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Conjugation-driven horizontal gene transfer in bacteria on antimicrobial surfaces

Bachelor's Thesis (12 ECTS)

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Conjugation-driven horizontal gene transfer in bacteria on antimicrobial surfaces

Abstract:

Conjugation is the main mechanism of horizontal gene transfer of antibiotic resistance genes between bacteria. There is evidence that the presence of trace amounts of antimicrobials in the environment may increase the conjugation frequency between bacteria and thus, potential spread of antibiotic resistance genes. However, to date the effect of stress conditions, including the presence of antimicrobials, on conjugation is relatively modestly studied and almost no studies are available about the effect of antimicrobial surfaces on conjugation frequency. This study first constructed an optimized conjugation protocol for solid surfaces. For that, a chromosomally marked strain of *Pseudomonas putida* KT2440 to be used as recipient strain in conjugation, was constructed. As a donor strain, *Escherichia coli* CSH26 with conjugative plasmid pKJK5 was used. The optimal experimental conditions: organic content, conjugation time, and donor-to-recipient ratio on conjugation frequency, were studied. For the conjugation experiments, a series of copper- silver- and quaternary ammonium-based surfaces that were expected to show an antimicrobial effect, were selected. Plastic and stainless-steel surfaces served as controls with no expected antimicrobial activity. Prior to conjugation, viability of donor and recipient on test surfaces was analyzed and the most toxic surfaces were not included in conjugation assay. On selected surfaces, conjugation experiments were carried out for 24 h and conjugation frequencies were calculated for each surface. The results showed that the presence of low level of copper on surface may promote conjugation while silver- and quaternary ammonium compounds-based surfaces did not significantly affect conjugation frequency.

Keywords:

Bacterial conjugation, *Escherichia coli, Pseudomonas putida*, horizontal gene transfer, plasmids, antibiotics resistance, metals, copper, silver, quaternary ammonium compounds

CERCS: B230 Microbiology, bacteriology, virology, mycology

Konjugatiivne horisontaalne geeniülekanne bakterirakkude vahel antimikroobsetel pindadel

Lühikokkuvõte:

Konjugatsioon on protsess, mis võimaldab geneetilise materjali liikumist rakust rakku ning mida on peetud üheks peamiseks horisontaalse geeniülekande viisiks bakteripopulatsioonides. Mõningad uuringud on näidanud, et antimikroobsete ühendite madalate kontsentratsioonide esinemine kekskonnas võib tõsta bakterite konjugatsioonisagedust ja seeläbi on võimalik ka antibiootikumiresistentsuse geenide leviku sagenemine. Tuleb aga tõdeda, et tänaseni on stressitingimuste, sealhulgas antimikroobsete ainete mõju, konjugatsioonile suhteliselt vähe uuritud ning konjugatsiooni kohta antimikroobsetel pindadel uuringud praktiliselt puuduvad. Käesoleva töö käigus koostati esmalt tahkete pindade jaoks sobivalt optimeeritud konjugatsiooniprotokoll. Konjugatsiooni retsipienttüveks konstrueeriti fluorestseeruvalt märgistatud ning sobivat resistentsusmarkerit kandev *Pseudomonas putida* KT2440. Doonortüvena oli kasutusel *Escherichia coli* CSH26, mis sisaldab konjugatiivset plasmiidi pKJK5. Selgitati välja ka optimaalsed katsetingimused: orgaanikasisaldus konjugatsioonikeskkonnas, konjugatsiooni aeg ning doonori ja retsipiendi suhe. Konjugatsiooni uurimiseks valiti rida vase, hõbeda ja kvaternaarse ammooniumi põhiseid pindu, millel kas oli varasemast teada antimikroobne toime või mida turustati kui antibakteriaalset toodet. Antimikroobse toimeta kontrollpindadena olid kasutusel plastikust ja roostevabast terasest pinnad. Enne konjugatsiooni hinnati donor- ja retsipientbakteri suremust antimikroobsetel testpindadel ning pindu, millel esines olulisel määral suremust, konjugatsioonikatsetesse ei kaasatud. Valitud pindadel viidi konjugatsioonikatsed läbi 24 tunni vältlel ning iga pinna jaoks arvutati välja konjugatsioonisagedused. Tulemused näitasid, et madal vasesisaldus pinna materjalis võib soodustada bakterite vahelist konjugatsiooni, samas kui hõbedal ja kvaternaarsetel ammooniumiühenditel põhinevad pinnad konjugatsioonisagedust oluliselt ei mõjutanud.

Võtmesõnad:

Konjugatsioon, *Escherichia coli, Pseudomonas putida*, horisontaalne geeniülekanne, plasmiidid, antibiootikumiresistentsus, metallid, vask, hõbe, kvaternaarsed ammooniumiühendid

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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INTRODUCTION

It is known that unfavorable environments, including exposure to antimicrobials, induce various stress response pathways in bacterial cells. The outcome of stress response pathways is a change in bacterial physiology, which, among other outcomes, may lead to higher rate of mutation or enhanced exchange of genetic information (Guo and Zhang, 2017; Jiao *et al.*, 2017; Jutkina *et al.*, 2018; Lu *et al.*, 2018, 2020; Wang *et al.*, 2018; Zhang *et al.*, 2018; Jia *et al.*, 2021; Tang *et al.*, 2022). Those processes may in turn increase bacterial resistance towards the specific unfavorable environment, including specific chemicals such as metals or antibiotics (Klümper *et al.*, 2017; Argudín, Hoefer and Butaye, 2019).

One key pathway how bacterial community may exchange genetic information, including a variety of resistance genes, is conjugation (von Wintersdorff *et al.*, 2016). Conjugation was discovered in 1946 by Edward Tatum and Joshua Lederberg, who revealed a directional exchange of genetic information between bacteria, mediated by a so-called F (Fertility) factor (Lederberg and Tatum, 1946). It was later realized that the F factor is a replicative extrachromosomal genetic element – a plasmid, that can be transferred across bacterial cells.

Conjugation involves a physical contact between a donor and a recipient bacterium, formation of sex pili between those cells, and transfer of conjugative plasmid from donor to recipient (von Wintersdorff *et al.*, 2016). Conjugation is one of the main mechanisms of horizontal gene transfer (HGT), and the reason why conjugation is so important is because it can occur not only among members of the same bacterial species but also between different bacterial species (Norman, Hansen and Sørensen, 2009; Klümper *et al.*, 2015; Alderliesten *et al.*, 2020). Moreover, often plasmids, that are transferred during conjugation contain genes conferring resistance towards metal ions, alternative metabolic pathway genes, and antibiotic resistance genes. This, in turn, may increase the presence of bacteria resistant to antibiotics or other antimicrobial compounds, and become a serious human health hazard (Bahl *et al.*, 2007, p. 20172201; Popowska and Krawczyk-Balska, 2013; Klümper *et al.*, 2017).

Despite the relatively high number of studies examining HGT in bacterial communities *via* conjugation, including in stress conditions, the methods used, and results obtained, vary greatly. Bacterial conjugation under various stressors, including antimicrobial exposure has thus far been studied mostly in liquid or filter mating formats. However, as the focus of the current study is on antimicrobial surfaces, a solid surface exposure protocol was first developed to study conjugation and necessary strains with chromosomally expressed resistance markers and green fluorescent protein (GFP) were constructed. In parallel with conjugation

experiments, bacterial viability on antimicrobial surfaces was assessed, conjugation time was optimized, and conjugation frequency calculations were carried out. Finally, conclusions on the potential of antimicrobial surfaces to enhance HGT and by that, exchange of potential resistance genes *via* conjugation were drawn.

1 LITERATURE REVIEW

1.1 Conjugation in bacteria

All living organisms can multiply – transfer their genetic information to the offspring. Bacteria are not an exception, multiplying by binary fission vertically transmitting genes from parental cells to daughter ones. However, one of the characteristic features of bacterial genetics is HGT, in which case genetic material is spread between bacterial cells without cellular multiplication (Burmeister, 2015). The three possible routes of HGT are transduction in which case genetic material is spread with the help of bacterial cells, transformation which refers to the uptake of free DNA from the environment, and conjugation in the case of which genetic information moves from cell to cell (Burmeister, 2015). The most common mode *via* which HGT and spread of antibiotic resistance genes (ARGs) occurs in healthcare settings is conjugation (Huddleston, 2014). During conjugation, usually "specific genes" within conjugative plasmids – extra-chromosomal DNA entities that bacteria can transfer among individuals independently from the chromosome, will be transferred from cell to cell (Lujan *et al.*, 2007; Rozwandowicz *et al.*, 2018). The result of conjugation is formation of a new cell with "specific features" that are carried by the conjugative plasmid.

Conjugative transfer of plasmid DNA is recognized as a main route for antibiotic resistance spread within and between bacterial strains (Lujan *et al.*, 2007; Graf *et al.*, 2019; Waksman, 2019; Alderliesten *et al.*, 2020; Palm *et al.*, 2022; Piscon *et al.*, 2023). Moreover, conjugative elements – plasmids, act as a reservoir to maintain antibiotic resistance in the bacterial population even in the absence of antibiotic pressure. Therefore, antibiotic resistance can quickly spread between bacteria of the microbiome and pathogens when selective pressure, e.g. antibiotics are introduced (Graf *et al.*, 2019).

Over the past several decades, numerous studies have shed light on the molecular mechanisms underlying conjugation in bacteria (Lujan *et al.*, 2007; Wallden, Rivera-Calzada and Waksman, 2010; Waksman, 2019). Conjugation requires a physical contact between a donor cell, carrying a conjugative plasmid and a recipient cell (von Wintersdorff *et al.*, 2016; Waksman, 2019). Conjugation process relies on a large membrane-anchored machinery type IV secretion system (T4SS) and a DNA processing complex – relaxosome (Lujan *et al.*, 2007; de la Cruz *et al.*, 2010; Wallden, Rivera-Calzada and Waksman, 2010; Waksman, 2019; Piscon *et al.*, 2023). The T4SS consists of a pilus, a transport apparatus, and a type IV coupling protein (T4CP). The relaxosome complex involves a relaxase protein and accessory

proteins that bind specifically to the origin of transfer of the plasmid. These components are encoded on the conjugative plasmid by transfer (*tra*) genes (Graf *et al*., 2019). According to the currently accepted model for Gram-negative bacteria, the general mechanism of conjugation is as follows (Figure 1):

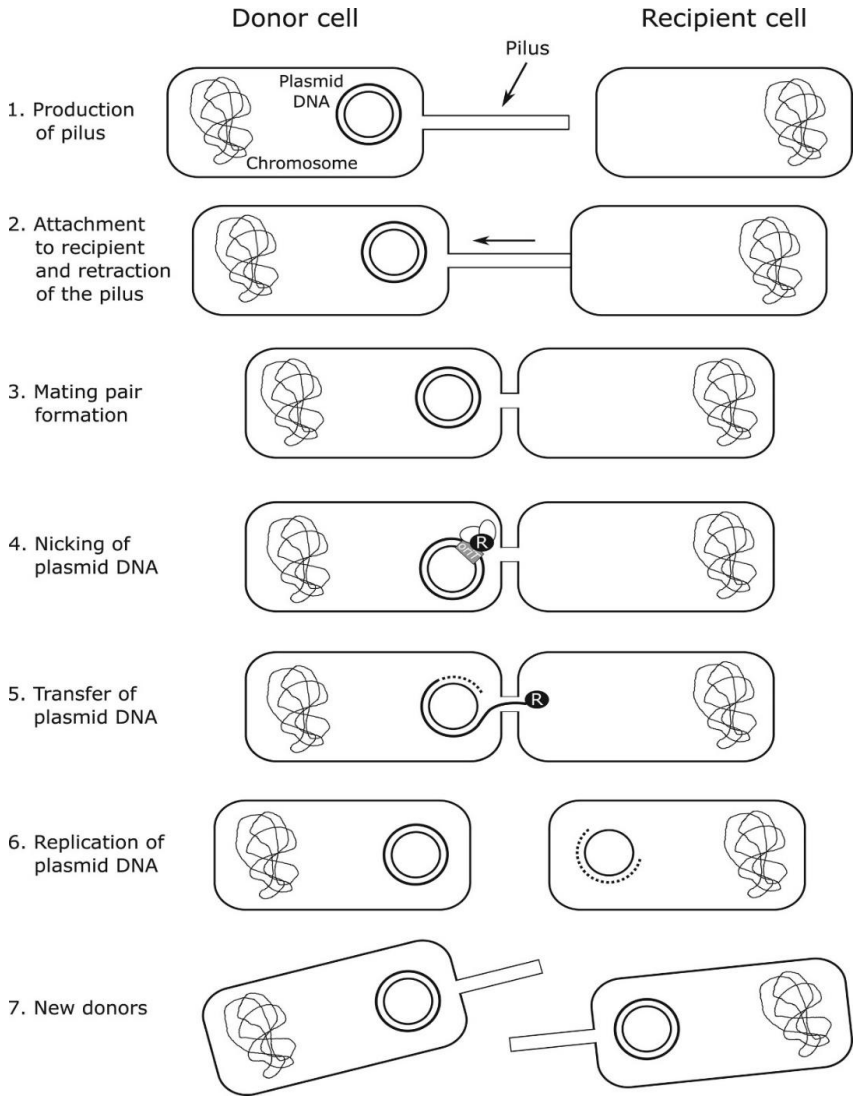

Figure 1. Overview of bacterial conjugation in Gram-negative bacteria. 1. A donor cell produces a pilus; 2. The pilus attaches to the recipient cell and retracts to bring the pair of cells together; 3. When the two cells are close together, a pore is formed between them, connecting them as a mating pair; 4. The relaxosome binds to the *oriT* ansd the relaxase nicks and covalently binds to one of the strands of the plasmid DNA; 5. The DNA–relaxase complex is transferred to the recipient cell while the DNA in the donor is replicated; 6. The relaxase then performs a reverse nicking reaction to recircularize the single-stranded DNA; 7. The DNA replicated in the recipient cell, resulting in two cells carrying the conjugative plasmid and being able to act as donors. Figure source – Graf *et al*., 2019 with permission.

In Gram-negative bacteria, at least 12 proteins compose the T4SS responsible for pilus biogenesis and transferring the conjugation cargo. The genetic framework of these systems is organized into two functional groups: DNA transfer and replication (Dtr) module and the mating pair formation (Mpf) system (de la Cruz *et al.*, 2010). The Mpf system is crucial for the production of conjugative pili and cell-to-cell junction formation, which enables the translocation of DNA-protein substrates (de la Cruz *et al.*, 2010).

Despite numerous studies on conjugation, several aspects remain poorly understood. For instance, the factors that determine the frequency and specificity of DNA transfer between bacterial strains are still unclear (S. Sørensen *et al.*, 2003).

1.1.1 Conjugative plasmids

Plasmids are extra-chromosomal DNA entities, self-replicating genetic elements, naturally found in bacteria (Actis, Tolmasky and Crosa, 1999). These small circular DNA molecules have been identified in a wide range of bacterial species and are known to play key roles in bacterial adaptation, evolution, and pathogenesis (Actis, Tolmasky and Crosa, 1999; Davison, 1999; Virolle *et al.*, 2020; Piscon *et al.*, 2023). However, nowadays, scientists can use them as molecular tools to clone, transfer, and manipulate genes. Plasmids, that are used experimentally for these purposes are called vectors. Researchers can insert DNA fragments or genes into a plasmid vector, creating a so-called recombinant plasmid. Recombinant plasmids can be introduced into a bacterium via the process called transformation.

Besides vectors, there are plasmids, that bacteria can directly transfer in natural conditions. These are conjugative plasmids. Such plasmids contain all necessary genes, required for conjugation to happen (Virolle *et al.*, 2020). A plasmid without these genes can get into a bacterium only by transformation. With the help of the conjugative plasmids, bacteria spread genes within and between species, resulting in genetic variation among individuals (Klümper *et al.*, 2015). Conjugative plasmids are well known for carrying “special” genes, that confer important survival properties, frequently necessary for bacteria under stress conditions and may give the bacterium an advantage in a highly competitive environment (Popowska and Krawczyk-Balska, 2013; Klümper *et al.*, 2015).

Traditionally, plasmids are grouped into incompatibility (Inc) groups based on their inability to be stably propagated together with other plasmids that share similar systems for replication and partitioning. In *Enterobacteriaceae* there are 28 Inc groups and, at least, four (IncF,

I, A/C, and H) are notoriously responsible for antibiotic resistance (ABR) (Rozwandowicz *et al.*, 2018). Inc groups are classifications that are based on the compatibility or inability to coexist within the same bacterial host due to the presence of specific replication and maintenance mechanisms. These Inc groups provide a way to categorize and classify plasmids based on their genetic properties.

There are numerous Inc groups identified for conjugative plasmids. Conjugative plasmids usually belong to groups such as:

IncF: *Enterobacteriaceae*-associated plasmids. They are often carry resistance to antimicrobial agents (Rozwandowicz *et al.*, 2018).

IncP: They are known for their broad host range. Low-copy-number plasmids ranging in size from 70 to 275 kb. They are often associated with resistance to antimicrobial agents such as (extended) spectrum b-lactams, sulphonamides, aminoglycosides and tetracyclines (Rozwandowicz *et al.*, 2018).

IncW: This group includes plasmids that are often found in *Pseudomonas* and have been associated with the spread of antibiotic resistance. Low-copy number, broad-host-range plasmids with sizes up to 40 kb. Was shown to carry genes conferring resistance to chloramphenicol, tetracyclines, sulphonamides, gentamicin and trimethoprim (Rozwandowicz *et al.*, 2018)

One example of conjugative plasmids, is pKJK5, a plasmid used in this study and belonging to IncP-1ε group (Bahl *et al.*, 2007; Popowska and Krawczyk-Balska, 2013).

Most of the conjugative plasmids are very large, reaching even hundreds of kb and carrying all the genes required for the conjugation process as well as specific, such as resistance genes (Norman, Hansen and Sørensen, 2009). Conjugation specific genes, e.g., those in *tra* and *trb* loci, are those required to create the physical contact between donor cell and recipient cell (Ochman, Lawrence and Groisman, 2000). *tra* and *trb* loci are about 33 kb long and consist of about 40 genes. The Tra1 region (*tra* genes) includes the pilin gene and regulatory genes, which together form pili on the cell surface – responsible for DNA transfer and replication. Tra2 region (*trb* genes) incorporates genes for the proteins that attach themselves to the surface of recipient bacteria and initiate conjugation – responsible for mating pair formation (Bahl *et al.*, 2007).

