Context-Dependent Acquisition of Antimicrobial Resistance Mechanisms

Submitted by Macaulay Ralph Winter to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Medical Studies, August 2023.

I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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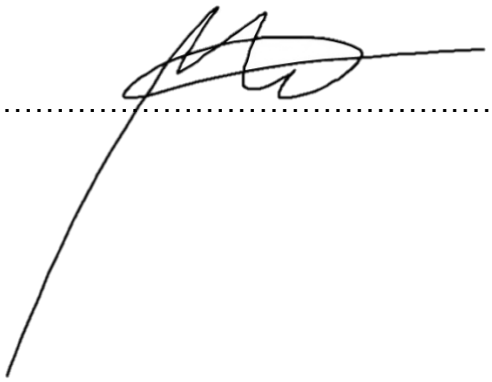

Abstract

Natural transformation is a process whereby bacteria actively take up free DNA from the environment while in a physiological state termed competence. Uptaken DNA is then recombined into the recipient's genome or reconverted into extra-chromosomal genetic elements. The inducing stimuli for competence vary widely between transformable species and competence induction is affected by a host of abiotic factors found in bacterial environments. Natural transformation is recognised to be responsible for the dissemination of antimicrobial resistance genes both within and between species, contributing to the global antimicrobial resistance crisis threatening modern medicine. Despite being the first mechanism of horizontal gene transfer discovered, the evolutionary benefits of natural transformation are still under debate.

This thesis is comprised of four standalone research chapters which aimed 1) to determine if chemotherapeutic compounds affect the transformation frequencies of transformable bacteria. This provides important information which can have implications on the contraction of a life-threatening infection in cancer patients. 2) to determine if other environmentally relevant bacteria affect the transformation frequencies of transformable bacteria. Understanding the contexts under which bacteria transform in their natural environments can help us to predict the spread of antimicrobial resistance mechanisms via natural transformation. 3) to produce a resource of genomic information for the scientific community, allowing researchers to improve our understanding of the *Acinetobacter* genus. And 4) to determine if environmentally relevant bacteria affect the transformation frequencies of transformable bacteria to find evidence for the sex hypothesis for natural transformation. This was performed by using biotic interactions as a selection pressure and DNA from a range of related species as a substrate for transformation. Together, these chapters provide information about the contexts under which transformation is both regulated and selected for in realistic environmental contexts. Enhancing our understanding of how and when bacteria naturally transform, in both natural and clinical environments, can help us to monitor and establish preventative measures to limit the spread of antimicrobial resistance genes between bacteria.

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Glossary

Term	Definition
Antibiotic	An antimicrobial substance used to kill a bacterium or inhibit its growth
Antibiotic Resistance	Loss of susceptibility by bacteria to antibiotics, leading to reduced effectiveness of antibiotic agents
Antimicrobial	A substance used to kill or inhibit the growth of microorganisms
Antimicrobial Resistance	Loss of susceptibility by microorganisms to antimicrobials, leading to reduced effectiveness of antimicrobial agents
Antimicrobial Resistance Gene	A gene which codes for a protein conferring a function which decreases the sensitivity of the microorganism to an antimicrobial agent
Capsid	A protein shell surrounding a virus particle
Competence	A physiological state whereby bacteria can take up DNA from their extracellular environment
Conjugation	Physical connection between to bacteria via a pilus which facilitates transfer of a plasmid from donor to recipient cell
Exonuclease	A DNA-degrading enzyme
Fitness	A measure of reproductive ability of an organism in a particular environment
Fitness Landscape	An illustrative metaphor describing the relationship between genotype (x axis, z axis) and fitness (y axis)
Gene Transfer Agents	Virus-like particles which can facilitate HGT between bacteria
Genetic Bottlenecking	Sharp reductions in population sizes, reducing genetic variation in the population
Homology	Similar in structure or function
Horizontal Gene Transfer	The exchange of genetic material from one organism to another independent of cell division
Immunocompromised	Describing an organism with a weakened immune system which may not effectively combat infections

Isogenic	Identical with respect to genetic characteristics
Malthusian Parameter	The average rate of increase of a population in a given time period
Membrane Vesicles	A self-contained structure filled with fluid surrounded by a phospholipid bilayer
Minimum Inhibitory Concentration	The minimum concentration of a compound required to inhibit growth of a microorganism
Minimum Selective Concentration	The minimum concentration of a compound in which resistant strains have a fitness advantage over sensitive strains
Natural Transformation	The genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surroundings through the cell membrane
Pathogenicity	The quality of being able to cause disease
Recombination	The rearrangement of genetic material within an organism
Second-Order Selection	Selection acting on the rates of mutation or recombination over the course of adaptation
Selection-Rate Constant	The difference in two Malthusian parameters calculated by subtraction
Transduction	Transfer of genetic material from one cell to another via a bacteriophage
Transformation Frequency	The fraction of cells which have transformed with marker DNA within a given time period
Type VI Secretion System	A molecular machine used by bacteria to predate upon other bacteria and increase virulence
Wildtype	The typical form of an organism or gene as it occurs in nature
Virulence	The ability of a pathogen to overcome a host's defences to cause disease

Abbreviations

Term	Definition
AMR	Antimicrobial Resistance
ANI	Average Nucleotide Identity
ARG	Antimicrobial Resistance Gene
ATK	Abelson Tyrosine Kinase
DNA	Deoxyribose Nucleic Acid
dsDNA	Double-Stranded DNA
DMSO	Dimethyl Sulfoxide
DUS	DNA Uptake Sequence
ENA	European Nucleotide Archive
EPI	Efflux Pump Inhibitor
GFP	Green Fluorescent Protein
GTA	Gene Transfer Agent
HGT	Horizontal Gene Transfer
MIC	Minimum Inhibitory Concentration
MSC	Minimum Selective Concentration
NDM-1	New Delhi Metallo-ß-Lactamase
PPMO	Peptide-Conjugated Phosphorodiamidate Morpholino Oligomers
R-M system	Restriction Modification System
PCR	Polymerase Chain Reaction
RFP	Red Fluorescent Protein
SOS response	Bacterial stress response pathway
ssb	Single Stranded Binding Protein
ssDNA	Single-Stranded DNA
T6SS	Type VI Secretion System
UV	Ultraviolet
WT	Wildtype

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Chapter 1: Thesis introduction

1.1 Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the transfer of genes between unicellular organisms independent of cell division and is a pervasive force driving bacterial evolution (Gogarten et al., 2002; Kuo & Ochman, 2009; Philippe & Douady, 2003). For instance, one study estimated that 81% ± 15% of genes in 181 genomes studied were horizontally transferred at least once during each species' evolution (Dagan et al., 2008). This likely is an underestimate, as some gene transfers will go undetected because they are subsequently lost or the received genetic material replaces identical DNA sequences (Soucy et al., 2015). Due to its ability to transfer whole clusters of functioning genes, HGT between strains and species is thought to have a greater impact on evolutionary diversification than the stepwise accumulation of mutations (Cohen et al., 2005; Jansen et al., 2013; Vos et al., 2015). There are three main mechanisms of HGT: conjugation, transduction, and transformation (although the boundaries between these mechanisms are sometimes blurred (Gillings, 2017; Figure 1)). Transformation, the focus of this thesis, will be discussed in more detail in the next section of this chapter.

Conjugation is the process where two neighbouring cells create a physical connection using a specialised pilus to allow for the transfer of genes, typically carried on a plasmid or integrative and conjugative element (ICE) (Aminov, 2011). Plasmids tend to be somewhat specialised to their hosts, but broad host range (BHR) plasmids can be passed to multiple genera (Jain & Srivastava, 2013). Plasmids can also be limited in their ability to spread if they are lacking the genes which code for conjugation machinery, typically relying on mobilisation genes located on other plasmids (Ramsay & Firth, 2017; Udo et al., 1992). Such plasmids are termed non-conjugative but mobilizable plasmids. Plasmids can sometimes not pass into both daughter cells upon cell division (Summers, 1991). If there is no strong selection for the plasmid such as with antimicrobial resistance, a significant fitness benefit the plasmid confers, or a toxin antitoxin system leading to cell death upon plasmid loss (Van Melderen, 2010), these accidental losses can lead to sustained loss of the plasmid across the population. However, plasmids can reduce the chance of being lost by the cell by precisely

distributing copies of the plasmid to each daughter cell upon cell division, and by carrying toxin-antitoxin genes which ensure cell death upon plasmid loss as the cell can no longer produce antitoxin to protect against the toxin (Zielenkiewicz & Cegłowski, 2001). Additionally, plasmids with high rates of infection through conjugation are more likely to be maintained in a population due to reinfection if a cell loses a plasmid (De Gelder et al., 2007). Hosts can also increase the likelihood that a plasmid is maintained through compensatory mutations which mitigate the fitness costs conferred by plasmids (Hall et al., 2020).

Transduction is when foreign DNA is introduced to bacterial cells via bacteriophages during their infection cycle. As bacteriophages insert their own DNA into the host chromosome and replicate within the infected host cell, they sometimes package host DNA alongside bacteriophage DNA during replication, leading to generalised or specialised transduction (Chiang et al., 2019; Von Wintersdorff et al., 2016). The spread of genetic material via phage infection cycles to other species is limited as phages are specialised to infect specific strains of bacteria (Koskella & Meaden, 2013). However, the contribution of transduction to HGT can be substantial as infected cells can contain 100-300 phage particles before lysing (Allers et al., 2013; Pavlova et al., 1997; Watters et al., 1997), providing opportunities for multiple regions of the host chromosome to be transferred to nearby cells upon infection. Similar to phages, gene transfer agents (GTAs) are phage-like elements which can take up host DNA into their capsids before the host lyses and releases its DNA into the surrounding environment (Bárdy et al., 2020). A recipient cell can then receive the DNA with a mechanism which uses homologues of some of the same genes (*comEC, comF,* and *comM*) that are used in natural transformation (Brimacombe et al., 2015).

Less-discussed, yet still likely highly significant mechanisms of HGT include membrane vesicles and nanotubes. Membrane vesicles are parts of the cell membrane which bud off from gram-negative cells, encapsulating their payload before releasing themselves (Mashburn-Warren et al., 2008). They are produced by all gram-negative bacteria and facilitate the transfer of proteins (Bonnington & Kuehn, 2014), signalling molecules (Mashburn-Warren et al., 2008), and DNA (Renelli et al., 2004), but can also be used to kill neighbouring cells (Evans et al., 2012; Kadurugamuwa &

Beveridge, 1996). This method of packaging DNA protects it from agents such as extracellular DNases which could facilitate degradation of the DNA (Renelli et al., 2004). Uptake of payloads from vesicles requires no active contribution from the recipient as it acts upon passive fusion with the recipient outer membrane (Kadurugamuwa & Beveridge, 1999), and thus can mediate transfer of DNA into any recipient bacteria. While not generally considered a main mechanism of HGT, membrane vesicles are understood to have a large contribution in exchanging DNA and highly soluble compounds between bacteria in marine ecosystems (Biller et al., 2014). Nanotubes act similarly to the pili used for conjugative transfer of plasmids and are typically used for bidirectional transfer of small molecules and signalling compounds (Dubey & Ben-Yehuda, 2011; Pande et al., 2015). Nanotubes can facilitate transfer of non-conjugative plasmids in some instances and can form membrane vesicles (Dubey et al., 2016; Dubey & Ben-Yehuda, 2011). Our understanding of which bacteria can utilise nanotubes and the phylogenetic limits of interspecies interactions using the mechanism are limited (Gillings, 2017; Pande et al., 2015), so it is hard to gauge how impactful this mechanism is for HGT.

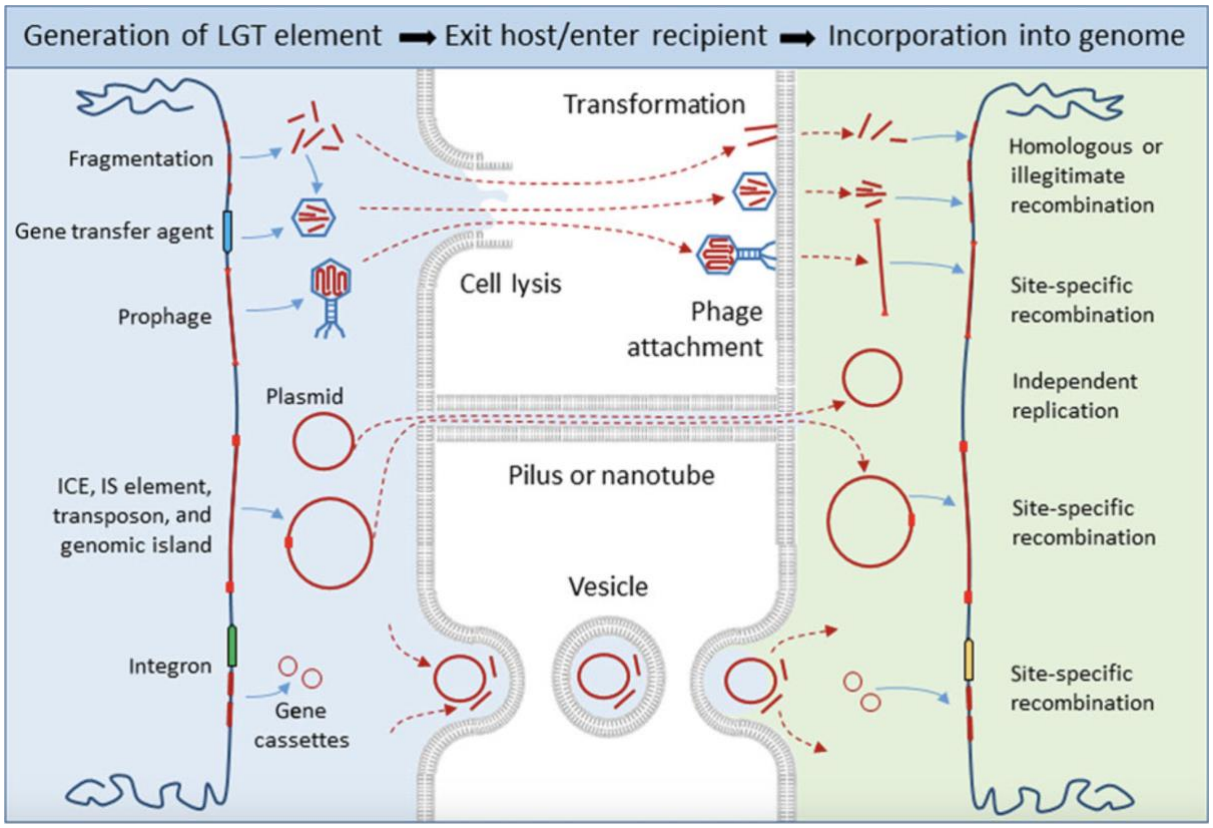

Figure 1. Illustration of variation between mechanisms of HGT (also termed lateral gene transfer, LGT). Direction of DNA transfer starts from mobilisation of the DNA from the donor (left) to the recipient (right) through the intercellular space (middle). Physical transfer of DNA is illustrated with red dashed lines, while blue arrows indicate excision from, mobilisation from, or recombination with the chromosome. Figure from Gillings, 2017.

1.2 Natural Transformation: Mechanisms and Regulation

Natural transformation describes the process whereby free, extracellular DNA is taken into a cell after which it can undergo recombination with the chromosome, be stored as an extrachromosomal element such as a plasmid, or be degraded (Winter et al., 2021). Bacterial natural transformation has been identified in only a relatively small number of species (around 80-90) (Blokesch, 2016; Johnston et al., 2014), and is well characterised in an even smaller cohort of species, such as *Bacillus subtilis* (Johnston et al., 2014)*, Acinetobacter baylyi* (Elliott & Neidle, 2011; Ray et al., 2009; Ray & Nielsen, 2005)*, Helicobacter pylori* (Baltrus et al., 2007; Bubendorfer et al., 2016; E. A. Lin et al., 2009; Nguyen et al., 2022) and *Vibrio cholerae* (Y. Sun et al., 2013).

Some of the pathways for homologous recombination via transformation vary between species with many of the details still unknown (Ambur et al., 2016; Dubnau & Blokesch, 2019; Johnston et al., 2014; Levin & Cornejo, 2009; Seitz & Blokesch, 2013). DNA in the extracellular space is taken into the cell by membrane-bound type IV pili when the cell is in a physiological state termed competence (Dubnau & Blokesch, 2019; Johnston et al., 2014). The only known exception to this rule is *Helicobacter pylori* which uses a type IV secretion system instead (Fischer et al., 2020). Gram-positive and gram-negative bacteria differ in the presence of an outer membrane, providing the need for a pilQ protein channel in gram-negative bacteria (Chen and Dubnau, 2004;Figure 2). Species such as *Acinetobacter baylyi* and *Neisseria gonorrhoeae* require the adhesin comC (or a homologue thereof) for natural transformation which is located on the end of type IV pili (Leong et al., 2017; Link et al., 1998), however *Vibrio cholerae* does not (Leong et al., 2017). Instead, *Vibrio* produces the pilus forming protein TcpJ which shows some homology to comC in *Bacillus subtilis* (Kaufman et al., 1991). Once captured by a pilus, one strand of the donor DNA is

pulled through the cell's inner membrane and the other strand is degraded within the intermembrane space by ssDNA-specific exonucleases (Harms et al., 2007; Johnston et al., 2014). Single-stranded DNA enters the cytoplasm in the 3' to 5' orientation (De Vries & Wackernagel, 2002), and is bound by ssb proteins and dprA which protect the ssDNA from degradation from intracellular exonucleases (Blokesch, 2016; Johnston et al., 2014; Vesel & Blokesch, 2021). The recFOR protein complex then assists drpA in displacing ssb with recA which facilitates loading of recA-bound ssDNA to the host genome (Blokesch, 2016; Johnston et al., 2014; Morimatsu & Kowalczykowski, 2003; Vesel & Blokesch, 2021). Plasmids can also be taken up by cells via natural transformation using these same mechanisms. RecA inhibits uptake of plasmids via natural transformation and RecA activity is downregulated by RecX to allow for plasmid uptake (Cárdenas et al., 2012).

1.2.1 Homologous Recombination Facilitated by Natural Transformation

Often, the entire site of recombination will be homologous to the incoming DNA fragment due to the proximity of kin to a recombining cell (Didelot & Maiden, 2010). However, if the incoming DNA strand has two sites of homology to the host genome which flank a heterologous DNA sequence, comM can assist in recombination of the two strands (Nero et al., 2018). Homologous flanking regions can be as small as 55 base pairs (Monier et al., 2007), but the size of the flanking regions will need to increase relative to inserted sequence length (Simpson et al., 2007). When a homologous region of DNA is found, strand invasion initiates with the use of radA (Marie et al., 2017) and a displacement loop (D-loop) is formed by locally separating the recipient dsDNA into two strands using redBCD (Spies & Kowalczykowski, 2006) to facilitate binding of donor and recipient strands. RadA is loaded onto recipient dsDNA on both sides of the D-loop to promote bidirectional extension of the D-loop and facilitate recA-mediated recombination (Marie et al., 2017). Interestingly, recQ helicase facilitates unwinding and rewinding of DNA helixes, but is also considered a "quality control enzyme" as it can arrest homologous recombination if it occurs in an undesirable orientation (Harami et al., 2017). If the incoming strand of DNA is not methylated, it is sensitive to restriction modification enzymes which can cleave the incoming fragment during recombination, leading to cell death (Johnston, Martin, et al., 2013; Johnston, Polard, et al., 2013). A DNA single strand break is required for separation of the intermediate complex, releasing a portion of recipient DNA for

degradation and DNA polymerase partially replicates DNA to fill any gaps in the site of recombination (Hülter et al., 2017). The newly formed DNA dimer is resolved with the involvement of XerCD and FtsK, occurring shortly before cell division (Castillo et al., 2017). Finally, ligation with DNA ligase seals the ends of the recombination section, finalising the recombination event (Kidane et al., 2012). Homologous recombination acts with little trace in the genome, so it is hard to determine what genetic material is acquired by this mechanism, especially retrospectively over evolutionary time. Recombination events can be identified by demonstrating different GC content or phylogeny of a region when compared to the rest of the genome (J. M. Smith, 1999). However, less-recent events may be hidden by mutations occurring on the recombination site which gradually change the GC content of the recombination site to that of the species' genome (Ochman et al., 2000). The most useful indicator of natural transformation involving donor DNA identical to the recipient strand is the generation of two single nucleotide polymorphisms on each end of the recombination site (Overballe-Petersen et al., 2013). Natural transformation can be observed experimentally in real time by using supplemented marker DNA conferring fluorescence or an antimicrobial resistance gene.

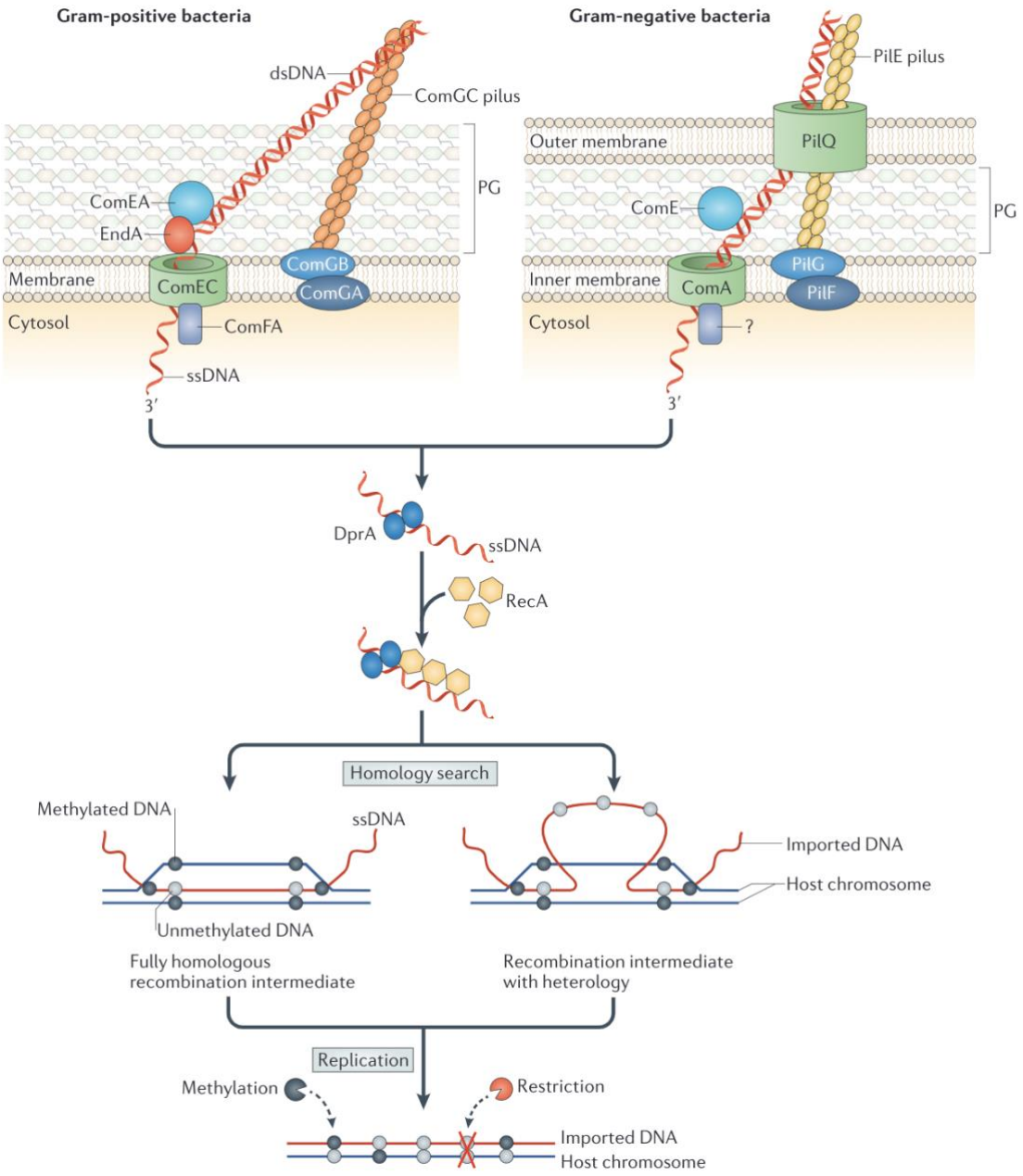

Figure 2. Illustration of the divergent mechanisms for natural transformation in gram-positive and gram-negative species. Notably, the key difference between the mechanisms is due to the presence of an outer membrane in gram-negative species requiring a pilQ secretin channel to facilitate movement of incoming DNA through the membrane. Most other transformation-associated proteins involved are homologues of those found in gram-positive species. Figure taken from Johnston *et al.,* 2014.

Table 1. Homologues of proteins involved in natural transformation in a range of bacteria. Table adapted from Nolan et al., 2020.

Competence protein	***Bacillus subtilis***	***Streptococcus pneumoniae***	***Haemophilus influenzae***	***Thermus thermophilus***	***Pseudomonas stutzeri***	***Neisseria gonorrhoeae***	***Pseudomonas aeruginosa****	***Acinetobacter baylyi***
Traffic NTPase(s)	ComGA	ComGA	PilB	PilF	PilT, PilU	PilF, PilT	PilB, PilT, PilU	PilB, PilT, PilU
Polytopic membrane protein	ComGB	ComGB	PilC	PilC	PilC	PilG	PilC	PilC
Pilins or pseudopilins	ComGC, -GD, -GE, -GG	CglC, CglD	PilA	PilA1, -A2, -A3, -A4	PilA1	PilE, ComP	PilA, -V, -W, -X, -E, FimT, FimU	ComF, ComE, FimT, PilX, ComB, PilV, FimU, ComP
Prepilin peptidase	ComC	CilC	PilD	PilD		PilD	PilD	PilD
Secretin/pilot	na	na	ComE	PilQ		PilQ/PilP	PilQ/PilP	PilQ
DNA translocation machinery								
DNA receptor	ComEA	ComEA		ComEA		ComE	PA3140	ComE
Membrane channel	ComEC	ComEC	Rec-2	ComEC	ComA	ComA	PA2984	ComA
ATP-binding protein	ComFA	ComFA			ExbB		PA2983	
Other								
			DprA (Smf)				PA0021	DprA
			TfoX (Sxy)				PA4703	
			CRP				Vfr	
			CyaA				CyaA, CyaB	
			ComM				PA5290	ComM
			ComF				PA0489	ComF
***Using *P. aeruginosa* PAO1 gene nomenclature.**								

While transformation appears to be highly conserved in its ability to actively take up DNA from the environment and facilitate its recombination with the chromosome, there is a great deal of variation between species in the proteins involved in natural transformation. Table 1 provides a brief example of variation in protein homologues which share the same function in natural transformation for separate species.

1.2.2 Non-Homologous Recombination Facilitated by Natural Transformation

Natural transformation leading to recombination with the chromosome requires sequence homology between the donor strand and the recipient chromosome for maximum efficiency (Majewski & Cohan, 1999), but can occur without any homology, albeit in drastically reduced frequency. For instance, recombination with non-homologous fragments (termed illegitimate recombination) occurs at 10^{-9} lower frequency in *A. baylyi* than homologous (legitimate) recombination (De Vries & Wackernagel, 2002). Illegitimate recombination may be assisted by one-sided neighbouring homology with as few as 183 nucleotides to increase the frequency of successful events by 10^5-fold (Figure 3; de Vries and Wackernagel, 2002).

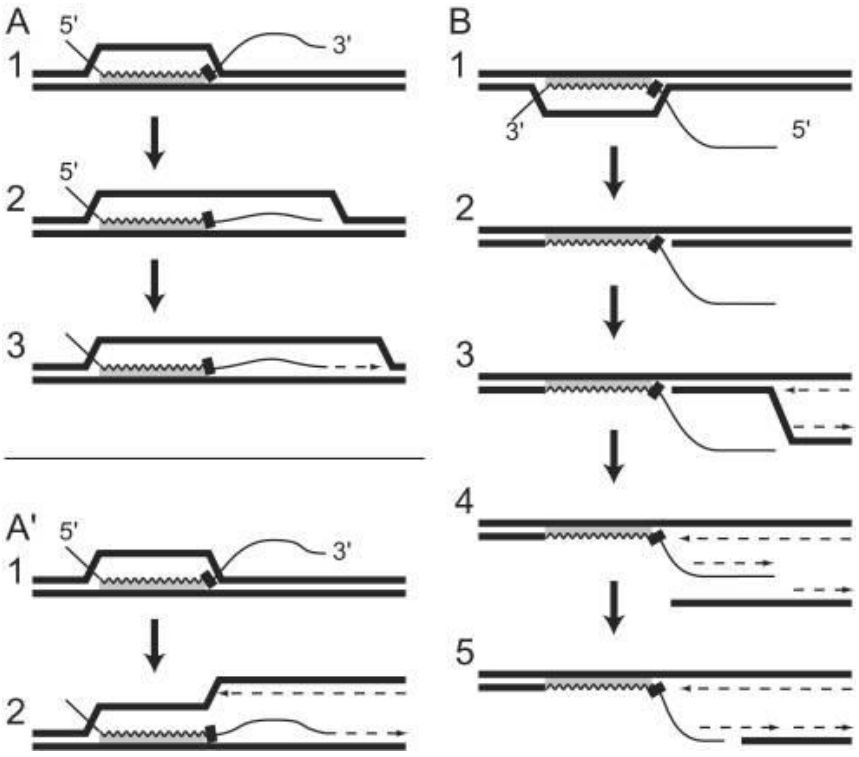

Figure 3. Illustrative diagrams of homology-facilitated recombination of homologous DNA with heterologous DNA at the 3' (A, A') and 5' (B) end. DNA homology is indicated with shading. Black bold lines indicate the recipient chromosome, homologous DNA

is indicated by waved lines, and newly synthesised DNA is indicated by dashed lines. Figure taken from de Vries and Wackernagel, 2002.

As previously mentioned, the fate of the incoming DNA molecule does not have to be chromosomal recombination. Plasmids can also be received via transformation (Winter et al., 2021; Yu et al., 2022), albeit less efficiently than chromosomal DNA (S. Knapp et al., 2017). The lack of efficiency is likely due to the need for two complimentary strands of the plasmid to enter separately (because only one single strand is taken into the cell per transformation event), making the uptake efficiency of complete plasmids inversely proportional to the square of the plasmid size (Sikorski et al., 1998). However, it has emerged more recently that there are species which can bypass this limitation by taking up plasmids via natural transformation as double stranded DNA elements such as *Pseudomonas aeruginosa* (Nolan et al., 2020) and *Escherichia coli* (D. Sun et al., 2006, 2009). However, the mechanisms have not yet been fully elucidated and may not be a genuine mechanism of natural transformation as they do not require type IV pili or pilQ secretin pores, indicating that the DNA passively enters and is not actively taken up by the cell.

1.2.3 Natural Competence: Inducing Stimuli and Regulatory Factors

There are a wide range of stimuli and environmental factors which induce competence of natural transformation. For this reason, the only fundamental method for determining natural competence and transformation in bacterial species is to supply cells with (typically homologous) cell-free marker DNA. If the cells take up and recombine with the DNA, they can be identified using the marker on the recombined DNA sequence which typically confers antimicrobial resistance or fluorescence (Ray & Nielsen, 2005). Some species will transform at all times, provided there is DNA available, while other species will only transform when certain conditions are met. For example, *Neisseria gonorrhoea* and *Neisseria meningitidis, Helicobacter pylori* and *Acinetobacter baylyi* are constitutively naturally competent (Blokesch, 2016; Elliott & Neidle, 2011; Moore et al., 2014; Seitz & Blokesch, 2013). Around 10-15% of a population of *Bacillus subtilis* 168 is typically competent (Berka et al., 2002; Johnsen et al., 2009), and the percentage of transforming *B. subtilis* cells in the population increases when the population reaches stationary phase due to increased production of ComK and its increased binding to its own promoter (Berka et al., 2002; Claverys

et al., 2006). Expression of over 100 genes occurs following the upregulated transcription of *comK*, making it the most significant regulator of competence in *B. subtilis* (Berka et al., 2002). Nutrient starvation is a known inducer of competence in *Haemophilus influenzae* (Maughan & Redfield, 2009) and *H. pylori* (Redfield, 1991). Competence in *Vibrio cholerae* is induced when high cell density is reached or when exposed to chitin which can be found on zooplankton or crab shells (Blokesch, 2012; Meibom et al., 2005; Y. Sun et al., 2013). *Streptococcus pneumoniae* uses cell-cell signalling to decide when to induce competence in response to environmental conditions (Johnston et al., 2014). *S. pneumoniae* (Engelmoer & Rozen, 2011) and *H. pylori* (Moore et al., 2014) induce competence in response to stress such as DNA damage or antibiotic challenge. *S. pneumoniae* and *Legionella pneumophila* induce competence as a response to genetic damage (Johnston et al., 2014). *H. pylori* is constitutively competent with upregulated transformation frequency in response to DNA damage, low pH, and low CO_2 conditions (Moore et al., 2014). These conditions are most like the space around gastric epithelial cells (Bury-Moné et al., 2008), suggesting that competence for transformation is upregulated in these hostile high-pH conditions as a stress response. This effect is also seen when colonising human stomachs, where *H. pylori* will also secrete proteins to encourage their kin to secrete DNA for recombination (Dorer et al., 2010). *Acinetobacter baumannii* engages in natural transformation as it moves across wet surfaces (Godeux et al., 2018; Wilharm et al., 2013). Recently, it has been discovered that pilus production (used for twitching motility and DNA uptake) is growth phase dependent (Vesel & Blokesch, 2021). The variety of stimuli which can induce competence in transformable bacteria appear to suit the niches they inhabit such as with chitin exposure for *V. cholerae*, and low pH conditions for *H. pylori*. The regulation of competence dictated by a species' environment suggests that transformation serves a specific function which is beneficial to the cell in certain contexts. However, exceptions such as the constitutively competent *A. baylyi* suggest that transformation is continuously serving a purpose for the cell.

Mobile genetic elements (MGEs) such as integrons, transposons, and viruses can affect transformation frequency in bacteria. One example is the bacteriophage CRAΦ. CRAΦ has been found to utilise the competence machinery *of A. baylyi* and compromise the host's ability to transform (Renda et al., 2016). Loss of competence

genes in *A. baylyi* confers complete resistance to this phage (Renda et al., 2016). MGEs can reduce the likelihood of transformable bacteria curing themselves of these MGEs by inducing greater secretion of DNAses, thereby reducing the concentration of extracellular DNA available for transformation (Croucher et al., 2016). MGEs can also insert themselves into parts of the genome which code for transformation machinery, thereby drastically reducing the efficiency at which transformation can be used to cure the cell of the inserted MGE (Croucher et al., 2016).

