.1. Effect of cell growth phases on plasmid transfer in plate matings using planktonically grown cells

Growth curves constructed for donor and recipient strains showed that the plasmid donor and recipient cells reached the early exponential growth phase after 6.5-7.5 h and the late stationary growth phase at 144 h (Figure 4.1 and Figure 4.2). The donor and recipient growth curves did not follow a typical growth curve pattern. After an initial rapid increase in cell number, the CFU/mL decreased and then plateaued. The CFU/mL of plasmid donor cells at 119.5 h and 169 h was 2.6×10^8 and 1.5×10^8, respectively. The CFU/mL of plasmid recipient cells at 147.5 h and 171.25 h CFU/mL was 2.6×10^8 and 2.2×10^8, respectively. Since the growth curves of our strains do not follow a typical pattern, we chose to assign the term "late stationary phase" to the phase of growth at 144 h or later. Finkel (2006) discussed the phenomenon of long term stationary phase cultures. After the death phase, survivor cells can be maintained for months in long term stationary phase conditions.
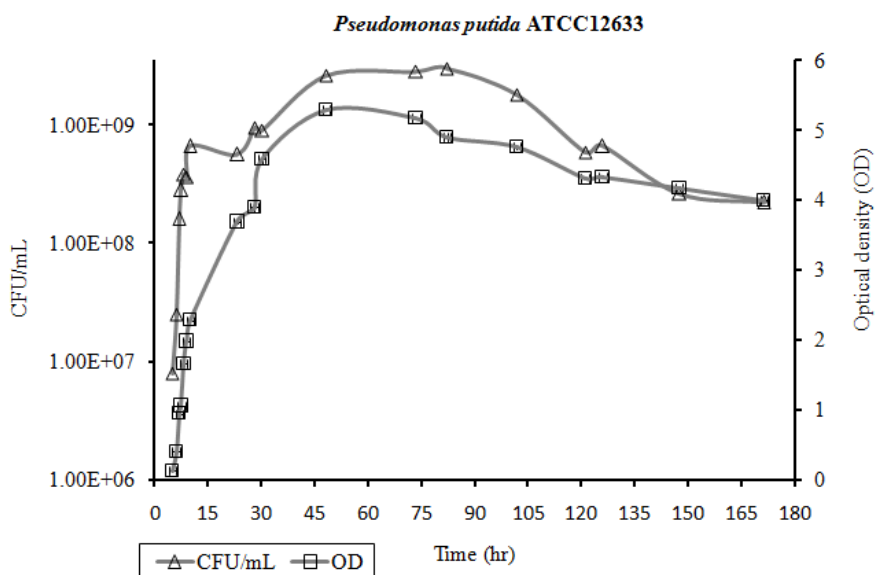
Figure 4.1. Growth curve of the plasmid recipient strain *Pseudomonas putida* ATCC12633. Optical density reading was obtained at 600 nm wavelength.
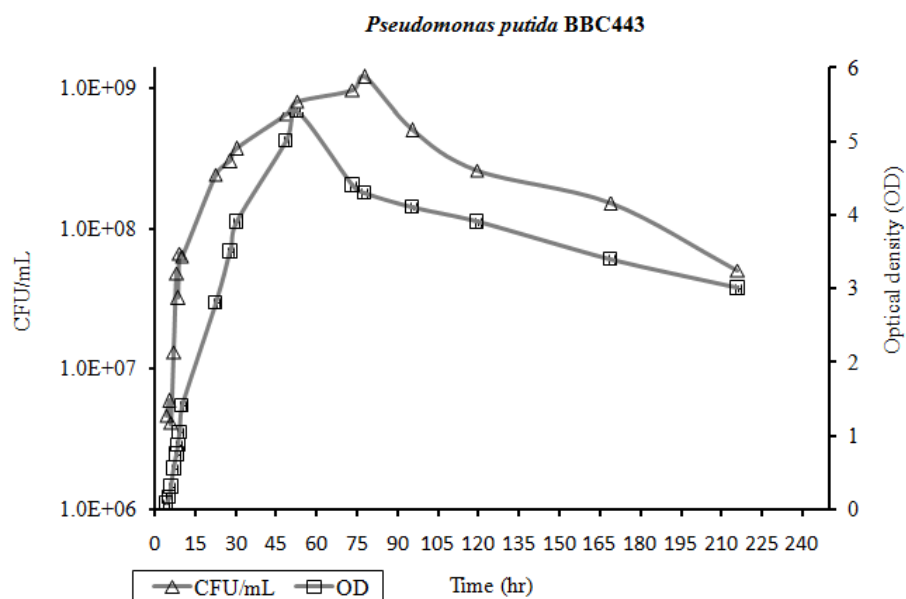
Figure 4.2. Growth curve of the plasmid donor strain *Pseudomonas putida* BBC443. Optical density reading was obtained at 600 nm wavelength.

The expression of GFP was repressed in the plasmid donor cells due to the action of *lacIq* gene. Figure 4.3, shows no detection of GFP in *P. putida* BBC443 by confocal laser scanning microscopy.
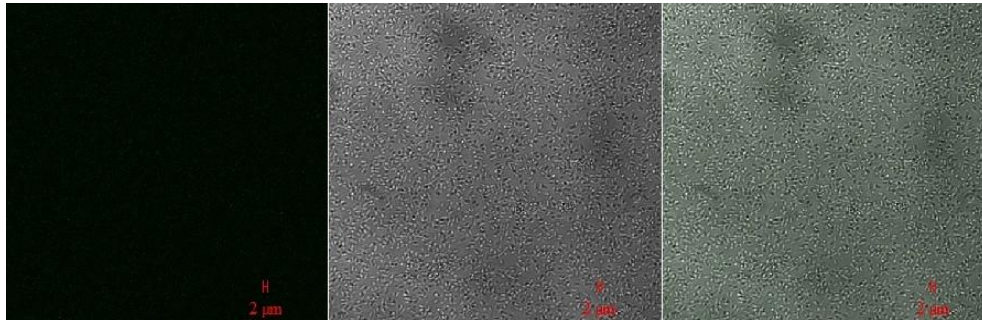
Figure 4.3. Microscopic image of plasmid donor *Pseudomonas putida* BBC443 cells. Donor contains the *lacI^q* repressor gene on its chromosome, which represses the expression of green fluorescence in donor cells.

Quantitative analysis (Table 4.1) of transconjugant cells and total cells derived from microscopic images (Figure 4.4 and Figure 4.5) followed by statistical analysis revealed that the transconjugants expressed as a percentage of all cells were the highest when both plasmid donor and recipient cells were harvested from the late stationary phase (Figure 4.5), whereas the lowest percentage of transconjugants was detected when the plasmid donor cells were grown to early exponential phase and the recipient cells were harvested from the late stationary phase (Table 4.1).

Table 4.1. Transconjugants in mating patches as a percentage of total cells.

Condition	Different growth phases of plasmid donor and recipient strains	Percentage of pWWO transconjugants in total cells
A	Plasmid donor and recipient cells were grown to early exponential phase	$8.0 \pm 3.0\%$
B	Plasmid donor and recipient cells were grown to late stationary phase	$20.0 \pm 7.6\%$
C	Plasmid donor cells were grown to early exponential phase and recipient cells were grown to late stationary phase	$2.1 \pm 1.0\%$
D	Plasmid donor cells were grown to late stationary phase and recipient cells were grown to early exponential phase	$7.3 \pm 2.5\%$

When both the donor and recipient cells were harvested from the early exponential phase, the percentage of transconjugants ($8.0 \pm 3.0\%$) was significantly lower (Figure 4.6) than when both the plasmid donor and recipient cells were harvested from the late stationary phase ($20.0 \pm 7.6\%$; Figure 4.5, Table 4.1). However, this result ($8.0 \pm 3.0\%$) was not significantly different from that obtained when the plasmid donor was grown to late stationary phase and the plasmid recipient was grown to early exponential phase ($7.3 \pm 2.5\%$; Figure 4.6).
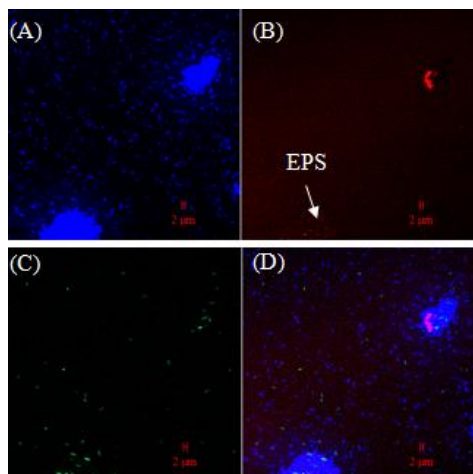
Figure 4.4. Microscopic images of plate mating patch of planktonically grown cultures (donor and recipient cells were grown to early exponential growth phase). (A) All cells were visualized with Syto 62 (blue), (B) EPS bound Con A-TRITC (red), (C) Transconjugants express GFP (green), (D) Overlay of all images.
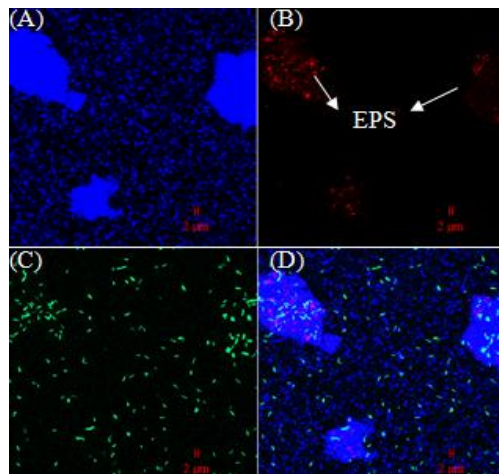
Figure 4.5. Microscopic images of plate mating patch of planktonically grown cultures (donor and recipient cells were grown to late stationary growth phase). (A) All cells were visualized with Syto 62 (blue), (B) EPS bound Con A-TRITC (red), (C) Transconjugants express GFP (green), (D) Overlay of all images.

