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EVOLUTION OF ANTIMICROBIAL RESISTANCE: BIOTIC  
AND ABIOTIC FACTORS AS DRIVERS OF NATURAL  
TRANSFORMATION

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## **Abstract**

To survive environmental stressors, such as DNA damage and the presence of antibiotics, bacteria have developed a mechanism of natural competence, leading to the acquisition of beneficial exogenous genes through natural transformation. Natural transformation is an understudied horizontal gene transfer (HGT) mechanism which plays an important role in the transmission and dissemination of eDNA in natural bacterial populations, providing a source of genetic diversity. However, there is a concern that this mechanism may also increase the prevalence of antimicrobial resistance (AMR) in environmental bacteria. In addition, there are some key environmental bacteria that have not yet been documented to be naturally transformable, such as *Escherichia coli* (*E. coli*), and its currently unknown whether biotic and/or abiotic stressors, such as the presence of competitors and availability of nutrients, can influence the natural transformation frequency. Recent literature suggests that more species than previously documented are capable of undergoing a competent state, and initial evidence has been generated here to support the ability of *E. coli* to perform natural transformation. However, further testing and replication are now required to fully understand and support this. In this study environmentally sampled *E. coli* strains were tested to see if they were naturally transformable. Then, using a model species system with *Acinetobacter baylyi* (*A. baylyi*), transformation frequency was assessed following exposure to different competitor species and altered nutrient concentration, with and without the addition of extracellular peptide filtrate. This work found that nutrient availability and the presence of certain competitor species have a significant effect on natural transformation efficiency of *A. baylyi*. Given that bacteria are extremely evolvable depending on their surrounding environment, it is crucial to understand the evolutionary drivers of mechanisms such as natural transformation and thus, reduce potential threats of environmental AMR emergence to One Health systems.

## **Abstract**

Per sopravvivere ai fattori di stress ambientali, come danni al DNA e alla presenza di antibiotici, alcuni batteri hanno sviluppato un meccanismo di competenza naturale che porta all'acquisizione di geni esogeni benefici attraverso il processo di trasformazione naturale. La trasformazione naturale è un meccanismo di trasferimento genico orizzontale (HGT) attualmente poco studiato che svolge un ruolo importante nella trasmissione e nella diffusione di DNA ambientale nelle popolazioni batteriche naturali, fornendo una fonte di diversità genetica. Se da un lato si tratta di una fonte di diversità, dall'altro questi meccanismi facilitano la trasmissione e la prevalenza della resistenza antimicrobica (AMR). Alcuni batteri ambientali chiave, per esempio *Escherichia coli* (*E. coli*), non sono stati ancora identificati come naturalmente trasformabili, inoltre attualmente non è ben chiaro se fattori di stress biotici e/o abiotici, come la co-presenza di specie (competitori) e la disponibilità di nutrienti, possano influenzare la frequenza di trasformazione naturale. Grazie a diversi studi, nuove specie vengono riconosciute come specie "naturalmente competenti" servendo da sostegno per supporre che *E. coli* possa essere incluso tra i batteri capaci di trasformazione naturale. Tuttavia, sono necessari ulteriori studi e repliche per comprendere e supportare pienamente questo fenomeno. In questo studio sono stati testati ceppi di *E. coli* da campioni ambientali per verificare se fossero naturalmente trasformabili. Inoltre, utilizzando una specie modello, *Acinetobacter baylyi* (*A. baylyi*), è stata valutata la frequenza di trasformazione in seguito alla co-esposizione a diverse specie (competitori) e a una concentrazione di nutrienti alterata, con e senza la presenza di peptidi extracellulari naturalmente presenti. Da questo lavoro è emerso che la disponibilità di nutrienti e la presenza di alcune specie hanno effetti significativi sull'efficienza di trasformazione naturale in *A. baylyi*. Poiché i batteri tendono a mutare e ad evolvere in base a diversi stimoli ambientali, è fondamentale comprendere i fattori evolutivi di meccanismi come la trasformazione naturale e ridurre così le potenziali minacce derivate dall'emergere dell'antibiotico resistenza nell'ambiente, considerando la questione da un punto di vista One Health.

## 1.Introduction

Since the evolution of visible life form was first discovered by Charles Darwin in “the Origin of Species” (*The Origin of Species*, n.d.), bacteria have been documented to both mutate and evolve, depending on various stressors and the surrounding environment. Further evidence for bacterial evolution was generated with the discovery of DNA and subsequently with the advent of next generation sequencing, proving that bacteria may gain and lose different genes that aid survival, but also that ecology and symbiotic relationship between microorganisms and their niche seems to be involved in this process (Smillie et al., 2011). These evolutionary processes correlate to fast time scale changes to microbiomes and ecology, which have been investigated by many researchers. Although it’s a vast and important topic, these studies are somewhat restricted to the timeframe, species and environment tested (Lenski et al., 2009).

Bacteria, Archaea, viruses (including phages) and even the eukaryotes have evolved and mutated/adapted in correlation to different selective pressures in the surrounding environment. To do so, beside from eukaryotic recombination and vertical gene transfer, the exchange of genetic material can take place also via horizontal gene transfer (HGT) mechanisms. Along with mutation, genetic drift, selection, and dispersal, HGT is a driving force of bacterial evolution. The freedom from sexual reproduction in archaea and bacteria, allows the sharing of genetic material even with distantly related organisms and the keeping of these genes through offspring (Arnold et al., 2022). HGT involves the transfer of genetic material from a cell to another and it is based on three different mechanisms, including conjugation, transduction and transformation. Conjugation involves the direct transfer of DNA through multiphase processes via direct cell-to-cell contact (e.g. using cell surface pili or adhesin). The transfer of DNA by transduction is the indirect result of bacteriophages replication (Von Wintersdorff et al., 2016). Finally, natural transformation refers to a specific physiological state called “competence”, in which prokaryotes are able to take up environmental DNA (eDNA) from their surrounding environment (Blokesch, 2016) and recombine it into their genome or reconvert it into extra-chromosomal genetic elements.

If from one side, HGT is a driving force of microorganism evolution, it is also a mechanism by which antimicrobial resistance genes (ARGs) are spread across taxa and between environments. Antimicrobial resistance (AMR) occurs when microorganisms no longer respond to antimicrobial compounds, such as antibiotics and fungicides, and so they become ineffective and diseases become increasingly difficult to treat. The lack of development of new antibiotics and increased resistance emergence are posing AMR as one of top list healthcare global threats. Indeed, a recent survey declared that “ an estimated 4·95 million (3·62–6·57) deaths associated with bacterial AMR in 2019, including 1·27 million (95% UI 0·911–1·71) deaths attributable to bacterial AMR” (Collaborators, 2022).

Antimicrobial overuse and misuse is a critical concern that not only involves the clinical setting but also agriculture, farming, wastewater treatment, and the wider natural environment. Scientists have recently discovered that, not only are diverse microbial communities present in the environment, but also different environments have different AMR resistance profiles (Williams et al., 2016). Furthermore, whilst discovering new genes is crucial for creating new and successful pharmaceuticals and preventing the spread of AMR, it is challenging due to the evolvability of microorganisms. Furthermore, there is an additional concern and potential exposure route to people consuming raw food, which has been a public health risk when AMR zoonotic bacteria are present in the food. Many ways in which food can be contaminated with ARGs and antimicrobial resistant bacteria (ARBs) have been identified (Verraes et al., 2013).

### **1.1 Horizontal Gene Transfer**

Traditionally, it was thought that bacteria could evolve and transfer genes only clonally, and this involved the transfer of only parenting genes, minimising the exchange of DNA to and from other diverse species. Now, more so with the development of genomic sequencing, it has been proven that even distant phylogenetically unrelated species share the same genes, indicating a high probability of HGT occurring (Jain et al., 2002).

As already discussed, there are three main mechanisms that facilitate HGT in prokaryotes (Figure 1): conjugation, transduction and transformation. These HGT mechanisms are described as canonical mechanisms. In addition to the canonical mechanisms, DNA can enter the recipient cell in others alternative “non canonical” mechanisms, such as via membrane vesicles, tiny pilus-like structure “nanotubes” or phase-like gene transfer agents. Besides from “non canonical” mechanisms, also some exception can take place, for example, conjugation process can occur not only with plasmid integration in chromosome DNA, but also mobile genetic elements (MGEs), such as integrative conjugative elements (ICEs) and transposons, can be integrated.

Transduction, which involves phage predation resulting in the integration of bacteria genome into phage genome, can be specialized or generalised. With specialisation, prophages specifically incorporate flanking host DNAs region, whereas with the generalisation, random pieces are incorporated during cell lysis (Soucy et al., 2015).

Once DNA, crosses the cell membrane in the above-mentioned ways and it is inside the cell, it may recombine into the host genome, which can be mediated via ubiquitous RecA protein or independently. The function of RecA protein is to mediate the recombination between the foreign DNA and the recipient genome by facilitating the search for homology sequence between them. The result of replacement with high sequence homology it is called

“homologous recombination”. Instead, the recombination without the RecA protein guide usually is the result of non-homologous DNA fragments hybridization during replication.

With “non canonical” mechanisms and exceptions (such as plasmid uptake and as cited above for conjugation processes) to recombine with the host genome, they use a site-specific recombinases or transposases. Although most recombinases and transposases require to have a certain percentage of sequence identity between the donor and the recipient DNA. The main difference between the last 2 processes is that the plasmids have origin of replication so they can persist in the bacteria cytoplasm, instead most ICEs lack this, so they need to recombine with the host genome (Arnold et al., 2022).

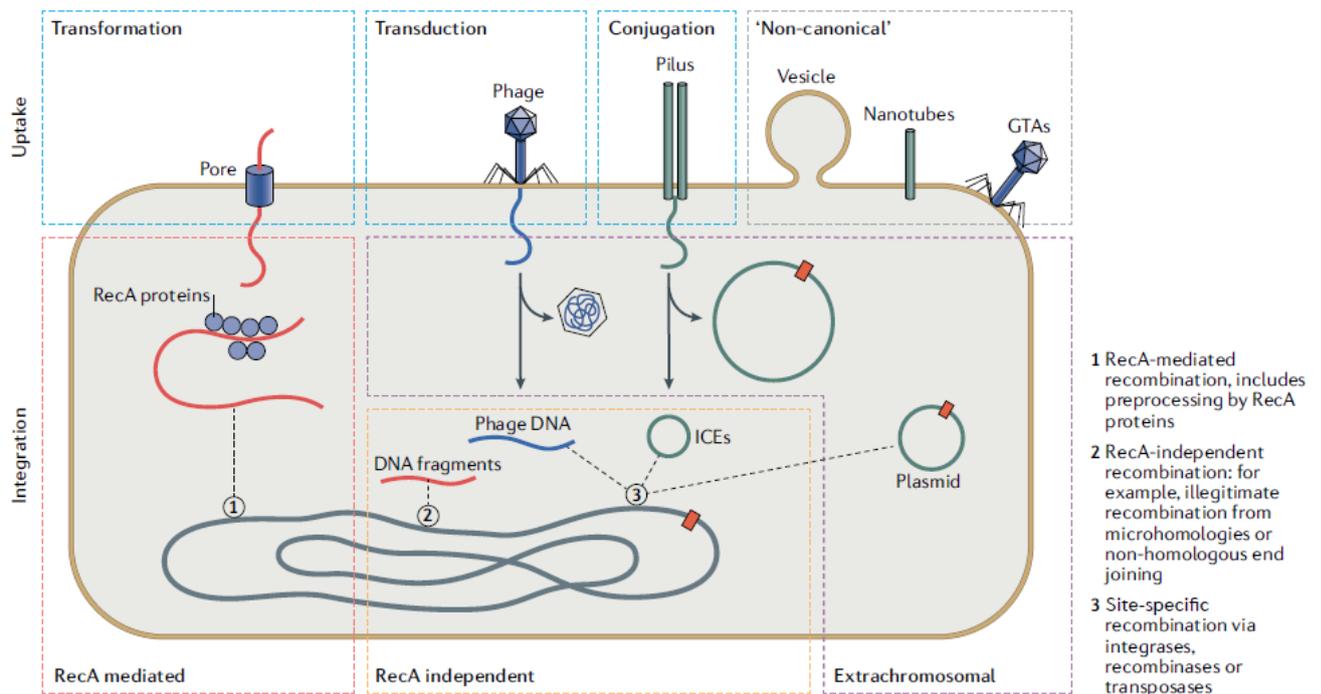


Figure 1 Three “canonical” mechanisms of HGT: transformation, transduction and conjugation; and “non canonical” HGT mechanisms such as vesicles, nanotube and GTA. ICEs: Integrative conjugative elements; GTAs: phage-like gene transfer agents. (Arnold et al., 2022)

## 1.2 Natural transformation

Natural transformation was first discovered by Frederick Griffith in 1928 whilst investigating how pathogens such as *Pneumococci* spp. exchange traits (Von Wintersdorff et al., 2016). From this, it has been documented that transformation contributes to the evolution of pathogenic species and to the proliferation of virulence factors. More than 80 species, including gram-positive and gram-negative bacteria, have been identified to be naturally competent (Blokesch, 2016). However, how they enter to physiological encoded competence state and what are the drivers for some it is still unknown, and it varies between species.

