CHALMERS
UNIVERSITY OF TECHNOLOGY

Effect of mutations in *E. coli* membrane components on bacterial conjugation

Master thesis in Biotechnology

Emelie Ahlstedt

DEPARTMENT OF LIFE SCIENCES

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2023
www.chalmers.se

Effect of mutations in *E. coli* membrane components on bacterial conjugation
EMELIE AHLSTEDT

© EMELIE AHLSTEDT, 2023

Supervisors: Anne Farewell, Alfred Fransson & Anders Lundgren, Department of Chemistry & Molecular biology, University of Gothenburg
Examiner: Johan Bengtsson-Palme, Department of Life Sciences, Chalmers University of Technology
Course code: BBTX60

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Life Sciences,
Chalmers University of Technology
SE-412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

Abstract

The rise of antibiotic resistant bacteria is a major threat to global health care as antibiotics are an important tool in modern medicine. Antibiotic resistance genes can spread between bacteria in a process called conjugation. Conjugation is a form of horizontal gene transfer where a donor cell transfers genetic material to a recipient cell. A possible method to stop the spread of antibiotic resistance genes is to target conjugation. In this project seven different single gene deletions were introduced in the donor. The genes are related to membrane components in *Escherichia coli* and their effect on conjugation was investigated. The genes of interest were *fabF, fabH, lpp, plsX, rfaD, rseA,* and *wzzE. fabF, fabH, plsX, rfaD,* and *wzzE* were investigated through liquid mating assays while *lpp* and *rseA* were investigated by a microfluidics method. However, this microfluidic method is not suited for large scale experiments and was therefore further developed in this project to enable a microfluidics system with four parallel channels. The liquid mating assay with mutant strains showed that *ΔfabH* and *ΔplsX* have a small effect on the transfer efficiency of the donor. *ΔfabF* and *ΔwzzE* were shown to have a moderate effect on transfer frequency while *ΔrfaD* showed a large effect on transfer frequency. *ΔfabF* and *ΔrfaD* were investigated further by a solid mating assay as these two mutations showed the biggest effect on transfer frequency. The results from the solid mating assay were similar to the liquid mating assay. It is hypothesised that the effects seen for *ΔfabF* and *ΔrfaD* could be due to altered membrane fluidity. Thus, the effect of altered membrane fluidity was investigated by treating the donor cells with the membrane softener pentanol in liquid mating assays. Pentanol concentrations of 0 M to 20 mM were examined. The liquid mating assay performed with pentanol showed that increased membrane fluidity decreases the transfer frequency of conjugation. Lastly, the development of the microfluidics method tested different surface modifications of the channels for the best adhesion of cells. A four-channel system was developed but needs further development to be used for conjugation experiments. As the microfluidics method showed problems with, for example, pressure sensitivity and adherence of cells, and issues with fluorescent markers in the mutant strains *Δlpp* and *ΔrseA*, conjugation with the two mutants were ultimately not investigated.

Table of contents

1 Introduction ... 1

1.1 Antibiotics and antibiotic resistance ... 1

1.2 Conjugation ... 1

1.2.1 Conjugative plasmid ... 2

1.2.2 The pili ... 2

1.3 How to combat spread of antibiotic resistance genes through conjugation ... 3

1.3.1 Escherichia coli ... 3

1.3.2 The gram-negative cell membrane ... 4

1.3.3 Can an altered cell membrane affect the donor cell's ability to conjugate? ... 5

1.4 Microfluidics ... 8

1.5 Aim ... 8

2 Methods ... 9

2.1 Conjugation assays ... 9

2.1.1 Liquid mating assay ... 9

2.1.2 Solid mating assay ... 10

2.2 Preparation of donor and recipient strains for microfluidics experiments ... 10

2.2.1 Making chemically competent cells ... 11

2.2.2 Heat shock transformation ... 11

2.3 Preparation of mutant strains for microfluidics experiments ... 12

2.3.1 P1 lysates ... 13

2.3.2 Transduction ... 13

2.4 Creating mutant strains that express a red fluorescent protein ... 13

2.4.1 λ-RED mediated gene replacement ... 13

2.4.2 FLP recombination ... 16

2.4.3 Transforming mutant strains with pEB2 ... 17

2.5 Microfluidics ... 17

2.5.1 Microfluidic channels ... 17

2.5.2 Surface modification of the channels ... 18

2.5.3 Microfluidics setup ... 19

3 Results ... 23

3.1 Conjugation assays ... 23

3.1.1 The effect of pentanol on conjugation ... 23

3.1.2 The effect of donors with single gene deletions on conjugation ... 24

3.1.3 Solid mating assay with *ΔfabF* and *ΔrfaD* ... 25

3.1.4 The effect of fimbriae on conjugation ... 26

3.1.5 Contaminations in mating assays 27
3.2 Microfluidics 27
3.2.1 Conjugation pairs for microfluidics experiments 28
3.2.2 Surface modification protocols 28
3.2.3 Single-channel system 31
3.2.4 Creating mutant cells expressing red fluorescent protein 34
3.2.5 Four channel system 34
4 Discussion 36
4.1 Conjugation assays 36
4.1.1 The effect of altered membrane fluidity on transfer frequency 36
4.1.2 The effect of single gene deletions on transfer frequency 36
4.2 Microfluidics 38
4.2.1 Creating red mutant cells 38
4.2.2 The different surface modification protocols 39
4.2.3 The four-channel system 40
5 Conclusion 41
6 References 42
7 Appendix 46
7.1 Appendix I 46
7.2 Appendix II 48

1 Introduction

1.1 Antibiotics and antibiotic resistance

Antimicrobial compounds are used to prevent and treat infections in humans, animals, and plants (1). Antibiotics are a subcategory of antimicrobials used for bacterial infections. When antibiotics were introduced in healthcare it revolutionised modern medicine (2). Today, antibiotics are used in a number of medical therapies including surgery, organ transplantation, cancer treatment and common bacterial infections. Most antibiotics used in the clinic today are produced by microbial fermentation or derived from natural antibiotic backbone structures (3). They target bacterial physiology and biochemistry leading to cell death or inhibited growth. Common targets of antibiotics include, for example, cell walls or membranes (e.g., beta-lactam), protein synthesis (e.g., tetracycline and chloramphenicol), and nucleic acid synthesis (e.g., fluoroquinolones).

Resistance against antibiotics is a natural mechanism in bacteria that is a result of evolution (3). Bacteria do not need to be exposed to a certain antibiotic for it to gain resistance. Antibiotics have been used by bacteria as signalling molecules and can give a competitive advantage as they can be used to eliminate competing microorganisms (3,4). Genes for the synthesis of antibiotics and genes encoding resistance against them have evolved over billions of years and were present long before antibiotics were used for medical purposes (3). However, antibiotic resistance is becoming a serious issue. Overuse and misuse of antibiotics by humans have contributed to the rise of antibiotic resistant strains (1,3,5). The rapid spread of antibiotic resistance in pathogenic bacteria have rendered many antibiotics ineffective in the last decades (3). This requires the discovery of new antibiotics but since the 1990s very few new classes have been discovered. With few new classes of antibiotics being discovered and bacteria becoming resistant to existing ones, the number of effective antibiotics is decreasing. This global threat to modern healthcare has been deemed to be one of the top 10 public health threats by the World Health Organization (1). Furthermore, it is estimated that the growing resistance will have caused 300 million premature deaths by 2050 (5).

1.2 Conjugation

There are three mechanisms through which bacteria can acquire resistance: transformation, transduction, and conjugation (2). Of these three mechanisms, only conjugation will be discussed further. Conjugation is a form of horizontal gene transfer that involves the transfer of a mobile genetic element such as a plasmid (2). The most well understood plasmid is the F-plasmid which is described in section 1.2.1.1. In short, conjugation involves a donor, which has a conjugative plasmid, and a recipient (6). The donor produces a conjugative pilus that identifies, attaches, and then pulls a recipient cell. This brings the cells into close contact with each other enabling the formation of a mating bridge or pore. The transport of DNA between the donor and recipient is then triggered. Once the DNA is in the recipient it will be recircularised, replicated, and established in its new host. The recipient is then called a transconjugant and can act as a donor.

Several components are necessary for conjugation to take place and the required genes clustered in the *tra* operon need to be expressed in the donor (6,7). The genes in this region encode all protein factors involved in the production of the conjugative pilus, the type IV secretion system, and the relaxosome components (7). The type IV secretion system is a protein complex required for formation of the mating pair and mediates transfer of the DNA while the relaxosome protein complex enables plasmid

processing before transfer to the recipient. In addition to these, there are several other processes that are related to conjugation like surface exclusion that prevent close contact between donors, entry expulsion that prevents transport of nucleoproteins between donors, and mating pair stabilisation that allows certain transfer systems to function in liquid and/or on solid media (6).

1.2.1 Conjugative plasmid

Through the process of conjugation, conjugative plasmids are transferred from a donor cell to a recipient cell (8). Conjugative plasmids have certain characteristics that makes them relevant with regards to antibiotic resistance (8). These plasmids often have a broad host range, which enables conjugation between bacteria of different species, genera, and kingdoms. They contain genes necessary for vertical gene transfer from mother to daughter cell, as well as the genes necessary for horizontal gene transfer between a donor and recipient (8). The genes required for the maintenance and transfer of the conjugative plasmid are often collectively called the plasmid backbone. In addition, conjugative plasmids can have genes that are not involved in the process of conjugation (8). These are genes that provide advantageous properties such as virulence, biofilm formation, and resistance to heavy metals and antibiotics.

Conjugative plasmids are characterised according to their plasmid backbone and the ATPases that take part in the conjugative mechanism (6). The conjugative plasmids are classified in incompatibility groups (Inc) where the classification is dependent on their replication mechanisms. In addition, the difference in the replication mechanism between the groups seems to be linked to the difference in their conjugation mechanism.

1.2.1.1 The F plasmid

The copy number of the F-plasmid (IncF) is around one to two plasmids per donor cell. This enables the donor to mate with up to two recipients at the same time (6). The F-system has a type IV secretion system but also contains extra genes that control pilus outgrowth and retraction (6). A cell with the F plasmid commonly has between one and five conjugative F pili in rich media at 37°C at mid-exponential phase in slightly anaerobic conditions (6). These pili are randomly arranged on the cell surface. Furthermore, the F system has a mating stabilisation property that ensures equally efficient mating in liquid and on solid media. Nine minutes after mixing the donor and recipient the transfer of the F-plasmid DNA can be detected. Within 30 minutes after mixing some of the recipients have become donors. The transfer of the F-plasmid itself takes approximately two minutes.

1.2.2 The pili

The role that the F pilus plays in conjugation has previously been studied (7). One function of the F pili is to act as an anchor that establishes contact with the recipient cell. It then retracts bringing the donor and recipient in close contact with each other, which enables the formation of a mating pair. The pili continuously undergoes a cycle of extension and retraction both in absence and presence of recipient cells (7). This allows the donor cell to probe its surrounding and when it finds a recipient cell a mating pair will be formed upon retraction. However, establishing close contact between the donor and recipient may not be the only function of the F-pilus (7). It has been proposed that it also serves as a channel for the single stranded DNA to travel through, thereby enabling transfer of the plasmid between

a donor and recipient. While the diameter of the pilus does accommodate a single stranded piece of DNA, the F pilus ability to transfer DNA has not been confirmed and is still debated.

Furthermore, the pili of the donor plays a central part in the mating preference for it to take place in liquid media or on solid media (6). The different Inc groups have pili with different morphology and mating preferences (7). There are three groups of conjugative pili based on their morphological properties: a thin flexible pili (found in IncI plasmids), a thick flexible pili (found in IncF plasmids), and a rigid pili (found in IncN plasmids) (6). The "universal" mating type mates equally well in liquid and on solid media and use both thin and thick flexible pili (6). The conjugative systems that prefer surface mating typically involve the thick flexible pili. The last mating type is surface obligatory and is characterised by the rigid pili. The preferred media has an impact on conjugation efficiency together with other factors (6). For example, IncF plasmids have higher transfer efficiencies in liquid media compared to solid media while the IncN plasmid has a higher transfer efficiency on solid media (9).

1.3 How to combat spread of antibiotic resistance genes through conjugation

Inhibition of the conjugation mechanism is a potential approach to hinder the spread of antibiotic resistance (10). This can be accomplished through several strategies. The first strategy would be to target the recipient. However, few genes have been discovered that have a substantial effect on the recipient's ability to conjugate (10). A second strategy is to target the conjugative plasmid itself. The relaxosome and type IV secretion system are crucial for the process of conjugation and could act as possible targets (10). A third option would be to target the donor. Mutations that influence conjugation in *Escherichia coli* with plasmids from the IncF family have been identified (11). The different mutations affect several processes within the cell including DNA replication, chaperones, and protein folding. Furthermore, many of the identified mutations that influenced conjugation were connected to the structure or the function of the cell membrane.

1.3.1 Escherichia coli

In this project *Escherichia coli* was used. *E. coli* is a gram-negative bacterium and is a facultative aerobe (12). It is rod-shaped and its size depends on the specific strain and growth conditions but is typically about 1 µm long and 0.35 µm wide. *E. coli* is commonly referred to as the "workhorse" of molecular biology due to its many advantages such as its fast growth in defined media, several molecular tools for manipulation, and the extensive knowledge of its genome (12). Most *E. coli* strains are harmless, with many considered biosafety 1, but pathogenic variants exist.

1.3.1.1 Fimbriae

Fimbriae are polymer organelles present on the surface of *E. coli* (13). Type 1 fimbriae are approximately 7 nm wide and 1 µm long and are made up by around 1000 subunits. The majority of type 1 fimbriae are made up of the protein FimA, but they also consist of the proteins FimF, FimG and FimH in small percentages. Fimbriae are responsible for the ability of *E. coli* to bind to D-mannosides and this allows the bacteria to adhere and colonise various epithelial surfaces. FimH is the subunit that is responsible for binding of fimbriae to D-mannose (14). Fimbriae's ability to bind to D-mannose is utilised in this project. The microfluidic channels are coated with D-mannose (see section 2.5.2 for surface modification protocols) to get bacteria to attach to the surface.

1.3.2 The gram-negative cell membrane

The cell membrane is crucial for cells to maintain the difference between the cytosolic environment and the extracellular environment (15,16). Bacteria can be divided into two groups: gram-positive and gram-negative. This grouping of bacteria is based on the Gram staining reaction where the structure of the cell membrane plays a major role (16). *E. coli* is a gram-negative bacterium and therefore only the gram-negative cell membrane, see Figure 1, will be discussed further.

The gram-negative cell wall has at least two layers (16). The inner membrane, also called the cytosolic membrane, consists of a lipid bilayer and its most important function is selective permeability (16). The most abundant lipid in the inner membrane is phospholipids and these are amphiphilic with a polar headgroup and two hydrophobic tails, usually fatty acids in bacteria (15). The lipid bilayer is dynamic and fluid in its structure and lipids can move around within the plane of a bilayer. The fluidity of the membrane is tightly regulated as certain cellular processes, such as membrane transport, are inhibited when the fluidity is altered. How fluid a membrane is depends on the composition of the membrane as well as temperature. In the case of bacteria, they adjust the composition of fatty acids to maintain a relatively constant fluidity. Apart from different lipids there are also membrane proteins with a variety of functions that span across the lipid bilayer.

One of the major functions of the outer membrane is to prevent harmful substances from entering the cell (15). Like the inner membrane, the outer membrane of a gram-negative cell also consists of a lipid bilayer, however, its composition differs (16). In addition to phospholipids and proteins, the outer membrane also contains polysaccharides that are made up by a core polysaccharide and O-specific polysaccharide. The lipids and polysaccharides form a complex and the outer membrane is therefore also called Lipopolysaccharide (LPS). The lipopolysaccharides are on the outside of the outer membrane while phospholipids are located on the inner leaflet of the outer membrane (15). Finally, enterobacterial common antigen (ECA) is a glycolipid that is present in all gram-negative enteric bacteria and is located on the cell surface and in the periplasm (17,18). ECA is present in three forms ECA_{PG}, ECA_{CYC}, and ECA_{LPS}. ECA_{PG} are polysaccharide chains of various lengths, ECA_{LPS} are characterised by the linkage of the ECA polysaccharide chains to LPS, and ECA_{CYC} is cyclic form (18). The function of ECA has not been fully elucidated, however it seems to be involved in regulating outer membrane permeability (19).

Neither the inner nor outer membrane confers much structural strength to the cell (16). The structural stability is provided by a porous and rigid layer of peptidoglycans that are in the periplasmic space between the inner and outer membrane (15,16). Strands of peptidoglycans forms a sheet where individual strands are bound by peptide cross-links. The outer membrane is linked to the peptidoglycan layer by a lipoprotein known as Braun lipoprotein. This protein spans between the LPS layer and the peptidoglycan layer (16).

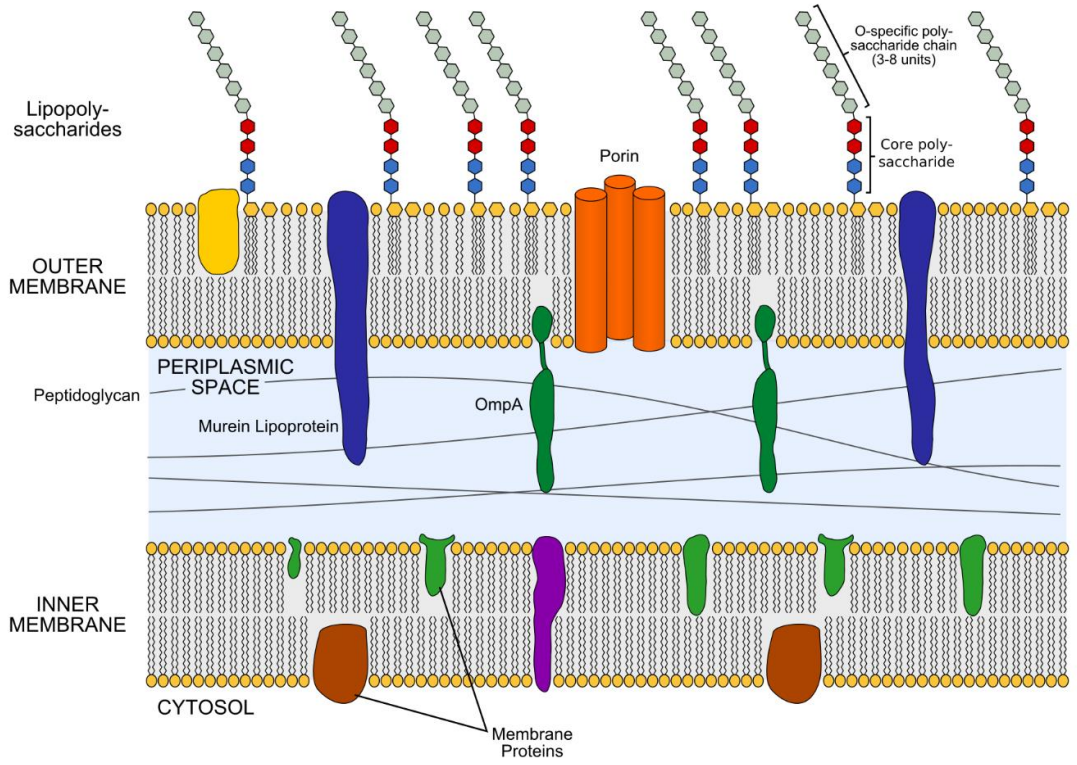

Figure 1. ***Gram-negative cell membrane.*** *Shows the cell membrane of gram-negative bacterium, such as Escherichia Coli, and some of its important structures* (20).