1.3 Why Does Transformation Occur?

It is likely that the ability to naturally transform comes with a fitness cost as it requires energy to maintain, utilise and produce the associated proteins after every cell division (Bacher et al., 2006), but is actively performed by a cell for motives separate to the selfish strategies by MGEs such as plasmids, transposons, and indirectly, bacteriophages (Von Wintersdorff et al., 2016). Indeed, transformation occurs in instances which are disadvantageous for the recipient cell. *A. baylyi* will transform with secreted prey DNA following successful lysis of competitors using type 6 secretion systems (T6SS), arresting its division, and lowering fitness in a period where rapid growth is vital for success in interspecies competition (L. Lin et al., 2019). The same study by Lin *et al*. found that non-competent *A. baylyi* outcompeted its competent counterpart, but only while lysis of prey via T6SS was occurring. The ability to transform has observed to be reduced in several experimental evolution studies (Bacher et al., 2006; Renda et al., 2015; Utnes et al., 2015). What benefit of transformation offsets the cost of maintaining the required machinery to prevent it being lost over evolutionary time in the natural world?

Natural transformation has been argued to provide three distinct potential benefits to cells which are not mutually exclusive, yet the benefits of transformation remain controversial (Ambur et al., 2016; Redfield, 2001; Vos, 2009). The first benefit could be that this incoming DNA is used as a source of energy or building blocks for DNA replication (Redfield, 1993; Redfield, 2001). Secondly, DNA fragments could be used as a template for repairing genetic damage (Seitz & Blokesch, 2013). Lastly, it is suggested that incoming DNA is a source of genetic novelty for the transforming

population, sometimes termed bacterial sex (Ambur et al., 2016; Blokesch, 2016; Vos, 2009).

1.3.1 Natural Transformation as a Mechanism for Food Acquisition

Redfield discusses that since *H. influenzae* has upregulated transformation frequency when in minimal medium, but not when it suffers DNA damage (which the second hypothesis argues against below), this DNA must be used for food (Redfield, 1991; Redfield, 1993). A study using the constitutively competent species *Acinetobacter baylyi* found that fitness (growth rate) of the species increases when in the presence of exogenous DNA (Hülter et al., 2017). This effect is a clear short-term benefit of transformation and acts independently of incoming DNA homology. In addition, some species such as *Bacillus subtilis,* and *Helicobacter pylori* induce competence during periods of nutrient deprivation (Herriott et al., 1970; Maughan & Redfield, 2009; Redfield, 1993b; Sinha et al., 2013). These findings support the hypothesis that at least one strand of the received dsDNA is degraded into single nucleotides and used for food. However, there is still no clear demonstration of pathways that lead to utilisation of received nucleotides in DNA synthesis.

Both *Neisseria* and *Helicobacter sp.* have DNA uptake sequences (DUS) on their pili which are complementary to motifs in their chromosomal DNA and therefore bias uptake of DNA to more homologous sequences (Mathis & Scocca, 1982). These phenomena seem contradictory; why are these species selective over the incoming DNA sequence homology if the intention is not to use it for recombination? The presence of a DUS throws doubt over the “transformation for food” hypothesis being the main function of transformation as the nucleotide sequence of the incoming DNA does not matter if its fate is to be broken down anyway. Further, the remainder of the recombination machinery needed for transformation-facilitated recombination with extracellular DNA would be redundant if transformation was used solely for food and would therefore disappear over evolutionary time.

However, even a DNA sequence used for recombination leads to an equal number of host nucleotides being displaced from the recipient’s genome and made available as food (Redfield, 1993). This argument does not suggest that the food hypothesis is the

primary function of transformation, but it could serve a convenient secondary benefit of natural transformation.

1.3.2 Natural Transformation as a Mechanism for DNA Repair

There are multiple factors which can induce DNA damage in a cell such as UV radiation, antibiotic challenge, and DNA replication (Charpentier et al., 2011). DNA damage can be lethal to bacterial cells, so avoiding a fatal amount of damage can be very beneficial. If a cell is able to replace damaged DNA using extracellular DNA, it will outcompete its competitors in hostile environments with genotoxic agents present. The SOS stress response in bacteria is activated in response to DNA damage and is induced by agents such as antibiotics (Charpentier et al., 2012; Quinn et al., 2018). This is consistent with the hypothesis suggesting that a function of transformation is for DNA repair using the incoming DNA and supported by the recruitment of the DNA repair pathway, including recombinational repair, as part of the SOS stress response (Michel, 2005). Another piece of evidence for this hypothesis is the observation that competence mitigates the growth inhibitory effect of the DNA-damaging agent mitomycin C in *S. pneumoniae*, suggesting that transformation is used to repair lesions on the affected cell's chromosome (Engelmoer & Rozen, 2011). Further, presence of the water disinfection product bromoacetic acid increases transcription of *recA* and *uvrB*, genes used in both DNA repair and transformation (Mantilla-Calderon et al., 2019).

DNA used for recombination needs homology to the host genome for maximum efficiency (Alexander et al., 1956; Majewski et al., 2000; Majewski & Cohan, 1999; Simpson et al., 2007). Recombination therefore preferentially occurs with DNA from the same or similar species to the transforming cell, where incoming DNA fragments are often near to identical to that of the transforming cell. Additionally, bacteria growing in solid phase, or a spatially structured environment will be surrounded by their own kin (Ambur et al., 2016), and so there will be a wealth of homologous DNA surrounding transformable cells, sometimes released and utilised after fratricide (Claverys & Håvarstein, 2007; Guiral et al., 2005).

Bacillus subtilis has been shown to increase rates of transformation following UV-induced damage (Michod et al., 1988). However, one consideration is that in a natural

environment, if a cell is suffering from DNA damage caused by UV radiation, that the extracellular DNA surrounding the cell will also be damaged. *B. subtilis* has been observed to increase in cell density by transforming with UV-damaged DNA, indicating that this DNA mitigates cell death through use of the DNA for repair and not all donor DNA is damaged as a result of UV damage (Hoelzer & Michod, 1991). In contrast to this observation, *Streptococcus thermophilus* engages its SOS stress response and concurrently downregulates transformation frequency in response to exposure to the DNA damaging agents mitomycin C and fluoroquinolones (Boutry et al., 2013). Additionally, the notion that incoming DNA is used for repair would imply mitigation of genotoxic damage. The elimination of competence in *H. pylori* and *L. pneumophila* saw no increased sensitivity to genotoxic stress (Charpentier et al., 2011; Dorer et al., 2010), and also for *H. influenzae* and *B. subtilis* (Redfield, 1993). As stated earlier, species such as *N. gonorrhoeae* and *H. influenzae* are discriminatory to incoming DNA since they have regions on the end of their pili which are complementary to motifs which are overrepresented in their genome (Seitz & Blokesch, 2013). This generates a strong bias for DNA compatible for homologous recombination, but only this small minority of species have been identified to have such mechanisms. This suggests that most other species don't require homology from incoming fragments and are accepting of less- or non-homologous DNA which would not be as suitable for use as templates for DNA repair. Further, *H influenzae* does not require site-specific sequence homology at the damaged site to use DNA for repair (Mongold, 1992). Instead, it is proposed that the establishment of a D-loop as part of the recombination process allows for replication of DNA for cell division, bypassing the limitation of having a damaged strand on the chromosome (Claverys et al., 2006). Further evidence against this hypothesis for the repair function are species such as *N. gonorrhoea, N. meningitidis,* and *A. baylyi* which are constitutively competent (Blokesch, 2016), and therefore do not require induction of a DNA damage repair pathway to initiate transformation.

1.3.3 Natural Transformation as a Mechanism of Sex

As much as 40% of ssDNA internalised by natural transformation is used for recombination (Kidane et al., 2012). This Figure is far too high to suggest that recombination is a by-product while transformation is primarily used for food. Additionally, the existence of proteins which protect ssDNA from degradation after it

has been taken into a cell via transformation suggests an intended purpose for the incoming ssDNA strand separate from food (Johnston et al., 2014). If transformable bacteria can acquire whole genes instead of relying on the stepwise accumulation of mutations, they may adapt faster than their competitors. Transformation-mediated recombination is hypothesised to increase genetic variation in a population which would allow for a better response to natural selection through faster adaptation to environmental conditions (Ambur et al., 2016; Hülter et al., 2017; Levin & Cornejo, 2009; Otto & Lenormand, 2002; Vos, 2009; Vos et al., 2015). An example of when natural transformation may be used to speed up adaptation to hostile environments is when *H. pylori* senses a change in pH and CO_2 in an environment such as a human stomach (Moore et al., 2014). *H. pylori* will also secrete proteins to encourage their kin to secrete DNA for recombination, presumably to increase genetic variation and encourage the dissemination of antibiotic resistance and virulence genes (Dorer et al., 2010). Natural transformation can lead to beneficial, neutral, or deleterious genetic change to the recipient (Baltrus, 2013; Hülter et al., 2017; Vos et al., 2015). Transformation with incoming DNA sequences can result in negative fitness effects by compromising the transcription of existing genes such as by inserting within chromosomal loci or completely replacing genes (Croucher et al., 2016), though insertion into a non-functional or non-essential gene will not have a significantly deleterious effect. Neutral and deleterious transformation events are hypothesised to be more frequent than beneficial transformation events due to the typically random nature of DNA received by the cell for recombination (Vos et al., 2015). However, other studies suggest that transformation events are typically neutral and act as a foundation for novel mutations (Knöppel et al., 2014). Recombination has been shown to facilitate rapid acquisition of genes which allow for the utilisation of different carbon sources or resistance to antimicrobials (Chu et al., 2017; Von Wintersdorff et al., 2016; Winter et al., 2021), thereby indicating that the benefits of transformation can act quickly on a cell to confer a fitness advantage. One recent hypothesis suggests that recombination with incoming DNA also has the function of curing the host genome of parasitic genetic elements, providing fitness increases by removal of genes rather than addition (Croucher et al., 2016). However, there is a strong bias to detect changes with a positive effect on fitness, simply because any changes with a negative effect on fitness might disappear before they can be observed.

Imagine that two cells in a population each acquire a beneficial mutation in two separate genes: one cell has the genotype Ab, while the second cell has the genotype aB. Without recombination, they would be unable to swap these genes between them to rapidly generate a fitter phenotype. Instead, they will have to each wait for a second reciprocal mutation to arise in at least one of the two cells before the fittest phenotype can be acquired. This phenomenon is the Hill-Robertson effect and demonstrates how sexual populations can evolve faster than asexual populations (Hill and Robertson, 1966; Otto, 2009; Figure 4). Further, if these two cells also carry deleterious genes, they will be unable to recombine to remove them, and will continue to associate their beneficial genes with the deleterious ones until a mutation alters one of the genes. The Fisher-Muller model suggests that sex can allow genes which have a positive effect on fitness to join across the "fitness landscape" via recombination instead of different alleles competing in individual clones, in a phenomenon termed clonal interference, potentially leading to the eradication of the less-fit genotype from the gene pool (T. F. Cooper, 2007; Kim & Orr, 2005; Vos, 2009). This model has been supported mathematically, suggesting that a recombining population can adapt faster than a non-recombining population and the ability to recombine is selected for, ensuring its maintenance in a population (Levin & Cornejo, 2009). Experimental support for this model has found that recombining strains are more adaptable to their environment than their less- or non-recombining experimental controls (T. F. Cooper, 2007; Nguyen et al., 2022). These studies test the adaptive benefits of sex by evolving a wildtype strain capable of recombination and a non-recombining mutant separately and later directly compete the strains to determine the fitness gains acquired by each strain.

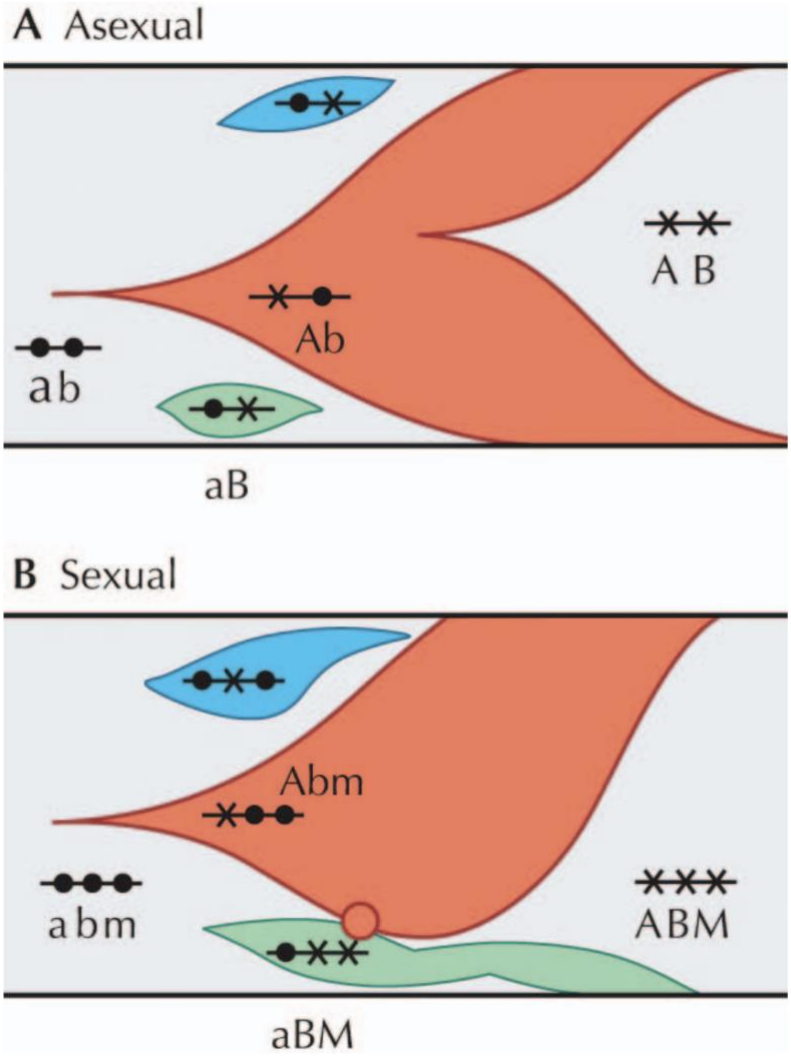

Figure 4. Illustration of the stepwise process which favourable mutations must be acquired in asexual populations (A). To increase in fitness to phenotype “AB”, alleles “a” and “b” must be mutated sequentially and clones with beneficial mutations will compete for fixation until an AB phenotype is reached in one clone and outcompetes the other phenotypes. The ability to acquire genes through sexual recombination (B) can facilitate faster adaptation by combining favourable mutations across cells (red circle). The ability to recombine “M” can also be selected for by hitchhiking on the fitness benefits recombination provides through adaption, also termed second-order selection. Figure from Barton, 2009.

Muller’s ratchet is a process whereby mildly deleterious mutations build up in the within a population due to selection not acting effectively because of small population sizes (Haigh, 1978). Examples of effected populations are ones subjected to strong genetic

bottlenecking, such as during host infection. Over time, the accumulation of deleterious mutations leads to irreversible fitness decreases in the population. The ability of a population to exchange chromosomal genetic information via natural transformation allows for genetic curing of these deleterious mutations and helps to preserve more beneficial alleles or mutations. Alleles which promote recombination or mutation may increase the probability that beneficial gene mutations will fixate under selective pressures (Colegrave, 2002; T. F. Cooper, 2007; Tenaillon et al., 2001). During this process, alleles promoting recombination would be selected for in a phenomenon termed second-order selection (Otto & Michalakis, 1998; Tenaillon et al., 2001). Bacteria typically exist in multispecies communities which are coevolving and reciprocally imposing greater selection pressures on their competitors upon acquisition of genes which confer fitness increases. The frequent changes in selection pressure caused by competitor coevolution provides a need for rapid adaptation to reduce the chances of out-competition by competitors with a fitness advantage. Thus, natural selection doesn't just select for those which evolve, it selects for those who evolve faster and more efficiently than their competitors through second-order selection (Buckling et al., 2009).

Interspecies predation via type VI secretion systems (T6SS) (R. M. Cooper et al., 2017; L. Lin et al., 2019), or phage lysis (Molina-Quiroz et al., 2020) may be a way to produce a food source, but it is also followed by transformation in some species. This is consistent with the hypothesis that the purpose of transformation is to acquire new genes and functions from prey bacterial cells in the surrounding environment. However, this is not always the case as some species may use T6SS as a defence to prevent conjugation-based transfer of MGEs due to the proximity between cells required for conjugation events (Dimitriu, 2022). Similarly, DNA is released into biofilms to increase their adhesive properties (Spoering & Gilmore, 2006; Whitchurch et al., 2002). Neighbouring transformable cells may use this resource to obtain an advantage by acquiring new genes from kin or nearby competitors which can provide a fitness increase.

One study found that exclusively heterologous DNA sequences can be used for transformation-based recombination in *S. pneumoniae* (Johnston, Polard, et al., 2013), and as they serve no function for genome maintenance and repair, the function

of this recombination may be to increase population-level genetic diversity. Heterologous DNA which enters a cell during transformation may not be methylated in the same manner as the host genome and is therefore sensitive to digestion by restriction modification system (R-M system) enzymes. To avoid digestion of incoming DNA, transforming species use DpnA which methylates ssDNA to protect it from the R-M system (Johnston, Martin, et al., 2013). If the function of DNA taken up via natural transformation was to be used as single nucleotides as food, DpnA would be redundant and would disappear from genomes over time due to negative selection. Additionally, the protection of non-homologous DNA must rule out the purpose of recombination for repair (Johnston, Martin, et al., 2013). Therefore, recombination with transformed DNA must provide a significant benefit to transforming species, suggesting its use for acquiring novel genes or curing the recipient genome of deleterious genes.

1.3.4 Species-Dependent Functions of Natural Transformation

As the mechanisms and inducing stimuli for natural transformation are so greatly varied across species, it is possible that all three hypotheses are correct and apply to most transformable species concurrently or that different combinations of these hypotheses apply to different species. Consequently, the relative importance of these functions will change depending on the species. The leading pieces of evidence suggesting that incoming DNA is used for food are that competence is induced during low nutrient conditions (Redfield, 1991, 1993b), and that fitness increases when DNA is available to transforming strains (Utnes et al., 2015). Additionally, the protected strand of incoming DNA displaces host DNA upon recombination which then gets degraded and potentially used as a nutrient source (Redfield, 1993b). Therefore, one could also suggest that the primary function of transformation in the case of nutrient deprivation is to attempt to acquire useful genes for fitness gains, while also benefitting secondarily from the nutrient source that is the ejected recipient DNA which is displaced by the donor DNA strand. *A. baylyi* clearly increases growth rate when DNA is present, supporting the food hypothesis as a function of transformation, but also transforms with heterologous DNA more frequently in response to UV damage causing lesions on DNA which can help start the recombination process (Hülter et al., 2017). *H. pylori* might predominately use transformation for recombination as it uses a more sophisticated mechanism for recombination compared to other species where it can

further segregate incoming DNA, sometimes separating beneficial and deleterious alleles for much faster fitness acquisition (Nguyen et al., 2022; Woods et al., 2020). *B. subtilis* may predominantly use transformation for DNA repair and benefit from the other two hypotheses secondarily (Hoelzer & Michod, 1991; Michod et al., 1988).

The species-specific differences in the perceived function of transformation may explain how transformation has evolved. DNA repair mechanisms likely existed before natural transformation and such mechanisms may be the evolutionary roots for the sex function of natural transformation. This is because many of the enzymes and mechanistic pathways are shared, differing mainly in the contexts under which these pathways are induced and regulated. Evolution of the ability to take up DNA with pre-existing pili on cell surfaces probably evolved for food, and then later adapted to using this DNA for repair using machinery already present. As previously discussed, the enzyme DpnA functions by changing the methylation structure of internalised foreign DNA to a similar structure to recipient DNA, increasing the chances it will avoid degradation by recipient restriction endonucleases (Johnston, Martin, et al., 2013; Johnston, Polard, et al., 2013). It seems likely that DpnA evolved as one of the final additions to this system to facilitate intake of heterologous DNA and the ability to recombine with foreign DNA. This is one of the strongest pieces of evidence for the use of transformation for sex as heterologous DNA is not useful for repair and DpnA protects internalised DNA from degradation which is not necessary for a cell which uses transformed DNA for food. If transformation was not used for sex, DpnA would be lost over evolutionary time as it would confer no benefit to the cells and would not be selected for. The maintenance of DpnA in bacterial genomes therefore suggests the enzyme confers a benefit which is selected for, supporting the sex hypothesis for transformation (provided it serves no other function to the cell).

The lines may always be blurred for the benefits of transformation as its induction is context dependent for most species. Indeed, transformation frequency (and the ability to transform itself) is widely varied within subgroups of the same genus or species such as *Pseudomonas stutzeri spp.* (Sikorski et al., 2002), *Haemophilus influenzae* spp. (Maughan & Redfield, 2009), and *Acinetobacter sp.* (Domingues et al., 2019; Hülter et al., 2017; Palmen et al., 1993; Quinn et al., 2019; Vesel & Blokesch, 2021), suggesting varied dependencies on transformation between closely related bacteria.

Additionally, there may be other functions of competence and transformation which we do not yet understand and are separate to the existing three hypotheses which are transformation for food, DNA repair, and sex. Treatment of *S. pneumoniae* populations with sub-inhibitive concentrations of the ribosome-targeting drug streptomycin observed a higher growth rate of competent cells than their non-competent counterparts (Engelmoer & Rozen, 2011). This finding does not support any of the previously described hypotheses as this effect is seen in the absence of DNA supplementation and is hence an effect of competence, not transformation.

Selection for the machinery required for competency and natural transformation may not always be selected for exclusively for the purpose of transformation; type IV pili in *Acinetobacter baylyi* are used for binding to extracellular dsDNA, but also twitching motility of the bacterium (Bakkali, 2013; Leong et al., 2017). This indicates that transformation ability can be indirectly selected for when components of transformation machinery confer a fitness benefit to the cell via functions which are not used during natural transformation. Transformation ability can therefore be maintained due to functions independent of natural transformation in bacteria, which can make strong evidence for the fitness and adaptive benefits of natural transformation harder to obtain. There is therefore no one answer defining the function of bacterial natural transformation, but future studies exploring the contexts under which transformation is performed and the functions it serves a transforming cell help to improve our understanding of bacterial evolution and the contribution transformation makes on both evolution and the dissemination of antimicrobial resistance.

1.4 Antimicrobial Resistance

In 1913, Paul Ehrlich presented his discovery of salvarsan, the first antibiotic, which was effective for treating syphilis infections (Gelpi et al., 2015). However, Ehrlich's achievements were overshadowed by Alexander Fleming's discovery of penicillin in 1928 (Fleming, 1929), and its subsequent purification by Abraham et al. (1941). Penicillin was quickly mass produced and distributed to soldiers in World War II, reducing mortalities associated with wound infections, and remained in high use after the war for the treatment of venereal diseases across Europe (Kardos & Demain, 2011). Shortly after its discovery, penicillin's effectiveness was observed to be

compromised as it was demonstrated to have no effect on a lab strain of *Escherichia coli* (Abraham & Chain, 1940), and four *Staphylococcus aureus* infections in hospital (Rammelkamp & Maxon, 1942). By 1970, over 80% of *S. aureus* infections in the USA were unresponsive to penicillin treatment (Lowy, 2003).

Antimicrobial resistance (AMR) is a phenomenon where microorganisms are less sensitive to the drugs used to prevent their growth or kill them. This has implications on healthcare as our reduced ability to cure infections with antimicrobials will lead to increased infection-based mortality. Antimicrobials are drugs which can be used to treat infections caused by viruses, fungi, and bacteria. In this thesis, the term antimicrobial will be used interchangeably with antibacterial, pertaining to bacteria, as fungi and viruses are not the focus of this study. Antimicrobial resistance genes (ARGs) are genes used by bacteria to confer resistance to antimicrobials. ARGs can be specific to a certain class of antimicrobial such as ß-lactamase encoding enzymes, which are used to degrade the ß-lactam ring in drugs such as penicillin (Babic, Hujer and Bonomo, 2006; Figure 4). An example of a more generalised resistance mechanism is efflux pumps such as ABC transporters which pump drugs back outside of a cell, concurrently reducing the ability of multiple classes of antibiotics to bind to their targets (Marquez, 2005; Figure 5).

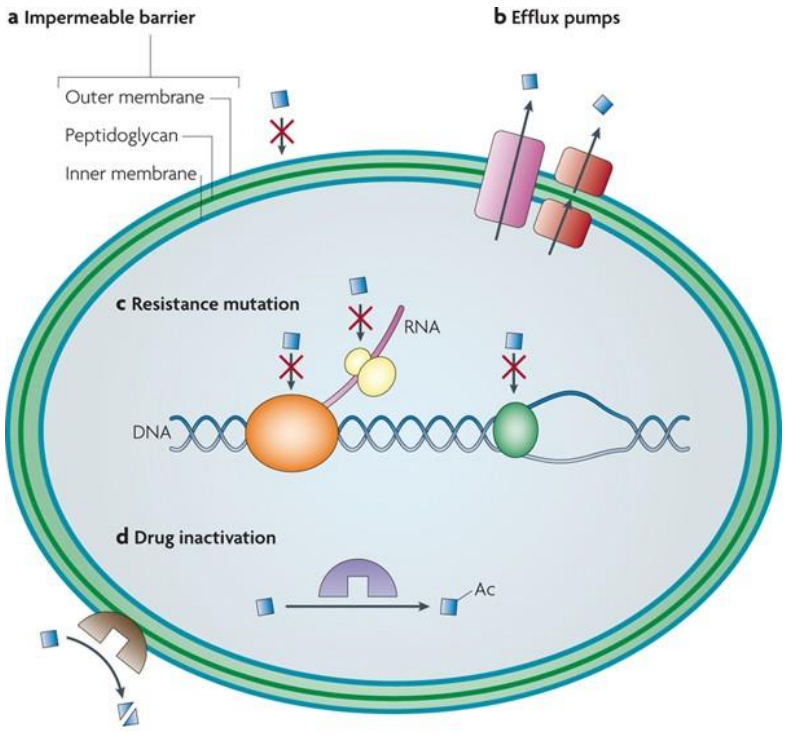

Figure 5. Illustration of the general mechanisms which can lead to increased resistance to antibiotics: a) presence of a cell membrane which cannot be penetrated by some antibiotics; b) efflux pumps which actively pump the antibiotic out of the cell; c) mutations which can lead to alterations in the binding sites of drugs which target components of the protein synthesis pathway; d) production of enzymes which catalyse the inactivation of antibiotics before they affect the cell. Figure taken from Allen et al., 2010.

Antimicrobial resistance genes can be acquired vertically (through cell division) or horizontally (not involving cell division). A key benefit horizontal gene transfer (HGT) has over its vertical counterpart is that novel genes (and therefore functions such as AMR) can be acquired at any stage of a bacterial cell's lifecycle. This makes the recipient genome more plastic and adaptable, allowing it to be modified in a context-dependent manner. HGT via conjugation is the most recognised mechanism contributing for large scale inter-species AMR dissemination as it relies on plasmids which exist outside of the chromosome and can replicate quickly, allowing the recipient to become a donor to other cells (Jain & Srivastava, 2013). This is especially relevant in the case of antibiotic challenge. When a bacterial population is challenged with an antibiotic at a sufficiently high concentration, sensitive bacteria will die or have their growth inhibited, while ones harbouring resistance gene(s) will survive or grow faster than inhibited kin or competitors. Therefore, prevalence of ARGs in a population can be increased in this context as a function of passive spread of a plasmid (or chromosomal element transferred by other HGT mechanisms), or because of selection via antibiotic challenge.

The rate at which the proportion of ARG-harbouring bacteria in a monoclonal population increases is correlated with the concentration of antibiotic used: the higher the concentration of antibiotic used, the more non-resistant bacteria are killed or unable to grow, allowing for a much faster rate of out-competition by the antibiotic-resistant bacteria (Sandegren, 2014). In multispecies communities, resistant strains may secrete extracellular degradation enzymes which can cross protect sensitive strains (A. K. Murray et al., 2018). However, net inhibition of population growth (through arresting cell division or killing a fraction of the population) isn't necessary for selection of AMR. Previously, the minimum inhibitory concentration (MIC), the

concentration at which a drug completely inhibits a population's growth (Kowalska-Krochmal & Dudek-Wicher, 2021), was considered the lowest concentration of an antibiotic needed to select for AMR. However, we now know that the concentration for AMR selection exists lower (by 100-fold in some cases) at the minimum selective concentration (MSC) (Gullberg et al., 2011). Selection for AMR occurs at concentrations at or above the MSC because of slight fitness differences between susceptible and resistant bacteria which are observed during antibiotic challenge. This reframes perspective on the thresholds considered tolerable for environmental concentrations of antibiotics and antibiotic-like compounds as they may be exposing bacteria to concentrations which are greater than the MSC. Relevant environmental domains include sewage (Schuster et al., 2022), agricultural soils (Xie et al., 2018), and freshwater systems (Davis et al., 2006; Sharma et al., 2021). Bacterial exposure to pharmaceutical products is occurring worldwide as it is present in sewage and surface water environments across the globe (Graumnitz & Jungmann, 2021), but our understanding of which pharmaceuticals select for AMR is still very limited.

The rate at which we see multidrug resistant pathogens is increasing, and the rate at which we discover novel antibiotics to treat these pathogens is insufficient (da Cunha et al., 2019). We should invest more money and time into discovering novel antimicrobials while also increasing our understanding of how AMR is acquired and disseminated to prevent its projected cataclysmic impact on modern medicine.

1.4.1 The Antimicrobial Resistance Crisis

Antimicrobial resistant pathogens are widespread across the world and infections caused by these pathogens were responsible for an estimated 4.95 million deaths worldwide in 2019 (C. J. Murray et al., 2022). Bacterial sepsis is the leading cause of death globally, accounting for 1 in 5 deaths (World Health Organisation, 2020). As 11 million of the 49 million most recent cases worldwide were fatal (World Health Organisation, 2020), there is clear potential for the number of deaths to increase uncontrollably if antibiotic resistant infections cannot be treated. Regression to an era where antibiotics no longer have their desired effect in treating infections is an intimidating prospect as around 30% of deaths in the USA before the availability of antimicrobials were due to infections (Fair & Tor, 2014). This problem is not likely to be solved quickly because ARGs are abundant in natural environments because of

high and prolonged use of antimicrobials since their discovery. Abundance of ARGs in soil has been increasing since the 1940s (C. W. Knapp et al., 2010), clearly demonstrating a rich reservoir where ARGs can persist and spread to other bacteria via HGT. Antibiotic use on livestock as growth promoters and prophylaxis against potential infections is prevalent across the world (Chand, 2021). Non-essential use of antimicrobials on livestock can lead to the selection of AMR bacteria in the livestock (Chand, 2021) or in farm soil, further selecting for AMR in the natural environment (Davis et al., 2006; Xie et al., 2018). Pesticides can also co-select for AMR (Alderton et al., 2021), making their use in agriculture problematic. Both overuse of and insufficient access to antibiotics are significant health problems (Mendelson et al., 2016). Poverty is a driver of antimicrobial resistance as it leads to underfunded national healthcare systems, inadequate access to effective drugs, poorly regulated dispensing of antimicrobial drugs, and sharing of antimicrobial courses between patients and friends (Planta, 2007). Between 0.6 and 1.51 billion people are estimated to be in poverty worldwide (Aguilar & Summer, 2020).

Antibiotics are prescribed needlessly to patients in some cases. A strong example is in the USA, where up to 50% of antimicrobials were prescribed without necessity in recent history (CDC, 2013). Antimicrobial stewardship refers to reserving use of antimicrobials for cases when they are likely to have their desired effect on a pathogen (Fishman, 2006). This preserves their effectiveness by limiting the number of instances where antibiotics are administered thereby limiting selection pressures for resistance. General surveillance and “rational” use of antimicrobial agents over a nine-year period in Sweden found a general reduction in antimicrobial-resistant pathogens (Mölstad et al., 2008). Targeted use of stewardship on a per-patient basis also appears to work. A US study found that rapid polymerase chain reaction (rPCR) testing on putatively methicillin-resistant *Staphylococcus aureus* infections in patients allowed clinicians to determine if infections were sensitive to methicillin or needed other treatment, saving an average of $21,387 per patient, and shortened hospital stays by an average of 6.2 days (Bauer et al., 2010). Indeed, its effect is appreciated and acted upon as at least 48% of US hospitals have adopted stewardship programmes to some degree (Fair & Tor, 2014). However, resistances to some antibiotics such as trimethoprim confer a negligible fitness cost, so stewardship does little to diminish prevalence of the respective ARG (Sundqvist et al., 2009). Importantly, it must be

recognised that effectiveness of stewardship or any strict medical policies is significantly reduced if it is not uniformly adhered to, as novel resistances in non-adhering areas will simply spread to areas that do adhere (Dickmann et al., 2017).

Another way to address the antibiotic crisis is to invest in research and development of new antibiotics that can effectively treat infections caused by resistant bacteria. In the case of bacterial infection, the concurrent use of multiple antibiotics isn't always applied but is often shown to be effective in suppressing and even reversing AMR in pathogens (Ocampo et al., 2014), particularly when using at least one bacteriostatic and one bactericidal drug such as tetracycline and ampicillin respectively. However, it appears that achieving synergy between antibiotics can be difficult, as using antibacterials with different mechanisms of action may have an antagonistic effect (Beppler et al., 2017). Beppler *et al.* (2017) found that therapy using three antimicrobial agents instead of two is more likely to have a suppressive effect and therefore less effective in treating the disease. However, there are arguments to suggest that this may slow the rate of antimicrobial resistance as the less intense cytotoxic effect of the therapy lowers the pressure for positive selection of a resistance gene in the microbial population (Hegreness et al., 2008). Therefore, it may be better to reduce the bacterial population to a size that will alleviate the symptoms while being populous enough for detection by the host's immune system to eradicate the remaining pathogens. Pathogens expressing New Delhi metallo-ß-lactamase (NDM-1) have been difficult to treat since there are no known ß-lactamase inhibitors which are effective against them (Gonzales et al., 2015). This is typically considered an inoperable pathogen, but use of peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) can selectively silence resistance genes within the pathogen to restore efficacy of the specific medication used (Sully et al., 2017). However, a pathogen with effective efflux pumps may confer resistance to PPMO treatment strategies due to active removal of the compounds from the cell. In this instance, efflux pump inhibitors (EPI) could be used as they disrupt the expulsion of antibiotics from the bacterial cell (Pagès & Amaral, 2009).

Combatting AMR requires global cooperation involving some unrealistic expectations from less developed countries which may not be able to afford different treatment regimens or a change of policy. An alternative is to predict where and when

resistances may develop and spread in bacteria. In order to achieve that, we need to delve into the contexts and frequencies surrounding bacterial acquisition of ARGs through HGT, including via natural transformation.

1.4.2 Transformation-Based Dissemination of Antimicrobial Resistance

Natural transformation is a mechanism of HGT which is likely underappreciated for its role in disseminating antimicrobial resistance (Winter et al., 2021). The ability to transform has been closely correlated with a species' notoriety as a multidrug resistant pathogen (Blokesch, 2017). In 2017, the WHO published a list of priority multidrug resistant pathogens researchers need to find new medicines to treat, and all listed pathogens have at least some conserved core genes for transformation if they haven't yet been experimentally proven to be able to transform (Blokesch, 2017; Nolan et al., 2020). However, this supposed correlation between multidrug resistance and natural transformation may be biased as these high priority pathogens are researched more than other less-relevant species.