Qualitative observations revealed that more EPS was present in the late stationary phase mating patch samples (Figure 4.5) than in those from the early exponential mating patch (Figure 4.4) and was mostly associated with cell clusters.
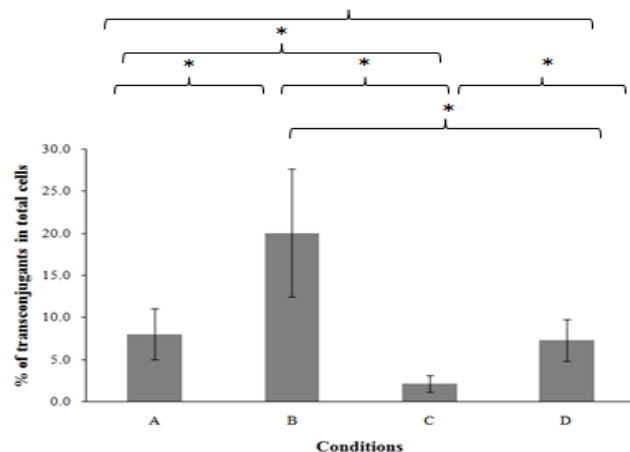
Figure 4.6. Data represent transconjugants expressed as a percentage of all cells for batch culture experiments. Condition A: plasmid donor and recipient cells were grown to early exponential phase, condition B: plasmid donor and recipient cells were grown to late stationary phase, condition C: plasmid donor cells were grown to early exponential phase and recipient cells were grown to late stationary phase, condition D: plasmid donor cells were grown to late stationary phase and recipient cells were grown to early exponential phase. Statistically significant groups are marked as *.

Qualitative observations also revealed that the plasmid donor strain culture at the late stationary phase exhibited increased cell aggregation and associated EPS than at the early exponential growth phase (Figure 4.7 and Figure 4.8, respectively).
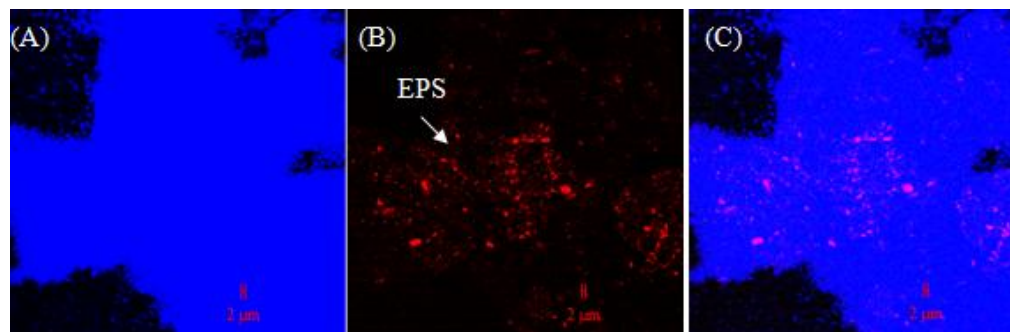
Figure 4.7. Microscopic image of planktonically grown donor cells to late stationary growth phase. Cells were stained with Syto 62 and Con A-TRITC. (A) All cells were visualized with Syto 62 (blue), (B) EPS bound ConA-TRITC (red), (C) Overlay of all images.
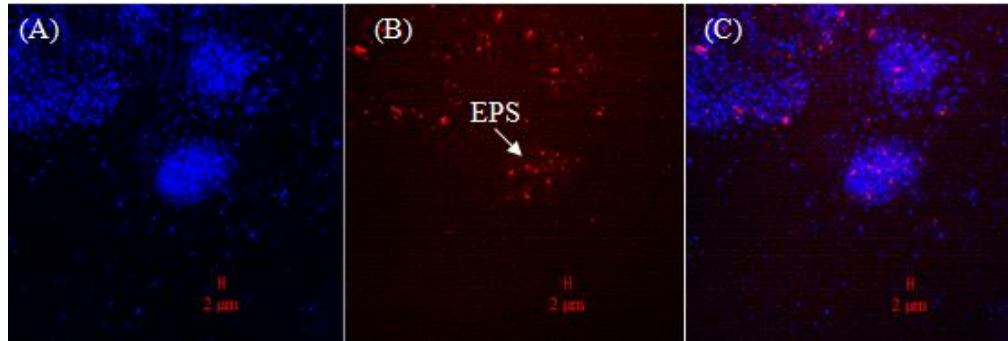
Figure 4.8. Microscopic image of planktonically grown donor cells to early exponential growth phase. Cells were stained with Syto 62 and ConA-TRITC. All cells were visualized with Syto 62 (blue), (B) EPS bound ConA-TRITC (red), (C) Overlay of all images.

While it was expected that the presence of EPS may obstruct conjugation by preventing direct cell to cell contact or may hinder the spread of donor cells in the inner layers of recipient biofilms (Merkey et al., 2011), our qualitative observations of EPS presence suggest that EPS does not impede gene transfer in plate matings. It was desired to find out if the higher percentage of transconjugants obtained from mating patches of both donor and recipient cells harvested from the stationary phase correlate with cell size. Results demonstrated that the cell length of both donor and recipient cells obtained from the early exponential phase was 2.4-2.6 fold greater than that of cells harvested from the late stationary phase (Table 4.2, Figure 4.9).
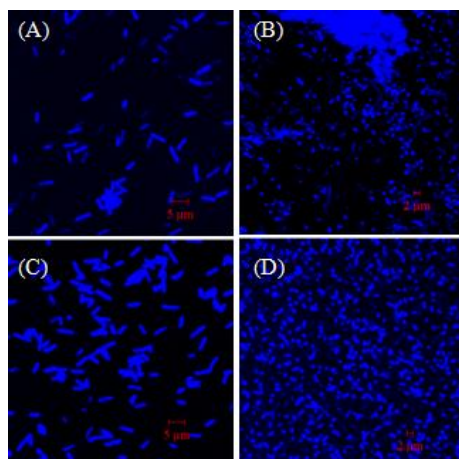
Figure 4.9. Microscopic images of plasmid donor and recipient cells grown to different growth phases. Cells were stained with Syto 62 (blue). (A) Donor cells grown to early exponential phase, (B) Donor cells grown to late stationary phase (C), Recipient cells grown to early exponential phase, (D) Recipient cells grown to late stationary phase.

Interestingly, the highest percentage of transconjugants was detected when both donor and recipient cells were harvested from the late stationary phase (Table 4.1), i.e. when cells were the smallest (Table 4.2).

Table 4.2. Average cell size at exponential and stationary growth phases of *P. putida* ATCC12633 and *P. putida* BBC443.

Strains and growth phases	Average cell size (µm)
Donor strain *P. putida* BBC443 at exponential phase	4.8 ± 0.56
Donor strain *P. putida* BBC443 at stationary phase	2.0± 0.36
Recipient strain *P. putida* ATCC12633 at exponential phase	4.9 ± 0.81
Recipient strain *P. putida* ATCC12633 at stationary phase	1.9 ± 0.35

In our study, the presence of the *tra*A gene (encoding pilin synthesis, Greated et al., 2002) in both early exponential (6.5 h) and late stationary phase cells (144 h) (Figure 4.10) by PCR.
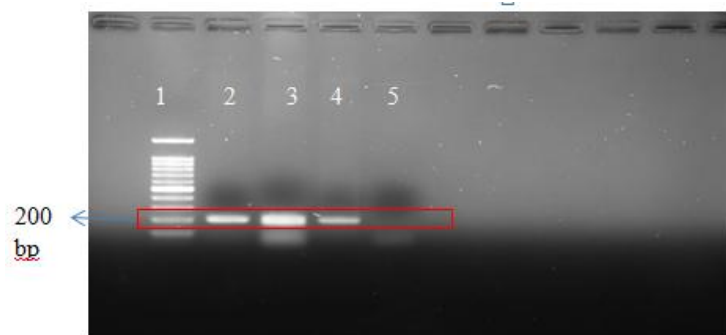
Figure 4.10. Agarose gel showing PCR-products of the *traA* gene (200 bp) Lane 1: marker, lane 2: positive control (PCR product for DNA extracted from donor strain *P. putida* BBC443 overnight culture), lane 3: PCR product for DNA extracted from donor strain *P. putida* BBC443 grown to early exponential growth phase, lane 4: PCR product for DNA extracted from donor strain *P. putida* BBC443 grown to late stationary growth phase, lane 5: negative control (PCR product for DNA extracted from recipient strain *P. putida* ATCC12633 overnight culture).

Using RT-PCR we detected expression of 16S rRNA gene (Figure 4.11) in donor cells grown to early exponential phase (6.5 h), late exponential phase (48 h) and late stationary phase (144 h). Expression of *traA* (which encodes pilus biosynthesis) was detected in donor cells grown to early exponential phase (6.5 h) and late exponential phase (48 h), but not for donor cells grown to late stationary phase (144 h) (Figure 4.12).
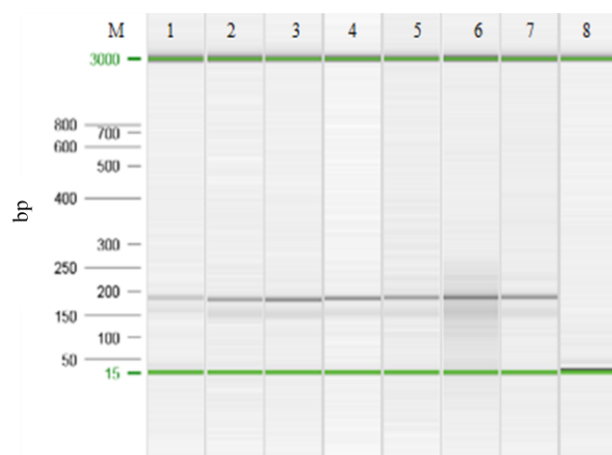
Figure 4.11. Gel electrophoresis for RT-PCR products (with 16S rRNA primers). RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases. Lane M: marker, lane 1: positive control (DNA), lanes 2 and 3: donor early exponential phase (6.5 h), lanes 4 and 5: donor 48 h, lanes 6 and 7: donor late stationary phase (144 h), lane 8: negative control (cDNA reagents). Expected product size was 200 bp.
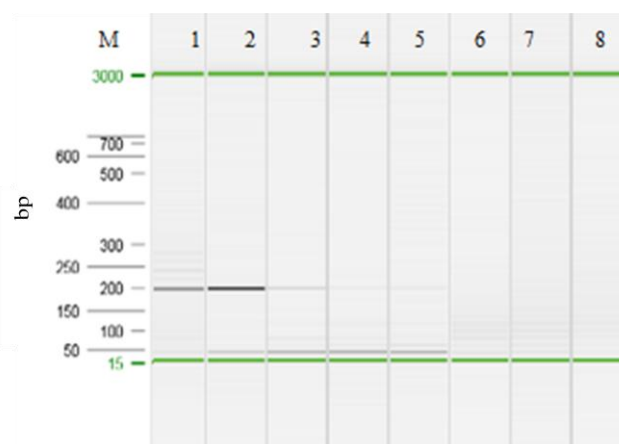
Figure 4.12. Gel electrophoresis for RT-PCR products (with *traA* primers). RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases. Lane M: marker, lane 1: positive control (DNA), lanes 2 and 3: donor early exponential phase (6.5 h), lanes 4 and 5: donor 48 h, lanes 6 and 7: donor late stationary phase (144 h), lane 8: negative control (cDNA reagents). Expected product size was 200 bp.