1.3.3 Can an altered cell membrane affect the donor cell's ability to conjugate?

This project includes the investigation of several mutant strains of *E. coli.* The original mutant strains are part of the Keio collection, which includes single gene knockouts of all nonessential genes in *E. coli* K-12 (21). The genes have been replaced with a kanamycin cassette that is flanked by FLP recognition target sites. The selected gene deletions are all involved in important components of the gram-negative cell membrane, such as the Braun lipoprotein and LPS synthesis. The reasoning behind the selection of these genes is that the conjugation mechanism has several steps that are linked to the membrane. For example, the type IV secretion system has components that are embedded in both the outer and inner membrane, the pilus on the donor cell is attached to the cell membrane, and there are also several proteins necessary in conjugation that interact with the cell membrane such as TraA and TraN (22–24). Apart from the gene deletions it was also hypothesised that membrane fluidity could affect the conjugation ability of the donor and the membrane softener pentanol was used to investigate this (25). Pentanol acts by causing disorder in the lipid bilayer upon incorporation (25).

1.3.3.1 Description of the genes of interest

The mutant strains selected for this project has gene deletions for the genes *fabF, fabH, lpp, plsX, rfaD, rseA,* and *wzzE,* respectively. A description of the genes of interest can be found below. Figure 2 shows a gram-negative cell membrane where the genes of interest have been included with arrows indicating which component of the membrane they are associated with. In addition, the figure has been modified to include ECA to give an idea of its location within the membrane. The gene deletions of *fabF, fabH, plsX, rfaD,* and *wzzE* were studied using traditional conjugation assays. Gene deletions

of *lpp* and *rseA*, on the other hand, were studied using microfluidics as these mutants are more well studied.

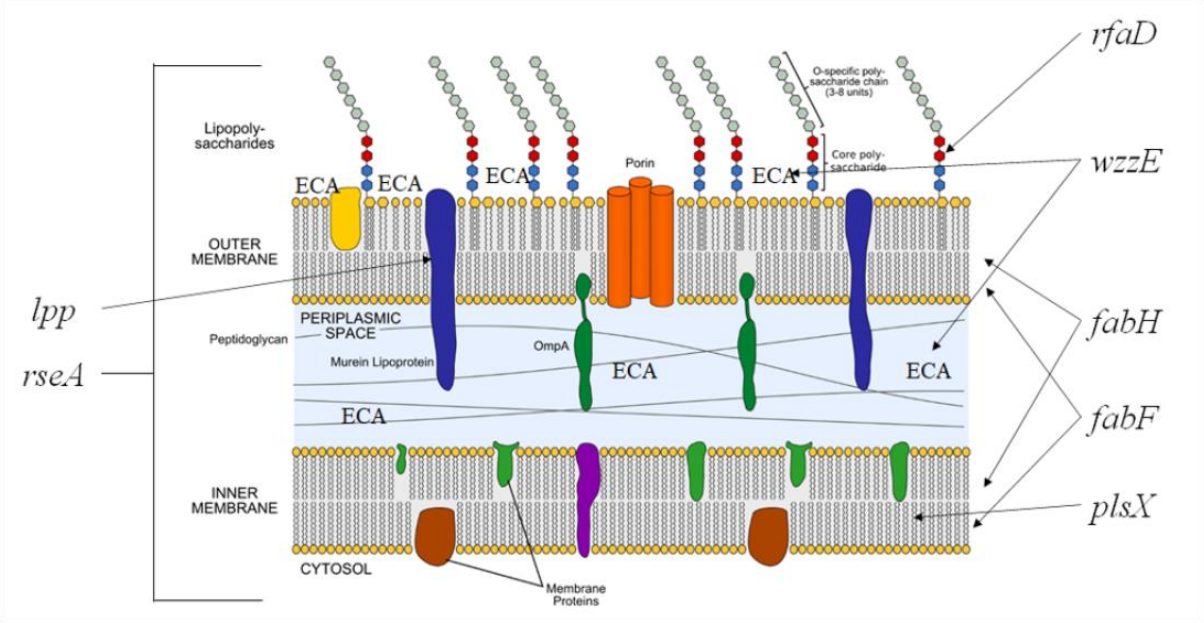

Figure 2. ***The gram-negative cell membrane where the affected components of the genes of interest shown.*** *Shows the gram-negative cell membrane* (20) with the addition of ECA to give an idea of its location in the cell membrane. In addition, the genes of interest have been included with arrows indicating which component in the membrane they are associated with.

1.3.3.1.1 fabF and fabH

In *E. coli* there are three β-ketoacyl-[acyl carrier protein]synthases (KAS), namely KASI, KASII and KASIII where KASII and KASIII are encoded by *fabF* and *fabH*, respectively (26). The KAS enzymes are all involved in fatty acid synthesis in *E. coli*. KASII is involved in type II fatty acid elongation cycle. KASIII, on the other hand, is involved in the initiation of fatty acid biosynthesis.

1.3.3.1.2 lpp

The gene *lpp* encodes a murein lipoprotein that is known as Braun lipoprotein (Lpp) (27). The protein is the most abundant lipoprotein in *E. coli*. There are two types of Lpp. The first binds the peptidoglycan layer to the outer membrane, and the second spans across the outer membrane (28). Cells that do not have the Lpp protein or have been mutated in ways that affect its attachment to the peptidoglycan layer show a deformed outer membrane and are sensitive to toxic compounds (29).

1.3.3.1.3 plsX

Phospholipids are prominent molecules in the cell membrane and glycerol-3-phosphate (G3P) is the backbone of all phospholipid molecules (30). The synthesis of G3P begins with two acylation steps. In *E. coli* G3P and 1-acylglycerol-phosphate acyltransferase are encoded by *plsB* and *plsC*, respectively. PlsB utilises acyl-ACP (acyl carrier protein) and acyl-CoA as acyl donors for synthesis of 1-acyl-G3P. In *Streptococcus pneumonia* an alternative two-step pathway that utilises a novel fatty acid for formation of phospholipid intermediates has been discovered (31,32). There, PlsX produce a fatty acid by catalysing the synthesis of fatty acyl-phosphate from acyl-ACP for PlsY to transform it from acyl-

phosphate to G3P. *plsX* has been proposed to be involved in fatty acid and/or phospholipid synthesis in a similar manner in *E. coli* as the gene is conserved in eubacteria (30).

1.3.3.1.4 rfaD

ADP-L-glycero-D-mannoseheptose-6-epimerase is encoded by *rfaD* (33). This enzyme is part of the last step in the synthesis of the ADP-heptose precursor and is required for lipopolysaccharide core biosynthesis. Lipopolysaccharides are located on the outer membrane and are required to prevent passive diffusion of hydrophobic solutes, like antibiotics and detergents, into the cell (34).

1.3.3.1.5 rseA

rseA encodes an anti-sigma factor, RseA, that inhibits σ^E. σ^E is involved in maintaining the integrity of the cell envelope during both normal cell growth and when the cell envelope is damaged (35). RseA functions by blocking the association of σ^E and RNA polymerase.

1.3.3.1.6 wzzE

The gene *wzzE* is located in a gene cluster that encodes enzymes that are needed for ECA synthesis and assembly (18). The protein encoded by *wzzE* adjusts the length of ECA_{PG} polysaccharide chains where most are about six to seven repeats long. Mutant *wzzE* strains have ECA_{PG} polysaccharides with varying chain lengths. Apart from being related to the length of polysaccharide chains the resulting protein is also required for synthesis of ECA_{CYC} (17).

1.3.3.2 Description of genes used as control strains

In addition to the genes of interest, a positive control and a negative control were also used in the project, both from the Keio collection. The positive control has a deletion of the gene *argC*. This deletion as no effect on conjugation and acts as a control donor that has the same background as the other mutants. The negative control has an *arcA* deletion and cannot conjugate. A description of these two genes can be found below.

1.3.3.2.1 arcA

arcA encodes the protein ArcA that is part of the Arc system together with ArcB. These two proteins regulate genes involved in the tricarboxylic acid cycle as a response to oxygen levels (36). Furthermore, ArcA is linked to conjugation with the F plasmid. ArcA together with TraJ are known activators of the *tra* operon promoter P_Y (37). Both proteins are required for efficient transfer of the F plasmid. However, donors where *arcA* has been knocked out show some limited conjugative transfer. This indicates that there is some ArcA independent activation of the *tra* operon (37).

1.3.3.2.2 argC

argC encode N-acetylglutamylphosphate reductase that is responsible for the third step in arginine biosynthesis (38,39). The enzyme more specifically catalyses the of N-acetylglutamyl-phosphate in a NADPH-dependent reduction that yields N-acetyl-L-glutamate-5-semialdehyde.

1.4 Microfluidics

The traditional ways of culturing cells have been with flasks, petri dishes, bioreactors, etc., and can preserve the sample of interest for the required period of time (40). When using these common cell culture methods the overall aim is to create an environment that is as close as possible to the natural cellular environment (41). In addition, these methods need to be simple enough so that replicates can be performed in order to acquire statistically significant results. These two goals commonly result in a trade-off. Furthermore, bulk assays provide a level of uncertainty due to natural cell-to-cell variability (42). These variabilities in, for example, protein expression levels may give rise to misleading and even inaccurate conclusions.

In this project microfluidics was used to study conjugation on a solid surface. Microfluidics is a technique that enable precise control of fluids and particles at the nanolitre scale (42). Microfluidic systems aim at creating a cellular environment that is more similar to *in vivo* systems by controlling flow rate and chemical gradients (40). The technique makes it is possible to study heterogeneities and cell dynamics in a microbial population and enables investigation of, for example, growth, cell-cell interactions, and gene expression (43). Furthermore, it can be paired with tools for visualisation, spatial analysis, and quantification of intracellular processes, which is commonly done with fluorescent markers (43).

Microfluidic approaches also have the potential for parallelisation since the dimensions are small (41,42). Multiple cell culture chambers can be used in a single device. This enables higher throughput while still retaining the level of accuracy and reproducibility without having a major impact on the amounts of reagents and chemicals needed. Because of the microfluidic device's small scale, flow within the channel is laminar (44). This implies that two streams inside a channel will flow in parallel without any turbulence. The only mixing between the streams is diffusion of molecules across the interface of the two fluids.

1.5 Aim

With the increasing number of antibiotic resistant bacteria, the spread of these genes through conjugation becomes an important subject for investigation. The aim of this work was to investigate conjugation donors with single gene deletions in order to evaluate the effect of these mutations on conjugation efficiency. In total, seven different mutants were investigated. The mutants selected are related to components in the cell membrane and are of interest since there are several connections between the conjugation mechanism and the cell membrane. Two of these mutants were studied using an existing microfluidic method. However, this method is time consuming and inefficient for large scale experiments. Therefore, part of the aim was to further develop the existing method to allow for a multichannel system that enables analysis of multiple conjugation pairs during one experiment. The remaining five mutants were studied using liquid and solid mating assays. Finally, the potential role of membrane fluidity in conjugative efficiency was examined.

2 Methods

2.1 Conjugation assays

Liquid mating assays were performed several times during this project but with different strains. All strains can be found in Table 1. The same general protocol was used each time with the strains and antibiotics varying. Both liquid and solid mating assays was performed twice on separate days for each mating set up. This is to verify the results.

2.1.1 Liquid mating assay

For the liquid mating assay the donor strains, control donor, and recipient strain were inoculated in 2 ml LB with appropriate antibiotics. At least two biological replicates were prepared for each donor strain. The cultures were grown over night at 37°C with shaking.

3 ml LB for each donor and control donor and 5 ml LB for each recipient were prewarmed to 37°C. 1 ml of donor cells and recipient cells were washed by centrifugation at 13300 rpm for 2 minutes. They were then resuspended in 1 ml LB without antibiotics. In order to determine the amount of cell culture to add to 3 ml LB for the donors and 5 ml LB for the recipients, the OD_{600} was measured. An OD_{600} of approximately 2 corresponded to 60 µl cell culture being added to the media. The cells were incubated at 37°C with shaking until an OD_{600} of 0.3-0.6 was reached. The OD_{600} was measured by diluting 100 µl of cell culture in 900 µl 1x M9 salts. 180 µl of pre-chilled 1x M9 salts was aliquoted in rows B to H and all columns in a 96-well plate that was stored in the fridge until further use.

When the cell cultures reached an appropriate OD_{600}, 500 µl of each donor were mixed with 500 µl of recipient. They were then incubated for 30 minutes at 37°C without shaking to allow mating. Negative controls were also prepared where 500 µl of donor culture was mixed with 500 µl of LB and 500 µl recipient was mixed with 500 µl LB. The mating was stopped by vortexing the mating mixture for 1 minute and were then placed on ice for at least 1 minute. The cell mixtures were kept on ice thereafter.

200 µl of each mixture was aliquoted in the first row of the previously prepared 96-well plate. Then a 1/10 serial dilution was performed where 20 µl was added to the 180 µl 1x M9 salts until a dilution of 10^{-7} was reached. 10 µl of each dilution was spotted on LB plates with appropriate antibiotics. The negative controls were also spotted. The plates were then incubated overnight at 37°C.

The donor and transconjugant colonies were then counted. The colony counts were then used to calculate the transfer frequency per donor cell. The number of donors and transconjugants were estimated by taking the highest number of colonies for a certain dilution factor. This was divided by 10 to get the number of donors or transconjugants per µl. It was then multiplied by the dilution factor to get the concentration of donors and transconjugants, respectively, in the original sample. This was then multiplied by 200 to get the number of cells in the 200 µl undiluted culture. The transfer frequency was determined by dividing the number of transconjugants by the number of donors.

2.1.2 Solid mating assay

Overnight cultures of the mutant strains *ΔfabF* and *ΔrfaD*, the control donor with *argC* deletion and the recipient HA4 were prepared. 2 ml of LB with the appropriate antibiotics was inoculated with a single colony of the different strains. Two biological replicates were prepared for each donor and the control donor.

The following day 1 ml of each culture was washed through centrifugation at 13300 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of LB. 75 µl of washed cells were added to 1,5 ml of LB without antibiotics. The cultures were left to grow until they reached an OD_{600} of 0.3-0.8. The OD_{600} was measured with 100 µl culture in 900 µl 1x M9 salts. 180 µl of pre-chilled 1x M9 salts was aliquoted in row A to F and column 2-8 in 96-well plate that was stored in the fridge until further use.

When an appropriate OD-value had been reached, 50 µl of donor was mixed with 50 µl recipient in an Eppendorf tube. 50 µl of the mix was spotted on filters (0.22 µm) that were placed on LB plates and left to mate for 30 minutes at 37°C. Negative controls were also prepared where 50 µl of donor culture was mixed with 50 µl of LB and 50 µl recipient was mixed with 50 µl LB. After mating, the filters were removed from the LB plates and placed in 1 ml of 1x M9 salts and vortexed for one minute to stop the mating before being placed on ice for at least two minutes. 200 µl of the mating mixture was then aliquoted in the first column, row A to F, of the previously prepared 96-well plate.

A 1/10 serial dilution was performed where 20 µl mating mixture was added to the 180 µl 1x M9 salts until a dilution of 10^{-7} was reached. 10 µl of each dilution was spotted on LB plates with appropriate antibiotics. The negative controls were also spotted. The plates were then incubated overnight at 37°C. The next day the colonies were counted. The colony counts were used to calculate the number of donors and transconjugants in 200 µl undiluted culture. The number of transconjugants was divided by the number of donors to get the transfer frequency per donor cell.

2.2 Preparation of donor and recipient strains for microfluidics experiments

The *E. coli* strain MG1655 was used for both the donor and recipient. MG1655 cells were made chemically competent and transformed.

Two conjugation pairs were made. The first conjugation pair consisted of a donor (ELA1) where an existing MG1655 strain with pPKL91 acquired the conjugative plasmid pLP8 through mating. pPKL91 upregulates the cell's fimbriae expression. pLP8 is the conjugative F plasmid with a green fluorescent protein (GFP). The recipient (ELA3) was the same MG1655 strain with pPKL91 that was transformed with pEB2. pEB2 contains a red fluorescent protein (mScarlet). The fluorescent markers enable easy detection with the microscope in the microfluidics experiment. With this set up the donors exhibited green fluorescence, the recipients exhibited red fluorescence and the transconjugants exhibited both red and green fluorescence. The general protocol for making competent cells and transformation can be seen in section 2.2.1 and 2.2.2, respectively. To create ELA3, an existing MG1655 strain with pPKL91 was made competent. Carbenicillin at a concentration of 50 µg/ml was added to the overnight (ON) culture. Once made competent, the cells were transformed with pEB2 and 2 µl plasmid was added to 100 µl cells and transformants selected on LB plates with carbenicillin and kanamycin.

A second conjugation pair was also prepared due to difficulties with the first conjugation pair, a further explanation can be found in section 3.2.1. To make the new donor, ELA3 was used as a base and was mated with ELA1 for it to acquire the F plasmid pLP8. The new donor (ELA8) contained three plasmids in total: pPKL91, pEB2 and the conjugative plasmid pLP8. The new recipient (ELA9) was a MG1655 strain with pPKL91 only. This set up has a green and red fluorescent donor, a recipient that was not fluorescent and a transconjugant that was green fluorescent. See Table 1 for a complete strain list.

2.2.1 Making chemically competent cells

Cells were made competent by using an overnight (ON) culture that was diluted 1:10 in LB media with appropriate antibiotics. The optical density at 600 nm (OD_{600}) was periodically checked until it reached a value between 0.5 and 0.7. Once an appropriate OD_{600}-value was reached the culture was placed on ice for 20 minutes. It was then centrifuged at 4000 rpm at a temperature of 4°C for 5 minutes. The supernatant was removed and the pellet was resuspended in cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES pH 7) to one fifth of the previous media volume, i.e. if the culture was 10 ml the pellet was resuspended in 2 ml cold $CaCl_2$ solution. The resuspended pellet was centrifuged again with the same settings. The supernatant was removed and the pellet was resuspended in half the amount of cold $CaCl_2$ solution, i.e. 1 ml following the previous example. The culture was placed on ice for 1-2 hours before aliquoting 100 µl of the cell suspension in sterile, pre-chilled Eppendorf tubes. The cells were either frozen or used immediately for transformation.

2.2.2 Heat shock transformation

Heat shock transformation was executed by adding 1 to 10 µl of plasmid to 100 µl chemically competent cells. The mixture of cells and plasmids was incubated on ice for approximately 45 minutes. The mixture was then heat-shocked for 30 seconds at 42°C. 1 ml of LB was added to the mixture and was left to incubate at 37°C for 1 hour. It was then centrifuged at 4000 rpm for 2 minutes. Most of the supernatant was removed while leaving approximately 100 µl, which was used to resuspend the cell pellet. The remaining cell suspension was spread on selective plates with appropriate antibiotics and incubated overnight at 37°C.

Table 1. ***Strain list.*** *Contains a list of the strains used in the project. The table includes if the strain has any plasmids, any relevant genotype, the specific antibiotic resistance, and the strain's name.*

Name	Strain Background	Plasmid(s)	Relevant genotype	Antibiotic resistance
ELA1	*Escherichia coli* MG1655	pPKL91, pLP8	-	Carbenicillin, Chloramphenicol, Tetracycline
ELA2	*Escherichia coli* MG1655	pPKL91, pLP8	*ΔfimA*	Chloramphenicol, Kanamycin, Tetracycline
ELA3	*Escherichia coli* MG1655	pPKL91, pEB2	-	Carbenicillin, Chloramphenicol, Kanamycin
-	*Escherichia coli* MG1655	pLP8	*ΔxylA*::KanR	Chloramphenicol, Kanamycin, Tetracycline
ELA8	*Escherichia coli* MG1655	pPKL91, pEB2, pLP8	-	Carbenicillin, Chloramphenicol, Kanamycin, Tetracycline
ELA9	*Escherichia coli* MG1655	pPKL91	-	Carbenicillin, Chloramphenicol
-	*Escherichia coli* MG1655	-	*ΔarcA::*KanR	Kanamycin
HA14	*Escherichia coli* MG1655	-	*ΔargC::*KanR	Kanamycin
-	*Escherichia coli* BW25113	F-plasmid	*ΔargC::*KanR	Kanamycin, Tetracycline
-	*Escherichia coli* BW25113	F-plasmid	*ΔfabF::*KanR	Kanamycin, Tetracycline
-	*Escherichia coli* BW25113	F-plasmid	*ΔfabH::*KanR	Kanamycin, Tetracycline
-	*Escherichia coli* MG1655	-	*Δlpp::*KanR	Kanamycin
-	*Escherichia coli* BW25113	F-plasmid	*ΔplsX::*KanR	Kanamycin, Tetracycline
-	*Escherichia coli* BW25113	F-plasmid	*ΔrfaD::*KanR	Kanamycin, Tetracycline
-	*Escherichia coli* MG1655	-	*ΔrseA::*KanR	Kanamycin
-	*Escherichia coli* BW25113	F-plasmid	*ΔwzzE::*KanR	Kanamycin, Tetracycline
HA4	*Escherichia coli* BW25113	-	*araA* + *araC* + *ΔaraB::*CamR	Chloramphenicol
LF1	*Escherichia coli* MG1655	pKD46	Encodes λ-RED	Carbenicillin, Kanamycin

2.3 Preparation of mutant strains for microfluidics experiments

In order to make the mutant donors P1 lysates for each mutation was created and P1 transduction was performed to introduce the mutations to the donor strain. The four mutations that were introduced were *ΔarcA*, *ΔargC*, *Δlpp*, and *ΔrseA*. *ΔarcA* is a strain that cannot conjugate and *ΔargC* will be used as a control as the mutation has no effect on conjugation.