Many conjugative plasmids carry, in addition to conjugation-specific genes, also all the genes required for their replication (Norman, Hansen and Sørensen, 2009). Studies of conjugative plasmids has revealed considerable diversity in terms of genetic properties and organization of plasmids (Virolle *et al.*, 2020). This diversity indicates, that different plasmids have various regulations, molecular reactions, and strategies to achieve productive conjugational transfer and maintenance (Virolle *et al.*, 2020). This means that being quite a universal process, conjugation, on other hand, is controlled differently in respect to bacterial strains, plasmids, and external conditions, meaning, that depending on environmental conditions different conjugative plasmids might modify host physiology differently with different outcome on conjugation rate (Thompson *et al.*, 2023).

1.1.2 The role of conjugation in horizontal gene transfer

Horizontal gene transfer, HGT – is a unique phenomenon, occurring in bacterial community, that allows bacterial cells to acquire genetic material either directly from the environment (transformation) or from other cells in the same population (conjugation). HGT may thus enable the acquisition of new features such as resistance or tolerance towards chemicals. As already described above, HGT can take place *via* three major canonical mechanisms: transformation, transduction and conjugation, and a fourth mode of intercellular DNA transfer called vesiduction, which involves secretion and uptake of extracellular vesicles, are known (Figure 2) (Alderliesten *et al.*, 2020; Piscon *et al.*, 2023).

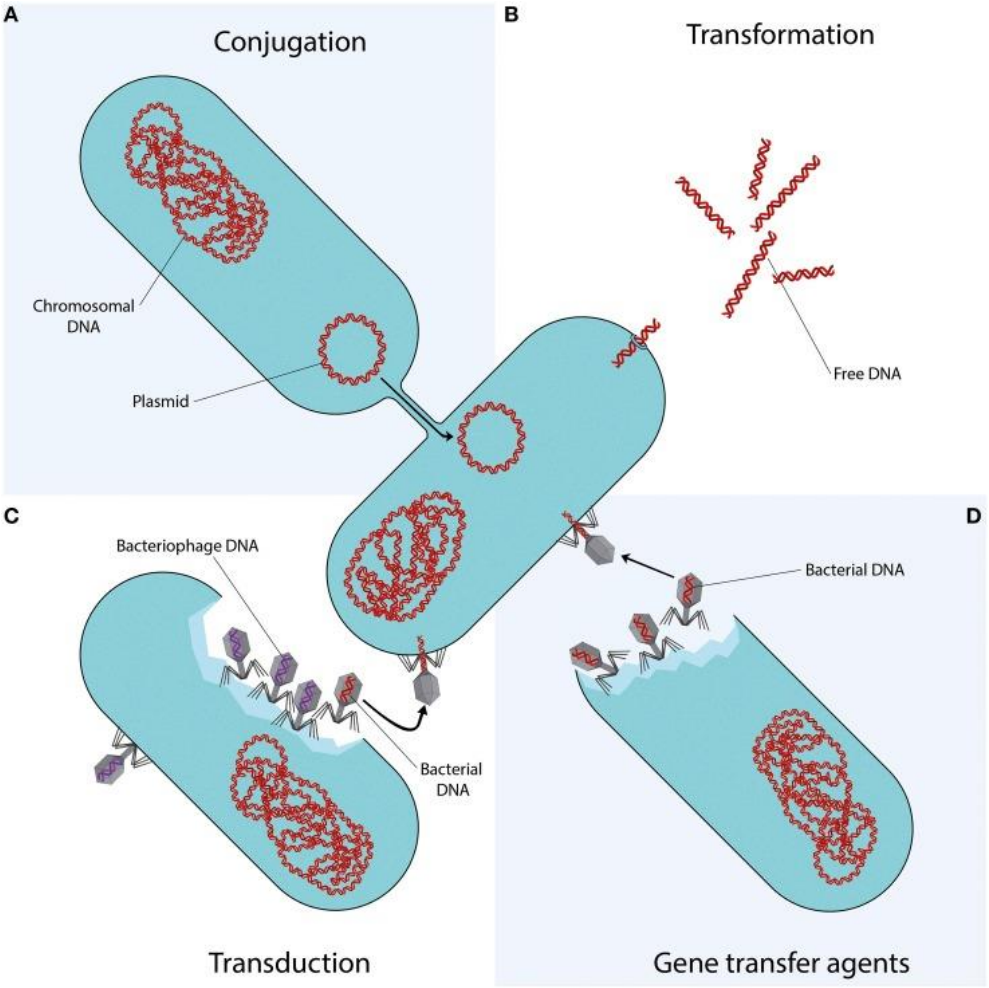

Figure 2. Mechanisms of horizontal gene transfer. Each sector represents one method of gene transfer. (A) – Conjugation; (B) – Transformation. Uptake, integration, and functional expression of naked fragments of extracellular DNA; (C) – Transduction. Bacteriophages may transfer bacterial DNA from a previously infected donor cell to the recipient cell; (D) – Gene transfer agents (GTAs). Bacteriophage-like particles that carry random pieces of the producing cell's genome. GTA particles may be released through cell lysis and spread to a recipient cell. Figure source – von Wintersdorff *et al.*, 2016 with permission.

One of the critical outcomes of HGT, mostly by conjugation, is the spread of ARGs among the bacterial communities. Conjugation is the main mechanism of ARGs spread due to several reasons: conjugation, in general, is a broad host process, meaning that it can occur between different bacterial classes and even families, conjugation is more safe for a bacterium and more precise than transformation or transduction (von Wintersdorff *et al.*, 2016;

Alderliesten *et al.*, 2020; Piscon *et al.*, 2023). Antibiotic resistance spread in bacteria is a growing crisis worldwide. In recent years, the role of HGT in the dissemination of ARGs in the environment has become a serious concern to public health officials and doctors. There is a growing concern that the presence of antibiotics in the environment can act as a stressor to induce HGT of ARGs (Headd and Bradford, 2018). According to previous research, in 2019 there were an estimated 4,95 million deaths associated with bacterial antimicrobial resistance, with 1,27 million deaths directly attributed to bacterial antibiotic resistance (Murray *et al.*, 2022). Some resistant strains have been classified as a critical priority by the World Health Organization, WHO (Tacconelli *et al.*, 2018).

Conjugation was shown to be a ubiquitous process that occurs in bacterial communities present in environments such as the soil, on plant surfaces, and in water and sewage, as well as in biofilms and bacterial communities associated with plant or animal hosts (Davison, 1999). Conjugation facilitates the adaptation of bacterial strains by establishing in the population advantageous metabolic properties, such as symbiotic lifestyle, virulence, or resistance to heavy metals and antimicrobials. Conjugation is, therefore, a major driver of the rapid evolution of bacterial genomes (Virolle *et al.*, 2020).

Despite several years of research, there is still a considerable lack of information on the importance of HGT in various bacterial communities that live in different environmental conditions (S. Sørensen *et al.*, 2003; Alderliesten *et al.*, 2020; Piscon *et al.*, 2023).

1.1.3 Conjugation in stress conditions

The environment is a dynamic system, with constantly changing parameters such as temperature, humidity, and other abiotic and biotic factors. As a result, every living organism, from bacteria to mammals, is subject to some level of stress, which can be defined as any suboptimal condition that arises in the environment. The term "stress" encompasses a broad range of physiological and molecular responses that enable an organism to cope with adverse conditions and maintain homeostasis. While some level of stress is necessary for the proper functioning of an organism, excessive or prolonged exposure to stress can pose a direct threat to the organism's well-being and activate stress-response mechanisms. Stress-response mechanisms are a complex network of physiological and molecular pathways that enable an organism to detect and respond to stress. These pathways involve the activation of stress sensors, such as heat shock proteins, transcription factors, and signaling molecules, which

trigger downstream responses that can include changes in gene expression, metabolism, and bacterial physiology.

Bacterial conjugation is a complex and energy-expensive process, which requires sensing and regulatory strategies to ensure accurate *tra* gene expression at the precise time and place (Piscon *et al.*, 2023). The expression of *tra* genes is tightly regulated by various plasmid and host factors that collaborate to suppress or activate the expression of conjugation genes when unfavorable conditions arise. It has been observed that quorum sensing, i.e., the co-presence of certain number of bacteria, and a response to specific environmental cues such as oxygen levels, nutrients, and temperature are generally required to induce *tra* gene expression (Piscon *et al.*, 2023). There is evidence, that the background presence of antimicrobial compounds and even non-antibiotic chemicals such as artificial sweeteners, nanomaterials, ionic liquids, and disinfectants may stimulate HGT *via* conjugation, because these environmental stimuli may induce a variety of stress responses such as formation of intracellular reactive oxygen species (ROS), followed by cell membrane damage, induction of SOS response, and the enhanced expression of conjugation-related genes (von Wintersdorff *et al.*, 2016; Klümper *et al.*, 2017; Li *et al.*, 2018; Graf *et al.*, 2019; Headd and Bradford, 2020; Ye *et al.*, 2022).

In earlier studies the effect of a variety of stressors on conjugation frequency has been investigated. Mostly such stressors have been chemical exposures or short wavelength illumination (Li *et al.*, 2018). In general, the effect of chemical or light exposure on conjugation frequency has been concentration-dependent and at low concentrations, conjugation has become more frequent while at high concentrations, the frequency of conjugation has decreased. For example, exposure of *Escherichia coli* to silver nanoparticles, iron minerals, graphene oxide nanostructures, copper oxide nanomaterials or zinc oxide nanoparticles, copper, silver or chromium ions, waste chemicals from textile industry, triclosan, chlorhexidine or pharmaceuticals such as acetaminophen suggested that at subtoxic concentrations conjugation process was enhanced (Guo and Zhang, 2017; Jiao *et al.*, 2017; Jutkina *et al.*, 2018; Lu *et al.*, 2018, 2020; Wang *et al.*, 2018; Zhang *et al.*, 2018; Jia *et al.*, 2021; Tang *et al.*, 2022). Also, at simulated sunlight, the frequency of conjugation has been shown to increase e.g., between the cells of *E. coli* (Chen *et al.*, 2019). Some of those publications also discussed the potential mechanism of action behind the elevated conjugation and most often production of ROS was proposed (Lu *et al.*, 2020). There have been also some conditions

that have inhibited conjugation, and such have been chlorine treatment at biocidal concentrations. (Lin *et al.*, 2016).

Inhibition of conjugation has been suggested as a way to decrease the rate at which antibiotic resistance spreads, and several compounds have been shown to affect conjugation (Graf *et al.*, 2019).

1.1.4 Methods to study conjugation in laboratory

Bacterial conjugation has been studied from many different perspectives, starting from mechanisms of mating-pair formation to effect of different conditions on conjugation efficiency. Conjugation efficiency is usually quantified by the ratio of the number of transconjugants at the end of the experiment to the number of donors or recipients at the beginning of the experiment or at the specific timepoint (Alderliesten *et al.*, 2020).

Usually in conjugation experiments differently marked donor and recipient cells have been used, the cells are mixed in liquid, or on agar medium, or on filters, and allowed to conjugate for a certain time. Then bacteria are spread using selective plates, that allow only new transconjugants – recipients that had obtained the conjugative genetic element, i.e., plasmid, to grow. Then simply colony forming units (CFU) are counted and the conjugation frequency is determined.

As already mentioned, conjugation may be carried out in different formats that are in more detail introduced below:

1. Conjugation on plate: donor and recipient strains are mixed and incubated on an agar plate. After incubation, the new plate is screened for the presence of transconjugants, which can be identified based on antibiotic resistance profiles of colonies (Piscon *et al.*, 2023). Transconjugants can also be identified on the basis of other markers than resistance, for example by differently marking the donors and transconjugants and the conjugative plasmid (S. Sørensen *et al.*, 2003). The transfer of conjugative DNA element between cells can also be confirmed by quantitative PCR where the copy numbers of those elements in donors and transconjugants are quantified (Klümper *et al.*, 2015).

2. Conjugation in liquid: donor and recipient are mixed in liquid media and incubated under appropriate conditions. Then, the mixture is plated to selective media and transconjugants are screened as in plate mating (Headd and Bradford, 2020).

3. Conjugation on filter: donor and recipient, pre-grown in liquid, are mixed on a filter that is placed onto the surface of a solid medium. Transconjugants are selected as described above (Pallares-Vega *et al.*, 2021).

Conjugation experiments have shown that the conjugation frequency is affected by various biotic and abiotic factors, such as growth phase, cell density, donor-to-recipient ratio, carbon and metal concentrations, temperature, pH, and mating time (Alderliesten *et al.*, 2020; Piscon *et al.*, 2023). Additionally, donor and recipient species, the plasmid, and the use of liquid mating, filter mating, as experimental method, also significantly influence the conjugation frequency (Alderliesten *et al.*, 2020).

One of the main challenges in laboratory studies of conjugation is that the process of conjugation is relatively inefficient, and the frequency of conjugation usually ranges between $1/10^5$ – $1/10^7$ per donor cells (Alderliesten *et al.*, 2020). This means that obtaining enough transconjugants for downstream analyses can be challenging, particularly if the donor and recipient strains are not well-matched or the experimental conditions are not optimal. Another challenge is that conjugation can be influenced by a wide range of environmental factors, as stated before, which can make it difficult to reproduce results between different experiments or laboratories, or to extrapolate findings from laboratory studies to natural environments. However, a so-called, rapid expansion process can occur, when newly occurring transconjugants became a donor, therefore increasing the conjugation rate and introducing variability into the data (Schmidt *et al.*, 2022).

Additionally, conjugation is often subject to a high degree of genetic and ecological variability, with different plasmids and bacterial strains exhibiting widely varying transfer rates and patterns (Alderliesten *et al.*, 2020; Huisman *et al.*, 2022). This can make it challenging to develop universal or generalizable models of conjugation that can be applied across different bacterial species or environments.

Overall, while laboratory studies of conjugation can be valuable for understanding the mechanisms and dynamics of gene transfer in bacteria, they require careful attention to experimental design and control.

1.2 Antimicrobial compounds

An antimicrobial agent is defined as a natural or synthetic substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, and algae (Burnett-Boothroyd and McCarthy, 2011). Antimicrobial agents may be of very wide origin. Those compounds may be natural and originate from plants, animals, or microbes, naturally occurring inorganic compounds, or such compounds may be synthetic and produced specifically to target and inhibit bacterial cells (Kohanski, Dwyer and Collins, 2010; Stan *et al.*, 2021).

1.2.1 Quaternary ammonium compounds (QACs)

Quaternary ammonium compounds (QACs) are biocidal or antiviral and antimicrobial compounds, that appear in form of a liquid or integrated as additive chemical (Daood *et al.*, 2020; Hora *et al.*, 2020; Mohapatra *et al.*, 2023). Because of their broad-spectrum antimicrobial properties, QACs are some of the most extensively used classes of biocides, disinfectants, sanitizers, antimicrobials, and cleaners. QACs are applied in household, food-processing, agriculture, and clinical settings to control the spread of environmentally transmitted pathogens. (Hora *et al.*, 2020). Often quaternary ammonium is integrated into products such as in fabric softeners, disinfectants, biocides, detergents, and hair cleaning products (Mohapatra *et al.*, 2023).

QACs contain a positively charged nitrogen atom linked to at least one hydrophobic hydrocarbon chain, i.e., alkyl groups, which are usually short chain substituents such as methyl or benzyl groups (Figure 3) (Mohapatra *et al.*, 2023).

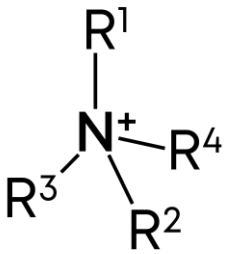

Figure 3. General structure of QACs.

1.2.1.1 Mechanism of action of QACs on bacterial cells

Due to the net negative charge on bacterial membranes driven by membrane lipoproteins in Gram-negative cells and teichoic acids in Gram-positive cells, positively charged QACs have a toxic effect on bacterial cells (Silhavy, Kahne and Walker, 2010). The ionic interaction between QACs and bacteria cell membrane destabilizes the cell membrane, causing leakage of intracellular low-molecular-weight material, proteins and nucleic acids, resulting in rapid cell lysis (Mohapatra *et al.*, 2023). It also has been reported, that QACs can destroy bacterial biofilm (Daood *et al.*, 2020). On the other hand, QACs have also been demonstrated to cause rapid development of tolerance upon repeated use (Nordholt *et al.*, 2021).

1.2.2 Metals

Metals that usually occur within naturally present inorganic compounds have been used as antimicrobial agents since antient times. The use of silver for the treatment or prevention of infection dates back to at least 4000 BCE (Politano *et al.*, 2013). For example, vessels made of copper and silver have been used for water disinfection and food preservation since the time of the Persian kings. This practice was later adopted by the Phoenicians, Greeks, Romans, and Egyptians. Settlers of North America dropped silver coins into transport containers to preserve water, wine, milk and vinegar, and a similar strategy was used by Japanese soldiers during the Second World War to prevent the spread of dysentery (Lemire, Harrison and Turner, 2013).

1.2.2.1 Mechanism of action of metals on bacterial cells

Some studies indicate that different metals cause injuries to microbial cells as a result of oxidative stress, protein dysfunction or membrane damage (Lemire, Harrison and Turner, 2013). Metals disrupt antibiotic-resistant biofilms, exert synergistic bactericidal activity with other biocides, inhibit metabolic pathways in a selective manner, and can kill multidrug-resistant bacteria. Bacteriotoxic doses of these metals disrupt processes needed for cell growth by interfering with biochemistry of intracellular biomolecules. (Lemire, Harrison and Turner, 2013). Many reports have shown that antimicrobial doses of certain metal ions can increase intracellular ROS, which leads to cell functions disruption and may lead to cell death (Figure 4).