The amplified products (200 bp) were not detected in negative controls that omitted reverse transcriptase (Figure 4.13 and Figure 4.14).
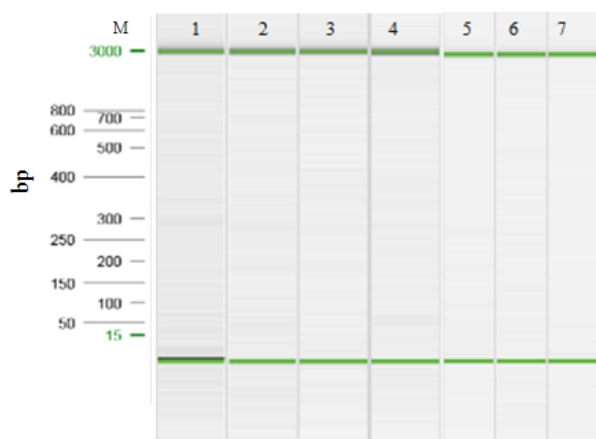
Figure 4.13. Gel electrophoresis for no RT control samples (with 16S rRNA primers). RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases without RT enzyme. Lane M: marker, lane 1: negative control (water as template), lanes 2 and 3: donor exponential phase (6.5 h), lanes 4 and 5: donor 48 h, lanes 6 and 7: donor late stationary phase (144 h).
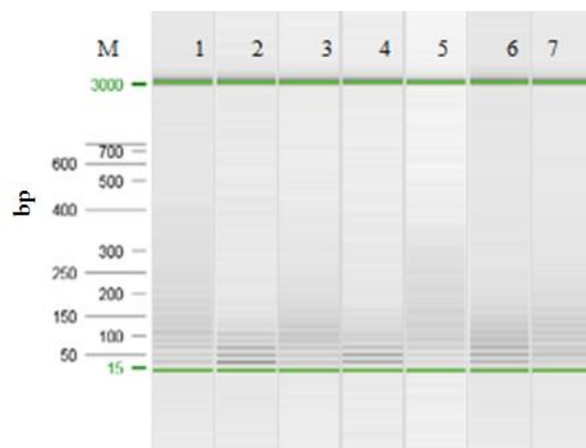
Figure 4.14. Gel electrophoresis for no RT control samples (*traA* primers). RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases without RT enzyme. Lane M: marker, lane 1: negative control (water as template), lanes 2 and 3: donor exponential phase (6.5 h), lanes 4 and 5: donor 48 h, lanes 6 and 7: donor late stationary phase (144 h).

4.1.2. Effect of plasmid donor growth phases on plasmid transfer in biofilms

Quantitative analysis of transconjugant cells and total cells derived from microscopic images revealed that transconjugants expressed as the percentage of all cells were $0.11 \pm 0.1\%$ and $0.20 \pm 0.2\%$ when donor cells grown to early exponential growth phase and late stationary growth phase were added to 24 h old recipient biofilm, respectively (Figure 4.15 and Figure 4.16, respectively).
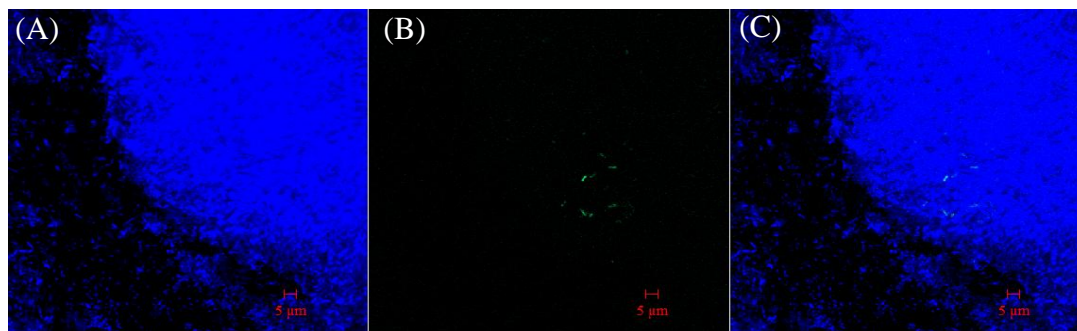
Figure 4.15. Microscopic image of transconjugants in recipient biofilms after plasmid donor cells grown to early exponential growth phase were added to 24 h old plasmid recipient biofilms. Image was taken after 72 h of donor cells addition. (A) All cells were visualized with Syto 62 (blue), (B) Transconjugants express GFP (green), (C) Overlay of all images.

However, these results were not significantly different following t-test analysis ($p = 0.27$).
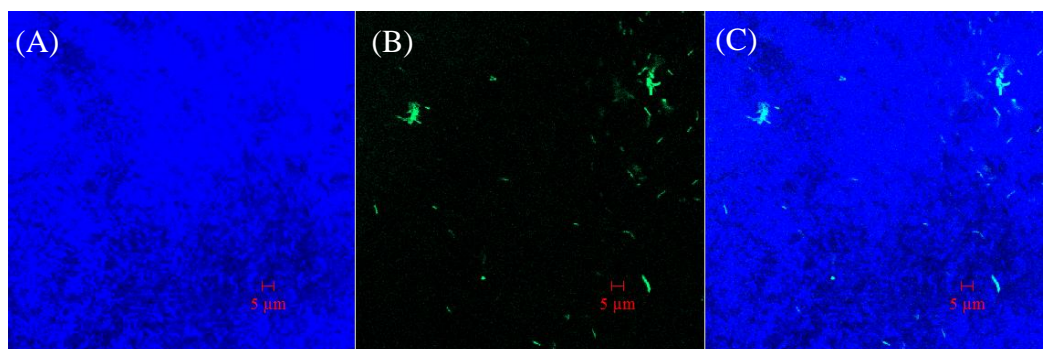
Figure 4.16. Microscopic image of transconjugants in the recipient biofilms after plasmid donor cells grown to late stationary growth phase were added to 24 h old plasmid recipient biofilms. Image was taken after 72 h of donor cells addition. (A) All cells were visualized with Syto 62 (blue), (B) Transconjugants express GFP (green) (C) Overlay of all images.

4.2. Optimization of RT-PCR to detect expression of *traA* in plasmid donor cells

RNA was extracted from *P. putida* BBC443 cells grown to different growth phases using the mini kit from GE health sciences (Figure 4.17 and Figure 4.18). cDNA was synthesized and RT-PCR reactions were performed to detect the presence of 16S rRNA and *traA* genes (Figure 4.19 and Figure 4.20, respectively).
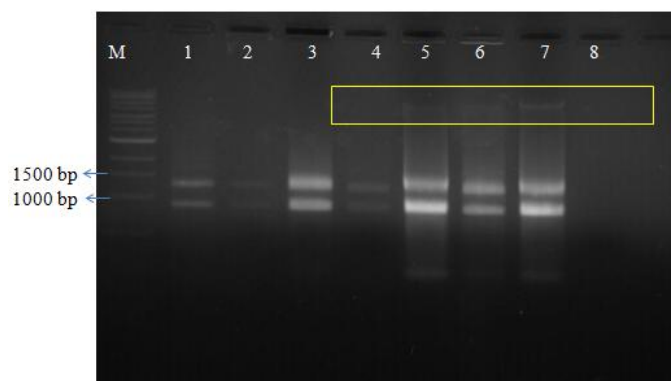
Figure 4.17. Gel electrophoresis for RNA samples. RNA was extracted from different growth phases of *P. putida* BBC443 donor cells. Illustra RNA spin mini kit from GE healthcare life sciences was used to extract RNA. Lane M: marker (1 kb), lanes 1-4: RNA was extracted from donor cells grown to early exponential phase (6.5 h, OD 0.6), lanes 5-7: RNA was extracted from donor cells grown to 24 h, lane 8: negative control (H2O, no RNA).
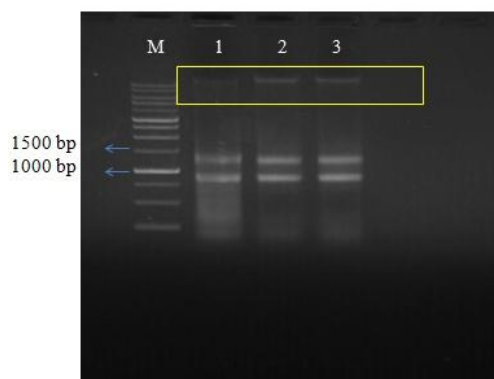
Figure 4.18. Gel electrophoresis for RNA samples. RNA was extracted from different growth phases of *P. putida* BBC443 cells. Illustra RNA spin mini kit from GE healthcare life sciences was used to extract RNA. Lane M: marker (1 kb), lane 1: RNA was extracted from donor cells grown to 72 h, lanes 2 and 3: RNA was extracted from donor cells grown to stationary phase (day 6).