2.3.1 P1 lysates

ON cultures were prepared with the strains containing the mutation of interest the day before the P1 lysates were made. 1 ml of LB was inoculated with a single colony of each strain and kanamycin was added to a concentration of 50 µg/ml. 25 µl of 1 M $CaCl_2$, 25 µl of the ON culture, 5 ml LB, 2 µl P1 stock and 50 µl 1 M $MgSO_4$ was added to a 50 ml tube for each of the mutant strains. The culture was allowed to grow at 37°C with shaking until complete lysis was observed. 10 drops of chloroform were added to each tube and vortexed. It was then left to incubate for 10 minutes at room temperature. 2 ml of the lysate was transferred to Eppendorf tubes and centrifuged at 13300 rpm for 2 minutes. The supernatant was transferred and 2 drops of chloroform were added. The tubes were incubated for 10 minutes before being centrifuged again at the same rpm for 2 minutes. The supernatant was transferred to new tubes while leaving the chloroform behind. It was centrifuged once more using the same settings and transferring the supernatant to new tubes. The resulting P1 lysates of *ΔarcA, ΔargC, Δlpp,* and *ΔrseA* were stored in the refrigerator.

2.3.2 Transduction

Transduction was performed for all four mutants. An ON culture of ELA1 was centrifuged at 4000 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in MC buffer (0.1 M $MgSO_4$, 5 mM $CaCl_2$ in water) at half the volume of the ON culture. 100 µl of cell culture was transferred to Eppendorf tubes where 30 µl of P1 lysate was added and the mixtures were left to incubate for 20 minutes at 37°C. 150 µl of 1 M Na Citrate was added as well as 1 ml of LB. They were then incubated for 1 hour at 37°C with shaking. The cell mixture was then centrifuged for 2 minutes at 13300 rpm. The supernatant was removed and the cells were resuspended in 100 µl LB and spread on selective plates with carbenicillin, kanamycin, and tetracycline. Controls of P1 lysates, MC buffer, LB and Na Citrate were also spotted on a LB plate without antibiotics to check for contaminations.

2.4 Creating mutant strains that express a red fluorescent protein

The second conjugation pair was used to enable easy detection of the donor, recipient and transconjugant cells, respectively, in the microfluidic channels. The donor cells express both GFP and RFP, the recipients have no fluorescent marker and the transconjugant express only GFP. However, when making donors with the gene deletions *Δlpp*, *ΔrseA*, *ΔargC*, and *ΔarcA* there was an overlap between the selection markers for the plasmid pEB2 and the mutants. The gene deletions have been replaced by a kanamycin cassette and pEB2 encodes kanamycin resistance. Therefore, three different methods were explored to get the mutant donors to express an RFP to enable easy distinction in the microfluidic experiments. The first method utilises a λ-RED recombinase for insertion of the RFP gene into the chromosome through homologous recombination (45), thereby creating a strain that express an RFP without using the plasmid pEB2. The second method used the plasmid pCP20 to flip out the kanamycin cassette from the mutants. This makes it possible to then transform the mutant cells with pEB2 and still use kanamycin as a selection marker. For the third method the pEB2 plasmid was transformed into the mutant cells without removing the kanamycin cassette first. Identification of successfully transformed colonies was based on the colour of the colonies.

2.4.1 λ-RED mediated gene replacement

The method developed by Fried, Lassak and Jung allow fast construction of *lacZ* fusion reporters in *E. coli* (45). By inserting a DNA cassette with a dominant wild type *rpsL* gene and kanamycin resistance in between *lacI* and *lacZ* in a MG1655 with a recessive mutated *rpsL* allele they created the strain called

LF1. LF1 is resistant to kanamycin, sensitive to streptomycin, and lac$^-$. The LF1 strain enables construction of practically any promoter-*lacZ* fusion by creating a DNA construct that is flanked by regions that are homologous to the chromosomal *lacI* and *lacZ* genes of LF1. The DNA construct, which in this project contains the gene encoding the RFP mScarlet, can be incorporated into the chromosome by homologous recombination. The resulting strain in this project is resistant against streptomycin and sensitive to kanamycin.

2.4.1.1 Preparation of host cells through transformation

First the pKD46 plasmid, which encodes the λ-RED system, needed to be extracted. For this an ON culture of MG1655 with pKD46 was prepared. The plasmid was extracted using Thermo Scientific's GeneJet Plasmid Miniprep Kit. The ON culture was centrifuged at 8000 rpm and the supernatant was removed. The cell pellet was then resuspended in 250 µl of Resuspension solution. The cell suspension was then transferred to an Eppendorf tube. 250 µl of Lysis Solution was added and the tubes were mixed by inverting them six times. Following this, 350 µl of Neutralization Solution was added and mixed immediately through inverting the tubes six time. The mixture was centrifuged for 5 minutes at 13300 rpm. The supernatant was transferred to a supplied GeneJet spin column being careful not to disturb the cell pellet. It was centrifuged for 1 minute at 13300 rpm and the flow through was discarded. 500 µl of Wash Solution was added and centrifuged for 1 minute, the flow through was discarded. The washing step was then repeated once. An additional centrifugation of 1 minute at 13300 rpm was performed to remove any remaining ethanol. The GeneJet column was transferred to an Eppendorf tube where 50 µl of Elution Buffer was added to elute the plasmid. It was incubated for 2 minutes at room temperature before being centrifuged at 13300 rpm for 2 minutes. The purified plasmid was stored at -20℃.

The LF1 strain was transformed with extracted pKD46. Chemically competent cells were prepared using the protocol in section 2.2.1 with the exception that the time for centrifugation was 10 minutes. The cell culture used was an ON culture of the strain LF1 grown at 37℃. Next, the competent LF1 cells were transformed through heat shock with pKD46. 1 µl, 5 µl and 10 µl pKD46 was added to 100 µl of cells in three different tubes. The protocol for heat shock transformation can be seen in section 2.2.2. The cells were, however, allowed to recover for 1 hour at 30℃ instead of 37℃ as pKD46 is heat sensitive. For the same reason, the plates were incubated overnight at 30℃. The cells were spread on LB plates with carbenicillin and kanamycin.

2.4.1.2 Construction of DNA fragment through PCR

5 PCR reactions of 50 µl each were used to amplify the mScarlet gene from pEB2. For each reaction 10 µl of 5x Phusion Plus buffer, 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 1 µl of 10 mM dNTPs, 1 µl of pEB2 in 4 µl water, 28.5 µl water, and 0.5 µl Phusion DNA polymerase was added. The primer sequences can be seen in Table 2 and the PCR program used can be seen in Table 3.

*Table 2. **Primer sequences.** Contains the sequences of the forward and reverse primer used to construct the DNA fragment of pEB2 containing the gene for mScarlet.*

Forward primer	GcacatctgaacttcagcctccagtacagcgcggctgaaatcatcattaaTCTGAGGTTCTTATG GCTCTTGT
Reverse primer	GcacatctgaacttcagcctccagtacagcgcggctgaaatcatcattaaTCTGAGGTTCTTATG GCTCTTGT

*Table 3. **PCR program.** Contains the PCR program used for creating the DNA fragment from pEB2.*

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	30	1
Denaturation	98	20	30
Annealing	60	20	
Extension	72	90	
Final extension	72	300	1

The resulting PCR mixes were then run on a gel to isolate the resulting DNA fragment of pEB2. A 1% agarose gel was prepared with SYBR safe dye. The PCR fragment was run on the gel as well as the original pEB2 plasmid and a DNA ladder. The five reactions from the PCR were pooled together and placed in a large well in the gel. The gel ran for 50 minutes at 100 V. The gel was placed under UV light to visualise the bands. The large PCR fragment band was cut out using a scalpel and gel extraction was performed using Thermo Scientific GeneJET Gel Extraction Kit. The cut-out fragment was sliced up and placed in five Eppendorf tubes of around 300 mg each. 300 µl of Binding Buffer was added to each tube. The tubes were then incubated at 50°C until the gel was completely dissolved. The tubes were mixed every few minutes. The solubilised gel was transferred to supplied GeneJET purification columns and centrifuged for 1 minute at 13300 rpm. Two columns were used and this step was repeated until all solubilised gel had been centrifuged. The flow through was discarded each time. 700 µl of Wash Buffer was added to the purification columns and centrifuged for 1 minute at 13300 rpm. The flowthrough was discarded and the tube was centrifuged once more to remove residual wash buffer. The column was transferred to an Eppendorf tube where 50 µl of Elution Buffer was added and centrifuged for 1 minute. The flowthrough was stored at -20°C.

2.4.1.3 Transformation of LF1 pKD46 with PCR fragment

Cells were first made electro competent. An ON culture of LF1 with pKD46 was prepared and the following day 4 tubes with 2 ml of LB with 50 µg/ml carbenicillin was inoculated with 20 µl of the ON culture. The 4 cultures had different conditions, see Table 4. Only tube 1 is expected to give the desired strain and the others are controls. The cells were grown until an OD_{600} of 0.1 was reached. Then 20 µl of 1 M L-arabinose was added to the respective tubes to reach a concentration of 10 mM. The L-arabinose was added to induce λ-RED expression on pKD46. The cells were then grown until an OD_{600} approximately 0.4. The cells were put on ice for 10 minutes before being centrifuged at 4°C with 4000 rpm for 10 minutes. The supernatant was removed, and the cell pellet was resuspended in 1 ml cold sterile MQ water. The cells were then centrifuged once more with the same conditions. The supernatant was removed, and the pellet was resuspended in 50 µl cold sterile MQ water.

Table 4. ***Conditions used for transformation of LF1 with pKD46.*** *Shows the different conditions used when transforming LF1 pKD46 with the pEB2 PCR fragment. Only tube 1 is expected to give the desired strain and the others are controls.*

Tube	L-arabinose	pEB2 PCR product
1	+	+
2	+	-
3	-	+
4	-	-

5 µl of the PCR product of pEB2 was added to the respective tubes. The culture was transferred to prechilled cuvettes for electroporation. The settings for the electroporation were 2.1 kV, 25 µF and 200 Ω. The cells were shocked and directly after 1 ml of LB was added before transferring the cells to Eppendorf tubes. The cells were then incubated for approximately 2 hours with shaking at 37℃. 100 µl was then plated on streptomycin plates and left to grow over night at 37℃. The successfully transformed colonies were then checked under the microscope to check if they exhibited red fluorescence.

2.4.2 FLP recombination

A method that removes the kanamycin resistance cassette from the mutated strains through FLP recombination with pCP20 was used to create mutant donor strains that express RFP. pCP20 has a temperature-sensitive origin of replication, resistance to carbenicillin and encodes a FLP recombinase. This protocol was used for all four mutant strains *Δarg*, *ΔarcA*, *Δlpp*, and *ΔrseA*.

First the plasmid needed to be extracted. For this an ON culture of MG1655 with pCP20 was prepared. The plasmid was then extracted using Thermo Scientific's GeneJet Plasmid Miniprep Kit. The ON culture was centrifuged at 8000 rpm. The supernatant was removed and the cell pellet was resuspended in 250 µl of Resuspension solution. The cell suspension was then transferred to an Eppendorf tube. 250 µl of Lysis Solution was added. The tubes were mixed by inverting them six times. Next, 350 µl of Neutralization Solution was added and mixed immediately through inverting the tubes six time. The mixture was centrifuged for 5 minutes at 13300 rpm. The supernatant was transferred to a supplied GeneJet spin column taking care not to disturb the pellet. It was centrifuged for 1 minute at 13300 rpm and the flow through was discarded. 500 µl of Wash Solution was added and centrifuged for 1 minute, the flow through was discarded. The washing step was then repeated. An additional centrifugation of 1 minute at 13300 rpm was performed to remove residual ethanol. The GeneJet column was transferred to an Eppendorf tube where 50 µl of Elution Buffer was added to elute the plasmid. It was incubated for 2 minutes at room temperature before being centrifuged at 13300 rpm for 2 minutes. The purified pCP20 plasmid was stored at -20℃.

Chemically competent cells with the respective mutation, without the F plasmid, were made using the protocol listed in section 2.2.1. Kanamycin at a concentration of 50 µg/ml was used for the ON culture. The mutant strains were transformed through heat shock with 10 µl pCP20 according to the protocol in section 2.2.2. They were allowed to recover for one hour at 30℃, as pCP20 is temperature sensitive. 100 µl of transformed cells were then spread on plates with carbenicillin and kanamycin and incubated at 30℃ overnight. The following day, a single colony was used to inoculate 5 ml of LB and was left to grow overnight at 43℃ to select for loss of pCP20. The next day a 10^{-6} dilution of the overnight culture was made and 50 µl of the dilution was spread on LB plates. The plates were incubated overnight at 30℃ to prevent partial loss of pCP20. Colonies were then screened for genomic recombination and loss

of pCP20. Individual colonies were streaked on LB + Kan, LB + Carb, and LB plates in that order. The LB-plates were incubated at 37℃ overnight while LB + Kan and LB + Carb were incubated overnight at 30℃.

2.4.3 Transforming mutant strains with pEB2

As a last resort to achieve donor cells that express RFP the pEB2 plasmid was directly transformed into the mutant cells. ON culture of two of the mutant strain (*Δlpp* and *ΔrseA*) were prepared and made chemically competent according to the protocol in section 2.2.1, and were transformed with the heat shock protocol described in section 2.2.2. 4 µl of pEB2 was used for transformation. As the cells could grow on kanamycin plates even if pEB2 was not successfully transformed into the cells, due to the kanamycin cassette in the mutant strains, the transformed cells were spread on plates with different kanamycin concentrations. The concentrations used were 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, and 400 µg/ml kanamycin. In addition, the cells were diluted to 10^{-2} to get individual colonies on the plates.

2.5 Microfluidics

2.5.1 Microfluidic channels

The microchannels were constructed by placing the glass capillary, with an inner diameter of 0.8 mm, on a microscope slide. They were glued together using optical glue. The glue was left to set under a UV light for 15 minutes. Two capillary pipette tips were adjusted in length and sandpaper was used to roughen up the surface in order for the glue to adhere better. The pipette tips were inserted into the capillary tubes, one at each end. The tips were then glued in placed and the whole slide was placed under a UV light for 2 hours to allow the glue to cure. See Figure 3 for a schematic of the single-channel slide. The newly made channels were stored with Hellmanex 2% until use.

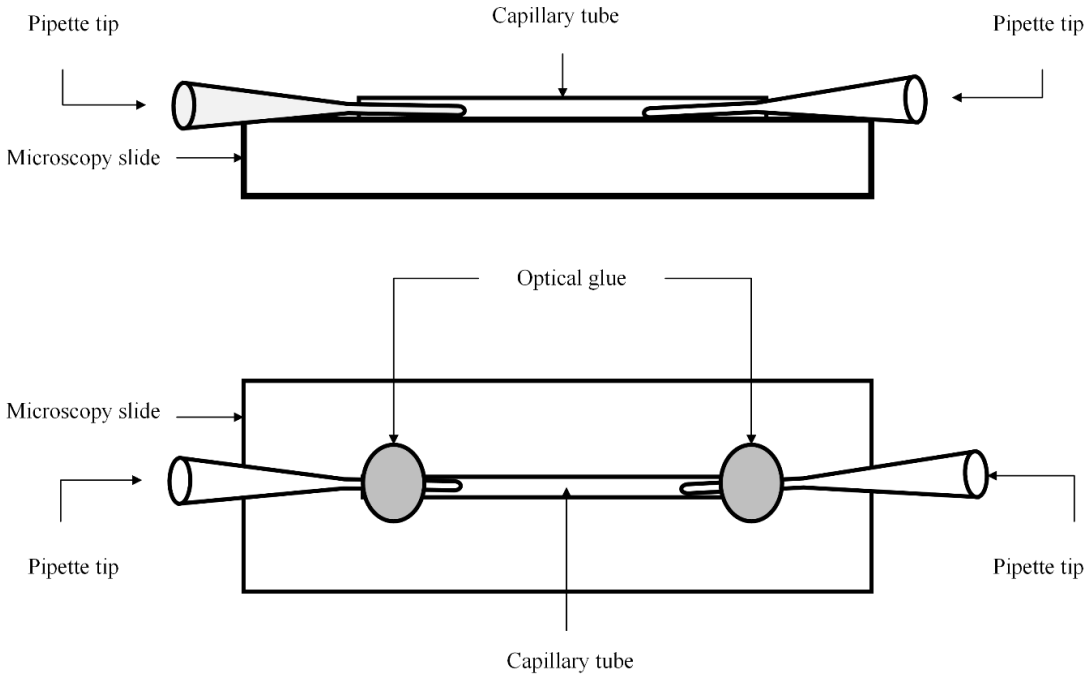

Figure 3. ***A microscope slide with a single channel.*** *A schematic image of a microscope slide with one microchannel. The first image shows a side view of the channel while the second shows the tope view of the channel.*

For the four-channel system, four glass capillaries were placed next to each other on a microscope slide. They were glued onto the slide using optical glue that was left to set under UV light for 15 minutes. Capillary pipette tips were adjusted in length and their surface was roughened up with sandpaper in order for the glue to adhere better. The pipette tips were then inserted into each capillary tube, one at each end, and glued in place. The glue was allowed to set for 2 hours under a UV light. Glue was then used to form a sort of pool around the middle of the four channels. This was to enable the column of water that was necessary for the microscope objective used, see Table 5 for details of the objective. Figure 4 show a schematic of the four-channel slide.