Antibiotic challenge itself can be a stimulus for inducing competence in bacteria (Charpentier et al., 2012; Von Wintersdorff et al., 2016). Because of this, microbiota in human guts are key sites for AMR dissemination as they are directly challenged by antibiotics during some disease treatment therapies due to ingestion of antibiotic tablets (Hobson et al., 2020). The ability to naturally transform with ingested ARGs can accelerate when conditions become selective for AMR in the gut. A study by Ding et al., (2020) showed that transformation-mediated uptake confers greater dissemination of plasmid-borne ARGs than conjugation in murine models.

Table 2. Examples of inter-species ARG transfer in human pathogens mediated by natural transformation. Adapted from Winter et al. (2021).

Recipient species	Donor Species	ARGs	Antibiotics	Description	Reference
A. baumannii	*K. pneumoniae* CRKp, *Providencia rettgeri* M15758 (*bla*$_{NDM-1}$) and methicillin-resistant *Staphylococcus aureus* 'Cordobes' clone (SAC) (*mecA*)	*bla*$_{TEM-1}$, *bla*$_{KPC-2}$, *bla*$_{SHV-11}$, *bla*$_{SHV-12}$, *bla*$_{KPC}$, *bla*$_{OXA-23}$, *bla*$_{NDM-1}$, *mecA*	Meropenem, Cefotaxime, Ampicillin	Interspecies cell-free DNA was added to *A. baumannii* cultures and successfully transformed. Resulting transformants were resistant to multiple antibiotics	(Traglia et al., 2019)
Enterococcus faecalis	*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella aberdeen*	Not specified	Kanamycin, Ampicillin, Tetracycline	Transfer of RP4 from chlorine-killed antibiotic-resistant bacteria to chlorine-injured bacteria via natural transformation	(Lu et al., 2020)
Haemophilus influenzae, ***H. suis,*** ***H. parainfluenzae***	*H. influenzae, H. suis, H. parainfluenzae*	Not specified	Streptomycin	DNA conferring streptomycin resistance from *Haemophilus* species was used to transform different species of *Haemophilus*	(Alexander et al., 1956)

S. pneumoniae, ***S. oralis,*** ***S. mitis***	*S. oralis, S. mitis,* *S. sanguis* and *S. constellatus, S. pneumoniae*	*parC* and *gyrA*	Fluoroquinolones (Pefloxacin and Sparfloxacin)	Cell-free DNA conferring fluoroquinolone resistance from *Streptococcus* species was used to transform multiple other *Streptococcus* species	(Janoir et al., 1999)
Neisseria meningitidis	*N. cinerea and N. flavescens*	*penA*	Penicillin	Cell-free DNA conferring penicillin resistance from *Neisseria* species was used to transform *N. meningitidis*	(Bowler et al., 1994)

Conjugation-mediated HGT has long been the focus when monitoring dissemination of AMR. Recent observations have highlighted that transformation is also an important driver of ARG dissemination across- or within species, yet evidence of transformation-mediated exchange of ARGs was demonstrated over 60 years ago (Alexander et al., 1956; Winter et al., 2021;Table 2). This is likely due to plasmids typically being considered to transfer via conjugation and not transformation, despite evidence demonstrating plasmids can be taken up via natural transformation (albeit at much lower frequencies than via conjugation) (Ding et al., 2020; Kothari et al., 2019; Yu et al., 2022). As stated previously, the reduced frequency of plasmid transfer though transformation compared to conjugation is likely due to the reduction of dsDNA fragments into ssDNA as they enter the transforming cell's cytoplasm. This creates a requirement to take up two complementary strands of the plasmid where they must recombine in the cytoplasm before they are functional (Figure 6). However, a significant advantage in transformation-mediated uptake of ARGs is that donors do not need to be temporally linked to recipients like they do in conjugation. This is because a fraction of DNA can stably exist for at least three days in soil (Sikorski et al., 1998), to over 43,000 years in permafrost (Overballe-Petersen et al., 2013). Bacterial resistance to antimicrobials predates the human discovery of antibiotics (Dcosta et al., 2011), indicating that there are ARGs in the environment available only to species which are capable of natural transformation, demonstrating a clear advantage of transformation over conjugation for ARG acquisition.

Interspecies interactions can play a significant role in rates of ARG dissemination via natural transformation as they can lead to the release of increased quantities of DNA (Figure 6). For example, type VI secretion systems (T6SS) as mentioned earlier are used by species such as *Acinetobacter baylyi* to predate upon other species such as *Escherichia coli* and then immediately transform with DNA from the lysed cell (Cooper, Tsimring and Hasty, 2017; Figure 6). Another example is the complicated "carry-back model" employed by Proteobacteria observed to be used to obtain the transposable element containing the chloramphenicol exporter gene *cmx* from actinobacteria (Jiang et al., 2017; Figure 6). Briefly, a conjugative plasmid is donated to the actinobacterium from the proteobacterium via conjugation, and the transposon later moves from the actinobacterial chromosome to the plasmid. The actinobacterium releases the plasmid upon cell division or cell death, making it available for uptake by the proteobacterium

via natural transformation. Due to the homologous regions on the plasmid flanking the inserted transposon, the proteobacterium chromosome can effectively recombine with a region of the plasmid, inserting an AMR gene in the process. Species such as *Vibrio cholerae* may induce lysis of kin of a different strain by using a bacteriophage which is lethal to its kin but not itself (Molina-Quiroz et al., 2020; Figure 6). The lysogenic predator strains in this example harbour a prophage region containing a gene which disrupts the lytic effect of phages produced by the gene. Kin without the prophage region are sensitive to the lytic effect of the phages and so are killed upon infection after they are released by nearby lysogenic strains. This phenomenon greatly increases the amount of cell-free DNA available for the lysogenic bacteria to transform with.

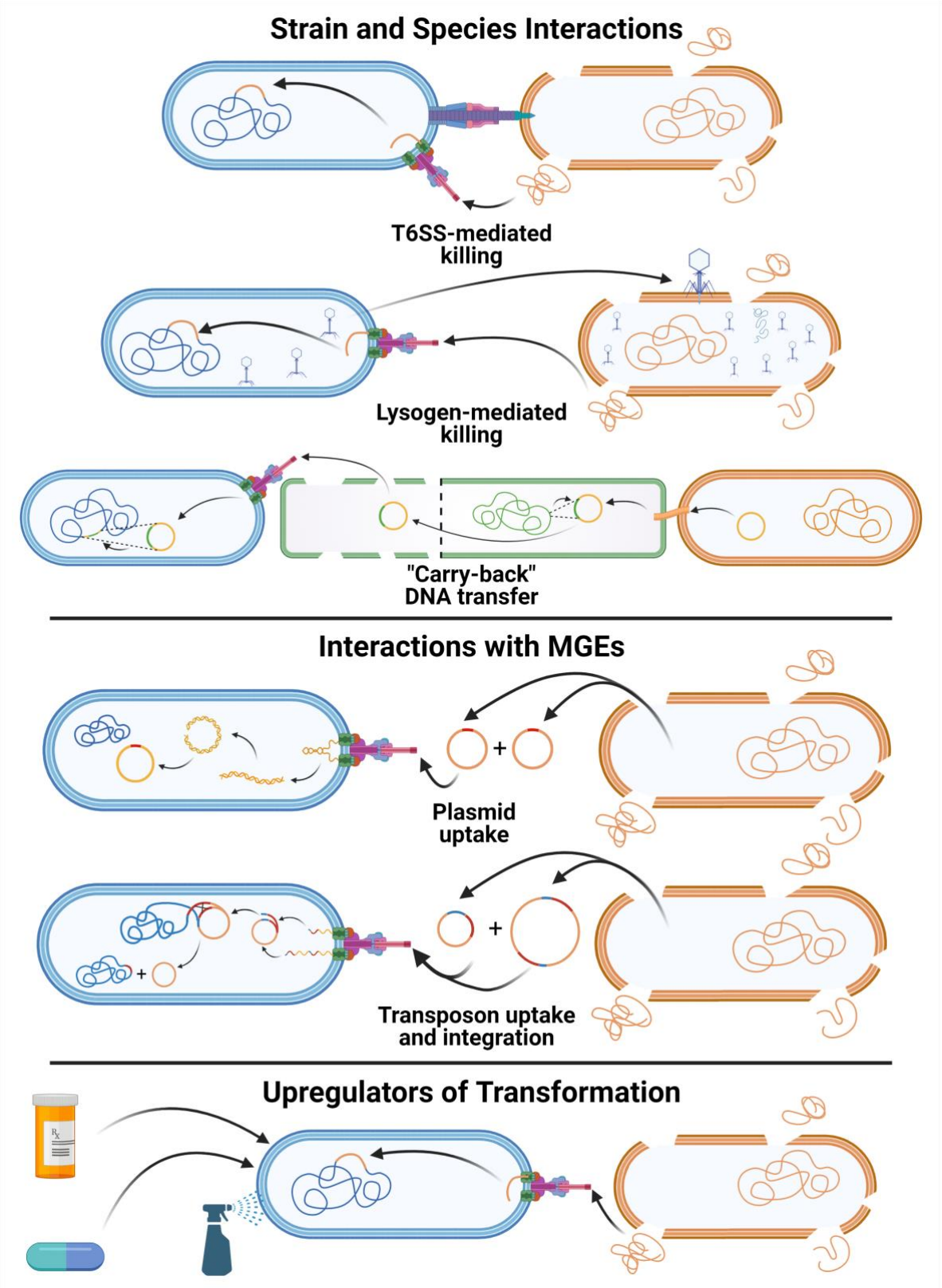

Figure 6. Illustration of some mechanisms involving species interactions, interactions with mobile genetic elements (MGEs), and anthropogenic factors which can increase frequencies of natural transformation in bacteria, thus increasing rates of ARG dissemination. Figure taken from Winter *et al.,* 2021.

1.5 Knowledge gaps

The problem of transformation-mediated acquisition of AMR is widespread and needs to be addressed by uncovering how it is regulated in transforming cells, and how or why it is maintained in species over evolutionary time. Previous studies (discussed later in this section) have explored how abiotic factors affect rates of transformation in bacteria, but the elements tested are only a small proportion of what may be ecologically relevant to environmental bacteria. Therefore, further work should be conducted to test the effects of other abiotic factors on natural transformation. Additionally, the short-term effect of other cocultured species on rates of transformation has never been explored yet remains to be potentially the most significant manipulator of natural transformation frequencies in natural environments due to the diverse range of secreted elements and interspecies interactions (Federle & Bassler, 2003; James et al., 1995; Kuramitsu et al., 2007; Tashiro et al., 2013; Traxler et al., 2013). Bacteria seldom live in single-species communities so if a significant impact of biotic interactions is found, it will be an important consideration for predicting rates of gene transmission via natural transformation in the environment. Further, interspecies interactions and coevolution of competitors may also be a valuable tool for providing selective pressure when testing the adaptive benefits of natural transformation and can help to explain why this mechanism of HGT is maintained in some species.

1.5.1 The Effect of Anthropogenic Pollutants on Natural Transformation

Naturally transformable bacteria are seen to be greatly affected by a host of abiotic factors which can change the transformation frequency of the bacterium (Winter et al., 2021). These abiotic agents include, but are not limited to: artificial sweeteners which increase membrane permeability, facilitating increased uptake of DNA (Yu et al., 2022); UV radiation causing DNA damage and inducing the SOS response for repair (Charpentier et al., 2011; Hülter et al., 2017); DNA-damaging antimicrobial agents (Charpentier et al., 2011, 2012; Prudhomme et al., 2006); non-antibiotic drugs such as the anti-inflammatory diclofenac (Wang et al., 2020) and antidepressants (Lu et al., 2022); free radicals caused by chlorination of drinking water (Jin et al., 2020); microplastics which lead to increased exposure to antimicrobials due to their adsorptive properties (Arias-Andres et al., 2018; Li et al., 2018); and both albumin and

calcium ions in serum which induce competence in *A. baumannii* (Quinn et al., 2019; Traglia et al., 2016). Ultimately, lethal levels of any compounds in the environment will lead to increased cell death and therefore greater amounts of DNA available for transformable species to use (Jin et al., 2020). The increase of transformation frequencies in response to exposure to agents such as the antibiotics and non-antibiotic drugs described in this section may lead to acquisition of antimicrobial resistance and pathogenicity genes.

Cancer chemotherapeutic drugs have not been investigated for their effect on natural transformation at concentrations pertaining to the environment or *in vivo* during treatment of malignancies. Cancer chemotherapeutic drugs are cytotoxic agents which are used to treat malignancies in humans because they interfere with cell division or DNA replication in human cells (Nygren, 2001). DNA damage is known to induce the SOS repair pathway in transformable bacteria and upregulate transformation frequency (Hoelzer & Michod, 1991; Michod et al., 1988; Mongold, 1992), suggesting that anthropogenic pollution of cancer chemotherapeutic drugs may upregulate transformation frequencies of environmental bacteria and transformable commensal bacteria within a patient who is administered a course of chemotherapy. Patients receiving chemotherapy have weakened immune systems caused by the cytotoxic effect of the chemotherapeutic agents and are administered prophylactic antibiotics to prevent infection (Zitvogel et al., 2008). This can lead to increased colonisation by pathogenic bacteria and increased ARG prevalence within those bacterial communities (Hobson et al., 2020; Meunier et al., 2019; Papanicolas et al., 2018). If transformation frequencies are upregulated in the presence of chemotherapeutic drugs, this may lead to increased dissemination of antimicrobial resistance and pathogenicity genes. Therefore, it is important to test for these effects to increase our understanding of the rates of dissemination of AMR and pathogenicity genes in the environment.

1.5.2 Biotic Dimensions of Transformation

A highly relevant ecological dimension of HGT is the effect of interspecies interactions on transformation which has not yet been studied. Bacteria almost always live in multispecies communities (Stubbendieck et al., 2016) so this effect must be investigated to understand the contexts under which bacteria alter their transformation

frequencies as it may shed light on the rates of uptake of antimicrobial resistance genes in natural environments.

Because some bacteria upregulate competence induction as a response to stress (Engelmoer & Rozen, 2011; Prudhomme et al., 2006; S. Zhang et al., 2021; X. Zhang et al., 2018) while others may upregulate transformation frequencies when they are experiencing higher growth rates (Utnes et al., 2015), it is hard to predict how interspecies interactions may affect rates of transformation. Bacteria engage in a range of intercellular interactions such as predation (R. M. Cooper et al., 2017; Molina-Quiroz et al., 2020; Veening & Blokesch, 2017), competition (Ortiz et al., 2021; Rosenberg et al., 2016; Zheng et al., 2013) and mutualism (cross-feeding) (D'Souza et al., 2018; Pande et al., 2015; N. W. Smith et al., 2019) between bacteria. These interactions may alter transformation frequencies due to induction of the stress response or higher growth rates, or by novel mechanisms which are not yet characterised. Because species interactions are complex and not yet fully understood, these effects on transformation must be tested for directly. To test for this, we must test how transformation frequencies of a focal species change when cocultured in the presence of other species. A range of species should be tested to provide insight on whether species relatedness has implications on transformation frequency of the focal strain. The likelihood that species will compete for the same resource increases when species are more related, while low relatedness could lead to reciprocal susceptibility to antagonistic mechanisms such as toxin-antitoxin systems but also mutualistic interactions such as cross-feeding. The nature of these interactions could be elucidated somewhat by determining if interactions are based on cell-cell contact, secreted substances, and if they are secreted substances, whether they are proteinaceous. This information would provide a foundation for future work testing for the effect of biotic interactions on natural transformation and potentially highlight significance for using biotic interactions in all future work testing natural transformation.

1.5.3 Testing the Sex Hypothesis for Natural Transformation

The sex hypothesis for natural transformation has been tested previously with mixed evidence which does not always support it. To test for the adaptive benefits (or sex hypothesis) of natural transformation, a transformable strain and a non-transforming

counterpart are observed as the strains adapt to a selection pressure. Varied approaches such as cycling antibiotics for selection (Perron et al., 2012), using bacteriophages as a selection pressure (McLeman et al., 2016), or adaptation solely to culture media (Bacher et al., 2006) have been used, but all studies have used experimental evolution techniques (Kawecki et al., 2012). If natural transformation functions to increase variation in a population and speed up adaptation in response to natural selection, this effect occurs over evolutionary time (Ambur et al., 2016; Hülter et al., 2017; Levin & Cornejo, 2009; Otto & Lenormand, 2002; Vos, 2009; Vos et al., 2015). Therefore, experiments testing the sex hypothesis for transformation must subject their test populations to enough generations in which a significant change in fitness can be observed. This change in fitness can be observed by comparing fitness of evolved clones to their ancestors, or by comparing fitness of an evolved wildtype transformable strain to an evolved non-transformable isogenic counterpart (Buckling et al., 2009; Colegrave & Collins, 2008; Kawecki et al., 2012). Selective pressure can be provided in the form of antibiotic challenge (Perron et al., 2012), opportunity to utilise a novel carbon source (Chu et al., 2018), and infection by a bacteriophage (McLeman et al., 2016). For long-term evolutionary scales, if selection pressures are not strong or fluctuating, transformability could diminish in the focal strain (Bacher et al., 2006). This is likely due to the focal strain losing the expensive transformation machinery as it is already presenting near-peak fitness with no need to acquire more genes through transformation since the fitness landscape is unchanging. The presence of competitor species has long been recognised as a selective pressure driving evolution in bacteria (Kawecki et al., 2012; Otto & Nuismer, 2004). The use of competitor species as a selective pressure may be superior to abiotic factors if species coevolve during coculture. Coevolution of competitors can provide a fluctuating selection pressure which alters the fitness landscape with time. Therefore, implementation of non-self-species competitors may provide a selection pressure which constantly provides a need for rapid adaptation, thereby selecting for the ability to naturally transform and demonstrating an adaptive benefit of natural transformation if it confers a fitness advantage over evolutionary time.

DNA is the substrate for natural transformation and should be a key consideration for the experimental design of studies testing the adaptive benefits of natural transformation. Without the provision of extracellular DNA containing novel genetic

information, natural transformation may not demonstrate a clear adaptive benefit as it must rely on the exchange of *de novo* mutations by kin and potential gene duplications or deletions via transformation. The existing published literature has mixed approaches for supplying DNA such as via cells which may eject DNA fragments while living or through death, releasing their whole chromosome (Baltrus et al., 2007; Johnsen et al., 2009). In some studies, DNA was not provided to transforming cells, again relying on the release of homologous DNA from cocultured cells (Bacher et al., 2006; Engelmoer et al., 2013). DNA is typically provided cell-free on a regular basis from conspecific sources (Baltrus et al., 2007; Johnsen et al., 2009; McLeman et al., 2016; Nguyen et al., 2022; Perron et al., 2012; Power et al., 2020; Renda et al., 2015; Utnes et al., 2015; Woods et al., 2020). Providing conspecific DNA is arguably the superior format for testing the sex hypothesis for transformation as it ensures some level of sequence homology between donor and recipient strain, increasing transformation efficiency (De Vries & Wackernagel, 2002), while also carrying some potentially relevant and compatible genes for the acquisition of increased fitness.

1.6 *Acinetobacter baylyi* as a Model Organism for Transformation

Acinetobacter baylyi is a ubiquitous gram-negative gamma-proteobacterium considered to be a soil-dwelling bacterial species (T. L. Chen et al., 2008; Watson & Carter, 2008). It is capable of natural transformation by using type IV pili situated on the cell membrane to pull exogenous DNA into the cell for integration within the genome (Blokesch, 2016). Like in other species, the mechanistic basis of transformation in *A. baylyi* is well understood and there is a number of functionally diverse proteins involved (Bacher, Metzgar and De Crécy-Lagard, 2006; Leong et al., 2017). *A. baylyi* is a model organism for bacterial transformation as it is constitutively naturally competent in solid or liquid growth media with peak transformation frequency when it is in the log phase of growth (Blokesch, 2016; Domingues et al., 2012; Elliott & Neidle, 2011; Hülter et al., 2017; Leong et al., 2017; Mantilla-Calderon et al., 2019; Overballe-Petersen et al., 2013; Palmen et al., 1993; Ray & Nielsen, 2005; Vaneechoutte et al., 2006; Watson & Carter, 2008). *A. baylyi* has no DNA uptake sequences in its type IV pili, and so does not identify whether an incoming sequence of DNA is homologous before uptake, also making it a useful model for illegitimate recombination (at several orders of magnitude lower frequency than homologous

recombination (De Vries & Wackernagel, 2002; Hülter & Wackernagel, 2008)), and for plasmid uptake via natural transformation (De Vries & Wackernagel, 2002; Elliott & Neidle, 2011). Exogenous DNA is readily available for *A. baylyi* as it releases 1-3µg of DNA per mL of liquid culture while growing, presumably for kin to use for transformation (Murin et al., 2012). Its ability to grow reliably at temperatures ranging from 15-37°C in an array of growth media makes this organism a very versatile model for most lab-based studies (Ray & Nielsen, 2005). Cloning organisms to change their genomes (and by extension, phenotypes) allows for well-controlled studies testing the function of a specific gene where an isogenic pair of strains are tested in the same study: one strain with a functioning gene copy, and one without. The high plasticity of the *A. baylyi* genome (Elliott & Neidle, 2011) lends itself to such studies, which is useful when testing the mechanistic basis and function of natural transformation in this species. Fortunately, this is not the first study which tests an aspect of transformation in *A. baylyi*, and indeed protocols for observing transformation in this species have been established which help ensure results are more reproducible both within and across labs (Ray & Nielsen, 2005).

A. baylyi is closely related to the notorious multidrug resistant pathogen *A. baumannii*. Indeed, *A. baylyi* itself has been found to cause disease in immunocompromised hospital patients (T. L. Chen et al., 2008), though the species is generally considered non-pathogenic and is suitable for use in biosafety level 1 rated labs, making it a safer and more convenient alternative to *A. baumannii*. The *Acinetobacter* genus has many transformable species within it (Domingues et al., 2019; Godeux et al., 2018; Hülter et al., 2017; Palmen et al., 1993; Traglia et al., 2016), and indeed links between transformability and human pathogenicity (through acquisition of antibacterial resistance and virulence genes) have been found (Domingues et al., 2019; Von Wintersdorff et al., 2016). Essential genes for competence for natural transformation are also highly conserved in the *Acinetobacter* genome (Touchon et al., 2014). Therefore, there is clear clinical and ecological relevance in exploring how the model organism *A. baylyi* evolves through use of natural transformation in any context.

1.7 Thesis summary

Natural transformation is affected by a host of abiotic factors present in natural environments such as freshwater systems and human bodies, yet there are many more abiotic agents which have not been tested for their effect on natural transformation. Further, a more environmentally relevant and continually present component of the bacterial natural environment is competitor bacterial species. The effect of other species on natural transformation has not yet been studied despite our understanding of intercellular interactions which can significantly affect neighbouring cells in a community. It is likely that competitor species affect rates of natural transformation in some bacteria and may also create a strong and fluctuating selection pressure which provides a need for rapid adaptation. Therefore, using competitor species in coculture with a transformable species may find evidence in support of the sex hypothesis for natural transformation.

The studies presented in this thesis aimed to gain a better understanding of the biotic and abiotic contexts affecting bacterial (most specifically *A. baylyi*) transformation frequencies, having implications for the rate at which bacteria could obtain ARGs. In Chapter 2, the short-term effects of chemotherapeutic agents used to treat human cancers were tested for their effect on natural transformation. Cancer treating drugs with six different mechanisms of action were tested at concentrations found in patients undergoing cancer chemotherapy to determine if they affected rates of natural transformation in *A. baylyi*. For drugs found to affect natural transformation, the mechanisms responsible were tested for using mutants which may respond differently to the respective drug.

To explore the biotic factors which affect rates of natural transformation, Chapter 3 tested the changes in transformation frequency of *A. baylyi* in response to coculture with a range of other species. Patterns explaining biotic effects in response to coculture with other species were tested for by identifying whether transformation frequency changes are linked to *A. baylyi* growth rate, and cocultured species relatedness. Additionally, the mechanistic basis of these effects was tested for by observing transformation frequency changes in the presence of another species, the supernatant of another species, or the boiled supernatant of another species. Bacteria in the environment can live in a range of nutrient availabilities, so the interaction between nutrient availability and observed biotic effects was also tested for.

As previously discussed, the sex hypothesis for natural transformation is contested and has mixed evidence supporting it. If a function of natural transformation for a cell is to provide genetic novelty from extracellular sources of DNA, this can lead to greater rates of adaptation, facilitating natural selection leading to rapid acquisition of antimicrobial resistance mechanisms. Chapters 4 and 5 sought to test the sex benefits for natural transformation by building on prior knowledge and adapting methodologies to account for insufficiencies in model systems used by other studies. The sex benefits of transformation were tested for by comparing a transformable *A. baylyi* strain with a non-transformable counterpart during adaptation to an environment containing bacterial competitors. Coculture with competitors has never been used in transformation experiments before and could lead to an ever-changing fitness landscape. This is because the contexts of selective pressures change over time due to reactive evolution by species following competitive interactions (Paterson et al., 2010). Additionally, heterologous DNA in saturating concentrations was provided from donors of the same genus as the focal species *A. baylyi*. Using non-self-DNA from a collection of *Acinetobacter* species as a substrate for transformation provides functionally diverse genes which can confer fitness benefits when acquired, while also offering homology for high rates of transformation (De Vries & Wackernagel, 2002). Fitness changes observed at the end of the evolution period were compared against an isogenic non-transforming counterpart which was subjected to the same evolutionary conditions over the same period.

The environment contains diverse ARGs and a host of factors which can select for the ability to naturally transform. Understanding the contexts under which bacteria transform and the functions transformation is used for can help identify why the ability to transform is maintained in bacterial species and can help to predict the rates of dissemination of antimicrobial resistance in the environment and the emergence of new pathogens.

Chapter 2: Effect of Chemotherapeutic Agents on Natural Transformation in *Acinetobacter baylyi*

2.1 Abstract

Bacterial natural transformation is the ability of a bacterial cell to take up extracellular DNA from the environment for recombination into the cell's chromosome or storage as an extrachromosomal element such as a plasmid. Like other mechanisms of horizontal gene transfer, natural transformation is recognised to be a significant driver for dissemination of antimicrobial resistance mechanisms between bacteria. Previous work has shown many pharmaceutical compounds such as antidepressants and anti-inflammatory drugs can upregulate transformation frequency in the model species for natural transformation, *Acinetobacter baylyi*. Chemotherapeutic compounds have been shown to increase abundance of antimicrobial resistance genes and increase colonisation rates of potentially pathogenic bacteria in patient guts, indicating an increased risk of infection and providing a pool of donor pathogenicity or resistance genes for transformable commensal bacteria. This study tested for the effect of six cancer chemotherapeutic compounds on *A. baylyi* natural transformation frequency and suggested mechanisms of action for their effects. Enhancing our understanding of how and when bacteria naturally transform, especially in clinical environments, can help us to monitor and establish preventative measures to limit the spread of antimicrobial resistance genes between bacteria.

2.2 Introduction

Antimicrobial resistance (AMR) is a global threat to modern medicine and is accelerated greatly by rapid dissemination of antimicrobial resistance genes (ARGs) via horizontal gene transfer (HGT) (Allen et al., 2010; X. Jiang et al., 2017; Ventola, 2015; Vinayamohan et al., 2022; Von Wintersdorff et al., 2016). The increased prevalence of AMR has severe consequences on modern medicine, for instance, AMR infections were responsible for an estimated 4.95 million deaths worldwide in 2019 (C. J. Murray et al., 2022). Natural transformation, the process whereby prokaryotes take up extracellular DNA from the environment (Johnsborg et al., 2007; Johnston et al., 2014; Seitz & Blokesch, 2013), is a potentially underrecognised driver of AMR

dissemination worldwide despite conferring the ability to acquire cell-free chromosomal DNA, plasmids, and transposons (Winter et al., 2021). While only approximately 80-90 species are known to be naturally transformable (Johnston et al., 2014), the WHO's list of priority multidrug resistant pathogens is composed mostly of transformable species, indicating that this mechanism could be important in the acquisition of resistance.

Transformation frequency in bacteria can be up or down regulated in response to a range of niche-specific stimuli (Johnston et al., 2014; Blokesch, 2016). A wide variety of anthropogenic pollutants including pharmaceutical products have been demonstrated to increase transformation frequencies, particularly in *Acinetobacter baylyi* (Jin et al., 2020; Johnston et al., 2014; Li et al., 2018; Wang et al., 2020; Yu et al., 2022; S. Zhang et al., 2021). For example, pharmaceutical compounds such as anti-inflammatory drugs (Wang et al., 2020) and antidepressants (Lu et al., 2022) increase rates of natural transformation between 2-3-fold. However, no data are available on the effect of chemotherapy compounds on natural transformation. Chemotherapy compounds are cytotoxic agents which target a range of cell functions which are often upregulated in- or unique to- malignant cells to induce cell death (Nygren, 2001). An estimated 17 million new cancer cases were diagnosed in 2022 and approximately 34 million cancers are expected to be diagnosed in 2070 (Soerjomataram & Bray, 2021). The global use of cancer chemotherapy compounds is therefore high and set to increase in the future. The use of chemotherapy compounds to treat malignancies in humans has been shown to increase levels of AMR in gut microbiota, increasing the risk of contracting a resistant bloodstream infection (Papanicolas et al., 2018; Meunier et al., 2019), a cause of death in approximately 1 in 10 cancer patients (Danai et al., 2006; Williams et al., 2004). A proposed mechanism for AMR acquisition in response to exposure to chemotherapy compounds is the induction of the SOS response pathway which leads to increased bacterial mutation rates (Bjedov et al., 2003; Do Thi et al., 2011; Meunier et al., 2019). The SOS stress response is also an inducer of competence for natural transformation in some species, potentially including *A. baylyi* (L. Lin et al., 2019), however the SOS response is atypical in *A. baylyi* as the species lacks essential genes for SOS response induction found in *Escherichia coli* such as *LexA* and *SulA* (Mantilla-Calderon et al., 2019; Robinson et al., 2010). Increased rates of transformation during chemotherapy

are additionally hazardous due to the routine use of last resort antibiotics as prophylactic treatment for cancer patients which increases the resistance of gut microbiota to those antibiotics (Hobson et al., 2020), and has been known to increase colonisation of potentially pathogenic bacteria in the gut (Van Vliet et al., 2009). This can lead to infection but could also potentially act as a donor pool of resistance or pathogenicity genes to resident species that are able to engage in natural transformation.

Here, the model organism *A. baylyi* was used to test the effects of chemotherapeutic drugs on natural transformation. *A. baylyi* is a ubiquitous environmental bacterium capable of opportunistic infection (T. L. Chen et al., 2008; Zhou et al., 2011), and is constitutively naturally transformable (Blokesch, 2016; Palmen et al., 1993). *Acinetobacter* species, particularly *A. baumananii*, can colonise human gastrointestinal systems (Ketter et al., 2018) and can cause severe and even fatal infections in patients undergoing cancer chemotherapy (Freire et al., 2016; Fukuta et al., 2013). *A. baumannii* is an infamous multidrug resistant nosocomial pathogen with the ability to naturally transform (Fournier et al., 2006; Godeux et al., 2018; C. J. Murray et al., 2022; Traglia et al., 2019; Wilharm et al., 2013).

Table 3. List of the drugs used in this study, their mechanisms of action, and concentrations reported for environmental and clinical samples.

Drug	**Mechanism of Action / Drug Target**	**Clinically relevant concentration**
Cytarabine	Cytosine analogue	Estimated blood plasma concentration - 17.8µg/mL (Slevin et al., 1983)
Daunorubicin	Topoisomerase II inhibitor	Concentration inside leukemic cells - 10.6µg/mL (Tidefelt et al., 2000)
Docetaxel	Disrupts microtubule function	Blood plasma - 2.42ng/mL (Morgan et al., 2009)
Exemestane	Aromatase inhibitor (oestrogen synthesis inhibitor)	Blood plasma - 4.1ng/mL (Luo et al., 2018)

Imatinib	Tyrosine kinase inhibitor	Blood plasma - 1µg/mL (Gotta et al., 2014)
Methotrexate	Folate synthesis inhibitor	Blood plasma - 13.63µg/mL (Hornung et al., 2008)

In this study, six chemotherapy compounds currently used to treat malignancies in humans were used which could affect cell viability or transformation frequency in *A. baylyi*. A dose-response analysis was conducted for the six compounds to test for their effect on transformation frequency and growth rate of *A. baylyi*. Each drug belongs to a different class to provide a range of mechanisms of action (Table 3). These drugs are: cytarabine, a cytosine analogue (Crisp et al., 1996); daunorubicin, a DNA and topoisomerase II inhibitor (Agrawal, 2020); docetaxel, a disruptor of microtubule function (Herbst & Khuri, 2003); exemestane, an aromatase inhibitor (Lombardi, 2002); imatinib, a tyrosine kinase inhibitor (De Kogel & Schellens, 2007); and methotrexate, a folate synthesis inhibitor (Cronstein, 1997). We proposed the cytosine analogue cytarabine could potentially cause lesions on DNA upon integrating with the replicating chromosome as in human cells (Cline & Osheroff, 1999). Daunorubicin acts on eukaryotic cells by topoisomerase II inhibition which could lead to an upregulated SOS response in *A. baylyi* if DNA gyrase (a topoisomerase II subclass; Reece, Maxwell and Wang, 1991) is also targeted by daunorubicin (Lu et al., 2022). Docetaxel targets microtubule function so may interfere with chromosomal segregation or cell division in *A. baylyi* (Herbst & Khuri, 2003; Ptacin et al., 2010). Exemestane inhibits oestrogen production in humans (Lombardi, 2002), but to our knowledge, no effect of oestrogen inhibition has directly been tested for any Gammaproteobacteria, so effects of this drug are unpredictable. Imatinib is an Abelson tyrosine kinase (ATK) inhibitor (De Kogel & Schellens, 2007). While no ATKs have been identified in bacteria, imatinib may act on a homologue of ATK and have unpredictable effects on transformation frequency as tyrosine kinases facilitate a wide range of cellular functions. Methotrexate inhibits folate synthesis similar to the antibiotic trimethoprim (Alqarni & Zeidler, 2020; Burchall et al., 1982). As folate is used for the synthesis of pyrimidines, transformation frequency may increase in response to depleting stores of DNA in the cytoplasm during incubation with methotrexate. The concentrations of chemotherapeutic compounds used in this study are near to or

greater than clinically relevant concentrations found in the body during chemotherapy (Table 3). As the varied pharmacokinetic properties of these six drugs can indicate differences in diffusion between blood plasma and tissues (Chillistone & Hardman, 2017), a range of concentrations spanning previously measured blood plasma concentrations were used.

