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Fitness cost and excision rate of the conjugative transposon Tn*916*

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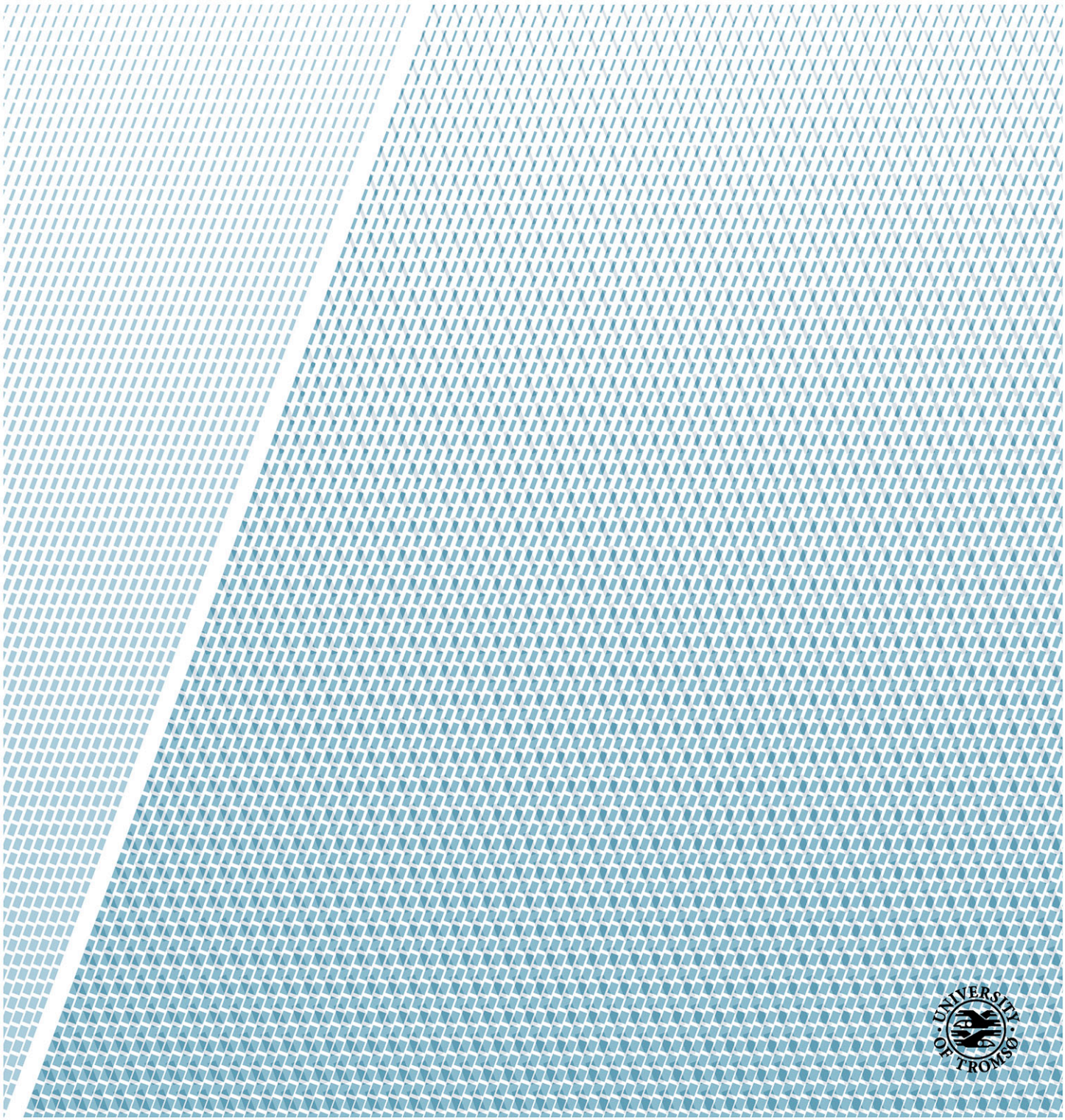

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1 ABSTRACT

Dental infections are getting more difficult to treat because of the increasing amount of antibiotic resistant oral bacteria. A bacterium can acquire resistance by horizontal gene transfer, this allows a bacterial population to acquire antibiotic resistance genes at a great rate.
Occasionally, newly acquired resistance can reduce bacterial fitness. However, bacteria can evolve quickly to compensate for any fitness loss.

Mobile genetic elements are genetic elements capable of translocation in the same genome, or move between bacterial species or cross bacterial populations. Mobile genetic elements are important from a clinical point of view because they can mediate the transfer of antibiotic resistance. Tn*916* is a type of mobile genetic element which can be transferred into a wide range of bacterial phyla.

This study aimed to investigate the fitness cost of newly acquired Tn*916* in *Enterococcus faecium* through investigation of growth curves of different strains of *E. faecium* with and without Tn*916*. This study will also investigate the excision rate of Tn*916*, using ddPCR.

2 INTRODUCTION

2.1 ANTIBIOTIC RESISTANCE

Antibiotics are drugs used to prevent and treat bacterial infections [1]. In 1928, Alexander Fleming discovered penicillin. After the introduction of antimicrobials for therapeutic use, the development and emergence of specific mechanisms to resist the action of antimicrobials have been observed [2].

Dental infections are getting more difficult to treat because of the increasing amount of antibiotic resistant oral bacteria [3]. A study by Al-Haroni and Skaug published in 2006 showed that 70.0 % of the authorized dentists in Norway issued antibiotic prescriptions [4]. Research shows that the incidence of resistant bacteria is higher in societies where the consumption of antibiotics are usually high [5].

A bacterium can acquire resistance by horizontal gene transfer or by mutation of its own genome. A bacteria can also be "born" resistant, this is called natural or inherent resistance and is due to their intrinsic properties. Bacteria can for example lack certain structures that serves as the target molecule for the antibiotic drug or lack metabolic processes essential for the activation of the antibiotic drug [3].

There are several measures to limit the acceleration of resistant development. WHO suggest that policymakers should develop guidelines for the use of antibiotics and that health workers should only prescribe antibiotics when they are truly needed and with the right dose and duration of the therapy [1].

2.2 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) is a method for amplifying DNA-sequences into millions of copies. Double-stranded DNA from any biological sample acts as a template in PCR. Before amplifying can take place, the double strands DNA has to be separated. Primers are short DNA sequences designed to recognize specific DNA-sequences. Primers bind to both strands of the DNA and serve as a guide for further DNA synthesis which is catalyzed by DNA polymerases. Polymerase is an enzyme that catalyzes addition of new nucleotides [6].