Initial experiments omitted a set up where no reverse transcriptase was used. This step is important to confirm cDNA was synthesized and RNA is free of genomic DNA (Park et al.,

2003). As shown in Figure 4.20 (lanes 4 and 5), *traA* was detected from donor cells grown to 72

h and day 6. For the mini kit, DNase 1 treatment was performed for 30 min and bands were

visualized above the RNA (indicated by the yellow box in Figure 4.17 and Figure 4.18),

suggesting the possibility of genomic DNA contamination. Therefore, RNA samples were

treated with additional DNase 1 and RT-PCR reactions were performed.
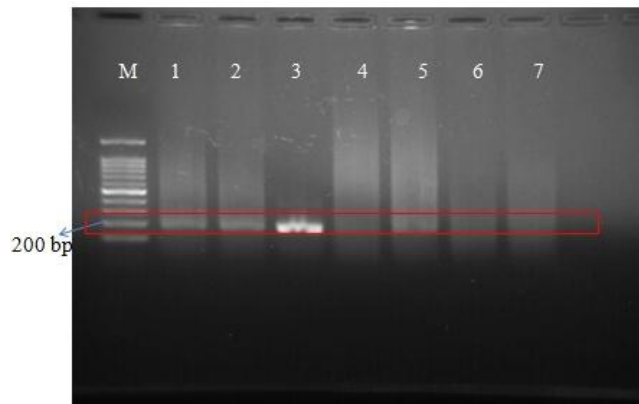
Figure 4.19. Gel electrophoresis for RT-PCR products. RT-PCR with cDNA (16S rRNA primers). RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases. Lane M: marker (100 bp), lane 1: positive control, lane 2: donor early exponential phase, lane 3: donor 24 h, lane 4: donor 72 h, lane 5: donor late stationary phase (day 6), lane 6: negative control (PCR reagents + cDNA reagents), lane 7: negative control (PCR reagents+ MiliQ water).

After additional treatment with DNase 1, 16S rRNA was detected for donor cells grown to

different growth phases, but *traA* was not detected by RT-PCR (Figures not shown). The

presence of *traA* that was detected by RT-PCR from donor cells grown to 72 h and day 6 (Figure

4.20) might indicate contamination of genomic DNA in the RNA samples.
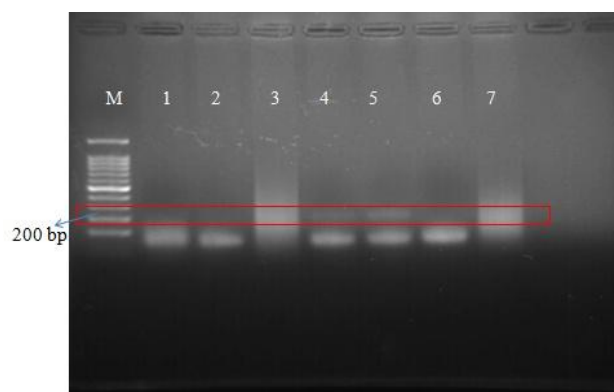
Figure 4.20. Gel electrophoresis for RT-PCR products. RT-PCR with cDNA (with *traA* primers) to detect the expression of *traA* in donor cells at different growth phases. RNA was extracted and cDNA was synthesized for donor cells harvested from different growth phases. Lane M: marker (100 bp), lane 1: positive control, lane 2: donor early exponential phase, lane 3: donor 24 h, lane 4: donor 72 h, lane 5: donor late stationary phase (day 6), lane 6: negative control (PCR reagents + cDNA reagents), lane 7: negative control (PCR reagents+ MiliQ water).

To optimize the RT-PCR method, RNA was extracted from different growth phases of donor cells by using FastPrep system (Figure 4.21) and cDNA was synthesized. The PCR program that was used to detect the presence of 16S rRNA gene was also used to detect *traA* by using cDNA as template. Different cycle numbers (30, 40, and 42) and different amounts of cDNA (0.1, 0.2, 0.4, 0.8, or 1.5 µg) were used to amplify *traA* by RT-PCR (cDNA as template). Different cycle numbers and amount of cDNA were tested to optimize the RT-PCR protocol to detect the expression of *traA* in donor cells grown to different growth phases. But when cDNA was used as template, *traA* was not detected by this PCR program.

To optimize the annealing temperature for the *traA* primers gradient PCR was performed by using the donor colony cells directly (colony PCR) and DNA extracted from donor cells (Figure 4.22 and Figure 4.23).

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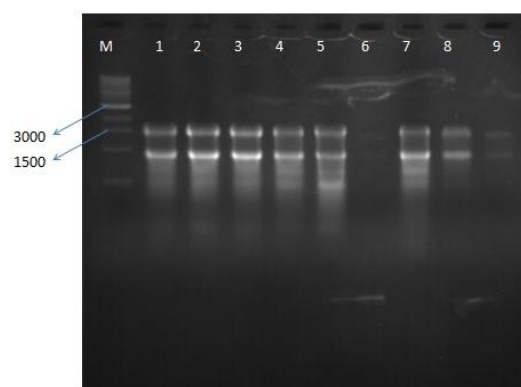
Figure 4.21. Gel electrophoresis for RNA samples. RNA was extracted from *P. putida* BBC443 donor cells grown to different growth phases. Lane M: marker (1 kb), lanes 1-3: donor RNA extracted from early exponential phase (6.5 h, OD 0.6), lanes 4-6: RNA extracted from donor cells grown to 48 h, lanes 7-9: RNA was extracted from donor cells grown to late stationary phase (day 6).

For gradient PCR, the best results were obtained from 55.5ºC, 56.75º C, 58º C and 59.25 º C annealing temperatures (Figure 4.22 and 4.23).
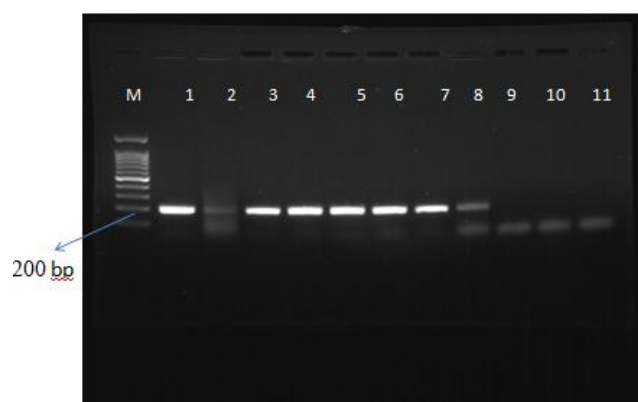
Figure 4.22. Gel electrophoresis for gradient PCR products. Gradient PCR with *P. putida* BBC443 colony cells. *traA* primers were used. Lane M: marker (100 bp), lane 1: annealing temperature 53º C, lane 2: annealing temperature 54.25º C, lane 3: annealing temperature 55.5º C, lane 4: annealing temperature 56.75º C, lane 5: annealing temperature 58º C, lane 6: annealing temperature 59.25 º C, lane 7: annealing temperature 60.5º C, lane 8: annealing temperature 63º C, lanes 9-11: negative control (H2O) at annealing temperatures 53º C, 55.5º C, 58º C.

Figure 4.22. shows the gel electrophoresis for gradient PCR products with *P. putida* BBC443 colony cells as template and Figure 4.23 shows the gel electrophoresis for gradient PCR products with DNA as template.
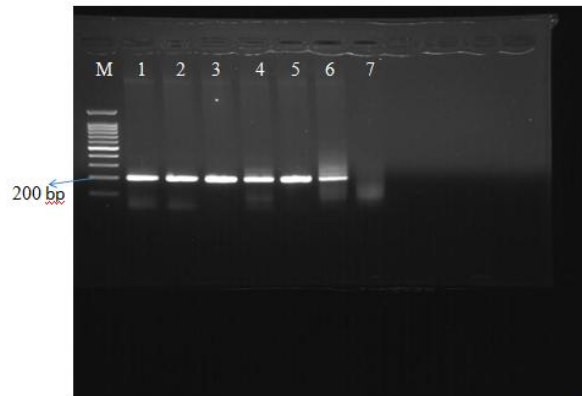
Figure 4.23. Gel electrophoresis for gradient PCR products. Gradient PCR with *P. putida* BBC443 DNA. *traA* primers were used. Left lane: marker (100 bp), lane 1: annealing temperature 53º C, lane 2: annealing temperature 54.25º C, lane 3: annealing temperature 55.3º C, lane 4: annealing temperature 56.75º C, lane 5: annealing temperature 58º C, lane 6: annealing temperature 59.25 º C, lane 7: plasmid recipient DNA as negative control at annealing temperature 55.5º C.

4.3. Effect of nutrient concentrations on the determination of minimum inhibitory concentration of antibiotics

In general, for both *Pseudomonas* strains, as the nutrient concentration decreased, the minimum inhibitory concentration for each antibiotic tested also decreased for both *P. putida* BBC443 and *P. putida* ATCC12633 (Table 4.3).

Table 4.3. Determination of MIC of kanamycin (kan), gentamicin (gen), tetracycline (tet), and rifampicin (rif) for *P. putida* BBC443 and *P. putida* ATCC12633 with varying nutrient concentrations.

| Nutrient concentrations (% LB) | MIC at different nutrient concentrations (µg/mL) | | | | | | | |
| | *P. putida* BBC443 | | | | *P. putida* ATCC12633 | | | |
	kan	gen	rif	tet	kan	gen	rif	tet
100	101.1	1.5	>270	2.0	2.3	3.6	11	2.0 – 2.2
50	Between 20.1 – 21.0	<1.0	>130	1.6	Between 0.8 - 1.5	<2.0	4.8	1.8-2.0
10	<4.0	Between 0.2 - 0.4	>50	1.2	<0.3	Between 0.5-0.8	3.8	1.2

In order to test if increased cell number provides protective action against gentamicin, variable cell numbers were added as inocula. As shown in Table 4.4, while MIC increased with increasing cell numbers, for all cell numbers tested, MIC again decreased with decreasing nutrient concentration.

Table 4.4. Effect of different cell numbers on minimum inhibitory concentration of gentamicin for *P. putida* BBC443 with varying nutrient concentrations.

| Nutrient concentration (% LB) | MIC (µg/mL) at different nutrient concentrations with variable cell numbers | | |
	10^3 CFU/mL	10^5 CFU/mL	10^7 CFU/mL
100	<1.5	1.5	Between 4.5 – 5.0
50	Between 0.4-0.8	<1.0	< 3.0
10	0.2	Between 0.2 – 0.4	Between 0.4 – 0.8

In order to detect if pH changes after incubation, and thus may influence MIC values, we measured pH when different cell numbers were added as inoculum (Table 4.5 and Table 4.6). For this study, 10^3 CFU/mL or 10^5 CFU/mL or 10^7 CFU/mL was used as inoculum. The pH of the solutions prior to incubation was 6.9. pH was measured at the end of the experiments. At the MIC and above MICs of gentamicin no visible growth was observed and the pH was $6.8 - 7.0$ (Table 4.5). When gentamicin was added at below MIC level ($0.5\times$ or near $0.5\times$ minimum

inhibitory concentration) turbidity or visible growth was observed and the pH was $7.1 - 7.7$

(Table 4.6).