Understanding underlying mechanisms for the effect of compounds on transformation frequency changes can help to suggest other compounds which may demonstrate similar effects and improve our understanding of the underlying mechanisms facilitating natural transformation. To investigate the mechanistic basis of effects on transformation frequency caused by chemotherapeutic compounds, isogenic *A. baylyi* mutants with trimethoprim resistance and a *xerC* deletion, respectively were constructed. We chose two chemotherapeutic compounds for further study on the molecular basis of effects: docetaxel, which demonstrated a significant effect on transformation frequency, and methotrexate, which appeared to not have an effect on transformation frequency. Using these two compounds, it was possible to test for the mechanism of action of docetaxel, and test whether the reason for not finding an effect of methotrexate was due to the genetic background of the focal strain. Therefore, using a mutant strain can increase the chances of observing an effect of methotrexate in a realistic context. We hypothesised that growth rates of *A. baylyi* may decrease in response to interaction with methotrexate as it shares a similar mechanism of action with the antibiotic trimethoprim (Kielhofner, 1990). As methotrexate interferes with folate synthesis (Alqarni & Zeidler, 2020; Cronstein, 1997), a trimethoprim resistant mutant was constructed to test if the effects of methotrexate are altered by the presence of dihydrofolate reductases (Burchall et al., 1982). A XerC-deficient strain was constructed to help determine the mechanistic basis of docetaxel's effect on transformation. Docetaxel targets microtubule function (Herbst & Khuri, 2003), which suggests spindle-like structures in bacteria may also be affected (Ptacin et al., 2010). Deletion of *xerC* results in improper sorting of chromosomes during cell division (Blakely et al., 1991; Castillo et al., 2017; Mei et al., 2021). Differences in response to docetaxel between the Δ*xerC* strain and the wildtype may therefore inform which component of the cell replication or DNA repair pathway is affected by docetaxel.

2.3 Methods

2.3.1 Chemotherapeutic drugs

Cytarabine (Abcam, UK), daunorubicin (Cayman Chemical Company, USA), docetaxel (Cambridge Bioscience, UK), exemestane (Merck, UK), imatinib (Cambridge Bioscience, UK), and methotrexate (Cayman Chemical Company, USA) were stored at -20˚C in single-use aliquots dissolved in DMSO (Fisher, USA) at 100x the concentration used in each treatment. Aliquots of drug stocks were added as 1% of the final volume of culture to ensure an equal final concentration of DMSO across all treatments. This kept the negligible cytotoxic effect of the DMSO consistent throughout, but there is no indication as to whether interactions between DMSO and these drugs contribute to the effects observed in this study.

2.3.2 Strains and culture conditions

For all experiments, a naturally transformable green-fluorescent and apramycin resistant construct of *A. baylyi* ADP1 was used. Methods of strain construction are described elsewhere (Winter, Harms, Johnsen, Buckling, et al., 2023). To establish a trimethoprim resistant mutant, cultures of the focal *A. baylyi* strain were plated on LB agar (Formedium, England) amended with 2µg/mL trimethoprim (Sigma-Aldrich, USA) (Schuster et al., 2022) to screen for trimethoprim resistance. Clones which grew on the selective antibiotic amended agar were passaged another three times on 2µg/mL trimethoprim LB agar to confirm the resistance phenotype. The XerC-deficient mutant was established in the focal *A. baylyi* strain by natural transformation with DNA sourced from a XerC-deficient mutant described in another study (Kloos et al., 2021) (see below for natural transformation conditions). The *xerC* gene in this strain is replaced by an *nptII::sacB* cassette conferring kanamycin resistance via the *nptII* gene which codes for neomycin phosphotransferase (Hülter & Wackernagel, 2008). Successful transformants were isolated by plating on agar amended with 10µg/mL kanamycin (Fisher, USA).

2.3.3 Transformation assay

Genomic DNA as a substrate for natural transformation was isolated from the isogenic *A. baylyi* labelled with red fluorescence and spectinomycin resistance (Winter, Harms,

Johnsen, Buckling, et al., 2023). The transformable green-fluorescent *apraR* wildtype *A. baylyi* ADP1 was grown overnight in LB broth (Formedium, England) and diluted 5-fold into 2mL of LB broth in a universal 30 mL container. Cultures were amended with chemotherapeutic drugs on a $\log_{10}$ dilution range. In the no-drug control, DMSO was added to match the 1% DMSO concentration in drug treatment groups. Isogenic spectinomycin resistance-conferring DNA was produced by lysis following the Qiagen® Genomic DNA Handbook (April 2012) protocol. DNA from the eluate was precipitated by adding two volumes of ice-cold isopropanol and centrifuged at 26000xg for 15 minutes to pellet the DNA. DNA was dissolved in TE buffer to a final concentration of 342.4ng/µL (Nanodrop 2000, Thermo Scientific) and frozen at -20˚C in single-use aliquots for addition to each experiment at a final concentration of 100ng/mL for each sample. Samples were incubated at 30˚C, 180rpm for 5 hours. Recipients and transformants were enumerated before and after incubation by plating on LB agar amended with 240µg/mL apramycin (Duchefa, The Netherlands), and LB agar amended with both 240µg/mL apramycin and 360µg/mL spectinomycin (Melford, UK), respectively. To determine the highest concentration of DMSO which had no observable effect on cell viability and growth, a transformation assay was conducted as above, but without chemotherapy compounds (5-fold dilution of *A. baylyi* into LB broth for 3 hours at 30˚C, 180rpm with 100ng/mL DNA and 10%, 1%, 0.01%, 0.001%, or 0% DMSO; data not shown). All treatments were sampled at a minimum of sixfold biological replication.

2.3.4 Statistical analyses

Generalised mixed linear models were used with block as a random explanatory variable as all six replicates for each drug concentration were tested on separate days. The glmmTMB package was used for model construction (Brooks et al., 2017). Models of transformation frequency were plotted with binomial distribution using transformed cell density per mL and transformed cell density per mL subtracted from total potential recipient cell density per mL. For analyses measuring growth rate, the Malthusian parameter was calculated and used as a response variable (Lenski et al., 1991). The Malthusian parameter is a variable which describes the average rate of increase in a population and has a unit of $5hrs^{-1}$ in this study. Model assumptions were checked for using the DHARMa package (Hartig, 2022). Normal distribution of the residuals for data used in statistical modelling was verified using Shapiro-Wilks tests, and visually

using histograms. Significance of drug concentration was tested for using the drop1 test function in R. Significant differences between drug concentrations were tested using pairwise estimated marginal means (EMMs) comparisons using model outputs as input data and the emmeans package v1.8.4-1 (Length, 2023). The Kenward-Roger method was used to estimate the degrees of freedom for EMM comparisons. In all analyses, a *p* value of less than 0.05 was considered significant. False discovery rate (FDR) adjustment for multiple testing was used in all instances where multiple tests were conducted in the same analysis (Benjamini & Hochberg, 1995).

2.4 Results

2.4.1 Contrasting effects of different chemotherapy drugs on natural transformation in *Acinetobacter baylyi*

The effect of six chemotherapeutic compounds on natural transformation and growth rate was tested at varied concentrations similar to that found in patient blood plasma. Cytarabine was not observed to have a significant effect on transformation frequency (χ^2 = 3.1519, *p* = 0.3688, df = 3; Figure 7A) or growth rate (χ^2 = 1.5266, *p* = 0.6762, df =3; Figure 7B). Daunorubicin was observed to significantly decrease transformation frequency (χ^2 = 77.866, *p* < 0.0001, df=3) and growth rate (χ^2 = 23.401, *p* < 0.0001, df =3). The control group significantly differed in transformation frequency to samples containing 5.63µg/mL and 0.583µg/mL daunorubicin (EMMs comparison, df = 45, *p* <0.05; Figure 7C; Supplemental Table 1; Supplemental table 2) and differed in growth rate to samples containing 56.3µg/mL and 5.63µg/mL daunorubicin (EMMs comparison, df = 45, *p* < 0.05; Figure 7D; Supplemental table 3; Supplemental table 4), respectively. Transformation frequency of *A. baylyi* in 56.3µg/mL daunorubicin was below the detectable limit (10^{-8}) for all replicates and so could not be accurately analysed with a GLMM but is significantly different to the control group (Wilcoxon paired test, *p* < 0.001). Docetaxel was observed to significantly decrease transformation frequency (χ^2 = 16.803, *p* < 0.001, df = 3; Figure 7E), but not affect growth rate (χ^2 = 3.629, *p* = 0.3044, df =3; Figure 7F). Significant differences in transformation frequency were found between 80.7µg/mL docetaxel and 0.807µg/mL docetaxel (EMMs comparison, t-ratio = 2.538, df = 46, *p* < 0.05; Figure 7E; Supplemental table 5; Supplemental table 6), and the control group (EMMs comparison, t-ratio = 4.339, df = 46, *p* < 0.001; Figure 7E; Supplemental table 5;

Supplemental table 6). Exemestane was not found to have a significant effect on transformation frequency (χ^2 = 4.2067, p = 0.24, df = 3; Figure 7G) or growth rate (χ^2 = 2.3735, p = 0.4986, df =3; Figure 7H). Imatinib appeared to have no significant effect on transformation frequency (χ^2 = 6.2509, p = 0.1, df = 3; Figure 7I) or growth rate (χ^2 = 1.0623, p = 0.7862, df =3; Figure 7J). Methotrexate appeared to have no significant effect on transformation frequency (χ^2 = 4.8585, p = 0.1825, df = 3; Figure 7K) or growth rate (χ^2 = 0.46005, p = 0.9276, df =3; Figure 7L).

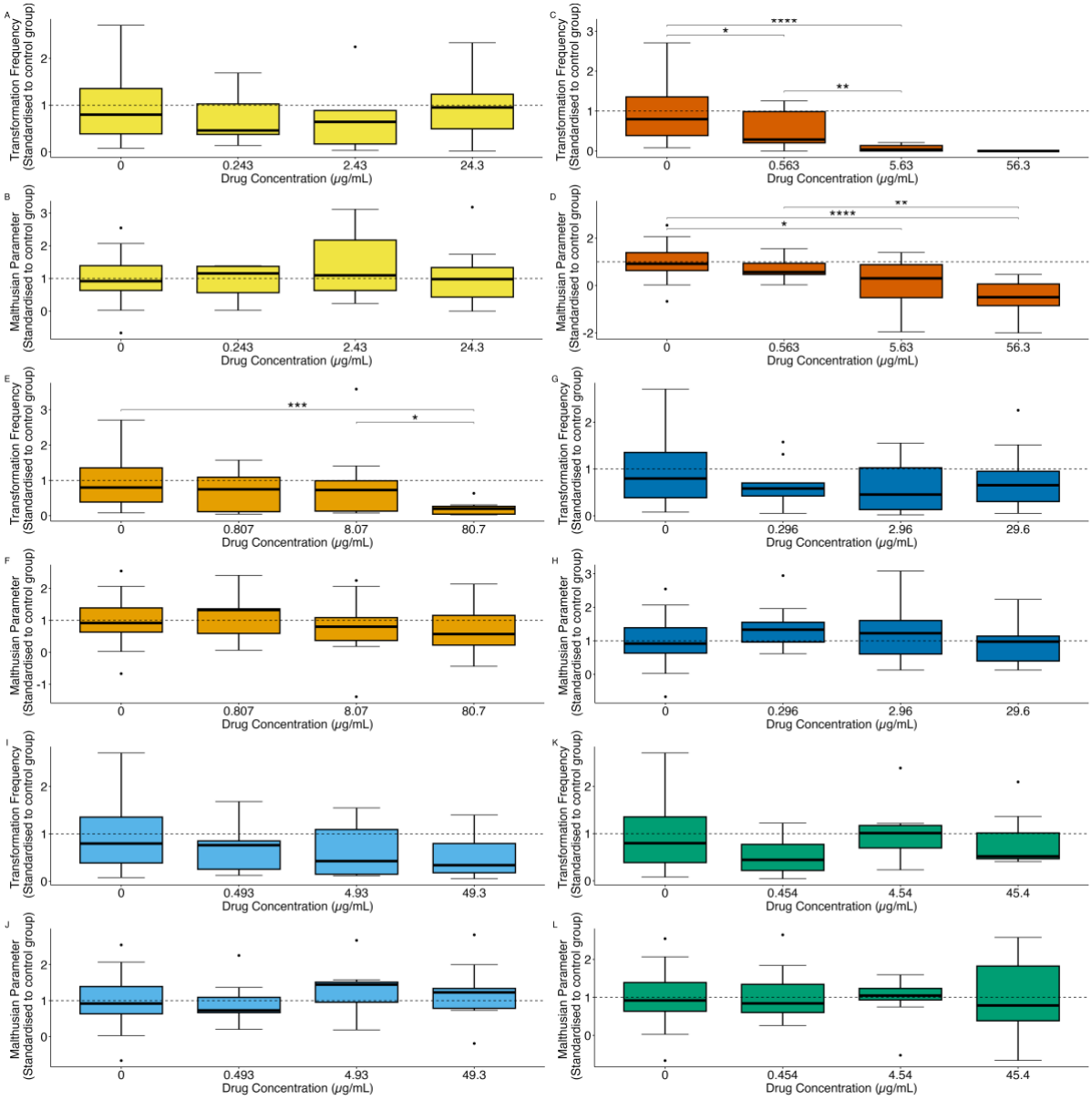

Figure 7. Effect of six chemotherapeutic agents on the transformation frequency and growth rate of *A. baylyi*. Effect of cytarabine on transformation frequency (A) and growth rate (B); daunorubicin on transformation frequency (C) and growth rate (D);

docetaxel on transformation frequency (E) and growth rate (F); exemestane on transformation frequency (G) and growth rate (H); imatinib on transformation frequency (I) and growth rate (J); methotrexate on transformation frequency (K) and growth rate (L). (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001)

2.4.2 Trimethoprim resistance does not alter *A. baylyi's* response to methotrexate

No significant effect of the interaction between methotrexate concentration and trimethoprim resistance was found on transformation frequency (Generalised linear mixed modelling, χ^2 = 2.1026, p = 0.5514, df = 3) or growth rate (χ^2 = 7.7626, p = 0.05118, df = 3). Methotrexate concentration was observed to have no overall effect on transformation frequency (χ^2 = 2.39, p = 0.4955, df = 3; Figure 8A) or growth rate (χ^2 = 3.944, p = 0.2676, df =3; Figure 8B) for the wildtype or trimethoprim resistant strain. Trimethoprim resistance is a significant predictor of transformation frequency (χ^2 = 106.4, p < 0.0001, df=1) and growth rate (χ^2 = 78.813, p < 0.0001, df =1). The trimethoprim resistant mutant was observed to have lower transformation frequency (EMMs comparison, t ratio = 19.814, df = 44, p < 0.0001; Supplemental table 7) and higher growth rate (EMMs comparison, t ratio = -14.140, df = 45, p < 0.0001; Supplemental table 8) than the wildtype ancestor, independent of methotrexate concentration.

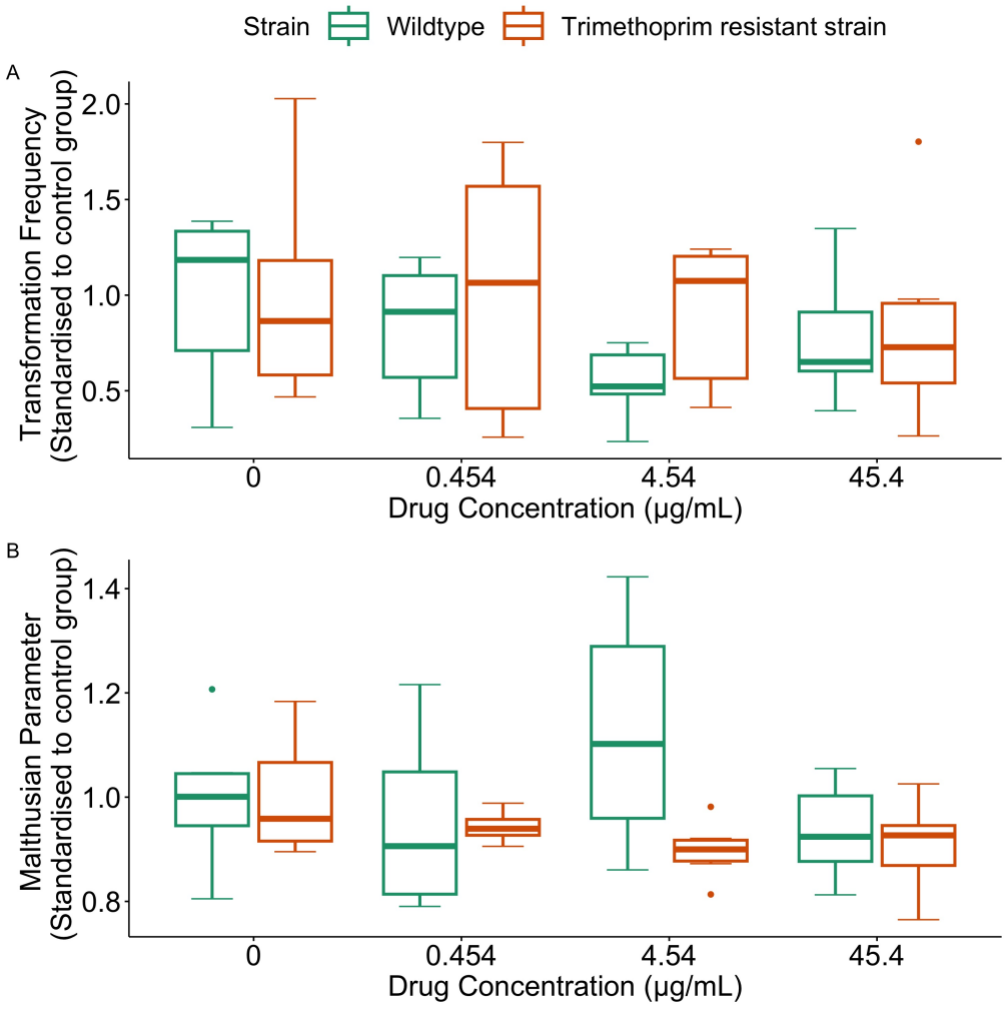

Figure 8. Effect of methotrexate on the transformation frequency (A) and growth rate (B) of a trimethoprim resistant (orange) and a wildtype *A. baylyi* strain (green).

2.4.3 Improper sorting of chromosomes as a tentative mode of action for hypersensitivity of *A. baylyi* to docetaxel

A Δ*xerC* mutant was used to test for the mechanism of action of docetaxel on transformation frequency. No dose-response effect of docetaxel for the mutant suggests XerC is the drug target, while increased sensitivity may suggest a synergistic effect between docetaxel's mechanism of action and improper chromosomal segregation. A significant effect was found for the interaction between docetaxel concentration and *xerC* deletion on transformation frequency (GLMM, χ^2= 21.312, df = 3, p <0.0001), but not growth rate (χ^2= 7.2884, df = 3, p = 0.06325). Both *xerC* deletion (χ^2 = 141.992, df = 1, p < 0.0001) and docetaxel concentration (χ^2 = 19.433,

df = 3, $p < 0.001$) significantly affect growth rate. Transformation frequencies of the Δ*xerC* strain were significantly lower than the wildtype in all treatments (EMMs comparison, $p < 0.0001$; Supplemental table 9; Supplemental table 10). Transformation frequency of the Δ*xerC* strain in 0.807µg/mL docetaxel increased relative to the control (EMMs comparison, t ratio = -2.233 , $p < 0.05$; Figure 9A; Supplemental table 9; Supplemental table 11), while the 80.7µg/mL treatment significantly decreased transformation frequency relative to the control, 0.807µg/mL, and 8.07µg/mL treatments (EMMs comparison, $p < 0.0001$; Figure 9A; Supplemental table 9; Supplemental table 11). Growth rates of the Δ*xerC* strain were significantly lower than the wildtype strain in all treatments (EMMs comparison, $p < 0.0001$; Figure 9B; Supplemental table 12; Supplemental table 13). Growth rates of the Δ*xerC* strain in the presence of 80.7µg/mL docetaxel were lower than the 0µg/mL, 0.807µg/mL, and 8.07µg/mL groups (EMMs comparison, $p < 0.001$; Figure 9B; Supplemental table 12, Supplemental table 14).

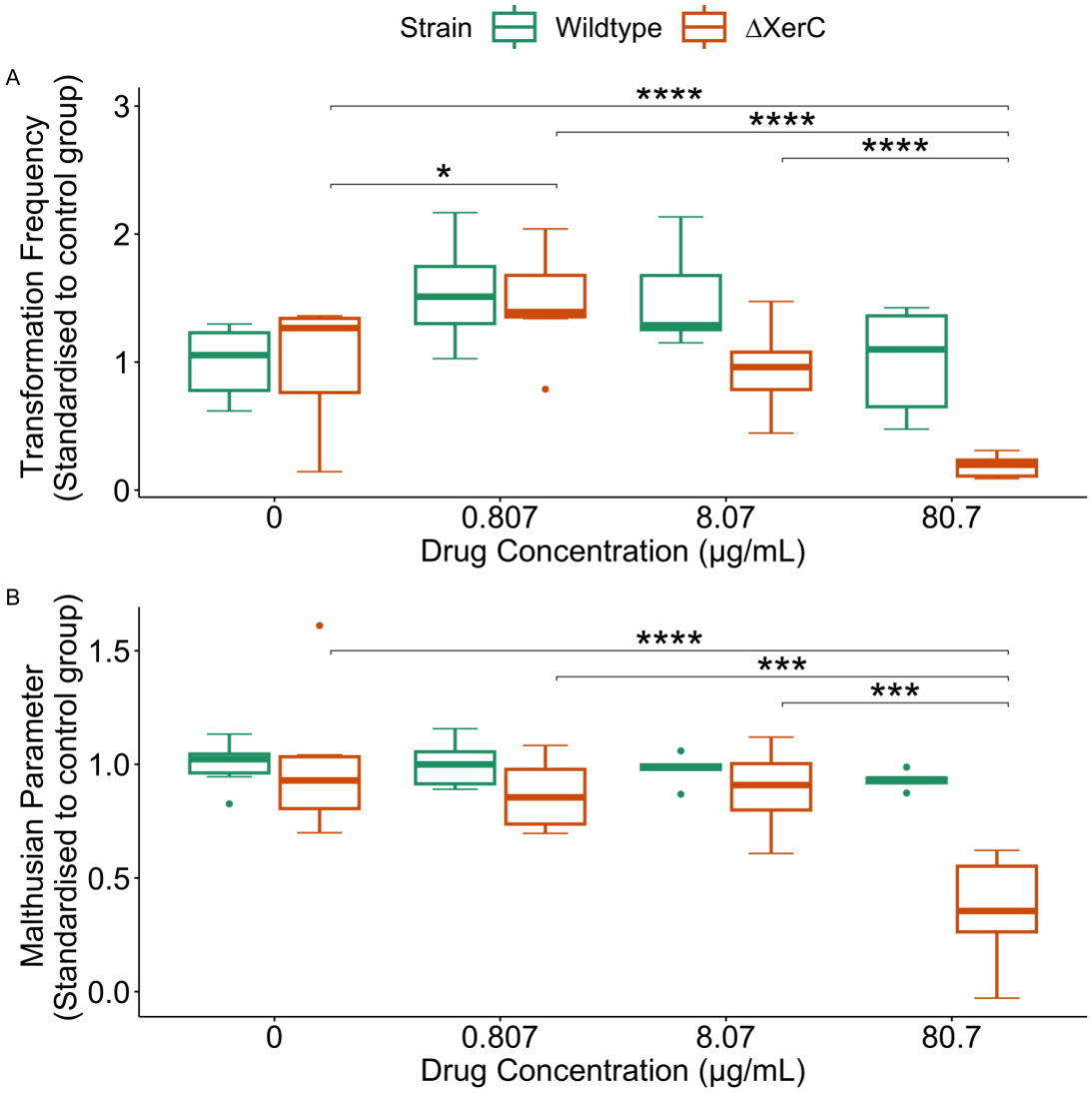

Figure 9. Effect of docetaxel on the transformation frequency (A) and growth rate (B) of a Δ*xerC* (orange) and a wildtype *A. baylyi* strain (green). (*, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$).

2.5 Discussion

Chemotherapy compounds increase rates of mutation in bacteria (Do Thi et al., 2011; Meunier et al., 2019), but it is not known if transformation frequency is affected by these compounds. Concurrent increases in transformation frequency would greatly accelerate dissemination of AMR, highlighting a significant risk for patient health. In this study, six chemotherapeutic drugs with diverse mechanisms of action were tested for their effect on natural transformation and growth rate in *A. baylyi.* Four compounds, cytarabine, exemestane, imatinib, and methotrexate, demonstrated no observable

effect on natural transformation or growth at clinically relevant concentrations, while two compounds (docetaxel and daunorubicin) caused a dose-dependent decrease in transformation frequency, with daunorubicin significantly decreasing growth rate. We conducted additional experiments testing the effects of two drugs, docetaxel and methotrexate, using mutants which we hypothesised would result in observing more extreme effects of the compounds. The consistent direction of effect of the wildtype and mutant strains suggested that these compounds are likely to have a similar effect on *A. baylyi* across a range of contexts.

Two chemotherapeutic drugs, docetaxel and daunorubicin, caused significant decreases in transformation frequency, and daunorubicin furthermore significantly decreased growth rate in *A. baylyi*. Docetaxel acts on eukaryotic cells by causing disruption of microtubule function leading to reduced cell proliferation (Herbst & Khuri, 2003). If cell proliferation in *A. baylyi* was arrested by docetaxel, a reduction in competence was expected as competence is linked to growth phase in this species (Palmen et al., 1993; Ray & Nielsen, 2005; Utnes et al., 2015). Interestingly, an effect of docetaxel was observed on transformation frequency, but not growth rate, suggesting that the mechanism of transformation frequency reduction by docetaxel is not closely associated with growth rate and may target a component of the DNA repair pathway or transformation machinery. Clinically relevant dosages of docetaxel are around 2.42 ng/mL (Morgan et al., 2009) and are therefore lower than those used in this study. No significant effects of docetaxel on growth or transformation frequency were observed at any concentration at or below 8.07µg/mL (Figures 7EF). The suppressive effect of docetaxel on transformation frequency at 80.7µg/mL was further investigated by engineering a Δ*xerC* strain which was expected to cause improper sorting of chromosomes during cell division (Blakely et al., 1991; Castillo et al., 2017; Mei et al., 2021). A Δ*xerC* strain should therefore be more sensitive to docetaxel than the wildtype counterpart as it is already compromised in an aspect of the cell division pathway or show no dose effect if XerC was the primary drug target. At the 80.7µg/mL concentration of docetaxel, transformation frequency and growth rate was observed to decrease for the Δ*xerC* strain but not the wildtype (Figure 9), indicating that *xerC* deletion increases drug sensitivity, and the drug target is likely to be another enzyme

involved in the replication pathway such as FtsZ (Bi & Lutkenhaus, 1991) or components of the DNA repair or transformation pathways.

Daunorubicin acts on eukaryotic cells by topoisomerase II inhibition and could act on bacterial cells in a similar mechanism to ciprofloxacin which binds to DNA gyrase, an enzyme belonging to the topoisomerase group (Reece et al., 1991). Exposure to daunorubicin could lead to altered rates of *gyrA* and *gyrB* transcription which are also upregulated in the SOS response in *A. baylyi* (Lu et al., 2022). Daunorubicin showed strong effects on both growth rate and transformation frequency where transformation frequency reduced to below detectable levels at 56.3µg/mL daunorubicin (Figures 7CD). Further, at lower concentrations, effects on transformation are independent to effects on growth rate and so the mechanism of action of daunorubicin may act uniquely on the transformation machinery. The mean concentration of daunorubicin found in leukemic cells during treatment is around 18.8µmol/L (Tidefelt et al., 2000), which is equivalent to 10.6µg/mL and is within the range tested in this study. Therefore, daunorubicin may have significant effects on rates of growth and transformation in *A. baylyi* at clinically relevant concentrations. However, it is unclear how concentrations of cancer chemotherapeutics in blood are related to those in blood serum. To our knowledge, only one study to date has attempted to estimate non-antibiotic drug concentrations in the gut (Maier et al., 2018), but exclusively considered oral drug administration. As both daunorubicin and docetaxel are administered intravenously, we cannot estimate the concentrations at which these drugs are found in the gut and thus the clinical relevance of the concentrations tested in this study. The mechanism for resistance to ciprofloxacin may also confer resistance to daunorubicin due to similar target enzymes. As such, upregulated *qnrS1* gene expression may confer resistance to daunorubicin in *A. baylyi* through non-competitive binding of Qnr proteins to DNA gyrase which may release daunorubicin from its target allowing re-ligation of DNA (Monárrez et al., 2018). However, it was not possible to test for this mechanism in this model system as all ciprofloxacin resistant mutants generated for this purpose displayed significantly reduced fitness making it incomparable to the wildtype strain (data not shown).

Cytarabine can pass through the blood-brain barrier and is found at concentrations of 347ng/mL to 1070ng/mL in cerebrospinal fluid depending on dosage administered, which is only 6-22% of the concentration found in plasma during treatment (Slevin et al., 1983). This suggests that plasma concentrations of cytarabine can reach up to 17.8µg/mL, which is within the range tested in this study. Any DNA damage caused by DNA lesions by cytarabine might lead to an induction of the SOS stress response (B. Jiang et al., 2020) and subsequent increase in DNA repair through natural transformation (Augsburger et al., 2019). However, no effect of cytarabine on transformation frequency or growth rate was observed in this study. This may be due to the presence of *pyrE* in the *A. baylyi* ADP1 genome (locus tag ACIAD_RS15940) which facilitates cytosine production and would hence compete with cytarabine for utilisation by DNA polymerase (Jensen, 1993).

Methotrexate interferes with B12 production in human cells by binding to dihydrofolate reductase and inactivating it (Alqarni & Zeidler, 2020). B12 is important for production of nucleotides and is used for treating malignancies as it causes stagnation of growth by preventing stores of B12 from being replenished following DNA replication (Cronstein, 1997). Transformation frequencies may therefore rise - in proportion to population size - as extracellular DNA fragments may have been used to extend replicating DNA fragments when nucleotides in the cytoplasm were depleted. Clinically relevant concentrations of methotrexate in blood plasma can reach 13.63µg/mL (Hornung et al., 2008), which is within the concentrations used in this study (Figure 7KL). This suggests that clinically relevant blood plasma concentrations of methotrexate do not influence growth rates or transformation frequency in *A. baylyi*. It is possible that the decrease in transformation frequency of the trimethoprim resistant mutant relative to its ancestor is due to the absence of selection for transformation during sequential passages on antibiotic selective agar. Other studies have observed reduction of transformation frequency in *A. baylyi* which support this hypothesis (Bacher et al., 2006; Renda et al., 2015; Utnes et al., 2015). The increase in fitness of the trimethoprim resistant strain is unexpected as a putative mutation in *folA* is not reported to increase fitness (Griffith et al., 2018). However, overexpression of *folA* to compensate for the inhibitory action of trimethoprim could have occurred in this strain (A. C. Palmer & Kishony, 2014) and may have led to the higher fitness through

increased pyrimidine production. It is possible that the combination of methotrexate and cytarabine may produce an effect on transforming cells as the reduced pyrimidine production caused by methotrexate may subsequently increase the reliance of cells on cytarabine for DNA replication while transforming or reproducing. However, there is no likely scenario in which these two compounds would be concurrently prescribed to a patient, so this effect was not tested for experimentally.

Exemestane is an aromatase inhibiting compound which leads to reduced oestrogen production in humans, making it effect in treating breast cancers which are responsive to oestrogen (Lombardi, 2002). It is not known if bacteria produce oestrogen, but they do respond to its presence. For example, vaginal flora is stable when oestrogen levels are high, but bacterial vaginosis can arise when oestrogen levels drop (Wilson et al., 2007). Gut bacteria also play a role in increasing bioavailability of oestrogen by secreting enzymes which deconjugate oestrogen into its active form (J. M. Baker et al., 2017). Our experiment tested for a direct interaction between exemestane and the rates of transformation and growth of *A. baylyi.* Since no literature exists testing for the direct effect of exemestane on any Gammaproteobacteria, no effect of the drug was expected, or an effect caused by a previously undescribed mechanism. Exemestane is present in plasma at concentrations of around 4.1ng/mL (Luo et al., 2018), which is markedly lower than those tested in this study (0.296µg/mL – 29.6µg/mL) and so suggests that clinically relevant concentrations of exemestane have no effect on transformation frequency or growth rate on *A. baylyi in vivo*. However, exemestane may have implications on growth of bacterial flora due to its suppression of oestrogen production (Wilson et al., 2007).

Imatinib acts as an Abelson tyrosine kinase (ATK) inhibitor in humans which leads to growth inhibition (De Kogel & Schellens, 2007). Since it is not known if bacteria produce or utilise ATKs, specific target could be suggested that the drug may act on for *A. baylyi*. Bacteria have a host of tyrosine kinases which facilitate a range of functions (Grangeasse et al., 2012), so shared affinity of the imatinib to human ATK and a bacterial homologue could have unpredictable effects. Imatinib is present in plasma at concentrations above 1µg/mL in clinical treatments (Gotta et al., 2014), which is within the range tested in this experiment (Figure 7IJ). This suggests that

clinically relevant concentrations of imatinib are unlikely to influence growth rate or transformation frequency in *A. baylyi*. ATKs are involved in invasion of human cells by bacteria such as *Salmonella enterica*, so inhibition of ATKs can lead to reduced rates of infection by such species (Ly & Casanova, 2009).

A. baylyi is a ubiquitous environmental bacterium which may come into contact with cancer chemotherapeutic pollutants in wastewater systems (Mantilla-Calderon et al., 2019; Schuster et al., 2022). Environmentally relevant concentrations of the drugs used in this study (Supplemental Table 15) are several orders of magnitude lower than those tested in this study, so it is unlikely that concentrations of these drugs in water systems are sufficient to affect rates of transformation or growth.

The limited effect these drugs have on the transformation frequency at clinically relevant concentrations is favourable, as it reduces or does not affect the rate of transformation events which can lead to increased pathogenicity (Johnston, Martin, et al., 2013), virulence (Frosch & Meyer, 1992), and antimicrobial resistance (Godeux et al., 2022; Hannan et al., 2010). However, more work should be done to confirm that other classes of drugs such as alkylating agents, mitotic inhibitors, corticosteroids, or compounds without groupings such as all-trans-retinoic acid and asparaginase do not influence rates of natural transformation. Additionally, synergistic interactions occurring between prescribed chemotherapeutics and other concurrently administered drugs cannot be ruled out. For example, fluoroquinolones have been demonstrated to increase transformation frequencies in bacteria (Boutry et al., 2013), and the anticancer drug mitoxantrone increases bacterial sensitivity to vancomycin when both drugs are administered concurrently (Da Silva et al., 2023), thereby acting as an antimicrobial adjuvant. More research must be conducted to properly understand the bioavailability of these drugs to gut bacteria, as bioaccumulation of drugs in the gut may lead to higher-than-expected levels of exposure of prescribed medications to gut bacteria as they may not correlate with drug plasma concentrations (Klünemann et al., 2021a), limiting possible inferences from this data. It is also important to test other species because not all transformable species react equally to genotoxic agents such as antibiotics (Charpentier et al., 2012) and species have different inducing stimuli for competence for natural transformation (Johnston et al., 2014), so naturally transformable species such as *Pseudomonas aeruginosa, Acinetobacter baumannii,*

Helicobacter pylori, Vibrio cholerae, Streptococcus pneumoniae, Legionella pneumophila, or *Bacillus subtilus* may react differently to *A. baylyi* in this system. Of those species, *V. cholerae, S. pneumoniae*, *B. subtilis,* and *L. pneumophila* have competence linked with classical SOS induction (following DNA damage) and may therefore be more likely to demonstrate transformation frequency changes during exposure to chemotherapeutic agents (Charpentier et al., 2011; Claverys et al., 2006; Rahman et al., 2022). Our data demonstrate the effect of some chemotherapeutic agents on natural transformation and growth rate of *A. baylyi* and identifies a need to future work to further investigate the effects and mechanisms of these and currently untested drugs to help to monitor and establish preventative measures to limit the spread of antimicrobial resistance genes between bacteria.