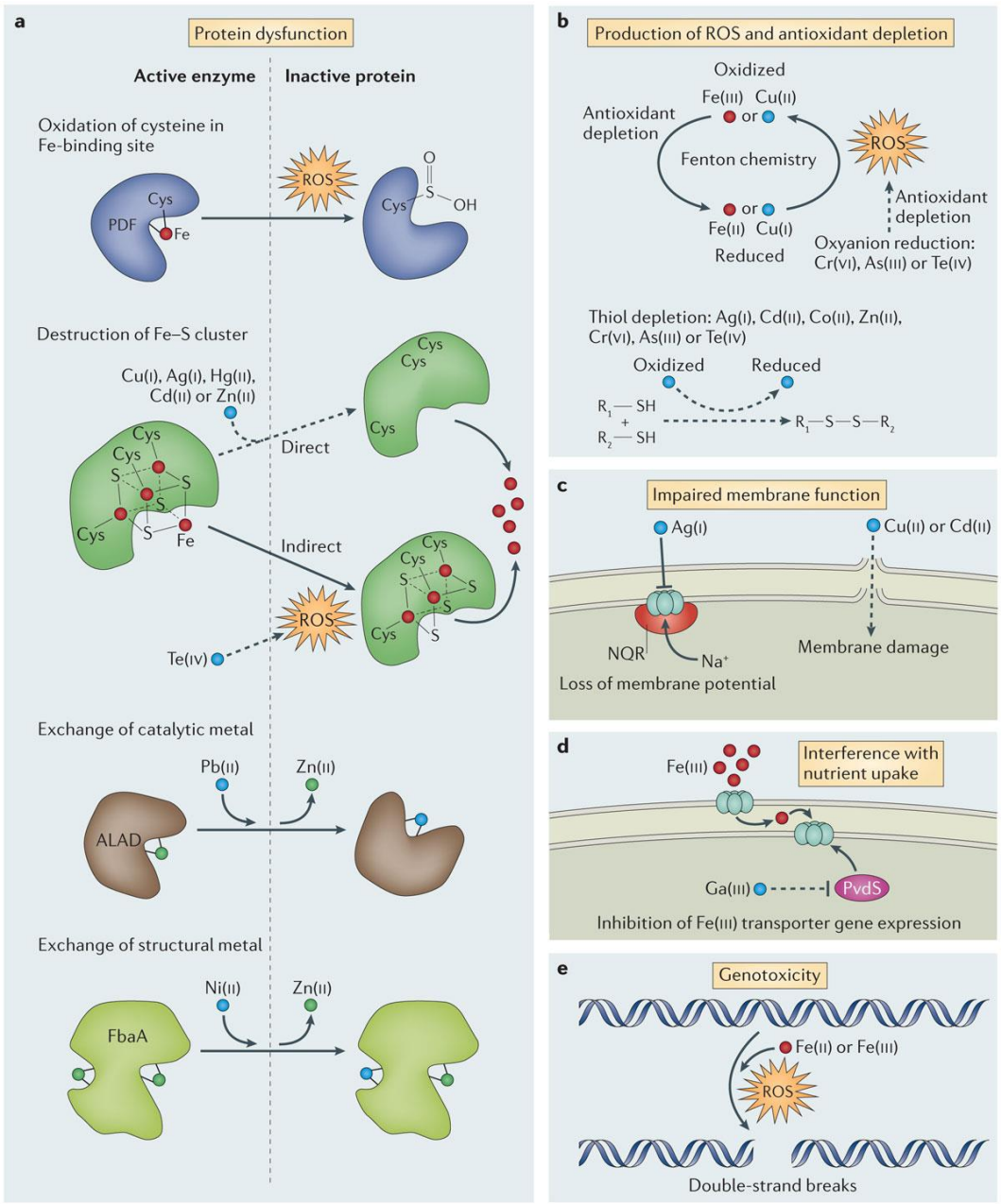

Figure 4. Antibacterial mechanisms of metals. The mechanisms of antibacterial action can be generally classified as: a – protein dysfunction, b – production of reactive oxygen species (ROS) and depletion of antioxidants, c – affecting the membrane function, d – interference with nutrient assimilation, e – genotoxicity. Solid arrows represent pathways in which the underlying biochemistry has been established, whereas dashed arrows represent a route of toxicity for which the underlying biochemical mechanism is still unclear. ALAD – δ-aminolevulinic acid dehydratase; FbaA – fructose-1,6-bisphosphate aldolase; NQR – NADH: quinone oxidoreductase; PDF – peptide deformylase; PvdS – a σ-factor (σ24) from *Pseudomonas aeruginosa*. Figure source – Lemire, Harrison and Turner, 2013 with permission.

It is known that silver affects bacterial cells by adhering to cell membrane and adhering to the proteins of the electron transport chain (Yin *et al.*, 2020). Similarly, membrane rupture has been suggested as one of the mechanisms of action of copper in bacteria but in case of copper also the formation of reactive oxygen species originating from interchanging redox forms of copper, has been suggested (Salah, Parkin and Allan, 2021). However, also some uncertainties have been reported with regard to the mode of action of those metals (Sudha *et al.*, 2012; Lemire, Harrison and Turner, 2013; Politano *et al.*, 2013).

1.2.3 Antimicrobial surfaces

One of the main sources of transmission of infectious disease is surface transfer of pathogenic bacteria (Kramer and Assadian, 2014). Therefore, surfaces decreasing microbial activity and viability and thus, the probability of transfer of potential pathogens, may have a significant contribution to the decrease of infectious diseases and improvement of public health (Muller *et al.*, 2016). There are two broadly different, but not mutually exclusive, strategies used in developing antimicrobial surfaces: biocidal surfaces that kill microbes, and anti-biofouling surfaces that reduce microbial adhesion and prevent subsequent biofilm formation (Cassidy *et al.*, 2020; Soni and Brightwell, 2022). Nowadays more attention is gaining the use of metallic copper as an antimicrobial surface. Copper-containing surfaces kill microorganisms in a process that has been termed contact killing and occurs on a timescale of minutes to hours (Mathews, Kumar and Solioz, 2015). Although, the underlying biochemistry is not entirely clear, loss of bacterial cell viability has been correlated with the uptake of Cu ions and increased production of ROS (Sudha *et al.*, 2012).

1.3 Bacterial chromosome modification by transposon systems

As previously mentioned in the section where different methods to study conjugation in laboratory were discussed, one common necessity for conjugation experiments is to be able to distinguish between donors, recipients and transconjugants. Usually, such a distinction is made by having different antibiotic resistance profiles for donors and recipients. For this reason, some strains must be genetically modified or chromosomally tagged, to have a resistance towards target antibiotic. In this study a recipient strain was constructed for conjugation experiments, therefore, a short overview is presented about the gene manipulation techniques.

Several gene manipulation techniques exist for introduction of a DNA fragment into bacterial chromosome. The goal of this gene manipulation is to have stable strain without any plasmid, but with chromosomal modification. The inserted fragment is, usually antibiotic resistance gene and/or fluorescent protein gene. Many systems are based on homologous recombination between an inserted fragment and part of bacterial chromosome, for example bacteriophage λ Red recombination system, *cre-lox* recombination system (Kües and Stahl, 1989; Sharan *et al.*, 2009). However, there are methods, involving transposons activity.

Transposons are DNA sequences that are able to change their position within the genome (Muñoz-López and García-Pérez, 2010). The simplest transposon consists of a gene encoding transposase and inverted repeats, located at the transposon ends. In addition to the transposase gene, many transposons also carry other genes, e.g., antibiotic, or heavy metal resistance genes, catabolic genes, and reporter genes such as GFP gene, e.g., Tn7 minitransposon (Figure 5).

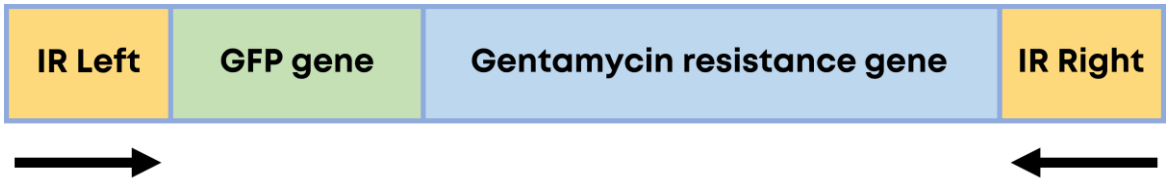

Figure 5. Tn7 minitransposon scheme. Components of the minitransposon: inverted repeats (IR) at the ends of the minitransposon, GFP gene, entamicin resistance gene.

The "jumping" of the transposon is carried out by protein transposase, which specifically binds to the inverted repeats, catalyzes DNA cleavage next to the transposon ends and rejoins a fragment to a new target site in the bacterial chromosome (Muñoz-López and García-Pérez, 2010). The transposon ends are joined to the target DNA usually in a staggered fashion, resulting in small gaps adjacent to both ends of the inserted transposon. These gaps are filled by DNA polymerase and the ends of the DNA are joined together by DNA ligase (Muñoz-López and García-Pérez, 2010). As a result, duplication of the target site is created on both sides of the inserted transposon. Depending on the transposon, they can be 2 - 14 base pairs long. Therefore, minimal requirements needed for transposon translocation are:

1) Transposase, which catalyzes the movement of the transposon,
2) Inverted repeats at the ends of the transposon

Differently from homologous recombination, transposition does not require homology between the transposon and the target sequence. Therefore, the insertion of the transposon can be random (depending on the transposon system) and may result in an accidental disruption of genes or regulatory regions.

In this research *Pseudomonas putida* KT2440 strain with chromosomal insertion was made using the mini-Tn7 transposon system.

1.3.1 Mini Tn7 transposon system

The mini-Tn7 system is a molecular biology tool that is used for site-specific integration of DNA into bacterial genomes (Waddell and Craig, 1989; Choi and Schweizer, 2006). It is derived from the larger Tn7 transposon system, which is a mobile genetic element found in bacteria.

In contrast to other transposable elements, which show little target site selectivity, the TnsABCD transposase components of Tn7 catalyze site- and orientation-specific insertion into bacterial chromosome at Tn7 attachment (*att*Tn7) sites (Figure 6), (Choi and Schweizer, 2006). With very few exceptions, these sites are located downstream of highly conserved *glmS* gene, which codes for an enzyme glutamine-fructose-6-phosphate aminotransferase, responsible for the formation of a compound essential for cell wall biosynthesis (Ferré-D'Amaré, 2010). Another advantage of mini Tn7 system is the fact that only one copy of the transposon is inserted to a chromosome and that the system has a broad host range (Choi and Schweizer, 2006).

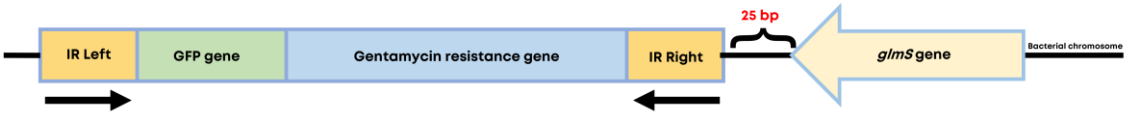

Figure 6. The schematic insertion of the Tn7 transposon into bacterial chromosome, near the *glmS* gene.

The mini Tn7 transposon system contains two plasmids - delivery and helper plasmids, which can be introduced into the modified bacteria by electroporation. Delivery plasmid carries a transposable element with target genes between inverted repeats. Helper plasmid

has transposase components (*tnsABCDE* genes) for minitransposon to insert from the plasmid into the bacterial chromosome. It is important for minitransposon to not encode a transposase. The lack of transposase gene ensures, that the movement of the transposon will occur only once in the target sequence. Both delivery and helper plasmid are suicide plasmids because they can only replicate in certain bacterial strains designed for this purpose and in all other strains such plasmids will disappear with time. This usage of suicide plasmids ensures that transposon mediates the transfer of the minitransposon only for short period of time.

2 THE AIMS OF THE THESIS

The main goal of this thesis was to study conjugation between *Pseudomonas putida* KT2440 as a recipient and *Escherichia coli* CSH26 as a donor for pKJK5 plasmid on selected antimicrobial surfaces. The detailed aims were:

- To construct a chromosomally marked strain of *Pseudomonas putida* KT2440 to be used as recipient strain in conjugation,
- To optimize the conditions for conjugation experiments on solid surfaces and construct a protocol for conjugation assay,
- To carry out conjugation experiments on selected solid surfaces that either exhibited antibacterial effect or were commercialized as antimicrobial and evaluate their effect on conjugation frequency.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strains and plasmids used in experiments

Strains and plasmids used in this study are listed in Table 1. *Pseudomonas putida* KT2440 was provided by Maia Kivisaar´s group (University of Tartu), and was used as a recipient strain in conjugation, and *Escherichia coli* strain CSH26 harboring plasmid pKJK5, provided by Marko Virta (University of Helsinki), was used a donor strain in conjugation. *Escherichia coli* strains DH5α and DH5α λ pir were used for plasmid cloning and were provided by Merike Jõesaar (University of Tartu). Bacterial cultures were routinely maintained on LB agar (5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl, 15 g/l agar) plates at 37 °C (*E. coli)* or 30 °C (*P. putida)* supplemented with proper antibiotics, if needed. Plasmids pUC18T-mini-Tn7T-Gm-gfpmut3 and pUX-BF13 were used for introduction of *gfp* and *aac* genes to *P. putida* KT2440 prior to conjugation experiments and were provided by Merike Jõesaar (University of Tartu). IncP-1ε pKJK5 (Sengeløv *et al.*, 2001) is a conjugative plasmid that was used in conjugation experiments and was provided by Marko Virta (University of Helsinki).

Table 1. Bacterial strains and plasmids.

Tc – tetracycline, Tp – trimethoprim, Su – sulfonamides, Sp – spectinomycin

Characteristics		***Reference***
Bacterial strains		
Pseudomonas putida KT2440	*cusA/czcA, cueR, cusA/czcA, arsR, arsC, arsO, arsH, arsB, arsN2* providing resistance towards copper, silver, and arsenic metals. Natural resistance to number of antibiotics such as chloramphenicol	(Bagdasarian *et al.*, 1981; Regenhardt *et al.*, 2002)
Escherichia coli CSH26	Common laboratory K12 strain, showing resistance to nalidixic acid	(Miller J.H., 1972; A. H. Sørensen *et al.*, 2003)
***E. coli* DH5 α λ pir**	Common strain for transformation and other laboratory modifications, pir gene from λ phage	(Platt *et al.*, 2000)
***E. coli* DH5 α**	Common strain for transformation and other laboratory modifications	(Taylor, Walker and McInnes, 1993)
Plasmids		
pUC18T-mini-Tn7T-Gm-gfpmut3	pUC18 *ori*, *aac* (gentamicin resistance) and *gfp*, located between Tn7L and Tn7R inverted repeats	(Choi and Schweizer, 2006)
pUX-BF13	R6K γ *ori*, transposase genes (*tnsABCDE*), *bla* gene for beta-lactamases resistance	(Roos, Werner and Loessner, 2015)
pKJK5	54383 bp long, IncP-1ε group conjugative plasmid. Providing resistance to Tc, Tp, aminoglycosides, Su, Sp, quaternary ammonium compounds (*tetA, tetR, dfrA1, aadA11b, sul1, qacE*Δ)	(Sengeløv *et al.*, 2001)

3.1.2 Preparation of recipient strain for conjugation experiments

Prior to conjugation experiments, *P. putida* KT2440 was chromosomally tagged to express green fluorescent protein (GFP) and gentamicin resistance using Tn7 minitransposon system. For that, two plasmids, pUC18T-mini-Tn7T-Gm-gfpmut3 and pUX-BF13 (Table 1), were used. pUC18T-mini-Tn7T-Gm-gfpmut3 is a pUC19 derivative that can replicate in *E. coli* and other *Enterics*, but not in *Pseudomonas* due to its ColE1 *ori* (Choi and Schweizer, 2006). The plasmid carries *gfp* gene and *aac* gene between the left and right sides of Tn7, Tn7L and Tn7R inverted repeats (Figure 7). Helper plasmid pUX-BF13 (Figure 8) is necessary as it encodes for transposase genes (*tnsABCDE*) enabling the insertion of the minitransposon to chromosomal DNA. In addition, pUX-BF13 plasmid encodes resistance to ampicillin and its replication is controlled by R6K γ *ori,* which can only be maintained if Pir protein is expressed, and, thus, for the replication of this plasmid any bacterium with chromosomal *pir* gene is suitable (Bao *et al.*, 1991; Rakowski and Filutowicz, 2013).

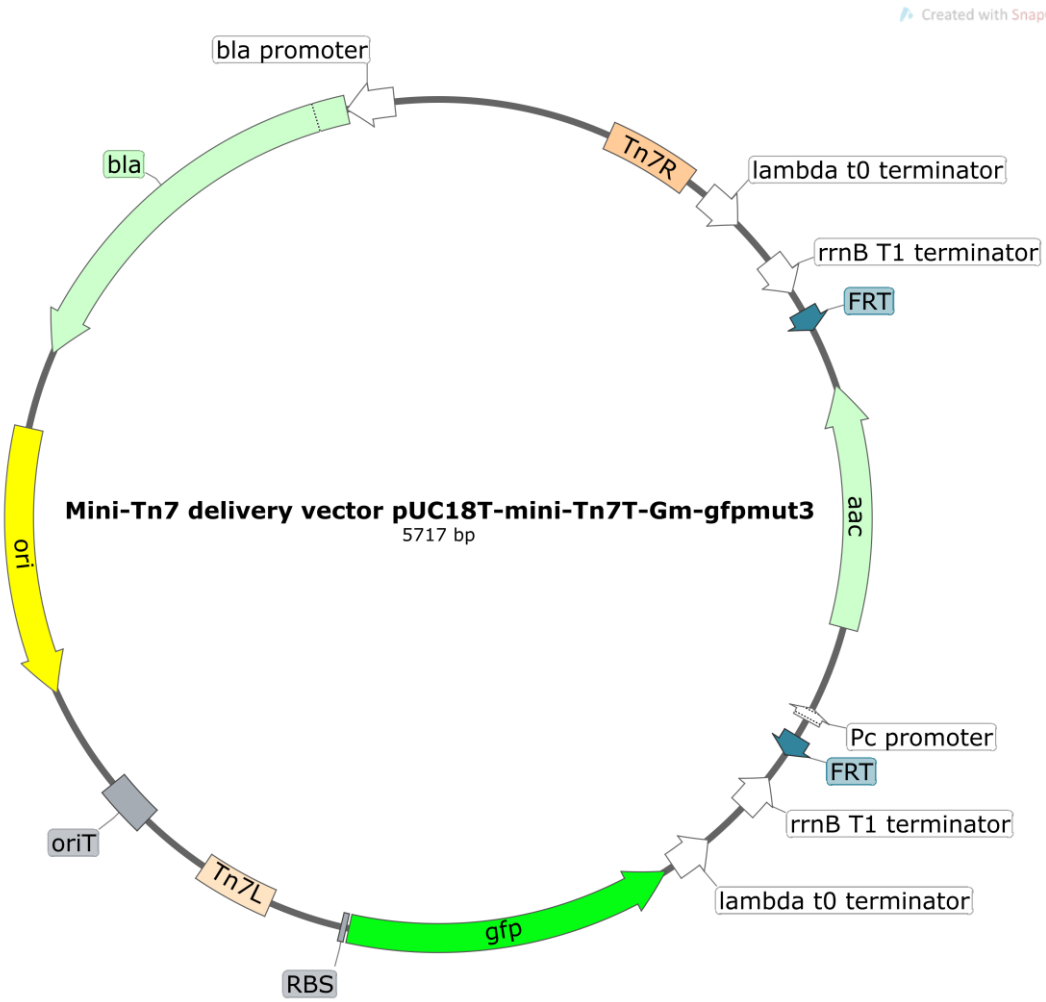

Figure 7. Delivery plasmid pUC18T-mini-Tn7T-Gm-gfpmut3, that encodes a mini-transposon. Components of the plasmid: mini-Tn7 minitransposon located between Tn7R and Tn7R and carries *gfp* and gentamicin resistance gene, ColE1 *ori*, origin of replication ensuring high copy number of the plasmid, and ampicillin resistance gene.