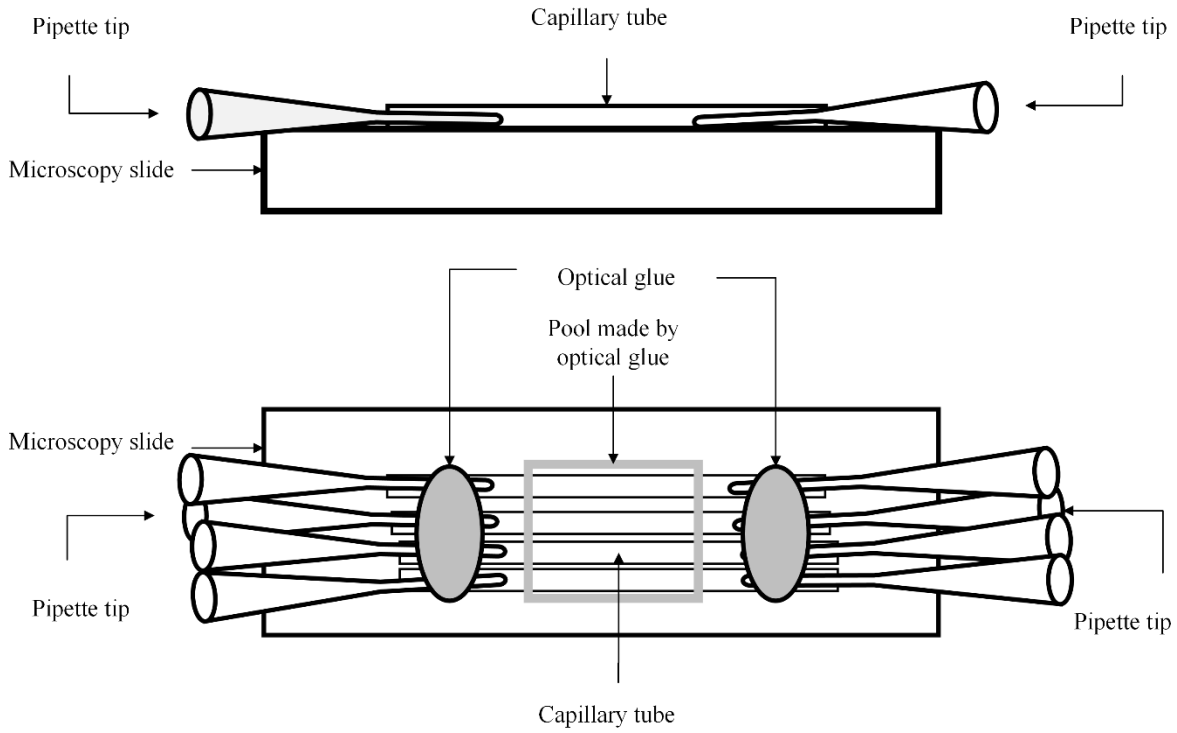

Figure 4. ***Microscope slide with four channels.*** *A schematic image of a microscope slide with four microchannels. The first image shows a side view of the channels while the second shows the tope view of the channels.*

2.5.2 Surface modification of the channels

The surface of the microchannel needed to be modified to ensure that the bacteria could adhere to the channel walls. Fimbriae on the cell's surface can bind to mannose and therefore the aim of the modifications is to coat the surface in mannose. Three different protocols were tested. All protocols were developed by the Lundgren group at the department of Chemistry and Molecular Biology at University of Gothenburg.

2.5.2.1 Ethanol protocol

20 ml syringes were used to wash through the channels and each side of the channel was washed twice with 3-5 ml of chemical. The channel was first washed with 2% Hellmanex before being rinsed with milliQ (MQ) water. Then 2 M H_2SO_4 was added and left to incubate for 30-60 minutes. The sulfuric

acid was rinsed with MQ water and the channel was washed with 99.5% ethanol. The ethanol was left to incubate for 5 minutes before 100 µl of 10% room temperature O-(Propargyloxy)-N-(triethoxysilylpropyl)urethane diluted in 99.5% ethanol was injected with a pipette in each side of the channel. The silane was left to incubate for 1 hour. The channel was then washed with 99.5% ethanol, MQ water and PBS buffer. A click reaction was then prepared. The click reaction was made by mixing 333 µl of 100 µM α-mannose PEG3 azide, 333 µl 750 µM THPTA (tris-hydroxypropyltriazolylmethylamine), 2.5 µl of 30 mM $CuSO_4$, 16.7 µl of 100 mM guanidine hydrochloride, and lastly 16.7 µl of 30 mM ascorbic acid. The volume was diluted to 1 ml with 1x PBS at pH 7.4. 200 µl click reaction was then injected in each end of the channel and left to incubate for 10 minutes before washing with MQ water. The channels were, if not used directly, stored on a plane surface in the fridge at 4°C with water in the channel and parafilm over the ends to prevent evaporation.

2.5.2.2 Methanol protocol

20 ml syringes were used to wash through the channels and each side of the channel was washed twice with 3-5 ml of chemical. The channel was first washed with 2% Hellmanex before being rinsed with MQ water. Then 2M H_2SO_4 was added and left to incubate for 30-60 minutes. The sulfuric acid was rinsed with MQ water and then washed with 99.9% methanol which was left to incubate for 5 minutes. 150 µl of 10% 3-Aminopropyldimethylethoxysilane diluted in methanol was added through one side of the channel with a pipette and left to incubate for 30-60 minutes. The channel was washed with 99.9% methanol with a 5-minute incubation before washing it with MQ water and PBS. 150 µl of 2.5% glutaraldehyde diluted in PBS was added with a pipette and was incubated for 15 minutes before rinsing with PBS. 50 µl of µg/ml of D-mannose-BSA was injected with a pipette through one side of the channel. It was incubated for 1 hour before injecting 150 µl of BSA with a pipette. The channels needed to incubate for at least 30 minutes before being used. If not used directly after 30 minutes, the ends of the channels were covered in parafilm and stored in the fridge with BSA (bovine serum albumin) until use.

2.5.2.3 PolyL-Lysine protocol

A click reaction mixture was prepared. First 100 µl of 0.8 mg/ml alkyne-BSA, with either 25 or 3 alkynes per BSA, was mixed with 32.5 µl of 1 mM azide-bound molecule (α-mannose PEG3 azide) in an Eppendorf tube. Then 250 µl of 750 µM of THPTA, 1.25 µl 30 mM $CuSO_4$, and 8.35 µl of 100 mM guanidine hydrochloride was mixed in a tube. These two solutions were mixed and 25 µl of PBS was added. Lastly, 83.3 µl of 30 mM ascorbic acid was added. The reaction was then added to filter tubes and centrifuged for 10 minutes at 5000 rpm. The flowthrough was discarded and 500 µl of PBS was added before centrifuging at the same conditions. Washing with PBS was done twice. 500 µl PBS was then added and pipetted up and down to collect the BSA-mannose from the filter before being moved to an Eppendorf tube. The channels were rinsed with MQ water. Then 2M H_2SO_4 was added and left to incubate for 30-60 minutes. The channels were washed with MQ water before 100 µl of 15 µg/ml PolyL-Lysine was added and left to incubate for 15-20 minutes. The BSA-mannose was then added and incubated for at least 30 minutes before use. This protocol was also done with store-bought BSA-mannose.

2.5.3 Microfluidics setup

The day before a microfluidics experiment ON cultures of the donor and recipient strain were prepared with LB and carbenicillin. To establish a protocol for a single-channel microfluidics system, both

conjugation pairs ELA1 and ELA3 as well as ELA8 and ELA9 were used. The only antibiotic used for the overnight cultures were carbenicillin, as the resistance is shared between the donor and recipient strains. The cells were grown in an incubator at approximately 32℃ to prevent the donor from aggregating. For the four-channel microfluidic system only ELA8 and ELA9 was used as donor and recipient, respectively. The growth conditions and antibiotics used were the same. Furthermore, for the four-channel system only the methanol protocol was used to modify the surface of the channels. Table 5Table 5 contains details about the microscope used, the accessories that were needed and the two different pumps used during the project.

Table 5. ***Details about the microscope setup.*** *Contains information about the pump, the microscope, and its accessories.*

Product	Model	Brand
Microscope	Axioskop 20	Zeiss
Objective	63x/1.0 VIS-IR water objective	Zeiss
Camera for microscope	Axiocam 305 colour	Zeiss
Heated microscope table	Axiovert 40 CFL	Zeiss
Pump 1	C20T-MAXI Peristaltic pump	Watson-Marlow Alitea
Pump 2	300 Syringe Pump	New Era Pump Systems, Inc.

2.5.3.1 Single-channel protocol

For the microfluidic set up the channel was placed on the microscope, which was equipped with a heating pad set to 37℃. The outlet tube was connected to a peristaltic pump (pump 1 in Table 5) and a falcon tube that collects the flowthrough waste. On the inlet side of there is a tube with LB or cell culture where suction allows flow through the channel. See Figure 5 for the setup.

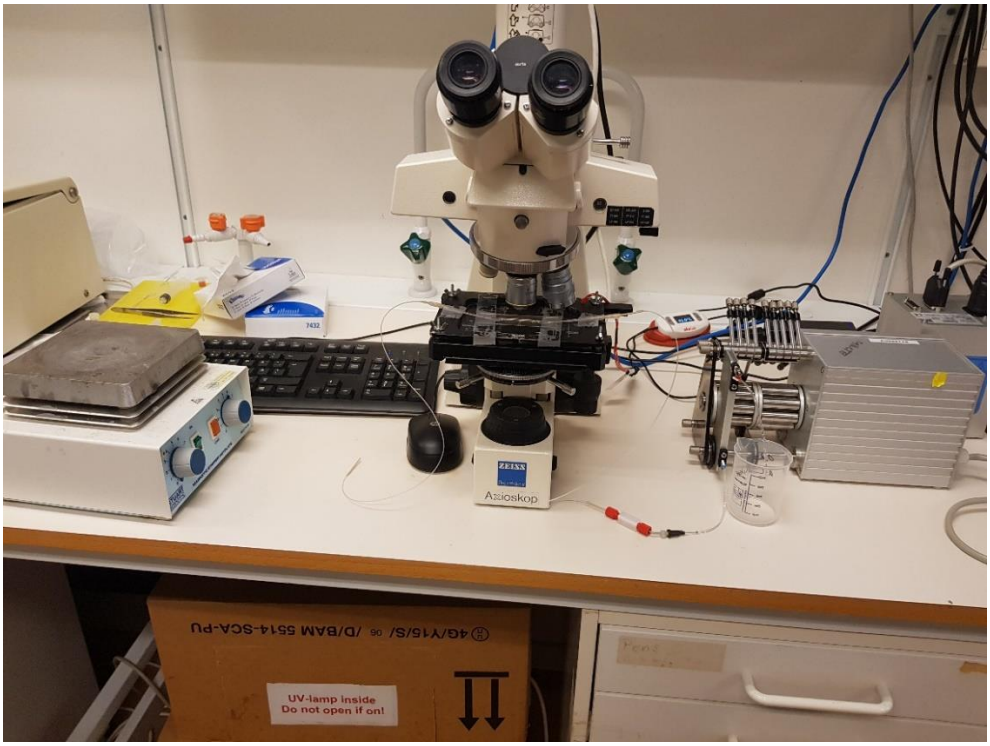

Figure 5. ***Single-channel microfluidics setup.*** *Shows the setup of a single-cannel microfluidics system. The peristaltic pump used can be seen on the right. The inlet tube (left) would be submerged in the liquid that is being flowed through the channel (e.g. LB, or cell culture) and the outlet tube (right) would be connected to a falcon tube for waste collection.*

First water was washed through the channel to ensure that the inlet tube and the channel itself were filled with liquid. Then LB media at approximately 37℃ was flowed through. The flow for the water and media was approximately 100 µl/min. During this the ON culture for the recipient was centrifuged at 4000 rpm for 2 minutes to get rid of any possible aggregates. The OD_{600} was measured and appropriate values were between 0.3 and 0.5. The recipient was then injected for 10-20 minutes at a flow of approximately 30 µl/min to allow the formation of an even coat of cells on the channel surface. When a sufficient number of recipients were on the surface the flow was increased to 100 µl/min and LB was flowed through for 20 minutes. This was done to allow the bacteria to adapt to the new environment and a temperature of 37℃. During this time the donor ON culture was prepared in the same manner as the recipient culture. It was then injected with a flow of 30 µl/min for 10-20 minutes to reach an adequate number of donors on the surface. After injection of the donors, LB was flowed through the system at 100 µl/min. The bacteria were then left to grow for 2 hours. The channel was recorded, red fluorescence images and brightfield images were taken every 20 minutes to monitor the growth. After 2 hours LB is switched for PBS that is flowed through the channel at 100 µl/min for a few minutes. After that the final brightfield, red fluorescence and green fluorescence images were taken. If too few bacteria adhered to the channel walls the experiment was cancelled after recipient or donor injection. After the experiment the channel was cleaned by first washing with 2% Hellmanex, then 95% ethanol and lastly water at a flow of 100 µl/min for approximately 10 minutes. After the experiment the channels are stored with 2% Hellmanex in room temperature and can be modified and reused for another experiment.

2.5.3.2 Four-channels protocol

For the microfluidic set up the channel was placed on the microscope, which was equipped with a heating pad set to 37℃. The syringe pump (pump 2 in Table 5) was placed on the inlet side of the channels. A splitter was connected to the syringe and split the flow into four tubes leading to the inlet of each channel. The outlet tubes were placed in falcon tubes for waste collection. See Figure 6 for the setup of the four-channel system.

Figure 6. ***Four-channel microfluidics setup.*** *Shows the setup of a four-cannel microfluidics system. The syringe pump used can be seen on the left. The syringe is connected to a splitter that splits the flow into four inlet tubes (left). The four outlet tubes (right) are connected to falcon tubes for waste collection.*

Water was washed through the four channels to ensure that the entire system was filled with liquid. The water was then switched for LB. The flow rate set on the pump was 400 µl/min, which results in a flow rate of 100 µl/min in each of the four channels. While LB was flowed through the channel the recipient culture was prepared by centrifugation at 4000 rpm for 2 minutes. This was done to try and remove bigger aggregates from the cell culture. The supernatant was later used for injection. The OD_{600} was measured before injection. The recipient was injected first into the channels with the flow rate at 120 µl/ml on the pump resulting in a flow of 30 µl/ml in each channel. The recipients were injected until an even layer covered the surface of the channel, usually for approximately 10 minutes, before the flow was increased to 100 µl/min per channel and switched to LB. While LB flowed through the system the donor culture was prepared in the same manner as the recipients. The donor culture was then injected at a flow rate of 30 µl/min in each channel. After injection of the donors, LB was flowed through each channel at 100 µl/min. The bacteria were left to grow and conjugate for 2 hours. One of the four channels was recorded. Red fluorescence images and brightfield images were taken of all four channels every 20 minutes to monitor the growth. After 2 hours PBS was flowed through the channel instead of LB at 100 µl/min for a few minutes. After that the final brightfield, red fluorescence and green fluorescence images of each channel were taken. After the experiment the channel was cleaned by first washing with 2% Hellmanex, then 95% ethanol and lastly water at 100 µl/min for approximately 10 minutes. The channels are stored with 2% Hellmanex in room temperature after the experiment and can then be modified and reused for another experiment.

3 Results

3.1 Conjugation assays

3.1.1 The effect of pentanol on conjugation

To investigate the connection between the fluidity of the cell membrane and conjugation a liquid mating assay was performed with the membrane softener pentanol (25). For this mating the donor HA14 and the recipient HA4 was used. Pentanol was added to the donor cultures during growth in different concentrations. First 20 mM, 30 mM, 50 mM, and 70 mM pentanol was added and a control with no pentanol. However, all pentanol concentrations except 20 mM were too high and caused cell death. Therefore, the concentrations were adjusted. The final concentrations that were investigated were 1 mM, 5 mM, 10 mM, 15 mM, 20 mM pentanol and a control without pentanol. When performing the mating equal amounts of donor and recipient cultures were mixed. As that would have diluted the pentanol concentrations, considering that the recipients did not grow in presence of pentanol, pentanol was added to ensure that the mating mixture had the same pentanol concentrations as the donor cultures.

Figure 7 shows the combined normalised transfer efficiency for the two liquid mating assays. The error bars show the standard deviation between biological replicates for each concentration. Note that four biological replicates were used for pentanol concentration 0 M to 10 mM. For 15 mM pentanol three biological replicates were used as the fourth replicate did not grow. In the case of 20 mM, three out of four biological replicates did not grow and only one is displayed in Figure 7. 100 % indicates the transfer frequency of the control, 0 M pentanol. Note that the y-axis has a log scale. Figure 7 shows a trend where an increasing concentration of pentanol corresponds to lower transfer frequencies, except for 15 mM where a slight increase can be seen. The significance of these differences was calculated with a Student's t-test where each pentanol concentration was compared to the control with 0 M pentanol. For every concentration except 1 mM, there is a significant difference in transfer frequency with p-values all below 0.05. In the case of 1 mM the p-value was approximately 0.44. Regarding 20 mM, the t-test cannot be used as there is only one biological replicate. The results indicate that the addition of pentanol influences conjugation efficiency.

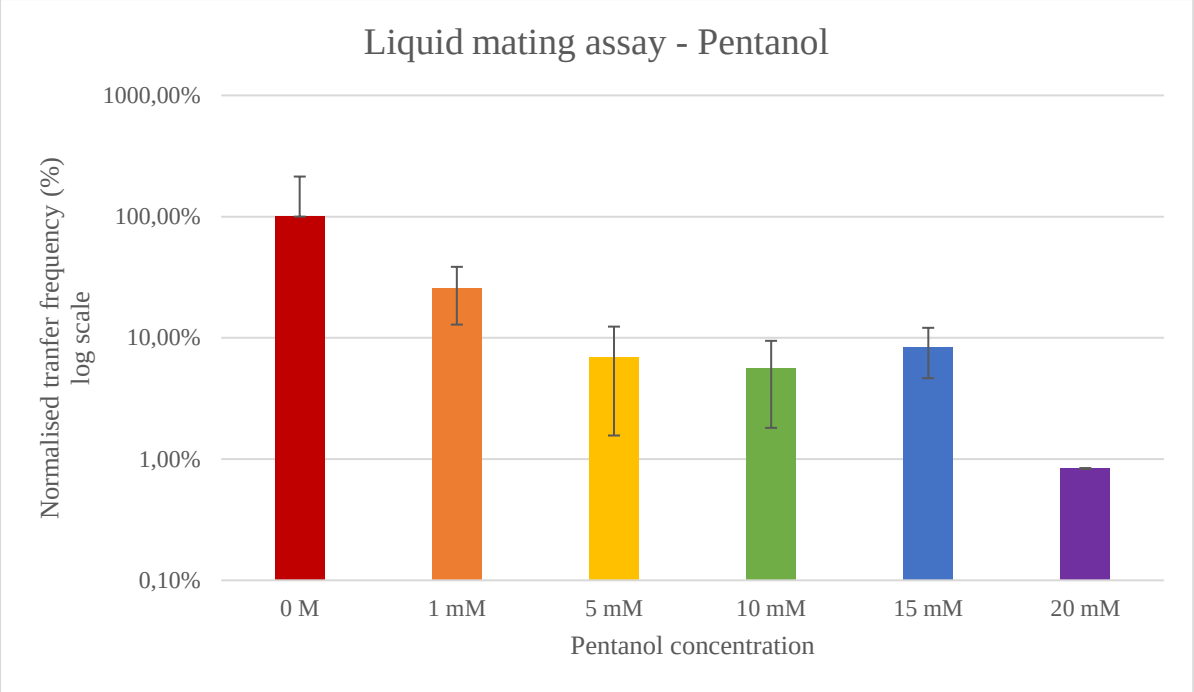

Figure 7. ***Pentanol decrease conjugation efficiency.*** *Shows the combined normalised transfer frequency of two liquid mating assay were pentanol was added to the donor cultures at 1 mM, 5 mM, 10 mM, 15 mM, and 20 mM. A control without any pentanol (0 M) is also shown.*

3.1.2 The effect of donors with single gene deletions on conjugation

To further investigate how the cell membrane influences conjugation a second liquid mating assay was performed with six different mutant strains acting as the donors. The mutants have one gene removed that has been replaced with a kanamycin cassette. The different mutant strains were *ΔfabH*, *ΔfabF*, *ΔplsX*, *ΔrfaD*, *ΔwzzE*, and *ΔargC*, where *ΔargC* (HA14) acted as a control donor. The recipient was HA4, which has resistance against chloramphenicol.

The combined normalised transfer frequency of the mutant strains from two liquid mating assays can be seen in Figure 8**.** Each mating had two biological replicates per donor, meaning that four biological replicates in total are included in the figure. The error bars show the standard deviation for the biological replicates and 100 % indicates the control's transfer frequency. *ΔfabH* and *ΔplsX* show transfer frequencies that are similar to the control *ΔargC. ΔfabF* and *ΔwzzE* show a decrease in the transfer frequency. *ΔrfaD* shows a prominent decrease in transfer frequency compared to the control. However, when the statistical significance was calculated using a Student's t-test, neither of the mutant donors showed a significant difference to the control with a cut-off of 0.05.