Table 4.5. pH at minimum inhibitory concentrations of gentamicin for *P. putida* BBC443 with different cell numbers.

Nutrient concentrations (% LB)	Cell numbers (CFU/mL)					
	10^3		10^5		10^7	
	Growth/no growth	pH	Growth/no growth	pH	Growth/no growth	pH
100	-	6.8	-	6.9	-	6.9
50	-	6.9	-	6.9	-	6.9
10	-	6.9	-	7.0	-	6.9

*growth (+), no growth (-)

Table 4.6. pH at $0.5\times$ or near $0.5\times$ minimum inhibitory concentrations of gentamicin for *P. putida* BBC443 with different cell numbers.

Nutrient concentrations (% LB)	Cell numbers (CFU/mL)					
	10^3		10^5		10^7	
	Growth/no growth	pH	Growth/no growth	pH	Growth/no growth	pH
100	+	7.2	+	7.5	+	7.4
50	+	7.4	+	7.5	+	7.7
10	+	7.1	+	7.1	+	7.5

*growth (+), no growth (-)

In order to investigate whether DNA presence increases the MIC of gentamicin, we tested the effect of extracellular DNA using *P. putida* BBC443. To determine the effect of extracellular DNA, 10^3 or 10^5 CFU/mL was used as inoculum and 10% LB was used as medium. When 10^5 CFU/mL was added as inoculum, the MIC of gentamicin for 10% LB was higher than when 10^3 CFU/mL was added as inoculum (Table 4.4). Our results showed that when 5 µg/mL or 10 µg/mL extracellular DNA was added, no visible growth was observed at MIC or higher gentamicin concentrations than MIC (e.g. 0.2, 0.4, 0.8 and 1.5 µg/mL) when 10^3 CFU/mL was added as inoculum. When 10^5 CFU/mL was added as inoculum visible growth was observed for

higher MIC of gentamicin (e.g. 0.4, 0.6 µg/mL) (Table 4.7). We also tested if biomass (biomass was heat inactivated cells and obtained by boiling the *P. putida* BBC443 cells) has a protective effect from gentamicin. When biomass was added, no growth was observed for higher concentrations than MIC for 10^3 CFU/mL inoculum with 10% LB. Visible growth was observed at MIC and higher concentrations than MIC gentamicin when 10^5 CFU/mL was added as inoculum (Table 4.8).

Table 4.7. Effect of extracellular DNA at either the MIC or above MIC of gentamicin for *P. putida* BBC443. 10^3 or 10^5 CFU/mL were used as inocula and 10% LB was used as growth medium.

Added DNA concentration (µg/mL)	Cell numbers	
	10^3 CFU/mL	10^5 CFU/mL
5	-	+
10	-	+
No DNA (control)	-	-

*growth (+), no growth (-)

Table 4.8. Effect of biomass at either the MIC or above MIC of gentamicin for *P. putida* BBC443. 10^3 or 10^5 CFU/mL were used as inocula and 10% LB was used as medium.

Biomass	Cell numbers	
	10^3 CFU/mL	10^5 CFU/mL
10^7 CFU/mL (heat-inactivated cells) was added	-	+
No biomass (heat inactivated cells) was added (control)	-	-

*growth (+), no growth (-)

We also measured the MBC for kanamycin, gentamicin, rifampicin and tetracycline for *P. putida* BBC443 and *P. putida* ATCC12633 and MBEC for *P. putida* ATCC12633 with 100% LB (Tables A2 and A3). The values of MBC and MBEC were higher than MIC values for all four antibiotics.

4.4. The effects of subinhibitory and minimum inhibitory concentrations of gentamicin on transconjugant formation in biofilms and plate matings

In plate mating experiments, a higher abundance of transconjugants expressed as a percentage of total cells (4.3± 0.62%) was detected when the donor cells were exposed to 0.5× minimum inhibitory concentration of gentamicin for 48 h and then additionally challenged with gentamicin at MIC for 1 h and no gentamicin was added to the plate on which plate matings were carried out (Table 4.9). This result was significantly different from the negative control (donor cells were not exposed to gentamicin and no gentamicin was added to the LB plate), where transconjugants comprised 2.5 ± 1.20% of total cells (Figure 4.24).

Table 4.9. Effect of gentamicin on transconjugant formation in plate mating experiment.

Conditions	Donor exposure to gentamicin	Mating on LB plates-gentamicin presence/absence	Transconjugants (% of total cells)
A (control)	None	None	2.5 ± 1.20
B	0.5× MIC (48 h)	None	2.9 ± 1.33
C	0.5× MIC (48 h)	0.5× MIC	3.5 ± 0.99
D	0.5× MIC (48 h), 1 h challenge with MIC	MIC	3.7 ± 1.57
E	None	0.5× MIC	3.8 ± 1.61
F	0.5× MIC (48 h), 1 h challenge with MIC	None	4.3 ± 0.62

*To grow plasmid donor cells 50 µg/mL kanamycin was added in all conditions.

Statistical analysis revealed that all other conditions did not yield significantly different results (Figure 4.24).
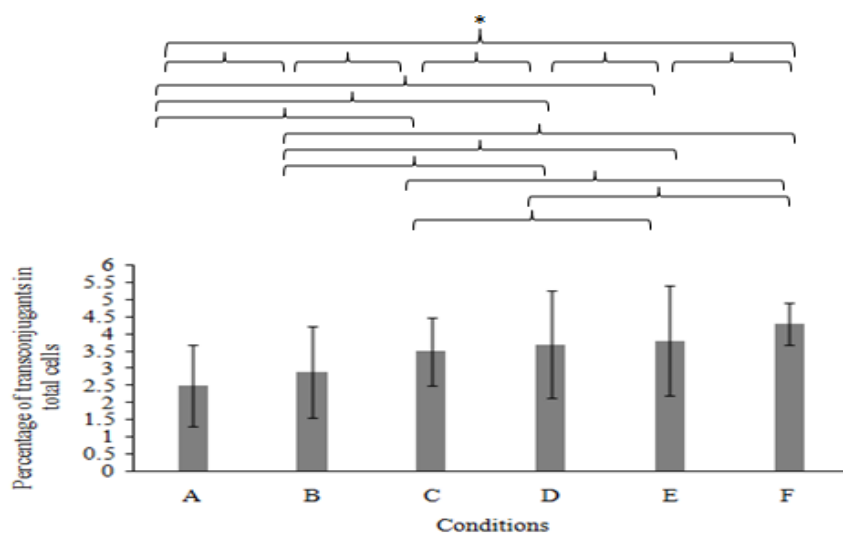
Figure 4.24. Data represent transconjugants expressed as percentage in total cells for batch culture experiments. Condition A: donor cells were not exposed to gentamicin and gentamicin was not added to the plate on which matings were carried out, condition B: donor cells were grown with 0.5× MIC gentamicin for 48 h and gentamicin was not added to the plate on which matings were carried out, condition C: donor cells were grown with 0.5× MIC gentamicin for 48 h and 0.5× MIC gentamicin was added to the plate on which matings were carried out, condition D: donor cells were grown with 0.5× MIC gentamicin for 48 h, additionally challenged with MIC gentamicin for 1 h and MIC gentamicin was added to the plate on which matings were carried out, condition E: donor cells were not exposed to gentamicin and 0.5× MIC gentamicin was added to plate on which matings were carried out, condition F: donor cells were grown with 0.5× MIC gentamicin for 48 h, additionally challenged with MIC gentamicin for 1 h and gentamicin was not added to the plate on which matings were carried out. Statistically significant group is marked as *.

In biofilms, transconjugant formation was not enhanced in the presence of 0.5× gentamicin (Table 4.10). The result was significantly different when the donor cells were grown with 0.5× MIC and 0.5× MIC gentamicin was added in the biofilms (percentage of transconjugants in the total cells was 0.014± 0.01%) and the control (gentamicin was not added in the biofilms, percentage of transconjugants in the total cells was 0.039 ± 0.02 %) (Table 4.10, Figure 4.25).

Table 4.10. Effect of 0.5× MIC of gentamicin on transconjugant formation in biofilms.

Conditions	Donor exposure to gentamicin	Mating in plasmid recipient biofilms-gentamicin presence/absence	Transconjugants (% of total cells)
A	None	None	0.037 ± 0.02
B	0.5× MIC (48 h)	0.5× MIC	0.014 ± 0.01
C	0.5× MIC (48 h)	None	0.039 ± 0.02

*To grow plasmid donor cells 50 µg/mL kanamycin was added in all conditions.

In comparison to the control (condition A, Table 4.10), transconjugant formation was not enhanced when the donor was exposed to 0.5× MIC of gentamicin (condition C, Table 4.10) or when both the donor and the mating mixture were exposed to 0.5 MIC of gentamicin (condition B, Table 4.10). However, donor and mating patch exposure to 0.5 MIC (condition B) resulted in significantly decreased abundance of transconjugants (Figure 4.25).
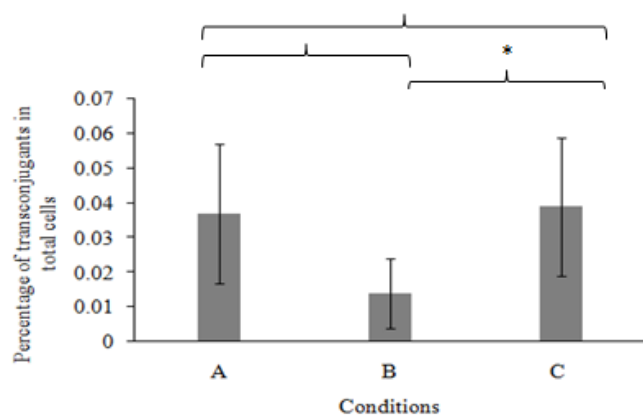
Figure 4.25. Data represent transconjugants expressed as percentage in total cells for biofilm experiments. Condition A: donor cells were not exposed to gentamicin and gentamicin was not added in the biofilms, condition B: donor cells were grown with 0.5× MIC gentamicin for 48 h and 0.5× MIC gentamicin was added in the biofilms, condition C: donor cells were grown with 0.5× MIC gentamicin for 48 h and no gentamicin was added in the biofilms. Statistically significant group is marked as *.