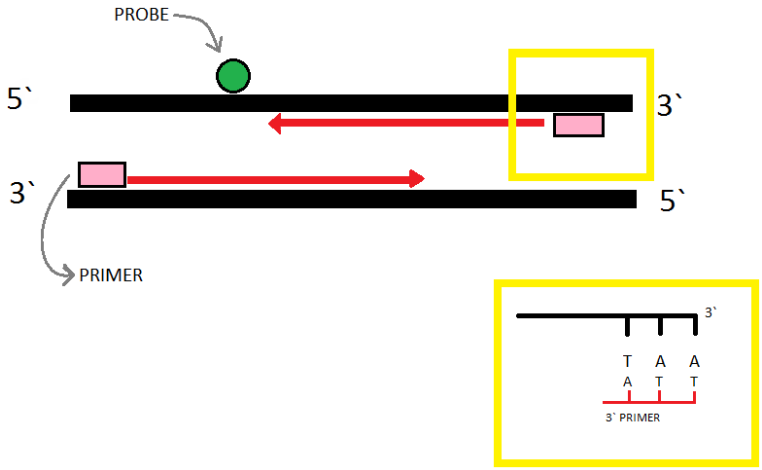

Figure 1: Polymerase chain reaction.

In our project we have used droplet digital PCR (ddPCR) to determine the excision rate of the mobile genetic element Tn*916* that carry the tetracycline resistance gene *Tet(M)*. In a ddPCR assay, primers and probe are used to amplify the target DNA molecule (Figure 1). The ddPCR provide high-precision, absolute quantification of nucleic acid target sequences by counting nucleic acid molecules encapsulated in volumetrically defined water-in-droplet partition.

In the ddPCR workflow, a given DNA sample is divided into 20.000 droplets. One droplet contains approximately one nanoliter. If a droplet contains the specific target sequence of interest, PCR will be performed in the droplet and the amplification reaction would emit a fluorescent signal and be classified as a positive droplet. A positive droplet can contain one or more copies of the target sequence. However, droplets which do not contain the specific DNA target will not emit fluorescence and be, therefore, classified as negative.

After DNA amplification, the droplet reader with its software counts the positive and negative droplets and uses Poisson distribution to calculate the absolute number of the target DNA in the reaction [7].

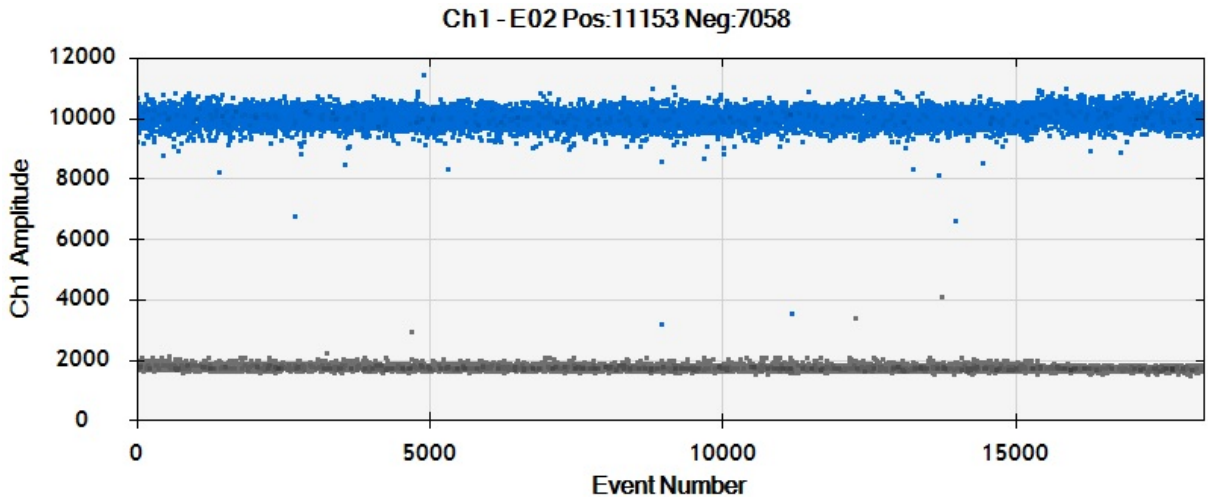

Figure 2: Screenshot from QuantaSoft.

The illustration above (Figure 2) shows a graph where all the droplets from a given sample are plotted. The droplets at the top are positive while the droplets at the bottom are negative.

2.2.1 Determination of target DNA Copy number in ddPCR

The ddPCR can determine the copy number of a given DNA target. For example, Tn*916* copy number in bacterial genome can be determined using a specific sequence in Tn*916* as a representative of its presence and compare that occurrence with a known number of a reference gene. However, when trying to determine the copy number there is a technical challenge regarding the copy number assessment. When copy numbers are consecutive, it is difficult to discriminate between them with statistical confidence. The ability to discriminate between the copy numbers consecutive states can be difficult because when the copy number state increases, the percentage difference in target genomic material states decreases [7]. A copy number of 2 is 100 % more abundant in concentration per genome than a copy number of 1. This can easily be discriminated. When the copy numbers states are high, the situation is different: for example, a copy number of 7 is only 16.7 % more abundant in concentration per genome than a copy number of 6. In the latter situation, the difference requires a much higher sensitivity and resolution to be assessed.

Many methods used to analyze copy number variation, like comparative genomic hybridization and quantitative PCR (QPCR). However, these methods lack the sensitivity and resolution that is needed to get an optimized quantitative discrimination. On the other hand, the enormous partitioning capability of ddPCR that

can make up to 20,000 droplets, enables the fine quantitative discrimination required to resolve consecutive copy number states [7].

After the droplets are transferred to a PCR plate and heat-sealed, the plate is placed in the thermal cycler for PCR amplification with an appropriate thermal cycling protocol due to temperature and type of assay. To ensure that each droplet reaches the right temperature for each step of the cycle, the protocol includes a 2.5°C/sec ramp rate. It is important that the annealing temperature of the PCR assay is optimized to get good results. Different factors affect the annealing temperature, including the buffer conditions as well as primer and probe concentrations. If the temperature is too low, the chances are that the results show amplification of non-specific PCR products. On the other hand, if the temperature is too high, this may reduce the yield of the desired PCR product.

To determine the optimal annealing temperature for an assay, a thermal gradient feature of the thermal cycle can be used. This is a variable step in the cycle (step 2) that enables testing of the assay with different temperatures simultaneously and thereby finding the best temperature for that specific PCR assay. A range of temperatures below and above the primers melting temperature (T_m) is usually tested. The results with the largest fluorescence amplitude difference between the positives and negatives and that avoids nonspecific amplification is the one with optimal annealing temperature.

Right amount of DNA-concentration is also important to get good results of the ddPCR. Too high or too low concentration will not get registered and will show as "No Call" on the QuantaSoft software. For detection of genes in human DNA, the amount of DNA should not be over 3ng/µl (60ng/20µl reaction) and intact DNA requires restriction digestion for optimal performance of the ddPCR [7]. For bacterial DNA, the load should usually not exceed 60 picogram.