Natural transformation usually results in DNA recombination and in particular cases, such as in *E. coli*, plasmid uptake (Arnold et al., 2022). Furthermore, transformation processes usually consist of beneficial DNA exchange and consequently higher DNA variability, but also other possible benefits are encountered, such as the use of the acquired DNA as a nutrient source or as a template for recombinational repair in cases of DNA damage (Bacher & Metzgar, 2006). This has been observed previously in *Bacillus subtilis*, *Legionella pneumophila* and *Streptococcus pneumoniae*, for example (Hülter et al., 2017). Although recent evolutionary bacterial approaches have uncovered the possibility to be also either neutral, e.g. removing deleterious mobile genetic elements, and/or context dependent (Hülter et al., 2017).

Also, various factors are considered to induce the bacterial recipient in a competence state. For example, in both *Haemophilus Influenzae* and in *S. pneumoniae* a competence state was generated by stress response, or that it could be related to the exposure of UV-light (Charpentier et al., n.d.). For *Vibrio cholerae*, a colonizer of aquatic system and not known to be naturally competent, the competence and the transformation was established while reaching high cell density when grown in chitin, a oligosaccharide present in crustacean exoskeletons (Meibom et al., 2016). Instead, for *Bacillus subtilis*, entering the competence state is closely related to cell-to-cell signalling pathways (Domingues, 2016). Or even in some cases neither of these processes are required, for example for *Neisseria gonorrhoeae* and *Helicobacter pylori*, where their competence state can take place during all phases of growth (Domingues, 2016). As shown, the competence induction and fitness are not only limited to closely related relationship between the species and a particular growth condition or quorum sensing (Mell & Redfield, 2014). It has been demonstrated that several antibiotics, such as aminoglycosides, fluoroquinolones and beta-lactams can activate the competence state of *S. pneumoniae* (Huang et al., 2021).

Some studies have also suggested that the transformation process is likely to decrease fitness when the eDNA is derived from a homologous strain exposed to UV-induced stress (Hülter et al., 2017). In addition, the fitness cost is likely to be related to the metabolic demand of synthesizing the protein transformation machineries needed to actively take up the DNA from the environment (Bacher & Metzgar, 2006). Lastly, due to the fact that DNA could be derived from a dead relative bacteria, recombination reduces fitness instead of increasing it, even if beneficial alleles are observed (Mell & Redfield, 2014). In contrary, a study with *S. pneumoniae* population observed higher fitness when exposed to periodic stress (Engelmoer et al., 2013).

If for some species, such as *Haemophilus influenzae* and *Neisseriaceae* family, the transfer of DNA is strictly related to the uptake of homologous DNA, for others such as *B. subtilis* and *A. baylyi*, it is the opposite (Hülter et al., 2017).

*A. baylyi*, a species used as a model in this study, is a gram-negative soil bacteria which contains a broad variety of metabolic genes, such a unique feature that gives it the name of “archipelago of catabolic diversity” for its characteristics (Bacher & Metzgar, 2006). Also, *A. baylyi* transformation is not related to DNA damage, instead, it is highly competent during exponential growth-phase, recombinogenic and it doesn't require high recombination homology as a requirement for DNA uptake. But instead, the fitness of *A. baylyi* increased with homologous and heterologous DNA uptake (Hülter et al., 2017).

Lastly, bacteria have evolved defence systems against genetic parasites that could affect transformation frequency (limit transformation) such shown in Bikard et al., (2012) study with short palindromic repeat (CRISPR) targeting the capsule virulence factor and preventing acquiring gene through transformation. Or another defence mechanism required for bacteria genome stability can be correlated the production of nucleases to digest exogenous DNA (Huang et al., 2021).

### **1.3 Mechanism of transformation: single stranded DNA (ssDNA) and double stranded DNA (dsDNA) transformation**

Natural transformation can occur either with linear but also with circular donor DNA (Domingues, 2016). The DNA uptake machineries described in gram-negative and gram-positive bacteria are quite similar from each other but also correlated to their cell wall characteristics. Gram negative bacteria consist of an outer membrane (OM) (unique from gram-positives) and an inner membrane (IM), instead gram-positive contain a peptidoglycan layer. The latter is a thicker and denser barrier for DNA crossing, indeed, before DNA transfer, it needs to be weakened to proceed. For that reason, dsDNA, such as plasmids, can only be pulled across gram-negative bacteria, instead ssDNA across the IM in both gram-positive and gram-negative bacteria (Sun, 2018).

Gram-negative bacteria contain a sophisticated protein complex in the OM responsible for binding and pulling exogenous DNA into periplasm through a pore cavity large enough to bring dsDNA. *E. coli*, not known to be naturally competent, just recently has been observed to transform either in nutrient-deficient and nutrient-rich agar plates (Sun, 2016). Besides from the others dsDNA machineries uptake required, *E. coli* doesn't seem to require any and instead use them as a function to use DNA as a nutrient source (Palchevskiy & Finkel, 2006). Also, other new proteins and transporters seem to be involved in this process, but more studies are required to assess this statement (Sun, 2018).

Instead, for *A. baylyi*, a gram-positive bacteria, natural transformation it is not only influenced by the surrounding environment, but also a set of core Type IV pilus proteins are necessary for this process and for switching (Leong et al., 2017). Once the DNA it is been translocated

in the cytoplasm, some of the base pair, between 500 and 1500 bp, get degraded before the integration in *A. baylyi*. The minimum amount of base-pair needed for initiate recombination is 183 bp (Domingues, 2016). Also, various studies have shown that *A. baylyi* natural transformation in soil can occur also with isogenic cell or lysate from *Pseudomonas fluorescens* or *Burkholderia cepacian* instead of the addition of purified DNA (Domingues, 2016).

#### **1.4 Aim of this study and research questions**

Currently natural transformation it is an understudied mechanism of HGT. Furthermore, in recent years the general concern about AMR specially in the environment has been highlighted up by many researchers. Unfortunately, research gaps remain regarding about how natural transformation in bacteria and the surrounding environment coexist and influence each other.

##### *1.4.a. E. coli plasmid transformation*

It is a current unknown whether *E. coli* is naturally transformable. Though it is known to not take up any ssDNA, recent studies have shown the possibility of dsDNA being transferred through natural transformation mechanism. In this study the initial hypothesis of *E. coli* being a naturally transformable species was tested and supported by recent studies (Sun et al., 2006) (Sun, 2018). Genomic analysis has also shown the presence of a homology of a complete set of gene recognised to be the classical DNA uptake machinery and to be conserved in most of the natural transformable bacteria (Sun, 2016). Also the presence of plasmid carrying ARGs that could transmit AMR through HGT in natural environment e.g. water it has been highly documented (Li & Zhang, 2022) . Starting from this understanding, environmental sewage *E. coli* community was tested to evaluate the possibility to perform plasmid natural transformation. Therefore, this question: **is *E. coli* capable of plasmid natural transformation?**

##### *1.4.b. A. baylyi natural transformation*

#### **Research question 2. Does nutrient availability influence the transformation frequency in *A. baylyi*?**

The effect of various factors on HGT have been documented in different environments. The effect of nanomaterials, metals, quorum sensing signal molecules and pollutant have been reported to effect conjugation (Li & Zhang, 2022). The effect of disinfectant on water treatment is to inactivate pathogenic microorganism, but whatever this influences HGT processes and how these pharmaceuticals effect the abundance of ARGs us also still yet not known. Also, factors, such as nanoparticles, chlorine and some organic pollutants have shown to increase transformation frequency (Al-Gashgari et al., 2023). So, with *A. baylyi* as a model species, the first step was to evaluate if nutrient availability could have an effect on natural transformation frequency.

**Research Question 3. Does the presence of competitor species and nutrient availability influence natural transformation in *A. baylyi*?**

It has been reported that signalling and the quorum sensing between community bacteria present plays an important role on understanding the evolution of cooperation. The social collaboration and signalling could help better to understand the physiological forces driving bacteria evolution. So in this study, it have been encountered the ability of bacteria to communicate with each other and for some to being suppressed by driving forces and stressors. In a previous unpublished Winter et. al. study, natural transformation was tested with a number of different competitor species. This was evaluated to see which surroundings species co-present in the same environment for example, could communicate and influence natural transformation of *A. baylyi*. However, it is still not known whether the interaction of both nutrient and the presence of a competitor will affect the transformation of *A. baylyi*.

**Research Question 4. Do surrounding proteins and metabolites influence transformation frequency in *A. baylyi*?**

As it was already seen, numerous of protein machinery and peptides are involved in the uptake of DNA from the surrounding. Various receptors, transporters, nucleases are known to mediate and facilitate the DNA uptake, but little it is still known on which, if and how do surrounding media environment proteins could affect natural transformation. Some of genes and consequently protein essential for natural transformation, encode also for bacteriocin transporter in *Streptococcus pneumoniae*; which means that there is a close correlation between natural transformation competence, quorum sensing and bacteriocin pathways activation (Shanker & Federle, 2017) . If these pathways encode for the production accessories protein, it is fair to question whichever free protein derived by surrounding bacteria lysis could influence natural transformation in *A. baylyi*.

## **2. Methods**

Two different species were used throughout this thesis. The rationale behind this was to test two different mechanisms of transformation: the uptake of a single stranded DNA (ssDNA) with *Acinetobacter baylyi* as a model species, and the understudied extracellular plasmid uptake mechanism using *Escherichia coli* as a model specie. *A. baylyi* was used as a model for natural transformation because it's one of the most well understood bacteria to be naturally competent and hence transform. The latter, *E. coli*, a faecal indicator species, is widely used interlaboratory model species, but it is not recognised as a naturally transformable and a competent gram-negative bacterium, despite fewer evidence for natural transformation in *E. coli* existing in published literature.

## 2.1 *E. coli* project

This bacterium was chosen from the base of lacking knowledge and literature of its natural transformation process and mechanism. Indeed, one of the fewer published papers, (Sun et al., 2006) research paper, was used as methods base for the following study. The main focus of the paper was firstly to investigate if a lab-based strain of *E. coli* could naturally transform and subsequently to evaluate the underlying mechanisms and conditions under which it enters the physiological state termed competence.

Starting from this knowledge, the aim of this study, using *E.coli* as a focal species, was to evaluate if an environmental sewage strain could be able to perform natural transformation.

### 2.1.1 Bacterial strains and plasmids

#### *Recipient strains*

- *Sewage samples*

Sewage influent samples were collected from a wastewater treatment plant(WWTP) in Falmouth, UK (serving a population of approximately 43,000) in February 2018. Samples were transported in cool boxes, then mixed 1:1 with 40% glycerol (Fisher, LOT: G/0600/17) and stored at -70°C (Murray *et al.*, 2020).