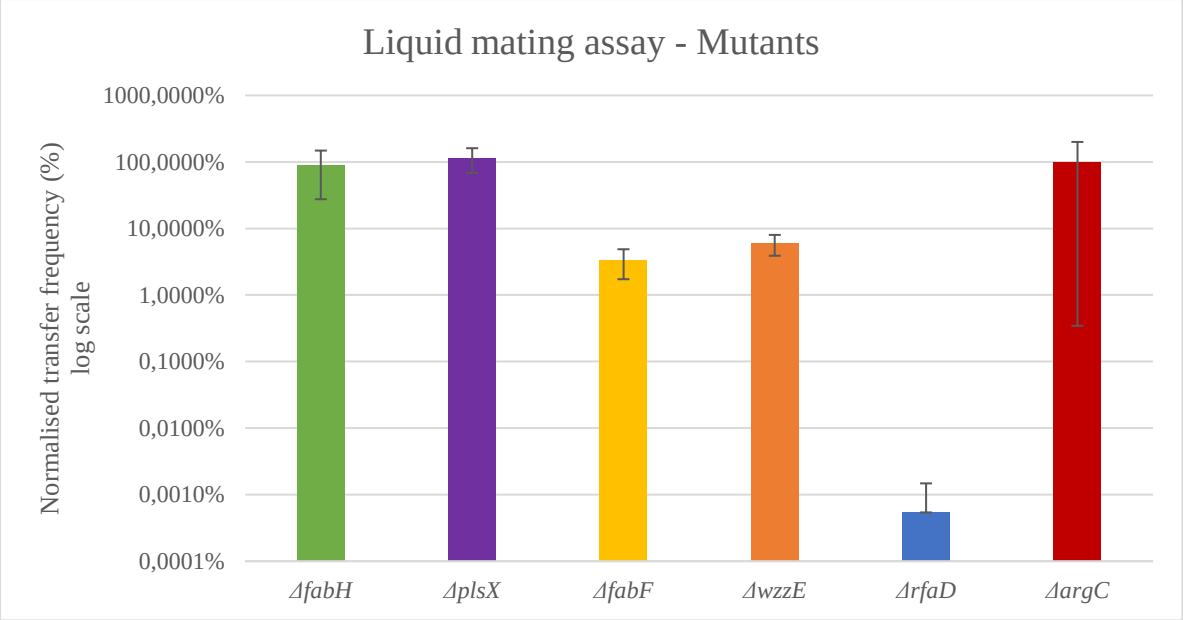

Figure 8. ***Five single gene deletions effect on transfer frequency in liquid mating assays.*** *Shows the combined normalised transfer frequency for the two liquid mating assays where the five donors had single gene deletions that affect membrane components in* E. coli. *The mutations investigated were* ΔfabH, ΔplsX, ΔfabF, ΔwzzE, ΔrfaD, *and the control* ΔargC.

3.1.3 Solid mating assay with *ΔfabF* and *ΔrfaD*

A solid mating assay was performed with *ΔfabF* and *ΔrfaD* as these showed the biggest effect on conjugation in the liquid mating assay. This was done to get further insight in how these mutations affect conjugation. It is known that mutants with affected mating pair stabilisation can mate on solid media but not as well in liquid (11). The same control donor as for the liquid mating assay with the mutants was used and HA4 was the recipient strain. The donor and control donor strains are resistant to kanamycin and tetracycline while the recipient is resistant to chloramphenicol.

Figure 9 shows the combined normalised transfer frequency for the two solid mating assays. The error bars show the standard deviation for the biological replicates. *ΔrfaD* has a very low transfer frequency indicating that almost no conjugation takes place. The transfer frequency for *ΔfabF* is lower than the control donor, but not as large as for *ΔrfaD*. A Student's t-test was used to calculate the significance and showed that the difference between *ΔfabF* and the control *ΔargC* is not significant, but the difference between *ΔrfaD* and *ΔargC* is significant with a p-value of approximately 0.049.

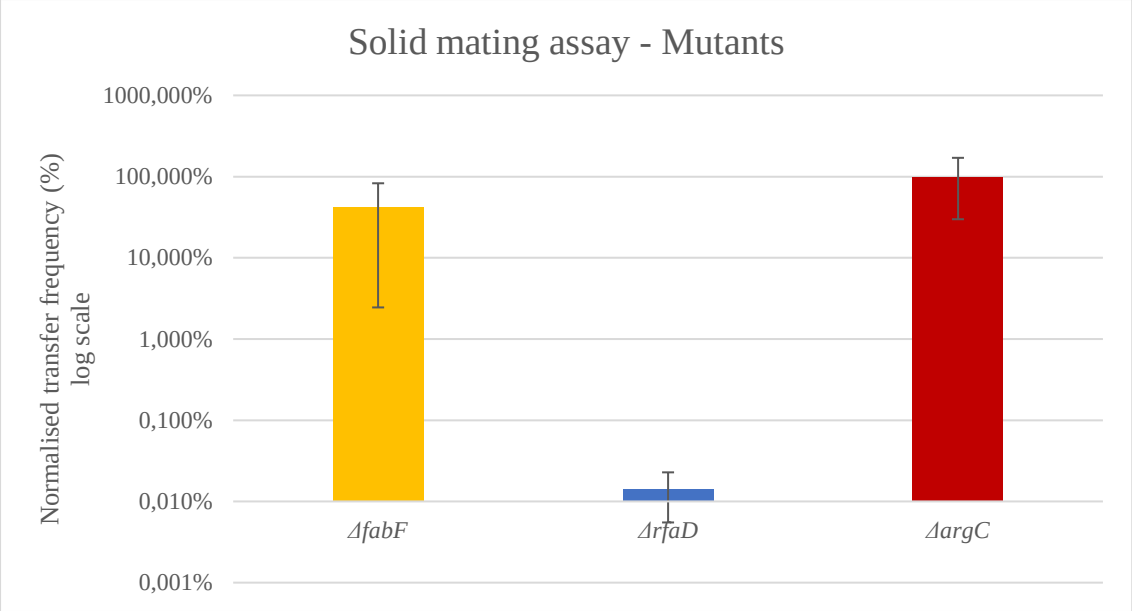

Figure 9. ***Two single gene deletions effect on transfer frequency in solid mating assays.*** *Shows the combined normalised transfer frequency for the solid mating assays where two donors had single gene deletions that affect membrane components in* E. coli. *The mutations investigated were* ΔfabF, ΔrfaD, *and the control* ΔargC.

3.1.4 The effect of fimbriae on conjugation

For the experiments where microfluidics was used to investigate conjugation on a solid surface both the donor strains and recipient strain have the plasmid pPKL91 that overexpress fimbriae. This is due to fimbriae being necessary for the bacteria to be able to adhere to the modified surface of the channel. To determine if fimbriae expression has any effect on conjugation a liquid mating assay was performed. Three different donors were used for this experiment. The first donor was ELA1 with pPKL91. The second donor (ELA2) was a MG1655 with a *fimA* deletion. This mutation prevents fimbriae formation. The third donor acted as a control and was a MG1655 with the gene *xylA* deleted and with kanamycin resistance cassette taking its place. The donors were then ELA1 that overexpress fimbriae, ELA2 with no fimbriae, and MG1655 *ΔxylA::*Kan that has wild type expression of fimbriae. The recipient used was ELA3.

Figure 10 shows the combined normalised conjugation frequency for the two liquid mating assays where the effect of fimbriae on conjugation was investigated. The control with wildtype fimbriae expression, MG1655 *ΔxylA*, was used for normalisation. The error bars show the standard deviation for the biological replicates. Each donor had four biological replicates and two technical replicates were performed for each mating. The transfer frequency for the donor that overexpress fimbriae (ELA1) is similar to the control. Overexpression of fimbriae do not seem to have any effect on transfer frequency. In the case of the *fimA*-mutant (ELA2), there is a slight decrease in transfer frequency. A Student's t-test was performed to see whether the difference in transfer frequency was significant between ELA1 and ELA2, respectively, and the control with wildtype fimbriae expression. The difference was not significant in either case. However, while the combined transfer frequencies for the two mating assays show no major difference between the three donors, the frequencies varied between the individual mating assays and therefore, it would be good to further investigate this.

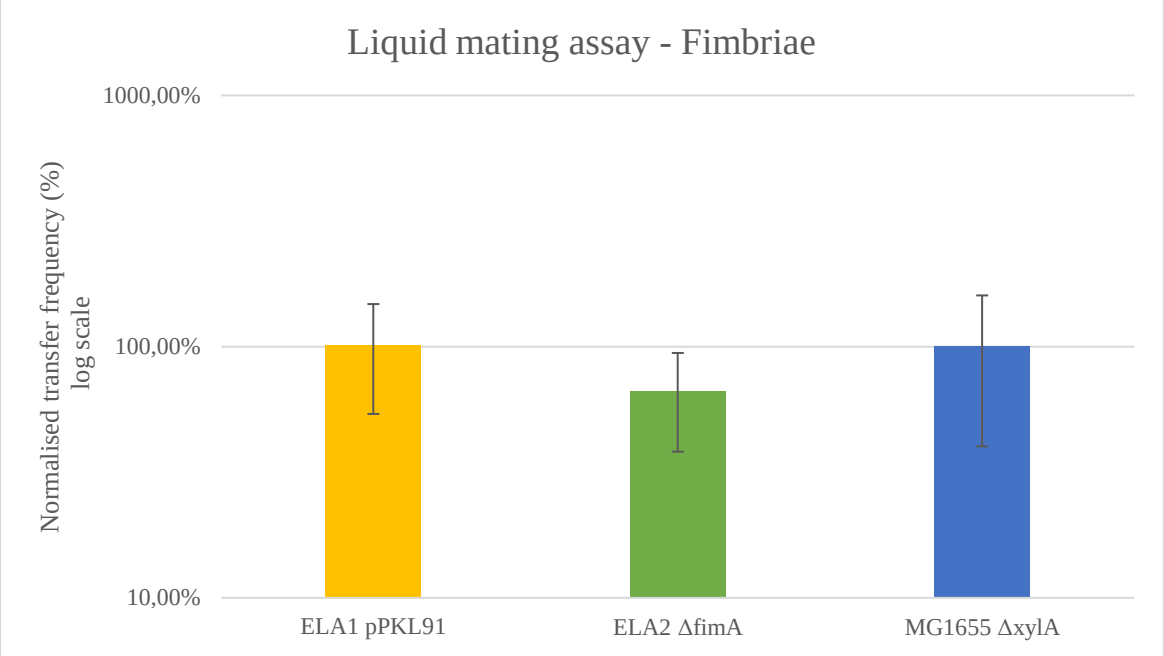

Figure 10. ***Fimbriae expression has no major effect on transfer frequency.*** *Shows the combined normalised transfer frequency for the liquid mating assays performed to investigate the effect of fimbriae expression on conjugation. The donors used are MG1655 with pPKL91 (ELA1), MG1655 with a fimA deletion (ELA2), and MG1655 ΔxylA::Kan and the recipient MG1655 with pPKL91 and pEB2. All donors have the same F-plasmid pLP8.*

3.1.5 Contaminations in mating assays

In three of the mating assays performed colonies grew for the negative control, indicating contamination. However, for each of these cases the number of colonies were small and countable of between 10-40 colonies. For calculations of the number of donors or transconjugants in the 200 µl aliquoted in the 96-well plates no colony counts above a dilution of 10^{-2} was used. That would mean that 0.1-0.4, and in most cases less as the more diluted samples were often used for calculations, colonies would be due to contamination. As these numbers are small the effect on the results is likely insignificant. The only exception to this is *ΔrfaD* in the solid mating assays as approximately 30 colonies in the undiluted sample was observed in the case of contamination. Contaminations would then account for approximately all colonies of the undiluted *ΔrfaD* sample meaning that *ΔrfaD* would cause complete inhibition of conjugation.

3.2 Microfluidics

Microfluidics experiments were performed to investigate conjugation of the two mutant strains *Δlpp* and *ΔrseA* on solid surfaces. *Δlpp* mutants were previously shown to mate well on solid surfaces but not in liquid, but *ΔrseA* is expected to be poor in both conditions (11). Different surface modification protocols were tested to achieve satisfactory adhesion of cells to the walls of the channel. A microfluidic protocol for a single-channel system was modified from an existing protocol to suit the strains and aim of this project. The single-channel system was then used to develop a four-channel system that can be used for the study of conjugation on solid surfaces.

3.2.1 Conjugation pairs for microfluidics experiments

Two different conjugation pairs were made for the single-channel microfluidic experiments. The first conjugation pair with ELA1 and ELA3 had a donor with GFP, a recipient with the RFP mScarlet, and transconjugant that with both GFP and mScarlet. The problem with this set up was that mScarlet and GFP have an overlap in the excitation wavelength. The recipient ELA3 thereby showed both red and green fluorescence. Consequently, it was difficult to separate the recipient and the transconjugant, which had not been producing GFP in a large enough amount to enable distinction between the two. Therefore, the second conjugation pair was created. The donor ELA8 have both GFP and mScarlet, the recipient has no fluorescent marker, and the transconjugant receives a GFP upon conjugation. The initial test showed that this set up was better suited for the microfluidics experiment.

3.2.2 Surface modification protocols

Conjugation is a contact dependent mechanism which implies that the surface in the channel needs to be evenly covered with donor and recipient cells in order for them to establish contact and conjugate. Three different protocols for surface modifications of the channels were tested to establish which protocol provided the best cell adhesion (described in section 2.5.2). Figure 11, ethanol and methanol protocol, and Figure 12, variants of PolyL-Lysine protocols,Figure 12 shows the average injections of donor and recipient cells for the different surface modifications.

In Figure 11 a comparison of the number of cells that attach to the surface of the channel can be seen for the surface modification protocols using ethanol, A and BFigure 11, and methanol, C and DFigure 11. In A and Figure 11C only the donor cells have been injected. In B and D both donor and recipient cells have been injected. Regarding the ethanol protocol the number of cells after injection of the donors is not satisfactory. After the recipients have been injected the number of cells on the surface seems to have decreased. This indicates that not only do the cells not attach in the degree that is required for this type of experiment, but they also fall off the surface and thereby the number of cells on the surface is decreasing over time. Furthermore, most of the cells that are present in Figure 11 Figure 11B can also be seen in Figure 11Figure 11 A indicating that these cells are donors and very few recipient cells have attached to the surface. Therefore, conjugation is unlikely to happen as there are too few cells present in the channel, and out of these cells few are recipients. In addition, the distance between the cells is large and as conjugation is a contact dependent process it is unlikely to occur.

Figure 11 C and D, where C has only donor cells and D both donor and recipient cells, depicts a channel that has been modified with the methanol protocol. Out of the different protocol used to modify the surface of the channel, the methanol protocol shows the highest number of attached cells. Furthermore, comparing C and D, the number of cells increased after injection of recipient cells. This indicates that not only do both cell types adhere to the channel, but the cells do not seem to be falling off in the same degree as for the ethanol protocol. As the methanol protocol showed the best results it was used for the remaining experiments in the project.

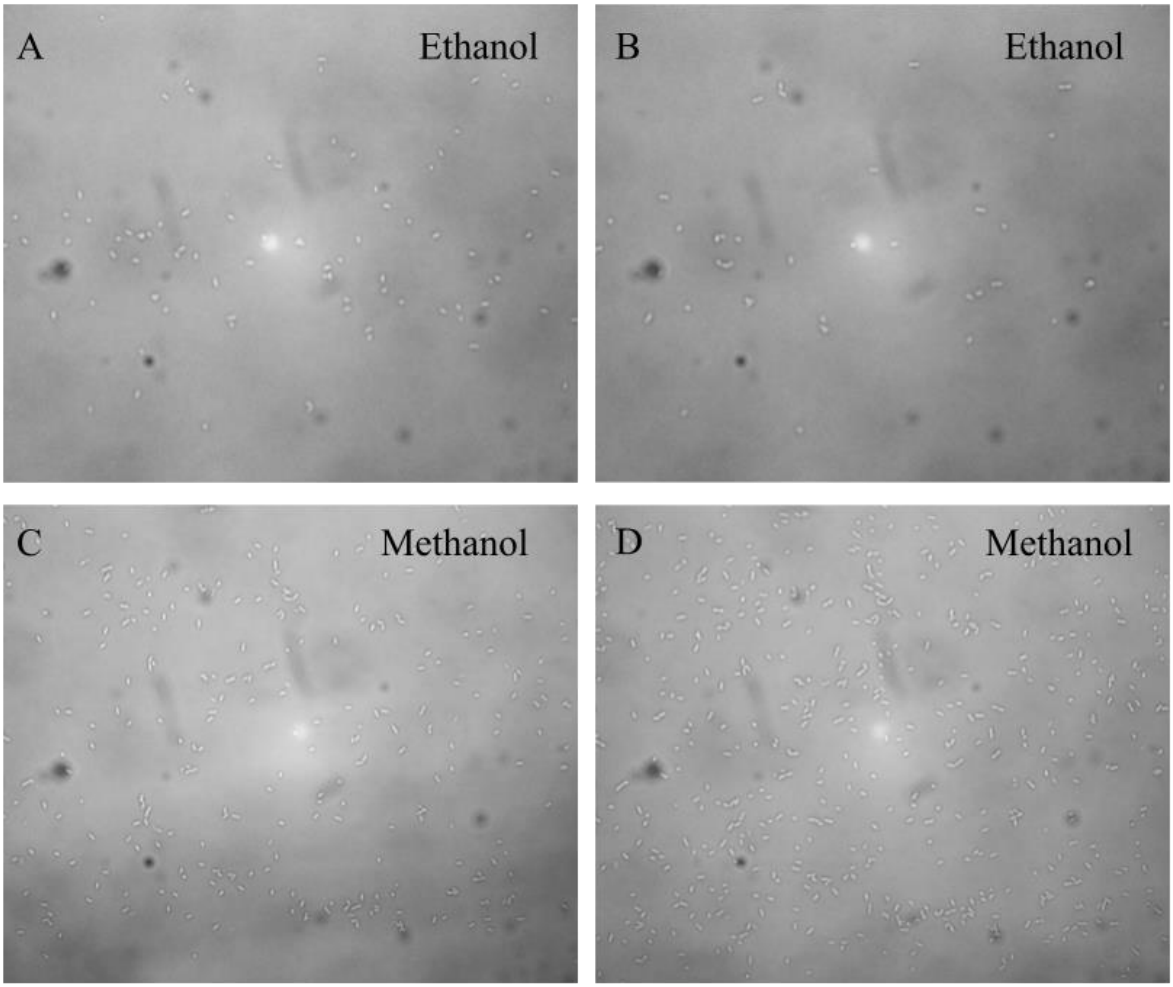

Figure 11. ***Comparison of cell adhesion with the ethanol and methanol surface modification protocols.*** *Shows the surface of the channel in two single-channel microfluidics experiments after injection of cells. In A and B, the channel has been modified with the ethanol protocol. In A, only donor cells have been injected, in B both donors and recipient are injected. For C and D, the methanol protocol has been used to modify the channel, C shows only donor cells while D shows both injected donor and recipient cells.*

For all variants of the PolyL-Lysine protocol, that can be seen in Figure 12, the number of cells that adhere to the surface of the channel is limited. In addition, no major difference in the number of cells when comparing only donor cells injected (A, C and E) to when both cell types have been injected (B and D) can be seen.