2.3 HORIZONTAL GENE TRANSFER

In the oral ecosystem, we have a complex bacterial composition. This consists of hundreds or thousands of bacterial species belonging to both residential and transient bacterial inhabitants where bacteria live primarily as complex, polymicrobial biofilms (dental biofilm) [8, 9, 10]. In the dental biofilm, the bacterial cells are in close

proximity to each other. This provides an excellent environment for horizontal gene transfer (HGT). This can lead to the spread of resistance genes amongst the inhabitants of the biofilm [11]. From human oral bacteria, several transmissible genetic elements carrying antibiotic resistance genes (e.g. plasmids and conjugative transposons) have been reported [4].

Acquired resistance in oral bacteria is generally achieved by two mechanisms: horizontal gene transfer, or, less frequently, chromosomal mutation in the pre-existing bacterial genome. Horizontal gene transfer has contributed to the spread of resistance to most antibiotics.

2.3.1 What are mobile genetic elements?

A mobile genetic element is a piece of DNA, also called a jumping gene [6]. Mobile genetic elements encode for all the genes that are necessary for their own movement. Their movement can occur within the same genome, between the same bacterial species or cross bacterial populations [9]. Conjugative transposons like Tn*916*, described later, are a type of mobile genetic elements.

2.3.2 How does horizontal gene transfer occur?

Horizontal gene transfer describes the situation where a mobile genetic element is transferred from the genome of one cell or from the surrounding environment to another cell. The term mobile genetic element usually refers to a short, specialized segment of DNA. These can move from one position in the genome to another. They typically encode a specialized recombination enzyme, which mediates the movement. Therefore, the mobile genetic elements does not require DNA sequence similarity to move [6].

The acquisition of a genetic material that could carry resistance genes into a bacterium occur via three mechanisms:

- **Transformation**: free exogenous segments of DNA carrying resistance genes are acquired by the bacteria from their environment, and the bacteria is required to become competent i.e. altered bacterial phenotype by which bacteria are able to take up and integrate exogenous free DNA from their environment.

- **Transduction**: transfer of genetic material from one bacterium to another by a virus (a bacteriophage), which carries both its own DNA and a fragment of DNA from the donor (which is a bacterium that the virus has recently infected). Recombination may occur.
- **Conjugation**: two bacteria are in direct contact with each other, which leads to an exchange of genetic material.

2.3.3 Conjugative transposons

Conjugative transposons (cTns) are broad host range gene transfer elements that encode all the necessary proteins for intracellular transposition and intercellular conjugation [9]. These genetic elements are capable of Excising from the chromosome of the donor genome, transfer to a recipient cell by conjugation, and insertion into the transconjugants genome (Figure 3).

2.3.4 Why are conjugative transposons important?

Conjugative transposons are very important from both a clinical and a basic scientific perspective. Clinically, they mediate the transfer of antibiotic resistance between a wide range of environmental, commensal and pathogenic bacteria. In a scientific perspective, they also provide significant genomic variation on which natural selection can act and are responsible for the dissemination of genes other than antibiotic resistance genes. The conjugative transposon Tn*916* is a prototype of conjugative transposons. It could be surprising that conjugative transposons have been found in bacteria from environments that have never been in contact with the antibiotics that they encode resistance for.

2.3.5 Horizontal gene transfer among oral bacteria

Evidences of horizontal gene transfer among oral bacteria have been accumulating [12]. Horizontal gene transfer is present in the oral cavity, where over 500 bacterial species can co-inhabit in the tongue, gingiva and/or the teeth. This makes DNA exchange between them possible [13]. Every cell has a *mobilome*, which is an agent of change that makes horizontal gene transfer possible. It consists and is referred to as all mobile genetic elements (MGEs) in any cell. Mobile genetic elements found in oral bacteria include plasmids, phages and transposons [14]. In prokaryotes, the movement of genetic material is not primarily confined within the genome as it is in eukaryotes, but includes elements that provide movement between different genomes.

The MGE is defined as a broader set of agents compared to what is in eukaryotes, where the eukaryotic mobilome mostly include intragenomic dynamics within somatic cells [14]. The transfer of genetic material can be done by three parasexual processes: conjugation, transduction and transformation [15].

Horizontal gene transfer allows the bacterial population to acquire antibiotic resistance genes at a rate significantly greater than would be afforded by mutation of chromosomal DNA. For the dissemination of antibiotic resistance gene or genes by horizontal gene transfer, a resistance gene may be inserted into mobile genetic elements like plasmids, transposons, and integrons and may be linked within them to other resistance genes.

2.4 THE CONJUGATIVE TRANSPOSON TN*916*

2.4.1 What is Tn*916*?

Tn*916* was first discovered in the late 1970s, and in 1981, Franke and Clewell became the first to identify a chromosomal drug-resistance transposon capable of conjugal transfer [16]. The study showed that the Tetracycline resistance determinant of the *E. faecalis* strain DS16 is capable of conjugal transfer, and that this determinant is located on Tn*916*. The study also demonstrated that Tetracycline resistance determinant from *E. faecalis* DS16 could transfer to *E. faecalis* strain JH2-2, in the absence of plasmids – but in the presence of Tn*916*.

Tn*916* is a type of mobile genetic elements (a conjugative transposon (CT) or integrative conjugative element (ICE)), capable of both transposing intracellularly and conjugating intercellularly [17]. It has been found in or been transferred into a wide range of bacterial phyla [16].

Since Franke and Clewells reported its presence [16], Tn*916* has been DNA sequenced and genetically analyzed in many studies. These have shown that Tn*916* has four regions in its genome that is devoted to a single function. These regions are called functional modules, and they are conjugation, transcriptional and translational regulation, recombination (excision and insertion), and accessory genes not involved in conjugative transposition (Figure 3). The latter is the functional module that contains tetracycline resistance. The gene that is encoding tetracycline resistance in

Tn*916* is called *tet*(M), and it is encoding *tet(M)*, which is a ribosomal protection protein [9].

Figure 3: Schematic view of the Tn916. Tn916 has four functional modules: conjugation (red), transcriptional and translational regulation (yellow), excision and insertion (green), and accessory functions (purple).

The Tn*916* family of conjugative transposons is large, but what they all have in common is the general modular organization described above, and their regulation and conjugation modules should be similar in both sequence and structure to the original Tn*916*.