2.7 Supplemental tables

Supplemental table 1. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in varying concentrations of daunorubicin. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Drug Concentration (µg/mL)	EMM	SE	df	lower.CL	upper.CL
0	-10.104	0.62847	45	-11.96	-8.2495
0.563	-11.581	0.76513	45	-13.839	-9.3224
5.63	-14.178	0.8177	45	-16.593	-11.764
56.3	-36.821	4440.19	45	-13144	13070

Supplemental table 2. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying concentrations of daunorubicin. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Drug Concentration (µg/mL)	Drug Concentration (µg/mL)	EMM	SE	df	T-ratio	P value
0	0.563	1.48	0.617	45	2.392	0.042
0	5.63	4.07	0.682	45	5.972	<.0001
0	56.3	26.72	4440	45	0.006	0.996
0.563	5.63	2.6	0.802	45	3.239	0.0068
0.563	56.3	25.24	4440	45	0.006	0.996
5.63	56.3	22.64	4440	45	0.005	0.996

Supplemental table 3. Estimated marginal means (EMMs) of *A. baylyi* growth rate in varying concentrations of daunorubicin. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Drug Concentration (µg/mL)	emmean	SE	df	lower.CL	upper.CL
0	0.6129	0.103	45	0.3088	0.9171
0.563	0.4295	0.1571	45	-0.034	0.8934
5.63	0.117	0.1662	45	-0.373	0.6077
56.3	-0.312	0.1653	45	-0.8009	0.175

Supplemental table 4. Estimated marginal means (EMMs) contrasts of *A. baylyi* growth rate in varying concentrations of daunorubicin. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Drug Concentration (µg/mL)	Drug Concentration (µg/mL)	EMM	SE	df	T-ratio	P value
0	0.563	0.183	0.17	45	1.078	0.2867
0	5.63	0.496	0.179	45	2.777	0.0159
0	56.3	0.926	0.177	45	5.223	<.0001
0.563	5.63	0.313	0.213	45	1.466	0.1794
0.563	56.3	0.743	0.213	45	3.481	0.0034
5.63	56.3	0.43	0.22	45	1.952	0.0858

Supplemental table 5. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in varying concentrations of docetaxel. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Drug Concentration (µg/mL)	emmean	SE	df	lower.CL	upper.CL
0	-10.09	0.5705	46	-11.77	-8.41
0.807	-10.88	0.6302	46	-12.74	-9.026
8.07	-10.64	0.6301	46	-12.49	-8.78
80.7	-11.86	0.646	46	-13.76	-9.959

Supplemental table 6. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying concentrations of docetaxel. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Drug Concentration (µg/mL)	Drug Concentration (µg/mL)	EMM	SE	df	T-ratio	P value
0	0.807	0.183	0.17	45	1.078	0.2867
0	8.07	0.496	0.179	45	2.777	0.0159
0	80.7	0.926	0.177	45	5.223	<.0001
0.807	8.07	0.313	0.213	45	1.466	0.1794
0.807	80.7	0.743	0.213	45	3.481	0.0034
8.07	80.7	0.43	0.22	45	1.952	0.0858

Supplemental table 7. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in the wildtype and trimethoprim resistant mutant across all tested concentrations of methotrexate. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Strain	emmean	SE	df	lower.CL	upper.CL
Wildtype	-3.785	0.0258	44	-3.849	-3.72
Trimethoprim resistant strain	-4.508	0.0258	44	-4.572	-4.444

Supplemental table 8. Estimated marginal means (EMMs) of *A. baylyi* growth rate for the wildtype and trimethoprim resistant mutant across all tested concentrations of methotrexate. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Strain	emmean	SE	df	lower.CL	upper.CL
Wildtype	2.199	0.069	45	2.025	2.373
Trimethoprim resistant strain	3.595	0.069	45	3.421	3.769

Supplemental table 9. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in the wildtype and Δ*xerC* mutant across all tested concentrations of docetaxel. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Drug Concentration (µg/mL)	Strain	emmean	SE	df	lower.CL	upper.CL
0	Wildtype	-5.62	0.176	39	-5.97	-5.26
0	Δ*xerC*	-7.22	0.177	39	-7.58	-6.86
0.807	Wildtype	-5.17	0.176	39	-5.53	-4.81
0.807	Δ*xerC*	-6.66	0.176	39	-7.02	-6.31
8.07	Wildtype	-5.21	0.176	39	-5.56	-4.85
8.07	Δ*xerC*	-7.11	0.177	39	-7.47	-6.76
80.7	Wildtype	-5.66	0.176	39	-6.01	-5.3
80.7	Δ*xerC*	-8.78	0.177	39	-9.13	-8.42

Supplemental table 10. Estimated marginal means (EMMs) contrasts of wildtype and Δ*xerC* mutant *A. baylyi* strain transformation frequencies in varying concentrations of docetaxel grouped by drug concentration. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Drug Concentration (µg/mL)	Strain	Strain	EMM	SE	df	T-ratio	P value
0	Wildtype	Δ*xerC*	1.6	0.25	39	6.429	<.0001
0.807	Wildtype	Δ*xerC*	1.49	0.25	39	5.981	<.0001
8.07	Wildtype	Δ*xerC*	1.91	0.25	39	7.64	<.0001
80.7	Wildtype	Δ*xerC*	3.12	0.25	39	12.495	<.0001

Supplemental table 11. Estimated marginal means (EMMs) contrasts of wildtype and Δ*xerC* mutant *A. baylyi* strain transformation frequencies in varying concentrations of docetaxel grouped by strain. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Strain	Drug Concentration (µg/mL)	Drug Concentration (µg/mL)	contrast	SE	df	T-ratio	P value
Wildtype	0	0.807	-0.4458	0.25	39	-1.786	0.1637
Wildtype	0	8.07	-0.4086	0.25	39	-1.637	0.1645
Wildtype	0	80.7	0.0412	0.25	39	0.165	0.8821
Wildtype	0.807	8.07	0.0373	0.25	39	0.149	0.8821
Wildtype	0.807	80.7	0.487	0.25	39	1.951	0.1637
Wildtype	8.07	80.7	0.4498	0.25	39	1.802	0.1637
Δ*xerC*	0	0.807	-0.5575	0.25	39	-2.233	0.047
Δ*xerC*	0	8.07	-0.1062	0.25	39	-0.426	0.6728
Δ*xerC*	0	80.7	1.5563	0.25	39	6.233	<.0001
Δ*xerC*	0.807	8.07	0.4512	0.25	39	1.808	0.094
Δ*xerC*	0.807	80.7	2.1138	0.25	39	8.466	<.0001
Δ*xerC*	8.07	80.7	1.6625	0.25	39	6.658	<.0001

Supplemental table 12. Estimated marginal means (EMMs) of *A. baylyi* growth rate for the wildtype and Δ*xerC* mutant across all tested concentrations of docetaxel. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

Drug Concentration (µg/mL)	Strain	emmean	SE	df	lower.CL	upper.CL
0	Wildtype	2.636	0.081	39	2.387	2.885
0	Δ*xerC*	0.938	0.081	39	0.689	1.187
0.807	Wildtype	2.639	0.081	39	2.39	2.888
0.807	Δ*xerC*	0.814	0.081	39	0.565	1.063
8.07	Wildtype	2.584	0.081	39	2.335	2.833
8.07	Δ*xerC*	0.835	0.081	39	0.586	1.084
80.7	Wildtype	2.453	0.081	39	2.205	2.702
80.7	Δ*xerC*	0.336	0.081	39	0.087	0.585

Supplemental table 13. Estimated marginal means (EMMs) contrasts of wildtype and Δ*xerC* mutant *A. baylyi* strain growth rates in varying concentrations of docetaxel grouped by drug concentration. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Drug Concentration (µg/mL)	Strain	Strain	EMM	SE	df	T-ratio	P value
0	Wildtype	Δ*xerC*	1.7	0.116	39	14.681	<.0001
0.807	Wildtype	Δ*xerC*	1.82	0.116	39	15.778	<.0001
8.07	Wildtype	Δ*xerC*	1.75	0.116	39	15.122	<.0001
80.7	Wildtype	Δ*xerC*	2.12	0.116	39	18.305	<.0001

Supplemental table 14. Estimated marginal means (EMMs) contrasts of wildtype and Δ*xerC* mutant *A. baylyi* strain growth rates in varying concentrations of docetaxel grouped by strain. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Strain	Drug Concentration (µg/mL)	Drug Concentration (µg/mL)	EMM	SE	df	T-ratio	P value
Wildtype	0	0.807	-0.00282	0.116	39	-0.024	0.9807
Wildtype	0	8.07	0.05194	0.116	39	0.449	0.7871
Wildtype	0	80.7	0.18272	0.116	39	1.58	0.3667
Wildtype	0.807	8.07	0.05476	0.116	39	0.473	0.7871
Wildtype	0.807	80.7	0.18554	0.116	39	1.604	0.3667
Wildtype	8.07	80.7	0.13078	0.116	39	1.131	0.5301
Δ*xerC*	0	0.807	0.12408	0.116	39	1.073	0.4349
Δ*xerC*	0	8.07	0.1029	0.116	39	0.89	0.4549
Δ*xerC*	0	80.7	0.60187	0.116	39	5.204	<.0001
Δ*xerC*	0.807	8.07	-0.02118	0.116	39	-0.183	0.8556
Δ*xerC*	0.807	80.7	0.47779	0.116	39	4.131	0.0004
Δ*xerC*	8.07	80.7	0.49897	0.116	39	4.314	0.0003

Supplemental table 15. Concentrations of chemotherapeutic compounds found in the environment.

Drug	Mechanism of Action / Drug Target	Environmental Concentration
Cytarabine	Cytosine analogue	Canadian untreated hospital sewage – 0.000µg/L – 2018 (Graumnitz & Jungmann, 2021)
		Canadian WWTP influent – 0.924µg/L – 2019 Canadian WWTP effluent – 0.217µg/L – 2019 (Graumnitz & Jungmann, 2021)
Daunorubicin	Topoisomerase II inhibitor	Treated Brazilian hospital sewage - 3.69µg/L – 2017 (Graumnitz & Jungmann, 2021)
		Canadian surface water (outlet to lake) – 0.011µg/L – 2014 (Graumnitz & Jungmann, 2021)
Docetaxel	Disrupts microtubule function	Treated Catalonian hospital sewage – 0.079µg/L – unknown date (Graumnitz & Jungmann, 2021)
		Catalonian WWTP inflow – 0.219µg/L – unknown date Catalonian WWTP outflow – 0.000µg/L – unknown date (Graumnitz & Jungmann, 2021)
Exemestane	Aromatase inhibitor (oestrogen synthesis inhibitor)	Swiss surface water (river/stream) – 0.01µg/L – 2007 (Graumnitz & Jungmann, 2021)
		Swiss WWTP effluent – 0µg/L -2007 (Graumnitz & Jungmann, 2021)
Imatinib	Tyrosine kinase inhibitor	UK WWTP treated effluent – 0.302µg/L – 2018 (Graumnitz & Jungmann, 2021)
		UK surface water (River) - 0.183µg/L – 2018 (Graumnitz & Jungmann, 2021)
Methotrexate	Folate synthesis inhibitor	Jordanian untreated hospital sewage – 699µg/L – Unknown date (Graumnitz & Jungmann, 2021)

		USA surface water (Creek) - 0.034µg/L – 2012 (Graumnitz & Jungmann, 2021)

Chapter 3: Biotic Interactions Can Significantly Affect Transformation Frequency in *Acinetobacter baylyi.*

3.1 Abstract

Natural transformation, a main mechanism of horizontal gene transfer, is the uptake of extracellular DNA used for chromosomal recombination or storage as an extrachromosomal element. Genes conferring resistance to antimicrobial agents can be disseminated between species via natural transformation, indicating the need to understand the contexts under which transformation frequencies may be altered in natural environments. While we understand many abiotic factors can affect transformation frequencies in bacteria, no work has yet been done to test the effect of other species on natural transformation. Twenty-six phylogenetically diverse species were tested for their effect on *A. baylyi* natural transformation through pairwise competition and estimated a strong negative effect of 16 species. To further provide insight into how biotic interactions affect natural transformation, two species belonging to the *Bordetella* and *Bacillus* genus, respectively, were tested to determine if effects are caused by cell contact or extracellular compounds. Presence of *Bordetella* was found to increase transformation frequency of *A. baylyi* by releasing extracellular compound(s) which are likely not proteinaceous. Next, the effect of competitor species presence on *A. baylyi* transformation changes was tested for as a function of nutrient availability, as nutrient availability is known to affect transformation frequencies in many species. *Bacillus* was found to have a strong negative effect on transformation frequency but only at low nutrient concentrations. This work highlights the significance of interspecies competition on transformation frequency changes and shows the importance of utilising ecologically-relevant nutrient availabilities when testing natural transformation as it can significantly affect the effect of species interactions.

3.2 Introduction

Horizontal gene transfer (HGT) is arguably the main driver of prokaryotic diversity (Gogarten et al., 2002; Kuo & Ochman, 2009; Philippe & Douady, 2003). The ability to take up alleles, genes and operons from other strains and species can greatly speed

up the acquisition of novel traits relative to the stepwise accumulation of mutations (Cohen et al., 2005; Jansen et al., 2013; Vos, 2009). One main mechanism of HGT is natural transformation, where bacteria develop a physiological state termed "competence" to take up cell-free DNA from the environment (Blokesch, 2016; Johnston et al., 2014). As much as 40% of ssDNA internalised by natural transformation is used for chromosomal recombination (Kidane et al., 2012) and extrachromosomal elements such as plasmids can also be taken up by natural transformation (Yu et al., 2022). Natural transformation is also recognised to disseminate antimicrobial resistance through the acquisition of antimicrobial resistance genes released by kin or other species in environmental and clinical contexts (Von Wintersdorff et al., 2016; Winter et al., 2021), and so understanding the circumstances under which bacteria transform can help us to predict the spread of resistance genes in the environment.

The environmental conditions that induce competence widely vary across species (Seitz & Blokesch, 2013), with some species being constitutively competent (Blokesch, 2016; Hülter et al., 2017; Moore et al., 2014; Ray & Nielsen, 2005; Vaneechoutte et al., 2006), whilst other species become competent in response to nutrients (Palmen et al., 1993; Nielsen, Bones and Van Elsas, 1997; Watson and Carter, 2008; Sun et al., 2013; Johnston et al., 2014) or stress (DNA damage, antibiotic treatment) (Charpentier et al., 2012; Engelmoer & Rozen, 2011; Hülter et al., 2017; Jin et al., 2020). Although we have an increasing understanding of the many abiotic factors affecting transformation, our understanding of biotic factors that might affect transformation is still very limited, with most studies investigating natural transformation utilising lab strains in isolation. Natural microbial communities are generally highly diverse and host a wide array of interspecific interactions, including predation (R. M. Cooper et al., 2017; Molina-Quiroz et al., 2020; Veening & Blokesch, 2017), competition (Ortiz et al., 2021; Rosenberg et al., 2016; Zheng et al., 2013) and mutualism (reciprocal cross-feeding) (D'Souza et al., 2018; Pande et al., 2015; N. W. Smith et al., 2019). It can be hypothesised that some of these species' interactions could affect cell physiology and the frequency of natural transformation, but this has not yet been tested.

This study used *Acinetobacter baylyi* – a model bacterium for natural transformation – to test how coculturing with a range of competitor species affects transformation rates. *A. baylyi* is constitutively naturally competent with peak transformation frequency at exponential growth (Elliott & Neidle, 2011; Hülter et al., 2017; Palmen et al., 1993; Ray & Nielsen, 2005). Its relatedness to *A. baumannii,* a notorious multidrug resistant pathogen (Brovedan et al., 2020; Fournier et al., 2006; Xiao et al., 2017), also makes *A. baylyi* a clinically relevant model for transformation-mediated transfer of antimicrobial resistance (AMR) genes.

A. baylyi was incubated in direct pairwise competition with 26 phylogenetically diverse soil bacterial isolates while providing conspecific DNA as a substrate for natural transformation (Figure 10). The effect of genetic relatedness of *A. baylyi* to competitor species was analysed in this study as genetic relatedness can have implications on species interactions, suggesting antagonistic (competition for similar resources, overlapping niches) or mutualistic effects (cross-feeding) (Dunny et al., 2008). The effects of biotic interactions on transformation rates were tested to determine if they can change across nutrient gradients such as those seen across different niches in the environment. Species can interact via direct cell-cell contact or indirectly through the release of compounds into the environment (D'Souza et al., 2018; James et al., 1995; Kuramitsu et al., 2007; Stubbendieck et al., 2016). Using supernatant instead of a cell culture, it could be tested whether the observed effects on natural transformation are largely mediated by cell contact or extracellular compounds. The effect of boiled supernatant was tested to determine if extracellular compounds affecting transformation frequency were likely to be proteinaceous.

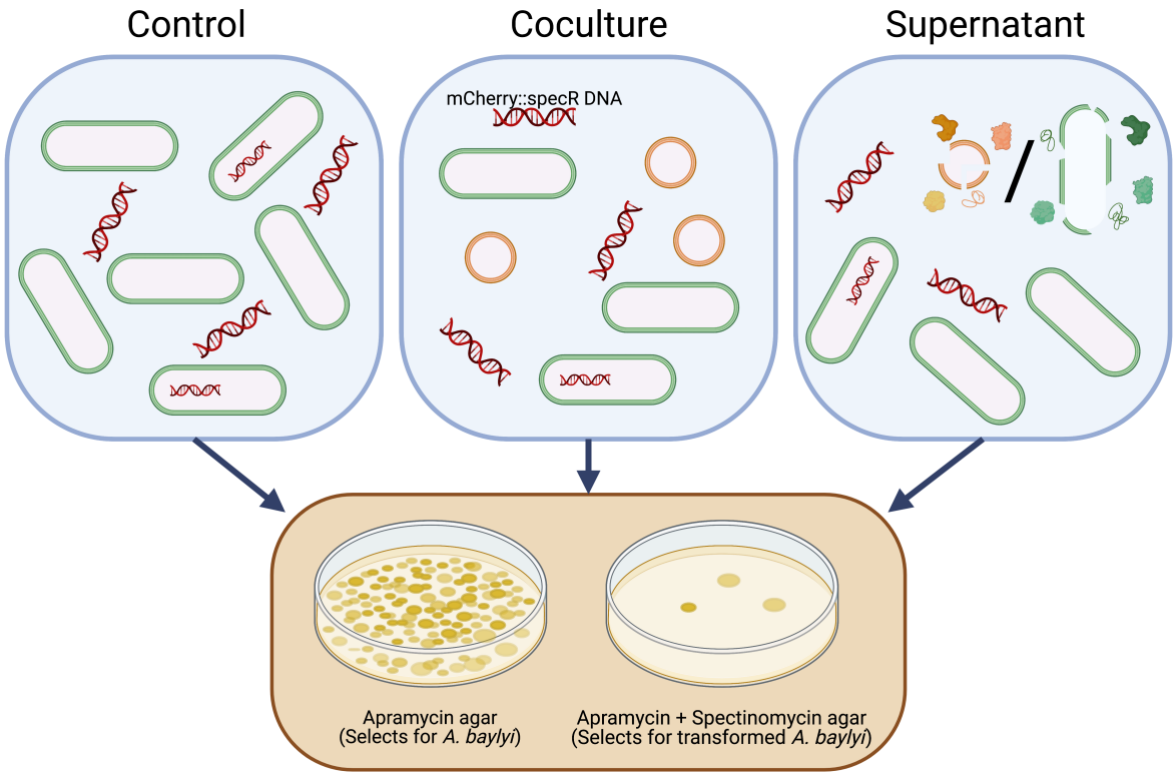

Figure 10. Schematic of the assays used to test for the effect of direct (competition) and indirect (supernatants) competitor interactions on the transformation frequency of *A. baylyi* (green cell). Each experimental condition (blue boxes) is tested in LB broth and supplemented with cell lysate containing copies of a spectinomycin resistance gene. Cultures are plated on selective agar to enumerate the starting cell densities (yellow box) and incubated at 28˚C for 5 hours before plating on selective agar (yellow box). For the abiotic control, double the volume of focal strain overnight culture was used to ensure the same number of possible generations were observed across treatments. Supernatants were also tested after boiling for 10 minutes at 100˚C. Figure created with Biorender.com.

3.3 Methods

3.3.1 Bacterial strains.

A green-fluorescent apramycin-resistant *A. baylyi* ADP1 was used as the focal strain for the natural transformation assays (Winter, Harms, Johnsen, Buckling, et al., 2023). A red-fluorescent spectinomycin-resistant *A. baylyi* ADP1 (WT-red) was used as a DNA donor as described below (Winter, Harms, Johnsen, Buckling, et al., 2023). Twenty-six bacterial environmental strains previously isolated from potting soil and

classified to the level of genus based on 16S rRNA sequencing (Hesse et al., 2018) were used as competitors. *A. baylyi* and competitor strain 16S rRNA gene sequences were aligned using the ClustalW algorithm, and genetic relatedness calculated in MEGA X v11.0.11 using the p-distance function (Kumar et al., 2018).

3.3.2 Transformation assays.

Freezer stocks of WT-green and non-focal strains were individually grown in LB broth (Formedium, England) for 48 hours at 28˚C. One hundred microliters of each overnight culture and 200µL of *mCherry::specR* heat-killed lysate were added to 1.6mL LB broth to yield a final volume of 2mL. Lysate was produced by heating overnight cultures of a red-fluorescent *A. baylyi* ADP1 at 70˚C for 1.5 hours and centrifuging to concentrate 50x. Lysate was confirmed to be sterile by plating on LB agar and stored at -20˚C for a maximum of one month before use in the transformation assay. For the abiotic control, 200µL of *A. baylyi* overnight culture was used to ensure total cell densities were constant between treatment and control samples. Transformation assays were performed for 5 hours at 28˚C and 180rpm. For assays involving supernatant in lieu of a second species, aliquots of a subset of strains' overnight cultures were centrifuged at 2700xg for 10 minutes and passed through a 0.22µm filter to be used as supernatant. Excess supernatant was added to LB broth and incubated in the above conditions for 24 hours to confirm sterility. Half of each supernatant sample was heated to 100˚C for 10 minutes before use to denature any heat-labile structures or compounds which may affect *A. baylyi* transformation frequency. When added, supernatant replaced 50% of the LB broth volume. To test whether the presence of another species modifies the effect of nutrients on transformation frequency, cultures were prepared as described above and added to 1.6mL of LB broth at 1%, 10%, or 100% strength with 200µL of *mCherry::specR* heat-killed lysate to yield a final volume of 2mL. Cultures in all assays were plated on LB agar supplemented with 240µg/ml apramycin (Duchefa, The Netherlands) to select for WT-green or 240µg/ml apramycin and 360µg/ml spectinomycin (Melford, UK) to select for transformants and incubated for 48 hours at 28˚C. All treatments were sampled at a minimum of sixfold replication. Transformation frequency was calculated by dividing the CFU/mL of the transformed population by the total population CFU/mL. Values from samples which involved competition were standardised to the monoculture control within the same block and assayed at the same nutrient concentration by dividing by the monoculture control.

3.3.3 Statistical analysis

Due to the unbalanced nature of the experimental design, a Bayesian modelling framework was used to test how the presence of competitors, and their identity, affected transformation frequency. In particular, how the presence of phylogenetically diverse competitors affected transformation frequency was tested using a hierarchical Bayesian linear model (warmup = 1000, post warmup = 3000, chains = 5, cores = 4 and weakly informed priors) with log-standardised transformation frequency as response variable, random intercepts fitted for competitors and blocks, separate standard deviation terms allowed to vary for each block, and default priors in 'brms' (v2.19.1; Bürkner, Weber, et al., 2023). Frequencies were standardized by dividing individual estimates by the mean of the abiotic control in each block; estimated values with credible intervals (CIs) not overlapping 0 (i.e. log(1)) are considered significant. R-hat values for all intercepts for the model selected for analysis were ≤1. Bulk and tail effective sample sizes were in excess of 2800 for all intercepts. The model above, was chosen over models of less (no group-specific variation) or greater complexity (testing the additional effects of *A. baylyi* Malthusian growth parameter and competitor genetic distance) after checking model behaviour (pp_check) and model selection by Pareto smoothed importance sampling leave one out (PSIS-LOO) cross validation (Vehtari et al., 2017). Region of practical equivalence (ROPE) analysis was used to determine if estimated values which were practically different to the monoculture control by determining the percentage of posterior values which were below 0. Expected values (estimated marginal means of posterior distribution; 'EMMs) were calculated to test (1) whether the presence of any competitor has an effect, independent of any modelled group effects; (2) whether competitor identity has an effect; and (3) to generalise predictions beyond the subset of species used, how the presence of a 'hypothetical' new species would affect expected transformation rate of *A. baylyi* was tested by taking random draws from fitted model estimates.

The Kenward-Roger method was used to estimate the degrees of freedom in EMMs comparison. The 95% CIs were calculated as the 95% highest posterior density interval (HPDI), with only one observation falling outside the predicted CIs. Bayesian modelling in R was performed using rstan v2.21.8 (The Stan Development Team, 2023), rstanarm v2.21.4 (Goodrich et al., 2023), cmdstanr v0.5.3 (Gabry & Češnovar,

2023), rethinking v2.31 (Ross et al., 2023), posterior v1.4.1 (Bürkner, Gabry, et al., 2023), and bayestestR v0.13.1 (Makowski et al., 2023).

To test for the effect of secreted elements and nutrient availability on transformation frequency in control populations of *A. baylyi*, a generalised linear mixed model (GLMM; (lmer() function in the 'lme4' package; Bates et al., 2015)) was used with the number of transformed vs non-transformed cells μL^{-1} as binomial response variable, and 'environment' (cell supernatant, boiled cell supernatant) and 'nutrient' (1%, 10%, 100% LB concentration) as fixed effects, respectively. To model non-dependency of observations, random intercepts for day of measurement were fitted. Residual behaviour was checked using the 'DHARMa' package (Hartig, 2022). To test whether the presence and identity of competitors changes the effect of spent media (competitor cell supernatant) on transformation frequency, a gaussian linear mixed model (LMM) was carried out with transformation frequency (calculated as the number of transformed cells divided by the total number of potential recipient cells) as a response variable and environment (interspecific competition, competitor cell supernatant, boiled competitor cell supernatant) and competitor species identity as fixed effects with no interaction. To test whether the presence and identity of competitors changed the effect of nutrients on transformation frequency, the number of transformed vs non-transformed cells μL^{-1} as binomial response variable was again used in a beta binomial GLMM to test for the interactive effect of competitor and nutrient availability on transformation frequencies. Based on the obtained simulation-based residual plots, treatment-specific dispersion parameters were included in the above model, using the glmmTMB() function in the glmmTMB package v1.1.3 (Brooks et al., 2017), with 'dispformula = ~ competitor' for the effect of competitor species, including the monoculture control. For all analyses, the most parsimonious model was arrived at by sequentially deleting terms and comparing model fits using χ^2-tests, after which pairwise contrasts were computed using the 'emmeans' packages v1.8.6 (Length, 2023), with $\alpha < 0.05$. Graphs were made using ggplot2 v3.3.6 (Wickham, 2016), bayesplot v1.10.0 (Gabry & Mahr, 2023), and gghalves v0.1.4 (Tiedemann et al., 2023). In all analyses, a *p* value of less than 0.05 was considered significant. *P* values were corrected for multiple testing, where appropriate, using false discovery rate (FDR) adjustments (Benjamini & Hochberg, 1995).

3.4 Results

3.4.1 Species presence generally decreases natural transformation frequency in *A. baylyi*

To test whether the presence of another species affected the transformation frequency of *A. baylyi*, pairwise direct competition assays were performed with 26 bacteria against *A. baylyi*, first investigating the overall effect of species presence. The presence of other species has a varied effect on *A. baylyi* transformation frequency, with most species decreasing transformation frequency in *A. baylyi* (Figure 11). The predicted average effect of competitors on transformation frequency results in a decrease in transformation frequency in *A. baylyi* (estimated average log transformation frequency change = -0.5, HDI = -1.06 to 0.06). Region of practical equivalence (ROPE) analysis showed 98.65% of expected observations are below 0, which is congruent with the above test suggesting a global negative effect of competitors on *A. baylyi* transformation frequency (ROPE limits = -6, 0). The model explained only 42.2% (R^2) of the variation in the data, implying that at least one important variable was likely missed. Despite this, the model predicts that the presence of other species generally decreases transformation frequency in *A. baylyi*.

Figure 11. Raw data values for *A. baylyi* transformation frequency changes in the presence of other species. Transformation frequencies are standardised to the monoculture control and log transformed. Left: raw data, right: box plot of data. The box in the box plot represents the interquartile range, which is the range between the first quartile and the third quartile with the mean value denoted as a horizontal line within the box. Box plot whiskers extend to the minimum and maximum values within 1.5x the interquartile range from quartiles 1 and 3, respectively.

Table 4. Posterior values for log transformation frequency changes in *A. baylyi* when in coculture with a competitor species. All highest density intervals (HDIs) have a width of 95%.

Competitor	**mean**	**Lower HDI**	**Upper HDI**	**Does HDI overlap with 0?**	**Genetic Distance from *A. baylyi* ADP1**
Achromobacter	-0.4217517	-0.8817283	0.07692945	Yes	0.2406
Acidivorax	-0.2958201	-0.7686026	0.2322497	Yes	0.2999
Acinetobacter	-0.6125236	-1.0527946	-0.1580506	No	0.032
Arthrobacter	-0.5316888	-1.0579501	-0.015402	No	0.3554
Bacillus	-0.9520111	-1.5384784	-0.3849614	No	0.355
Bordetella 1	-0.1646202	-0.5055205	0.20519077	Yes	0.2461
Bordetella 2	-0.6507922	-1.1607138	-0.1417619	No	0.217
Brevundimonas	-0.4112773	-0.7668816	-0.0512367	No	0.2646
Candidimonas	-0.8912287	-1.2289539	-0.5255694	No	0.2092
Devosia	-0.7224445	-1.097733	-0.3804707	No	0.2416
Flavobacterium	-0.5022226	-0.8480336	-0.1497704	No	0.4059
Lysinibacillus	-0.2450847	-0.6106463	0.12851955	Yes	0.3578
Microbacterium	-0.4348228	-0.7634075	-0.089799	No	0.3506
Ochrobactrum	-0.4776054	-0.837421	-0.0980509	No	0.2378
Paenibacillus 1	-0.9505372	-1.4100036	-0.5156304	No	0.3689
Paenibacillus 2	-0.2275202	-0.6383149	0.20851875	Yes	0.3951
Paracoccus 1	-0.2599163	-0.687385	0.17323045	Yes	0.26
Paracoccus 2	-0.5576629	-0.9683251	-0.1470195	No	0.2548
Pigmentiphaga	-0.6362081	-0.9994013	-0.2435373	No	0.245
Pseudomonas	-0.675631	-1.0563808	-0.2857402	No	0.231
Pusillimonas	-0.6169995	-1.0141186	-0.2205662	No	0.2162
Rhizobium	-0.285254	-0.653731	0.08362465	Yes	0.2592
Rhodococcus	-0.1421422	-0.5619001	0.26323791	Yes	0.3551
Shinella	-0.48419	-1.0114804	0.02645189	Yes	0.2611
Staphylococcus	-0.78158	-1.2704623	-0.2770507	No	0.4463
Variovorax	-0.322599	-0.7866923	0.19258363	Yes	0.2831

3.4.2 The effect of species presence on *A. baylyi* natural transformation frequency is species dependent

As the overall effect of species on *A. baylyi* natural transformation frequency was weakly negative, the effect of species-specific interactions was investigated. Bayesian posterior predictions for species-specific effects on *A. baylyi* transformation frequency were validated by comparing against observed data (Figure 12). Most observed data points fall within the 95% credibility interval (CI) for each competitor, validating model

predictions (Figure 12). The estimated effect of competitor is varied, with multiple species having a negative effect on *A. baylyi* transformation frequency, suggested by CIs not overlapping 0 (EMMs comparison; Table 4). Posterior predictions of sixteen competitors were estimated to have a strong effect on decreasing transformation frequency of *A. baylyi* during coculture, with *Bacillus* showing the strongest negative effect (Figure 13; Table 4). Inclusion of genetic distance into Bayesian modelling did not significantly improve model fit or help to explain transformation frequency changes in *A. baylyi* (PSIS-LOO, ELPD difference = -0.6, SE difference = 0.4). Similarly, inclusion of *A. baylyi* growth rate (reported as the Malthusian parameter) did not significantly improve model predictions or explain transformation frequency changes in *A. baylyi* (PSIS-LOO, ELPD difference = -1.3, SE difference = 0.7). These data suggest that the effects of competitor species presence on *A. baylyi* natural transformation frequency are varied across competitors and might be driven by factors other than genetic distance and growth rate changes.

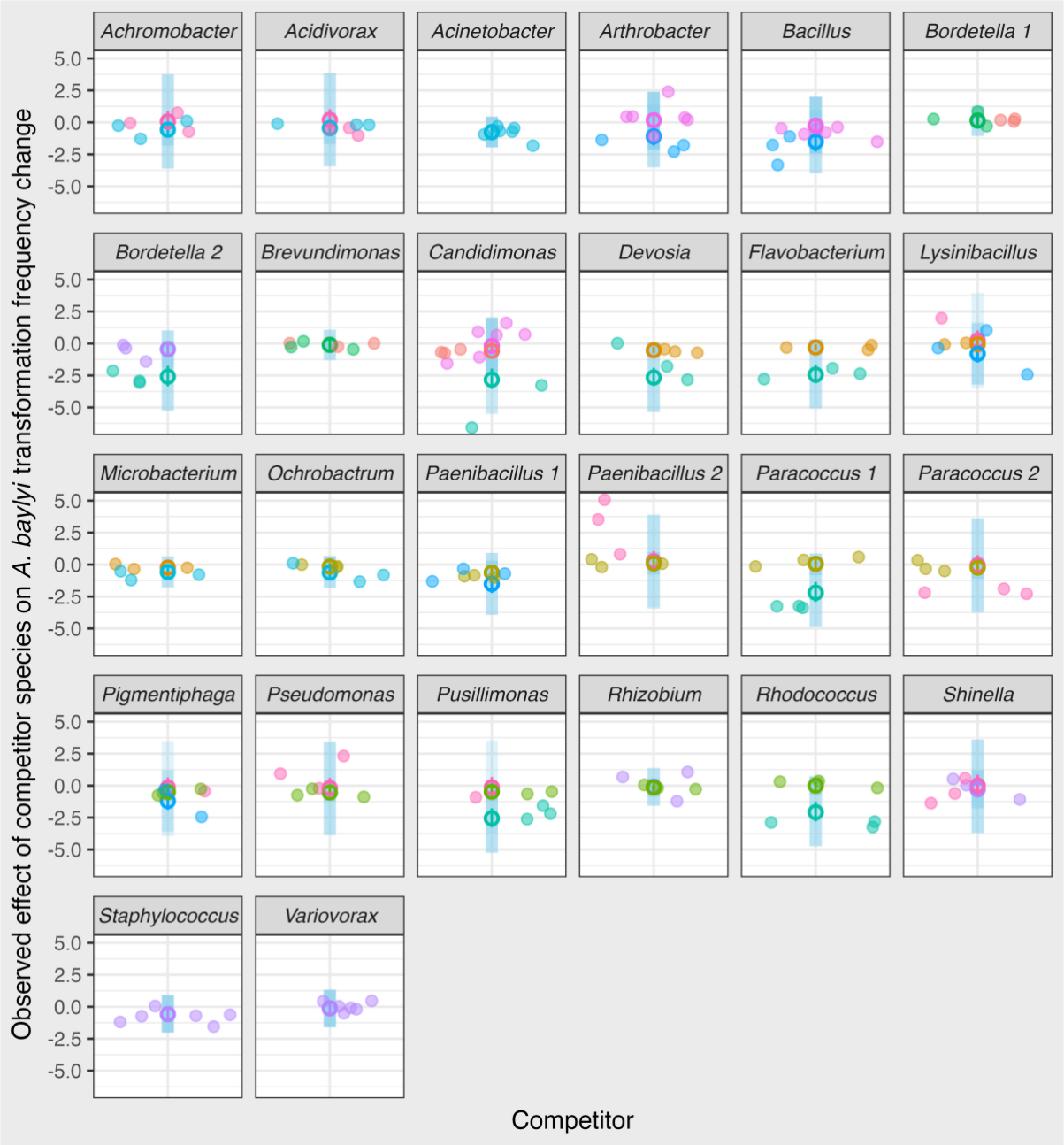

Figure 12. Comparison of posterior estimations against observed standardised transformation frequency values. Posterior check for individual species. Key: closed circles, observed values; open circles, estimated posterior mean values with 95% confidence intervals (lines); blue bars, posterior estimated value range; colours, block. Points are scattered left to right for improved visibility only.