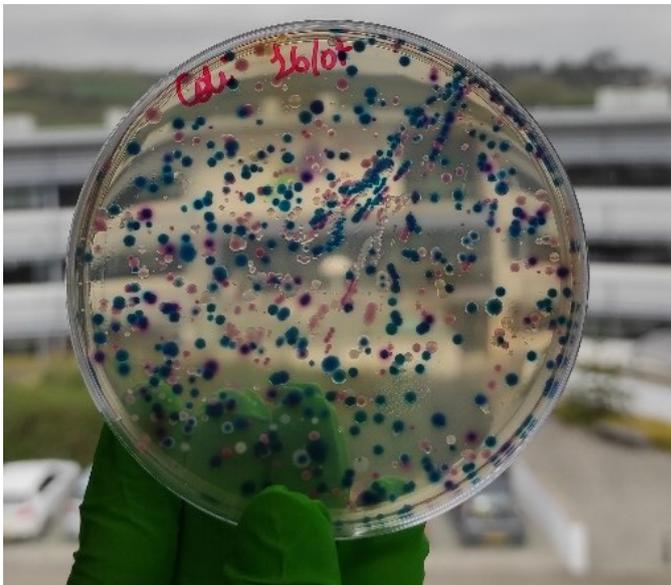


Figure 2 *E. coli* colonies plated from sewage.

In order to isolate the chosen bacteria, the sewage stocks were streaked on a selective Coliform Chromoselect agar (Merk, LOT: 81938) and incubated (Panasonic, Sanyo, Serial No. 08100035) at 37° for at least 24 hours (Figure 1). From this, only blue colonies (presumptive *E. coli*), were picked into 96-well-plates containing 100µL of sterile Luria-Bertani (LB) broth (Fisher Bioreagent, LOT: 187444), and incubated at 37°C overnight, then 100µL of 50% glycerol was added and stored at -70°C. To select antibiotic susceptible *E. coli* colonies from frozen stocks, each colony was inoculated onto fresh coliform and LB agar (Formedium, LOT: FM0322/11769) supplemented with 100 µg/mL of ampicillin and incubated at 37°C overnight. Colonies that were unable to grow in the presence of ampicillin were deemed to be susceptible and were stored in 25% glycerol at 70°C before downstream analysis (Figure 2).

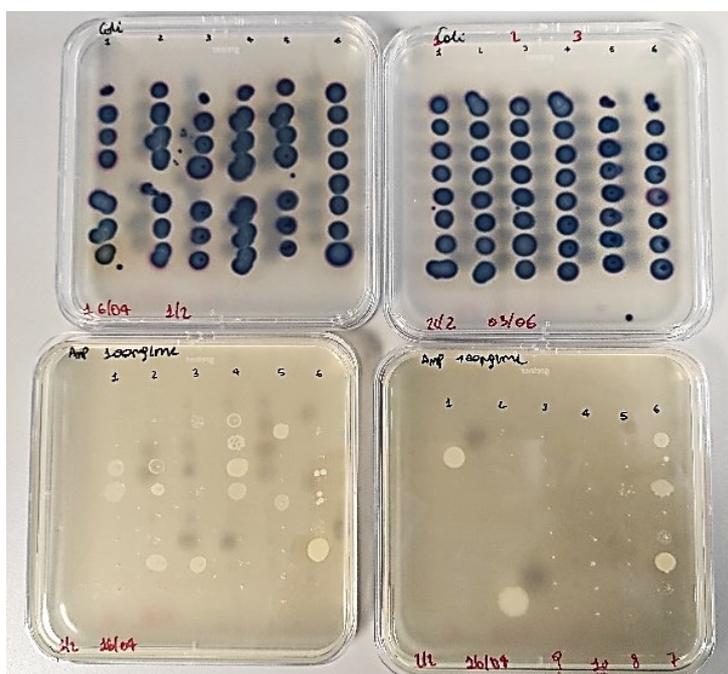


Figure 3 *E. coli* colonies picked from plate Figure one

- *Mixture of environmental and laboratory strain samples:*  
Lab based strains and environmentally sampled strains of *E. coli* were used as recipients during transformation assay (Table 1).

Table 1 Laboratory and environmental strains used as recipient strains

Isolate ID	Date of sample collection	Source	Location	Kraken identification number
6TV-28	20.09.12	Environmental (bathing water)	River Parrett at Somerset Bridge	<i>Escherichia coli</i> 042 (taxid 216592)
TV7.12	08.08.12	Environmental (bathing water)	Nant Y Talwg	<i>Escherichia coli</i> SMS-3-5 (taxid 439855)
6ZP-70	20.09.12	Environmental (bathing water)	River Parrett at Somerset Bridge	<i>Escherichia coli</i> (taxid 562)
6TV-28	20.09.12	Environmental (bathing water)	River Parrett at Somerset Bridge	<i>Escherichia coli</i> 042 (taxid 216592)
ZBI-2		Environmental (sewage water)	Falmouth	<i>Escherichia coli</i> SMS-3-5 (taxid 439855)
ZBI-3		Environmental (sewage water)	Falmouth	<i>Escherichia coli</i> O157:H7 (taxid 83334)
ZVI-11		Environmental (sewage water)	Falmouth	<i>Escherichia coli</i> 042 (taxid 216592)
6TP-19	20.09.12	Environmental (bathing water)	River Seaton D/S Trout Mill	<i>Escherichia coli</i> O157:H16 (taxid 930406)
S17		Lab strain		<i>Escherichia coli</i> (AOGN01000119)
SID		Lab strain		<i>Escherichia coli</i>
DH5ALFA		Lab strain		<i>Escherichia coli</i> DH5a pOPS03678

Table 2 Donor strains used in this study.

<i>E. coli</i> strain	Molecule size (kb)	Accession number	Plasmid	Fluorescence marker	AMR marker	Application
K12	~50		PKJK5	green	tetracycline	2.1.2a
DH5α			PASG-1	Green (sfGFP)	apramycin, ampicillin	2.1.2
S17			pOPS03678	/	kanamycin	2.1.2a
DH5α	2,686	M77789	pUC19	/	ampicillin	2.1.2.c
DH5α	5,087	EU603324	pHERD20T	/	ampicillin	2.1.2.c & 2.1.3

Strains and plasmids screened in these experiments are listed in Table 2. All strains were sub-cultured from frozen stock. Stocks were prepared from overnight batch cultures at 180 rpm, 37 °C (Informs HT, Serial No. S-000126581-003) grown in LB broth supplemented with approximately 100µg/mL of antibiotic depending on *E. coli* tested.(Table 2) Aliquots were then frozen 1:1 with 50% glycerol and stored at -70 °C.

*Preparation and storage of transforming plasmid DNA isolation*

The DNA of DH5α strains harbouring pUC19 and pHERD20T plasmids were extracted using PureYield™ Plasmid Miniprep and Maxiprep Kit (Promega, Italy) following protocols as described in respective manuals. DNA sample concentrations were obtained using a Nanodrop 2000 (Thermo Scientific, Serial No. F351) and samples were subsequently stored at -20°C until use.

*Preparation and storage of transforming plasmid DNA lysate*

400mL of overnight cultures of DH5α and K12 strains harbouring, pASG-1 and pKJK5 plasmids respectively, were centrifuged (Thermo Fisher, Serial No. 41419089) at 3600xg for 10-15 minutes to collect bacterial pellet and supernatant was discarded. Cultures were resuspended in LB broth at a 30-fold increased concentration and placed in a 70°C water bath (Stuart, CAT No SWB15D) for 90 minutes in order to release the transforming DNA. The solution was aliquoted in sterile eppendorf tubes and stored at -20°C. Lysate was confirmed to be sterile by incubating on LB agar at 37°C overnight and checked for colony forming units (CFU).

*Gel electrophoresis for plasmid selection*

Products of plasmid miniprep and cell lysate were run on 1% agarose gel (Figure 3) at 90 volts for 70 minutes with Gene Ruler™ 1kb Plus DNA Ladder (Thermo Scientific and using Fisher Scientific machine No. 5390712).

*Table 3 Antibiotics used in this study*

<b>Antibiotic</b>	<b>Stock conc. (mg/mL)</b>	<b>Solvent</b>	<b>Application- strain</b>
<b>tetracycline</b>	30	Sterile DI water	2.1.2a - K12
<b>ampicillin</b>	10	sterile DI water	2.1.2a- DH5α
<b>kanamycin</b>	40	sterile DI water	2.1.2a - S17
<b>ampicillin</b>	100	sterile DI water	2.1.2c - DH5α - pUC19 & DH5α - pHERD20T
			2.1.3 -

Table 4 Antibiotics stock adopted in this study

Antibiotic	Code	Class	Store d (°C)	Brand	LOT number	CAS	Product No
Apramycin	APR	aminoglycoside	4	Duchefa Biochemie	14826.02	65710-07-8	A0164.0025
Ampicillin	AMP	beta-lactam	4	Melford Sigma-	57572-58681	69-52-3	A40040-5.0
Ampicillin	AMP	beta-lactam	4	Aldrich	59349-100	69-53-4	1E+08
Spectinomycin	SPT	aminocyclitol	4	Melford Fisher	41048-50813	32-8	S23000-25.0
Streptomycin	STR	aminoglycoside	4	Bioreagents Fisher	187951	3810-74-0	BP910-50
Kanamycin	KAN	aminoglycoside	4	Bioreagents Fisher	192444	25389-94-0	BP906-5
Tetracycline	TET	tetracycline	-20	Formedium	20/MFM/2580	64-75-5	TET02

## 2.1.2 Pilot work

### 2.1.2.a Fluorescent plasmid transformation

Overnight cultures of the selected *E. coli* single colonies were inoculated 1:100 into 5mL fresh LB broth and incubated at 37°C, 180rpm. After 12 hours, 50µL was transferred into sterile eppendorfs covered with a sterile, breathable membrane and incubated in static conditions at 37°C for a further 10 hours. Cultures were subsequently diluted with sterile 0.85% saline and mixed 1:1 separately with two different cell lysates of *E. coli* strains harbouring pASG-1 and pKJK5 and plated on LB agar supplemented with 100 µg/mL ampicillin and tetracycline, respectively. Plates were inverted and incubated at 37°C overnight. Negative controls were included by plating lysate and cultures alone. Positive controls were also included by mixing an isogenic pair of *E. coli* strains with and without the antibiotic plasmid marker (isolate S17 from Table 1 and S17 harbouring pOPS03678 plasmid as showed in Table 2). This and the following protocols were designed and adjusted following (Sun et al., 2006) methods paper.

### 2.1.2.c Plasmid electroporation protocol

#### Competent cells

Each sewage *E. coli* sample colony was taken from 50% frozen glycerol stocks and placed into 10 mL LB broth in 50mL Falcon tubes, incubated overnight at 37°C and shaken at 250rpm,

to ensure good aeration. After 24 hours 1:100 dilutions into 25 mL of pre-warmed fresh LB were made and placed at 37°C and shaken at 250rpm. After 6-7 hours, until the cultures reached to an OD600 (measured by Biotek Serial No. 254462) they were placed onto ice for 15 minutes. From this point all culture samples remained chilled. Cultures were transferred into chilled 50 mL falcon tubes, centrifugated at 3500 rpm for 20 minutes and the supernatant was decanted. 25 mL of 10% glycerol (pre-chilled) was added to the 50mL falcon tubes and resuspend by pipetting. Two rounds of centrifuging, decanting and washing were repeated, and then the pellet was resuspended in 10% glycerol for a total volume of ~800  $\mu$ L and stored in 100  $\mu$ L aliquots at -70°C. Plated electrocompetent cells with 100 $\mu$ g/mL ampicillin selective agar were made to exclude colonies already harbouring ampicillin resistant plasmids. Negative controls (electrocompetent cells with no plasmid DNA added) were plated into 100 $\mu$ g/mL ampicillin selective plates as well to make sure that the competent colonies did not already harbour ampicillin resistant plasmids.

### *Electroporation*

From freezer stocks, the cultures were placed on ice and before they had thawed, 500ng of plasmid DNA (using two different strains of DH5 $\alpha$  harbouring pUC19 and pHERD20T respectively) were added and incubated for 20 minutes on ice. The competent cells were pipetted in pre chilled cuvettes and electroporated at 1.8 kV (Biorad Micropulser™ Serial No.411BR11228). Immediately, pre-warmed LB broth with the electroporated cells were placed at 37°C for at least 30 minutes. Finally, the electroporated cells were placed into pre-made 100ug/mL ampicillin selective plates and incubated overnight at 37°C. As well as selective antibiotic plates, LB agar plates were made and used as positive control, to ensure that the colonies were still alive after such a shock and comparing electroporated cells to see whatever the cells were able to take up the plasmid or not.