2.4.2 Why is Tn*916* important?

Tn*916* is important both because it makes a big impact on the host bacteria, but also because of its widespread nature. While some families of conjugative transposons seem to be confined to a particular species or genera of bacteria, Tn*916* is much more widespread: members of the Tn*916*/Tn*1545* family are found in over 30 different genera of bacteria [9]. The superfluity of transfer mechanisms (transformation and conjugation) is considered as one of the main reasons for this. Tn*916* is a promiscuous element which can integrate at different sites – both to the host chromosome and into plasmids and larger integrative and conjugative elements [9]. Because of this ability, Tn*916* has a higher chance of successful transfer than conjugative transposons that only have the ability to conjugate. Because of the big impact on the host bacteria and its widespread nature, Tn*916* is very important from a clinical view. Tn*916* has a high success rate of transfer to other bacterial strains, and therefore, tetracycline resistance is spreading too. Conjugative transposons like Tn*916* is often found in pathogenic bacteria, and because they spread antibiotic resistance, they represent a serious medical problem [18]. Antibiotic resistance is considered by the World Health Organization as an "increasingly serious threat to global public health" [19].

Tn*916*-like elements may cause multiple heritable changes, as stated by Roberts and Mullany [17]: The elements can disrupt target genes through insertion of Tn*916*. They can generate gene fusions upon insertion, introduce foreign DNA present at the coupling sequences, and remove DNA from target sites upon excision. Tn*916* can be

reversed, it can have upstream or downstream effects on transcription of host genes, and it can introduce foreign DNA in the form of accessory transposons entering the chromosome within Tn*916*. *Tet(M)* can be left immobile present within a genome after deletion events. Tn*916* can interact with other genetic elements such as Tn*5386*, and transactivation with other copies of Tn*916* or mobilization of other mobile elements in the same cell can happen.

This clearly states that conjugative transposons may have a profound effect on the genomic structure in their host cells. This may lead to variation between bacteria, on which natural selection can act. This will in turn contribute to the evolution of bacteria.

2.4.3 How does Tn*916* transfer between bacteria?

Excision of the transposon from the donor DNA is the first step in conjugative transposition of Tn*916* [20]. Staggered endonucleolytic cuts are made at each end of the element. This results in 6 bp protruding 5'-hydroxyl ends called coupling sequences. Then the transposon circularizes and the ends are ligated. In other words, the Tn*916* has now formed a circular intermediate (CI). The target site (from which Tn*916* has excised from) is also ligated. While the excised Tn*916* is single stranded, another strand is synthesized so that the circular intermediate is double-stranded [17]. This ensures that the circular intermediate is capable of insertion in both the donor and recipient. The circular intermediate is transferred by cell-cell contact. A site-specific recombinase is then responsible for inserting Tn*916* into the recipient genome [21]. Tn*916* can insert into a large number of targets in different hosts, but it prefers A:T-rich targets and it has a strongly preferred insertion site in some hosts [17]. Because of this preference for T-rich targets, it has been difficult to distinguish between T's belonging to the transposon, to the coupling sequence, and to the target [18]. More recent research has stated that the coupling sequence consists of 6 base pairs [22].

Mobile genetic elements have evolved to minimize their transcriptional and translational activity, so they only move when they need to in order to ensure their own perpetuation within the cellular population. The advantage of this is that it reduced the burden on the cell carrying the elements. These regulatory mechanisms

are poorly understood, however, a model for regulation of transcription of the genes involved in Tn*916* conjugative transposition has been proposed.

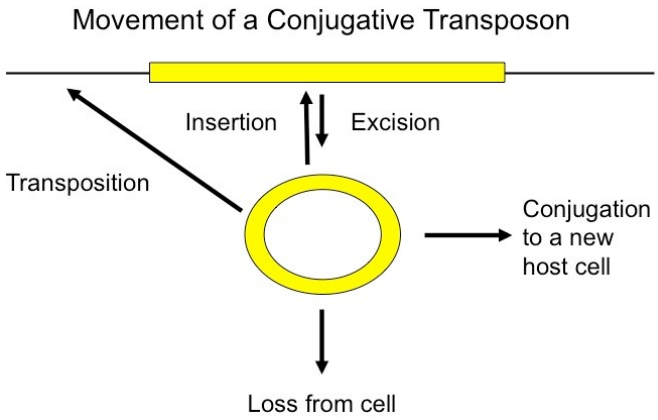

Figure 4: Movement of a conjugative transposon.

2.5 THE BIOLOGICAL COST

Fitness is a measure of the relative survival and reproductive success of a given individual or genotype [23]. Newly acquired resistances can reduce bacterial fitness when compared to their antibiotic-susceptible counterparts – this is termed the biological cost. In the 1980s scientists assumed that antibiotic resistance would disappear from bacterial populations because they thought that in the absence of selective pressure from antimicrobial agents, less fit bacteria would be outcompeted by their more fit susceptible counterparts in the population. An experiment by Irina Starikova *et al.,* showed that acquisition of Tn*916* in *E. Faecium* reduced the relative fitness of the new host by 17% [24] and *E. Faecium* with acquired resistance keep the mechanism of resistance and alleviate the fitness cost through compensatory evolution [24]. Tn*916* has a tight regulation of its conjugative and transposition functions and once acquired, they integrate into the genome [17].

3 HYPOTHESIS

We hypothesized that when *E. faecium* acquires Tn*916*, it will result in a reduced fitness to the bacteria and make *E. faecium* less fit (the element has a fitness cost). This can be tested by investigation of growth curves of different strains of *E. faecium* before and after the acquisition of Tn*916*.

Second, we have a hypothesis that the bacteria will try to get rid of extra Tn*916* elements if it has more than one, in order to reduce the fitness cost. This means that they will carry only one copy of Tn*916*. To investigate this, we will determinate the excision rate of Tn*916*, using ddPCR.

4 MATERIALS AND METHODS (PROTOCOL)

4.1 CULTIVATION OF BACTERIA ON LB AGAR PLATES AND IN LIQUID LB

In these experiments, we used the bacterium *Enterococcus faecium* with the following designations: *E. faecium*$_{4E}$, *E. faecium*$_{A3}$, *E. faecium*$_{A3b}$, *E. faecium*$_{B7}$, *E. faecium*$_{B7a}$, *E. faecium*$_{C1}$ and *E. faecium*$_{C1b}$. All these strains, except *E. faecium*$_{4E}$ are tetracycline resistant. The tetracycline resistance gene is carried on Tn*916*. All necessary preparations for bacteria for the different experimental procedures were done in a safety cabinet to reduce any risk of contamination.

Bacteria were cultivated in LB agar plates prepared by mixing 25 grams LB Broth, 13 grams agar and all dissolved in 1 liter of distilled water. This was mixed well and autoclaved at 121°C for 15 min. When the agar had cooled, the media was poured onto petri dishes and then placed in a refrigerator for future use. When necessary, bacteria were cultivated in LB agar plates with or without 10 µg of tetracycline using a 10-µl inoculation loop. The plates were placed in a 37 °C incubator to grow bacteria for about 24 hours. After 24 hours, if the colonies look normal, the bacteria were further cultivated in LB broth media. Bacteria were grown overnight in 20 ml LB broth at 37 °C.