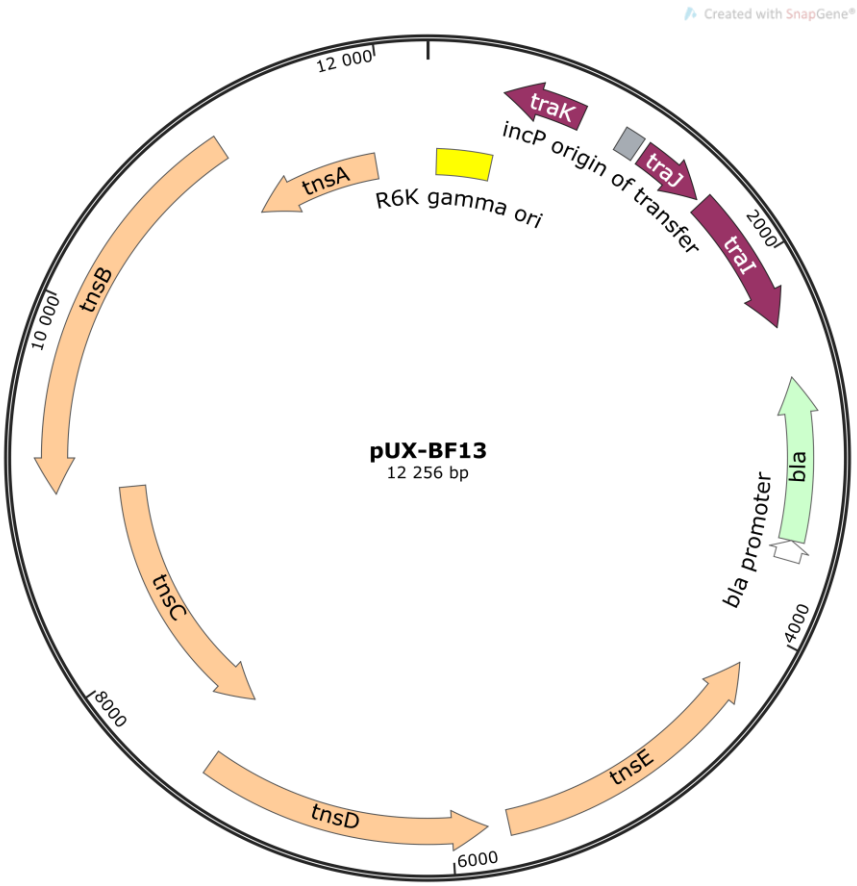

Figure 8. Helper plasmid pUX-BF13. Components of the plasmid: genes, necessary for transposase activity *tnsABCDE*, *R6K γ ori*, ampicillin resistance gene, genes, necessary for the plasmid conjugal transfer *traKJI*, incP origin of transfer

The plasmids were transformed to and maintained in suitable *E. coli* strains: *E. coli* DH5 α (pUC18T-mini-Tn7T-Gm-gfpmut3) and in *E. coli* DH5 α λ pir (pUX-BF13). Plasmids were then purified prior to their transformation to *P. putida* KT2440.

For transformation of the plasmids to the strains, the following procedure was used: bacterial strains were inoculated to 6 ml of LB (5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl) in a 14 ml tube and incubated for 16-18 hours at 37°C and 250 rpm. The culture was diluted to 0.5-0.7 optical density (OD_{600}), and by 1 ml was divided into 5 pre-cooled microcentrifuge tubes

and placed on ice. Cells were then centrifuged at 2300 g for 10 min, supernatant was removed. Then, 1 ml of Sterile deionized water was added to wash the cells by resuspending the cells and centrifuging them at 2300 g. The washing procedure was repeated twice with 1 ml of ice cold 10% glycerol and finally 250 µl of ice cold 10% glycerol solution by pipetting up and down. 100 µl of electrocompetent cells were transferred to a cold 2 mm gap-width electroporation cuvette and 2.5 µl (ca 30-75 ng) of helper or delivery plasmid was added to the cells and mixed by pipetting. No plasmid was added for negative transformation control. Electroporation settings were: 1.7-2.5 kV, 200 Ωs and 25 µF. Straight, 1 ml of 37°C prewarmed LB was added to the electroporated cells and mixture was transfer to a microcentrifuge tube. The cells were incubated in the 37°C incubator-shaker for 3 h. The cells were put on selection plates (LB + 100 µg/ml of ampicillin). The plates were incubated overnight at 37°C.

From obtained transformants, plasmid minipreps were prepared using GeneJET Plasmid Miniprep Kit (Thermoscientific), according to the manufacturer's manual. Plasmid DNA concentration was determined at 260 nm using NanoDrop. To verify the identity of the plasmids, overnight restriction analysis was carried out. Plasmid DNA restriction was performed with FastDigest Restriction Enzymes (Thermo Fisher Scientific) as in Table 2. Restriction was allowed to proceed overnight at 37°C. After that, the restriction mix was loaded onto 1% agarose Tris-actetate ethylene-diaminetetraacetic acid (TAE) gel (1% agarose, 0.5% TAE buffer, about 0.5 µg/ml of ethidium bromide), followed by electrophoresis and the restricted DNA was visualized on UV transilluminator.

Table 2. Enzymes and reaction conditions used for restriction analysis of plasmids.

Reagent	***pUC18T-mini-Tn7T-Gm-gfpmut3***	***pUX-BF13***
Nuclease-free water	15 µl	15 µl
Buffer	2 µl 10x O buffer (both enzymes)	2 µl 10x O buffer (SalI) or 10x R buffer (HindIII)
Plasmid	2 µl (ca 30 ng)	2 µl (ca 75 ng)
Enzyme	1 µl Acc65I or NotI	1 µl SalI or HindIII

After verification of correctness of the plasmids, electroporation of *P. putida* KT2440 was performed according to Bao *et al*. (1991). For that, 5 ml of LB medium in a 14 ml culture tube was inoculated from a fresh plate of *P. putida* KT2440 and incubated for 16-18 hours at 30°C and 250 rpm. After 16-18 hours incubation, liquid culture OD was adjusted to 0.5-0.6 and cooled on ice. Then, 1 ml of the culture was pipetted into pre-cooled microcentrifuge tubes and cells were harvested at 2300 g for 10 min. Supernatant was removed and 1ml of Sterile deionized water was added to wash the cells from LB to each of the centrifuge tubes and the cells were completely resuspended by pipetting up and down. Cells were again centrifuged at 2300 g for 10 min. This washing step was repeated two times with Sterile deionized water and once with pre-cooled 300 mM sucrose. In the end, cells were resuspended in 100 µl of 300 mM sucrose. Finally, 2.5 µl (ca 30-75 ng) of both helper and delivery plasmid was added to the cells in electroporation cuvette, mixed electroporation at 1.7-2.5 kV, 200 Ω and 25 µF was performed. No plasmid was added for negative transformation control. Straight, 1 ml of 30°C pre-warmed LB was added to the electroporated cells and mixture was transferred to a microcentrifuge tube. The cells were incubated in the 30°C incubator-shaker for 3 hours and then plated to selection agar plates: LB with 20 µg/ml of gentamicin. The plates were incubated overnight at 30°C. After at least 30hours of growth, transformants were screened for GFP signal under a UV lamp. For additional verification of the insert in *P. putida* KT2440 chromosome, colony PCR was performed as in Tables 4 and 5 with primers, which were designed according to (Choi and Schweizer, 2006):

Tn7-*glmS*, forward 5'-AATCTGGCCAAGTCGGTGAC-3' and

Tn7-Gm, reverse 5'-ATATCGACCCAAGTACCGCC-3'.

Before PCR, the colonies were resuspended in 200 µl of Sterile deionized water and boiled in thermostat for 1 minute in 95°C (Green and Sambrook, 2019).

Table 3. Colony PCR reaction mixture.

Reagent	*Volume*
DMSO (5%)	1 µl
dNTP (0.2 mM)	0.4 µl
10x Taq buffer	2 µl
$MgCl_2$ (2.5 mM)	1 µl
Taq polymerase (2.5 units/µl)	0.4 µl
Sterile deionized water	11.2 µl
Both primers (5 µM)	2+2 µl
Template (colony extract)	as less as possible (c.a. 0.1 µl)
Total w/o template	20 µl

Table 4. Colony PCR program.

Step	*Temperature (°C)*	*Time*	*Number of cycles*
Initial denaturation	95	5	1
Denaturation	95	30	30
Annealing	53	30	
Extension	72	1	
Final extension	72	2	1

Following colony PCR, the obtained PCR mixture was loaded on 1% agarose TAE gel (1% agarose, 0.5% TAE buffer, about 0.5 µg/ml of ethidium bromide), followed by electrophoresis and visualization under UV light to verify the site-specific chromosomal integration of target genes into chromosome of *P. putida* KT2440. The resulting *P. putida* KT2440 strain with *gfp* and *aac* (Gm^R) genes was further designated as *P. putida* KT2440::*gfp-aac*.

3.1.3 Cell counts optimization

In order to be able to calculate the frequency of conjugation, a precise number of bacteria in the initial inoculum must be known. To determine the cell number (the amount of colony forming units, CFU) in bacterial suspension, OD of both the donor and recipient strains was related with its CFU number. For that, bacterial suspensions were prepared from fresh overnight cultures on LB agar medium supplemented with Tc 20 µg/ml in case of *E. coli* CSH26/pKJK5 and Gm 20 µg/ml in case of *P. putida* KT2440::*gfp-aac*. Bacterial mass was collected from the plates with a sterile inoculation loop and suspended in Sterile deionized water by vortexing. Optical density (absorbance at 600 nm) of the culture was measured from 10-fold dilution of this suspension and a series of parallel dilutions in 96 well microplate was prepared in phosphate-buffered saline (PBS; 0.8 g/l NaCl, 0.02 g/l KCl, 0.144 g/l Na_2HPO_4, 0.02 g/l KH_2PO_4; pH 7.1), and 20 µl of six consecutive dilutions was plated on agar square plate supplemented with proper antibiotic. Plates were incubated overnight at 30°C for optimal growth. After 16-18 hours, colonies were counted and CFU number was calculated using the following formula:

Equation 1: $$CFU\ per\ ml = \frac{number\ of\ colonies \times dilution\ factor}{volume\ plated\ (ml)}$$

CFU count was expressed as $\log_{10}$ CFU/ml. All experiments were conducted in class II biosafety cabinet (BSC2).

3.1.4 Pre-treatment and reusing of materials and media

To compare the conjugation frequency on antimicrobial surfaces, i.e., in the presence of antimicrobial stress compared with conjugation frequency without antimicrobial stress, five test surfaces with previously shown or claimed antimicrobial effect were selected and two control surfaces were used. All the surfaces were round coupons with 2 cm diameter. The materials used are the same as in Kaur *et al.*, (2023) and as shown in Table 5. Silver paint (AgP) and Si-Quat (SQ) coating were covered onto stainless steel coupons. All media and buffers were sterilized by autoclaving at 121°C for 15 minutes. Single-use surfaces and glass coverslips were sterilized with 70% ethanol and dried in a biosafety cabinet.

Table 5. Properties of surfaces used as substrates for conjugation.

Surface	***Properties***	***Source***
Test surfaces with antimicrobial effect		
Metallic Cu (Cu100)	99% Cu	Metroprint OY, Estonia
Metallic 10% Cu (Cu10)	8-10% Cu in stainless steel	Collaboration with Prashanth Konda Gokuldoss (Tallinn University of Tecnology)
Metallic Ag (Ag)	99.9% Ag	Surepure Chemetals, USA
Silver paint (AgP)	0.027 mg Ag in g of paint	TOUCH Antimicrobial, UK
Si-Quat (SiQ)	Quaternary ammonium compound-based surface paint	Affix Labs, Finland
Control surfaces		
Plastic (PP)	Polypropylene	Etra OY, Finland
Stainless steel (SS)	AISI 304 SS, major elements in addition to Fe were 18% of Cr, 8% of Ni, 1.4% of Mn; 2B finish	Aperam Stainless, France

Ag, Cu100, Cu10 and SS coupons were re-used between the experiments and, therefore, those surfaces were always cleaned using the following procedure: a three-step shaking (2 min), sonication (15 min) and shaking (2 min) procedure in 5% citric acid, 100% acetone and 70% ethanol with 0.3 mm glass beads. Between every wash procedure, the coupons were rinsed with distilled water and after the last step, were sterilized at least for 15 min by UVC in a biosafety cabinet.

3.1.5 Bacterial viability on surfaces

Prior to conjugation experiments, survival of bacteria on test surfaces was checked separately for *E. coli* CSH26/pKJK5 and *P. putida* KT2440::*gfp-aac*.

For bacterial viability analysis a modified ISO 22196 protocol as described in Kaur *et al.* (2023) was used. For that, inoculums of *E. coli* CSH26/pKJK5 and *P. putida* KT2440::*gfp-aac* were photometrically adjusted to the target 1.5×10^9 - 3.0×10^9 CFU/ml in Sterile deionized water and mixed 1 to 1 with soil load (**SL**; final concentration: bovine serum albumin 2.5 g/l, yeast extract 3.5 g/l, mucin 0.8 g/l (EPA, 2022)) or in 500-fold diluted nutrient broth of ISO 22196 (**NB**; final concentration of ingredients: 0.006 g/l meat extract, 0.02 g/l peptone, 0.01 g/l NaCl (ISO, 2011)), and 15 µl of this inoculum was pipetted onto each surface. NB and SL represent organics poor, i.e., oligotrophic, and organic rich environments respectively and are important to demonstrate bacterial behavior on surfaces in clean and dirty conditions (discussed later). The surfaces were then covered by 25×25 mm coverslips with an overhang but avoiding inoculum leakage and incubated at room temperature (22°C) in a >90% relative humidity environment for 1, 2, 6 and 24 hours. >90% relative humidity was achieved by incubating the samples in a closed sterile contained in which the surfaces were suspended above sterile water on a 3D printed plastic grid. After exposure, the surfaces were placed into 10 ml of SCDLP neutralizing medium (17 g/l casein peptone, 3 g/l soybean peptone, 5 g/l NaCl, 2.5 g/l Na_2HPO_4, 2.5 g/l glucose, 1.0 g/l lecithin, 7.0 g/l Tween80 (ISO, 2011)), surfaces with cells were vortexed for 30 seconds for cell recovery, serial dilutions were made in PBS and 20 µl of bacteria from six consecutive dilutions was plated onto LB agar plates with appropriate antibiotics. Plates were incubated overnight at 30°C for optimal growth. Colony counts (CFU) were registered after 16-18 hours of growth. CFU per surface was calculated using the following formula:

Equation 2: $$CFU\ per\ surface = \frac{number\ of\ colonies \times dilution\ factor \times washoff\ volume\ (ml)}{volume\ plated\ (ml)}$$

CFU count was expressed as $\log_{10}$ CFU/surface. Having 3 colonies were considered to be the limit of detection (LOD) and equals to 3.17 $\log_{10}$ CFU/surface. At least 3 biological and 3 technical repeats were analyzed. All experiments were conducted in class II biosafety cabinet (BSC2).

3.1.6 Bacterial conjugation on surfaces

Conjugation was carried out between donor strain *Escherichia coli* CSH26 harboring IncP-1ε pKJK5 (Sengeløv *et al.*, 2001) (strain: *E. coli* CSH26/pKJK5) conjugative plasmid (Table 1) and the recipient strain *P. putida* KT2440::*gfp-aac* constructed in this study. Those strains express different antibiotic resistance and, thus, *E. coli* CSH26/pKJK5 was grown in the presence of tetracycline (Tc 20 µg/ml) and *P. putida* KT2440::*gfp-aac* in the presence of gentamicin (Gm 20 µg/ml).

For conjugation on surfaces donor and recipient strains were prepared as for viability experiments, i.e., pre-grown on LB agar media and resuspended in Sterile deionized water. Serial dilutions were made from those resuspended donor and recipient suspensions in PBS and plated to antibiotic-selective LB agar plates to later analyze the CFU counts. The prepared bacterial suspensions were mixed in different donor-to-recipient ratios, depending on the experiment (1:1, 1:10 or 1:100) with 2-fold concentrated medium, i.e., SL for organics rich environment and NB for oligotrophic environment. From 1:1, 1:10 or 1:100 conjugation mix, serial dilutions were made immediately in PBS and plated to antibiotic-selective LB agar plates to later analyze the CFU counts. In this case, recipient cells were expected to grow on LB + Gm 20 µg/ml plates and donors on LB + Tc 20 µg/ml plates. Then, 15 µl of conjugation mix was pipetted onto each surface and the surfaces were covered by ethanol-sterilized 25×25 mm coverslips. It was visually inspected that the mix was equally distributed on the surfaces. Surfaces were then incubated at the same conditions as for viability experiments for 1-24 hours to allow conjugation to happen. After incubation time, surfaces were placed to 10 ml of SCDLP neutralizing medium in 50 ml tubes and vortexed for 30 sec, as in viability experiments. Serial dilutions were made from the wash-offs in PBS and 20 µl of six consecutive dilutions were plated to selection plates: LB + Tc 20 µg/ml for donor *E. coli* CSH26/pKJK5, LB + Gm 20 µg/ml for recipient *P. putida* KT2440::*gfp-aac*. From the

wash-off, 50 and 500 µl was also plated to LB Gm 20 µg/ml + Tc 20 µg/ml for enumeration of transconjugants. In addition, the wash-off was centrifuged for samples with expected low numbers of transconjugants. For this, firstly, glass and surface were removed by sterile tweezers after which the samples were centrifuged at 4430 g for 10 minutes. Supernatant was removed in a way, to leave ca 800 µl of cell pellet, which was then suspended gently by pipetting and plated to LB (Gm 20 µg/ml + Tc 20 µg/ml) plates. LB plates with seeded bacteria were allowed to grow for 16-18 h at 30°C (*E. coli* CSH26/pKJK5, *P. putida* KT2440::*gfp-aac* and transconjugants) for optimal growth. Log_{10} CFU/surface was calculated as in equation 2. At least 3 biological and 3 technical repeats were analyzed.