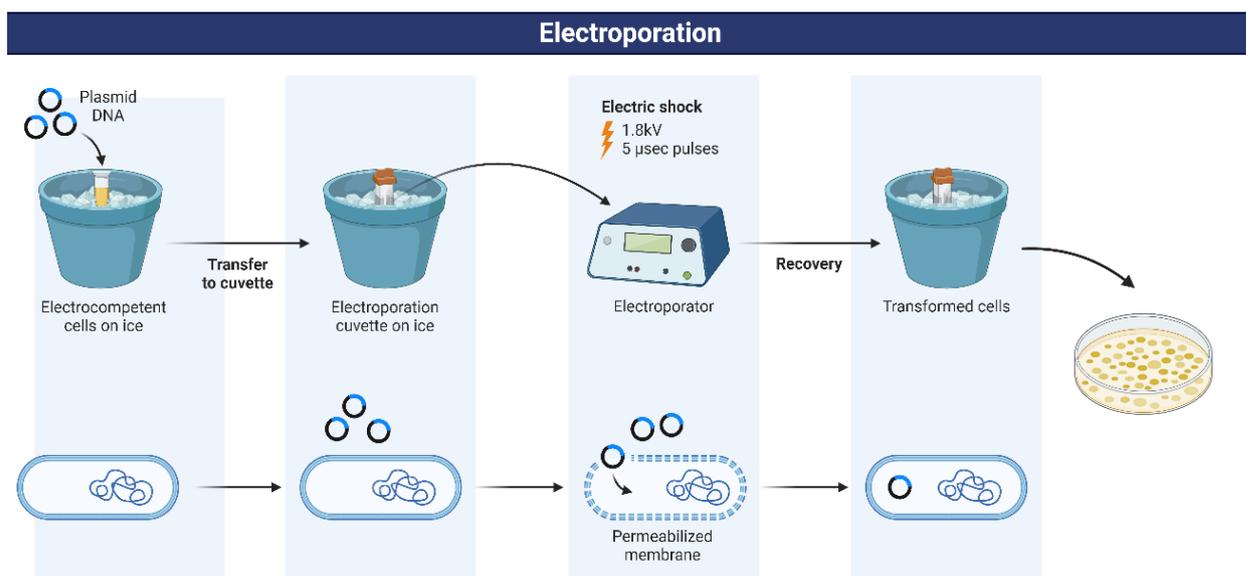


Figure 4 Electroporation protocol (made using Biorender.com)

### 2.1.3 Natural transformation protocol

The same samples used in electroporation pilot work were taken from 50% glycerol stock and placed into 10mL of fresh LB broth in a 50mL Falcon tubes and incubated overnight at 37°C at 200 rpm for 12 hours. 50µL and 200µL of the overnight culture were transferred into 2 mL Eppendorf left open (“open system”) by using a breathable membrane and incubated in a static system at 37°C for 10 hours (Figure 5). Respectively, 2µL and 8µL of plasmid DNA (from *E. coli* DH5α harbouring pHERD20T) were added to 50µL tubes and 200µL tubes solution, 100µL

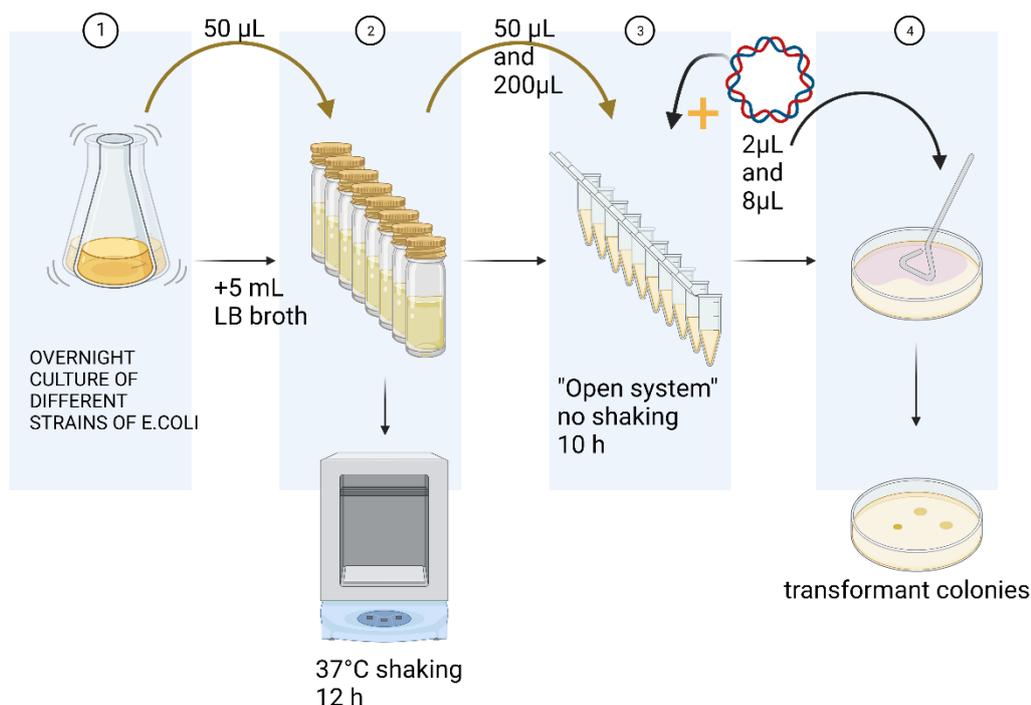


Figure 5 Natural transformation workflow (made with Biorender.com)

were taken and spread on 100µg/mL ampicillin supplemented plates. For each sample 3 replicates were made, and an extra replicate was made from step 3 (Figure 4) and 100µL of the final solution without plasmid DNA were plated in 100µg/mL ampicillin selective plates as a “negative control”.

## 2.2 *Acinetobacter baylyi* project

### 2.2. A. baylyi donor and recipient

Two variants of wildtype *A. baylyi* ADP1 strain were previously naturally transformed with two different chromosomally encoded fluorescence cassettes; *sfGFP::apraR* cassette conferring green fluorescence and apramycin resistance, hereby referred to as “green wildtype/GWT”, and the latter *mCherry::specR* cassette conferring red fluorescence and spectinomycin resistance, referred to as “red wildtype (RWT)”. Successful transformants were isolated using selective agar supplemented with 240µg/mL apramycin (Duchefa, The Netherlands), and 360µg/mL spectinomycin (Melford, UK).

### Preparation and storage of *mCherry::specR* DNA lysate

400 mL of overnight cultures of RWT, were centrifuged (by Thermo Fisher, Serial No. 41419089) at maximum speed for 10-15 minutes, to collect bacterial pellet and supernatant was discarded. The pellet was resuspended in remaining supernatant and mixed by vortexing, and placed in a 70°C water bath for 90 minutes in order to release the transforming DNA. The solution was aliquoted in sterile eppendorf tubes and stored at -20°C. To ensure no live cells remain, 100µL of the solution were plated on LB agar plates, incubated at 37°C overnight and checked for colony forming units.

The following protocols were based on previous unpublished research from Diamond et al. which evaluated the effect of competitor species on transformation frequency using *A. baylyi* as a model species. A system of two variants of *A. baylyi* ADP1 strain was established, consisting of one expressing a red fluorescence marker, the RWT, as a donor eDNA strain and a green fluorescence marker, the GWT, as the recipient strain in natural transformation process. By using this system combined with their antibiotic resistance marker, it was possible to estimate the effect of 48 competitor species on transformation frequency. The findings from this highlighted competitor strains responsible for either increasing or decreasing transformation frequency. Consequently, the following study aimed to adopt similar protocols to assess the influence of the combined effect of nutrient and competitor species on *A. baylyi* transformation frequency.

#### **2.2.a Nutrient pilot protocol**

Three different nutrient concentrations were adopted to assess if nutrient had an effect on transformation frequency: 1%, 10%, 100%. To generate this gradient LB broth was diluted with sterile water.

In 30mL universal tubes, 1,6mL of 1%, 10%, 100% LB broth, 200µL of GWT overnight culture, 200µL of RWT lysate were added and incubated at 28°C. At this stage, samples were diluted and plated on 240µg/mL apramycin selective LB agar plates. After 5 hours, 200µL from each tube was transferred in pre-chilled 96 well-plates, diluted and plated on 240µg/mL apramycin, and on 240µg/mL apramycin combined with 360µg/mL spectinomycin selective LB plates. All plates were incubated at 28°C for two days.

#### **2.2.b Nutrient assay protocol**

Diamond et al. (2022, unpublished) highlighted that the presence of a *Bordetella spp.* could increase transformation frequency, instead *Bacillus spp.* showed the opposite effect (Figure X). Thus, these strains were chosen to test the effect of a competitor species in combination with varied nutrient concentrations on transformation frequency of *A. baylyi*. A similar protocol

to the nutrient pilot (2.2.a) was employed with the addition of either *Bordetella* or *Bacillus* as competitors (Figure 6). Instead of the previous 200µL of GWT overnight culture, 100µL of each competitor species and GWT were added.

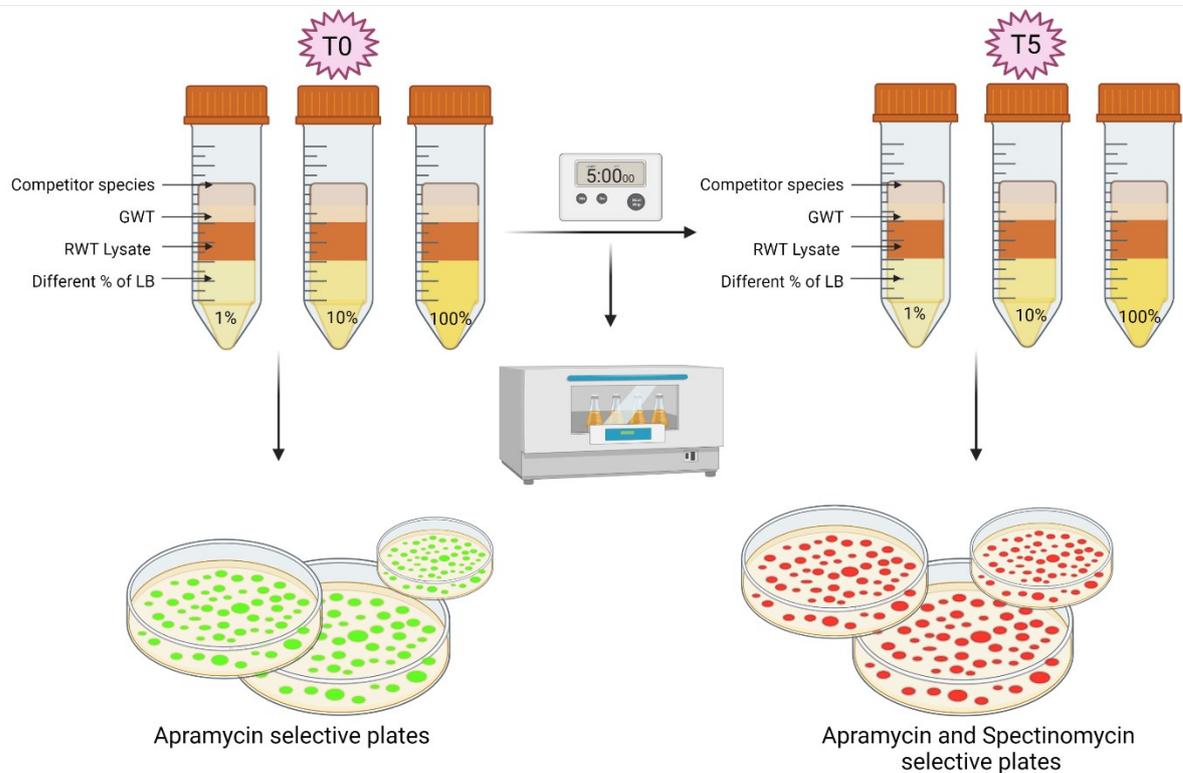


Figure 6 Schematic overview of experimental design. T0: time point 0, T5: time point 5 hours, GWT: green wild type, RWT: red wild type, LB: Luria-Bertani Broth (made with Biorender.com).