4.2 GROWTH CURVE FOR THE BACTERIAL STRAINS

To determine if bacterial fitness was affected by acquisition of Tn*916*, bacterial growth curve was analyzed before and after acquisition. In brief, 14 850 µl of LB liquid were distributed into 8 tubes, one for each bacteria plus one negative control, and properly marked with type of bacteria and date of experiment. Approximately 150 µl of the overnight culture was pipetted into the tubes containing fresh LB broth and mixed well to get a homogeneous suspension. Then 200 µl is distributed into a 96-microtiter plate, with the following arrangement:

Table 1: Arrangement of bacterial samples in the microtiter plate.

Well:	1	2	3-5	6	7
Bacteria:	LB	LB	Empty	*E. faecium*$_{4E}$	*E. faecium*$_{A3}$

Well:	8	9	10	11	12
Bacteria:	*E. faecium*$_{A3b}$	*E. faecium*$_{B7}$	*E.* $_{B7}$a	*E. faecium*$_{C1}$	*E. faecium*$_{C1b}$

The plate was placed in the MultiSkan Go microtiter plate reader to read the optical density (OD) over the cultivation period with a reading interval of every 10 minutes (a total of 144 readings per 24th hour). The OD readings were transferred into SkanIt Software 3.0 and the growth curve of each strain was established.

4.3 BACTERIAL DNA EXTRACTION

DNA was extracted from *E. faecium*$_{4E}$, *E. faecium*$_{A3}$, *E. faecium*$_{A3b}$, *E. faecium*$_{B7}$, *E. faecium*$_{B7a}$, *E. faecium*$_{C1}$ and *E. faecium*$_{C1b}$. After 3.5, 7 hours, and overnight growth (mid-log, late-log and stationary phase) to measure the excision rate of Tn*916* by ddPCR at different time point of the growth curve. In brief, an overnight culture of 25 ml was used to transfer 250 µl bacteria into fresh LB media. Bacteria was mixed well and placed in the incubator at 37 °C. After 3.5 hours, 1.5 ml is removed into eppendorf tube, marked with the bacteria name. The 1.5 ml is then centrifuged to pellet bacteria at 3260 G for 10 minutes. Supernatant was then removed and the bacterial pellet was used to extract DNA.

The QIAcube was used to extract DNA using the QIAamp DNA mini-protocol according the manufacturer's instructions. The different tubes and the rotor adaptor were marked properly in each run. After the extraction was finished, the DNA tubes were placed in the freezer (-18°C) for further analysis. The same procedure was repeated for all DNA extractions at the different time points of bacterial growth curve.

4.4 MEASUREMENTS OF DNA CONCENTRATION

The Qubit Fluorometer from Invitrogen Life was used to measure the extracted DNA from all bacteria. Two of the extractions were from 3.5 hours of bacterial growth, at two different days, and two extractions after 7 hours of growth, also at two different

days. The instruction for the machine's user manual was followed to make the standard assays, the different sample assays and to take readings from the assay tubes. A total of 28 sample-tubes were marked with bacterial name and time of extraction.

4.5 AGAROSE GEL ELECTROPHORESIS

The yields of DNA after extraction were checked by agarose gel electrophoresis. In brief, TAE-buffer was made by mixing 20 g TAE buffer (20x) and 1 liter sterilized water. This was done to get the buffer-concentration down to 1x. Agarose gel was made with 1 g agarose and 100 ml TAE buffer.

The solution was mixed well before heated up in a microwave oven for about 90 sec – until the two components had dissolved and the color was transparent. Let it cool down before adding 6 µl DNA stain (gel red) to the solution. Some of this solution was pipetted on the walls and corners of the template of the *gel electrophoresis-unit* to seal it. The rest was poured in from the middle. A comb was placed in the "red zone" at the floor of the template – to make wells for the samples. The gel needed about 30 min to set, and the samples were made while waiting.

The samples was prepared as follows:

Table 2: Preparation of DNA samples for agarose gel electrophoresis

1		
2	10 µl DNA (*E. faecium*$_{A3}$)	2µl loading buffer
3	10 µl DNA (*E. faecium*$_{A3b}$)	2µl loading buffer
4	10 µl DNA (*E. faecium*$_{B7}$)	2µl loading buffer
5	10 µl DNA (*E. faecium*$_{B7a}$)	2µl loading buffer
6	10 µl DNA (*E. faecium*$_{C1}$)	2µl loading buffer
7	10 µl DNA (*E. faecium*$_{C1b}$)	2µl loading buffer

When set, the gel was placed in the electrophoresis-unit chamber and the chamber were filled with TAE-buffer, so it covered the gel. 5 µl ladder was then pipetted in the gel's first well, to determine the size of the different DNA-samples placed in the next 6 wells (well 2-7). This procedure was done in the same order as the overview of the sample preparation. 10µl of each sample were pipetted in the wells. The gel

electrophoresis unit was started with settings; 120 V for 50 min. After the 50 minutes, the gel was placed on the *ChemiDoc touch imaging system* with settings:

- Image size: Medium
- Application: DNA
- Stain: Gel Red.

The picture was taken and visualized.

4.6 ddPCR FOR BACTERIAL STRAINS AT 3.5 HOURS, 7 HOURS AND OVERNIGHT CULTURE

The bacterial DNA extracted after 3.5 hours was diluted down from 1:1 to 1:100, and the bacterial DNA extracted after 7 hours were diluted down from the concentration 1:1 to the concentration 1:500, using the following steps:

Table 3: Dilution of DNA samples

Dilution 1 (D1); 1:10	**Dilution 2 (D2); 1:100**	**Dilution 3 (D3); 1:500**
90 µl Pure Water	90 µl Pure water	80 µl Pure Water
10 µl DNA	10 µl D1	20 µl D2

When making master mix, it was made for 34 samples (because of the 8 wells in the cartridge for the droplet generator and an extra 2µl to ensure enough master mix after splitting/pipetting the master mix). All the components, except Pure Water, are stored on ice and mixed well before pipetting the right amounts to the master mix. The master mix was also stored on ice while it was made.

