Effects of biocides on processes underlying resistance evolution

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently
and that I have not used any sources and aids other than those indicated by me.
This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

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List of Abbreviations

A. baylyi	*Acinetobacter baylyi*
ALE	adaptive laboratory evolution
AMR	Antimicrobial resistance
BAC	benzalkonium chloride
B. subtilis	*Bacillus subtilis*
cfu	colony forming units
CHX	chlorhexidine digluconate
DDAC	didecyldimethylammonium chloride
dw	dry weight
E. coli	*Escherichia coli*
HGT	horizontal gene transfer
IPBC	3-Iodo-2-propynyl N-butylcarbamate
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
mL, L	milliliter, liter
QAC	quaternary ammonium compound
S	Evolutionary treatment with selection for survival only
SG	Evolutionary treatment with selection for survival and growth
P	Evolutionary treatment with selection for persistence
γ	plasmid transfer rate (mL $cell^{-1}$ h^{-1})
µg, g	microgram, gram
μ_g	mutation rate per genome per replication

Preface

This thesis is a study on the effects of selected chemicals used as active substances in biocides on processes that underlie the development of resistance in selected bacteria. To investigate this topic, I chose a range of biocides used in different applications and performed laboratory experiments with a set of model bacteria. These laboratory experiments were selected covering known microbial resistance evolution and acquisition mechanisms to study the effect of biocides on those mechanisms. Our research findings show that biocides can affect resistance development mechanisms under specific conditions. These findings contribute to our understanding of biocide effects on resistance development and are a significant contribution to existing biocide research literature.

Summary

Antimicrobial resistance (AMR) is a global health problem. It is well known that antibiotics can drive evolutionary processes that underlie antimicrobial resistance (AMR) evolution and spread in clinical and environmental settings. In contrast, less is known about the effects of antimicrobial substances that are used as biocides (i.e. disinfectants and preservatives) on AMR evolution and spread. Biocides are present in various settings, interacting with diverse microbial communities. Therefore, it is crucial to evaluate their role in the evolution and dissemination of antimicrobial resistance. Biocides occur in a wide range of concentrations in various environmental settings. By examining how the various concentrations affect selection mechanisms, we gain insights into potential developments related to antimicrobial resistance. The aim of this PhD thesis is to investigate the effects of biocides on processes underlying resistance evolution. Specifically, the work focused on key mechanisms for resistance spread, resistance evolution, and the effect of selection pressures on evolved resistance mechanisms. The thesis is structured around three major objectives: (i) to determine the effect of biocides on the evolution of resistance by affecting the rate of occurrence of de novo mutations, (ii) to determine the effect of biocides on the spread of resistance genes by modifying the rate of horizontal gene transfer (HGT) processes, and (iii) to investigate the selective drivers of the emergence of antimicrobial resistance in adaptive laboratory evolution (ALE) experiments.

De-novo mutations are spontaneous mutations that occur at a certain rate in microorganisms. The effect of biocides at subinhibitory environmentally relevant concentrations on the mutation rate in *Acinetobacer baylyi*, *Bacillus subtilis* and *Escherichia coli* was assessed with the fluctuation assay. The results showed that biocides affected mutation rates in a species and substance dependent matter. The bisbiguanide chlorhexidine digluconate, the quaternary ammonium compound didecyldimethylammonium chloride, the metal copper, the pyrethroid-insecticide permethrin, and the azole-fungicide propiconazole increase mutation rates in *E. coli*, whereas no increases were identified for *B. subtilis* and *A. baylyi*.

Horizontal gene transfer refers to diverse mechanisms that mediate the transfer of mobile genetic elements between microorganisms. This work focused on conjugation and transformation. Conjugation is a process whereby a conjugative plasmid is transferred from a donor cell to a recipient cell. Transformation is a process whereby exogenous donor DNA is taken up into a recipient cell and integrated into the recipient's' genome. The effects of subinhibitory environmentally relevant biocide concentrations on the conjugation rate of *E. coli* and the transformation rate of the naturally competent organisms *A. baylyi* in were assessed. The results showed that benzalkonium chloride (BAC), chlorhexidine and permethrin increased conjugation in *E. coli*, while none of the biocides increased transformation rates in *A. baylyi*.