All experiments were conducted in aseptic conditions in class II biosafety cabinet (BSC2).

3.1.6.1 Calculation of conjugation frequency

Conjugation frequency was calculated in 4 different ways: by (1) dividing the log_{10} CFU/surface of transconjugants on a surface by initial log_{10} CFU/surface of donor cells, (2) dividing the log_{10} CFU/surface of transconjugants on a surface by final log_{10} CFU/surface of donors at the final conjugation timepoint, (3) dividing the log_{10} CFU/surface of transconjugants on a surface by initial log_{10} CFU/surface of recipients, or (4) by dividing the log_{10} CFU/surface of transconjugants on a surface by final log_{10} CFU/surface of recipients at the final conjugation timepoint. This was done to illustrate the dependency of the conjugation frequency on mortality of either donor or recipient on a given surface. Importantly, such calculations do not consider the fact that newly occurred transconjugants may also be able to transfer the plasmid (Huisman *et al.*, 2022) and therefore the true number of donors may not be equal to number of initial *E. coli* CSH26/pKJK5 donors. However, we consider that the number of transconjugants passing on the conjugative plasmid are significantly less than the original donors.

3.1.7 Statistical analysis

Statistical analysis of the data was performed with GraphPad Prism 9.5.0 (GraphPad Software, San Diego, USA). One-way ANOVA followed by multiple comparisons at $\alpha = 0.05$.

3.2 RESULTS

3.2.1 Transformation of *P. putida* KT2440

To create chromosomally determined GFP and Gm^R phenotype for recipient bacterium *P. putida* KT2440, pUC18T-mini-Tn7T-Gm-gfpmut3 and pUX-BF13 plasmids were simultaneously transformed to *P. putida* KT2440. On selective medium 29 colonies were obtained from transformation that expressed GFP under UV lamp. 3 colonies of those were chosen for colony PCR to prove the chromosomal insertion of *gfp* and *aac* (gentamicin resistance) genes into *P. putida* KT244 near *glmS* gene into *att*Tn7 site.

Using primers Tn7-*glmS* and Tn7-Gm, ~ 520 bp fragment was expected as *gfp* and *aac* genes were integrated to the chromosome of *P. putida* KT2440 after the *glmS* gene into the *att*Tn7 sites. Interestingly, out of three colonies tested, only one resulted in a clear PCR product(s) and out of those, the ~500 bp fragment was considered to be correct (Figure 9). The other fragments from colony 2 (Figure 9) are considered as results of non-specific binding of primers to genomic DNA. It is suggested that the amount of DNA in PCR reaction from colonies 1 and 3 did not provide sufficient chromosomal DNA or inhibition of the PCR reaction occurred or in the chromosome of those colonies there was no mini Tn7 transposon insertion. Plasmid controls, i.e., the original plasmids transformed to *P. putida* KT2440 did not show expected specific bands and those weak bands visible are likely the initial plasmids and their supercoiled forms. As a result, we consider that colony 2 is the expected strain *P. putida* KT2440::*gfp-ac* with chromosomally integrated *gfp* and *aac* genes and this strain was selected for following conjugation experiments.

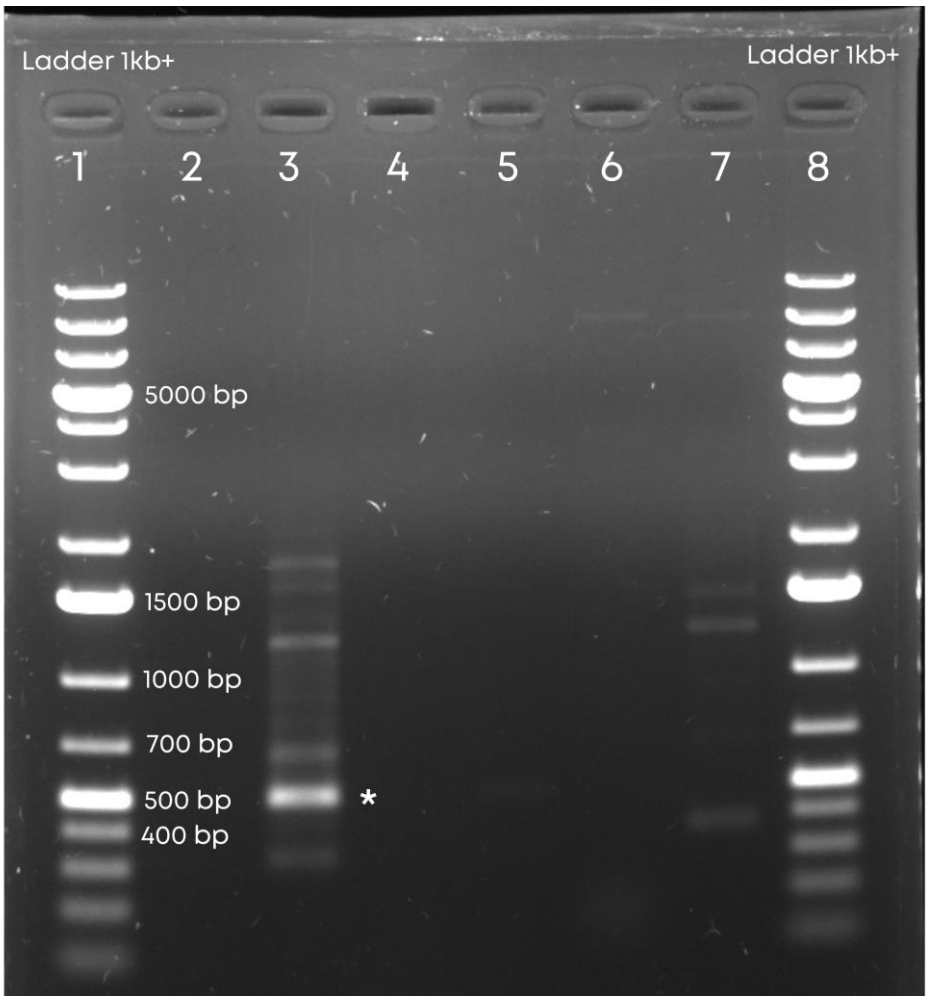

Figure 9. Verification of site-specific chromosomal integration of *gfp* and *aac* genes into *P. putida* KT2440. Results of colony PCR covering the region between chromosomal *glmS* gene and newly chromosome-inserted *aac* gene. GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) marked as 1 and 6; Transformed samples 2-4 designate three different colonies on transformation plates; 5 – untransformed colony of *P. putida* KT2440; 6 and 7 – plasmid controls, 30 ng of pUC18T-mini-Tn7T-Gm-gfpmut3 and 70 ng of pUX-BF13. Desired band that was considered as the proof for chromosomal insertion of the mini-Tn7 transposon with *gfp* and *aac* genes marked with (*).

3.2.3 Determination of bacterial cell number for conjugation

To be able to reliably demonstrate both increase and decrease of conjugation frequency as a result of exposure to antimicrobial surfaces, the number of bacterial cells in conjugation should be sufficient. According to previous studies, in experiments with liquid conjugation, conjugation frequency has been $10^{-4.97}$ and, on filter, conjugation frequency has been $10^{-5.38}$ (Alderliesten *et al.*, 2020). Therefore, approximately $1/10^5$ cells are expected to acquire a plasmid from donors. In case of conjugation on solid surfaces used in this study, only 15 µl of bacteria were exposed to surfaces and in order to have detectable results of transconjugants, the cell density should be relatively high. For example, one valid standard for analysis of antibacterial activity of solid surfaces, ISO 22196, suggests having 10^4 cells/cm^2, which in case of surfaces used in this study (3.14 cm^2 surface area) would be around 10^5 cells/surface. Considering that every 10^{5th} cell would become transconjugant, the detection of transconjugants would be challenging. Therefore, the aim was to have at minimally, 10^7 cells/surface, which would translate to 1.5×10^9 - 3.0×10^9 CFU/ml. For easier determination of the cell number before the experiment, OD (absorbance at 600 nm) of the donor *E. coli* CSH26/pKJK5 and the recipient *P. putida* KT2440::*gfp-aac* was adjusted to the desired CFU count (Figure 10). It was found that OD of 4 in case of *E. coli* CSH26/pKJK5 and OD of 5 in case of *P. putida* KT2440::*gfp-aac* resulted in the desired cell count.

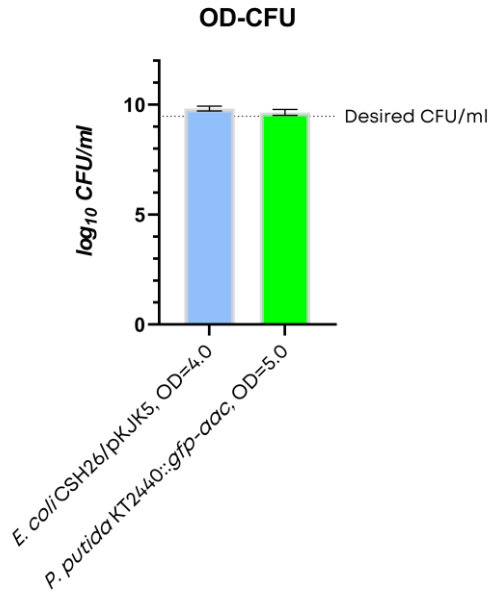

Figure 10. OD (optical density based on absorbance at 600 nm) and CFU/ml ratio for donor strain *E. coli* CSH26/pKJK5 and recipient strain *P. putida* KT2440::*gfp-aac*. The dashed line represents the target CFU/ml value.

3.2.4 Bacterial viability on test and control surfaces during 6 hours of exposure

Prior to conjugation experiments, bacterial viability on surfaces should be assessed to estimate the potential mortality and its effect on conjugation. Substantial mortality level plays a critical role in conjugation experiments, not only as a stress condition marker, but it affects the observed conjugation frequencies with respect to final number of recipient vs final number of donors. Therefore, toxicity of every surface in different conditions must be considered before conjugation experiments. Because of to the plan, for estimating the conjugation frequencies in organics rich environment representing "dirty" surfaces and nutrient-poor oligotrophic environment representing "clean" environment, bacterial viability on the test and control surfaces was firstly determined on those surfaces over time. At first, 6 hours was considered as the maximal time of conjugation and, therefore, was chosen as the maximal timepoint for bacterial viability. It is important to note that on plastic inert control surface no significant mortality of bacteria, during 6 hours of exposure, was observed (Figure 11). Similarly, SQ surface that contains quaternary ammonium compounds as active agent, did not kill the donor or recipient in the used conditions. On the other hand, metallic copper (Cu100) surfaces caused 100% mortality of both donors and recipients already after 1 hour of incubation both, in organics rich and in oligotrophic conditions (Figure 11 D). Therefore, Cu100 surfaces were considered not suitable for conjugation and in following experiments were replaced by Cu10 (surfaces with 10% metallic copper and 90% stainless steel (Table 5.) Also, on metallic silver (Ag) and in oligotrophic conditions high mortality of the donor *E. coli* CSH26/pKJK5 was observed starting from 2 hours of exposure (Figure 11 C). Therefore, oligotrophic exposure medium was considered not suitable for conjugation on Ag surfaces.

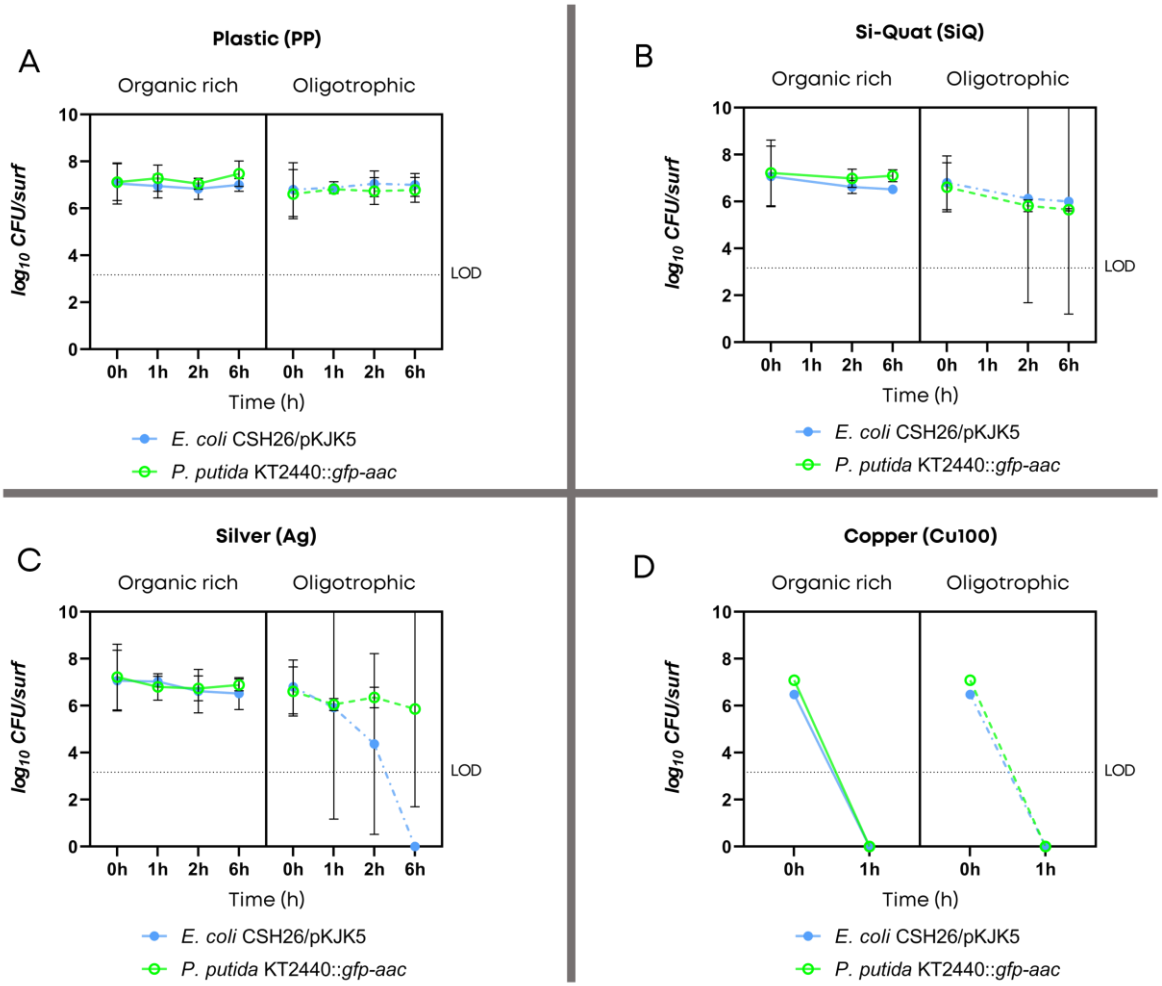

Figure 11. Viability of the donor strain *E. coli* CSH26/pKJK5 **and recipient strain** *P. putida* KT2440::*gfp-aac* **on surfaces in organic rich and oligotrophic conditions.** Dashed line represents limit of determination (LOD) below which bacterial numbers cannot be reliably determined. Error bars represent standard deviation of at least three biological replicates.

On Figure 11 it can be clearly seen that the donor strain *E. coli* CSH26/pKJK5 was more sensitive towards surfaces and was inactivated faster than the recipient strain *P. putida* KT2440::*gfp-aac*. A bioinformatic search conducted using the chromosomal DNA of *P. putida* KT2440 revealed that this strain contained several genes responsible for metal tolerance while such genes are not present in the genome of *E. coli* CSH26. Therefore, in conjugation experiments one would be more worried about the toxicity of surfaces towards the donor strain than towards recipient strain.

Table 6. Genetic determinants of increased tolerance to copper, silver, and arsenic in *P. putida* KT2440. SnapGene was used to identify genes according to annotated genome of *P. putida* KT2440 (Genbank: GCA_024662315.1).

Metal	*Genes*	*Function, protein*
Copper	*cusA/czcA, cueR*	Heavy metal efflux transporter, Cu(I) – responsive transcriptional regulator
Silver	*cusA/czcA*	Heavy metal efflux transporter
Arsenic	*arsR, arsC, arsO, arsH, arsB, arsN2*	Transcription factor, arsenate reductase, arsenate monooxygenase, resistance protein ArsH, arsenical efflux pump membrane protein, N-acetyltransferase

3.2.5 Optimization of protocol for conjugation on surfaces

As stated in materials and methods, the conjugation protocol was based on bacterial exposure to surfaces as suggested in ISO 22196 protocol and according to its modifications as described in Kaur *et al.*(2023). According to this method a suitable amount of liquid containing the desired number of bacteria was placed onto a surface, covered, and incubated in humidified environment during specified time. After that bacteria were washed from the surfaces, plated and enumerated (Figure 12). In conjugation process, some of the ISO 22196 conditions slightly deviated. Specifically, instead of approximately 10^6 CFU per surface, in conjugation, at least 10^7 CFU per surface was used. Also, while in ISO 22196 protocol, plastic foil is used to cover bacteria on surface, in conjugation experiments 25x25 mm glass coverslips were used. The latter modification was used to ensure the full coverage of surface and maximal contact between bacteria and the surface. In addition to the previously listed testing conditions, conjugation also depends on the ratio at which donors and recipients are mixed together (Alderliesten *et al.*, 2020). Therefore, the effect of donor and recipient ratio on conjugation frequency was studied in detail. Also, conjugation frequencies on surfaces in organics rich (SL) and oligotrophic conditions (NB) was compared. All those optimizations were carried out on inert plastic control surfaces.