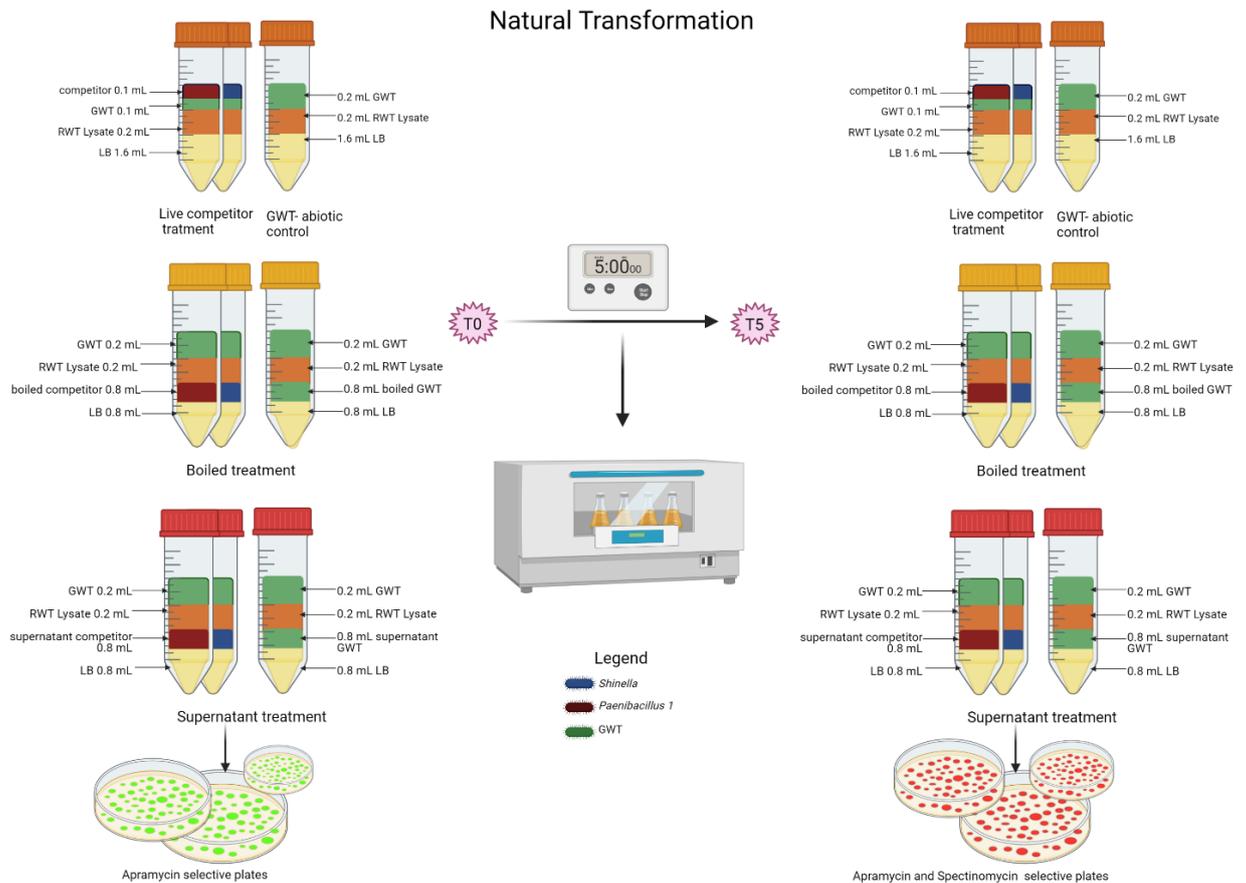
### 2.2.c Supernatant, boiled and live cells protocol

Three different strains were used for this assay: *Bordetella 1*, *Bacillus* and the GWT of *A.baylyi*. Overnight cultures in triplicate were made in LB broth, incubated at 28°C and shaken at 180rpm. After two days, the overnight culture was aliquoted into three treatments: supernatant, boiled and live cells.

For supernatant and boiled treatments, 10 mL of each overnight cultures were centrifuged at 2700xg for 10 minutes and then filtered using 0.22µm syringe filters. 5 mL aliquots were made, and half of the supernatant aliquots were boiled using a block heater (Stuart, Serial No. R000104648) at 100°C for 10 minutes.

Each treatment was prepared according to Figure 5. 100 µL of all treatment of each strain combined treatment used were plated at  $10^{-4}$  on 240µg/mL apramycin selective LB plates.

After 5 hours, 100  $\mu\text{L}$  of each treatment were plated on 240 $\mu\text{g}/\text{mL}$  apramycin on  $10^{-5}$  dilution and  $10^{-3}$ , on 240 $\mu\text{g}/\text{mL}$  apramycin and 360 $\mu\text{g}/\text{mL}$  spectinomycin supplemented LB plates. All the plates were incubated at 28°C for 48 hours before CFUs were enumerated.



**Figure 7 Schematic diagram illustrating experimental protocol (6 biological replicates per treatment). Supernatant and boiled treatments: 200 $\mu\text{L}$  of GWT overnight culture, 800 $\mu\text{L}$  of LB broth, 800 $\mu\text{L}$  of supernatant or boiled culture, and 200 $\mu\text{L}$  of lysate. Abiotic control: 200 $\mu\text{L}$  of GWT overnight culture, 200 $\mu\text{L}$  lysate, 1.6 $\text{mL}$  LB broth. Live competitor: 200 $\mu\text{L}$  of lysate, 100 $\mu\text{L}$  of GWT and competitor overnight culture and 1.6 $\text{mL}$  LB broth. Made using Biorender.com.**

## 2.2.d. Statistical analysis

Transformation fraction is the number of cells which have transformed, calculated by the number CFU/mL after transformation (after 5 hours) divided by the number of CFU/mL before transformation.

Transformation frequency was calculated by dividing the (CFU/mL)/1000 of the transformed population by the total population (CFU/mL)/1000 (Winter et al., 2023).

In all the statistical analysis, p-value < 0.05 were considered significant.

## *E. coli* electroporation data

Normal distribution of data was assessed with Shapiro-Wilks test, finding the data to be not normally distributed ( $p = 0.0002264$ ,  $p < 0.001$ ). Transformant fraction was calculated as above. Data generated was visualized with ggboxplot (R studio v2023.09.0).

#### *Nutrient Data*

Normal distribution of the data were assessed with Shapiro-Wilks test. Because the data was found to be not normally distributed ( $p = 0.003342$ ), non-parametric pairwise multiple comparison test was performed with Kruskal–Wallis test, followed by Dunn's post-hoc test. The result was plotted with ggplot (R studio v2023.09.0) for data visualisation (Figure 10).

#### *Nutrient and competitor species present data*

Normal distribution of the data was evaluated with Shapiro-Wilks test. The data resulted not normally distributed ( $p = 7.492e-15$ ) so the data was square-root transformed.

To test the effect of LB concentration, and competitor species on transformation frequency of the focal *A. baylyi* populations, a comparative test of means (T-test) was used to see the difference between the mean of nutrient availability and the mean of competitor species group. The result was plotted with ggplot for data visualisation (Figure 11).

#### *Supernatant and competitor species data*

Transformation frequency was calculated using the following equation:

$$y = (\text{Transformed cfu.mL}/1000) / (\text{total number of cfu.mL}/1000) - (\text{Transformed cfu.mL}/1000)$$

Because multiple factors are included in this experiment, such as species in monoculture, co-culture and different treatments, such as boiled supernatant, alive cells and supernatant, a 2-way test could not be proceeded with.

Instead, generalized linear mixed models were made in order to analyse this data. The models are fitted using maximum likelihood estimation via 'TMB' (Template Model Builder). Random effects are assumed to be Gaussian on the scale of the linear predictor and are integrated out using the Laplace approximation. Different model and interaction were tested.

The first model was made comparing the interaction between the behaviour of the different environment (alive cell, supernatant, boiled supernatant) and the competitor species present. The second model was made to analyse the effect of the environment's behaviour on transformation not considering the species involved. Then, in order to determine if it was a statistical difference between this two model research results, ANOVA (analysis of variance) was employed. Also an Emmeans (estimated marginal means) was employed, to do a

comparison on which species competitor has an effect on transformation frequency. To visualize the data, (Figure 12), set `geom_dotplot` was used.

### 3.Results & Discussion

#### 3.1 *E. coli*

Besides from well-known chemical and physical (high  $\text{Ca}^{2+}$  and electroporation respectively) transformation process in bacteria, natural transformation is an understudied process, especially with *E. coli*. To investigate natural transformation of *E. coli*, different environmental and clinical isolates were collected (Table 1) and tested with 2 different cell lysate strains containing fluorescently tagged plasmid DNA (pASG-1 and PKJK5). By using a fluorescence detecting “lamp”, no transformant colonies were found in both of different antibiotic selective plates, so it can be concluded that no DNA uptake by transformation was achieved. It could be correlated to the fact that cell lysate could potentially create heterogeneous DNA fragment sizes that could not contain the origin of replication, a region were mostly of the necessary genes required for competence initiation (Huang et al., 2021).

Also various forces, such as ecology and phylogenetic distance, between the donor and the recipient, could regulate and thus be negatively correlated to transformation efficiency (Smillie et al., 2011) (Labroussaa et al., 2016).

But still, various factors or stressors, such as antibiotic presence or DNA damage, and changing of specific physiological conditions may effect natural transformation efficiency, depending on bacterium specie and taxa involved (Huang et al., 2021).

To overcome the possibility that the donor DNA cell lysate would be damaged after such stress, or not be appropriate for *E. coli* natural transformation process, plasmid DNA purification was performed and the differences between DNA stability and size(bp) was assessed with gel electrophoresis (Figure 3). It appears clear that extraction and lysate of plasmid DNA processes produce different bands, indeed, it can be immediately visualized that there is a sizeable difference between plasmid DNA extraction (from A to D) and DNA cell lysate (from E to G). Where the first, have higher intact size plasmid DNA, and may therefore struggle to pass through the pores of the gel matrix. Additionally, from E forward, lower sized, fragmented, and damaged DNA is observed, likely due to heat-shock from by lysate process. Interestingly, C section (corresponding to pHERD20T plasmid DNA) appears to have 2 bands, so it can be suggested that the stronger and bigger band could be identified as a supercoiled covalent circular plasmid DNA form (compact double-stranded DNA twisted around itself) and the latter band could be identified as the circular plasmid DNA.

Even though the same plasmids were tested not diluted (e.g. A and E, C and G) and 10x diluted (e.g. B and F, D and H), they have produced different bands depending on the isolation

method. Indeed, the extraction method have assessed the right bp for both plasmids tested, pUC19 and pHERD20T, such as ~2kb and ~5kb respectively, supported by scale DNA 1kb Plus ladder (Figure 8). Instead, the lysis method has produced small, fragmented plasmid DNA, not high quality and damaged DNA so it was chosen to not be appropriate for natural transformation with *E. coli*.

For instance, plasmid extraction was chosen to be the method of plasmid isolation for further experiments.