To further understand the molecular mechanisms underlying the effects on mutation and conjugation rates, I investigated the induction of the *RpoS*-mediated general stress and the *RecA*-linked SOS response upon biocide exposure. The results show a link between the general stress and the SOS response with increased rates of mutation and conjugation, but not for all biocides.

One major approach to study the evolutionary response of bacteria to antimicrobials are ALE experiments with growth at subinhibitory concentrations linked to serial subculturing over many generations. Such experiments have been used to study resistance evolution to antibiotics and biocides. However, previous work showed that adaptation to biocide stress may be mediated by different evolutionary drivers. Here, I investigated the contributions of evolution for increased survival as opposed to improved growth in ALE experiments with E. coli exposed to subinhibitory BAC concentrations. Two distinct evolutionary treatments selecting for survival only or survival and growth led to specific evolutionary adaptations apparent in the phenotypes and genotypes of the evolved populations. Populations growing in the presence of BAC evolved increased fitness in the presence of BAC associated with higher resistance to BAC and cross-resistance to antibiotics, while this was not the case for populations evolving for increased survival only. Genotypic characterization by whole genome sequencing of the evolved populations revealed parallelism in mutated genes among replicate populations and distinct differences across treatments. Treatments selecting for survival and growth showed mutations in stress response related genes (*hslO* and *tufA*), while selection for survival led to mutations in genes for metabolic regulation (*cyaA*) and cellular structure (flagella *fliJ*).

In summary, this thesis shows that biocides affect AMR evolution and emphasizes the importance of understanding of how biocides impact the molecular and evolutionary process that underlie AMR evolution.

Zusammenfassung

Antibiotikaresistenz stellt ein globales Gesundheitsproblem dar. Es ist bekannt, dass Antibiotika evolutionäre Prozesse befördern können, die der Entwicklung und Verbreitung antimikrobieller Resistenzen (AMR) in Krankenhäusern und der Umwelt zugrunde liegen. Im Gegensatz dazu ist über die Auswirkungen antimikrobieller Substanzen, wie Biozide (z.B., Desinfektions- und Konservierungsmittel), auf die Entwicklung und Verbreitung von AMR weniger bekannt. Biozide treten in verschiedenen Umgebungen auf und interagieren mit verschiedenen mikrobiellen Gemeinschaften. Daher ist es von entscheidender Bedeutung, ihre Rolle bei der Entstehung und Verbreitung von Antibiotikaresistenzen zu evaluieren. Biozide können in weiten Konzentrationsbereichen in verschiedenen Umweltkontexten auftreten. Die Untersuchung, wie diese verschiedenen Konzentrationen Selektionsmechanismen beeinflussen, ermöglicht Einblicke in die potenzielle Entstehung von AMR im Zusammenhang mit der Verwendung von Bioziden. Das Hauptziel dieser Doktorarbeit ist die Untersuchung der Auswirkungen von Bioziden auf die Prozesse, die der Resistenzentwicklung zugrunde liegen. Die Arbeiten haben sich speziell auf die Hauptmechanismen für die Resistenzevolution, die Ausbreitung von Resistenzen und die Auswirkungen des Selektionsdrucks auf entwickelte Resistenzmechanismen konzentriert. Die Arbeiten hatten drei Hauptziele: (i) die Bestimmung der Auswirkungen von Bioziden auf die Resistenzentwicklung durch Beeinflussung der Häufigkeit des Auftretens von *De-novo*-Mutationen, (ii) die Bestimmung der Auswirkungen von Bioziden auf die Ausbreitung von Resistenzgenen durch Veränderung der Häufigkeit des horizontalen Gentransfers (HGT) und (iii) die Untersuchung der selektiven Triebkräfte für die Entstehung antimikrobieller Resistenzen in Experimenten zur adaptiven Laborevolution (ALE).