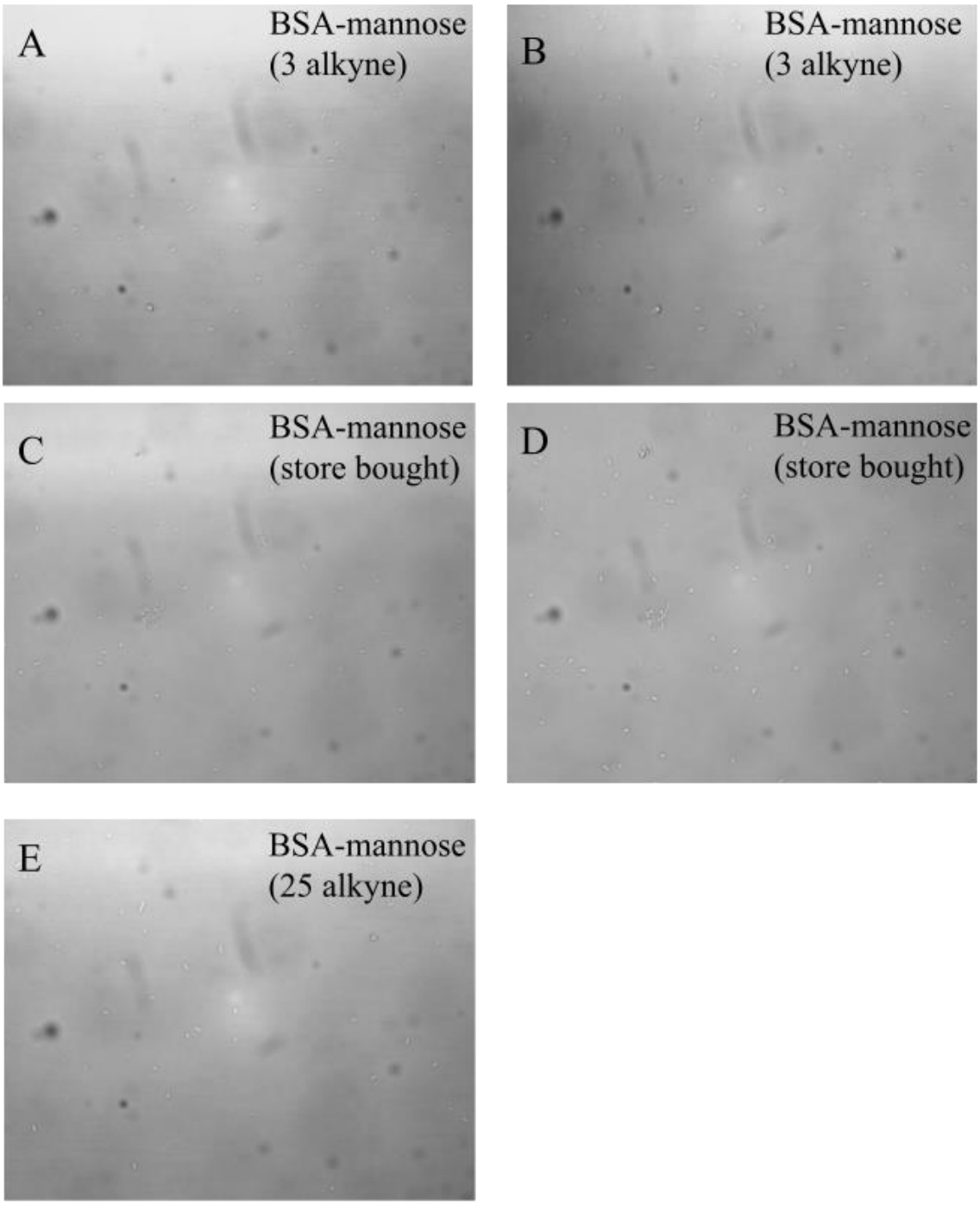

Figure 12. ***Comparison of cell adhesion with the different variant of the PolyL-Lysine surface modification protocol.*** *Shows the surface of the channel in two single-channel microfluidics experiments after injection of cells. In A and B, 3 alkyne BSA was used to make BSA-mannose. C and D used store bought BSA-mannose, in E 25 alkyne BSA was used o make BSA-mannose. The first column shows only donors, in the second column both donors and recipients have been injected.*

In conclusion, the ethanol protocol and the PolyL-Lysine protocol achieves approximately the same number of cells that adhere to the channel's wall. The surfaces that are modified using the methanol protocol have the best adhesion ability. Therefore, the methanol protocol was used for the remaining experiments.

3.2.3 Single-channel system

Microfluidics experiments were performed to investigate conjugation of the two mutant strains *Δlpp* and *ΔrseA* on solid surfaces. In order to do this a single-channel system was first evaluated to get a functioning protocol that could be scaled up to a four-channel system. For the single-channel system the donor ELA8 and the recipient ELA9 were used, where ELA8 has the fimbriae plasmid pPKL91, pEB2 for expression of mScarlet and the F plasmid with a GFP, and ELA9 only has pPKL91. The order of injection into the channel was first investigated. Table 6 contains the cell counts for two experiments where the donor culture was injected first in one experiment and recipient cells were injected first in the other. It also contains the percentage of donors and recipients for easy comparison. For each of these experiments the majority of the cells precent on the surface are of the type of which was injected first. Figure 13 shows the surface of the channel after injection of both cell types from the same two experiments. Figure 13 A and B come from an experiment where donors were injected into the channel first, then followed by recipients. In Figure 13 C and D another experiment is shown where the recipients were injected first and the donors were injected second. B and D show the red fluorescence image where only donors are visible for the two experiments, respectively. There is an obvious difference between the number of donors depicted inFigure 13 B and D.

Table 6. ***Comparison of the number of donors vs. recipients on a surface after cell injection.*** *Contains the number of donor and recipient cells present on the channel surface for two different experiments where the donor cells were injected first and the recipients were injected first, respectively. See Figure 13.* ***Comparison of the number of donors vs. recipients on a surface after cell injection.*** *Shows the surface right after injection of both donor and recipient cells for two different single-channel microfluidics experiments after injection of cells. In experiment 1, A and B, donor cells were injected first. A show the brightfield image of all cells precent on the surface. B shows the red fluorescence image and only depicts donors expressing the RFP mScarlet. In experiment 2, C and D, the recipients were injected first. C is a brightfield image and shows all cells present in the channel. D is the red fluorescence image of the same cells but only depicts the donors as these express mScarlet. The cells have been counted and the counts are presented in Table 6Table 6.Figure 13 for images of the surface for these two experiments after injection cells.*

	Donors	Recipient
Donor injected first	235 (88 %)	33 (12 %)
Recipient injected first	96 (25 %)	283 (75 %)

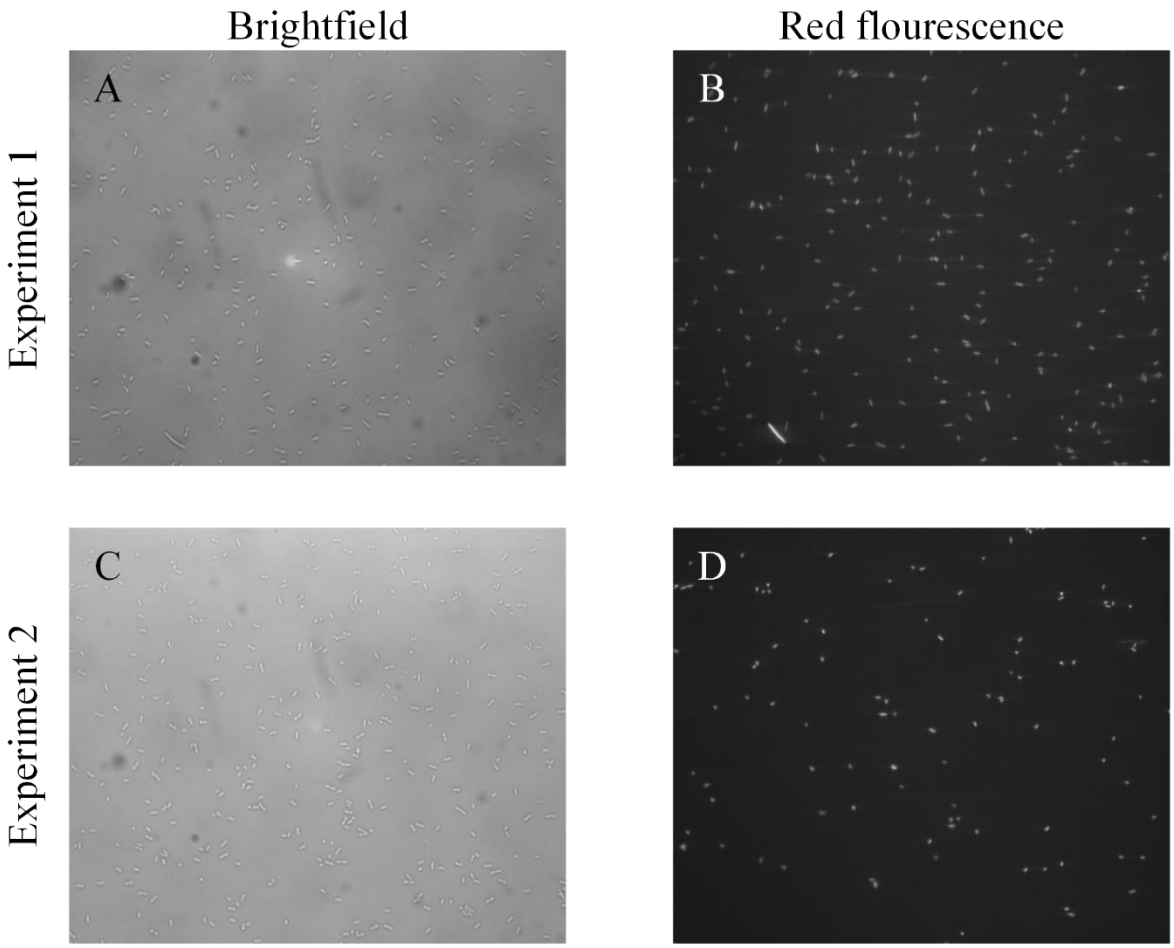

Figure 13. ***Comparison of the number of donors vs. recipients on a surface after cell injection.*** *Shows the surface right after injection of both donor and recipient cells for two different single-channel microfluidics experiments after injection of cells. In experiment 1, A and B, donor cells were injected first. A show the brightfield image of all cells precent on the surface. B shows the red fluorescence image and only depicts donors expressing the RFP mScarlet. In experiment 2, C and D, the recipients were injected first. C is a brightfield image and shows all cells present in the channel. D is the red fluorescence image of the same cells but only depicts the donors as these express mScarlet. The cells have been counted and the counts are presented in Table 6Table 6.*

The images in Figure 14 shows the surface of the channel at the end of the experiments two hours after the start and are from the same experiments seen in Figure 13. The first column shows the experiment where the donors was injected first, and the second column show the experiment where the recipients were injected first. The rows show a brightfield image, a red fluorescence image, a green fluorescence image, and the same green fluorescence image where transconjugants have been identified. Transconjugants were identified based on fluorescence. The donor has both an RFP and a GFP, the recipients have no fluorescent protein, and the transconjugant will have a GFP. Cells that show green fluorescence, but not red fluorescence, are counted as transconjugants.

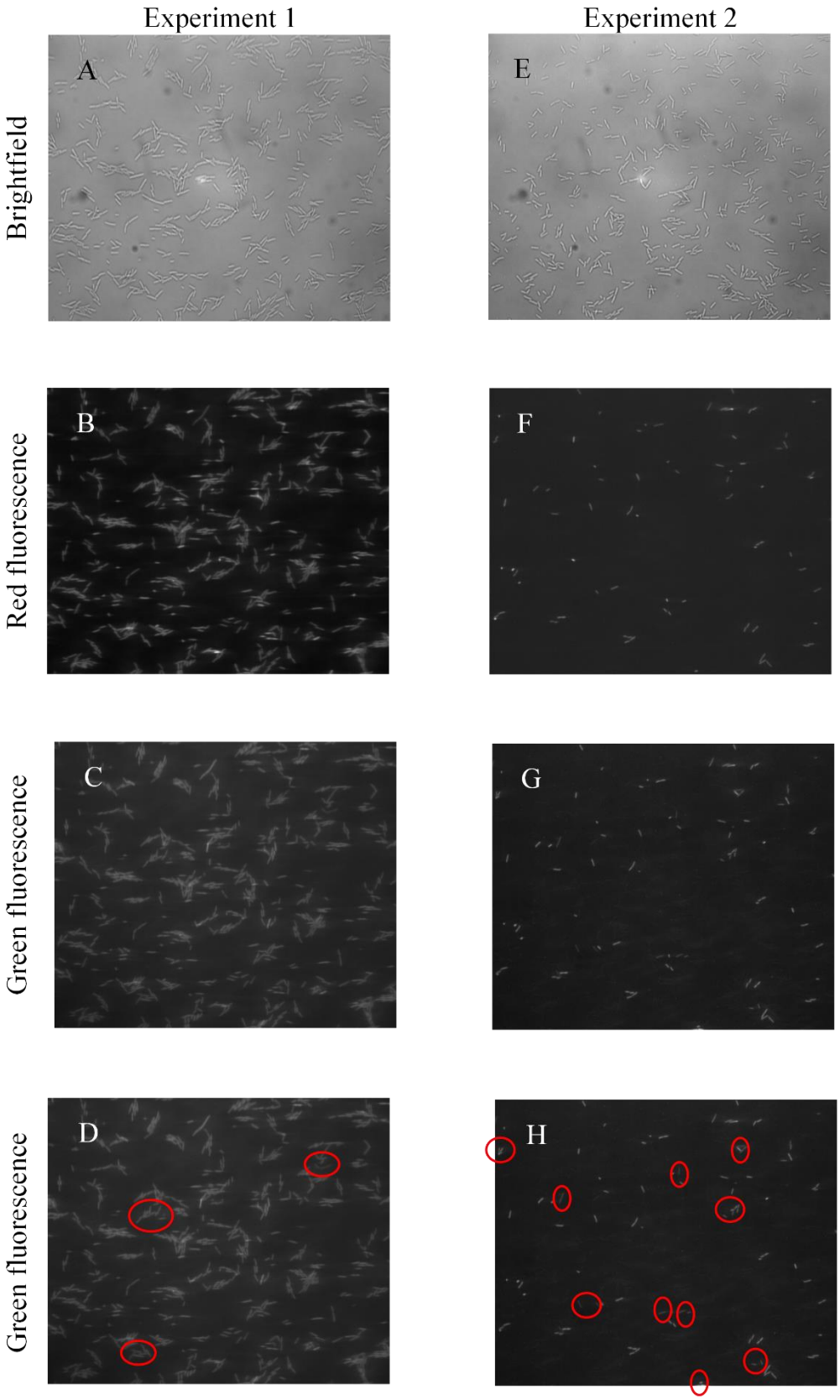

Figure 14. ***Comparison of the number of donors, recipients and transconjugant between two experiments.*** *Shows the surface of the channel in two single-channel microfluidics experiments at the end of the experiments (2 hours from start). In experiment 1 the donor cells were injected first and in experiment 2 the recipient cells were injected first. The images are, top to bottom, the brightfield image where all cell types are visible, red fluorescence image where only donors are visible, green fluorescence image where donors and transconjugants are visible, and a green fluorescence image where transconjugants have been identified.*

3.2.4 Creating mutant cells expressing red fluorescent protein

To enable investigation of *ΔarcA, ΔargC, Δlpp* or *ΔrseA* the cells needed to express an RFP. Three different protocols were tested to achieve red fluorescent donor strains, but none of the protocols resulted in red colonies. The first protocol that utilised a λ-RED system seemed to work as bacteria grew on streptomycin plates, which indicates incorporation of the PCR product. However, when examining the cells under the microscope, no expression of RFP could be seen. For the second protocol the kanamycin resistance marker needed to be recombined out, but no colony passed the screening step despite being performed multiple times. Kanamycin was present in all colonies and only pCP20 had been lost. The third protocol relying only on screening for the correct strain through colour did not give rise to any red colonies.

3.2.5 Four channel system

While the conjugation with mutants *ΔarcA, ΔargC, Δlpp* or *ΔrseA* as donors could not be investigated due to difficulties with fluorescent markers, a four-channel microfluidic system was still developed to make future analysis of them easier. The same donor and recipient as for the single channel experiments, ELA8 and ELA9, were used for the four-channel experiments. Figure 15 shows the surface of the four channels where the rows correspond to the different channels. In the first column only recipients have been injected and in the second column both cell types have been injected. The adherence of cells to the channel surface varies between the four channels. Channel one, A and B, has the highest number of cells that have stuck to the surface. There also seems to be an increase in the number of cells if one compares recipients only (A) to the image with both recipients and donors (B). The second channel has fewer cells, but still a sufficient number. As with the first channel, an increase in the number of cells can observed between only recipients and both cell types in C and D, respectively. In channel two other structures can also be seen. These are located under the channel and not inside. In the third channel few bacteria are present. Furthermore, neither recipients nor donors seem to adhere to the surface. In the last channel few recipients stick to the surface, Figure 15 G. However, there is an increase in the number of cells after injection of donors meaning that these cells can bind to the surface (Figure 15 H). Unfortunately, no transconjugants were seen for any channel at the end of the experiment.

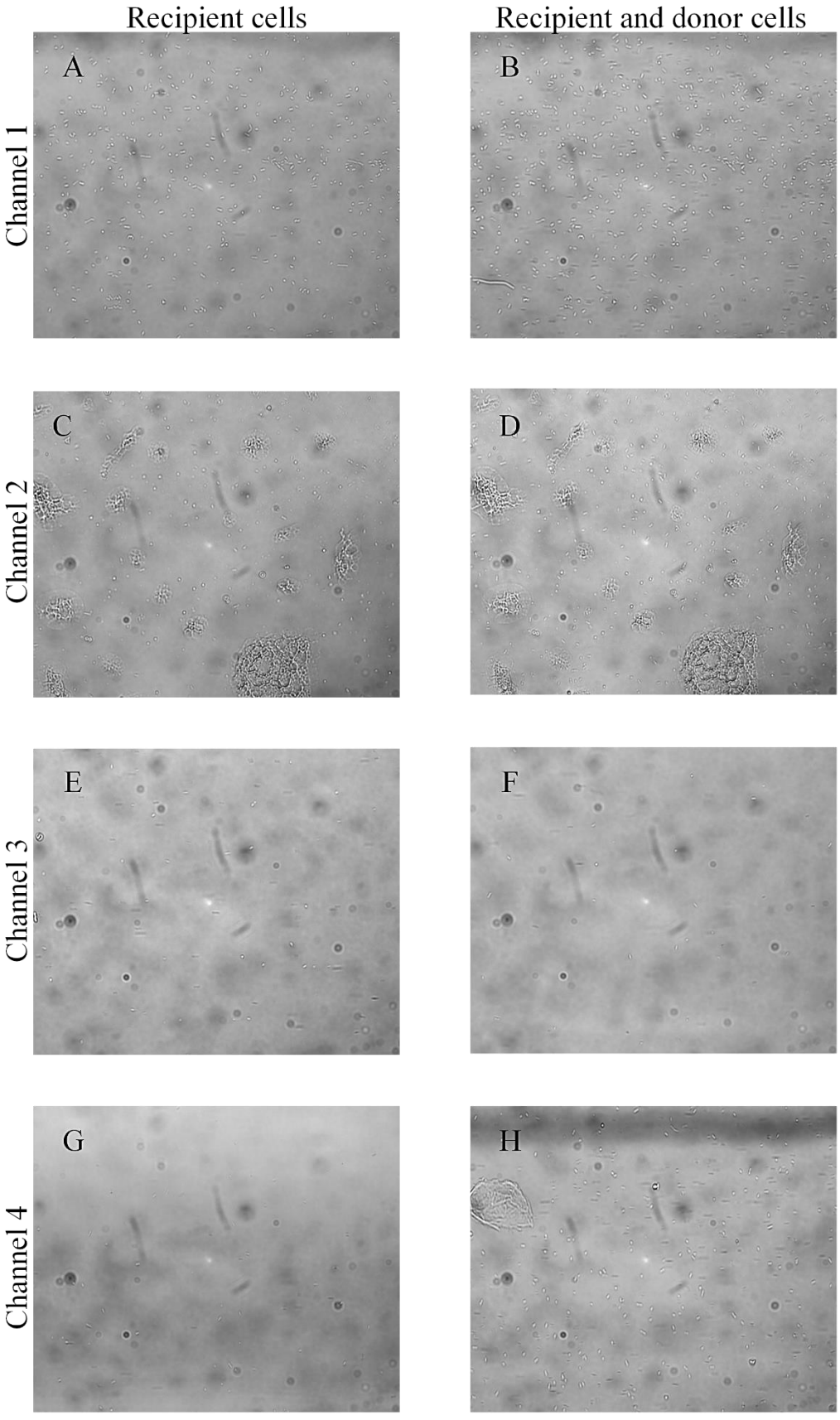

Figure 15. ***Comparison of the number of donors and recipients for a four-channel system after injection of cells.*** *Shows brightfield images of the surface of the four channels after injection of recipients, first column, and both recipients and donors, second column. The first row corresponds to the first channel, the second row to the second channel, and so on.*

4 Discussion

4.1 Conjugation assays

4.1.1 The effect of altered membrane fluidity on transfer frequency

In Figure 7 the effect of an increasing concentration of pentanol on conjugation can be seen. There is a trend that higher concentrations of pentanol lead to a lower transfer frequency. Membrane fluidity is altered with the addition of pentanol, and a higher concentration leads to a more fluid membrane (25). The results indicate that membrane fluidity influences conjugation. It is possible that the increased fluidity prevents correct incorporation of proteins into the membrane or other components of the conjugation mechanism that are necessary for efficient transfer.