Preliminary experiments indicate that transconjugant formation was enhanced when the donor cells were grown with 0.5× MIC of gentamicin and additionally challenged at MIC of gentamicin for 1 h and gentamicin at MIC was added to the biofilms in comparison to when donor cells were not grown with gentamicin but challenged with MIC of gentamicin prior to adding in the recipient biofilms and no gentamicin was added in the biofilms (Table 4.11).

Table 4.11. Effect of MIC of gentamicin on transconjugant formation in biofilms.

Plasmid donor exposure to gentamicin	Mating in recipient biofilms – gentamicin presence / absence	Transconjugants (% of total cells)
0.5× MIC (48 h), 1 h challenge at MIC	MIC	0.90 ± 0.69
1 h challenge at MIC	None	0.03 ± 0.03

*To grow plasmid donor cells 50 µg/mL kanamycin was added in all conditions.

Figure 4.26 shows the presence of a greater amount of transconjugants in biofilms exposed to MIC of gentamicin (A) in comparison to no exposure to gentamicin (B).
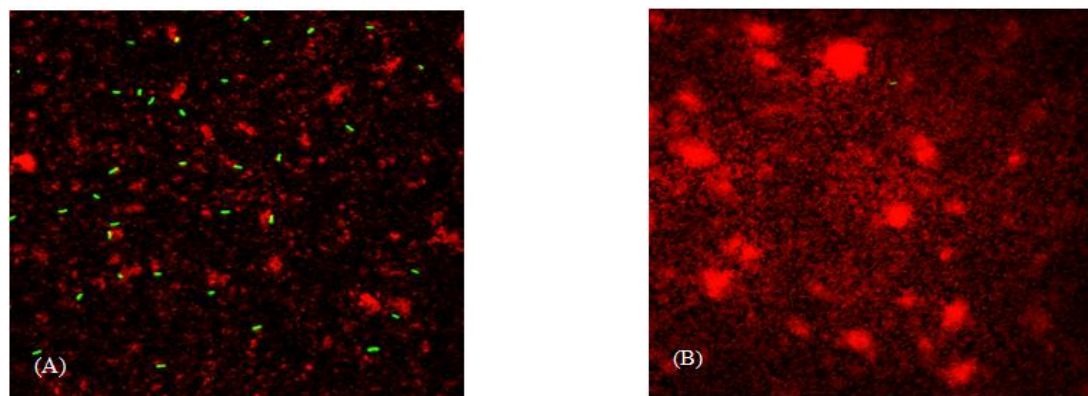
Figure 4.26. Microscopic images of biofilm cells exposed to MIC of gentamicin (A) and without exposure to gentamicin (B). Green cells represent transconjugant cells, red cells represent total cells. (63× magnification was used)

Chapter 5

5. Discussion

5.1. Effect of cell growth phases on transconjugant formation using planktonic cultures and in biofilms

In this study, the effect of donor and recipient growth phases on transconjugant formation in plate matings using planktonically grown batch culture cells and biofilms was investigated.

5.1.1. Effect of cell growth phases on plasmid transfer in plate matings using planktonically grown cells

Our results showed that transconjugants expressed as a percentage of all cells was the highest when both plasmid donor and recipient cells were harvested from the late stationary phase (144 h; Table 4.1, Figure 4.6), when the cell size of both donors and recipients was smaller than that observed during the exponential growth phase (Table 4.2, Figure 4.9), whereas the lowest percentage of transconjugants was detected when the plasmid donor cells were grown to early exponential phase (6.5-7.5 h) and the recipient cells were harvested from the late stationary phase. The results did not support our hypothesis. Our hypothesis was the abundance of transconjugants will be higher in the exponential growth phase than in the stationary growth phase of the donor and recipient cells in plate matings.

Growth curves for the recipient and donor strains are shown in the Figures 4.1 and 4.2. In our study, the cell length of both donor and recipient cells obtained from the early exponential phase was 2.4-2.6 fold greater than that of cells harvested from the late stationary phase. When grown in batch culture, cells face nutritional limitation upon entry into the stationary phase (Gefen et al., 2014). When bacterial cells are subjected to nutrient limitation, the cell size becomes smaller,

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and cells become more resistant to environmental stress (Trevors, 2011, Kolter et al., 1993). Contrary to Seoane et al. (2011), who suggested that cell elongation may facilitate conjugation, Muela et al. (1994) observed the lowest values of plasmid transfer frequency when donor cells were obtained from the mid-exponential phase when the cell size of the plasmid donor cells was maximum. In their study, no significant differences in plasmid transfer frequency were detected along the growth phase of the recipient strains. The authors suggested that the energy available to donor cells may be involved in the processes of biosynthesis, thus hindering the plasmid transfer process. Conjugation is an energy dependent process as energy is required for DNA replication and protein synthesis in donor and recipient cells (Rittmann et al., 1995, Król et al., 2011). ATP may also be required for maintenance of sex pili on the cell surface (Yamamoto et al., 1981). In our study, the highest percentage of transconjugants was obtained when the plasmid donor and recipient cells were harvested from the late stationary phase and when the cell size of the plasmid donor and recipient was smaller. Because less energy was needed for cellular processes, we speculate that the energy available to the donor and recipient cells was used to facilitate plasmid transfer process in the stationary growth phase. In long term stationary phase, the expression of stress response genes is necessary for the survival of bacterial cells (Finkel, 2006). We speculate that in the late stationary growth phase genes (other than *traA*) involved in mating pair formation in plasmid donor and recipient cells may have been expressed, enhancing plasmid transfer and transconjugant formation.

The number of transconjugants formed may be also influenced by the physiological status of the donor cells while the physiological state of recipient cells did not seem to impact plasmid transfer (Muela et al., 1994, Arana et al., 1997, Sudarshana and Knudsen, 1995). In contrast to our results, Smets et al. (1993) showed that plasmid transfer rates were the highest with *P. putida*

(TOL) donor cultures harvested from the exponential phase, and lower rates were obtained with stationary phase donor cultures. Frost and Manchak (1998) investigated whether the F transfer apparatus breaks down at the stationary phase in *Escherichia coli* strains and their results showed that the plasmid transfer frequency decreased at the mid exponential phase while proteins involved in transfer functions, such as TraA (pilin synthesis), TraD (DNA transport), TraT (surface exclusion) remained constant in the stationary phase. Under normal physiological conditions, production of F pili by the donor is needed for the mating pair formation and the maximum piliation is attained at the end of the exponential growth phase (Novotny and Lavin, 1971).

Seoane et al. (2011) reported that plasmid conjugation is more likely to occur through the lateral cell walls rather than through the cell pole regions, and showed that the number of plasmid transfer events increased with recipient cell elongation. The microscopic images from Babic et al. (2008) also supported conjugation occurred through the lateral walls when they visualized the conjugation mechanism in *Escherichia coli* by using fluorescent protein SeqA-YFP. According to Bauer et al. (2011) in gram positive *Bacillus subtilis* plasmid pLS20 was found at the cell pole indicating that transfer occurred at the pole. VirD2 (relaxes) and VirD4 (coupling protein) were also located at the cell pole. The authors concluded "pole is the preferred site for assembly of active conjugation apparatus".

According to Gilbert et al. (2016) among 205 R27 genes, 33% showed growth phase-dependent expression. A total of 42 genes had higher expression in the exponential phase, whereas 26 genes were expressed more in the early stationary phase. Among the genes with altered expression, 21 belonged to the tra operon (*tra* genes). In their study, increased conjugation frequency detected in exponentially growing cells, 18 of the 21 *tra* genes were induced in actively growing cells,

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including those encoding crucial proteins for conjugation. They also detected the expression of *tra* genes *traH, trhR, trhF* in the exponential phase but not in the early stationary phase (except for *trhR*).

In our study, we monitored expression of *traA*, a gene which encodes the 13-kDa precursor protein needed for pilin subunit synthesis (Maneewannakul et al., 1993). The results showed that *traA* expression was detected by RT-PCR in samples collected from early and late exponential phases, but not in samples collected from the late stationary phase (Fig. 4.12). This was surprising, as we anticipated that a higher transconjugant percentage will correspond to increased expression of *traA*. Since the highest percentage of transconjugants was detected in the stationary phase (when expression of *traA* was not detected), but expression of *traA* was pronounced during early exponential phase, we concluded that expression of *traA* in donor cells does not coincide with enhanced transconjugant abundance. It is possible that once pilin subunits are synthesized, they may persist into the stationary phase where they mediate conjugation. Further investigation can be performed using proteomics or western blotting to characterize proteins that may be involved in conjugation in cells grown to stationary phase.

Similarly to our results, Frost and Manchak (1998) showed that the *traA* transcript decreased to below detectable levels approximately 2 hours after the mid-exponential phase of an *E. coli* strain, while the levels of TraA persisted at constant levels in the stationary phase. We speculate that factors (e.g. distance between the cells, cell position) other than *traA* expression associated with donor and recipient cell growth phases affect plasmid transfer and transconjugant formation.

5.1.2. Effect of plasmid donor growth phases on plasmid transfer in biofilms

The quantitative data obtained from microscopic images revealed that transconjugants expressed as the percentage of all cells were $0.11 \pm 0.1\%$ and $0.20 \pm 0.2\%$ when donor cells grown to early exponential growth phase and late stationary growth phase were added to 24 h old recipient biofilm, respectively. These results were not significantly different. The results did not support our hypothesis as no significant difference in transconjugant formation was obtained when donor cells grown to early exponential phase or late stationary phase were added to 24 h old recipient biofilms.

We chose to use 24 h old biofilms because 24 h old biofilms presented a confluent layer of cells; earlier experiments performed in our lab showed that older biofilms were thicker (> 60 µm), possessed a greater abundance of EPS and hindered plasmid transfer (data not shown). Presence of EPS may inhibit conjugative pilus reach between plasmid donor and potential recipient cells in biofilms (Merkey et al., 2011). Seoane et al. (2011) identified parameters (e.g. conjugation rate, donor–recipient distance and positioning) that may control bacterial conjugation in biofilms at the individual cell level while investigating the inability of the pWWO TOL plasmid to invade *P. putida* microcolonies and biofilms. Their results showed that pWWO transfer occurs not only between cells in direct cell to cell contact, but also between two distant cells since plasmid transfer took place when donor-recipient cells were separated by a distance of 5-10 µm. Their results also revealed that conjugation occurs more frequently through the lateral wall of the donor cells than through the poles as they obtained highest number of successful mating pairs when plasmid donor and recipient cells attached with lateral wall. They suggested that plasmid transfer within biofilms is limited due to spatial constraints which exist within organized

microbial communities such as biofilms. Similarly, cell positioning, distance between plasmid donor and recipient cells may have influenced the outcome of our experiments.