De-novo-Mutationen sind spontane Mutationen, die mit einer bestimmten Rate in Mikroorganismen auftreten. Die Auswirkung von Bioziden in subinhibitorischen, umweltrelevanten Konzentrationen auf die Mutationsrate in *Acinetobacter baylyi*, *Bacillus subtilis* und *Escherichia coli* wurde mittels der Fluktuationstest untersucht. Die Ergebnisse zeigten, dass Biozide die Mutationsraten je nach Art und Substanz beeinflussen. Chlorhexidin, Didecyldimethylammoniumchlorid, Kupfer, Permethrin und Propiconazol erhöhen die Mutationsraten in *E. coli*, während bei *B. subtilis* und *A. baylyi* keine Erhöhung festgestellt wurde.

Horizontaler Gentransfer bezeichnet die verschiedenen Mechanismen, die den Transfer mobiler genetischer Elemente zwischen Mikroorganismen ermöglichen. Diese Arbeit konzentrierte sich auf die Untersuchung von Konjugation und Transformation. Konjugation ist ein Prozess, bei dem ein konjugatives Plasmid von einer Spenderzelle auf eine Empfängerzelle übertragen wird. Transformation ist ein Prozess, bei dem exogene Spender-DNA von einer Empfängerzelle aufgenommen und in das Genom des Empfängers integriert

wird. Es wurden die Auswirkungen subinhibitorischer, umweltrelevanter Biozidkonzentrationen auf die Konjugationsrate von *E. coli* und die Transformationsrate des natürlich-kompetenten Organismus *A. baylyi* untersucht. Die Ergebnisse zeigten, dass Benzalkoniumchlorid (BAC), Chlorhexidin und Permethrin die Konjugationsrate von *E. coli* erhöhten, während keines der Biozide die Transformationsrate von *A. baylyi* erhöhte.

Um die molekularen Mechanismen, die den Auswirkungen auf die Mutations- und Konjugationsraten zugrunde liegen, besser zu verstehen, wurde die Induktion des RpoS-vermittelten allgemeinen Stresses und der RecA-verknüpften SOS-Reaktion bei Biozidexposition untersucht. Die Ergebnisse zeigen eine Verbindung zwischen dem allgemeinen Stress und der SOS-Reaktion mit erhöhten Mutations- und Konjugationsraten, jedoch nicht für alle Biozide.

Ein wichtiger Ansatz zur Untersuchung der evolutionären Reaktion von Bakterien auf antimikrobielle Substanzen sind ALE-Experimente, bei denen das Wachstum bei subinhibitorischen Konzentrationen in Verbindung mit serieller Subkultivierung über viele Generationen durchgeführt wird. Solche Experimente wurden zur Untersuchung der Resistenzentwicklung gegenüber Antibiotika und Bioziden bereits in der Literatur beschrieben. Frühere Arbeiten haben jedoch gezeigt, dass die Anpassung an Biozidstress durch verschiedene evolutionäre Prozesse vermittelt werden kann. In dieser Studie wurden die Einflüsse der Selektionsdrücke zur Verbesserung des Überlebens im Vergleich zur Verbesserung des Wachstums in ALE-Experimenten mit *E. coli* Kulturen, die subinhibitorischen BAC-Konzentrationen ausgesetzt waren, untersucht. Zwei unterschiedliche evolutionäre Behandlungen, die entweder auf das Überleben oder auf das Überleben und das Wachstum selektierten, führten zu spezifischen evolutionären Anpassungen, die sich in den Phänotypen und Genotypen der entwickelten Populationen zeigten. Populationen, die in Anwesenheit von BAC wuchsen, entwickelten eine erhöhte Fitness in Gegenwart von BAC, was mit einer höheren Resistenz gegen BAC und einer Kreuzresistenz gegen Antibiotika einherging. Dies war bei Populationen, die nur auf besseres Überleben selektiert wurden, nicht der Fall. Die genotypische Charakterisierung der entwickelten Populationen durch Sequenzierung des gesamten Genoms ergab Parallelen bei den mutierten Genen zwischen den Replikaten der Populationen und deutliche Unterschiede zwischen den Behandlungen. Behandlungen, die auf Überleben und Wachstum selektierten, zeigten Mutationen in Genen, die mit der Stressreaktion (*hslO* und *tufA*) in Verbindung stehen, während die Selektion auf Überleben zu Mutationen in Genen für die Stoffwechselregulation (*cyaA*) und die Zellstruktur (Flagellen *fliJ*) führte. Zusammenfassend zeigt diese Arbeit, dass Biozide die AMR-Evolution beeinflussen und unterstreicht die Bedeutung von vertieftem Wissen über die molekularen und evolutionären Prozesse, die der AMR-Evolution durch Biozide zugrunde liegen.