3.4.3 Compounds produced by competitors are responsible for transformation frequency changes in *A. baylyi*

To determine the effect of direct vs indirect competition on transformation frequency changes in *A. baylyi,* two competitors were focussed on: *Bacillus,* which significantly

decreased *A. baylyi* transformation frequency when present (Figure 13) and *Bordetella 1* which produced a weak negative effect on *A. baylyi* transformation frequency (Figure 13). *A. baylyi* was cultured in the presence of supernatant from *Bacillus* or *Bordetella*, and in the presence of boiled competitor supernatant to test for the effect extracellular compounds on natural transformation and to determine if effects are caused by heat labile (and therefore potentially proteinaceous) compounds, respectively. Samples without competitor species or their products were used as controls (*A. baylyi* in monoculture, *A. baylyi* in autologous spent media, and *A. baylyi* in boiled autologous spent media). There was no significant effect of *A. baylyi* spent media on *A. baylyi* transformation frequency (pairwise EMMs comparison, $p > 0.05$).

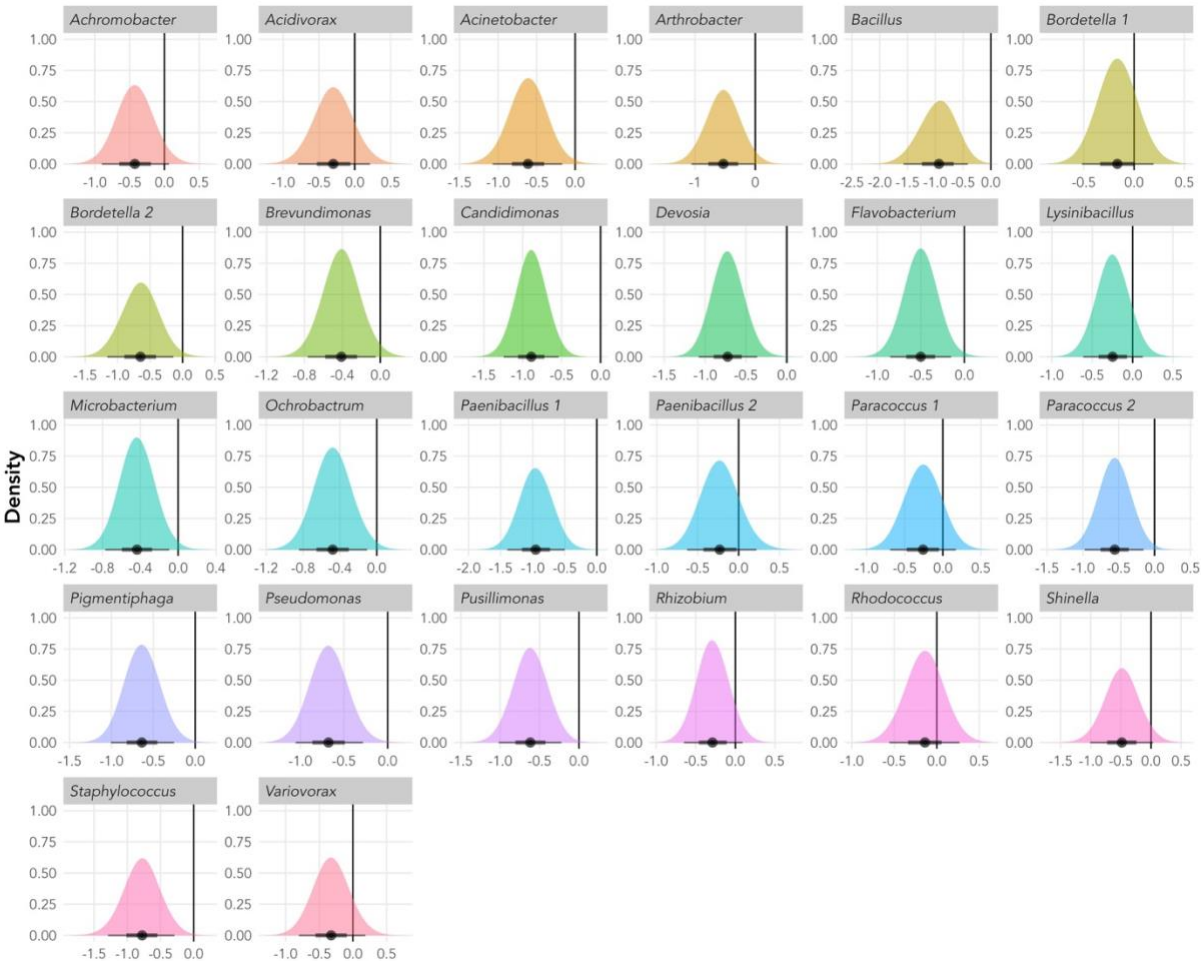

Figure 13. Effect of individual species on transformation frequency in *A. baylyi*. Distributions represent posterior estimations of log transformation frequency changes (x axis) during coculture with the respective competitor species. The vertical black line represents the mean of *A. baylyi* transformation frequency in monoculture. Thick and

thin black lines represent the 80% and 95% credible intervals, respectively. The black circle represents the mean estimated effect on transformation frequency change.

For treatments involving the two competitor species, the effect of competitor environment (interspecies competition, supernatant, boiled supernatant) on transformation frequency was consistent across competitor species, evidenced by no observed significant interaction between test environment or competitor species was found (LMM, χ^2 = 2.8629, df = 4, p = 0.581). Competitor species identity strongly affected transformation frequency in *A. baylyi* (LMM, χ^2 = 13.224, df=2, p < 0.01), but test environment did not (LMM, χ^2 = 0.9902, df = 2, p = 0.6095). Transformation frequency of *A. baylyi* was observed to increase in competition with *Bordetella* or *Bordetella* cell products compared to the monoculture control (pairwise contrast, t-ratio = 3.711, df = 49, p < 0.01; Figure 14; supplemental table 16; supplemental table 17), and competition with *Bacillus* or *Bacillus* cell products (pairwise contrast, t-ratio = 2.695, df = 49, p < 0.05; Figure 14; supplemental table 16; supplemental table 17). Transformation frequency of *A. baylyi* was not affected by competition with *Bacillus* or *Bacillus* cell product (pairwise contrast, t-ratio = 1.016, df = 49, p > 0.3145; Figure 14; supplemental table 16; supplemental table 17).

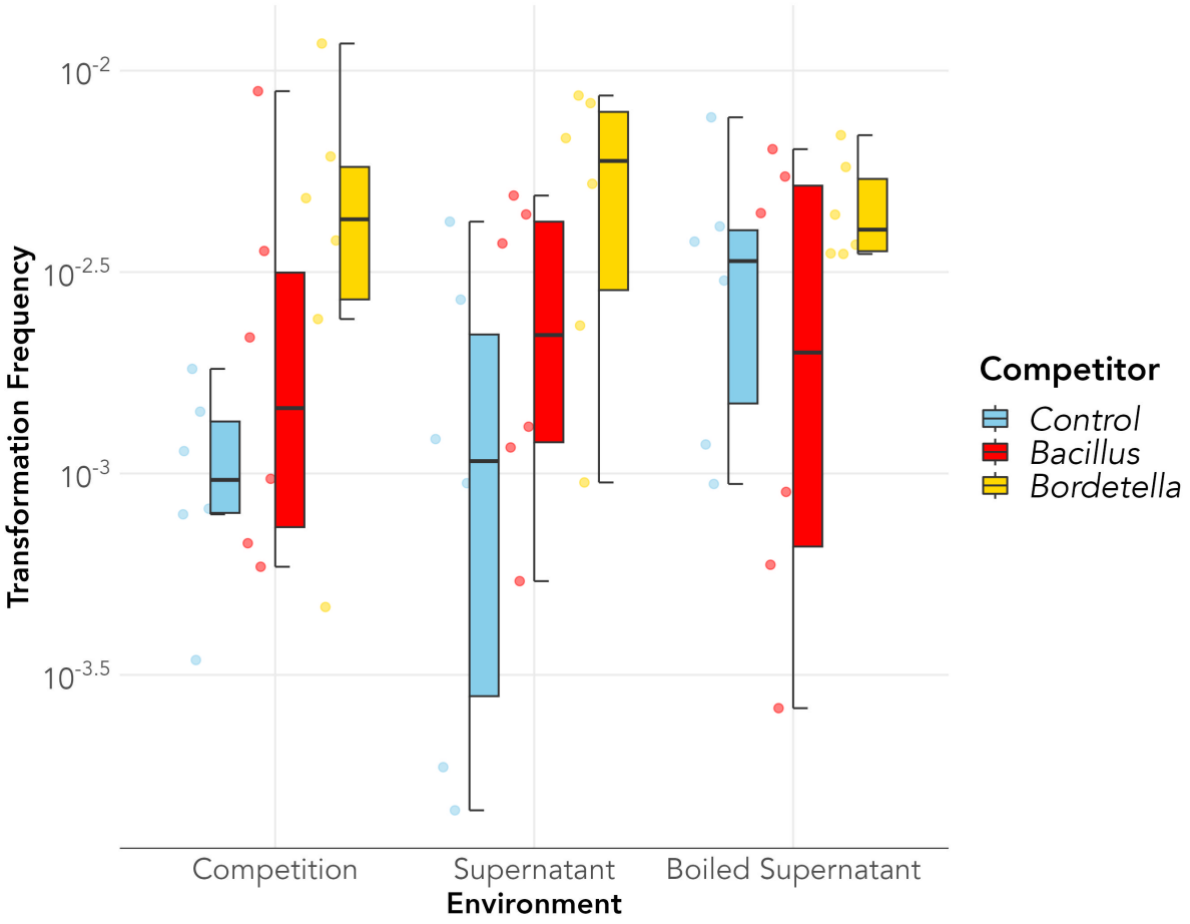

Figure 14. Transformation frequency of *A. baylyi* after 5 hours of incubation with a live competitor, competitor supernatant, or boiled competitor supernatant from *Bacillus*, *Bordetella*, or *A. baylyi* itself (Control).

3.4.4 Increased nutrient availability decreases transformation frequency in *A. baylyi* and alters the effect of species interactions.

Biotic (such as competitor species) and abiotic environments (such as nutrient availability) may interact to affect transformation frequency of *A. baylyi*. To test for this effect, the effect of *Bordetella* and *Bacillus* presence was tested for in a range of nutrient availabilities and compared to a monoculture control. While overall transformation frequency did not differ as a function of nutrient availability (supplemental table 18; supplemental table 14), the effect of competitor on *A. baylyi* transformation frequency changes as a function of nutrient availability, evidenced by a significant interaction between competitor species identity and nutrient availability (LMM, $\chi^2 = 25.61$, df = 4, $p < 0.0001$). A strong generalised effect of competitor species

presence was observed for both Bacillus and Bordetella ($p < 0.0001$; supplemental table 20; supplemental table 21).

In the absence of a competitor, transformation frequency of *A. baylyi* was observed to be highest under low nutrient (1% LB) conditions (EMMs pairwise test, z-ratio = 3.522, df = infinite, $p < 0.01$; Figure 15; supplemental table 22; supplemental 23). This effect changes when *Bacillus* is present; presence of *Bacillus* was observed to not affect *A. baylyi* transformation frequency at 100% LB (EMMs pairwise test, z-ratio = 0.936, df = infinite, $p = 0.3492$; Figure 15; supplemental table 22; supplemental table 23), but significantly decreased transformation frequency at 10% and 1% LB (EMMs pairwise tests, $p < 0.001$; Figure 15; supplemental table 22; supplemental table 23). Presence of *Bordetella* increases transformation frequency of *A. baylyi* in all concentrations of LB (EMMs pairwise tests, $p < 0.001$; Figure15; supplemental table 22; supplemental table 24). Transformation frequency of *A. baylyi* did not change as a function of nutrient availability when comparing 10% and 100% LB concentration in the absence of a competitor or in the presence of another species (EMMs pairwise tests, $p > 0.05$; Figure15; supplemental table 22; supplemental table 23). However, *A. baylyi* transformation frequency did change as a function of nutrient availability when comparing 1% and 100% LB in monoculture conditions and the presence of other species (EMMs pairwise tests, $p < 0.05$; Figure 15; supplemental table 22; supplemental table 23).

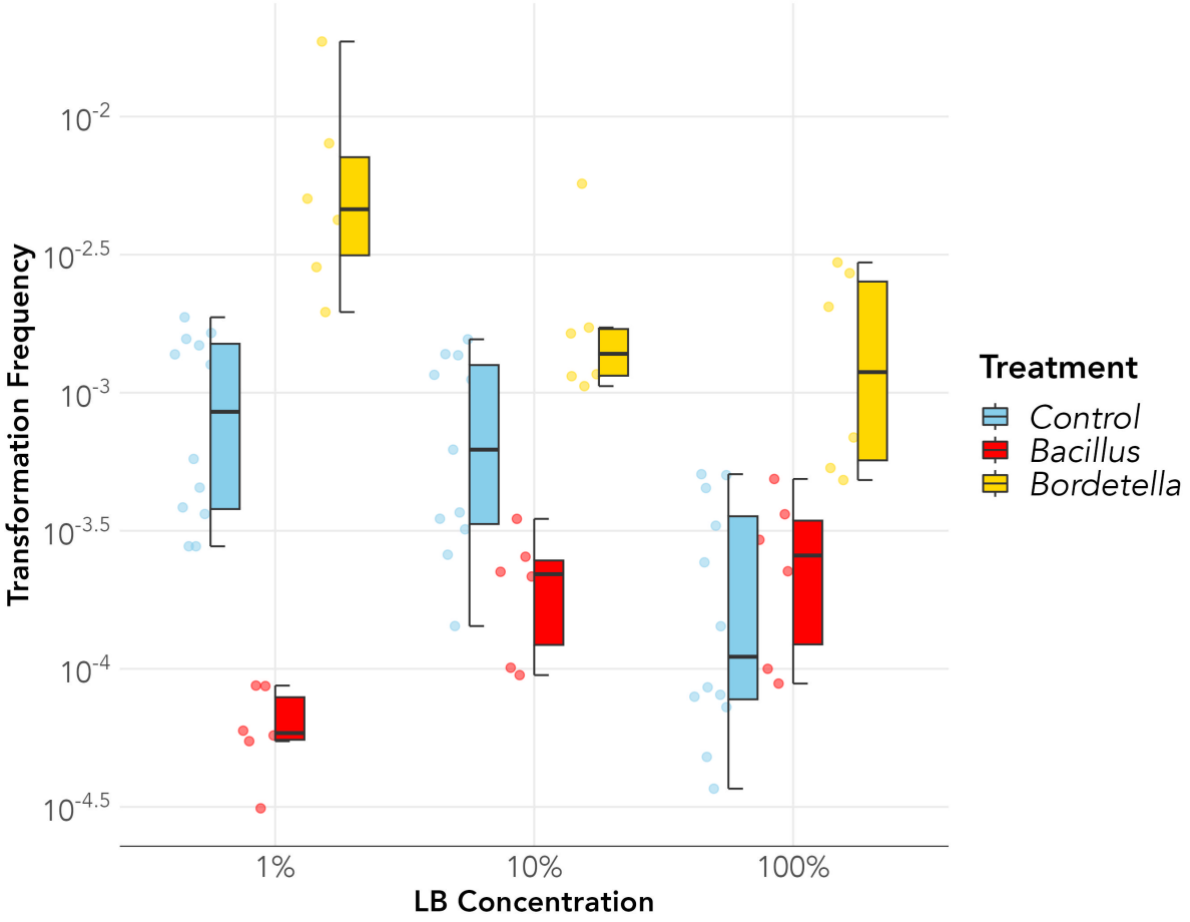

Figure 15. Transformation frequency of *A. baylyi* measured after 5 hours of culture in varying concentrations of LB (1%, 10% or 100%) in the presence of a competitor or in monoculture (control).

3.5 Discussion

This study tested for the short-term effects of biotic interactions on the frequency of natural transformation in *A. baylyi.* We conducted transformation assays during pairwise coculture of *A. baylyi* and 26 bacterial species. In general, species coculture trended towards a decrease in *A. baylyi* natural transformation frequency, though this was not significant overall due to mixed effects of species. Further, the large experimental design led to a large block effect which increased uncertainty of model predictions. The model cannot find a general consensus on how other species generally affect transformation frequency in *A. baylyi,* likely because transformation frequency estimates appear to be highly variable, even for the same competitor species in some cases. It is therefore likely that transformation frequency changes are sensitive to an unknown condition.

The effect of biotic interactions on *A. baylyi* transformation frequency were observed to change as a function of competitor species identity, with 16 of 26 species estimated to exhibit a strong negative effect. To test for the underlying cause of this species-dependent effect, *A. baylyi* growth rate and competitor species genetic relatedness (16S rRNA based distance) were tested for their effect on *A. baylyi* transformation frequency. Our rationales for testing these parameters are twofold: 1) Increased *A. baylyi* growth rate is observed in other studies to increase transformation frequency (Elliott & Neidle, 2011; Hülter et al., 2017; Palmen et al., 1993; Ray & Nielsen, 2005), and hence is a general mechanism by which competition may affect transformation frequency. Therefore, species interactions affecting *A. baylyi* fitness may indirectly affect transformation frequency. And 2) genetic relatedness of the focal strain to competitor species can have implications on the sign and strength of species interactions. This can be caused by direct competition for resources (Hibbing et al., 2009), or interference competition (e.g. via type VI secretion systems (T6SS)) (Bingle et al., 2008; Ho et al., 2014). As neither of these parameters improved model fit when included, they are not likely to be associated with the effects seen in this study.

To explore possible mechanisms by which interactions affect transformation, assays were carried out using a subset of competitor species. We selected "*Bacillus"* and "*Bordetella 1"* as these species were estimated to have strong and weak negative effects on *A. baylyi* transformation frequency, respectively. First, the mechanistic basis of these effects were tested for by observing *A. baylyi* transformation frequency in the presence of a competitor species, competitor supernatant, or boiled competitor supernatant. As *A. baylyi* transformation frequency increases in both the presence of *Bordetella* and *Bordetella* supernatant which has been boiled, the increase in transformation frequency may be due to non-heat-labile (therefore not proteinaceous) molecules produced by *Bordetella* (Bischof & He, 2006). As *A. baylyi* can upregulate transformation frequency in response to increased growth rate and stress, the data gathered here are not sufficient to suggest whether these effector compounds in *Bordetella* supernatant cause transformation frequency changes through antagonistic or beneficial effects on *A. baylyi*. Such compounds are unlikely to be secreted by *Bacillus* as there was no observed change in *A. baylyi* transformation frequency in

contexts where *Bacillus* was able to interact with *A. baylyi* via cell-cell contact, or extracellular products.

There is a lack of corroboration in the effect of coculture with *Bacillus* on *A. baylyi* natural transformation frequency between Figures 13, 14 and 15. Figures 14 and 15 show no effect of *Bacillus* on transformation frequency, while Figure 13 suggests a strong negative effect of *Bacillus*. This may be caused by random chance in Figure 13, where an inaccurate set of observations were analysed. As Bayesian modelling does not correct for multiple testing, there is a higher likelihood of generating false positive results. It is therefore likely that *Bacillus* has no strong effect on *A. baylyi* transformation frequency during coculture. Further, Figure 13 suggests a weak negative effect of *Bordetella 1* estimated by the Bayesian model, while five of six observed values are in the positive direction (Figure 11). Additionally, Figures 14 and 15 both suggest positive effects of *Bordetella 1* coculture with *A. baylyi* transformation frequency. Taken together, it is likely that *Bacillus* has no strong effect on *A. baylyi* transformation frequency during coculture while *Bordetella 1* may have a positive effect, but repeated testing may be necessary to confirm this suggestion.

Bacteria in natural populations are likely to experience wildly varying nutrient availability. Increasing nutrient availability decreased the transformation frequency of *A. baylyi* in monoculture and in the presence of *Bordetella* in this system. Presence of *Bordetella* increased *A. baylyi* transformation frequency relative to the monoculture control in all tested LB concentrations. Interestingly, transformation frequency of *A. baylyi* when cocultured with *Bacillus* demonstrated an inverse effect of nutrient availability compared to *A. baylyi* tested in monoculture; coculture with *Bacillus* in 1% LB decreased transformation frequency relative to the monoculture control while coculture with *Bacillus* in 100% LB did not change *A. baylyi* transformation frequency relative to the monoculture control. These findings demonstrate that transformation frequency changes as a function of competitor species presence can be highly context dependent and highlights the importance for testing transformation frequency changes at low nutrient conditions as they are more environmentally relevant and can significantly change treatment effects. Further, what has not been explored in this study is how species may interact over nutrient resources by outcompeting for resources (Hibbing et al., 2009), decontamination of the local environment (Ma, 2019),

or producing metabolic by-products which can be utilised by other species (D'Souza et al., 2018). These interactions have been shown to affect transformation frequency in this study in the case of resource depletion and extracellular compounds from other species, and in other studies through testing the effect of anthropogenic pollutants on natural transformation (Lu et al., 2022; Wang et al., 2020; Yu et al., 2022), and so should be considered for future work testing bacterial natural transformation.

Taken together, these findings demonstrate an effect of biotic interactions on natural transformation and highlight a need to utilise interspecies competition when studying natural transformation in future studies. Further, the effect of biotic interactions observed in low nutrient availability suggest that future work should use competitor species but also low nutrient availability to ensure findings are more representative of ecological contexts. The inclusion of competitor species into transformation assays may also modify the natural or contaminated environment, for instance by breaking down anthropogenic compounds implicated in transformation frequency changes (Lu et al., 2022; Wang et al., 2020; Yu et al., 2022). If cocultured species can degrade these compounds, the compounds' effect on transformation may be lost or change to a different effect caused by a metabolic by-product. Additionally, if competitors do not directly interact with these pollutants, their respective effects may act synergistically on the transforming species to change transformation frequency in unexpected ways. Understanding the ecologically relevant conditions under which transformation occurs can help us to predict and monitor the dissemination of genes between bacteria in the environment.

3.6 Supplemental tables

Supplemental table 16. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in varying environmental conditions (direct competition, competitor cell supernatant, boiled competitor cell supernatant) and competitor species. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit

Genus	EMM	SE	df	lower.CL	upper.CL
Monoculture	0.00202	0.000561	49	0.000896	0.00315
Bacillus	0.00283	0.000561	49	0.001702	0.00396
Bordetella	0.00497	0.000561	49	0.003839	0.00609

Supplemental table 17. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying environmental conditions (direct competition, competitor cell supernatant, boiled competitor cell supernatant) and competitor species, grouped by environment. Degrees of freedom were calculated using the Kenward-Roger method. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom.

Contrasts	**EMM**	**SE**	**df**	**t.ratio**	**p.value**
Monoculture - *Bacillus*	-0.000806	0.000793	49	-1.016	0.3145
Monoculture - *Bordetella*	-0.002943	0.000793	49	-3.711	0.0016
Bacillus - *Bordetella*	-0.002137	0.000793	49	-2.695	0.0144

Supplemental table 18. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in varying nutrient availabilities. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. Results are averaged over the three levels of competitor species and given on the logit scale. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit.

LB Concentration	EMM	SE	df	asymp.LCL	asymp.UCL
1%	-7.16	0.137	Inf	-7.43	-6.89
10%	-7.29	0.142	Inf	-7.57	-7.01
100%	-7.53	0.155	Inf	-7.84	-7.23

Supplemental table 19. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying nutrient availabilities. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom. Results are averaged over the three levels of competitor species and given on the logit scale.

LB Concentration Contrast	EMM	SE	df	z.ratio	p.value
1% - 10%	0.13	0.191	Inf	0.68	0.4963
1% - 100%	0.367	0.198	Inf	1.852	0.1919
10% - 100%	0.238	0.198	Inf	1.201	0.3448

Supplemental table 20. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in the presence of varying competitor species. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit. Results are averaged over the three levels of nutrient availability and given on the logit scale.

Competitor species	EMM	SE	df	asymp.LCL	asymp.UCL
Control	-7.46	0.149	Inf	-7.75	-7.17
Bacillus	-8.74	0.138	Inf	-9.01	-8.47
Bordetella	-5.78	0.182	Inf	-6.14	-5.43

Supplemental table 21. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in in the presence of varying competitor species. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom. Results are averaged over the three levels of nutrient availability and given on the logit scale.

Competitor Species Contrast	**EMM**	**SE**	**df**	**z.ratio**	**p.value**
Control - *Bacillus*	1.28	0.203	Inf	6.316	<.0001
Control - *Bordetella*	-1.68	0.235	Inf	-7.124	<.0001
Bacillus - *Bordetella*	-2.96	0.229	Inf	-12.933	<.0001

Supplemental table 22. Estimated marginal means (EMMs) of *A. baylyi* transformation frequency in varying nutrient availabilities and competitor species identity. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom; CL, confidence limit. Results are given on the logit scale.

LB Concentration	Competitor Species	EMM	SE	df	asymp.LCL	asymp.UCL
1%	Control	-6.94	0.183	Inf	-7.3	-6.58
1%	*Bacillus*	-9.43	0.301	Inf	-10.01	-8.84
1%	*Bordetella*	-5.12	0.212	Inf	-5.54	-4.71
10%	Control	-7.41	0.226	Inf	-7.86	-6.97
10%	*Bacillus*	-8.47	0.193	Inf	-8.84	-8.09
10%	*Bordetella*	-6	0.306	Inf	-6.6	-5.4
100%	Control	-8.03	0.276	Inf	-8.57	-7.49
100%	*Bacillus*	-8.33	0.174	Inf	-8.67	-7.99
100%	*Bordetella*	-6.23	0.333	Inf	-6.88	-5.58

Supplemental table 23. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying nutrient availabilities and competitor species identity, grouped by competitor species identity. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom. Results are given on the log odds ratio scale.

Competitor Species	LB Concentration Contrast	EMM	SE	df	z.ratio	p.value
Control	1% - 10%	0.473	0.278	Inf	1.704	0.0885
Control	1% - 100%	1.088	0.309	Inf	3.522	0.0013
Control	10% - 100%	0.614	0.331	Inf	1.853	0.0885
Bacillus	1% - 10%	-0.96	0.349	Inf	-2.751	0.0089
Bacillus	1% - 100%	-1.092	0.34	Inf	-3.216	0.0039
Bacillus	10% - 100%	-0.132	0.256	Inf	-0.516	0.6059
Bordetella	1% - 10%	0.876	0.358	Inf	2.446	0.0217
Bordetella	1% - 100%	1.107	0.379	Inf	2.924	0.0104
Bordetella	10% - 100%	0.231	0.421	Inf	0.549	0.5832

Supplemental table 24. Estimated marginal means (EMMs) contrasts of *A. baylyi* transformation frequency in varying nutrient availabilities and competitor species identity, grouped by LB concentration. Transformation frequency is standardised to the monoculture transformation frequency in the respective treatment. Degrees of freedom (df) were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE = standard error, df = degrees of freedom. Results are given on the log odds ratio scale.

LB Concentration	Competitor Species Contrast	EMM	SE	df	z.ratio	p.value
1%	Control - *Bacillus*	2.485	0.352	Inf	7.059	<.0001
1%	Control - *Bordetella*	-1.817	0.28	Inf	-6.487	<.0001
1%	*Bacillus* - *Bordetella*	-4.302	0.368	Inf	-11.698	<.0001
10%	Control - *Bacillus*	1.051	0.297	Inf	3.541	0.0004
10%	Control - *Bordetella*	-1.415	0.38	Inf	-3.72	0.0003
10%	*Bacillus* - *Bordetella*	-2.466	0.362	Inf	-6.816	<.0001
100%	Control - *Bacillus*	0.305	0.326	Inf	0.936	0.3492
100%	Control - *Bordetella*	-1.798	0.432	Inf	-4.159	<.0001
100%	*Bacillus* - *Bordetella*	-2.103	0.376	Inf	-5.596	<.0001

Chapter 4: Collection of Annotated Acinetobacter Genome Sequences

4.1 Abstract

The genus *Acinetobacter* contains environmental species as well as opportunistic pathogens of humans. Several species are competent for natural transformation, an important mechanism of horizontal gene transfer. Here, the genome sequences of 19 *Acinetobacter* strains used in past and upcoming studies of natural transformation are presented.

4.2 Announcement

Acinetobacter species are ubiquitous and are found in both terrestrial and clinical environments (T. L. Chen et al., 2008). Many *Acinetobacter* strains are able to acquire new genetic information via natural transformation, and this ability is thought to be at least partly responsible for some clinically relevant *A. baumannii* strains to become multidrug resistant (Von Wintersdorff et al., 2016). Here, the genomes of 19 *Acinetobacter* strains from a diverse set of environmental samples given by David M. Young (Harvard University) were sequenced. These strains were previously used in a study investigating the efficiency of DNA uptake in different genomic locations by the model species for natural transformation *A. baylyi* (Ray et al., 2009).

All strains are culturable on LB agar or in LB broth at 37°C at 180rpm. Genomic DNA of the strains was extracted from single colonies grown on agar using a Qiagen DNeasy kit (Qiagen, Germany). Sequencing was carried out by the Centre for Genomic Research at the University of Liverpool. Short read Illumina sequencing (1-150bp paired end) sequence data was generated for each strain using the NEBNext Ultra II FS kit with an Illumina MiSeq. The trimmed reads were quality controlled using FastQC (Andrews, 2010), and *de novo* assembled using Unicycler v0.4.8 (Wick et al., 2017a, 2017b) using default parameters and rotated to start at *dnaA*. Contigs under were omitted from downstream analyses. QUAST v5.2.0 was used to generate quality metrics of the assemblies (Mikheenko et al., 2018). Genomic features were annotated using prokka v1.13 (Seemann, 2014). All strains had a linear genomic topology.

Taxonomic classification of the strains was defined by topology and ANI using GTDBTk v1.7.0 (Chaumeil et al., 2020).

4.3 Data availability statement

The raw and annotated read data for the 19 strains were deposited at the European Nucleotide Archive (ENA) under BioProject accession number PRJEB55833. Hyperlinked accession numbers for raw reads and annotations are found in Table 5.

Table 5. List of strains used in this study. Raw read and annotation accession numbers are hyperlinked to BioProject accession number PRJEB55833 at the European Nucleotide Archive (ENA).

Strain name	Genome size (bp)	No. contigs	Largest contig (bp)	N50	GC content (%)	fastANI reference	fastANI taxonomy (ANI match %)	Predicted genes (Unique)	No. reads	Average read length	Number of sequenced bases	Whole Genome Coverage (x)	Raw read accessions	Annotation accessions
A. sp. 01B0, KmR isolate 2.	4012069	99	390125	119727	38.93	GCF_009759685.1	*Acinetobacter baumannii* (97.75)	3851	80498431	111.9	9007774429	2245.16937	ERR10167739	GCA_947331425
A. sp. 26B2, isol. 3.	4124650	18	1229448	1063596	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.84)	3817	74338568	114.25	8493181394	2059.127779	ERR10167740	GCA_947044995
A. sp. 423D, isol. 3.	4125066	17	1229448	1063963	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.84)	3816	61075879	121.7	7432934474	1801.894679	ERR10167741	GCA_947044985
A. sp. 48A1, isol. 3.	3838866	27	730290	340355	38.97	GCF_009759685.1	*Acinetobacter baumannii* (97.62)	3616	50129031	116.55	5842538563	1521.943867	ERR10167742	GCA_947044945
A. sp. 511B, isol. 5.	4124706	18	1229448	1063596	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.87)	3817	61442508	114	7004445912	1698.168527	ERR10167743	GCA_947045055
A. sp. 56A1, isol. 3.	3954990	94	390027	123641	38.96	GCF_009759685.1	*Acinetobacter baumannii* (97.72)	3789	51944110	114.25	5934614568	1500.538451	ERR10167744	GCA_947044975
A. sp. 62A1, isol. 3.	3800072	22	1667336	478807	38.6	GCF_900520355.1	*Acinetobacter calcoaceticus C* (98.02)	3526	55955595	114.1	6384533390	1680.108532	ERR10167745	GCA_947045035
A. sp. 63A1, isol. 7.	3822479	33	515714	240423	38.53	GCF_900520355.1	*Acinetobacter calcoaceticus C* (96.21)	3525	63908045	102.75	6566551624	1717.87775	ERR10167746	GCA_947044905
A. sp. 66A1, isol. 1.	3738809	27	855844	380638	38.9	GCF_009759685.1	*Acinetobacter baumannii* (97.96)	3440	45304263	118.3	5359494313	1433.476359	ERR10167747	GCA_947045065
A. sp. 71A1, isol. 4.	3834289	38	853248	465306	38.8	GCF_009759685.1	*Acinetobacter baumannii* (98.16)	3608	51498217	114.25	5883671292	1534.488217	ERR10477801	GCA_947366405
A. sp. 81A1, isol. 2.	3738813	29	855844	380638	38.9	GCF_009759685.1	*Acinetobacter baumannii* (97.94)	3438	43162178	119.05	5138457291	1374.355254	ERR10167749	GCA_947044935
A. sp. 85A1, isol.3.	4124656	18	1229448	1063596	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.86)	3818	62181982	114.95	7147818831	1732.949083	ERR10167750	GCA_947045025
A. sp. A06, isol. 7.	4124674	18	1229448	1063596	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.85)	3814	44354854	116.8	5180646947	1256.013675	ERR10167751	GCA_947044965
A. sp. A3-6, isol. 3.	3738812	27	857074	380638	38.9	GCF_009759685.1	*Acinetobacter baumannii* (97.96)	3440	40888945	118.3	4837162194	1293.769837	ERR10167752	GCA_947045075
A. sp. AD512A, isol. 4.	3834471	37	853248	465306	38.8	GCF_009759685.1	*Acinetobacter baumannii* (98.14)	3609	49769835	120.6	6002242101	1565.337722	ERR10477802	GCA_947366465
A. baumannii AZR3410, isol. 1.	3645238	99	187218	78501	39.09	GCF_009759685.1	*Acinetobacter baumannii* (97.87)	3488	57196478	116.3	6651950391	1824.832944	ERR10167754	GCA_947045015
A. sp. AZR54, isol. 8.	3897882	20	1303607	433975	38.59	GCF_000368965.1	*Acinetobacter calcoaceticus* (99.98)	3666	45034632	112.45	5064144368	1299.204124	ERR10167755	GCA_947044915
A. calcoaceticus AZR583, isol. 9.	3787947	38	445412	238481	38.56	GCF_000368965.1	*Acinetobacter calcoaceticus* (97.19)	3566	54949893	112.75	6195600436	1635.609061	ERR10167756	GCA_947044925
A. sp. P1-6, isol. 3.	4124672	18	1229448	1064826	38.48	GCF_000196795.1	*Acinetobacter oleivorans* (96.88)	3818	47141068	117.85	5555574864	1346.913128	ERR10167757	GCA_947044955

Chapter 5: Testing for the fitness benefits of natural transformation during community-embedded evolution.