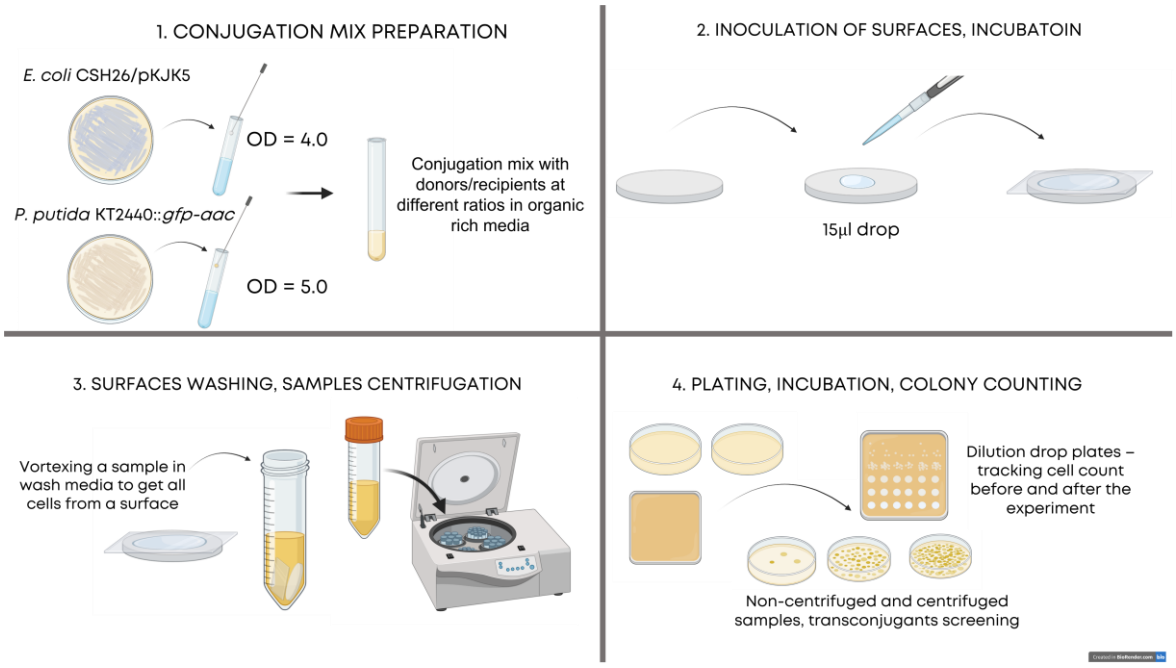

Figure 12. Main steps of the conjugation protocol on surface. This protocol was used in all surface conjugation experiments discussed in this work. The protocol allows to examine conjugation on various types of surfaces and different bacteria can be tested in different nutrient conditions, in different donor-to-recipient ratios.

It is important to note also that the surface testing protocol followed in this study has certain limitations. Specifically, in the case of surfaces with high toxicity, due to substantial mortality level on them, transconjugants cannot be enumerated. In case of toxic surfaces, the concentration of the active agent on surfaces cannot be generally adjusted and thus, conjugation frequencies can be analyzed only on surfaces presenting moderate antimicrobial activity. Therefore, conjugation frequency can be challenging to calculate.

3.2.5.1 Effect of donor-to-recipient ratio on conjugation frequency

As described before, the recipient strain in this study was newly constructed *P. putida* KT2440::*gfp-aac*, and as a donor, *E. coli* CSH26/pKJK5 was used. The donor strain with broad-host conjugative plasmid pKJK5 was used due to its wide use in previous conjugation experiments (S. Sørensen *et al.*, 2003; Bahl *et al.*, 2007; Røder *et al.*, 2013; Klümper *et al.*, 2017; Pallares-Vega *et al.*, 2021; Palm *et al.*, 2022). For estimation of the effect of donor-to-recipient ratio on number of transconjugants, conjugation experiments with different donor-to-recipient initial ratios were conducted on plastic surfaces.

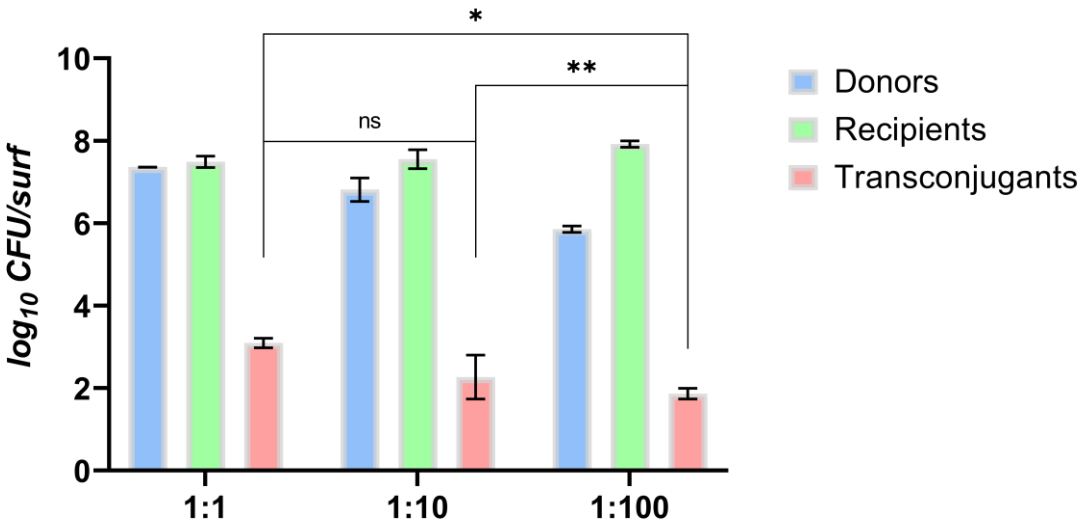

Figure 13. The effect of donor-to-recipient ratio on transconjugant numbers. The number of donors, recipients and transconjugants were determined at the end of 6-hour conjugation experiment in organics-rich environment on plastic surfaces. Data represents the mean of three experiments and their standard deviation. Statistical significance between groups (*Post hoc* Tukey test, $p < 0.05$) are marked with: ns ($p = >0.05$); * ($p = <0.05$), ** ($p = <0.01$).

The donor-to-recipient ratio had an effect on the number of transconjugants and lower donor-to-recipient ratios resulted in lower number of transconjugants (Figure 13). These data are in agreement with similar experiment from Pallares-Vega *et al.* (2021), where same conjugative plasmid, as in this study, was used. Clearly the highest number of transconjugants was obtained in case of 1:1 ratio and this ratio is also the most studied in previous research papers (Alderliesten *et al.*, 2020). As transconjugant count from 1:1 donor-to-recipient ratio was not different from transconjugant count 1:10 donor-to-recipient ratio, the latter was chosen as it was considered to be more relevant for real-life conditions and in case of HGT scenario. 1:100 ratio was omitted due to significantly lower transconjugant count and possible of substantial mortality (Figure 11) of donors on antimicrobial surfaces, resulting in undetectable donor and transconjugant counts. Some of the recent studies have indicated that in a random environment, i.e., not in an inflectional hot spot, e.g., in human excrement samples, the frequency of antibiotic resistant bacteria was less than 15% (Omulo *et al.*, 2022). It has also been shown that if 30% of people carry *Staphylococcus aureus*, then 1-2% carry the resistant variant of this bacterium, MRSA (Gorwitz *et al.*, 2008). Therefore, it may be considered that

the frequency of antibiotic resistant bacteria in a community is not more than 10%, however, it may be strongly dependent on the sample location. Considering these findings on antibiotic resistant strains in natural communities, observed data on conjugation frequencies (Figure 13), and the fact, that substantial mortality level of donors on some surfaces (Figure 11) will lower the potential transconjugants cell count, was decided to use 1:10 donor-to-recipient ratio in this study to illustrate the spread of the plasmid.

3.2.5.2 Effect of exposure environment and time on conjugation frequency

To reveal the effect of organic rich and oligotrophic conditions on conjugation frequency, conjugation experiments in SL and NB environments were conducted on inert plastic coupons. Despite the results showing no statistically significant difference in conjugation frequencies in oligotrophic and organic rich conditions after 2 and 6 hours (Figure 14), organic rich conditions were chosen for further surface conjugation experiments due to higher viability of donors and recipients on antimicrobial surfaces in these conditions (Figure 11). However, the negative effect of nutrient limitations on transfer rate of conjugative plasmid pKJK5 has been shown in earlier studies (Pallares-Vega *et al.*, 2021). Due to the low number of replicates in these pilot experiments (Figure 14, 2 and 6 hours), it might have not been possible to demonstrate statistically significant differences between small changes in effect size. In organics rich environment (SL) conjugation frequency increased with time, however, the frequencies obtained after 6 hours of conjugation (approximately 10^{-5} if calculating transconjugants per recipients and $10^{-4.5}$ if calculating transconjugants per donors) were relatively low and in case of the initial cell counts used in this study, would result in 50-100 transconjugant CFU per surface. Also, earlier studies have shown that conjugation frequency and the amount of transconjugants increase in time up till at least 15 hours (Huisman *et al.*, 2022) . Thus, also in this study, the time for conjugation was increased to 24 hours. Indeed, a significant increase in transconjugant numbers was observed (Figure 14). Interestingly, however, the conjugation frequency calculated for 24 hours was slightly lower than for 16 hours. However, due to practical considerations, 24 hours timepoint was chosen as the timepoint for all subsequent conjugation experiments.

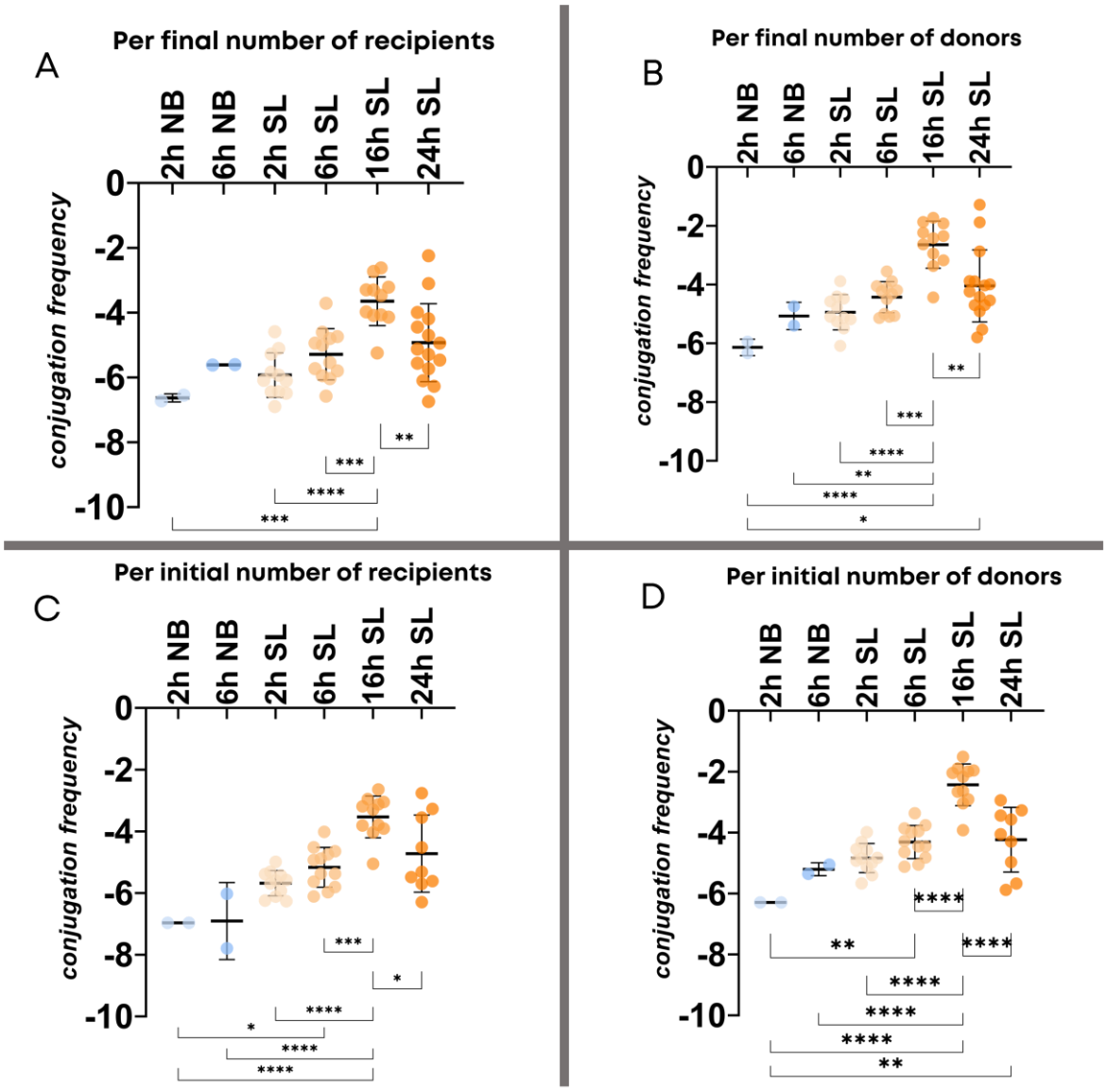

Figure 14. Conjugation frequency in organic rich (SL) and oligotrophic (NB) conditions on plastic coupons for 24 hours. Donor-to-recipient ratio 1:10 was used and conjugation frequency was calculated in comparison with initial or final number of donors or recipients. Only statistically significant differences (*Post hoc* Tukey test, $p < 0.05$) are shown: * ($p = <0.05$), ** ($p = <0.01$), *** ($p = <0.001$), **** ($p = <0.0001$).

3.2.6 Bacterial viability on test and control surfaces during 24 hours of exposure

Considering the results, indicating 24 hours as the most suitable time for conjugation, findings on substantial antibacterial effect of Cu100 surfaces and potential bacteriotoxicity of Ag surfaces (Figure 11), additional test surfaces, Cu10 (10% Cu and 90% steel), AgP (silver containing paint) and SS (stainless steel as a control for metal surfaces) were selected, and additional viability tests with extended duration to 24 hours were performed (Figure 15). Generally, for metal surfaces the same viability pattern as discussed in 3.2.4 was observed. *P. putida* KT2440::*gfp-aac* showed higher tolerance compared with the donor *E. coli* CSH26/pKJK5 (Figure 15 A and B). *E. coli* CSH26/pKJK5 showed notably higher sensitivity towards Cu10 and Ag surfaces compared with *P. putida* KT2440::*gfp-aac.* As discussed previously (Table 6), *P. putida* KT2440 carries several genes responsible for metal tolerance, making this bacterium more viable in the presence of Cu and Ag. Although, the donor strain *E. coli* CSH26/pKJK5, carries a plasmid-borne gene that may contribute to increased tolerance of quaternary ammonium compounds (Table 1), only 0.6 log difference ($p = 0.03$, Students T-test) in viability of *P. putida* KT2440::*gfp-aac* and *E. coli* CSH26/pKJK5 exposed to SiQ surfaces (Figure 15 A and B) was detected after 24 hours.

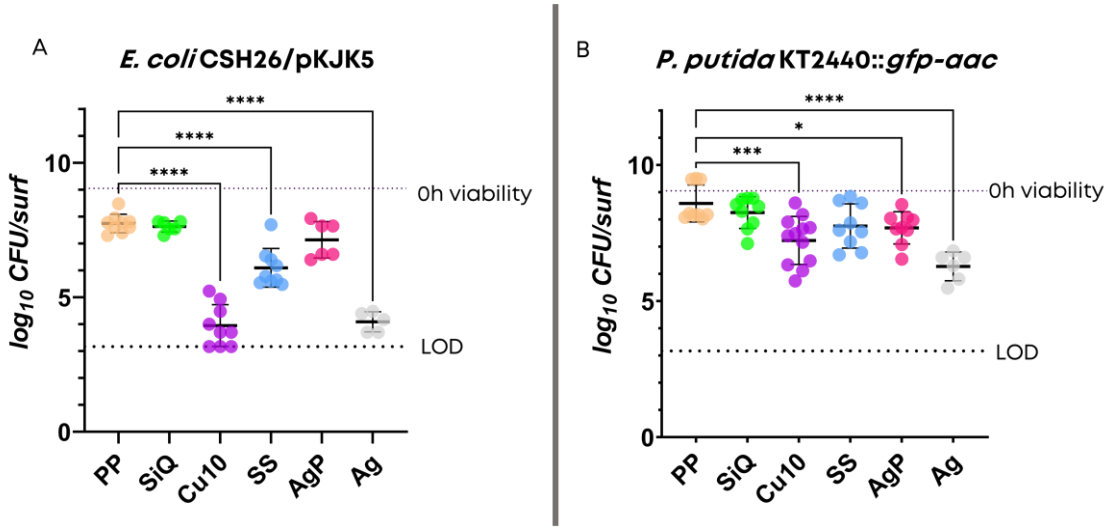

Figure 15. Viability of the donor strain *E. coli* CSH26/pKJK5 and recipient strain *P. putida* KT2440::*gfp-aac* on surfaces in organics rich conditions during 24 hours. Fine dashed line represents cell counts in the beginning of the experiment and coarse dashed line shows the limit of detection (LOD). Values of at least five individual measurements with standard deviation on PP (plastic), SiQ (Si-quaternary ammonium surface), Cu10 (10% copper in steel), SS (stainless steel), Ag (metallic silver) and AgP (silver paint) are shown. Only statistically significant differences (*Post hoc* Dunnett's test, $p < 0.05$) are shown: * ($p = <0.05$), *** ($p = <0.001$), **** ($p = <0.0001$).

3.2.7 Bacterial conjugation on antimicrobial active surfaces

After optimizing the test method for surface conjugation, conjugation frequency between the donor strain *E. coli* CSH26/pKJK5 and recipient strain *P. putida* KT2440::*gfp-aac* was determined on a selected set of surfaces that have a history for antimicrobial use or that have been specified as antimicrobial. As specified in Table 5, such surfaces were either based on metals: Cu10 (10% Cu in stainless steel), Ag (metallic Ag), AgP (Ag containing paint), or quaternary ammonium compound (SiQ). As controls, polypropylene (PP) plastic and stainless steel (SS) were used while the former was considered as an inert surface and the latter as a control for metal surfaces. Metal surfaces were preferred for the study not only due to the widespread use of Cu and Ag metals in antimicrobial applications (Codiţă *et al.*, 2010), but also due to the fact that metals are known to co-select for antibiotic resistance also in the process of conjugation (Argudín, Hoefer and Butaye, 2019; Palm *et al.*, 2022). SiQ was selected due to its different mode of action: while antibacterial effect of metal ions is based on their binding to proteins, quaternary ammonium compounds affect directly bacterial membranes (Tambosi *et al.*, 2018; Schrank, Minbiole and Wuest, 2020).