Chapter 1: General Introduction

Antimicrobial substances

Antimicrobials are used to protect human and animal safety and hygiene, nonetheless a rise in usage of antimicrobials and the associated global health treat of antimicrobial resistance [1–5] and thus increasing research activity was conducted on this topic in the recent years [6,7]. The term “antimicrobials” is often used interchangeably in the literature to refer to antibiotics or antimicrobial biocides, which might cause confusion. Antibiotics are mainly used to combat harmful infections in humans and animals [8]. Antimicrobial biocides are applied to control unwanted microorganisms in various settings such as clinical environments, agriculture, in industrial processes and domestic households [9]. However, both, antibiotics and antimicrobial biocides contribute to the global threat of the antimicrobial resistance problem [1,7].

Antibiotics

Antibiotics are naturally or synthetically produced chemical compounds that are used to treat infections in humans and animals [10,11]. Thereby, antibiotics targets essential processes or structures in bacteria and might act bacteriostatic, inhibiting the bacterial growth or act bactericidal, killing bacteria [12]. Antibiotics are classified into antibiotic classes based on their mode of action and chemical structure [11]. Further information on antibiotics from various antibiotic classes used in this thesis can be found in Table 1.

Humankind have already been in contact with antibiotics in ancient times as chemical analysis of skeletal remains from 350-550 CE prove [13,14], but an active usage of antibiotics for treating infections occurred more recently in the 20th century [15]. Before the industrial production of antibiotics infectious disease were the leading cause of human deaths [11]. The discovery of penicillin [16] and the large scale production of antibiotics made them available to the public for treating infectious diseases and enabling medical procedures [17]. Today, antibiotics are used to treat humans and animals [18], but antibiotics are also used in agriculture [19] and there is an increasing trend in the worldwide antibiotic consumption [5].

Table 1 Background information on antibiotics used in this thesis

Antibiotic class	Example	Molecular Target	Resistance mechanism	Reference
ß-Lactams	Ampicillin	Cell wall synthesis	Target alteration, hydrolyzing enzymes, efflux or exclusion	[20–22]
Diaminopyrimidines	Trimethoprim	Folate synthesis	overproduction, Target mutation, efflux	[22]
Aminoglycosides	Gentamicin	Protein synthesis (30S ribosomal subunit)	Enzymes, efflux, limited uptake, target alteration	[12,22]
Fluoroquinolones	Ciprofloxacin	DNA synthesis	Target mutation, efflux/exclusion	[22–24]
Ansamycins	Rifampicin	DNA synthesis	Target mutation	[11,23]
Cyclic polypeptides	Colistin	Membrane	Mutation, change of membrane charge	[25]

Antibiotics in the environment

The presence of antibiotics and the evolution of antibiotic resistance genes in the environment has a long evolutionary history, since antibiotics occur as natural products in microbial communities [26]. In contrast, antibiotics are artificially introduced into the environment at a broad concentration range via various pathways [27]. Thus, antibiotics can be found in soils [28–30] and aquatic environments [31]. In those environments, the native microbial community is exposed to antibiotic residues and antibiotic resistance might be selected [32,33].

Biocides

The term biocide refers to a biocidal product containing chemical active substances or refers directly to the chemical active substance itself, which is used to control target organisms [34]. The risk of a chemical active substance is evaluated prior to its usage [34]. Hereby, the risk evaluation of chemical active substances is not harmonized all over the world, but the OECD provides guidelines and test protocols for chemical risk assessments [35]. Active substances usage in biocides is regulated based on their intended purpose. For instance, in Europe there are different product categories, each with different requirements to a chemical active substance. There are 22 product types categorized into 4 main groups: i) disinfectants, ii) preservatives, iii) pest control and iv) other biocidal products [34,36]. Disinfectants are

employed in various context to promote hygiene and ensure safety for human and animals [37,38]. Preservatives are used to protect materials and products from biological decay caused by microorganisms or other organisms to ensure material integrity or shelf life [39–41]. Pest control products are employed to control a range of unwanted higher organisms, including insects and mammals [42,43]. Biocides in the main group ´other biocidal products´ are used in special contexts not covered by the other three main groups [44]. One active chemical substance can have a broad activity and might be used in various applications but for that it needs to be approved in the relevant product type for a specific application.