While the results strongly indicate that membrane fluidity impacts conjugation it is also possible that there is another effect of pentanol that affect the transfer efficiency, especially for the higher concentrations. At high concentrations of pentanol the cell growth is greatly affected indicating that the cells are negatively affected. Before the donor and recipient cultures were mixed in equal amounts, pentanol was added to the recipients as to not change the concentration during the mating. It is possible that some recipient cells became nonviable when the higher concentrations of pentanol, mainly 20 mM but to a certain degree also 15 mM, was added. If the concentration of recipients decreased, the transfer efficiency would be lower as there are fewer recipient cells that can conjugate. For future investigations of the effect of pentanol on conjugation it would be advisable to measure OD_{600} after the addition of pentanol to the recipients to ensure that the cells do not die. Furthermore, other membrane softeners could be examined to determine if the effect on conjugation is due to pentanol or the altered membrane fluidity. It would also be of interest to investigate to what degree the fluidity has been altered for the different concentrations of pentanol. Performing solid mating assays with pentanol could also give an insight in how membrane fluidity affects mating pair stabilisation in conjugation. Furthermore, it would be of interest to investigate compounds that decrease the fluidity in the membrane in order to see whether it would have a similar effect on conjugation.

4.1.2 The effect of single gene deletions on transfer frequency

None of the mutant donors showed a significant decrease in transfer frequency compared to the control in the liquid mating assays according to the calculated t-test. The p-values were all above the cut-off of 0.05 with the p-values for *ΔfabF, ΔwzzE,* and *ΔrfaD* around 0.15. Effects in conjugation efficiency can be observed in Figure 8, however, due to the small set of data this is not supported by the statistics. As the sample population is small, variations between the data points have a large impact on the result. This is likely the case for the liquid mating assay with mutant donors. To get higher confidence in the results, more mating assays need to be performed to increase the statistical power, not only for the liquid mating assay with mutant donors but also for the other mating assays performed in this project.

The liquid mating assay *ΔfabH* and *ΔplsX* show transfer frequencies that are similar to that of the control *ΔargC* in Figure 8, with *ΔfabH* having the lowest transfer frequency out of the three at around 90%. A possible reason for this small decrease could be that unsaturated fatty acids are known to inhibit conjugation and a deletion of *fabH,* which is involved in the initiation step of fatty acid biosynthesis, leads to a higher proportion of unsaturated fatty acids (46,47). It is also possible that the observed decrease in transfer efficiency is due to a change in membrane fluidity. The membrane would become more fluid if there is a larger amount of unsaturated fatty acids in the membrane (48).

As can be seen in Figure 8, *ΔrfaD* show a large decrease in transfer frequency compared to the control *ΔargC* in the liquid mating assays. *ΔfabF* was also affected in the liquid mating assay. These two mutants were selected to further investigate through solid mating assays. Mating assays on solid medium are less sensitive to effects on mating pair stabilisation (11). This means that some mutants can recover the mating efficiency on solid medium. The results for the solid mating assays, see Figure 9, are similar to the liquid mating assays for *ΔrfaD*. However, *ΔfabF* show a higher transfer frequency in solid mating assay of about 40 % compared to an approximate 10 % for the liquid mating assay.

Regarding *ΔrfaD*, the mutation has a large impact on conjugation efficiency. Furthermore, no difference can be observed for conjugation in liquid and on solid media. The fact that the mutant cannot mate in either medium suggest that it is not mating pair stabilisation that is affected by the mutation. If mating pair stabilisation was the affected mechanism conjugation should be inhibited in liquid media, but the mutated donor should be able to conjugate on solid media as that removes the issue of stabilisation.

The gene *rfaD* encodes an enzyme involved in the synthesis of the lipopolysaccharide core and the mutant thus has a deficient LPS (33). There are studies that have found that LPS is a conjugation factor in the recipient (49–51). However, it seems like it is still unclear of whether this is a direct impact or if it is due to an indirect effect on proteins in the outer membrane. It is possible that the mechanism in which a deficient LPS affects the recipient's ability to conjugate is the same effect observed in this project for the donor. Another study found that *ΔrfaD* increase the fluidity of the outer membrane (52). The experiments performed with the membrane softener pentanol indicate that increased membrane fluidity has a negative effect on transfer frequency. Perhaps the effect on conjugation caused by pentanol and *ΔrfaD* is due to the same mechanism caused by increased membrane fluidity. It would be of interest to investigate other genes that are related to LPS as *ΔrfaD* to determine how the decrease in conjugation efficiency is a direct effect of the deficient LPS or not.

In the case of the *fabF*-mutant the composition of unsaturated and saturated fatty acids in the membrane could be responsible for the observed effected on conjugation efficiency. The three fatty acids that exist in the phospholipids in *E. coli*'s lipid bilayer are the saturated palmitic (hexadecenoic) acid and the two unsaturated fatty acids palmitoleic (*cis*-9-hexadecenoic) acid and *cis*-vaccenic (*cic*-11-octadecenoic) acid. These three acids require the enzymes that are the results of *fabA*, *fabB* and *fabF* for synthesis (48). *E. coli* that lacks the gene *fabF* cannot properly control synthesise of *cis*-vaccenic acid nor the composition of fatty acids as a response to growth temperature. It is possible that this lack of control of the fatty acid composition affects membrane fluidity and that in turn affects the donor's ability to conjugate. It would be interesting to examine membrane fluidity of the *fabF*-mutant to see if it differs from a control. This could help establish if altered membrane fluidity could be a possible cause to the decreased conjugation efficiency.

Furthermore, an increase in the transfer frequency of *ΔfabF* can be seen when comparing frequency of the liquid and solid mating assay, see Figure 8 and Figure 9. The transfer frequency increase from 10 % in liquid media to around 40 % on solid media. As an increase can be seen between the different mating assays, it is possible that mating pair stabilisation is affected resulting in a large effect on conjugation in liquid media but a smaller effect for solid media.

Lastly, *wzzE* encodes a protein that adjust the length of ECA_{PG} and is involved in the synthesis of ECA_{CYC} (17,18). Mutant strains deficient in conjugation have been seen to lack cyclic ECA (53). For future research it would be interesting to perform a solid mating assay with the mutant *ΔwzzE* as it showed a large effect on transfer frequency.

4.2 Microfluidics

Early on in the development of the microfluidics protocol cellular aggregates were observed for the donor strain. Since the recipient cells showed no aggregation tendencies the conclusion was that the aggregates were linked to the F plasmid, and potentially the F pilus. Aggregates is a problem for microfluidics methods as it does not allow a view of single cells. The aggregates may also be 3D-shapes, which makes it hard to focus the microscope for imaging. Larger aggregates also pose as obstacles for the flow of media and may cause currents in the channel that are unwanted. The first method used to counteract this issue was centrifugation of the donor culture. However, it did not remove all aggregates and with smaller aggregates often remaining. Another solution was growing the cells at a lower temperature. If the donor cells are grown at a lower temperature fewer pili form on the cell surface (54,55). Therefore, the cells were grown at approximately 32°C and this reduced the number of aggregates while still allowing expression of the F-pilus.

The optimal injection time is difficult to establish as it depends on, for example, the cell concentration of the culture and how good the surface modification is. In an ideal situation there would be equal amounts of donors and recipients on the surface. In addition, they should also be evenly spread out over the surface so that contact can be established between a donor and a recipient. An excess of donors is, however, worse than an excess of recipients. If the surface is primarily coated by donor cells conjugation will be less likely to take place. With fewer recipients there are fewer cells that can accept the conjugative plasmid and it is more likely that the majority of donor cells will not be in the proximity of a recipient. Having slightly more recipients on the surface could be advantageous since donors are more likely to encounter recipient cells and conjugation is more likely to occur.

4.2.1 Creating red mutant cells

For the investigation of *ΔarcA, ΔargC, Δlpp, and ΔrseA* there was an overlapping resistance to kanamycin between the mutants, replaced by a kanamycin cassette, and the RFP-encoding plasmid pEB2. To be able to separate the three cell types, two fluorescent markers were necessary. However, due to the overlap in kanamycin resistance three different methods were used to try and get mutant donor cells that express an RFP.

The first method utilised a λ-RED mediated gene replacement that was meant to insert an RFP into the chromosome. The method seemed to be successful with colonies having the correct selection marker, resistance to streptomycin. However, after looking at colonies under the microscope no red fluorescence was observed. It is possible the pEB2 PCR fragment was not successfully inserted into the chromosome. This has not been confirmed through PCR. Another possibility is that the PCR fragment was successfully inserted but the expression of RFP is too low to detect. The second method used FLP recombinase to remove the kanamycin cassette in the mutants. Afterward the cells would be transformed with pEB2. Unfortunately, when the screening for loss of the pCP20 plasmid, encoding carbenicillin resistance, and loss of the kanamycin cassette only sensitivity to carbenicillin was observed. No colony had lost kanamycin resistance. The exact reason as to why this method did not

succeed is not known. The last method directly transformed the kanamycin resistant mutant strains with pEB2. No red colonies were observed.

The fact that mutant strains with an RFP were not successfully created meant that these mutants could not be investigated through microfluidics. It would be of interest to confirm whether the PCR product of pEB2 was successfully inserted into the chromosome to determine if the λ-RED mediated method worked and the RFP expression was too low. If the PCR product has not been inserted correctly it could be tested again to see if red mutants can be accomplished.

4.2.2 The different surface modification protocols

The protocol with ethanol has previously worked for cells with the same fimbriae plasmid pPKL91 the donors and recipients have in this project. However, for an unknown reason, the cells did not adhere to the channel walls when using this modification protocol. A small number of donors adhere to the surface of the channel but not in the amount that is necessary for a contact dependent experiment. The same was observed for the protocol using PolyL-Lysine. Some donor cells do adhere but not in a satisfactory amount. For both the PolyL-Lysine and ethanol protocol the adhesion of recipient cells is very poor. The exact reason for why these protocols did not work in this project is not known.

The surface modification protocol using methanol showed the best adhesion. The cells stick to the walls in a satisfactory amount and both donor and recipient cells adhere to the channel walls. The downside of this protocol is that it takes a slightly longer time to prepare the channels, which is not ideal when aiming to develop a system with four channels. Furthermore, methanol is more demanding than ethanol to work with and the protocol also includes glutaraldehyde which is toxic. Preferably, the ethanol protocol can be revaluated to investigate at what step it failed so that it can be used. It would also be of interest to do the same with the PolyL-Lysine protocol.

Apart from the surface modifications affecting the adhesion of cells, a difference in the ability to adhere was observed between donor cells and recipient cells. Throughout the project donors have been observed to attach in a larger extent than recipients. Both donor and recipient cells have the same background, namely MG1655, and the same fimbriae plasmid. The only difference between the two cell types is that the donor also has pEB2, which is unlikely to affect the adhesion of the cells, and the F-plasmid. Furthermore, pEB2 can be excluded as the reason since the first conjugation pair ELA1 and ELA3 used in this project had a recipient cell with pEB2 and the donor was better at adhering to the surface in this conjugation pair as well. This leaves the F plasmid as the most plausible cause for for what was observed. One possibility is that the F pili allows for better adherence to the surface of the channel (56).

With regards to adherence another observation was made. As can be seen in Table 6, the cell type that is injected first will be present on the surface to a larger degree. The second injection of cell culture is generally worse with fewer cells adhering. While the donor cells seem to have a better adhesion ability than the recipient cells, part of the issue is likely that the first cell type takes the best binding spots in the channel. It will therefore be harder for the second cell type to find good binding spots. As the donors have a better ability to stick to the surface of the channel, it is better to inject it second and allow the recipient that is worse at adhering to take the better binding spots and be injected first. Furthermore, it

is better to have a larger proportion of recipient cells on the surface than donors which further support the decision to inject recipients first.

4.2.3 The four-channel system

No transconjugants were found in either of the four channels at the end of the experiment. There are many possible causes for this. Firstly, the adherence of cells on the surface varies between the channels as seen in Figure 15. It is interesting that the difference in cell adhesion between the channels can be this prominent as the four channels were modified with the same protocol, the methanol protocol, on the same day. While the exact reason for this difference is hard to conclude it is likely the insertion of silane that is the cause of the variation as this is the most critical step in the protocol.

For channel 3 no conjugation took place as there were too few cells on the surface to enable conjugation. Regarding channel 4 the majority of cells were donors. If few recipient cells are present conjugation is unlikely to occur. Furthermore, if there are transconjugants these will be hard to detect as they are hidden among all the donors.

Channel 1 was the most promising regarding detection of transconjugants as there seemed to be a satisfactory amount of both donors and recipients. However, the fluorescence images show a light phenomenon. The phenomenon was observed for channel 1 and 4 raising suspicion that it has to do with the glue around the channels. The fluorescence light was likely reflected by the glue around the channels, and this caused the phenomenon observed in Figure 16 in Appendix II. To counteract this an initial check with red fluorescence should be done at the start of the experiment, after injection of both recipients and donors, to ensure that the cells investigated are not too close to the outer channel wall next to the glue.

For channel two the structures observed made it difficult to properly assess the fluorescence images, see Figure 17 in Appendix II. The exact cause of these structures is unclear, but they are likely underneath the channel and not inside. The channels were washed in 95% ethanol prior to the experiment to try and remove any dirt around them. However, this did not seem to have worked. The solution for this would be to build a new four channel slide.

During an experiment it is desired to monitor the same cells through the entirety of the experiment. This is done to monitor growth and conjugation. In this project imaging of the channels is done manually. The issue with four channels is that the objective must be moved between the channels and therefore it is hard to capture the exact same spot every time. Ideally, the imaging process could be automated so that the camera/objective takes the images at the same place in each channel every 20 minutes to make analysis easier. Furthermore, the channels on the slide used were not level and the objective needed to be adjusted in between each channel. It would be preferred if the channels were all on the same level to make imaging easier. This can possibly be done by taking extra care when gluing the channels onto the microscope slide and ensuring that all channels are in contact with the slide.

Furthermore, the four-channel system is extremely sensitive to differences in pressure between the four channels. The peristaltic pump that was used during the beginning of the project offered the possibility

to have four separate tubes that allowed for parallel flow through each channel. However, the peristaltic pump broke halfway through the project. The syringe pump, which replaced the peristaltic pump, was used for the four-channel system and had the limitation that only one tube could be connected to it. Therefore, a splitter was used to divide the flow. The one tube that was connected to the syringe was split into four tubes going to the four channels. The split tubing system was very sensitive to differences in pressure between the channels. For example, if one channel or tube was placed slightly higher than the others it could stop the flow of one or more of the channels. To lessen the issue of pressure sensitivity a different tube with a diameter 102 µm was added to each outlet. The added tube made the system less sensitive, however, it is still a factor to consider. Another way to help get flow through all four channels is to fill each tube with water before inserting it into the channel.

Leaks in the tubing or in the connections between the tubes and the channels is a problem related to the pressure sensitivity. If the leak is on the outlet the consequences are smaller. It will likely lead to loss of flow through one channel or slower flow. If the leak occurs in one of the inlet tubes, it can cause the flow to stop completely in all channels. Another reason as to why leaks can be problematic is that air bubbles may enter the channels. Air may cause the cells to release from the surface of the channel and wash out the cell, see Figure 18 in Appendix II. How many cells that are lost varies, likely due to how well the channel surface has been modified, and does not always lead to a large loss of cells. However, as there is a risk of losing cells air bubbles should be avoided.

5 Conclusion

From the liquid mating assays performed with different concentrations of pentanol it is possible to conclude that increased membrane fluidity seems to affect a donor's ability to properly conjugate. Out of the five mutants that were investigated *ΔrfaD* together with *ΔfabF* and *ΔwzzE* showed the biggest effect on transfer frequency with *ΔfabF* and *ΔwzzE* showing a moderate effect on conjugation frequency. However, none of these conclusions were statistically significant as the number of samples were too few. Mating on a solid surface did not improve *ΔrfaD's* ability to conjugate indicating that mating pair stabilisation is not the primary cause of the difference in transfer frequency compared to the control donor. *ΔfabF*'s ability to conjugate did improve on solid medium and mating pair stabilisation may be the affected mechanism for this mutant. Furthermore, the effect could also be due to altered membrane fluidity for the mutants *ΔrfaD* and *ΔfabF*. In this project a four-channel microfluidics protocol has been developed for investigation of conjugation on a solid surface. However, for the method to be a reliable and reproducible alternative for conjugation experiments it needs further optimisation.

6 References

1. World Health Organization. Antimicrobial resistance [Internet]. 2021 [cited 2022 Sep 12]. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

2. Hoffman SB. Mechanisms of Antibiotic Resistance. Compendium on Continuing Education for the Practicing Veterinarian. 2001 May;23(5):464–72.

3. Sengupta S, Chattopadhyay MK, Grossart HP. The multifaceted roles of antibiotics and antibiotic resistance in nature. Vol. 4, Frontiers in Microbiology. Frontiers Research Foundation; 2013.

4. Davies J. What are antibiotics? Archaic functions for modern activities. Mol Microbiol. 1990;4(8):1227–32.

5. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: A global multifaceted phenomenon. Vol. 109, Pathogens and Global Health. Maney Publishing; 2015. p. 309–18.

6. Arutyunov D, Frost LS. F conjugation: Back to the beginning. Vol. 70, Plasmid. 2013. p. 18–32.

7. Virolle C, Goldlust K, Djermoun S, Bigot S, Lesterlin C. Plasmid transfer by conjugation in gram-negative bacteria: From the cellular to the community level. Vol. 11, Genes. MDPI AG; 2020. p. 1–33.

8. Dionisio F, Conceição IC, Marques ACR, Fernandes L, Gordo I. The evolution of a conjugative plasmid and its ability to increase bacterial fitness. Biol Lett. 2005 Jun 22;1(2):250–2.

9. Soltysiak MPM, Meaney RS, Hamadache S, Janakirama P, Edgell DR, Karas BJ. Trans-kingdom conjugation within solid media from Escherichia coli to Saccharomyces cerevisiae. Int J Mol Sci. 2019 Oct 1;20(20).

10. Graf FE, Palm M, Warringer J, Farewell A. Inhibiting conjugation as a tool in the fight against antibiotic resistance. Vol. 80, Drug Development Research. Wiley-Liss Inc.; 2019. p. 19–23.

11. Alalam H, Graf FE, Palm M, Abadikhah M, Zackrisson M, Boström J, et al. A High-Throughput Method for Screening for Genes Controlling Bacterial Conjugation of Antibiotic Resistance. mSystems. 2020 Dec 22;5(6).