Observed results from 24 hours conjugation experiments on the selected surfaces indicated that except for Cu10 surfaces, in which case the conjugation frequency was calculated for the final number of donors, no surface changed significantly the conjugation frequency compared with control surfaces of PP or SS (Figure 16). This finding may suggest that low presence of Cu content in surfaces may promote conjugation. However, it should be considered the substantial donor's mortality level and the possibility, that already formed transconjugants may further contribution to the spread of the plasmid. The finding of increased conjugation frequency on Cu containing surfaces was somewhat surprising as in earlier studies with copper (usually soluble salts) this metal has caused decrease in conjugation frequency (Zhang *et al.*, 2019). To the present knowledge, the only article available about bacterial conjugation on solid surfaces is by Warnes, Highmore and Keevil, 2012. In this study Cu-based surfaces were used and due to toxicity of such surfaces the authors could not detect any transconjugants, therefore concluding that the conjugation frequency was low.

On the other hand, the fact that conjugation frequency did not change in the presence of AgP was slightly surprising. However, that can be explained by the extremely low concentration of silver in the paint itself. Earlier studies have indicated that silver ions and also silver nanoparticles increased conjugation frequency (Lu *et al.*, 2020). In this study, pure Ag surfaces could not be used due to their high toxicity to bacterial cells and mortality of donor and

recipient cells (Figure 15). In the future, potentially a new Ag containing surface will be designed and used to study conjugation frequency.

It was also somewhat surprising that SiQ, that contains quaternary ammonium compounds did not affect the conjugation frequency. Earlier studies have indicated that quaternary ammonium compounds may significantly enhance conjugation but only at certain concentrations (Jutkina *et al.*, 2018). For future proof of the effect of quaternary ammonium on conjugation, surface coatings containing such compounds at different concentrations such compounds at different concentrations should be tested.

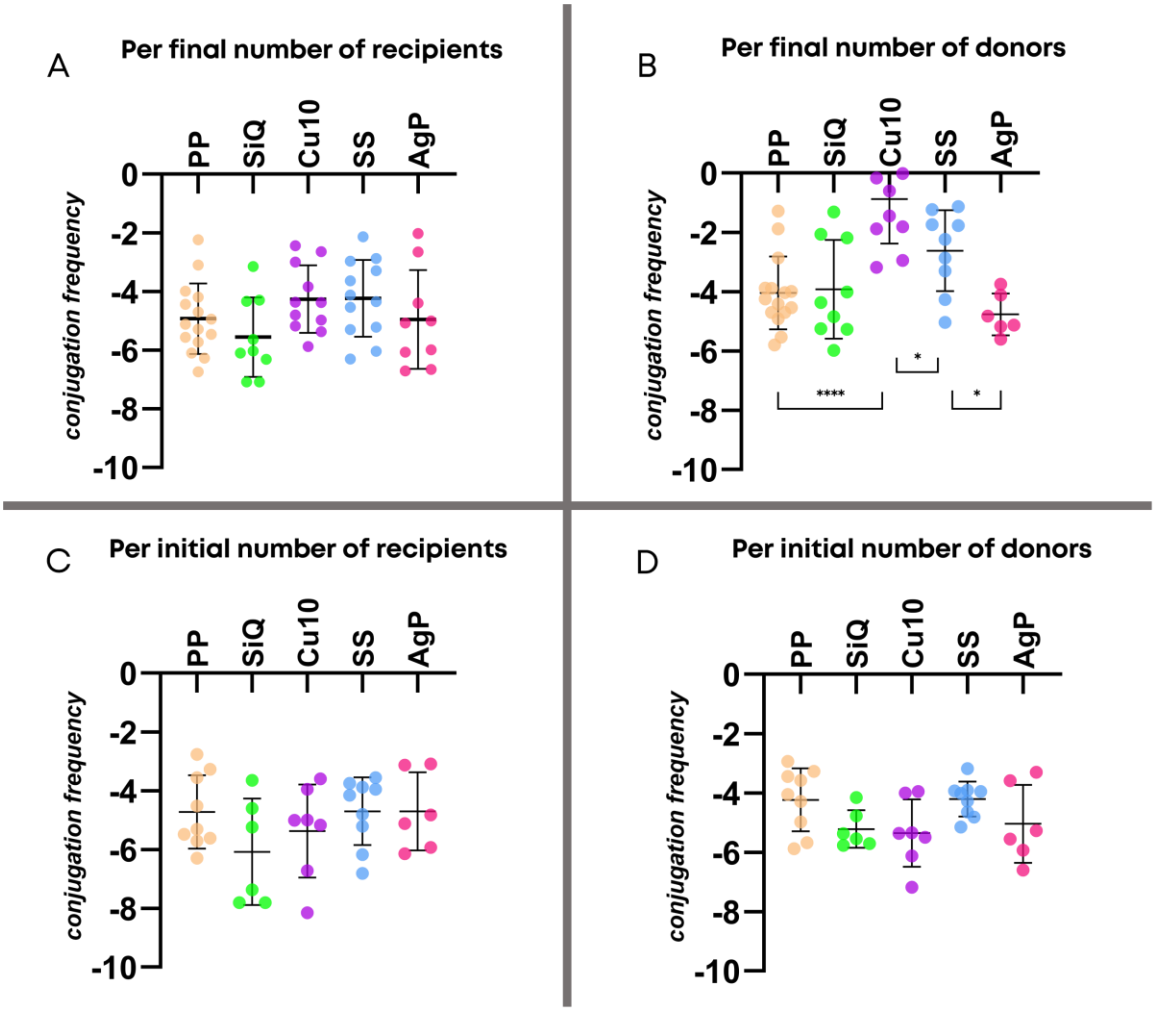

Figure 16. Conjugation frequency on antimicrobial active test surfaces and control surfaces for 24 hours. The donor-to recipient ratio was 1:10; Conjugation was in organic rich conditions and conjugation frequency was calculated in comparison with initial or final number of donors or recipients. Values of at least six individual measurements with standard deviation on PP (plastic), SiQ (Si-quaternary ammonium surface), Cu10 (10% copper on steel), SS (stainless steel), Ag (metallic silver) and AgP (silver paint) are shown. Statistical significance between groups (*Post hoc* Šídák's test, $p < 0.05$) are marked with: * ($p = <0.05$), **** ($p = <0.0001$).

3.3 DISCUSSION

There is evidence on the positive effect of stress conditions on bacterial conjugation and potential increase of HGT. Although conjugation frequencies in the presence of various stressors have been studied in liquid conditions, only one article has been published about conjugation frequency on antimicrobial surfaces. In this articles by Warnes et al. (2012). *E. coli* and *K. pneumoniae* were conjugated in the presence of copper, phosphor bronze, copper nickel, cartridge brass, nickel silver, muntz metal, stainless steel surfaces. The research reports that transfer of a plasmid from *E. coli* and *K. pneumoniae* occurred immediately in the suspension at a frequency of approximately $3*10^{-5}$ and did not increase after 2 hours. Similar results were observed on stainless steel, but no transconjugants were recovered from copper after 2 hours of exposure, likely due to bactericidal properties of this surface. Transfer from *K. pneumoniae* occurred at a lower frequency of approximately $3*10^{-7}$, and, although, transfer did occur immediately when the cells were mixed in suspension and on copper, the transconjugants were not stable and died on subculturing. However, after 2 hours, stable transconjugants were produced on stainless steel and suspension. The result from the paper suggests that HGT readily occurs on dry touch surfaces such as stainless steel but on copper surfaces this process may be inhibited mainly due to their toxicity. In general, the results of this previous study agree with the result of this work. Although Cu100 surfaces were not tested in conjugation experiments, viability experiments showed that substantial mortality level on those surfaces (more than 3 log reduction of CFU) occurred within 1 hour (Figure 11). Due to this fact it was not possible to study the conjugation on Cu100 surfaces but only on designed Cu10 surfaces that contained 10% of copper.

Surface conjugation experiments on Cu10 indicated increased conjugation frequency when transconjugants were calculated per final number of donors (Figure 16). Therefore, was suggested that release of Cu ions from surfaces in relatively low concentration may promote conjugation in organic rich environment and, thus, may induce the spread of conjugative plasmids and antibiotic resistance genes in bacterial community (Zhang *et al.*, 2019; Song *et al.*, 2021).

Conclusions from observations of this study suggest that:

1) Substantial mortality on antimicrobial surfaces affects the conjugation frequency, similar to the observation by Warnes *et al.* (2012);
2) Conjugation occurred in detectable levels in organic rich conditions within 24 hours on touch surfaces such as plastic, silver paint, and stainless steel;

3) A relatively low concentration of Cu ions may increase the conjugation frequency of pKJK5 conjugative plasmid between *E. coli* CSH26/pKJK5 and *P. putida* KT2440::*gfp-aac*;
4) Quaternary ammonium coating used in this study did not affect conjugation frequency compared to plastic and steel control surfaces.

One of the issues encountered during the study was the variability of data for conjugation frequencies. This observed variability in the data can be explained by the randomness of the conjugation process and the processes such as the probability of mating-pair formation, influenced by the density of donors and recipients, their motility, and the structure of the biofilm, where a donor has to meet a recipient bacterium, form a conjugative pilus, and attach to the surface of the recipient bacterium. Also temperature and humidity fluctuations, may affect conjugation frequency (Alderliesten *et al.*, 2020). A recent paper, where overview of conjugation studies was conducted, reported conjugation frequencies that varied over 11 orders of magnitude (Alderliesten *et al.*, 2020). Also, conjugation studies are complicated more because of the high level of diversity in conjugation systems, which makes one study unrelatable or partly relatable for other types of conjugation systems. Despite this fact, conjugation studies are still important, allowing to uncover the common principles in conjugation and developing new antimicrobial strategies, fighting with the spread of ARGs.

One suggestion for further research of conjugation would be to construct a one-way system, where plasmid can only be transferred from a donor strain to the recipient and newly occurred transconjugants would not be able to become donors. This system could provide more accurate insights on the conjugation frequency in respect to only donors. However, such a system is not accurate to represent a more-or-less natural community, where transconjugants also contribute to the spread of plasmid.

SUMMARY

In this work the conjugation on antimicrobial surfaces was conducted to assess the transfer rate of conjugative plasmid pKJK5 between *E. coli* CSH26/pKJK5 as donor and *P. putida* KT2440::*gfp-aac* as recipient.

A protocol for surface conjugation experiment was constructed and a series of optimization experiments, such as determination of cell count, viability assessment was conducted.

Altogether, mortality levels were estimated for Cu100 (99% metallic Cu), Cu10 (10% Cu in stainless steel), Ag (metallic Silver), AgP (silver-containing paint), SiQ (quaternary ammonium compound surface), stainless steel, and plastic surfaces, revealing that Cu100 and Ag exhibited mortality above the limit of tolerance for the conjugation assay. Mortality levels on surfaces were notably different between *E. coli* CSH26/pKJK5 and *P. putida* KT2440::*gfp-aac*, which may in turn have an effect on conjugation frequency.

The study of organic content of the exposure medium, time and donor-to-recipient ratio on conjugation frequency showed organic content did not significantly affect conjugation frequency within 6 hours. Observed data suggest that 6 hours was not sufficient time for conjugation and the frequency of conjugation increased within 16 hours. The lower the donor-to-recipient ratio, the less effective conjugation within the studied timeframe. Based on indications regarding the frequency of antibiotic resistant bacteria in natural communities and the obtained results, 1:10 donor-to-recipient ratio was selected for conjugation on antimicrobial active surfaces Cu10, SiQ, AgP, and control surfaces of plastic and stainless steel. Among the tested antimicrobial surfaces only Cu10 surface was found to increase the conjugation frequency significantly, where conjugation frequency was calculated per the final number of donors. This suggests that the low presence of Cu on surfaces may increase the conjugation frequency between bacteria and thus, also increase HGT, despite the fact that transconjugants also can act as donors.

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Figure 12 was created using BioRender.

REFERENCES

Actis, L.A., Tolmasky, M.E. and Crosa, J.H. (1999) 'Bacterial plasmids: replication of extrachromosomal genetic elements encoding resistance to antimicrobial compounds', *Frontiers in Bioscience: A Journal and Virtual Library*, 4, pp. D43-62. Available at: https://doi.org/10.2741/actis.

Alderliesten, J.B. *et al.* (2020) 'Effect of donor-recipient relatedness on the plasmid conjugation frequency: a meta-analysis', *BMC Microbiology*, 20(1), p. 135. Available at: https://doi.org/10.1186/s12866-020-01825-4.

Argudín, M.A., Hoefer, A. and Butaye, P. (2019) 'Heavy metal resistance in bacteria from animals', *Research in Veterinary Science*, 122, pp. 132–147. Available at: https://doi.org/10.1016/j.rvsc.2018.11.007.

Bagdasarian, M. *et al.* (1981) 'Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas', *Gene*, 16(1), pp. 237–247. Available at: https://doi.org/10.1016/0378-1119(81)90080-9.

Bahl, M.I. *et al.* (2007) 'The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established alpha, beta and delta sub-groups', *Plasmid*, 58(1), pp. 31–43. Available at: https://doi.org/10.1016/j.plasmid.2006.11.007.

Bao, Y. *et al.* (1991) 'An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria', *Gene*, 109(1), pp. 167–168. Available at: https://doi.org/10.1016/0378-1119(91)90604-A.

Burmeister, A.R. (2015) 'Horizontal Gene Transfer', *Evolution, Medicine, and Public Health*, 2015(1), pp. 193–194. Available at: https://doi.org/10.1093/emph/eov018.

Burnett-Boothroyd, S.C. and McCarthy, B.J. (2011) '13 - Antimicrobial treatments of textiles for hygiene and infection control applications: an industrial perspective', in Brian J. McCarthy (ed.) *Textiles for Hygiene and Infection Control*. Woodhead Publishing (Woodhead Publishing Series in Textiles), pp. 196–209. Available at: https://doi.org/10.1533/9780857093707.3.196.

Cassidy, S.S. *et al.* (2020) 'Antimicrobial surfaces: A need for stewardship?', *PLoS Pathogens*, 16(10), p. e1008880. Available at: https://doi.org/10.1371/journal.ppat.1008880.

Chen, X. *et al.* (2019) 'Antibiotic-resistance gene transfer in antibiotic-resistance bacteria under different light irradiation: Implications from oxidative stress and gene expression', *Water Research*, 149, pp. 282–291. Available at: https://doi.org/10.1016/j.watres.2018.11.019.

Choi, K.-H. and Schweizer, H.P. (2006) 'mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa', *Nature Protocols*, 1(1), pp. 153–161. Available at: https://doi.org/10.1038/nprot.2006.24.

Codiţă, I. *et al.* (2010) ‘Antimicrobial activity of copper and silver nanofilms on nosocomial bacterial species’, *Roumanian Archives of Microbiology and Immunology*, 69(4), pp. 204–212.

de la Cruz, F. *et al.* (2010) ‘Conjugative DNA metabolism in Gram-negative bacteria’, *FEMS microbiology reviews*, 34(1), pp. 18–40. Available at: https://doi.org/10.1111/j.1574-6976.2009.00195.x.

Daood, U. *et al.* (2020) ‘A quaternary ammonium silane antimicrobial triggers bacterial membrane and biofilm destruction’, *Scientific Reports*, 10, p. 10970. Available at: https://doi.org/10.1038/s41598-020-67616-z.

Davison, J. (1999) ‘Genetic Exchange between Bacteria in the Environment’, *Plasmid*, 42(2), pp. 73–91. Available at: https://doi.org/10.1006/plas.1999.1421.

EPA (2022) ‘Test Method for Evaluating the Efficacy of Antimicrobial Surface Coatings MB-40-00’. Environmental Protection Agency, US.

Ferré-D’Amaré, A.R. (2010) ‘The glmS ribozyme: use of a small molecule coenzyme by a gene-regulatory RNA’, *Quarterly reviews of biophysics*, 43(4), pp. 423–447. Available at: https://doi.org/10.1017/S0033583510000144.

Gorwitz, R.J. *et al.* (2008) ‘Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004’, *The Journal of Infectious Diseases*, 197(9), pp. 1226–1234. Available at: https://doi.org/10.1086/533494.

Graf, F.E. *et al.* (2019) ‘Inhibiting conjugation as a tool in the fight against antibiotic resistance’, *Drug Development Research*, 80(1), pp. 19–23. Available at: https://doi.org/10.1002/ddr.21457.

Green, M.R. and Sambrook, J. (2019) ‘Screening Colonies by Polymerase Chain Reaction (PCR)’, *Cold Spring Harbor Protocols*, 2019(6). Available at: https://doi.org/10.1101/pdb.prot095224.

Guo, M.-T. and Zhang, G.-S. (2017) ‘Graphene oxide in the water environment could affect tetracycline-antibiotic resistance’, *Chemosphere*, 183, pp. 197–203. Available at: https://doi.org/10.1016/j.chemosphere.2017.04.145.

Headd, B. and Bradford, S.A. (2018) ‘Physicochemical Factors That Favor Conjugation of an Antibiotic Resistant Plasmid in Non-growing Bacterial Cultures in the Absence and Presence of Antibiotics’, *Frontiers in Microbiology*, 9, p. 2122. Available at: https://doi.org/10.3389/fmicb.2018.02122.

Headd, B. and Bradford, S.A. (2020) ‘The Conjugation Window in an Escherichia coli K-12 Strain with an IncFII Plasmid’, *Applied and Environmental Microbiology*. Edited by C.M. Dozois, 86(17), pp. e00948-20. Available at: https://doi.org/10.1128/AEM.00948-20.

Hora, P.I. *et al.* (2020) ‘Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications’, *Environmental Science & Technology Letters*, p. acs.estlett.0c00437. Available at: https://doi.org/10.1021/acs.estlett.0c00437.

Huddleston, J.R. (2014) ‘Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes’, *Infection and Drug Resistance*, 7, pp. 167–176. Available at: https://doi.org/10.2147/IDR.S48820.

Huisman, J.S. *et al.* (2022) ‘Estimating plasmid conjugation rates: A new computational tool and a critical comparison of methods’, *Plasmid*, 121, p. 102627. Available at: https://doi.org/10.1016/j.plasmid.2022.102627.

ISO (2011) ‘22196:2011 Measurement of antibacterial activity on plastics and other non-porous surfaces’. International Organization for Standardization.