Master mix, M1:

Table 4: Mastermix for ddPCR

Components	**Sample**	**Total amount in master mix**
10 µl supermix for probes	X 34	= 340 µl supermix for probes
1 µl Tat(M)	X 34	= 34 µl Tat(M)
1 µl Tn*916*-CI	X 34	= 34 µl Total Bacteria
1 µl DNA (1:500 or 1:100 concentration)	X 34	= 34 µl DNA
6 µl pure water	X 34	= 238 µl pure water

20 µl of each master mix was pipetted to the eight lower wells of a cartridge. In the middle wells of the cartridge, 70 µl Oil for Probes are pipetted. When finished with the pipetting, a gasket was attached over the cartridge and placed in the droplet generator that automatically started when the lid closes. The gasket were removed from the cartridge and the solution in the upper wells were checked to have a "milky" color. 40 µl of the milky solution were slowly pipetted to a microtiter plate (first eight samples to the first eight vertical wells of the microtiter plate, and the next three at approximately well 2, 3 and 4). These processes with the droplet generator and microtiter plate were repeated three more times (same process for all the four DNAs). After all the four milky solutions was transferred, the microtiter plate was heat-sealed with a pierceable foil at 180°C. The plate was placed in the PCR machine with settings:

Table 5: PCR amplification program

Program:	**Temperature at step 2:**	**Time:**
DDPCR60	60°C	1:48h

When the PCR machine was finished, the microtiter plate was transferred to the Droplet Reader machine with the following parameters:

Table 6: Settings used in QuantaSoft software

Experiment:	**ABS (absolute quantity)**
Supermix:	ddPCR for probes (no dUTP)
Target 1:	Tat(M)
Channel 1:	Unknown
Target 2:	Tn*916*-CI
Channel 2:	Unknown

The results were read on a computer connected to the droplet reader machine.

5 RESULTS

5.1 CULTIVATION OF BACTERIA

The LB agar plates with *E. faecium*$_{4E}$, *E. faecium*$_{A3}$, *E. faecium*$_{A3b}$, *E. faecium*$_{B7}$, *E. faecium*$_{B7a}$, *E. faecium*$_{C1}$ and *E. faecium*$_{C1b}$ were inspected for bacteria growth. The colonies were of uniform size and shape, and no contamination was seen. No contamination was seen in liquid media and all bacterial growth was ok.

5.2 MEASUREMENT OF DNA CONCENTRATIONS OBTAINED FROM BACTERIAL SPECIES

Results from the DNA concentration measurements are presented in the following tables:

Table 7: DNA concentration of bacterial strains at 3.5 and 7 hours measured at day 1 A and day 2 B.

A)

Samples, 3.5 h day 1	**Results ng/ µl**	**Samples, 7 h, day 1**	**Results ng/ µl**
1. ***E. faecium*$_{4E}$**	0,104	***8.*** ***E. faecium*$_{4E}$**	5,26
2. ***E. faecium*$_{A3}$**	0,047	***9.*** ***E. faecium*$_{A3}$**	0,59
3. ***E. faecium*$_{A3b}$**	0,177	***10.*** ***E. faecium*$_{A3b}$**	4,20
4. ***E. faecium*$_{B7}$**	0,086	***11.*** ***E. faecium*$_{B7}$**	5,04
5. ***E. faecium*$_{B7a}$**	0,064	***12.*** ***E. faecium*$_{B7a}$**	4,48
6. ***E. faecium*$_{C1}$**	0,072	***13.*** ***E. faecium*$_{C1}$**	5,24
7. ***E. faecium*$_{C1b}$**	0,168	***14.*** ***E. faecium*$_{C1b}$**	5,34

B)

Samples, 3.5 h, day 2	**Results ng/ µl**	**Samples, 7 h, day 2**	**Results ng/ µl**
15. **E. *faecium*$_{4E}$**	0,094	**22.** **E. *faecium*$_{4E}$**	5,6
16. ***E. faecium*$_{A3}$**	0,049	**23.** ***E. faecium*$_{A3}$**	0,528
17. ***E. faecium*$_{A3b}$**	0,212	**24.** ***E. faecium*$_{A3b}$**	4,42
18. ***E. faecium*$_{B7}$**	0,158	**25.** ***E. faecium*$_{B7}$**	6,14
19. ***E. faecium*$_{B7a}$**	0,116	**26.** ***E. faecium*$_{B7a}$**	4,44
20. ***E. faecium*$_{C1}$**	0,087	**27.** ***E. faecium*$_{C1}$**	5,18
21. ***E. faecium*$_{C1b}$**	0,170	**28.** ***E. faecium*$_{C1b}$**	4,88

5.3 AGAROSE GEL ELECTROPHORESIS

Figure 5 shows agarose gel electrophoresis of 15 bacterial DNA extractions. The first lane represents 1 Kb ladder.

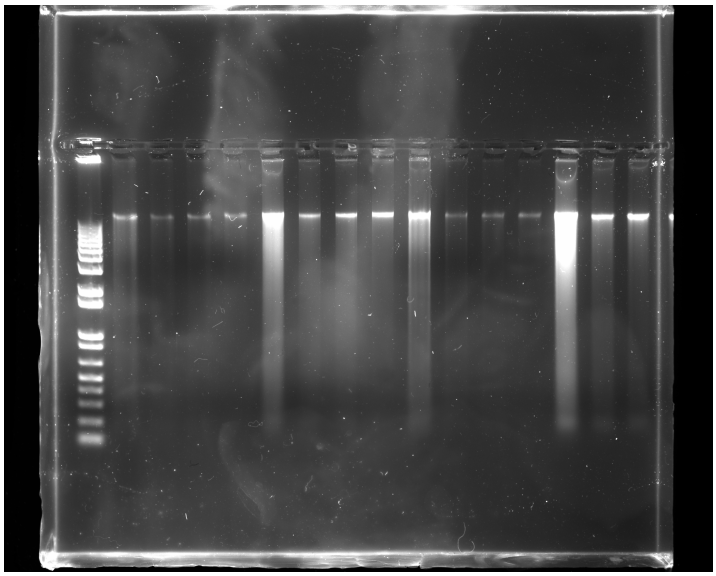

Figure 5: Results from agarose gel electrophoresis.

5.4 GROWTH CURVES

The blue line represents *E. faecium*$_{4E}$, the green line represents *E. faecium*$_{A3}$, the red line represents *E. faecium*$_{B7}$, and the yellow line represents *E. faecium*$_{C1}$. The growth curves showed reduce fitness of *E. faecium*$_{A3}$ and *E. faecium*$_{C1}$ that contain Tn*916* while better fitness for *E. faecium*$_{B7}$ that contains the same element compared to *E. faecium*$_{4E}$ that harbor no Tn*916*.