In summary, this project discussed the effect of cell growth phases on transconjugant formation in plate matings on agar plates using planktonically grown cultures and in 24 h old recipient biofilms grown on microscopic slides. Our results showed that in plate matings, the highest percentage of transconjugants was obtained when both plasmid donor and recipient cells were harvested from the stationary phase, when the cell size of both the donor and recipient cells was the smallest. The lowest percentage of transconjugants was obtained when donor cells were grown to exponential growth phase and recipient cells were grown to stationary growth phase. In biofilms, the percentage of transconjugants was not significantly different whether the donor was harvested from the early exponential or the late stationary phase and added to 24 h old recipient biofilms. These results show that the growth phase of the plasmid donor did not influence plasmid transfer frequency in biofilms, and while no clear trends exist for the plate mating experiments, the findings indicate that when either the donor or the recipient or both were harvested from the exponential phase, transconjugant formation was significantly lower than when both the donor and recipient were harvested from the stationary phase.

5.2. Optimization of RT-PCR to detect expression of *traA* in plasmid donor cells

To optimize the RT-PCR method, RNA was extracted from different growth phases of donor cells by using FastPrep system and cDNA was synthesized. Also gradient PCR was performed to optimize the annealing temperature. For gradient PCR colony cells or DNA was used as template. Our results showed that when donor DNA was used as template (Figure 4.23) at 55.3º C annealing temperature, a bright band was observed and there was no presence of primer

dimers. Based on these gradient PCR results, 55.3º C was used as the annealing temperature for *traA* primers.

To optimize RT-PCR for the detection of *traA* expression, it was necessary to determine the optimal primer annealing temperature. To confirm that cDNA was synthesized and RNA samples were free from genomic DNA, a negative control (no reverse transcriptase enzyme added during the cDNA synthesis from RNA) was included for all samples. Replicates were included for each sample. Also, to prevent the degradation of RNA; nuclease free water, pipette tips were used for RNA extraction and cDNA synthesis. By using the annealing temperature of 55.3º C, the presence of *traA* was detected by RT-PCR in *P. putida* BBC443 cells grown to early exponential phase (6.5 h) and late exponential phase 48 h (chapter 4, section 4.1.1).

5.3. Effect of nutrient concentrations on the determination of minimum inhibitory concentration of antibiotics

For kanamycin, gentamicin, rifampicin and tetracycline the minimum inhibitory concentrations decreased as nutrient concentration (% LB) decreased (Table 4.3). The minimum bactericidal concentration of gentamicin also decreased for *P. putida* BBC443 with decreased nutrient concentration (Figure A1). Kanamycin and gentamicin are aminoglycoside antibiotics, which bind to the 30S subunit of bacterial ribosome and inhibit protein synthesis (Jana and Deb, 2006, Mingeot-Leclercq et al., 1999). Tetracycline antibiotics inhibit bacterial protein synthesis by preventing the association of aminoacyl tRNA with the bacterial ribosome (Chopra and Roberts, 2001). Rifampicin inhibits RNA polymerase, this enzyme is involved in transcription of DNA to RNA (Wehrli, 1983). Greulich et al. (2015) investigated the effect of ribosome binding antibiotics (streptomycin, kanamycin, tetracycline and chloramphenicol) on *Escherichia coli.*

Their results revealed that for some of the antibiotics faster growth increases susceptibility to the antibiotics. For irreversibly binding antibiotics (streptomycin and kanamycin), faster growing cells are less susceptible to antibiotics. For reversibly binding antibiotics (tetracycline and chloramphenicol), faster growing cells are more susceptible to antibiotics. The authors mentioned that in the presence of high nutrients, fast growing cells have a larger ribosome pool and higher ribosome synthesis rate, so that they are able to tolerate a higher rate of antibiotic than slow-growing cells. In our study, for all ribosome binding antibiotics (kanamycin, gentamicin and tetracycline) susceptibility increased with lower nutrient concentrations. We tested the effect of cell numbers, presence of extracellular DNA and biomass on MIC of gentamicin to determine if these have protective effect and increase the MIC. We also measured the pH when different cell numbers were added as inoculum, because aminoglycoside antibiotics are less effective in lower pH (Jana and Deb, 2006). We tested the effect of cell numbers as with the higher nutrients cells grow to greater cell densities than at lower nutrient environments. One of our objectives was to test if MIC increases with higher cell number.

Our results showed that with the different cell numbers e.g. 10^3, 10^5, 10^7 CFU/mL as inoculum MIC of gentamicin decreased with the lower nutrient concentrations (Table 4.4). MICs of gentamicin for different nutrient concentrations (100, 50 and 10%) were higher when 10^7 CFU/mL was added as the inoculum than when 10^3 or 10^5 CFU/mL was used as inocula. The increase in the number of bacterial cells (10^7 CFU/mL) led to an increase of the minimum inhibition concentration of gentamicin; more antibiotic was required to inhibit the growth of *P. putida* cells.

We tested the effect of extracellular DNA and biomass on MIC of gentamicin if these provide protective effect from gentamicin in the planktonic cells. Extracellular DNA can increase MICs

due to the binding of anionic DNA with positively charged aminoglycosides (Chiang et al., 2013, Drew et al., 2009). Our results showed that when 5 or 10 µg/mL extracellular DNA and 10^5 CFU/mL were added together with cells at the beginning of the MIC assay, growth was observed at MIC and higher MIC of gentamicin (Table 4.7). However, when 10^3 CFU/mL was added as inoculum no growth was observed in the presence of extracellular DNA at higher MIC of gentamicin, suggesting that the concentrations of DNA used in our study did not provide a protective effect with lower cell numbers as inocula. Similar results were obtained when heat inactivated biomass (10^7 CFU/mL dead cells) was added. Heat inactivated biomass was added to test if it provides protective effect from gentamicin, since the positively charged gentamicin molecules could bind to the negatively charged cellular components resulting in a reduction of available antibiotic. For 10^5 CFU/mL (as inoculum) growth was observed at MIC and higher MIC of gentamicin; growth was not observed for 10^3 CFU/mL as inoculum (Table 4.8). As the aminoglycosides are polycationic molecules, our results suggest that DNA and biomass may provide protective effect when combined with high enough cell numbers (as inocula), possible through binding of gentamicin by the negatively charged DNA and cellular components.

Uptake of the gentamicin by bacteria depends on surrounding pH (Eisenberg et al., 1984, Henry Stanley et al., 2014, Baudoux et al., 2007). According to Baudoux et al. (2007), acidic pH reduced the activity of gentamicin in *Staphylococcus aureus* as the MIC was 70 times higher at pH 5.0 than at pH 7.4. For our experiments, pH was measured before and after the incubation.

Before incubation the pH of the medium was 6.8. After 18 h of incubation, the pH at MIC (no visible growth was observed) of gentamicin remained at 6.8-6.9. At a gentamicin concentration lower than MIC (0.5× MIC), growth was observed and the end-of incubation pH was 7.1-7.7 (Table 4.5 and Table 4.6), however, we do not consider this increase in pH to be significant. For

decreased nutrient concentration (100%, 50% and 10% LB), MIC also decreased in our experiments and it was not dependent on pH as the pH change during incubation was insignificant.

According to Henry Stanley (2014) *Staphylococcus aureus* biofilms grown with 3× TSB (supplemented with glucose) had increased resistance to gentamicin and streptomycin than biofilms grown with 1/3× and 1× TSB (supplemented with glucose or without glucose). The authors confirmed that for 3× TSB (supplemented with glucose) pH was 4.85-5.10. The acidic condition helped to decrease the inhibitory effect of gentamicin at higher nutrient concentration.

Hancock et al. (1981) found in both *Escherichia coli* and *P. aeruginosa* aminoglycoside uptake occurs in three steps, (1) initial electrostatic binding, (2) slow uptake phase, (3) rapid uptake phase. The initial binding is energy independent, whereas the last two steps are energy dependent and require active electron transport system. Our results showed that inhibition of *P. putida* BBC443 by gentamicin is nutrient dependent as the MICs decreased with lower nutrient concentration. *P. putida* BBC443 is more susceptible to gentamicin at lower nutrient concentrations. Our results also showed that as for gentamicin, the MICs for kanamycin, rifampicin and tetracycline decreased with decreased nutrient concentrations for *P. putida* strains. These results supported our hypothesis. Our hypothesis was the values of minimum inhibitory concentration of antibiotics will decrease with decreasing nutrient concentrations.

For *P. putida* BBC443, MICs of gentamicin at different nutrient concentration increased with the increased cell numbers. After incubation, pH did not change drastically at MIC or lower than MIC of gentamicin for *P. putida* BBC443 and thus did not have a role in the decrease of MICs of gentamicin with lower nutrient concentration. We also conclude that heat inactivated biomass and extracellular DNA might have protective effect from gentamicin as visible growth was

observed at MIC and higher than MIC when 10^5 CFU/mL was added as inoculum. Further investigation should be directed to study the effect of antibiotics in biofilms with varying nutrient concentration.

5.4. The effects of subinhibitory and minimum inhibitory concentrations of gentamicin on transconjugant formation in biofilms and plate matings

In plate mating experiments, using planktonically grown cultures, transconjugant formation was higher when the donor cells were grown with 0.5× MIC of gentamicin and additionally challenged with MIC of gentamicin for 1 h and no antibiotic was added in the LB plate (Table 4.9). This result was significantly different from when donor cells were not exposed to gentamicin and no gentamicin was added to the LB plate (transconjugants comprised 2.5± 1.20 % of total cells) (Table 4.9, Figure 4.24). These results supported our hypothesis. Our hypothesis was the number of transconjugants will be greater following gentamicin exposure.

The transconjugant formation was similar when the plate mating was done in the presence of 0.5× MIC of gentamicin and plasmid donor cells were additionally challenged with MIC for 1 h, mating plate contained MIC of gentamicin (percentages of transconjugants in the total cells were 3.5 ± 0.99 and 3.7 ± 1.57, respectively) (Table 4.9).