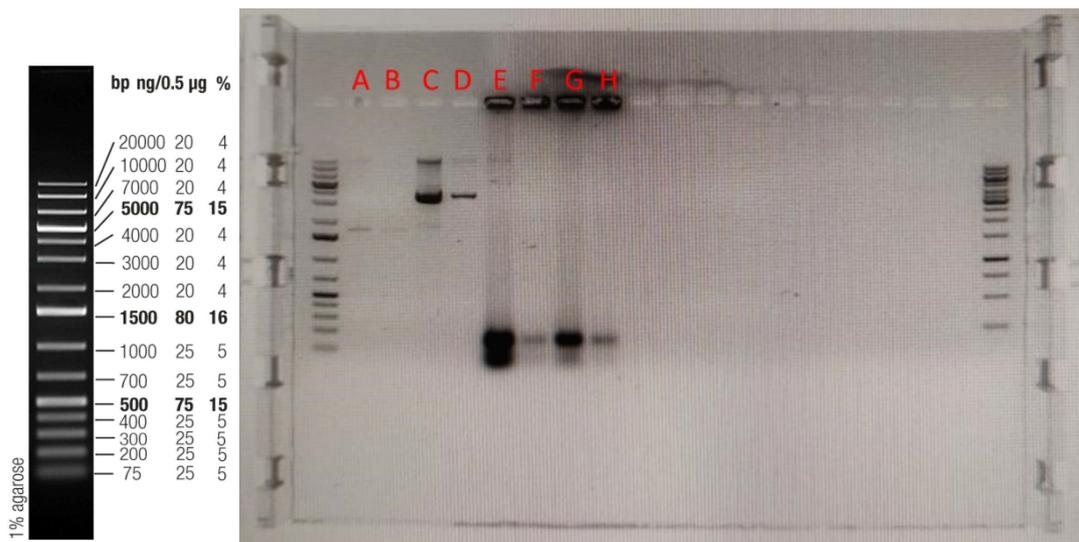


Figure 8 On the left: Scale DNA GeneRuler™ 1kb Plus: gives info about sizing and approximate quantification of a wide range of double-stranded DNA. Source: <https://www.fishersci.it/shop/products/fermentas-generuler-1kb-plus-dna-ladder/p-4529751>.

On the right; visualization of plasmid DNA on gel electrophoresis. A and E: *E. coli* harbouring pUC19. B and F: *E. coli* harbouring pUC19 dilute 10x. C and G: *E. coli* harbouring pHERD20T. D and H: *E. coli* harbouring pHERD20T diluted 10x. A,B,C,D were isolated with plasmid miniprep; E,F,G,H were isolated with cell lysate

To be more environmentally relevant, a mixed sewage community was chosen instead of the mixed community previously tested for natural fluorescent transformation assay. To validate if the *E. coli* community could undergo to a process of transformation, electroporation was performed with two *E. coli* donor strains, previously visualized with gel electrophoresis (Figure 3), harbouring pUC19 and pHERD20T plasmids respectively.

Even though it was expected to generate more electro transformation colonies with the smaller size plasmid DNA (pUC19), the result has shown the contrary when tested with environmental sewage isolates. The transformation efficiency in electroporation is directly proportional to size of homologous flanking region but inversely related to higher size of nonhomologous insert (Kung et al., 2013). Compared to the positive control, isolate DH5a, the environmental isolate D6 have show to have the same electroporation efficiency when PHERD20T was used.

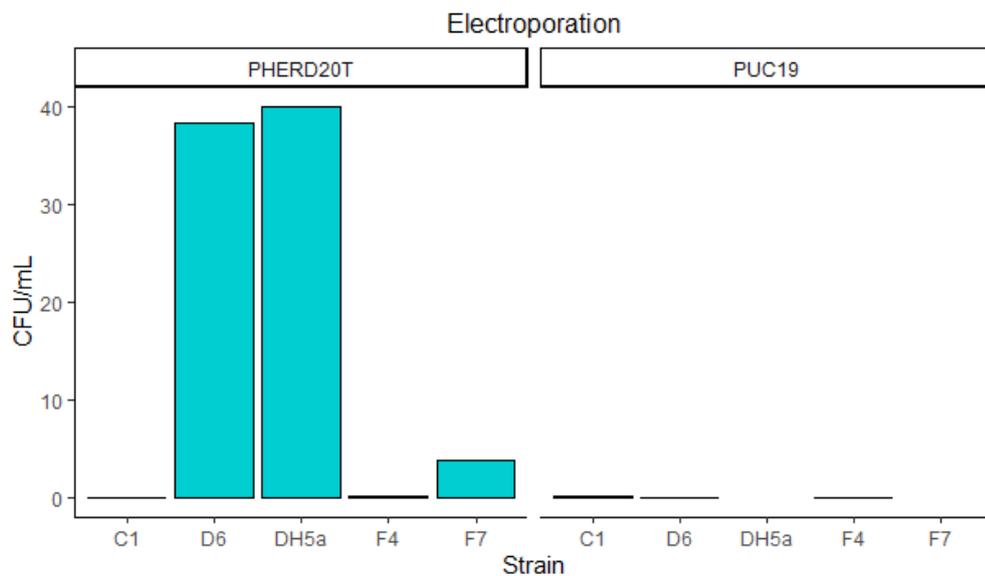


Figure 9 Electroporation of sewage *E. coli* isolates; It was performed adopting and testing two different previously isolated plasmid, pUC19 and pHERD20T, the last one showed more transformant colonies than the first one.

After, understanding the right recipient environmental isolate and plasmid with this pilot work, the main *E. coli* natural transformation experiment was performed. Even though the assignment was repeated multiple times, including 6 replicates per colony, and using different amount of plasmid isolated previously, the same number of transformations was found. It can be said that *E. coli* was able to transform, but the number of colonies shown in the plates was not enough to be reproducible. More studies need to be done to better understand the process of *E. coli* natural transformation.

## 3.2 *Acinetobacter Baylyi*.

### 3.2.1 Nutrient

To clarify if increasing concentration of nutrient availability could have an effect on natural transformation frequency, the recipient bacterium *A. baylyi* was cultured in three different concentrations of LB agar (1%, 10%, 100%). Transformation frequency of *A. baylyi* was significantly affected by nutrient concentration, with significantly greater transformation frequency at 100% nutrient than both 10% and 1% ( $p < 0.05$ ) (Figure 10). Nutrient availability at 1% and 10% seemed to have a significant effect on transformation frequency (1%:  $p$ : 0.00294, 10%:  $p$ : 0.00686). It has been shown in recent study, to support research question 2, with *Riemerella anatipestifer* species, different components of the media, such as iron, glucose, peptone etc, influence transformation frequency. For example, when iron chelator was supplemented in the media, the transformation frequency decreased by 10 fold to gradually increase when  $\text{Fe}(\text{NO}_3)_3$  was restored (Zhang et al., 2021). It could therefore be speculated that it is the reduction of compounds like these in the lower nutrient concentrations that is leading to the lower transformation frequencies observed.

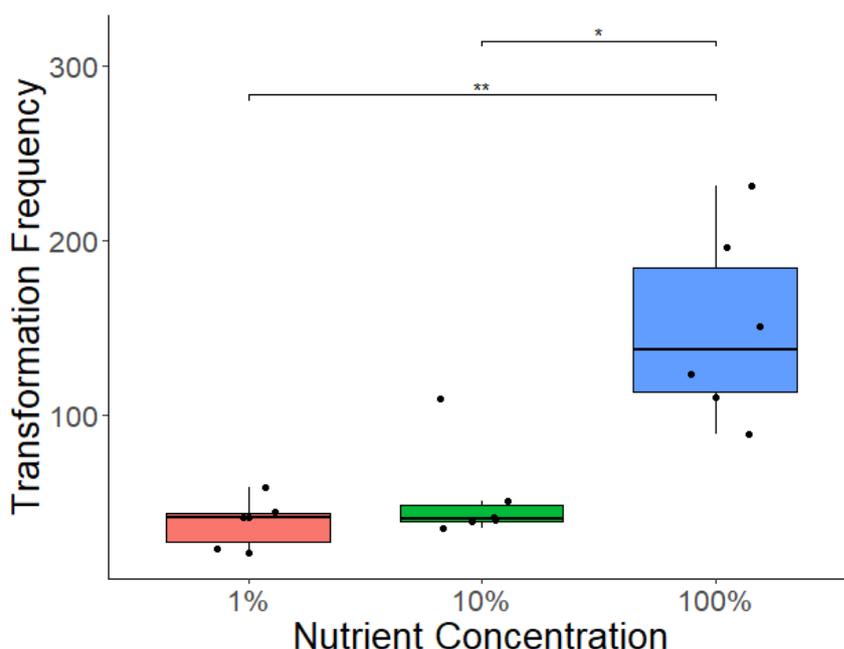


Figure 10 *A. baylyi* natural transformation frequency with three different nutrient availability, 1% LB nutrient, 10% LB nutrient and 100% LB nutrient.

### 3.2.2 Nutrient and competitor

Natural transformation takes place in complex microbial communities, and within this complex habitat, abiotic, such as nutrient availability, soil, temperature, sunlight, and biotic, such as organic compounds, zooplankton, and microorganisms in general are key drivers of this mechanisms (Al-Gashgari et al., 2023).

Adding to this, environmental pollutants may also stimulate competence response in competent bacteria. The effect of co-occurrence of those factors has been already evaluated on HGT (Al-Gashgari et al., 2023). In this study, nutrient availability, as a abiotic factor, and species present, as biotic factors were taken into account on natural transformation frequency in *A. baylyi*. With this it was able to investigate if 1% nutrient could affect transformation frequency when also competitor species were present and so on with 10% and 100% (Figure 11). The presence of *Bordetella* competitor specie increased natural transformation frequency at 1% but not significantly (p: 0.0887). Instead, the presence of *Bacillus* at the same nutrient availability rate decreased natural transformation frequency significantly (p: 0.00350). 100% nutrient increased transformation frequency with both species competitors tested, but the result wasn't significant (*Bordetella* p: 0.078; *Bacillus* p: 0.6). Instead, the presence of *Bacillus* with 10% nutrient availability decreased natural transformation frequency significantly (p: 0.0225).

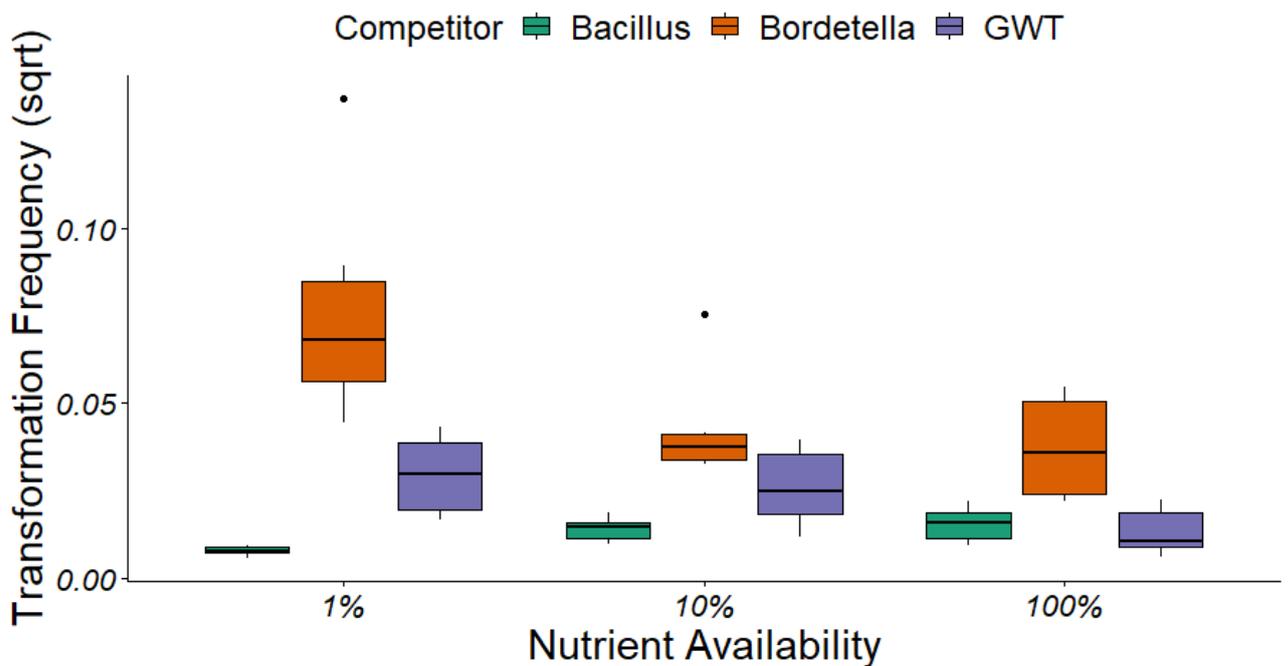


Figure 11 Effect of competitor species on natural transformation according to nutrient availability

So, it can be declared that the presence of *Bacillus* as a competitor species had an effect on transformation frequency, especially when minimum nutrient media was used. This could be related to adaptation to new environments, as some bacteria, in order to survive respond to stressors, exchange DNA in order to gain new defence properties, such as in this case gaining new ARGs, or to use it as a source of ATP (Seitz & Blokesch, 2012). The complex and interconnected competence regulatory system is induced only when physiological and environmental factors (such as abiotic and biotics) reach a critical point (Fontaine et al., 2015). In this way bacteria have optimized and increased the potential benefits and cost of undergoing to such energy-consuming process (Fontaine et al., 2015). HGT processes, specifically natural

transformation, have been reported in several environmental settings, such as WWTP, soil, plankton, marine water and sediments (Paul et al., 1991) (Paget & Simonet, 1994) (Chamier et al., 1993). Although, variables are involved in these environments. Nielsen et al., have addressed some of the abiotic factors, such as nutrient enrichment, showing that transformation frequency of *A. calcoaceticus*, was highly influenced and increased by the addition of LB and increasing concentration of phosphorous to the soil. But also, that moisturising and adjusting the soil with water influenced transformation frequency. Instead, the addition of  $Mg^{2+}$  and  $Ca^{2+}$  did not influenced natural transformation. This result shows a clear influence of the surrounding environment on transformation mechanisms as was already supported and questioned by this study. Furthermore, it is know that the induction of competence, in gram-negative bacteria is usually related to a stress challenge (Fontaine et al., 2015), that can presumably be the absence or the scarce presence of nutrient in the surrounding environment, as researched in this study.