Antimicrobial biocides are biocides with antimicrobial activity used mainly as disinfectants, preservatives and antifouling paints [45]. For instance, such an active chemical substance is copper, which is approved for various product types in the main group of disinfectants, preservatives and antifouling and the same goes for copper derivates as well [46]. There are various chemical classes of active substances being used as biocides [47]. However, the introduction focusses on biocides used in this thesis. The chemical classes of biocides used in this thesis are quaternary ammonium compounds (QAC), bisguanides, metals, carbamates, pyrethroids and azoles.

Bisguanides and quaternary ammonium compounds are both cationic antimicrobial agents. Cationic antimicrobial agents are a class of substances with a similar mode of action, which is based on the interaction with the cell envelope of microorganism, but differ in the specific nature of the interaction with cell envelopes [48].

Chlorhexidine digluconate (CHX), which belongs to the chemical class of bisguanides, was used in experiments in this study. CHX is active against a wide range of bacteria and fungi and used for disinfection such as in clinical or household settings and for preservation such as in personal care products [48,49]. CHX consists of a two differently charged sites, the cationic phospholipid binding site and the hydrophobic regions, consisting of hexamethylene group [48]. Thereby the cationic site interacts and adsorbs to the anionic site of the cell membrane, displacing anionic charges and decreasing fluidity and protein function of the membrane [48].

Benzalkonium chloride (BAC) and didecyldimethylammonium chloride (DDAC) have been selected as representatives of QACs in this study. QACs have a broad antimicrobial activity controlling various bacterial and fungal species and are used in various settings as active substances in product types in the main categories of disinfectants and preservatives [49]. For instances QACs are used as disinfectant in clinical and household settings [49,50] or used as preservative in personal care products [51]. QAC generally consist of two parts, a positively charged head group containing the differently quaternary bound nitrogen and nonpolar alkane group tails with varying chain lengths [48,52]. BACs are produced from natural heterogeneous

mixtures of fatty acids, thus the resulting BACs are a mixture of various n-alkyl groups with variable chain length and the relative abundance of those can vary [48,53]. Hereby, the antimicrobial efficacy of BACs are affected, since the chemical structure especially the chain length and the specific abundance have an effect on the antimicrobial efficacy [48,49,53]. The mode of action of QACs is based on interaction with microbial cell envelopes exerting growth inhibiting or lethal effects. Hereby, the QAC adsorbs to the membrane surface, then the hydrophobic tail is integrated into the phospholipid membrane, causing disruption of intermolecular bonds and interferes with the membrane fluidity and functions causing membrane damage which ultimately leads to cell leakage and lysis.[48].

The cationic surfactants CHX, BAC and DDAC have a mode of action being membrane active, however there is a difference between the bisguanide CHX and the QACs BAC and DDAC that QACs solubilize cell membrane and forms QAC-phospholipid micelles, while CHX does not [48]. Resistance mechanisms against cationic surfactants can be conferred by various mechanisms such as resistance genes, efflux pumps, cell membrane changes, biofilm formation, biodegradation [49,54]. Those resistance mechanisms, which confer resistance to a specific QAC such as often observed with BAC might confer resistance to other QACs such as DDAC as well [49]. Resistance to cationic surfactants can be associated with those mechanisms in isolates and confer cross-tolerance to some antibiotics and some biocides such as triclosan and peroxide [49]. Microbial growth in solutions containing BAC and DDAC have been observed and were associated with outbreaks in health care associated environments [49]. This underscores that cationic surfactants can have an impact on the susceptibility of microorganisms and can affect the transmission of resistance genes [55,56].