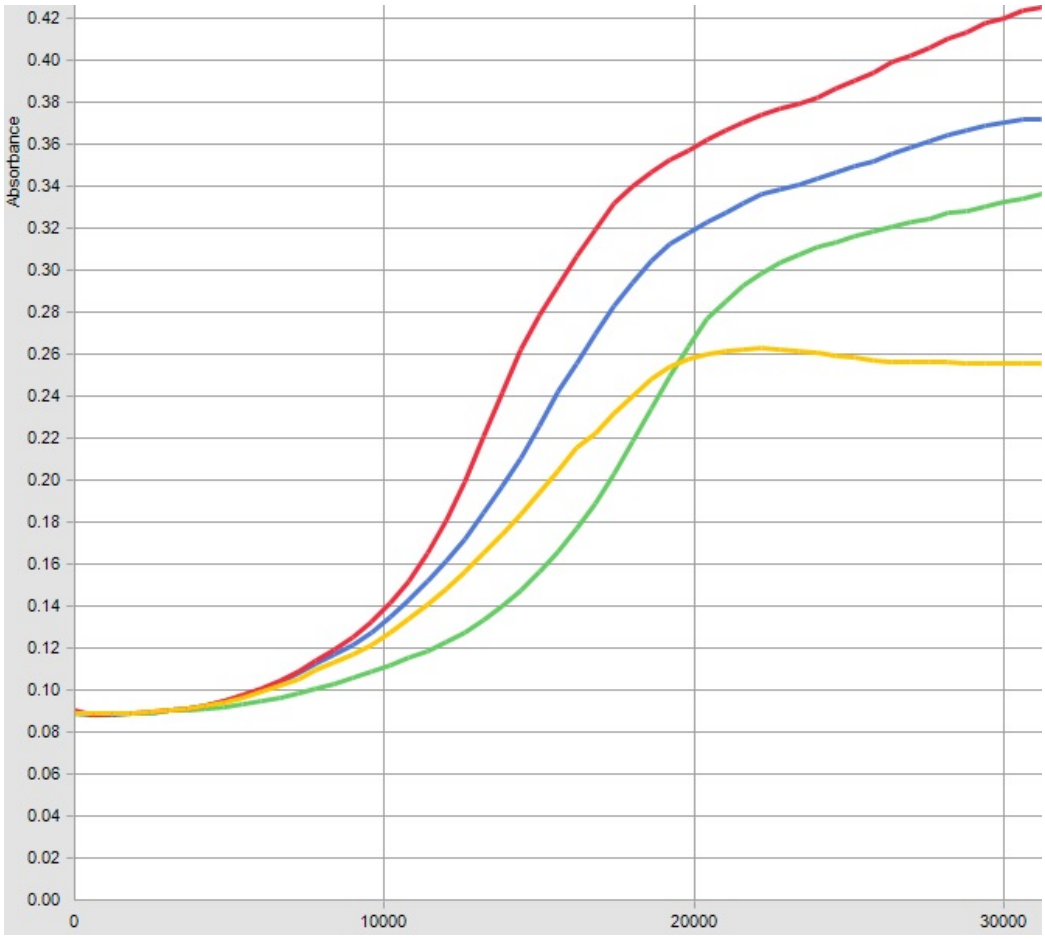

Figure 6: Growth curves of E. faecium$_{4E,}$ *E. faecium*$_{A3,}$ *E. faecium*$_{B7}$ *and E. faecium*$_{C1,}$ *E. faecium*$_{A3}$ *shows the most reduced fitness while E. faecium*$_{B7}$ *seems to be more fit than its ancestor*

5.5 OPTIMIZATION OF DDPCR

Optimization of the digital droplet PCR has been done many times with different temperatures and DNA concentrations. If the DNA concentration is too high, the only result in QuantaSoft will be "No Call". In that situation, there are too many positive droplets and too few negative droplets. Some primers and probes are also very sensitive to the temperature, as seen in figure below. In this example, we used *Bacillus subtilis* with the primer *Tn916-CI* with a temperature gradient ranging from

53-59 °C. Positive droplets are green and the negative droplets are black. The separation gradually gets better from left to right; F07, G07 and H07 shows good separation, while the separation in A07, B07 and C07 is very bad. The optimization was done multiple times until the results were acceptable.

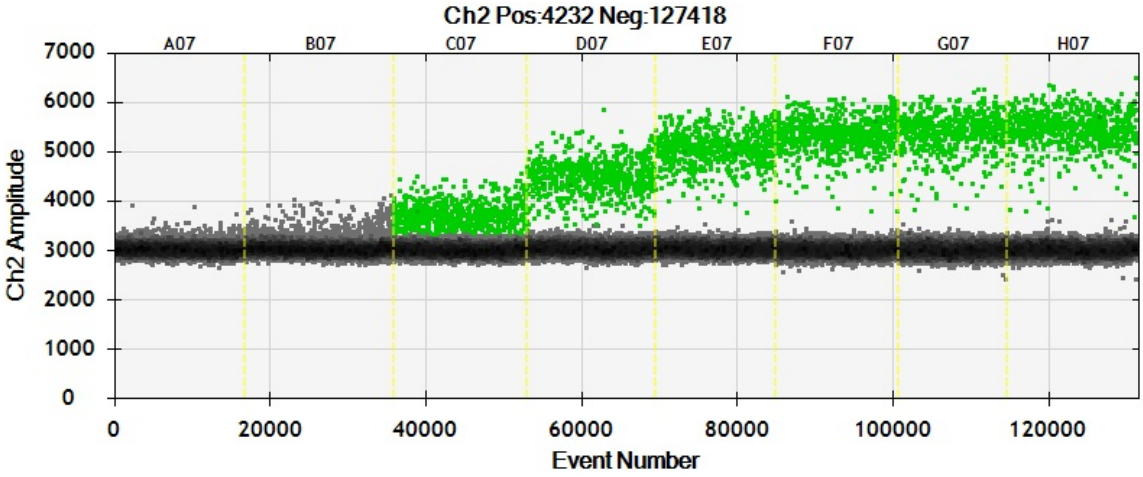

Figure 7: A screenshot from QuantaSoft is showing optimization the annealing temperature to separate between positive and negative droplets.

5.6 EXCISION OF TN916 IN BACTERIAL STRAINS

We used 20 picogram of DNA to investigate the excision rate of Tn916 in our bacterial strains at 3.5, 7 and 24 hours. We decided to work only with 7 and 24 hours because it was difficult to get DNA from the 3.5 hour samples. In fact, DNA from bacterial samples at 3.5 and 7 hours would be the same because they are in the same growth phase (log phase). Figure 8 present the excision rate of Tn*916* at 7 and 24 hours of from *E. faecium*$_{A3,}$ *E. faecium*$_{B7}$ *and E. faecium*$_{C1}$, *E. faecium*$_{A3b,}$ *E. faecium*$_{B7a}$ *and E. faecium*$_{C1b}$.