On the other side, the complex biotic network interaction in different ecosystems, such as parasitism and predation, are known to create biodiversity and play a role in the evolution of microorganisms, (Shanker & Federle, 2017). Although in many studies, quantifying this interaction using single species approaches might not correspond to innate evolutionary bacteria community dynamics. Nevertheless, these approaches are useful to understand the genetic mechanism underling these processes. When exposed to the abiotic environment, bacteria community may result to physiological competence state development, leading to an evolution of competent species and thus correlating to changes in species abundance and diversity (Lawrence et al., 2012).

### 3.2.3. *Competitor and supernatant*

Fewer studies have looked at how microorganisms interact with each other and how they communicate without requiring a direct contact. It was demonstrated that not only *Pseudomonas aeruginosa*, is able to interact with bacteria and fungi, e.g. inhibiting their growth, but also to increase virulence when co-cultured. Recently, a specific mechanism was investigated by which a particular toxin (gliotoxin) produced by the surrounding community could inhibit *P. aeruginosa* biofilm formation (Reece et al., 2018). Different ecological habitat correlated to the induction of competent specie-specific requirements varies between species (Fontaine et al., 2015). For this reason, natural transformation frequency of *A. baylyi* was assessed in three different simulated environments: live cell competitors, spent supernatant media competitor, and boiled supernatant competitor media, and compared to the control for each treatments (GWT) (Figure 11). This was to evaluate if proteins and metabolites present in live cell media and supernatant spent media could influence the transformation frequency of the model species. To do so, fewer statistical model were screened to evaluate if, for

example, just the environment could influence natural transformation. The first model created included the behaviour of the environment and the species, present with no interaction between them, showed significant effect when the live *Bordetella* competitor species was present ( $p: 0.000180$ ), supporting the result of the second research question and findings from Figure 11.

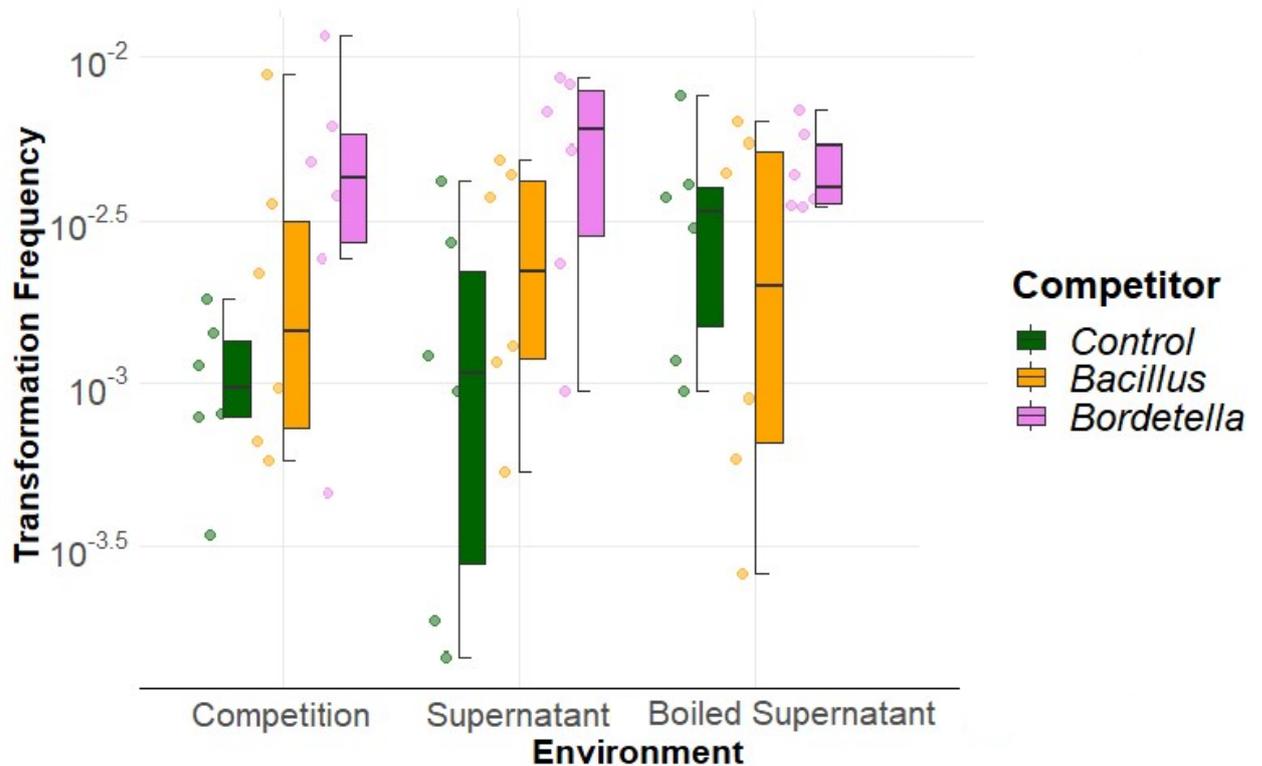


Figure 12 Natural transformation in *A. baylyi* exposed to different environmental habitat and to species competitor.

But, when another model expressing the interaction between the environment and the species competitor was compared to the first model for significance, it didn't show any significant effect on transformation frequency ( $p: 0.6095$ ), so it was concluded that the interaction between the different environments in synergy with the competitor species present does not have an effect on transformation frequency of *A. baylyi*. Furthermore, to make sure that the assumption obtained by this statistical model was robust, another model was created investigating the presence of competitor species not including the influence of the environment, and again, the species *Bordetella* showed a significant effect on transformation frequency ( $p: 0.000206$ ). An emmean was assessed to compare if the species present in this study had an effect on transformation frequency. The result (Table 4), validate the hypothesis that species are significantly different in their effects. Specifically, when compared between *Bordetella- Bacillus* and *Bordetella- A.baylyi* (respectively,  $p: 0.0144$ ;  $p:0.0016$ ). Instead, no significant effect was found when *A.baylyi* was exposed to *Bacillus* competitor specie ( $p:0.03145$ ). Although, this finding does not show a particular effect of the environment (boiled supernatant and

supernatant treatment) on natural transformation frequency, in contrast with the last hypothesis stated.

Table 4 Emmeans pairwise comparison between species

Species pairwise	estimate	SE	t.ratio	p-value
<i>A.baylyi(self)</i> - <i>Bacillus</i>	-0.000806	0.000793	-1.016	0.3145
<i>A.baylyi(self)</i> - <i>Bordetella</i>	-0.002943	0.000793	-3.711	0.0016
<i>Bacillus</i> - <i>Bordetella</i>	-0.002137	0.000793	-2.695	0.0144

It was supposed that different bacterial interactions and various compounds or stressors could have an effect on natural transformation. In previous works, for example, bromoacetic acid, a drinking water disinfection byproduct could stimulate natural transformation in *A. baylyi* (Mantilla-Calderon et al., 2019). Furthermore, eDNA and metabolites are a component of freshwater, posing a health problem at first instance, secondly a challenge to wastewater treatment establishments. This study supports the idea that eDNA and metabolites could be present in surrounding environments and playing a role in competence induction, even if no significant effect was encountered. The co- occurrence of multiple factors, such as disinfection byproducts and UV irradiation, has been shown to lead to a stress response mutagenic mechanism via reactive oxygen species in which lead to the increase of transformation (Augsburger et al., 2019). Also Lawrence et al., show a clear higher positive interaction between polyculture isolated than compared with the ancestral and monoculture. This result might be related to the fact that species adapted more easily if sources and compounds were produced and released by metabolisms of dead cells. The main sources of components found in polyculture were glucose, choline, succinate, followed by formate, acetate and lactate, leading to a cross-feeding interaction.

#### 4. Findings, limitations and future work

Firstly, *E. coli* WWTP community was able to transform with electroporation by adding PHERD20T plasmid. Though, even if different experiments and adjustment of protocols were made, only low percentage of plasmid natural transformation was assessed. Futher, after contacting the authors of the methods paper taken in consideration, it was discovered that the protocol needed adjustments that could not be assessed at that time. Thus, plasmid natural transformation with environmental *E. coli*, is a process that it is already happening in the environment but that needs further studies to be assessed and discovered.

This study has evaluated new key points how natural transformation could be regulated in the natural environment, developing our understanding of the putative drivers of this natural processes. It was firstly discovered that nutrient availability significantly effect transformation

frequency in *A. baylyi*. Afterwards, investigating co-culture and nutrient availability, found that some species do have more effect compared to others, specifically when *A. baylyi* is co-cultured with *Bacillus* in low percentage LB media, the transformation frequency tended to decrease significantly. Although the last hypothesis of the study, in which supernatant spent media compounds, metabolites and proteins present in species, such as *Bordetella* and *Bacillus*, could influence and have a role on the uptake of eDNA by natural transformation, no significant effect was found. But instead, when *Bordetella* species was present as a competitor species, it showed to up-regulate transformation frequency significantly. So, it was concluded that in this study, different environments do not have an effect on transformation frequency, but the species in co-culture does.

Of course, there are limitations present in this study. Firstly, this work required more time to process the literature review, in order to design a more quality protocol, especially for the *E. coli* project as it's unknown plasmid natural transformation process.

Using a model lab-based strain and an isogenic donor strain doesn't represent the environment in which bacteria could face natural transformation process. Although this study was necessary to begin to understand what the mechanisms and the interactions are involved in natural transformation. Furthermore, using ideal medium and even decreasing concentration of the LB media doesn't seem to mediate the absence or the low concentration of nutrient in the environment, but in future studies it would be ideal using supernatants, wastewater and soil samples as a treatment to study. Also, instead of using a lysate isogenic strain as a donor, fewer screening methods, such as flowcytometry targeting eDNA with fluorescence markers for example, could be ideal to target eDNA from the environment and assess natural transformation mechanism. Fewer studies suggest that bacterial evolution, especially with an evolutionary source such natural transformation, should not be investigated with simple population genetic model, because the features of bacteria ecology and evolution might influence the process (Arnold, 2022). Lastly, few bacteria are currently known to express natural competence in the environment, such as in soil, and just as little is know about the regulation and induction involved in natural transformation in these settings, so further research is required.