12. Idalia VMN, Bernardo F. Escherichia coli as a Model Organism and Its Application in Biotechnology. Recent Advances on Physiology, Pathogenesis and Biotechnological Applications [Internet] [Internet]. 2017 [cited 2023 Feb 8]; Available from: https://www.intechopen.com/chapters/53916

13. Klemm P, Krogfelt KA, Hedegaard L, Christiansen G. The major subunit of Escherichia coli type 1 fimbriae is not required for D-mannose-specific adhesion. Mol Microbiol. 1990;4(4):553–9.

14. Krogfelt,' KA, Bergmans H, Klemm' P. Direct Evidence that the FimH Protein Is the Mannose-Specific Adhesin of Escherichia coli Type 1 Fimbriae [Internet]. Vol. 58, INFECTION AND IMMUNITY. 1990. Available from: https://journals.asm.org/journal/iai

15. Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of the Cell. 6th ed. Wilson J, Hunt T, editors. W.W. Norton & Company; 2017.

16. Madigan MT, Bender KS, Buckley DH, Sattley WM, Stahl DA. Brock Biology of Microorganisms. 15th ed. Beauparlant S, Neelakantan KK, editors. Harlow: Pearson Education Limited; 2019.

17. Kajimura J, Rahman A, Rick PD. Assembly of cyclic enterobacterial common antigen in Escherichia coli K-12. J Bacteriol. 2005 Oct;187(20):6917–27.

18. Barr K, Klena J, Rick PD. The Modality of Enterobacterial Common Antigen Polysaccharide Chain Lengths Is Regulated by o349 of the wec Gene Cluster of Escherichia coli K-12 [Internet]. Vol. 181, JOURNAL OF BACTERIOLOGY. 1999. Available from: https://journals.asm.org/journal/jb

19. Rai AK, Mitchell AM. Enterobacterial Common Antigen: Synthesis and Function of an Enigmatic Molecule. 2020; Available from: https://doi.org/10.1128/mBio

20. Dahl J. Gram negative cell wall [Internet]. Wikimedia. 2008 [cited 2023 May 31]. Available from: https://commons.wikimedia.org/wiki/File:Gram_negative_cell_wall.svg

21. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol. 2006 May 16;2.

22. Costa TRD, Ilangovan A, Ukleja M, Redzej A, Santini JM, Smith TK, et al. Structure of the Bacterial Sex F Pilus Reveals an Assembly of a Stoichiometric Protein-Phospholipid Complex. Cell. 2016 Sep 8;166(6):1436-1444.e10.

23. Low WW, Wong JLC, Beltran LC, Seddon C, David S, Kwong HS, et al. Mating pair stabilization mediates bacterial conjugation species specificity. Nat Microbiol. 2022 Jul 1;7(7):1016–27.

24. Low HH, Gubellini F, Rivera-Calzada A, Braun N, Connery S, Dujeancourt A, et al. Structure of a type IV secretion system. Nature. 2014;508(7497):550–3.

25. Salinas-Almaguer S, Mell M, Almendro-Vedia VG, Calero M, Robledo-Sánchez KCM, Ruiz-Suarez C, et al. Membrane rigidity regulates E. coli proliferation rates. Sci Rep. 2022 Dec 1;12(1).

26. Janßen HJ, Steinbüchel A. Fatty acid synthesis in Escherichia coli and its applications towards the production of fatty acid based biofuels [Internet]. 2014. Available from: http://www.biotechnologyforbiofuels.com/content/7/1/7

27. Asmar AT, Ferreira JL, Cohen EJ, Cho SH, Beeby M, Hughes KT, et al. Communication across the bacterial cell envelope depends on the size of the periplasm. PLoS Biol. 2017 Dec 19;15(12).

28. Inouye M, Shaw J, Shen C. The assembly of a structural lipoprotein in the envelope of Escherichia coli. Journal of Biological Chemistry. 1972 Dec 25;247(24):8154–9.

29. Yem DW, Wu HC. Physiological Characterization of an Escherichia coli Mutant Altered in the Structure of Murein Lipoprotein [Internet]. Vol. 133, JOURNAL OF BACTERIOLOGY. 1978. Available from: https://journals.asm.org/journal/jb

30. Yoshimura M, Oshima T, Ogasawara N. Involvement of the YneS/YgiH and PlsX proteins in phospholipid biosynthesis in both Bacillus subtilis and Escherichia coli. BMC Microbiol. 2007;7.

31. Lu YJ, Zhang YM, Grimes KD, Qi J, Lee RE, Rock CO. Acyl-Phosphates Initiate Membrane Phospholipid Synthesis in Gram-Positive Pathogens. Mol Cell. 2006 Sep 1;23(5):765–72.

32. Lu YJ, Zhang F, Grimes KD, Lee RE, Rock CO. Topology and active site of PlsY: The bacterial acylphosphate:glycerol-3- phosphate acyltransferase. Journal of Biological Chemistry. 2007 Apr 13;282(15):11339–46.

33. Kneidinger B, Marolda C, Graninger M, Zamyatina A, McArthur F, Kosma P, et al. Biosynthesis pathway of ADP-L-glycero-β-D-manno-heptose in Escherichia coli. J Bacteriol. 2002;184(2):363–9.

34. Zhang G, Meredith TC, Kahne D. On the essentiality of lipopolysaccharide to Gram-negative bacteria. Vol. 16, Current Opinion in Microbiology. 2013. p. 779–85.

35. Ades SE. Control of the alternative sigma factor σE in Escherichia coli. Vol. 7, Current Opinion in Microbiology. 2004. p. 157–62.

36. Nizam SA, Zhu J, Ho PY, Shimizu K. Effects of arcA and arcB genes knockout on the metabolism in Escherichia coli under aerobic condition. Biochem Eng J. 2009 May 15;44(2–3):240–50.

37. Lu J, Peng Y, Wan S, Frost LS, Raivio T, Mark Glover JN. Cooperative function of TraJ and ArcA in regulating the F plasmid tra operon. J Bacteriol. 2019 Jan 1;201(1).

38. Parsofa C, Boyen A, Cohena GN, Glansdorffb N. Tel. 33.145688000; b Microbiologic, Vrije Universiteit Brussel, 1, ave. E. Gryson, B-1070 Brussels (Belgium) Tel. 32.2.523.28.80(313) and. Vol. 68, Gene. 1988.

39. Baich A, Vogel HJ. N-acetyl-y-glutamokinase and N-acetylglutamic y-semialdehyde dehydrogenase: Repressible Enzymes of Arginine Synthesis in Escherichia Coli. Vol. 7. 1962.

40. Coluccio ML, Perozziello G, Malara N, Parrotta E, Zhang P, Gentile F, et al. Microfluidic platforms for cell cultures and investigations. Microelectron Eng. 2019 Mar 1;208:14–28.

41. Young EWK, Beebe DJ. Fundamentals of microfluidic cell culture in controlled microenvironments. Chem Soc Rev. 2010 Feb 24;39(3):1036–48.

42. Mehling M, Tay S. Microfluidic cell culture. Vol. 25, Current Opinion in Biotechnology. Elsevier Ltd; 2014. p. 95–102.

43. Dusny C, Grünberger A. Microfluidic single-cell analysis in biotechnology: from monitoring towards understanding. Vol. 63, Current Opinion in Biotechnology. Elsevier Ltd; 2020. p. 26–33.

44. Whitesides GM. The origins and the future of microfluidics. Vol. 442, Nature. 2006. p. 368–73.

45. Fried L, Lassak J, Jung K. A comprehensive toolbox for the rapid construction of lacZ fusion reporters. J Microbiol Methods. 2012 Dec 1;91(3):537–43.

46. Yu F, Liu X, Tao Y, Zhu K. High saturated fatty acids proportion in Escherichia coli enhances the activity of ice-nucleation protein from Pantoea ananatis. Vol. 345, FEMS Microbiology Letters. 2013. p. 141–6.

47. Fernandez-Lopez R, Machón C, Longshaw CM, Martin S, Molin S, Zechner EL, et al. Unsaturated fatty acids are inhibitors of bacterial conjugation. Microbiology (N Y). 2005 Nov;151(11):3517–26.

48. Mansilla MC, Cybulski LE, Albanesi D, De Mendoza D. Control of membrane lipid fluidity by molecular thermosensors. Vol. 186, Journal of Bacteriology. 2004. p. 6681–8.

49. Anthony KG, Sherburne C, Sherburne R, Frost LS. The role of the pilus in recipient cell recognition during bacterial conjugation mediated by F-like plasmids. Mol Microbiol. 1994;13(6):939–53.

50. Manoil C, Rosenbusch JP. Conjugation-Deficient Mutants of Escherichia coliDistinguish Classes of Functions of the Outer Membrane OmpA Protein. Vol. 187. 1982.

51. Skurray RA, Hancock REW, Reeves P. Con-Mutants: Class of Mutants in Escherichia coli K-12 Lacking a Major Cell Wall Protein and Defective in Conjugation and Adsorption of a Bacteriophage [Internet]. JOURNAL OF BACTERIOLOGY. 1974. Available from: https://journals.asm.org/journal/jb

52. Storek KM, Vij R, Sun D, Smith PA, Koerber JT, Rutherford ST. The Escherichia coli -barrel assembly machinery is sensitized to perturbations under high membrane fluidity. J Bacteriol. 2019 Jan 1;201(1).

53. Dimovska Nilsson K, Palm M, Hood J, Sheriff J, Farewell A, Fletcher JS. Chemical Changes On, and Through, the Bacterial Envelope in Escherichia coli Mutants Exhibiting Impaired Plasmid Transfer Identified Using Time-of-Flight Secondary Ion Mass Spectrometry. Anal Chem. 2019 Jun 2;91(17):11355–61.

54. Novotny CP, Lavin K. Some Effects of Temperature on the Growth of F Pili [Internet]. Vol. 107, JOURNAL OF BACTERIOLOGY. 1971. Available from: https://journals.asm.org/journal/jb

55. Walmsley RH. Temperature Dependence of Mating-Pair Formation in Escherichia coli [Internet]. JOURNAL OF BACTERIOLOGY. 1976. Available from: https://journals.asm.org/journal/jb

56. Maier B, Wong GCL. How Bacteria Use Type IV Pili Machinery on Surfaces. Vol. 23, Trends in Microbiology. Elsevier Ltd; 2015. p. 775–88.

7 Appendix

7.1 Appendix I

The unnormalised transfer frequencies for each biological replicate of the liquid mating assay performed to investigate the effect of fimbriae on conjugation can be seen in Table 7. In Table 8 the unnormalised transfer frequencies for each biological replicate in the liquid mating with pentanol can be seen. Table 9 and Table 10 contains the normalised transfer frequencies for each biological replicate in the liquid and solid mating assay performed with mutant donors.

Table 7. ***Transfer frequencies from liquid mating assays examining the effect of fimbriae on conjugation.*** *Shows the average transfer frequency between technical replicates for each biological replicate in the liquid mating assay performed with the three donors MG1655 with pPKL91 and pLP8 (ELA1), MG1655 with a fimA deletion (ELA2), and MG1655 ΔxylA::Kan and the recipient MG1655 with pPKL91 and pEB2.*

	Liquid mating assay Fimbriae 1	Liquid mating assay Fimbriae 2
ELA1 Biological replicate 1	0.15	0.12
ELA1 Biological replicate 2	0.10	0.021
ELA1 Biological replicate 3	0.16	0.030
ELA1 Biological replicate 4	0.10	0.11
ELA2 Biological replicate 1	0.048	0.076
ELA2 B Biologial replicate 2	0.087	0.11
ELA2 Biologial replicate 3	0.039	0.079
ELA2 Biological replicate 4	0.022	0.055
MG1655 *ΔxylA::*Kan biological replicate 1	0.027	0.070
MG1655 *ΔxylA::*Kan Biological replicate 2	0.051	0.17
MG1655 *ΔxylA::*Kan Biological replicate 3	0.053	0.17
MG1655 *ΔxylA::*Kan Biological replicate 4	0.066	0.18

Table 8. ***The transfer frequencies from liquid mating assays examining the effect of pentanol concentration on conjugation.*** *Shows the calculated transfer frequencies for both liquid mating assays where pentanol at a concentration of 0mM, 1 mM, 5mM, 10 mM, 15 mM and 20 mM was added, respectively. The transfer efficiency is reported for all biological replicates.*

	Liquid mating assay no. 1		Liquid mating assay no. 2	
Pentanol concentration	Biological replicate 1	Biological replicate 2	Biological replicate 1	Biological replicate 2
0 mM	0.062	0.082	0.042	0.53
1 mM	0.068	0.056	0.0074	0.054
5 mM	0.024	0.019	0.0012	0.0062
10 mM	0.016	0.023	0.0023	0.0044
15 mM	0.024	-	0.0073	0.014
20 mM	0.0015	-	-	-

Table 9. ***The transfer efficiencies from liquid mating assays with five mutant donors.*** *Shows the transfer frequencies for two liquid mating assays with the mutant donors ΔfabH, ΔplsX, Δ fabF, ΔwzzE, ΔrfaD, and the control ΔargC. The transfer efficiency is reported for all biological replicates.*

	Liquid mating assay no. 1		Liquid mating assay no. 2	
Mutant strains	Biological replicate 1	Biological replicate 2	Biological replicate 1	Biological replicate 2
ΔfabH	0.067	0.57	0.039	0.19
ΔplsX	0.061	0.091	0.19	0.13
ΔfabF	0.00069	0.0049	0.0036	0.0042
ΔwzzE	0.0057	0.0042	0.0095	0.0047
ΔrfaD	0	0	2.2*10^-6	0
ΔargC (control donor)	0.077	0.0033	0.27	0.055

Table 10. ***The transfer efficiencies from liquid mating assays with two mutant donors****. Shows the transfer frequencies for two solid mating assays with the mutant donors Δ fabF, ΔrfaD, and the control ΔargC. The transfer frequencies are reported for all biological replicates of each mating assay.*

	Solid mating assay no. 1		Solid mating assay no. 2	
	Biological replicate 1	Biological replicate 2	Biological replicate 1	Biological replicate 2
ΔfabF	0.023	0.010	0.53	0.56
ΔrfaD	0.00015	4.2*10^-5	3.1*10^-5	0.00015
ΔargC (control donor)	0.13	0.26	1.1	1.1

7.2 Appendix II

The figures show the surface of different channels from a microfluidics experiment where the four-channel system was tested. In Figure 16 the effect of the glue on fluorescence images for the outer channels of a four-channel system is seen. Figure 17 shows channel two in the four-channel experiment where some structure can be seen to be fluorescent. Channel 1 in the four-channel experiment can be seen in Figure 18 before and after air passed through the channel.

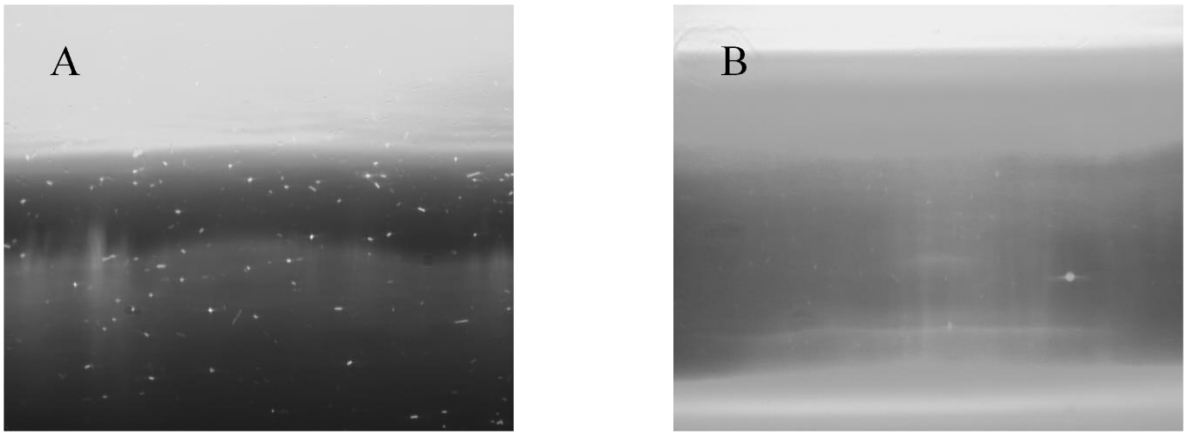

Figure 16. ***Light phenomenon on fluorescence images caused by the glue around the channel.*** *Shows red fluorescence images for channel 1 (A) and 4 (B) from a four-channel experiment where light is reflected by the glue used to form a pool for the water objective.*

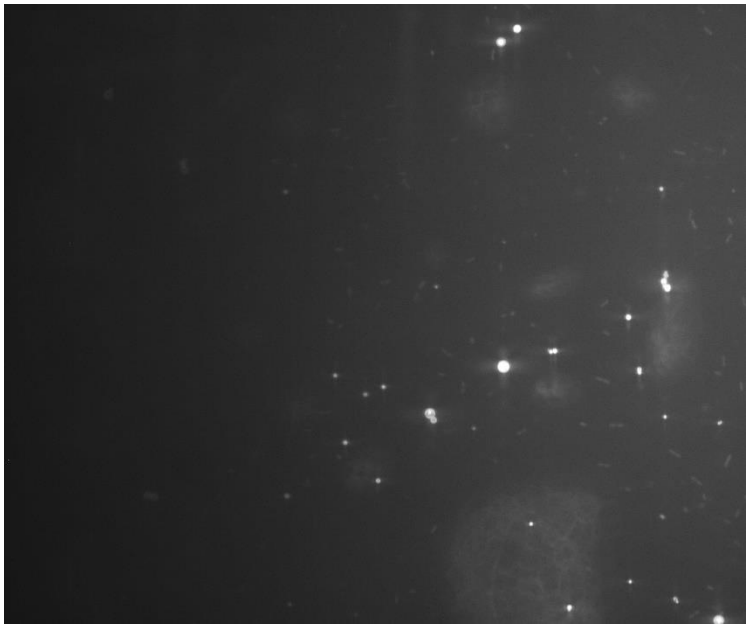

Figure 17. ***Structures under the channel interfere with fluorescence imaging.*** *Shows a red fluorescence image of channel 2 from a four-channel experiment where structures underneath the channel can be seen to be fluorescent and interfere with imaging of the cells that are attached to the channel surface.*

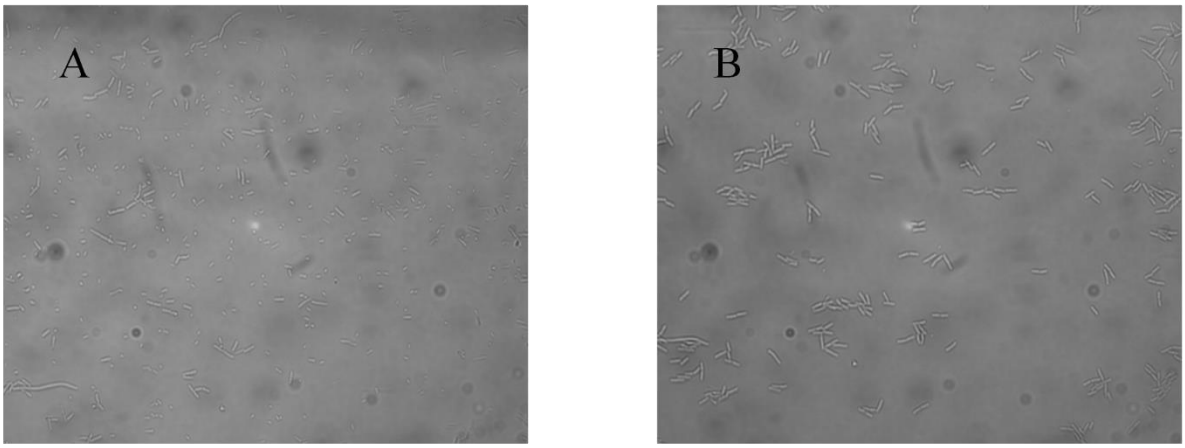

Figure 18. ***Comparison of a channel before and after air entered the system.*** *Shows channel 1 from a four-channel experiment before (A) and after (B) an air bubble passed through the channel. A decrease in the number of cells can be seen between A and B.*