Copper has been used in this study to represent the chemical class of metals. Copper has a broad antimicrobial activity against a range of bacteria, fungi and even against viruses and is used in various applications such as for disinfection e.g. in livestock farming or for preservation of materials e.g. as wood preservatives [57,58]. In pig farming in Denmark copper is even used as food supplement to control intestinal microorganisms [59]. The mode of action of copper is based on the interaction with cell components such as thiol groups as active center of enzymes and the formation of reactive oxygen species (ROS) inducing oxidative stress to macromolecules [58,60,61]. Copper tolerance is found in isolates from various environments, especially in Enterococci [62]. Copper resistance can be conferred via specific efflux pumps or by specific plasmids [63–66]. Copper tolerance or resistance can be found in isolates from various environments [67,68]

3-iodo-2-propynyl butyl carbamate (IPBC) represents the chemical class of carbamates in this study. IPBC has insecticidal and fungicidal activity and is used as preservative in material

protection and personal care products [69,70]. IPBC consists of an iodine being bound to propargyl butyl carbamate (PBC) [71]. Hereby, the iodine of IPBC can confer broad antimicrobial activity [72], while the carbamate have a specific activity inhibiting the acetylcholinesterase in insects [73]. IPBC can be introduced into the environment during application to protected materials, but also through weathering where IPBC can leach out [70].

Propiconazole represents the chemical group of triazoles. Propiconazole has fungicidal activity and is used as preservative [74]. Propiconazole inhibits the ergosterol biosynthesis of fungi [74]. Propiconazole can be found in environments such as wastewater treatment plants and water bodies [75,76]. Natural soil microbial communities were affected by propiconazole exposure [77].

Permethrin represents the chemical group of pyrethroids. Permethrin has insecticide activity as it exerts neural toxicity in insects and is used for pest control [78,79]. Permethrin can be found in various environments such as soil and water [75,80].

While the effect of cationic surfactants and metals on bacteria has been more investigated the effect of IPBC, propiconazole and permethrin on those remains vague. However , there have been reports of environmental microorganisms or isolates being able to degrade propiconazole and permethrin [81–83].

Biocides in the environment

Biocides have a long application history, since they already have been used for centuries and were used even before antibiotics such as penicillin, which have been clinically introduced in the 1940s [84]. Biocides are used in various environments such as healthcare settings [4] but also in public or community setting being in direct contact with humans [85]. The long usage history of biocides paired with the omnipresent usage of biocides in diverse applications explains the emission of biocidal residues into the environment. Biocide residues can be found in various environments such as waste water treatment plants, but also natural environments such as soil and water [75,86].

Antimicrobial resistance mechanisms

The effect of antimicrobial substances such as antibiotics and biocides on mechanisms underlying resistance can be studied in laboratory experiments. Hereby, laboratory evolution experiments are used to understand the evolutionary pathways of resistance evolution (Fig. 1).