Jia, Y. *et al.* (2021) ‘Acetaminophen promotes horizontal transfer of plasmid-borne multiple antibiotic resistance genes’, *Science of The Total Environment*, 782, p. 146916. Available at: https://doi.org/10.1016/j.scitotenv.2021.146916.

Jiao, Y.-N. *et al.* (2017) ‘Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems’, *Chemosphere*, 184, pp. 53–61. Available at: https://doi.org/10.1016/j.chemosphere.2017.05.149.

Jutkina, J. *et al.* (2018) ‘Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations’, *Science of The Total Environment*, 616–617, pp. 172–178. Available at: https://doi.org/10.1016/j.scitotenv.2017.10.312.

Kaur, H. *et al.* (2023) ‘Antibacterial activity of solid surfaces is critically dependent on relative humidity, inoculum volume and organic soiling’. bioRxiv, p. 2023.03.28.534510. Available at: https://doi.org/10.1101/2023.03.28.534510.

Klümper, U. *et al.* (2015) ‘Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community’, *The ISME Journal*, 9(4), pp. 934–945. Available at: https://doi.org/10.1038/ismej.2014.191.

Klümper, U. *et al.* (2017) ‘Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner’, *The ISME Journal*, 11(1), pp. 152–165. Available at: https://doi.org/10.1038/ismej.2016.98.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. (2010) ‘How antibiotics kill bacteria: from targets to networks’, *Nature reviews. Microbiology*, 8(6), pp. 423–435. Available at: https://doi.org/10.1038/nrmicro2333.

Kramer, A. and Assadian, O. (2014) ‘Survival of Microorganisms on Inanimate Surfaces’, *Use of Biocidal Surfaces for Reduction of Healthcare Acquired Infections*, pp. 7–26. Available at: https://doi.org/10.1007/978-3-319-08057-4_2.

Kües, U. and Stahl, U. (1989) ‘Replication of Plasmids in Gram-Negative Bacteria’, 53.

Lederberg, J. and Tatum, E.L. (1946) ‘Gene recombination in Escherichia coli’, *Nature*, 158(4016), p. 558. Available at: https://doi.org/10.1038/158558a0.

Lemire, J.A., Harrison, J.J. and Turner, R.J. (2013) ‘Antimicrobial activity of metals: mechanisms, molecular targets and applications’, *Nature Reviews Microbiology*, 11(6), pp. 371–384. Available at: https://doi.org/10.1038/nrmicro3028.

Li, L. *et al.* (2018) ‘Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community’, *Environmental Science & Technology Letters*, 5(5), pp. 260–265. Available at: https://doi.org/10.1021/acs.estlett.8b00105.

Lin, W. *et al.* (2016) ‘Reduction in horizontal transfer of conjugative plasmid by UV irradiation and low-level chlorination’, *Water Research*, 91, pp. 331–338. Available at: https://doi.org/10.1016/j.watres.2016.01.020.

Lu, J. *et al.* (2018) ‘Triclosan at environmentally relevant concentrations promotes horizontal transfer of multidrug resistance genes within and across bacterial genera’, *Environment International*, 121, pp. 1217–1226. Available at: https://doi.org/10.1016/j.envint.2018.10.040.

Lu, J. *et al.* (2020) ‘Both silver ions and silver nanoparticles facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes’, *Water Research*, 169, p. 115229. Available at: https://doi.org/10.1016/j.watres.2019.115229.

Lujan, S.A. *et al.* (2007) ‘Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase’, *Proceedings of the National Academy of Sciences*, 104(30), pp. 12282–12287. Available at: https://doi.org/10.1073/pnas.0702760104.

Mahanta, U., Khandelwal, M. and Deshpande, A.S. (2021) ‘Antimicrobial surfaces: a review of synthetic approaches, applicability and outlook’, *Journal of Materials Science*, 56(32), pp. 17915–17941. Available at: https://doi.org/10.1007/s10853-021-06404-0.

Mathews, S., Kumar, R. and Solioz, M. (2015) ‘Copper Reduction and Contact Killing of Bacteria by Iron Surfaces’, *Applied and Environmental Microbiology*, 81(18), pp. 6399–6403. Available at: https://doi.org/10.1128/AEM.01725-15.

Miller J.H. (1972) ‘Experiments in Molecular Genetics’.

Mohapatra, S. *et al.* (2023) ‘Quaternary ammonium compounds of emerging concern: Classification, occurrence, fate, toxicity and antimicrobial resistance’, *Journal of Hazardous Materials*, 445, p. 130393. Available at: https://doi.org/10.1016/j.jhazmat.2022.130393.

Muller, M.P. *et al.* (2016) ‘Antimicrobial surfaces to prevent healthcare-associated infections: a systematic review’, *Journal of Hospital Infection*, 92(1), pp. 7–13. Available at: https://doi.org/10.1016/j.jhin.2015.09.008.

Muñoz-López, M. and García-Pérez, J.L. (2010) ‘DNA Transposons: Nature and Applications in Genomics’, *Current Genomics*, 11(2), pp. 115–128. Available at: https://doi.org/10.2174/138920210790886871.

Murray, C.J.L. *et al.* (2022) ‘Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis’, *The Lancet*, 399(10325), pp. 629–655. Available at: https://doi.org/10.1016/S0140-6736(21)02724-0.

Nordholt, N. *et al.* (2021) ‘Persistence against benzalkonium chloride promotes rapid evolution of tolerance during periodic disinfection’, *Nature Communications*, 12(1), p. 6792. Available at: https://doi.org/10.1038/s41467-021-27019-8.

Norman, A., Hansen, L.H. and Sørensen, S.J. (2009) 'Conjugative plasmids: vessels of the communal gene pool', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1527), pp. 2275–2289. Available at: https://doi.org/10.1098/rstb.2009.0037.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000) 'Lateral gene transfer and the nature of bacterial innovation', *Nature*, 405(6784), pp. 299–304. Available at: https://doi.org/10.1038/35012500.

Omulo, S. *et al.* (2022) 'Estimating the population-level prevalence of antimicrobial-resistant enteric bacteria from latrine samples', *Antimicrobial Resistance & Infection Control*, 11(1), p. 106. Available at: https://doi.org/10.1186/s13756-022-01145-4.

Pallares-Vega, R. *et al.* (2021) 'Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild Escherichia coli Strains', *Frontiers in Microbiology*, 12, p. 656250. Available at: https://doi.org/10.3389/fmicb.2021.656250.

Palm, M. *et al.* (2022) 'The Effect of Heavy Metals on Conjugation Efficiency of an F-Plasmid in Escherichia coli', *Antibiotics*, 11(8), p. 1123. Available at: https://doi.org/10.3390/antibiotics11081123.

Piscon, B. *et al.* (2023) 'The Effect of Outer Space and Other Environmental Cues on Bacterial Conjugation', *Microbiology Spectrum*, p. e0368822. Available at: https://doi.org/10.1128/spectrum.03688-22.

Platt, R. *et al.* (2000) 'Genetic system for reversible integration of DNA constructs and lacZ gene fusions into the Escherichia coli chromosome', *Plasmid*, 43(1), pp. 12–23. Available at: https://doi.org/10.1006/plas.1999.1433.

Politano, A.D. *et al.* (2013) 'Use of Silver in the Prevention and Treatment of Infections: Silver Review', *Surgical Infections*, 14(1), pp. 8–20. Available at: https://doi.org/10.1089/sur.2011.097.

Popowska, M. and Krawczyk-Balska, A. (2013) 'Broad-host-range IncP-1 plasmids and their resistance potential', *Frontiers in Microbiology*, 4, p. 44. Available at: https://doi.org/10.3389/fmicb.2013.00044.

Rakowski, S.A. and Filutowicz, M. (2013) 'Plasmid R6K Replication Control', *Plasmid*, 69(3), pp. 231–242. Available at: https://doi.org/10.1016/j.plasmid.2013.02.003.

Regenhardt, D. *et al.* (2002) 'Pedigree and taxonomic credentials of Pseudomonas putida strain KT2440', *Environmental Microbiology*, 4(12), pp. 912–915. Available at: https://doi.org/10.1046/j.1462-2920.2002.00368.x.

Røder, H.L. *et al.* (2013) 'The impact of the conjugative IncP-1 plasmid pKJK5 on multispecies biofilm formation is dependent on the plasmid host', *FEMS microbiology letters*, 344(2), pp. 186–192. Available at: https://doi.org/10.1111/1574-6968.12175.

Roos, K., Werner, E. and Loessner, H. (2015) 'Multicopy integration of mini-Tn7 transposons into selected chromosomal sites of a Salmonella vaccine strain', *Microbial Biotechnology*, 8(1), pp. 177–187. Available at: https://doi.org/10.1111/1751-7915.12187.

Rozwandowicz, M. *et al.* (2018) 'Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae', *Journal of Antimicrobial Chemotherapy*, 73(5), pp. 1121–1137. Available at: https://doi.org/10.1093/jac/dkx488.

Salah, I., Parkin, I.P. and Allan, E. (2021) 'Copper as an antimicrobial agent: recent advances', *RSC Advances*, 11(30), pp. 18179–18186. Available at: https://doi.org/10.1039/D1RA02149D.

Schmidt, S.B.I. *et al.* (2022) 'Biocides used as material preservatives modify rates of de novo mutation and horizontal gene transfer in bacteria', *Journal of Hazardous Materials*, 437, p. 129280. Available at: https://doi.org/10.1016/j.jhazmat.2022.129280.

Schrank, C.L., Minbiole, K.P.C. and Wuest, W.M. (2020) 'Are Quaternary Ammonium Compounds, the Workhorse Disinfectants, Effective against Severe Acute Respiratory Syndrome-Coronavirus-2?', *ACS Infectious Diseases*, 6(7), pp. 1553–1557. Available at: https://doi.org/10.1021/acsinfecdis.0c00265.

Sengeløv, G. *et al.* (2001) 'Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette Between Pseudomonas Strains in the Rhizosphere and Spermosphere of Barley Seedlings', *Current Microbiology*, 42(3), pp. 160–167. Available at: https://doi.org/10.1007/s002840010197.

Sharan, S.K. *et al.* (2009) 'Recombineering: A Homologous Recombination-Based Method of Genetic Engineering', *Nature protocols*, 4(2), pp. 206–223. Available at: https://doi.org/10.1038/nprot.2008.227.

Silhavy, T.J., Kahne, D. and Walker, S. (2010) 'The Bacterial Cell Envelope', *Cold Spring Harbor Perspectives in Biology*, 2(5), p. a000414. Available at: https://doi.org/10.1101/cshperspect.a000414.

Song, Z. *et al.* (2021) 'Copper Ions Facilitate the Conjugative Transfer of SXT/R391 Integrative and Conjugative Element Across Bacterial Genera', *Frontiers in Microbiology*, 11, p. 616792. Available at: https://doi.org/10.3389/fmicb.2020.616792.

Soni, A. and Brightwell, G. (2022) 'Nature-Inspired Antimicrobial Surfaces and Their Potential Applications in Food Industries', *Foods*, 11(6), p. 844. Available at: https://doi.org/10.3390/foods11060844.

Sørensen, A.H. *et al.* (2003) 'Conjugative plasmid conferring resistance to olaquindox', *Antimicrobial Agents and Chemotherapy*, 47(2), pp. 798–799. Available at: https://doi.org/10.1128/AAC.47.2.798-799.2003.

Sørensen, S. *et al.* (2003) 'Direct Detection and Quantification of Horizontal Gene Transfer by Using Flow Cytometry and gfp as a Reporter Gene', *Current microbiology*, 47, pp. 129–33. Available at: https://doi.org/10.1007/s00284-002-3978-0.

Stan, Diana *et al.* (2021) 'Natural Compounds With Antimicrobial and Antiviral Effect and Nanocarriers Used for Their Transportation', *Frontiers in Pharmacology*, 12, p. 723233. Available at: https://doi.org/10.3389/fphar.2021.723233.

Sudha, V.B.P. *et al.* (2012) 'Storing Drinking-water in Copper pots Kills Contaminating Diarrhoeagenic Bacteria', *Journal of Health, Population, and Nutrition*, 30(1), pp. 17–21.

Tacconelli, E. *et al.* (2018) 'Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis', *The Lancet Infectious Diseases*, 18(3), pp. 318–327. Available at: https://doi.org/10.1016/S1473-3099(17)30753-3.

Tambosi, R. *et al.* (2018) 'Silver and Copper Acute Effects on Membrane Proteins and Impact on Photosynthetic and Respiratory Complexes in Bacteria', *mBio*, 9(6), pp. e01535-18. Available at: https://doi.org/10.1128/mBio.01535-18.

Tang, H. *et al.* (2022) 'Effects of iron mineral adhesion on bacterial conjugation: Interfering the transmission of antibiotic resistance genes through an interfacial process', *Journal of Hazardous Materials*, 435, p. 128889. Available at: https://doi.org/10.1016/j.jhazmat.2022.128889.

Taylor, R., Walker, D. and McInnes, R. (1993) 'E. coli host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing', *Nucleic acids research*, 21(7). Available at: https://doi.org/10.1093/nar/21.7.1677.

Thompson, C.M.A. *et al.* (2023) 'Plasmids manipulate bacterial behaviour through translational regulatory crosstalk', *PLOS Biology*, 21(2), p. e3001988. Available at: https://doi.org/10.1371/journal.pbio.3001988.

Virolle, C. *et al.* (2020) 'Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level', *Genes*, 11(11), p. 1239. Available at: https://doi.org/10.3390/genes11111239.

Waddell, C.S. and Craig, N.L. (1989) 'Tn7 transposition: recognition of the attTn7 target sequence.', *Proceedings of the National Academy of Sciences of the United States of America*, 86(11), pp. 3958–3962.

Waksman, G. (2019) 'From conjugation to T4S systems in Gram-negative bacteria: a mechanistic biology perspective', *EMBO reports*, 20(2), p. e47012. Available at: https://doi.org/10.15252/embr.201847012.

Wallden, K., Rivera-Calzada, A. and Waksman, G. (2010) 'Type IV secretion systems: versatility and diversity in function', *Cellular Microbiology*, 12(9), pp. 1203–1212. Available at: https://doi.org/10.1111/j.1462-5822.2010.01499.x.

Wang, X. *et al.* (2018) 'Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes', *NanoImpact*, 10, pp. 61–67. Available at: https://doi.org/10.1016/j.impact.2017.11.006.

Warnes, S.L., Highmore, C.J. and Keevil, C.W. (2012) 'Horizontal Transfer of Antibiotic Resistance Genes on Abiotic Touch Surfaces: Implications for Public Health', *mBio*, 3(6), pp. e00489-12. Available at: https://doi.org/10.1128/mBio.00489-12.

von Wintersdorff, C.J.H. *et al.* (2016) 'Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer', *Frontiers in Microbiology*, 7, p. 173. Available at: https://doi.org/10.3389/fmicb.2016.00173.

Ye, C. *et al.* (2022) ‘Dormancy induced by oxidative damage during disinfection facilitates conjugation of ARGs through enhancing efflux and oxidative stress: A lagging response’, *Water Research*, 221, p. 118798. Available at: https://doi.org/10.1016/j.watres.2022.118798.

Yin, I.X. *et al.* (2020) ‘The Antibacterial Mechanism of Silver Nanoparticles and Its Application in Dentistry’, *International Journal of Nanomedicine*, 15, pp. 2555–2562. Available at: https://doi.org/10.2147/IJN.S246764.

Zhang, S. *et al.* (2019) ‘Copper nanoparticles and copper ions promote horizontal transfer of plasmid-mediated multi-antibiotic resistance genes across bacterial genera’, *Environment International*, 129, pp. 478–487. Available at: https://doi.org/10.1016/j.envint.2019.05.054.

Zhang, Y. *et al.* (2018) ‘Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment’, *Environmental Pollution (Barking, Essex: 1987)*, 237, pp. 74–82. Available at: https://doi.org/10.1016/j.envpol.2018.01.032.

APPENDIX

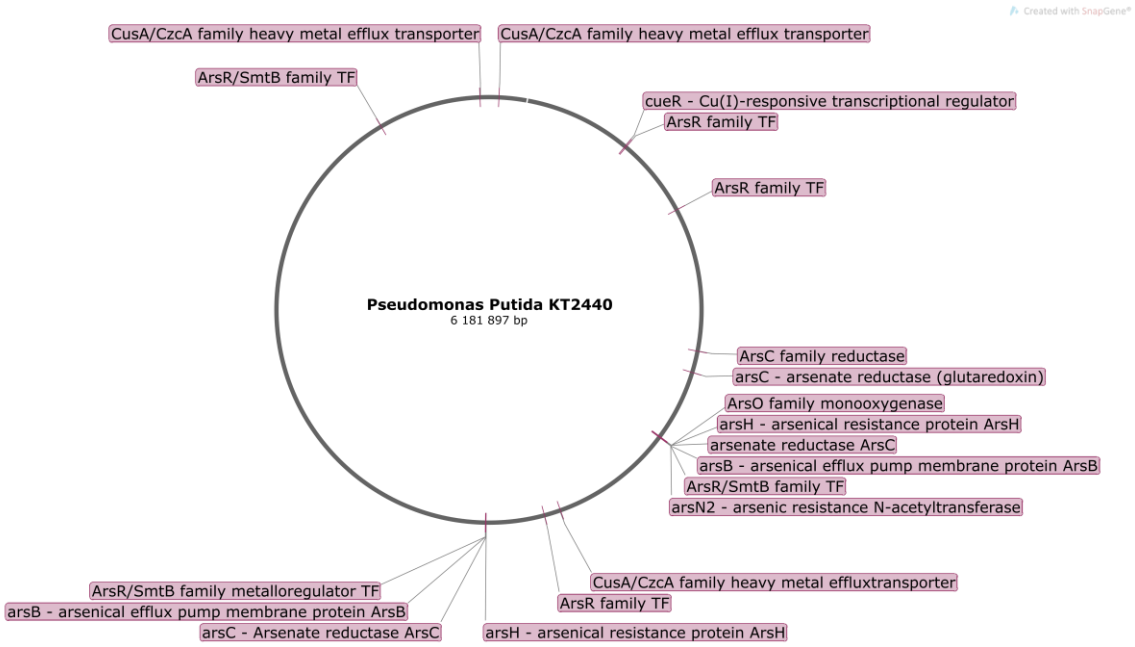

Figure 17. *P. putida* KT2440 genes and transcription factors responsible for copper, silver, and arsenic resistance. Genes and TFs were identified from the chromosomal sequence data, using SnapGene.

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