5.1 Abstract

Natural transformation is a process where bacteria actively take up DNA from the environment and recombine it into their genome or reconvert it into extra-chromosomal genetic elements. The evolutionary benefits of transformation are still under debate. One main explanation is that foreign allele and gene uptake facilitates natural selection by increasing genetic variation, analogous to meiotic sex. However, previous experimental evolution studies comparing fitness gains of evolved transforming- and isogenic non-transforming strains have yielded mixed support for the "sex hypothesis." Previous studies testing the sex hypothesis for natural transformation have largely ignored species interactions, which theory predicts provide conditions favourable to sex. To test for the adaptive benefits of bacterial transformation, the naturally transformable wildtype *Acinetobacter baylyi* and a transformation-deficient Δ*comA* mutant were evolved for five weeks. To provide strong and potentially fluctuating selection, *A. baylyi* was embedded in a community of five other bacterial species. DNA from a pool of different *Acinetobacter* strains was provided as a substrate for transformation. No effect of transformation ability on the fitness of evolved populations was found, with fitness increasing non-significantly in most treatments. Populations showed fitness improvement in their respective environments, with no apparent costs of adaptation to competing species. Despite the absence of fitness effects of transformation, wildtype populations evolved variable transformation frequencies that were slightly greater than their ancestor which potentially could be caused by genetic drift.

5.2 Introduction

Natural transformation is a process whereby bacteria actively take up free DNA from the environment during a physiological state termed competence, followed by the

recombination of this DNA into the recipient's genome (or its reconversion into extra-chromosomal genetic elements). Natural transformation has been demonstrated in 80+ species across divergent bacterial lineages (Johnston et al., 2014) but is likely to be present in more species. Natural transformation can mediate the cell-to-cell transfer of large tracts of DNA, including virulence (Frosch & Meyer, 1992), antibiotic resistance (Blokesch, 2016; Winter et al., 2021) and metabolic genes (Tumen-Velasquez et al., 2018), making it one of the main prokaryote horizontal gene transfer (HGT) mechanisms. The uptake of free DNA from the environment has been argued to provide three distinct (but not mutually exclusive) potential benefits to cells. First, as a source of nucleotides to be used for energy or building blocks (with recombination or maintenance of extrachromosomal DNA being a by-product) (Hülter et al., 2017; Redfield, 1993b, 2001), second, to serve as templates for repairing genetic damage (Ambur et al., 2016; Guiral et al., 2005; Hoelzer & Michod, 1991; Hülter et al., 2017; Michod et al., 1988; Mongold, 1992; Steinmoen et al., 2002), and third as a mechanism to create genetic variation (Ambur et al., 2016; Vos, 2009).

The genetic variation or 'sex' function of natural transformation has received most attention. Unlike many other HGT mechanisms, natural transformation is not mediated by Mobile Genetic Elements but is solely under the control of the recipient cell (Dubnau & Blokesch, 2019; Johnston et al., 2014; Seitz & Blokesch, 2013) and therefore could be assumed to be adaptive. By recombining adaptive alleles and genes in the same genomic background, natural transformation can result in the avoidance of clonal interference, allowing populations to adapt more rapidly. Indeed, both mathematical modelling (Engelstädter & Moradigaravand, 2013; Levin & Cornejo, 2009; Peabody et al., 2017) and laboratory evolution experiments (Baltrus et al., 2007; Nguyen et al., 2022; Woods et al., 2020) have supported this hypothesis. However, there remains controversy about the sex function of transformation, and not all experimental studies have found that fitness of recombining wildtype cells increased after evolution compared to isogenic, non-recombinogenic mutants. For instance, evolution experiments utilising the model system *Acinetobacter baylyi* found transformation-mediated fitness benefits either to be present (Perron et al., 2012), equivocal (Renda et al., 2015; Utnes et al., 2015) or absent (Bacher et al., 2006; McLeman et al., 2016). Multiple studies found the ability to transform was lost during experimental evolution, indicating that any potential recombination-mediated fitness benefits were outweighed

by the cost of maintaining the molecular machinery involved (Bacher et al., 2006; Renda et al., 2015).

Studies to date lack interactions with multiple competitors that almost certainly characterise most natural situations. This could be an important shortcoming, as for sex to remain selectively advantageous it is necessary for selection to be strong and dynamic (Burt, 2000; Charlesworth, 1993), and interspecific competitors could greatly influence both these requirements (Kawecki et al., 2012; Otto & Nuismer, 2004). For example, if species evolve niche divergence, then the strength of interaction will decline over time (in contrast to host-parasite coevolution, where strength is maintained) (Macarthur & Levins, 1967). However, while interspecific competition can create fluctuating conditions, it can also constrain evolution (Luján et al., 2022). Experimental evolution approaches have hitherto relied on evolving recombining clones in isolation. To study the evolutionary benefits of natural transformation in the context of species interactions, a transformable *A. baylyi* wildtype and a non-transformable isogenic Δ*comA* mutant were experimentally evolved for five weeks in the presence of other species (*i.e.,* under biotic conditions) or alone (*i.e.,* under abiotic conditions). The *comA* gene encodes the transmembrane protein ComA which facilitates transport of ssDNA through the inner membrane into the cytoplasm (Leong et al., 2017). Deletion of *comA* reduces transformation frequency in *A. baylyi* below the detectable limit (Leong et al., 2017). Specifically, a system of five bacterial species which have been previously shown to stably coexist was used (Castledine et al., 2020; Newbury et al., 2022; Padfield et al., 2020). This study's experimental evolution approach allows for the testing of 1) whether the evolved wildtype strain will be fitter than its non-recombining counterpart, specifically after evolution under biotic conditions and 2) whether the transformation rate of the evolved wildtype has changed in response to these treatments.

5.3 Material and Methods

5.3.1 *Acinetobacter baylyi* ADP1 constructs

Two variants of the wildtype *A. baylyi* ADP1 strain with chromosomally encoded GFP and RFP, respectively were constructed using fluorescence::AMR cassettes derived

from strains gifted by the Charpentier lab (Claude Bernard University, Lyon), and Hasty lab (University of San Diego, California), respectively. Briefly, the *sfGFP::apraR* cassette (Charpentier and Laaberki, unpublished) and *mCherry::specR* (R. M. Cooper et al., 2017) cassettes were amplified via PCR with 1kb flanking regions homologous to the *att*Tn*7* locus and a putative prophage p4 region, respectively. Primers used for the *sfGFP::apraR* and *mCherry::specR* cassettes were 5'3 AAAGCCAATCGCTGACAGATGGTGG-3', 5'-TTGGTCAGTGCCTGTCTTGCTGGTGAGCCGGTACGC-3', and 5'-TCACCTGCATCCACTCAAGTGTCGTTT-3', 5'-AAAGCCAATCGCTGACAGATGGTGG-3', respectively (Integrated DNA Technologies, USA). PCR products were added to *A. baylyi* in LB Miller broth (Formedium, England) at 37˚C and 180rpm for 24 hours at 1µg/mL to allow for chromosomal recombination of PCR amplicons via natural transformation. Transformants were isolated by plating on LB agar containing 240µg/mL apramycin (Duchefa, The Netherlands), or 360µg/mL spectinomycin (Melford, UK), respectively. Next, non-competent counterparts of each fluorescent strain were generated by deletion of the *comA* gene via sequential natural transformation with linearised plasmids pKHNH6 and pKHNH3. Plasmids pKHNH6 and pKHNH3 were linearised prior to transformation using restriction enzymes EcoRV (Promega, USA) and KpnI (Fisher, USA), respectively. pKHNH6 carried a *comA*$^{+}$::(*nptII sacB*) allele embedded in its natural flanking regions, and natural transformation of the respective fluorescence-marked *A. baylyi* strains by linearized plasmid DNA resulted in transformation-proficient isolates that were kanamycin-resistant and sensitive to 50g/L sucrose. Transformation of those respective isolates by pKHNH3 (carrying a Δ*comA* allele) resulted in a sucrose-resistant, kanamycin-susceptible and transformation-deficient strains, respectively. Deletion of *comA* was verified using agar containing 50g/L sucrose, agar containing 10ug/mL kanamycin, and by PCR using primers 5'-TTGGTGTGATTGGTACGGTGGCTGGTGC-3' and 5'-CTTGCAGACGATTGCTTACCTCAGCACTCGG-3'. The non-competent *A. baylyi* strains were confirmed to be non-transformable at the detectable limit (10^{-7}) in all (6) technical replicates.

Table 6. List of strains used in this study.

Strain	*Fluorescence label*	*Naturally Competent?*	*Antibiotic resistance marker*	*Used as*
Wildtype A. baylyi ADP1	sfGFP (green)	Yes	Apramycin (480ug/mL)	Focal strain in evolution experiment
ΔcomA A. baylyi ADP1	sfGFP (green)	No	Apramycin (480ug/mL)	Focal strain in evolution experiment
ΔcomA A. baylyi ADP1	mCherry (red)	No	Spectinomycin (600ug/mL)	Common competitor for fitness assays
Achromobacter	None	No	N/A	Community member
Ochrobactrum	None	No	N/A	Community member
Pseudomonas	None	No	N/A	Community member
Stenotrophomonas	None	No	N/A	Community member
Variovorax	None	No	N/A	Community member
A. sp. 01B0, KmR isolate 2.	None	No	N/A	DNA Donor
A. sp. 26B2, isol. 3.	None	Yes	N/A	DNA Donor
A. sp. 423D, isol. 3.	None	Yes	N/A	DNA Donor
A. sp. 48A1, isol. 3.	None	No	N/A	DNA Donor
A. sp. 511B, isol. 5.	None	No	N/A	DNA Donor
A. sp. 56A1, isol. 3.	None	No	N/A	DNA Donor
A. sp. 62A1, isol. 3.	None	Yes	N/A	DNA Donor
A. sp. 63A1, isol. 7.	None	Yes	N/A	DNA Donor
A. sp. 66A1, isol. 1.	None	No	N/A	DNA Donor
A. sp. 71A1, isol. 4.	None	No	N/A	DNA Donor

A. sp. 81A1, isol. 2.	None	No	N/A	DNA Donor
A. sp. 85A1, isol.3.	None	Yes	N/A	DNA Donor
A. sp. A06, isol. 7.	None	Yes	N/A	DNA Donor
A. sp. A3-6, isol. 3.	None	No	N/A	DNA Donor
A. sp. AD512A, isol. 4.	None	No	N/A	DNA Donor
A. baumannii AZR3410, isol. 1.	None	No	N/A	DNA Donor
A. sp. AZR54, isol. 8.	None	Yes	N/A	DNA Donor
A. calcoaceticus AZR583, isol. 9.	None	Yes	N/A	DNA Donor
A. sp. P1-6, isol. 3.	None	Yes	N/A	DNA Donor
A. baumannii clinical isolate FZ21	None	Unknown	N/A	DNA Donor

5.3.2 Five-species community

The five-species community was composed of compost isolates belonging to the genera *Pseudomonas, Achromobacter, Variovorax, Ochrobactrum* and *Stenotrophomonas* (as identified by 16S rRNA sequencing) (Castledine et al., 2020; Padfield et al., 2020) (Table 6).

5.3.3 Evolution experiment

The apramycin resistant wildtype and Δ*comA A. baylyi* ADP1 focal strains were separately propagated in three distinct competition environments with sixfold replication. Experimental treatments included competition against none, or all five of the five-species community concurrently (Figure 16). Cultures were grown in 25% TSB medium at 28˚C in static conditions in glass microcosms with one layer of ColiRoller glass beads (Millipore, Merck, USA) covering the base of the microcosm to provide additional spatial structure, potentially increasing the frequency of interspecies interactions. Instead of transferring a small volume to new microcosms, spent nutrient broth was replaced with fresh broth in the same microcosms, resulting in

approximately 34-fold daily dilutions. This approach maintained spatial structure and saved on glass and plastic waste (Alves et al., 2021). Every 7th day, cultures were vortexed, diluted 10-fold and plated on LB agar plates containing 240µg/ml apramycin to select for the green-fluorescent wildtype or Δ*comA A. baylyi* focal strain. After 24 hours of incubation at 37˚C, the bacterial lawn from each individual replicate was scraped off with a sterile loop and transferred to a new microcosm for overnight growth (reaching stationary phase). Overnight cultures of the five competitor species were also made in LB broth at this time from -70˚C freezer stocks. Equal volumes of overnight cultures of the focal *A. baylyi* strain and competitor species (where appropriate; Table 6) totalling 100µL was added to a new microcosm containing 9.9mL 25% TSB, commencing the next week of transfers. DNA as a substrate for transformation sourced from a pool of 20 *Acinetobacter* strains was added at the point of nutrient replenishment each day for each treatment (see next section). At the end of the final week of transfers, cultures were plated on LB agar supplemented with 240µg/ml apramycin where 100 colonies were picked and pooled for overnight growth and later frozen at -70˚C in 25% glycerol prior to use in subsequent competition and transformation assays (Figure 16).

5.3.4 Donor DNA

Twenty *Acinetobacter* strains (Table 6; Ray and Nielsen, 2005; Alseth et al., 2019) were cultured separately in LB broth overnight. Cultures were then pooled in equal volumes and lysed following the Qiagen® Genomic DNA Handbook (April 2012) protocol. Combined DNA from the eluate was precipitated by adding two volumes of ice-cold ethanol and centrifuged at 26000xg for 15 minutes to pellet the DNA. DNA was dissolved in TE buffer to a final concentration of 227.2ng/µL (Nanodrop 2000, Thermo Scientific) by heating at 50˚C and stirring for 16 hours. DNA was frozen at -20˚C in single-use aliquots for addition to each daily transfer. When used during the evolution period, DNA was diluted to a final concentration of 250ng/ml (the saturating concentration of genomic DNA for transformation in *A. baylyi* (Overballe-Petersen et al., 2013)). The raw and annotated DNA sequencing data for 19 of the strains were deposited at the European Nucleotide Archive under BioProject accession number PRJEB55833 (Winter, Harms, Johnsen, & Vos, 2023).

5.3.5 Competition assays

For all replicates of the five concurrent species community treatment and the abiotic treatment, 100 evolved green-fluorescent wildtype and Δ*comA A. baylyi* clones were picked, pooled and frozen at –80˚C before use. A mixture containing equal volumes of the green-fluorescent *A. baylyi* pool, a Δ*comA* red-fluorescent *A. baylyi*, and members of the five species community (where appropriate) was produced for each replicate. One hundred microlitres of mixed culture was immediately inoculated to 9.9ml of 25% TSB and grown for 24 hours in glass microcosms at 28˚C with no agitation. Each of the six replicate evolved populations per treatment were competed with the differentially marked Δ*comA* strain in conditions identical to treatments in the evolution experiment (i.e., containing either all or none of the five competitor species). Samples were plated 0 hours and 24 hours after inoculation on LB agar supplemented with either apramycin (240µg/ml) or spectinomycin (360µg/mL) to determine the densities of the evolved (green)- and Δ*comA* (red)-fluorescent *A. baylyi*, respectively (Figure 16).

5.3.6 Transformation assays

Red fluorescent, spectinomycin-resistance conferring marker DNA was obtained by heating overnight cultures of the *A. baylyi* ADP1 *mCherry::specR* at 70˚C for 1.5 hours and centrifuging at 2500xg for 15 minutes and resuspending in reduced volume to produce a 50x concentration of lysate. Lysate was stored at -20˚C for up to one month before downstream application. Freezer stocks of 100 pooled clones for each endpoint population were inoculated in LB broth and grown overnight at 37˚C. Cultures were diluted ten-fold with LB broth supplemented with cell lysate at a final concentration of 2.5x maximum cell density and incubated for 3 hours at 37˚C and 180 rpm. Cells were then plated on LB agar supplemented with 240µg/ml apramycin and 360µg/ml spectinomycin, and non-selective LB agar and incubated for 48 hours at 28˚C. Transformation frequency was calculated by dividing the CFU/mL of the transformed (doubly fluorescent and dually-AMR) population by the total population CFU/mL. As a control for spontaneous spectinomycin resistance mutations, a treatment including no *mCherry::specR* DNA was added.

5.3.7 Statistical analysis

Normal distribution of the residuals for data used in statistical modelling was verified using Shapiro-Wilks tests. To determine the relative fitness of evolved lines relative to the unevolved control in competition experiments, the selection-rate constant was calculated as described in (Lenski et al., 1991). Selection-rate constant estimates were analysed with estimated marginal means tests using estimated values from a linear model as input data (treatments tested within assay conditions and grouped by genotype and evolution treatment conditions). Because relative fitness values were often negative (i.e., the common competitor displayed greater fitness than the evolved focal strains), analyses in this assay were conducted using the selection-rate constant in lieu of the relative fitness parameter (Lenski et al., 1991). As ancestral populations were not significantly different to each other in fitness as a function of genotype (paired t-tests; biotic assay conditions, $p = 0.35$, abiotic assay conditions, $p = 0.74$), fitness measurements of the evolved populations were standardised to ancestors by subtracting the ancestral selection-rate constant (averaged for both genotypes) from that of the evolved population in all analyses. Standardised selection rates significantly different to 0 demonstrate fitness change.

Generalised statements about treatment effects observed in fitness assays were made using linear models. Model selection was achieved using backward stepwise regression. Model residuals were checked using the DHARMa package v0.4.5 (Hartig, 2022). Estimated marginal means (EMMs) comparisons were conducted using the emmeans package v1.8.4-1 (Searle et al., 1980). The Kenward-Roger method was used to estimate the degrees of freedom in EMM comparisons. All biological replicates for the selection-rate constant analyses and transformation frequency measurements were measured in triplicate and averaged before downstream analyses. Transformation frequencies were analysed non-parametrically with Wilcoxon tests as the data were not normally distributed (Shapiro-Wilks tests, $p<0.001$). The mean transformation frequency of the wildtype ancestor was subtracted from the mean transformation frequencies of all samples and the resulting values were tested for significant differences to 0. T-tests, Wilcoxon, Kruskal-Wallis and Levene testing was conducted using the rstatix package v0.7.0 (Kassambara, 2021). In all analyses, a p value of less than 0.05 was considered significant. Multiple testing was corrected for with false discovery rate (FDR) correction (Benjamini & Hochberg, 1995).

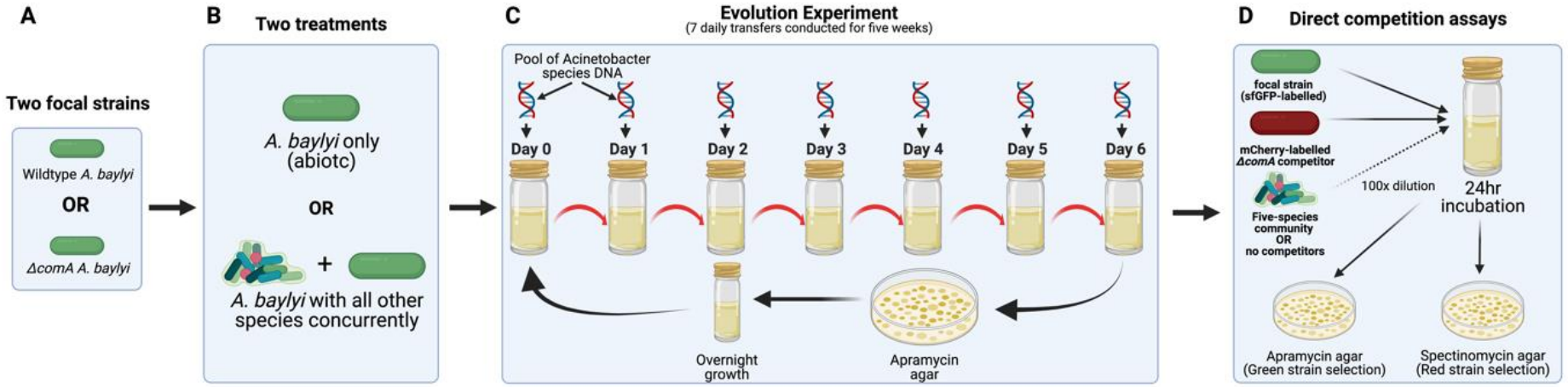

Figure 16. Illustration of the evolution experiment and direct competition assays. A: Two focal strains (competent wildtype and isogenic non-competent counterpart) were cultured separately in their respective treatment conditions (B). B: Focal strains were subjected to two treatment conditions: monoculture (abiotic) or co-culture with five competitor species. C: Cultures were propagated by replacing spent broth with fresh media resulting in an approximately 34-fold dilution each passage. After the 6th passage, all species except the focal strain are killed off using LB agar amended with apramycin. The focal strain and freezer stocks of the competitors were allowed to grow to maximum density in LB broth separately before being inoculated together for another week of passaging. D: After five full weeks of passaging, the focal strains are selected for again with use of apramycin agar and frozen in 25% glycerol at -70˚C until tested against a common competitor to measure relative fitness. Figure created with BioRender.com.

5.4 Results

Table 7. Estimated marginal means (EMMs) of standardised selection-rate constants for assay conditions, genotypes, and evolution conditions. Degrees of freedom were calculated using the Kenward-Roger method. A 0.95 confidence interval was used for all samples. SE, standard error; df, degrees of freedom CL, confidence limit.

Evolution Conditions	*Assay Conditions*	*Genotype*	*EMM*	*SE*	*df*	*lower.CL*	*upper.CL*
Abiotic	Abiotic	Wildtype	2.17	0.254	42	1.654	2.681
Biotic	Abiotic	Wildtype	3.11	0.266	42	2.569	3.643
Abiotic	Biotic	Wildtype	0.41	0.254	42	-0.104	0.923
Biotic	Biotic	Wildtype	1.35	0.266	42	0.811	1.885
Abiotic	Abiotic	*ΔcomA*	3.17	0.254	42	2.658	3.685
Biotic	Abiotic	*ΔcomA*	4.11	0.254	42	3.597	4.624
Abiotic	Biotic	*ΔcomA*	1.41	0.254	42	0.901	1.928
Biotic	Biotic	*ΔcomA*	2.35	0.254	42	1.839	2.866

5.4.1 Natural transformation does not provide fitness benefits in a community context

To test whether natural transformation favours adaptation in a biotic community context compared to an abiotic environment, a recombining wildtype *A. baylyi* and an isogenic non-recombining Δ*comA* mutant were evolved with DNA supplementation extracted from a pool of conspecific strains as a substrate for natural transformation. This five species community has been shown to stably coexist in 1/64th strength tryptic soy broth (TSB), and 28˚C with weekly passages (Padfield et al., 2020), but was found to also exist with *A. baylyi* stably in 25% TSB medium with daily transfers (results not shown). After five weeks of evolution (resulting in approximately 180 generations of log phase growth), changes in fitness of each evolved line relative to a single unevolved, differentially marked Δ*comA* strain were measured using pairwise competition assays. Populations were assayed in both presence and absence of competitors. All populations increased in fitness relative to ancestral populations (1 sample t-tests: $p < 0.01$, corrected for multiple testing, Figure 17, Table 7), except for the wildtype strains evolved in abiotic and biotic conditions when assayed in biotic conditions ($p = 0.486$, and $p = 0.058$, respectively; Figure 17, Table 7).

Table 8. Pairwise comparisons of evolved populations' relative fitness gains after 5 weeks' evolution in biotic or abiotic conditons (EMMs comparison). Evolved populations were tested for fitness gains in biotic and abiotic conditions. SE, standard error, EMM, estimated difference in relative fitness gain; df, degrees of freedom.

Evolution Condition	*Assay Condition*	*Genotype*		*Evolution Condition*	*Assay Condition*	*Genotype*	*EMM*	*SE*	*df*	*t.ratio*	*p.value*
Biotic	Abiotic	Wildtype	-	Abiotic	Abiotic	Δ*comA*	-0.0662	0.372	42	-0.178	0.8599
Biotic	Biotic	Wildtype	-	Abiotic	Biotic	Δ*comA*	-0.0662	0.372	42	-0.178	0.8599
Abiotic	Abiotic	Wildtype	-	Biotic	Biotic	Δ*comA*	-0.1856	0.439	42	-0.423	0.7266
Abiotic	Abiotic	Wildtype	-	Abiotic	Biotic	Δ*comA*	0.7529	0.364	42	2.069	0.0501
Biotic	Abiotic	Wildtype	-	Biotic	Biotic	Δ*comA*	0.7529	0.364	42	2.069	0.0501
Abiotic	Abiotic	Wildtype	-	Biotic	Biotic	Wildtype	0.8191	0.364	42	2.251	0.0361
Abiotic	Abiotic	Δ*comA*	-	Biotic	Biotic	Δ*comA*	0.8191	0.364	42	2.251	0.0361
Abiotic	Abiotic	Wildtype	-	Biotic	Abiotic	Wildtype	-0.9385	0.258	42	-3.644	0.001
Abiotic	Biotic	Wildtype	-	Biotic	Biotic	Wildtype	-0.9385	0.258	42	-3.644	0.001
Abiotic	Abiotic	Δ*comA*	-	Biotic	Abiotic	Δ*comA*	-0.9385	0.258	42	-3.644	0.001
Abiotic	Biotic	Δ*comA*	-	Biotic	Biotic	Δ*comA*	-0.9385	0.258	42	-3.644	0.001
Biotic	Abiotic	Wildtype	-	Abiotic	Biotic	Δ*comA*	1.6914	0.453	42	3.738	0.0009
Abiotic	Abiotic	Wildtype	-	Abiotic	Abiotic	Δ*comA*	-1.0046	0.258	42	-3.901	0.0006
Biotic	Abiotic	Wildtype	-	Biotic	Abiotic	Δ*comA*	-1.0046	0.258	42	-3.901	0.0006
Abiotic	Biotic	Wildtype	-	Abiotic	Biotic	Δ*comA*	-1.0046	0.258	42	-3.901	0.0006
Biotic	Biotic	Wildtype	-	Biotic	Biotic	Δ*comA*	-1.0046	0.258	42	-3.901	0.0006
Biotic	Biotic	Wildtype	-	Abiotic	Abiotic	Δ*comA*	-1.8237	0.453	42	-4.03	0.0005
Abiotic	Abiotic	Wildtype	-	Abiotic	Biotic	Wildtype	1.7575	0.257	42	6.837	<.0001
Abiotic	Abiotic	Wildtype	-	Biotic	Abiotic	Δ*comA*	-1.9431	0.356	42	-5.46	<.0001
Biotic	Abiotic	Wildtype	-	Abiotic	Biotic	Wildtype	2.696	0.364	42	7.409	<.0001

Biotic	Abiotic	Wildtype	-	Biotic	Biotic	Wildtype	1.7575	0.257	42	6.837	<.0001
Abiotic	Biotic	Wildtype	-	Abiotic	Abiotic	Δ*comA*	-2.7621	0.364	42	-7.591	<.0001
Abiotic	Biotic	Wildtype	-	Biotic	Abiotic	Δ*comA*	-3.7006	0.439	42	-8.43	<.0001
Abiotic	Biotic	Wildtype	-	Biotic	Biotic	Δ*comA*	-1.9431	0.356	42	-5.46	<.0001
Biotic	Biotic	Wildtype	-	Biotic	Abiotic	Δ*comA*	-2.7621	0.364	42	-7.591	<.0001
Abiotic	Abiotic	Δ*comA*	-	Abiotic	Biotic	Δ*comA*	1.7575	0.257	42	6.837	<.0001
Biotic	Abiotic	Δ*comA*	-	Abiotic	Biotic	Δ*comA*	2.696	0.364	42	7.409	<.0001
Biotic	Abiotic	Δ*comA*	-	Biotic	Biotic	Δ*comA*	1.7575	0.257	42	6.837	<.0001

Selection of the most parsimonious linear model was achieved by backward stepwise regression. Linear models revealed no interactions between explanatory variables genotype, evolutionary conditions, and assay conditions when predicting fitness increases ($p > 0.05$). Genotype, assay conditions, and evolution conditions are significant explanatory variables for model predictions ($F = 15.215$, df =1, $p < 0.001$; $F = 46.751$, df=1, $p < 0.0001$; and $F = 13.277$, df =1, $p < 0.001$, respectively). All evolved population groups demonstrated greater adaptation to the abiotic environment than the biotic environment irrespective of evolution conditions (EMMs pairwise testing, $p < 0.0001$; Figure 17, Table 8). Both wildtype and Δ*comA* strains evolved in the biotic environment are better adapted to both test environments than their counterparts evolved in the abiotic environment (EMMs pairwise testing, $p < 0.001$; Figure 17; Table 3). The evolved Δ*comA* populations are better adapted to the experimental environments relative to the evolved wildtype when assayed in either environment (EMMs pairwise testing, $p < 0.001$; Figure 17, Table 8). This observation applies to evolution in either environment.

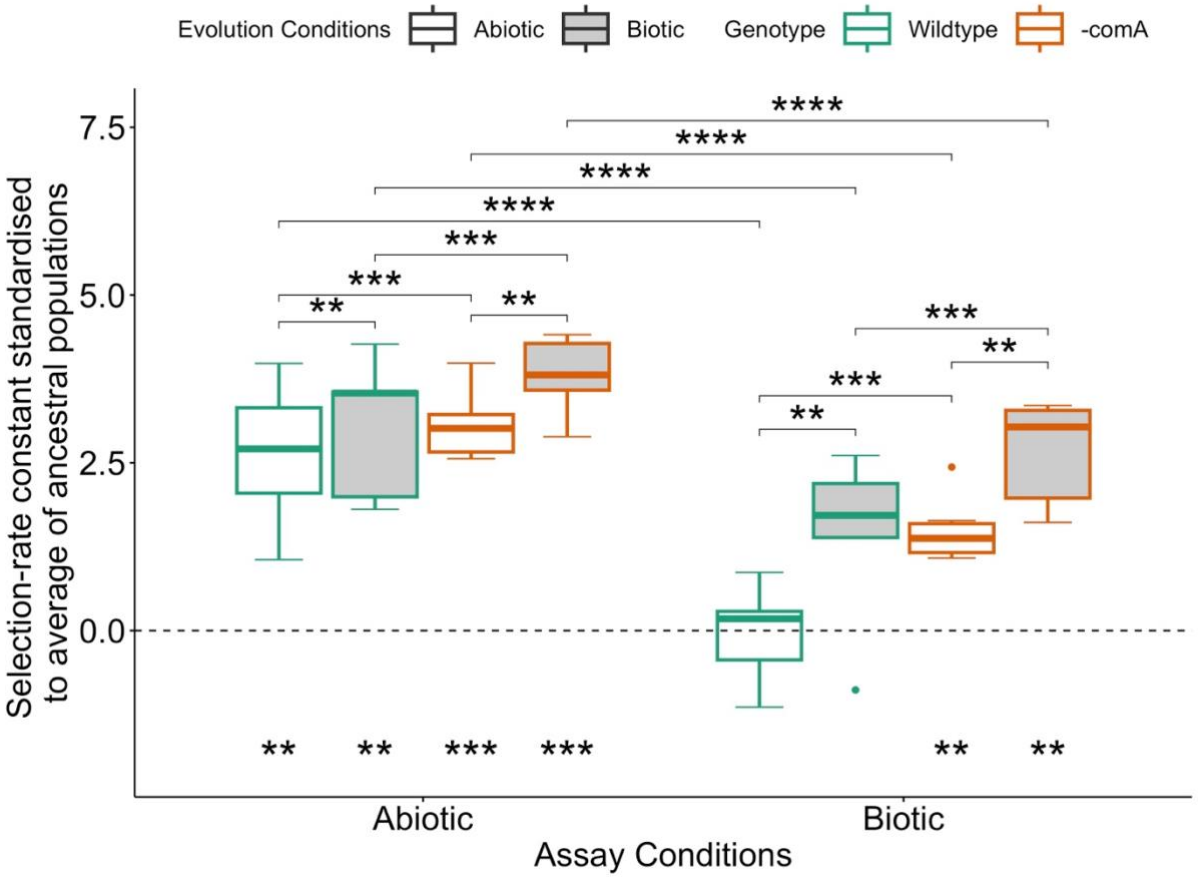

Figure 17. Selection-rate constants of evolved populations standardised to ancestral populations in respective assay conditions (y=0). Asterisks describe significant differences (EMMs comparison) between evolved populations within the same assay conditions. Asterisks (bottom) describe significant differences (t-test) between evolved populations and 0. Top, middle, and bottom bands of the boxes denote the 75% quartile, the median, and the 25% quartile, respectively. Box whiskers are 1.5x the interquartile range. Fitness differences of each of the six biological replicates per treatment were measured in triplicate. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

5.4.2 Transformation frequency did not decrease after evolution

To test whether the lack of fitness difference between the wildtype and mutant strains stemmed from a possible loss of natural transformation, transformation assays were conducted for the abiotic treatment and the five species community treatment (Figure 16). No significant differences in transformation frequency were found between the evolved wildtype lineages across the two evolution environments or the ancestor (Wilcoxon one sample test, $p > 0.05$; Figure 18). While transformation frequency did not change significantly, observed transformation frequencies were higher in all evolved wildtype populations than in the ancestor. Variance of transformation frequencies of evolved biological replicates was not found to be significant between treatment groups (Levene test). Kruskal-Wallis testing found that transformation frequencies of biological replicates were significantly different within the no competitor treatment assayed with heat-killed lysate ($p < 0.05$, Figure 19). Post-hoc analysis of the two treatment groups using Wilcoxon paired testing showed no pairwise significant differences between biological replicates.