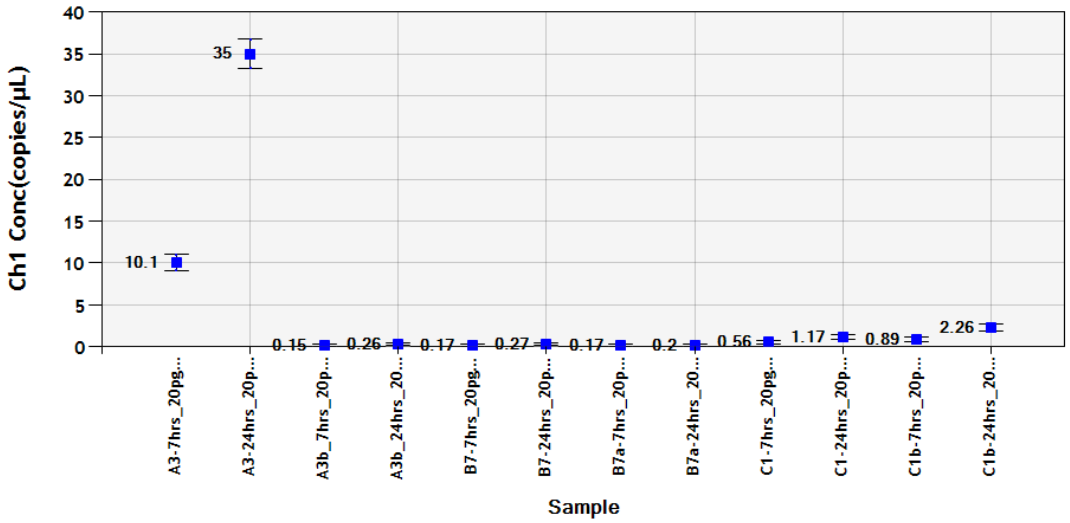

Figure 8: Excision of Tn*916* from *E. faecium*$_{A3,}$ *E. faecium*$_{B7}$ *and E. faecium*$_{C1}$*, E. faecium*$_{A3b,}$ *E. faecium*$_{B7a}$ *and E. faecium*$_{C1b}$

6 DISCUSSION

6.1 DOES TN*916* REDUCE THE FITNESS OF *E. FAECIUM*?

We hypothesized that acquisition of Tn*916* will result in a reduced fitness to *E. faecium* and make it less fit. The investigation of growth curves of *E. faecium*$_{4E}$ (which does not have the element Tn*916*), *E. faecium*$_{A3}$, *E. faecium*$_{B7}$, *E. faecium*$_{C1}$ shows us that the growth can be downregulated *or* upregulated. In our growth curves, the growth rates of *E. faecium*$_{B7}$ are obviously higher than that of *E. faecium*$_{4E}$. At the same time, the growth of *E. faecium*$_{A3}$ and especially *E. faecium*$_{C1}$ is considerably lower than that of *E. faecium*$_{4E}$. In other words, the *E. faecium*$_{A3}$ and *E. faecium*$_{C1}$ seems to be less fit than *E. faecium*$_{4E}$, while *E. faecium*$_{B7}$, seems to be more fit than *E. faecium*$_{4E}$.

If our hypothesis was right, *E. faecium*$_{4E}$ should be the fastest-growing strain, e.g. it should be the fittest bacterium. When Tn*916* is acquired, there is a possibility that it can insert itself into sites where it will disturb essential genes – in this case in genes that are responsible for (down regulation of) the growth rate. In bacteria, there are genes with different functions and responsibilities: some are responsible for speeding the growth, others for slowing the growth. In a limited environment where there is no food, the bacteria should down regulate the growth to survive more. When there are enough nutrients, then there is no point to down regulate the growth rate. If the element inserts itself into a region with genes responsible for speeding down the growth, and thereby disturb this function, the bacteria will grow slower. To find out what genes are affected in our bacteria, it will be necessary to sequence the whole genome of the bacteria. Because we know the sequence for Tn*916*, it is possible to locate it and find out which genes it may have been disturbed.

Regarding the second part of our hypothesis, it appears that only *E. faecium*$_{A3}$ and *E. faecium*$_{C1}$ became less fit because of acquiring Tn*916*, most probably because of multiple copies of the element. The bacteria with the newly acquired *Tn916* might get rid of extra copies of the element as a probable mechanism to restore fitness. The higher excision rate we found in *E. faecium*$_{A3}$ might suggest that the bacterium, indeed, is trying to get ride off extra Tn*916* inserted in its genome. These results confirm the second part of our hypothesis, at least in part, that a bacteria will try to get

rid of extra element, in order to reduce the fitness cost, this means that they will carry less copies of Tn*916*.

Because of this result, these are the two strains that should be investigated through ddPCR, together with the *E. faecium*$_{4E}$, which does not contain Tn*916*.

Clinically these findings are of great importance because they show that bacteria exposed for antibiotics will develop resistance and that they will find methods to cope with the fitness cost of having extra genes within 40 days and still carry the genes coding for antibiotic resistance, while they are becoming more fit again.

An explanation for the increased fitness of the evolved bacteria is the possibility that the evolved bacteria have muted. The genome of the non-evolved and the evolved bacteria have to be sequenced to know this for sure.

6.2 COMMENTS ON THE METHODOLOGY

6.2.1 Optimizing temperature in ddPCR

Before PCR amplifying can take place, the DNA strands need to be separated. This happens at 95 degrees. The second step is the most critical step in the ddPCR and is called annealing process. In order to get the primers and probe to sit at the right place on the separated DNA strands the process need an optimal temperature. Different DNA from different bacteria, and different primers and probes need optimal temperature for this procedure.
The third step in the amplification process is called synthesizing when nucleotides binds to the separated strands. We run the cycle 40 times and if we started with one molecule in the reaction we could ended up with approximately 2^{40} copies of the target DNA. This amount of DNA is of a sufficient number for presenting representable data to be detected by the methodology implemented in the current project.

In this project, we faced a challenge when the software after ddPCR gave the result “no call”. This could be either related to the concentration of DNA, or to the temperature used in the annealing process was not optimal.

6.2.2 Optimizing DNA concentration in ddPCR

In order to get good results of the ddPCR, optimal amount of DNA input into the system is very important to accurately absolute quantification of the target molecule in ddPCR.. The system is really sensitive for DNA concentration in order to the calculations. We measured the concentration of our DNA and diluted it to get the optimal input amount.

7 CONCLUSION

We hypothesized that when *E. faecium* acquires Tn*916*, it will result in a reduced fitness to the bacteria and make *E. faecium* less fit (the element has a fitness cost). The result from the growth curve determination indicates that this is not necessarily the case for every situation: in our study, two bacterial strains which newly had acquired Tn*916* became apparently less fit, while one bacterial strain actually seemed to become more fit.

Second, we had a hypothesis that the bacteria will try to get rid of extra Tn*916* elements if it has more than one in order to reduce the fitness cost. This means that they will carry only one copy of Tn*916*. Our result indicates that this is true. Of course we cannot generalize these findings to other bacteria than *E. faecium* and to other mobile genetic elements other than Tn*916* because each bacteria and each element with resistance genes act differently. This is the reason that further research on this theme is of great importance.

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