A study done by Zhang et al. (2013) showed that the conjugative transfer of plasmids pRK2013, pSU2007 and RP4 was enhanced between *Escherichia coli* strains in the presence of combination of MIC of kanamycin and streptomycin (MICs were added to the plates). Their study demonstrated that in the presence of kanamycin and streptomycin 60 proteins were downregulated and 14 proteins were upregulated in the conjugation of *E. coli* strains after 12 h mating incubation. Two proteins, oligopeptide-binding protein (OppA) and ribose-binding

protein (RbsB) were involved in the enhanced conjugation of *E. coli* in the presence of kanamycin and streptomycin. Conjugative transfer of the plasmid was also greatly increased by the presence of low doses of both antibiotics in the mating medium (Xia et al., 2008).

It has been reported in a few studies that in the presence of subinhibitory concentration of antibiotics gene transfer can be enhanced. Sublethal concentration of a DNA binding agent, mitomycin C induced SOS response and stimulated gene transfer. SXT, a conjugative element encoding resistance to chloramphenicol, sulphamethoxazole, trimethoprim and streptomycin in *Vibrio cholerae* was transferred and integrated at a much higher efficiency when the donor was exposed to subinhibitory concentration of mitomycin C (Beaber et al., 2003).

According to Salcedo et al. (2015), 100 ppb and 1,000 ppb of tetracycline exposure induced biofilm formation in *Escherichia coli* which contained plasmid pB10. Tetracycline resistance was encoded on the plasmid pB10. But in *Pseudomonas aeruginosa* which lacked plasmid pB10, biofilm formation was not induced in the presence of tetracycline. Their results also showed that in the mixed culture of *E. coli* and *P. aeruginosa*, transconjugant formation varied with different concentrations of antibiotics. The highest numbers of transconjugants were obtained in the presence of 100 and 1000 ppb of cephradine, streptomycin, gentamicin, and tetracycline. For ciprofloxacin and amoxicillin, the highest numbers of transconjugants were obtained in the presence of 1 and 10,000 ppb concentrations of antibiotics. The authors concluded that with the exposure to different antibiotics, transcription pattern varies as antibiotics act as signaling molecules. Subinhibitory concentration of a β lactam antibiotic, penicillin also stimulated plasmid transfer in *Staphylococcus aureus* (Barr et al., 1986).

Scornec et al. (2017) also found that subinhibitory concentration of some ribosome-targeting antibiotics (e.g. tetracycline) can induce the transfer of conjugative transposon Tn916 in

Enterococcus faecalis. The authors tested the ability of different classes of antibiotics to modulate the activity of promoter P_{orf12} controlling the transfer of Tn916 by developing a promoter- reporter (pSHlux::P_{orf12}) fusion method. Their results showed that inducibility of $P_{orf12}$was two to three folds higher in the presence of spectinomycin, tetracycline, doxycycline, lincomycin and clindamycin antibiotics. These five antibiotics stimulated the transfer of Tn916 with maximum transfer frequency. Maximum transfer frequency was obtained due the maximum induction of $P_{orf12.}$

The subinhibitory concentration of a biocide can also enhance gene transfer. Seier-Petersen et al. (2013) reported that subinhibitory concentration of ethanol enhanced transposon Tn916 in *Bacillus subtilis*. The transcriptional response of *tet*(M) (involved in recombination and conjugation of the element) to the biocides started at 2 h after exposure for ethanol, 1.5 h for hydrogen peroxide, 0.5 h for chlorhexidine digluconate and 1.5 h for sodium hypochlorite. Subinhibitory concentrations of antibiotics (e.g. fluoroquinolones, β-lactams and aminoglycosides) have been shown to induce the SOS response and the RpoS induction. Induction of the SOS response by sublethal antibiotic concentrations was also shown to increase the rate of horizontal gene transfer by prophage induction, integrative conjugative elements and transposition (Andersson and Hughes, 2014).

In our study, in plate matings a higher abundance of transconjugants expressed as the percentage of transconjugant in total cells ($4.3 \pm 0.62\%$) was detected when the donor cells were exposed to $0.5\times$ MIC of gentamicin, additionally challenged with MIC of gentamicin for 1 h and no gentamicin was added to the plates (Table 4.9). Our experiments with plasmid transfer in biofilms did not show an increase in transconjugant formation when either donor cells were grown with $0.5\times$ MIC gentamicin or when additionally $0.5\times$ MIC gentamicin was added to the

biofilms. The presence of 0.5× gentamicin in the donor/recipient wells was associated with a lower transconjugant abundance in comparison to the control (no gentamicin addition in the biofilms). Preliminary results indicate that an additional challenge of the donor strain at MIC and addition of gentamicin at MIC to recipient biofilms (following the addition of donor cells) enhanced transconjugant formation in biofilms. Additional studies should be performed to determine if exposure to antibiotics at MIC changes expression of genes relevant for conjugation.

Chapter 6

6.1. Conclusions

Subinhibitory concentrations of antibiotics are environmentally relevant due to their extensive human and veterinary applications. Antibiotic resistance is one the most serious problems for public health. The spread of antibiotic resistance in microbes by horizontal gene transfer is a serious hindrance to therapeutic effectiveness. To study the effect of subinhibitory concentrations of antibiotics on transconjugant formation in both planktonic cultures and biofilms will shed light on their effect on the spread of antibiotic resistance in environments.

One of the objectives of this research project was to study the effect of cell growth phases (exponential and stationary) of plasmid donor and recipient cells on transconjugant formation in planktonic cell batch cultures and to study the effect of growth phases of plasmid donor cells on transconjugant formation in biofilms. This may explain the importance of cell growth activity for plasmid spread. Our results showed that in plate mating experiments, the highest percentage of transconjugants was obtained when both plasmid donor and recipient cells were harvested from the late stationary phase. The lowest percentage of transconjugants was obtained when donor cells were grown to exponential growth phase and recipient cells were grown to stationary growth phase.

In biofilms, the percentage of transconjugants was not significantly different whether the donor was harvested from the early exponential or the late stationary phase and added to 24 h old recipient biofilms. These results showed that the growth phase of the plasmid donor did not influence plasmid transfer frequency in biofilms.

Expression of *traA* (involved in pilus biosynthesis) was detected from the plasmid donor cells grown to early exponential and late exponential phase by performing RT-PCR, indicating that *traA* expression did not coincide with greater transconjugant abundance.

Another objective of this project was to determine the minimum inhibitory concentration (MIC) of four antibiotics (kanamycin, gentamicin, rifampicin, and tetracycline) at 100%, 50%, and 10% LB broth for *Pseudomonas putida* strains. Our results showed that with decreased amount of nutrient concentration, MIC also decreased for both strains. Heat inactivated biomass and extracellular DNA may have a protective effect from gentamicin as visible growth was observed at MIC and above MIC of gentamicin when 10^5 CFU/mL was added as inoculum. Future studies will be performed to determine the effect of varying nutrient concentration on antibiotics in *P. putida* biofilms.

Another objective was to study the effect of subinhibitory concentration and minimum inhibitory concentration of gentamicin on transconjugant formation in planktonic cell batch cultures and in biofilms. In plate mating experiments, a higher abundance of transconjugants expressed as a percentage of total cells was detected when the donor cells were exposed to $0.5\times$ MIC gentamicin, additionally challenged with gentamicin at MIC for 1 h and no gentamicin was added to the plate on which plate matings were carried out. Preliminary results indicate that an additional challenge of the donor strain at MIC and addition of gentamicin at MIC to recipient biofilms (following the addition of donor cells) enhance transconjugant formation in biofilms. More controlled studies, together with RNA sequencing, should be performed to determine how exposure to antibiotics at MIC changes gene expression in donor cells and recipient biofilms.

6.2. Recommendations

For future work, transconjugant formation frequency can be studied in the presence of kanamycin, rifampicin, and tetracycline. For kanamycin, resistance is encoded on the plasmid pWWO, resistance for rifampicin is encoded on the donor chromosome. Further studies to investigate the effect of kanamycin and rifampicin on transconjugant formation will provide information if transconjugant formation frequency will be enhanced in the presence of these antibiotics due to the encoded resistance. Tetracycline is commonly found in the environment. The donor and recipient (used in this research project) strains do not carry resistance against tetracycline. Yet its mode of action is similar to that of kanamycin and gentamicin (inhibition of protein synthesis). Thus investigating the effect of tetracycline on plasmid transfer would allow us to draw conclusions about the effect of antibiotics with a similar mode of action but with different susceptibility profiles in donor and recipient cells. Also, it would be important to determine if plasmid transfer frequency differs in single species recipient as opposed to mixed recipient community exposed to antibiotics as in the environment microorganisms are found in mixed communities.

RNA sequencing can be used to perform differential gene expression analysis in the presence of subinhibitory and minimum inhibitory concentration of antibiotics. RNA sequencing will provide data about expression of other genes involved in plasmid transfer in plasmid donor and recipient cells. RNA sequencing can also provide information if genes (for example, metal resistance and antibiotic resistance genes) may be co-regulated upon exposure to antibiotics.

Microarrays can also be used to measure the expression level of known genes in the presence or absence of antibiotics.

Appendices

Appendix A: Effect of nutrient concentrations on minimum inhibitory concentration of antibiotics.

Table A1. Minimum bactericidal concentration (MBC) of gentamicin for *P. putida* BBC443 with varying nutrient concentrations, 10^5 CFU/mL was added as inoculum.

Nutrient concentrations (LB)	Minimum bactericidal concentrations (µg/mL)
100%	Between 30 - 40
50%	Between 5 - 10
10%	Between 5 -10

Table A2. Determination of minimum bactericidal concentrations (MBC) for *P. putida* BBC443 and *P. putida* ATCC12633 with 100% LB.

Antibiotics	Strains	Minimum bactericidal concentration (µg/mL)
Kanamycin	*P. putida* BBC443	>400
	P. putida ATCC12633	>100
Gentamicin	*P. putida* BBC443	Between 30-40
	P. putida ATCC12633	>70
Rifampicin	*P. putida* BBC443	>160
	P. putida ATCC12633	>80
Tetracycline	*P. putida* BBC443	>60
	P. putida ATCC12633	>70

Table A3. Determination of minimum biofilm eradication concentration (MBEC) of *P. putida* ATCC12633 with 100% LB.

Antibiotics	Minimum biofilm eradication concentration
Kanamycin	Between 900-1000 µg/mL
Gentamicin	<900 µg/mL
Rifampicin	>10,000 µg/mL
Tetracycline	Between 2000-2500 µg/mL

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