## **5. Conclusions**

The use and misuse of antimicrobials and their increasingly driven demand in clinical settings, agricultural soil, WWTPs and animal breeding settings are posing AMR to an increased position in the scale of a worldwide public health threat. The effect of AMR is wide-ranging, starting from the antimicrobial being non-effective for pathogenic and common bacteria, to the mutating microbial community and its correlated dissemination through HGT around the globe. In this context, natural transformation plays a leading role in the rapid spread, evolution and

adaptation of bacteria (Seitz & Blokesch, 2012). In order to mitigate the spread of AMR, it is fundamental to improve our understanding and study the metabolism and genetic interaction and fundamental sources involved in this process (Winter et al., 2021) . Whether free DNA is needed as a nutrient, damage repair or for evolution, it is essential to understand the balances underlining a microorganism's community quorum sensing. This study poses that the surrounding bacteria community is an important factor influencing natural transformation, so further studies are essential to investigate these processes from both an evolutionary aspect but also a from a AMR One health perspective.

## References-

- Al-Gashgari, B., Mantilla-Calderon, D., Wang, T., de los Angeles Gomez, M., Baasher, F., Daffonchio, D., Laleg-Kirati, T.-M., & Hong, P.-Y. (2023). Impact of chemicals and physical stressors on horizontal gene transfer via natural transformation. *Nature Water*, 1(7), 635–648. <https://doi.org/10.1038/s44221-023-00110-8>
- Arnold, B. J. (2022). *Horizontal gene transfer and adaptive evolution in bacteria*. 20(April). <https://doi.org/10.1038/s41579-021-00650-4>
- Arnold, B. J., Huang, I. T., & Hanage, W. P. (2022). Horizontal gene transfer and adaptive evolution in bacteria. *Nature Reviews Microbiology*, 20(4), 206–218. <https://doi.org/10.1038/s41579-021-00650-4>
- Augsburger, N., Mantilla-calderon, D., Da, D., & Hong, P. (2019). *Acquisition of Extracellular DNA by Acinetobacter baylyi ADP1 in Response to Solar and UV - C 254nm Disinfection*. <https://doi.org/10.1021/acs.est.9b01206>
- Bacher, J. M., & Metzgar, D. (2006). *Rapid Evolution of Diminished Transformability in Acinetobacter baylyi* □. 188(24), 8534–8542. <https://doi.org/10.1128/JB.00846-06>
- Bikard, D., Hatoum-aslan, A., Mucida, D., & Marraffini, L. A. (2012). *Article CRISPR Interference Can Prevent Natural Transformation and Virulence Acquisition during In Vivo Bacterial Infection*. 177–186. <https://doi.org/10.1016/j.chom.2012.06.003>
- Blokesch, M. (2016). Natural competence for transformation. *Current Biology*, 26(21), R1126–R1130. <https://doi.org/10.1016/j.cub.2016.08.058>
- Chamier, B., Lorenz, M. G., & Wackernagel, W. (1993). Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. *Applied and Environmental Microbiology*, 59(5), 1662–1667. <https://doi.org/10.1128/aem.59.5.1662-1667.1993>
- Charpentier, X., Polard, P., & Claverys, J. (n.d.). Induction of competence for genetic transformation by antibiotics : convergent evolution of stress responses in distant bacterial species lacking SOS? *Current Opinion in Microbiology*, 15(5), 570–576. <https://doi.org/10.1016/j.mib.2012.08.001>
- Collaborators, A. R. (2022). *Articles Global burden of bacterial antimicrobial resistance in 2019 : a systematic analysis*. 399. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0)
- Domingues, S. (2016). Horizontal Gene Transfer : Uptake of Extracellular DNA by Bacteria ☆. In *Reference Module in Biomedical Sciences* (Issue 1). Elsevier Inc.

<https://doi.org/10.1016/B978-0-12-801238-3.99485-6>

- Engelmoer, D. J. P., Donaldson, I., & Rozen, D. E. (2013). Conservative Sex and the Benefits of Transformation in *Streptococcus pneumoniae*. *PLoS Pathogens*, *9*(11), 1–7. <https://doi.org/10.1371/journal.ppat.1003758>
- Fontaine, L., Wahl, A., Fléchar, M., Mignolet, J., & Hols, P. (2015). Regulation of competence for natural transformation in streptococci. *Infection, Genetics and Evolution*, *33*, 343–360. <https://doi.org/10.1016/j.meegid.2014.09.010>
- Huang, M., Liu, M., Huang, L., Wang, M., Jia, R., Zhu, D., Chen, S., Zhao, X., Zhang, S., Gao, Q., Zhang, L., & Cheng, A. (2021). The activation and limitation of the bacterial natural transformation system: The function in genome evolution and stability. *Microbiological Research*, *252*(May), 126856. <https://doi.org/10.1016/j.micres.2021.126856>
- Hülter, N., Sørum, V., Borch-pedersen, K., Liljegren, M. M., Utnes, A. L. G., Primicerio, R., Harms, K., & Johnsen, P. J. (2017). Costs and benefits of natural transformation in *Acinetobacter baylyi*. 1–10. <https://doi.org/10.1186/s12866-017-0953-2>
- Jain, R., Rivera, M. C., & Moore, J. E. (2002). Horizontal Gene Transfer in Microbial Genome Evolution. *495*, 489–495. <https://doi.org/10.1006/tpbi.2002.1596>
- Kung, S. H., Retchless, A. C., Kwan, J. Y., & Almeida, R. P. P. (2013). Effects of DNA size on transformation and recombination efficiencies in *xylella fastidiosa*. *Applied and Environmental Microbiology*, *79*(5), 1712–1717. <https://doi.org/10.1128/AEM.03525-12>
- Labroussaa, F., Lebaudy, A., Baby, V., Gourgues, G., Matteau, D., Vashee, S., Sirand-Pugnet, P., Rodrigue, S., & Lartigue, C. (2016). Impact of donor-recipient phylogenetic distance on bacterial genome transplantation. *Nucleic Acids Research*, *44*(17), 8501–8511. <https://doi.org/10.1093/nar/gkw688>
- Lawrence, D., Fiegna, F., Behrends, V., Bundy, J. G., Phillimore, A. B., Bell, T., & Barraclough, T. G. (2012). Species Interactions Alter Evolutionary Responses to a Novel Environment. *10*(5). <https://doi.org/10.1371/journal.pbio.1001330>
- Lenski, R. E., Maloy, S. R., Pringle, A., & Bohannan, B. J. M. (2009). *Microbial Evolution*. May 2015.
- Leong, C. G., Bloomfield, R. A., Boyd, C. A., Dornbusch, A. J., Lieber, L., Liu, F., Owen, A., Slay, E., Lang, K. M., & Lostroh, C. P. (2017). The role of core and accessory type IV pilus genes in natural transformation and twitching motility in the bacterium *Acinetobacter baylyi*. 1–25.
- Li, W., & Zhang, G. (2022). Detection and various environmental factors of antibiotic resistance

- gene horizontal transfer. *Environmental Research*, 212(PB), 113267. <https://doi.org/10.1016/j.envres.2022.113267>
- Mantilla-Calderon, D., Plewa, M. J., Michoud, G., Fodelianakis, S., Daffonchio, D., & Hong, P. Y. (2019). Water Disinfection Byproducts Increase Natural Transformation Rates of Environmental DNA in *Acinetobacter baylyi* ADP1. *Environmental Science and Technology*, 53(11), 6520–6528. <https://doi.org/10.1021/acs.est.9b00692>
- Meibom, K. L., Blokesch, M., Dolganov, N. A., Wu, C., & Schoolnik, G. K. (2016). *American Association for the Advancement of Science*. 310(5755), 1824–1827.
- Mell, J. C., & Redfield, R. J. (2014). Natural competence and the evolution of DNA uptake specificity. *Journal of Bacteriology*, 196(8), 1471–1483. <https://doi.org/10.1128/JB.01293-13>
- Nielsen, K. M., Bones, A. M., & Van Elsas, J. D. (1997). Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. *Applied and Environmental Microbiology*, 63(10), 3972–3977. <https://doi.org/10.1128/aem.63.10.3972-3977.1997>
- Paget, E., & Simonet, P. (1994). On the track of natural transformation in soil. *FEMS Microbiology Ecology*, 15(1–2), 109–117. <https://doi.org/10.1111/j.1574-6941.1994.tb00235.x>
- Palchevskiy, V., & Finkel, S. E. (2006). *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. *Journal of Bacteriology*, 188(11), 3902–3910. <https://doi.org/10.1128/JB.01974-05>
- Paul, J. H., Frischer, M. E., & Thurmond, J. M. (1991). Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. *Applied and Environmental Microbiology*, 57(5), 1509–1515. <https://doi.org/10.1128/aem.57.5.1509-1515.1991>
- Reece, E., Doyle, S., Greally, P., Renwick, J., & McClean, S. (2018). *Aspergillus fumigatus* inhibits *Pseudomonas aeruginosa* in co-culture: Implications of a mutually antagonistic relationship on virulence and inflammation in the CF airway. *Frontiers in Microbiology*, 9(JUN), 1–14. <https://doi.org/10.3389/fmicb.2018.01205>
- Seitz, P., & Blokesch, M. (2012). *Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria*. <https://doi.org/10.1111/j.1574-6976.2012.00353.x>
- Shanker, E., & Federle, M. J. (2017). *Quorum Sensing Regulation of Competence and Bacteriocins in Streptococcus pneumoniae and mutans*. <https://doi.org/10.3390/genes8010015>

- Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A., & Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature*, *480*(7376), 241–244. <https://doi.org/10.1038/nature10571>
- Soucy, S. M., Huang, J., & Gogarten, J. P. (2015). Horizontal gene transfer: Building the web of life. *Nature Reviews Genetics*, *16*(8), 472–482. <https://doi.org/10.1038/nrg3962>
- Sun, D. (2016). Two different routes for double-stranded DNA transfer in natural and artificial transformation of *Escherichia coli*. *Biochemical and Biophysical Research Communications*, *471*(1), 213–218. <https://doi.org/10.1016/j.bbrc.2016.01.137>
- Sun, D. (2018). Pull in and push out: Mechanisms of horizontal gene transfer in bacteria. *Frontiers in Microbiology*, *9*(SEP), 1–8. <https://doi.org/10.3389/fmicb.2018.02154>
- Sun, D., Zhang, Y., Mei, Y., Jiang, H., Xie, Z., Liu, H., Chen, X., & Shen, P. (2006). *Escherichia coli* is naturally transformable in a novel transformation system. *FEMS Microbiology Letters*, *265*(2), 249–255. <https://doi.org/10.1111/j.1574-6968.2006.00503.x>
- The origin of species*. (n.d.).
- Verraes, C., Boxstael, S. Van, Meervenue, E. Van, & Coillie, E. Van. (2013). *Antimicrobial Resistance in the Food Chain: A Review*. June, 2643–2669. <https://doi.org/10.3390/ijerph10072643>
- Von Wintersdorff, C. J. H., Penders, J., Van Niekerk, J. M., Mills, N. D., Majumder, S., Van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology*, *7*(FEB), 1–10. <https://doi.org/10.3389/fmicb.2016.00173>
- Williams, M. R., Stedtfeld, R. D., Guo, X., & Hashsham, S. A. (2016). *Antimicrobial Resistance in the Environment*. *88*(10), 1951–1967. <https://doi.org/10.2175/106143016X14696400495974>
- Winter, M., Buckling, A., Harms, K., Johnsen, P. J., & Vos, M. (2021). Antimicrobial resistance acquisition via natural transformation: context is everything. *Current Opinion in Microbiology*, *64*, 133–138. <https://doi.org/10.1016/j.mib.2021.09.009>
- Winter, M., Harms, K., Johnsen, P. J., Buckling, A., & Vos, M. (2023). *Testing for the fitness benefits of natural transformation during community- - embedded evolution*. 1–10. <https://doi.org/10.1099/mic.0.001375>
- Zhang, L., Huang, L., Huang, M., Wang, M., Zhu, D., Wang, M., Jia, R., Chen, S., Zhao, X., Yang, Q., Wu, Y., Zhang, S., Huang, J., Ou, X., Mao, S., Gao, Q., Tian, B., Cheng, A., & Liu, M. (2021). Effect of Nutritional Determinants and TonB on the Natural Transformation

of *Riemerella anatipestifer*. *Frontiers in Microbiology*, 12(August), 1–15.  
<https://doi.org/10.3389/fmicb.2021.644868>