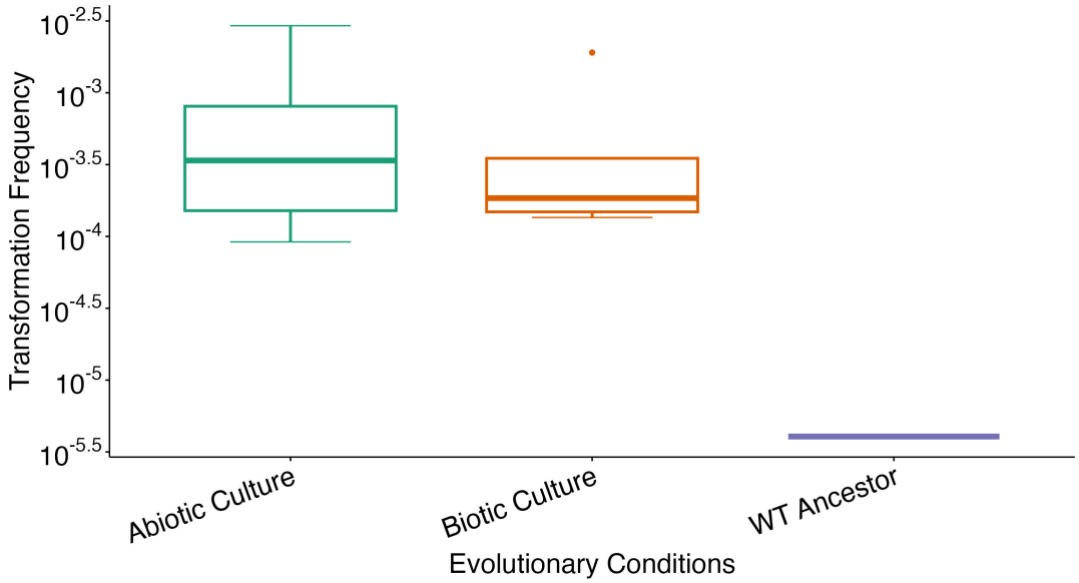

Figure 18. Treatment-level transformation frequencies of ancestral and evolved wildtype populations using heat-killed cell lysate. Transformation frequencies were measured in triplicate per biological replicate. The Abiotic Culture, Biotic Culture, and wildtype (WT) ancestor treatments had 6, 5, and 1 biological replicates, respectively.

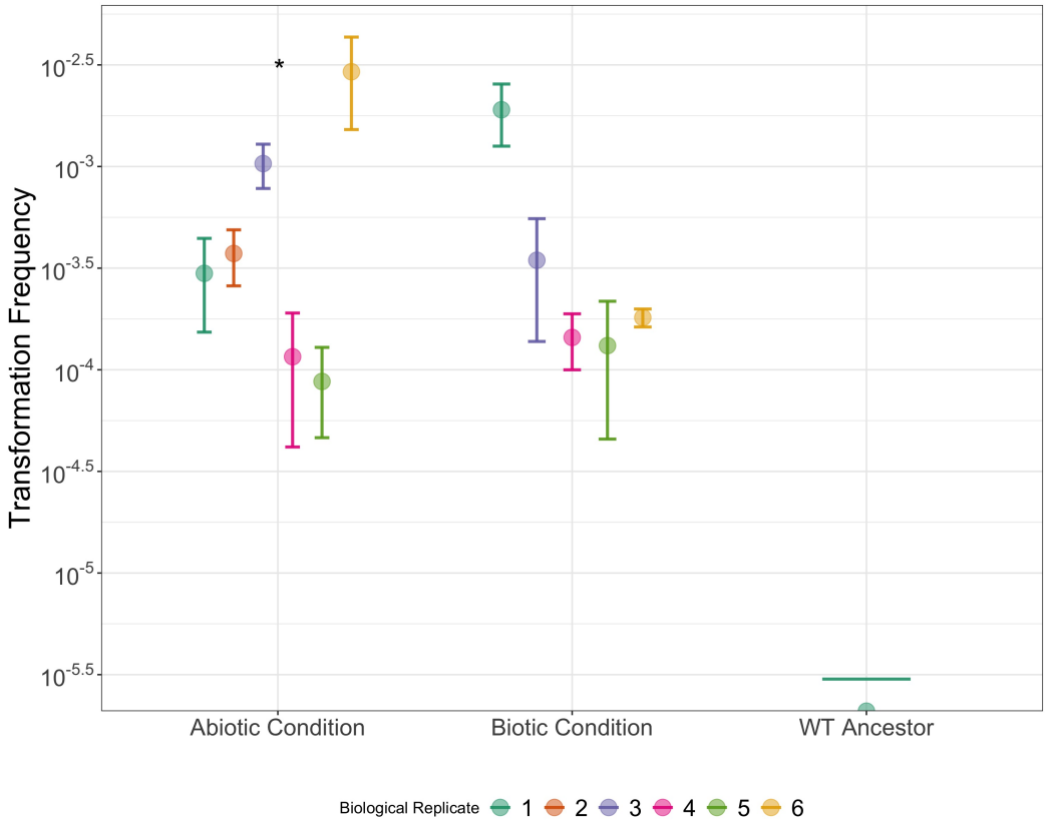

Figure 19. Variation of transformation frequencies of biological replicates within evolutionary treatment groups for heat-killed cell lysate. Transformation frequencies were measured in triplicate per biological replicate. Points are biological replicates and

bars are means ± one standard error. Asterisks denote significantly different transformation frequencies between intra-treatment biological replicates (Kruskal-Wallis, $p<0.05$).

5.5 Discussion

This study tested if recombination mediated by natural transformation in *A. baylyi* provided adaptive benefits under biotic and abiotic experimental conditions. All replicate populations in either treatment increased fitness compared to their ancestors when assayed in abiotic conditions (Figure 17). Populations of the non-recombining (Δ*comA)* genotype also show increased fitness relative to their ancestor when assayed under biotic conditions regardless of evolutionary conditions. This contrasts with the recombining wildtype, which does not show significant increases in fitness after evolution in the biotic environment, contrary to the hypothesis that sex is advantageous in the presence of other species. However, the absence of significant fitness increases observed in the wildtype which was evolved and tested in biotic conditions are probably due to a single tested population which is less fit than the ancestor, lowering the estimation of the mean (Figure 17). Further, no significant differences in fitness between evolved linages were found when comparing across genotypes and assaying in biotic conditions. Therefore, this study has not found a clear and significant beneficial (or deleterious) effect of natural transformation on adaptative evolution.

There is clear trade-off asymmetry displayed by evolving the wildtype *A. baylyi* in the presence or absence of the five competitor species (biotic and abiotic conditions, respectively). Evolution of both genotypes under biotic conditions did not constrain adaptation to an abiotic environment, but the converse is not true (Figure 17). This is because the factors in abiotic conditions (growth media, temperature, oxygen availability, spatial structure) were also consistent in the biotic condition and are therefore selected for in the biotic environment. However, there may be some adaptive mutations that arise under biotic conditions which are not advantageous in the abiotic environment, and therefore were not selected for during evolution in abiotic conditions (Gomez et al., 2022). These data show that evolution in a biotic environment best

prepares a population for a biotic environment, and does not constrain adaptation to an abiotic environment, regardless of the population's ability to naturally transform.

Interestingly, mean transformation frequencies of all tested populations increased relative to the transformable ancestor, although this was not statistically significant. This contrasts with previous studies where transformation frequencies declined markedly after experimental evolution (Bacher et al., 2006; Utnes et al., 2015). The maintenance, and potential increase of transformability in all evolved populations is not consistent with a selective benefit. As the ability to transform was not selected against either, it is possible it may have increased because of genetic drift. The increase of transformation frequency at varying rates per population (Figure 19) suggests that significant differences both within and between treatment groups may occur after a more prolonged evolution period.

Taken together, these findings show no significant evolutionary benefits of transformation in *A. baylyi*. This does not mean that no bacterial species use transformation to facilitate faster adaptation, as the physiological contexts and molecular mechanisms of natural transformation vary across species (Johnston et al., 2014). Specifically, there is convincing evidence supporting the sex hypothesis for transformation in *H. pylori* (Baltrus et al., 2007; Nguyen et al., 2022; Woods et al., 2020). There is no doubt that transformation can give an immediate adaptive benefit to *A. baylyi* such as in the contexts of antimicrobial resistance acquisition (Domingues et al., 2012; Hülter et al., 2017; Mantilla-Calderon et al., 2019; Perron et al., 2012), but its long-term benefits remain uncertain. The selection exerted by other species in this study may not have been sufficiently strong, or benefits derived from natural transformation might have accrued only at the start of the experiment before fitness was assayed. It is possible that future experiments utilising different growth conditions, interactions with live DNA donors, and reduced doubling rates of cells (limiting the frequency and contribution of mutation for adaptation), could still demonstrate the adaptive benefit of natural transformation in *A. baylyi*.

Footnote

One treatment sample containing the wildtype strain in the presence of five concurrent competitors was lost near the end of the experimental evolution period. In the interest of ensuring all samples were directly comparable by experiencing identical conditions,

the lost sample was not replaced by evolving it independently to the other samples. This explains why the replication level for one treatment is 5 instead of 6.

Chapter 6: Thesis Discussion

6.1 Thesis Summary

This thesis is comprised of four standalone research chapters which aimed 1) to determine if chemotherapeutic compounds affect the transformation frequencies of transformable bacteria. This provides important information which can have implications on the contraction of a life-threatening infection in cancer patients. 2) to determine if other environmentally relevant bacteria affect the transformation frequencies of transformable bacteria. Understanding the contexts under which bacteria transform in their natural environments can help us to predict the spread of antimicrobial resistance mechanisms via natural transformation. 3) to produce a resource of genomic information for the scientific community, allowing researchers to improve our understanding of the *Acinetobacter* genus. And 4) to determine if environmentally relevant bacteria affect the transformation frequencies of transformable bacteria to find evidence for the sex hypothesis for natural transformation. This was performed by using biotic interactions as a selection pressure and DNA from a range of related species as a substrate for transformation. Together, these chapters provide information about the contexts under which transformation is both regulated and selected for in realistic environmental contexts. Enhancing our understanding of how and when bacteria naturally transform, in both natural and clinical environments, can help us to monitor and establish preventative measures to limit the spread of antimicrobial resistance genes between bacteria.

6.1.1 Transformation Frequency Changes Caused by Cancer Chemotherapeutic Agents (Chapter 2)

Many pharmaceutical pollutants in the environment have been recognised to affect rates of natural transformation in bacteria including artificial sweeteners (Yu et al., 2022), antidepressant drugs (Lu et al., 2022), and anti-inflammatory drugs (Wang et al., 2020). However, the abiotic factors tested in previous studies thus far only represent a fraction of the compounds which bacteria are regularly exposed to in the environment. Chapter 2 is the first study to investigate the effects of cancer chemotherapeutic compounds on natural transformation or growth in any species. Six chemotherapeutic compounds with unique mechanisms of action were chosen to provide a range of possible mechanisms by which transformation may be affected.

Although the results of Chapter 2 saw no effect for most of the cancer chemotherapeutic compounds tested on natural transformation at clinically relevant concentrations, docetaxel and daunorubicin were observed to significantly affect transformation frequencies. As the effects observed in this chapter led to a decrease in transformation frequency (or no change), this is an overall positive finding from a clinical perspective as decreased rates of natural transformation lead to reduced rates of transformation-mediated dissemination of antimicrobial resistance genes (ARGs). Further, the reduction of growth rate in *A. baylyi* at clinically relevant concentrations of daunorubicin is also a positive outcome in the context of AMR dissemination, but if daunorubicin leads to cell death of commensal bacteria, cancer chemotherapy patients may be more at risk of colonisation by pathogenic bacteria (Abt & Pamer, 2014; Mueller & Macpherson, 2006). As the reduced transformation frequency observed in *A. baylyi* in the presence of daunorubicin and docetaxel occurs without changes in growth rate, the mechanisms responsible are not linked to growth phase as is seen in other studies (Palmen et al., 1993; Utnes et al., 2015; Vaneechoutte et al., 2006). A Δ*xerC* strain was constructed to determine if docetaxel affected the segregation of chromosomes during cell division. XerC works with XerD to catalyse the formation and reduction of Holliday junctions for chromosomal segregation (Barre & Sherratt, 2007). Because the Δ*xerC* strain presented with increased sensitivity to docetaxel than its wildtype counterpart, XerC is confirmed to not be the target of docetaxel's mechanism of action. The drug target is therefore likely to be another component of the cell replication pathway which can be compensated for via XerC function. A putative target for docetaxel is FtsZ which regulates the function of XerCD (Aussel et al., 2002; Hallet et al., 1999), but the target may instead be another enzyme involved in DNA replication or repair. All chemotherapeutic drugs were observed to have no effect on transformation or growth in *A. baylyi* at concentrations found in water systems in the environment, suggesting no significant effect of these drugs when released into the environment (Figure 7, Supplementary table 15). The results presented in Chapter 2 suggests two cancer chemotherapeutic drugs do not increase, and may decrease, rates of natural transformation in *A. baylyi*. While no mechanisms of action were identified in this study, it is important to continue such research as understanding of the mechanistic basis of abiotic factors such as chemotherapeutic agents on natural transformation can suggest the importance of investigation of other abiotic compounds with similar chemical structures.

6.1.2 Transformation Frequency Changes Caused by Biotic Interactions (Chapter 3)

While some past studies have investigated the effect of abiotic factors on rates of natural transformation, no work has been done testing the effect of biotic interactions on transformation frequency. Bacteria seldom live in single-species communities and interact with other species through a variety of mechanisms (James et al., 1995; Kuramitsu et al., 2007; Stubbendieck et al., 2016; Tashiro et al., 2013). Chapter 3 aimed to test the effect of biotic interactions on the transformation frequency of *A. baylyi* using 26 bacterial strains from the rhizosphere. The overall effect of these species was a weak effect causing a decrease in transformation frequency of the cocultured *A. baylyi*. Sixteen species were estimated to have a strong effect in causing decreases of transformation frequency in *A. baylyi*. As species relatedness can have implications on how species interact such as my competing for the same resources (Foster & Bell, 2012; J. D. Palmer & Foster, 2022; Peterson et al., 2020), or interbacterial toxin systems (J. D. Palmer & Foster, 2022; Peterson et al., 2020), we tested for the effect of species relatedness by comparing 16S rRNA sequence data of the competitors with the *A. baylyi* focal strain. As species relatedness did not improve the Bayesian model's explanation of observed data, species relatedness was not considered a significant predictor of a competitor's effect on *A. baylyi* transformation frequency. Similarly, as *A. baylyi* transforms more frequently during log phase growth (Hülter et al., 2017; Palmen et al., 1993), observed decreases in transformation frequency could have been caused by outcompetition for nutrients, decreasing *A. baylyi* growth rate and thus transformation frequency. However, as the inclusion of growth rate into the Bayesian model, did not improve model predictions, the estimated effects are likely to not be correlated with growth rate changes.

Two competitors, *Bordetella 1* and *Bacillus*, were used for more focussed testing for the mechanistic basis of their predicted effects on *A. baylyi* natural transformation. Presence of *Bordetella* and boiled *Bordetella* supernatant were observed to increase *A. baylyi* transformation frequency, suggesting that transformation frequency changes are caused by an extracellular compound which is not heat labile and therefore likely not proteinaceous. *Bacillus* was observed to not affect transformation frequency of *A. baylyi* in high nutrient conditions (100% LB), but significantly decreased transformation

frequency at lower nutrient availability, showing a significant context-dependence of the effect of species. Conversely, *Bordetella* significantly increased transformation frequency of *A. baylyi* at all nutrient availabilities tested. These results give insight in the mechanism of transformation frequency changes caused by competitor species and highlight the significance of testing transformation frequency changes in low nutrient conditions as they are more akin to nutrient availabilities in realistic environmental contexts.

6.1.3 Genome Announcement Describing Acinetobacter Genomes (Chapter 4)

The genome announcement produced in Chapter 4 is a resource for the scientific community providing novel information about the *Acinetobacter* genus. The most significant contribution is the sequencing of *Acinetobacter baumannii* genomes which have high clinical significance due to its notoriety as a multidrug resistant pathogen responsible for nosocomial infections and was estimated to be responsible for 400,000 deaths worldwide in 2019 (C. J. Murray et al., 2022). Using full genome sequences of *A. baumannii* helps researchers understand the prevalence of antibiotic resistance genes in the species and can help us to understand gene dissemination within the species and understand epidemiological traits of the species (Wright et al., 2014). Further, by using a greater number of full genome sequences, core and accessory genomes can be more accurately defined for species and genera (Bohlin et al., 2017).

6.1.4 Effect of Natural Transformation on Rates of Adaptation in a Community Setting (Chapter 5)

Chapter 5 aimed to test the adaptive benefits of natural transformation using a transformable focal strain and a non-transformable (Δ*comA*) isogenic counterpart evolved separately in either a community setting or in abiotic conditions. No significant adaptive benefit of natural transformation was observed in either environmental condition. This may be because the benefits of natural transformation acted upon the transforming population early in the experiment but the optimum phenotype for the experimental environment was reached before the end of the five-week evolution period. As fitness gains of the non-transforming Δ*comA* counterpart were greater than that of the wildtype, the wildtype transformable *A. baylyi* strain appeared to be less adaptable. However, as only *comA* was removed from the wildtype counterpart, the transcription of many competence and transformation genes can still occur but is

redundant without a functioning ComA transmembrane protein which can facilitate DNA uptake into the cell. Therefore, if the fitness peak is reached quickly in the experimental environment, the non-transforming strain may develop missense mutations in genes coding for transformation machinery which are selected for by increased growth rate due to redundant proteins which are no longer being synthesised. Loss of non-crucial transformation genes such as *comEA*, *pilG*, and *fimU* (Leong et al., 2017) may be restored via natural transformation in the wildtype strain, but not the Δ*comA* counterpart, suggesting fitness gains from transformation machinery loss may accumulate faster in a non-transforming strain than in a transforming strain.

The two strains were measured for fitness prior to the start of evolution period to ensure that they were isogenic and there was a negligible fitness difference created by the deletion of *comA*. However, it is unknown as to whether there are epistatic interactions which might interact with *comA*. The loss of such interactions may force the Δ*comA* strain to follow a different evolutionary trajectory to the wildtype ancestor. Further, an early benefit of natural transformation is suggested by the non-significant increase of transformation frequency in all evolved wildtype populations, which contrasts with the decrease in transformation frequency observed in past studies (Bacher et al., 2006; Renda et al., 2015; Utnes et al., 2015). Transformation frequency in this experiment may have been increased or maintained through second-order selection (Tenaillon et al., 2001), and not subjected to strong negative selection once the fitness peak had been reached. This is likely because the ability to transform does not incur a strong fitness cost as was demonstrated prior to the evolution period using the wildtype and Δ*comA* strains. Ultimately, this study did not provide strong evidence to support or reject the sex hypothesis for transformation. However, this study offers valuable insight on how future studies should design experiments testing this hypothesis, such as by frequently measuring fitness and transformation frequency changes for the duration of the evolution period and demonstrating how the use of coculture with competitor species can act as an ecologically relevant selective pressure.

6.2 Thesis limitations

6.2.1 Transformation Frequency Changes Caused by Cancer Chemotherapeutic Agents (Chapter 2)

This chapter provided an insight into the effects of cancer chemotherapeutic drugs on natural transformation, showing an effect of two compounds with unique mechanisms of action. Further, little is known about the accumulation of drugs in the body and the implications that will have on the transforming cell due to localised increases of drug concentration. Drug concentrations relevant to bacteria may be higher than concentrations measured in patient serum or intercellular fluid if accumulation occurs in the gut (Chillistone & Hardman, 2017), and neighbouring species may metabolise these drugs into compounds which may have a different effect on transformable species (Klünemann et al., 2021b).

Docetaxel shows different effects on the wildtype in experiments shown in Figures 7EF and 9 where the highest concentration of docetaxel decreases transformation frequency in the experiment shown in Figures 7EF but does not significantly affect transformation frequency in the experiment shown in Figure 9. Results for both experiments are considered to be valid as Figures 7EF include six replicates for each treatment sampled on separate days, while data collection for Figure 9 has been repeated twice with similar results (data not shown). The most likely explanation for this is where these experiments were conducted. Data for Figure 7 were collected in the Arctic University of Norway, Tromsø, and data for Figures 8 and 9 were produced in the Cornwall campus of The University of Exeter. It was noted after data collection in Tromsø that the pH of the deionised water used for producing LB broth was pH 8-8.5, while the deionised water in the ESI lab in Cornwall is pH 4.5-5 when tested using pH indicator strips (VWR, USA). Deionised water cannot be measured for pH as it has no ions and scrubs ions from any detector which is used to measure it. The pH of deionised water should therefore always be 7, but if there are faults in equipment used to produced deionised water, this cannot be tested for by measuring pH. This has substantial implications for experiments testing for natural transformation as pH values greater than 7 are known to decrease rates of natural transformation in *A. baylyi* (Palmen et al., 1993). The effect of transformation frequency reduction by docetaxel may have been augmented by the putatively higher pH in the Tromsø lab and caused the significant effect seen in Figures 7EF which is not seen in Figure 9. Indeed, water

characteristics can vary wildly within a single lab, leading to one study using Volvic mineral water instead of deionised water (Glücksman et al., 2010). Reproducibility of experimental work in science is widely recognised to be poor, with 70% of scientists reporting inability to replicate published work and 50% of scientists not being able to reproduce their own results (M. Baker & Penny, 2016). The lack of corroboration of data between experiments in this study leads to inconclusive results on the effect of docetaxel on the wildtype.

6.2.2 Transformation Frequency Changes Caused by Biotic Interactions (Chapter 3)

The high number of species tested for their effect on natural transformation necessitated the use of multiple blocks within the first experiment in Chapter 3. This limited the interpretation of the data as it generated large amounts of variation within treatments and led to more conservative analysis of data than experiments conducted in only one block. Indeed, the Bayesian model explained only 42.2% (R^2 value) of the variation in the data, implying that an important variable was likely missed, and outcomes cannot be generalised. As a result, some species tested may have a significant effect on natural transformation, but their effect could not be accurately estimated due to the unbalanced nature of the experimental design. An example is *Bordetella* which appeared to increase transformation frequency in experiments testing the effect of nutrient availability and mechanistic basis of effects but was not observed to significantly affect natural transformation frequency when also testing the effect of 25 other species. Use of 16S rRNA sequences to determine species relatedness of cocultured species to the focal *A. baylyi* strain is generally considered appropriate for species identification but is limited in representing overall genomic divergence. However, when full genome sequences would become available for all strains, it is not likely to change the overall result. The findings of Chapter 3 cannot be extrapolated to all natural environments as it used only one transformable focal species and competitor species from the rhizosphere. Species from other niches must be tested for their effect on natural transformation before any extrapolations can be made with confidence.

6.2.3 DNA Sequencing of a Collection of *Acinetobacter* Species (Chapter 4)

This study used short read Illumina MiSeq sequencing data which yields high accuracy reads but can cause difficulty when connecting contigs to form a full chromosome sequence. For example, the number of contigs for the strains in Chapter 4 range from 17 to 99 and cannot be confidently assembled into a full chromosome. This is also demonstrated by the assembly output describing these strains as having linear genomic topology like most eukaryotes, as opposed to circular topology like most prokaryotes (and indeed *Acinetobacters;* Favale et al., 2022; Traglia et al., 2014). Increasing the length of reads from 150bp to 250-300bp can greatly improve accuracy of reads and can greatly improve contig assembly in full genome sequencing (Sameith et al., 2017). Long read sequencing (sequencing of reads greater than 7kbp) can produce “closed” genomes which are a single contig of the chromosome, showing the location of genes on the chromosome with albeit with reduced accuracy than short read sequencing and no gaps (Koren & Phillippy, 2015). However, long read sequencing technology such as PacBio Single-Molecule Real-Time can assemble genomes with over 99.99% accuracy (Brown et al., 2014; Harhay, McVey, et al., 2014; Harhay, Murray, et al., 2014; Satou et al., 2014). In the near future, metagenome assembled genomic analysis will make possible the sequencing of multiple strains within the same sample to identify a bacterial community without isolating and sequencing each strain individually (Meziti et al., 2021). This will increase ease of sequencing protocols and increasing the convenience of using environmentally sampled communities for experiments instead of synthetic communities created in lab environments.

6.2.4 Effect of Natural Transformation on Rates of Adaptation in a Community Setting (Chapter 5)

Fitness gains in Chapter 5 were not measured over the course of the evolution period which made it impossible to determine if relative fitness gains of the two focal strains were different at separate timepoints in the experiment. Frequent sampling such as that conducted in other studies (Nguyen et al., 2022; Woods et al., 2020) can help identify how rates of adaptation fluctuate in evolution experiments. The loss of a single replicate for the transformable wildtype population evolved in the presence of the five competitors reduced the statistical power of the transformation assay analyses. Additionally, evolved populations were measured in triplicate and then an average was

taken, which was considered an accurate estimation of the population without unfair inflation of replication. As there was only one biological replicate of the ancestral population, it was treated as an evolved population and thus only having one biological replicate. This loss of statistical power led to no significant increase in transformation frequency observed, in spite of all observations of evolved population transformation frequency being higher than that of the ancestor.

6.3 Recommendations for future research

6.3.1 Transformation Frequency Changes Caused by Cancer Chemotherapeutic Agents (Chapter 2)

The findings presented in Chapter 2 demonstrate that cancer chemotherapy drugs affect rates of natural transformation in bacteria, but a very limited number of drugs and drug classes have been tested for this effect. As many more mechanisms of action remain untested, this highlights the importance of testing more chemotherapeutic compounds to determine if any cause transformation frequency increases which may be problematic for patients. Future work should be conducted to test the effect of cancer chemotherapy drugs belonging to other classes such as alkylating agents, corticosteroids, mitotic inhibitors, or non-grouped drugs such as all-trans retinoic acid to generate a more comprehensive understanding of the effects these drugs have on bacterial natural transformation. As cancer chemotherapy drugs act on different targets within a cell such as metabolism with asparaginase (Verma et al., 2008), DNA with busulfan (Patel & Tadi, 2022), and gene expression with prednisone (Cronstein et al., 1992), the findings in Chapter 2 are not sufficient to extrapolate to all other cancer chemotherapy drugs. Moreover, cancer chemotherapy regimens involve administering prophylactic antibiotics which may interact with the cancer treating agents administered to the patient, producing a different effect to that seen in Chapter 2, so must be investigated. Chapter 3 demonstrated that effects caused by biotic interactions are different to control groups when tested at varying nutrient availabilities. Testing the effects of these drugs in more environmentally relevant conditions, and in the presence of multiple species are important for making conclusions about the implications of these drugs in natural environments. Additionally, as transformable species are highly varied in the contexts under which competence is induced

(Johnston et al., 2014), future work should be done to test if the effects of these compounds are consistent for other transformable species.

6.3.2 Transformation Frequency Changes Caused by Biotic Interactions (Chapter 3)

The findings presented in this study demonstrate the importance of exploring the effects on natural transformation caused by biotic interactions. This relationship is highly ecologically relevant as most bacterial ecosystems are multispecies communities with a host of dynamic interactive processes occurring continually. This study tested a relatively small group of bacteria which are all known to exist in the rhizosphere, thereby suggesting that while the species must have some niche differentiation to be able to coexist, they are similar enough to successfully persist in in a rhizosphere environment. More work should be conducted testing a host of bacteria from a range of environmental niches to provide the chance to observe more mutualistic and antagonistic interactions. Studies investigating competitor species from a range of different niches may observe patterns which change transformation frequency in a predictable manner. Further, future studies should test the effect of biotic interactions on other transformable species such as *Vibrio cholerae*, *Helicobacter pylori*, or *Bacillus subtilis,* as it is unlikely that all other species will respond as *A. baylyi* did in this study. The use of other focal species brings greater relevance for testing a range of niches as *H. pylori* is a human stomach pathogen which may not interact with other bacterial species in gastric environments. Testing whether transformation frequency changes upon interacting with species of other taxonomical kingdoms such as a host-pathogen interaction during infection by *H. pylori* may offer novel insight into the pathogenesis.

This chapter tested the effect of pairwise competitions involving the focal strain. This does not inform whether more complex communities which host more species will have different effects as such species may interact with each other, suppressing or synergistically augmenting their effects on natural transformation of the focal species (Barraclough, 2015). Further, providing spatial structure similar to a soil environment, or embedding the cultures in soil during assay periods may alter the effects seen in this study which would be an important observation informing the experimental design of future studies. Interactions between species may change if competitors are in the

same biofilm, and secreted elements affecting transformation frequency may not be able to penetrate the biofilm to reach the bacteria within. This study demonstrated greater effects of species interactions when in low nutrient conditions. We may see different effects when species are cocultured for extended periods of time in stationary phase as physiological processes change as a function of growth rate (Kolter et al., 1993). Further, invasion of a focal species from rare or starting at a high cell density relative to its competitors may alter the regulation of transformation machinery.

The varied metabolic processes of bacterial species also imply that certain bacteria can metabolise compounds which others cannot, which may have implications on pre-existing discoveries on the effect of abiotic factors on natural transformation. If cocultured bacteria can metabolise abiotic factors into other compounds, those by-products may affect transforming cells in ways which have not been observed. Alternatively, abiotic factors in the environment may alter the physiological functions of community members which in turn affect the transforming cell. Therefore, it is pertinent to conduct future studies and revise prior studies with the implementation of bacterial communities to test for interactions between biotic and abiotic factors. Anthropogenic pollutants may therefore have different effects on transforming cells depending on the bacterial community they are embedded in.

The observed effect of biotic interactions on natural transformation has highlighted the significance of a previously ignored aspect of bacterial ecology. This may be crucial in testing the current hypotheses explaining the function of natural transformation and potentially leading to the establishment of new hypotheses which pertain to the ecological dimension of bacterial functions.

The effectiveness of analyses using Bayesian modelling were likely limited by the measurement of samples in separate blocks. Additionally, the model identified a large amount of inter-block variation. Ensuring that equal numbers of replicates of each treatment group are measured within the same block can help to minimise variation in the data generated by sampling methodology (Bernstein, 1927). Future studies should consider number of treatments in experimental design to ensure that the block effect can be controlled for by measuring equal numbers of every treatment per block.

6.3.3 DNA Sequencing of a Collection of *Acinetobacter* Species (Chapter 4)

The strains described in Chapter 4 can be used to further investigate the *Acinetobacter* genome and can be used as a convenient resource for future work testing the adaptive benefits of bacterial sex. These strains can be given to any lab and have already been full genome sequenced. This means that a future study using the strains described in Chapter 4 as a donor DNA pool will not need to sequence the donor genomes to understand what genes can be acquired by a transforming strain from donor DNA.

6.3.4 Testing for the Adaptive Benefits of Natural Transformation (Chapter 5)

More work needs to be conducted utilising biotic interactions when testing for the adaptive benefits of natural transformation as they can provide the most accurate conditions for ecological relevance which are likely to significantly affect transformation frequency. Biotic interactions can affect transformation frequencies of other bacteria as demonstrated in chapter 3, but they might also provide strong and fluctuating selection pressure. Indeed, recent work has suggested that species which are already ecologically associated will preserve their ecological stability in coculture by constraining adaptation which may otherwise lead to out-competition (Barber et al., 2022). Similarly, if species evolve niche divergence, the rates of coadaptation will decrease over time, while a scenario involving a host-parasite interaction will maintain coevolution (Macarthur & Levins, 1967). These arguments may explain why adaptation appeared to have stopped before the sampling period in Chapter 5, as all species used in the experiment are soil-dwelling and therefore share the same niche and may have coevolved over long periods of evolutionary time. In contrast, coevolving two species which are likely to never interact in the natural environment provides the possibility of out-competition and the forcing of one competitor into extinction, or for the species to adapt niche differentiation to facilitate coexistence (Barber et al., 2020). The use of multispecies communities in experimental work is still a recent approach and further optimisations need to be conducted before the most appropriate model systems can be applied in studies which would benefit from their use. Basic aspects such as culture media, temperature, agitation, spatial structure, transfer frequency and volume, and focal/competitor species can all greatly affect the function and integrity of the model system and so must be optimised prior to any experimental work.

6.4 Overall Conclusions

The studies presented in this thesis provide novel evidence to demonstrate that the transformation frequency of *A. baylyi* is affected by abiotic and biotic factors which are relevant to clinical environments in the case of cancer chemotherapeutic drugs in, and potentially any environment with multispecies bacterial communities. Moreover, the work done in this thesis provided a novel resource which can be used for analysing the *Acinetobacter* genome and tested for the sex benefits of natural transformation.

Chapter 2 describes positive news in a clinical perspective due to reduced or unaffected transformation frequencies in response to exposure to cancer chemotherapeutic drugs. A small handful of categorised mechanisms of action, and indeed drugs, were tested in this study, so extrapolation to all cancer chemotherapeutic drugs is unwise, indicating a need for future work to explore the effect of other chemotherapy drugs and prophylactic antibiotics to provide a more comprehensive understanding of these drugs which can inform best practice for clinicians to mitigate the spread of antimicrobial resistance and pathogenicity genes in clinical environments via natural transformation.

Chapter 3 outlines a previously unexplored dimension of the natural environment of bacteria which has implications on most contexts of transformation frequency changes in the environment as bacteria seldom live in single species communities. Additionally, the implications of the effect of bacterial community on natural transformation are likely not limited to typical processes of cells. Anthropogenic pollutants may be metabolised differently by different species which may lead to the release of by-products or altered cell function which affect transforming cells in different manners to those seen in previous studies (Lu et al., 2022; Wang et al., 2020; Yu et al., 2022) or lead to an effect where there was previously no effect observed.

The work in Chapter 4 was pre-emptively conducted to provide a resource to determine the source of novel genes acquired by the transformable focal strain in Chapter 5 but was not utilised due to no obvious adaptive benefit of natural transformation being found in Chapter 5. However, Chapter 4 is a published article in

Microbiology Resource Announcements (Winter, Harms, Johnsen, & Vos, 2023), an open access journal which presents full genome sequences of interest for the scientific community. This published data can aid studies which seek to better characterise species in the genus including *Acinetobacter baumannii* which is of high interest for its capacity to cause multidrug resistant infections in humans (Fournier et al., 2006; Fukuta et al., 2013).

The evidence for the sex hypothesis for natural transformation is mixed and this may be due in part to suboptimal experimental design. Chapter 5 aimed to build on the findings of past studies testing this hypothesis and optimise experimental design to provide clear evidence to support or reject the sex hypothesis. While the findings of Chapter 5 are not in clear support of the sex hypothesis of transformation, the appearance of increased transformation frequencies suggest there was no strong selection against the ability to transform, contrasting with past studies which saw transformation decreases over evolutionary time (Bacher et al., 2006; Renda et al., 2015; Utnes et al., 2015). Therefore, the results in Chapter 5 do not directly suggest a deleterious effect of natural transformation on adaptation and may demonstrate an adaptive benefit of natural transformation if more frequent testing of fitness changes were measured. These results provide insightful information informing the experimental design of future studies to improve the chances of finding strong evidence to support or reject the sex hypothesis for *A. baylyi*, and potentially other species. Chapter 5 is published with the open access journal *Microbiology* (Winter, Harms, Johnsen, Buckling, et al., 2023).

Taken together, the studies presented in this thesis provide novel information and inform direction for future work in further determination of the effect of abiotic and biotic factors on natural transformation, the sex hypothesis for natural transformation, and genomic analysis of the *Acinetobacter* genus.

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