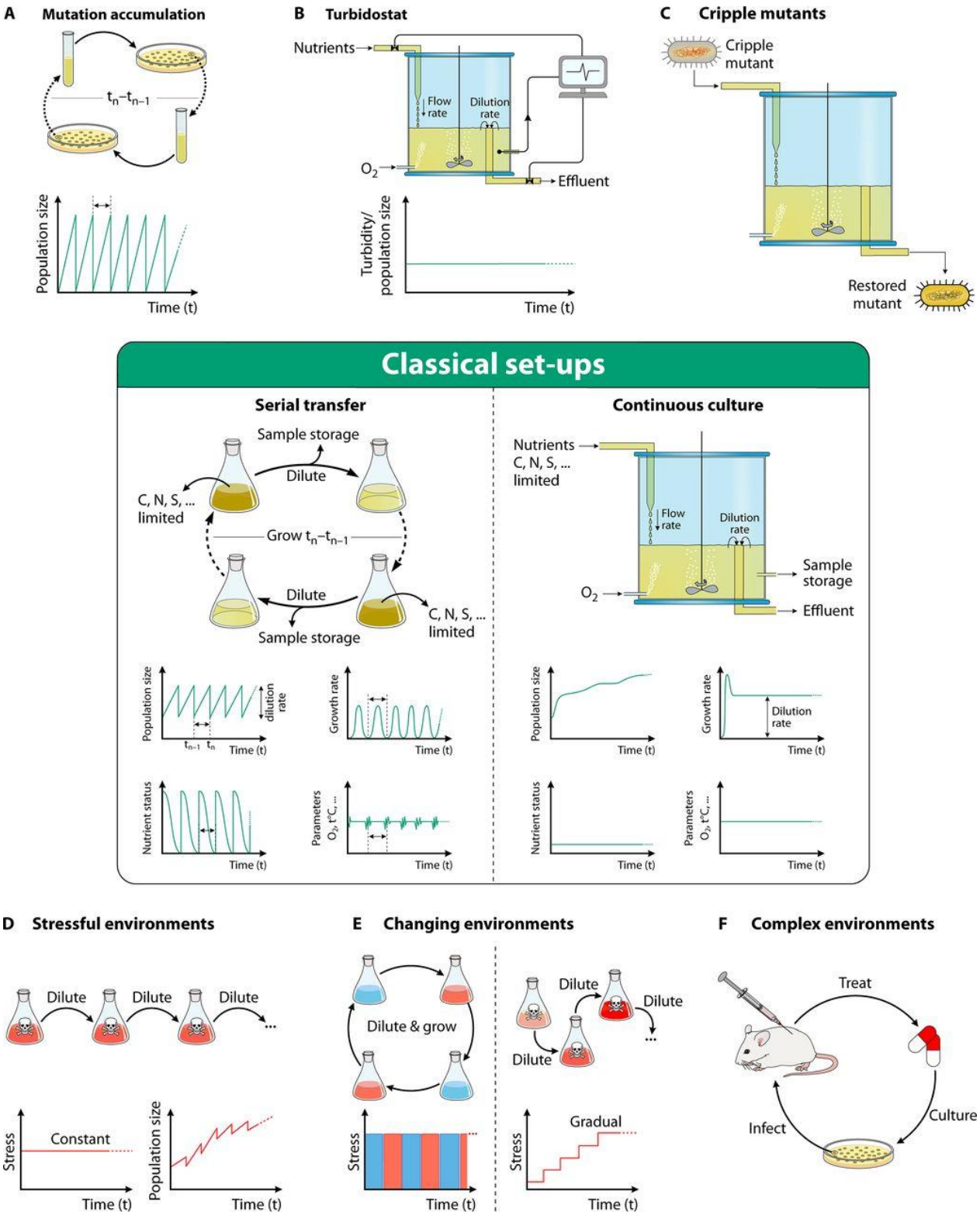

Figure 1 from [87] **Laboratory evolution experiments**

A frequently used laboratory evolution experiment is the serial transfer evolution experiment [88,89] and there is a prominent example the E. coli long term evolution experiment (LTEE) [90,91]. Another frequently used laboratory evolution experiment are continuous culture setups such as morbidostats [92–94]. While the mentioned methods are done in liquid cultures, there

is another evolutionary experiment which uses solid media with a antimicrobial gradient – the gradient plate [95–97].

Antimicrobial resistance describes the phenomenon of an organism not being hindered to grow by an antimicrobial substance. This antimicrobial resistance might be naturally intrinsic present in the organism, developed or acquired [22]. There are other mechanism conferring a decreased effect of an antimicrobial substance, observable as a specific phenotype such as tolerance or persistence [98].

Intrinsic resistance can be based on the basic structure and functions of a microorganisms, hereby especially the outer membrane structure and composition, as well as basic functions and regulation such as reduced uptake and active efflux can confer the intrinsic antimicrobial resistance [22,99].

Development of resistance can be enabled by mutation of an area in the genomic background of an organisms. This frequency of mutation is at a certain level for each organisms but can be affected by various factors such as culturing conditions [100] or by the exposure to antimicrobial substances [101]. The selection pressure is hereby essential, since mutations can confer a decrease in microbial fitness and thus higher selection pressures would benefit the resistant mutants [100].

Acquired resistance describes the phenomenon where the uptake of a mobile genetic element (MGE) confers antimicrobial resistance. The acquisition of MGE is conferred by are various mechanisms of horizontal gene transfer (HGT) (Fig. 2). The major mechanisms of HGT are transduction, transformation and conjugation. Transduction is the transfer of phage DNA mediated by phages. Transformation is the uptake and integration of DNA from the environment. Conjugation is the transfer of a conjugative plasmid from one cell to another.

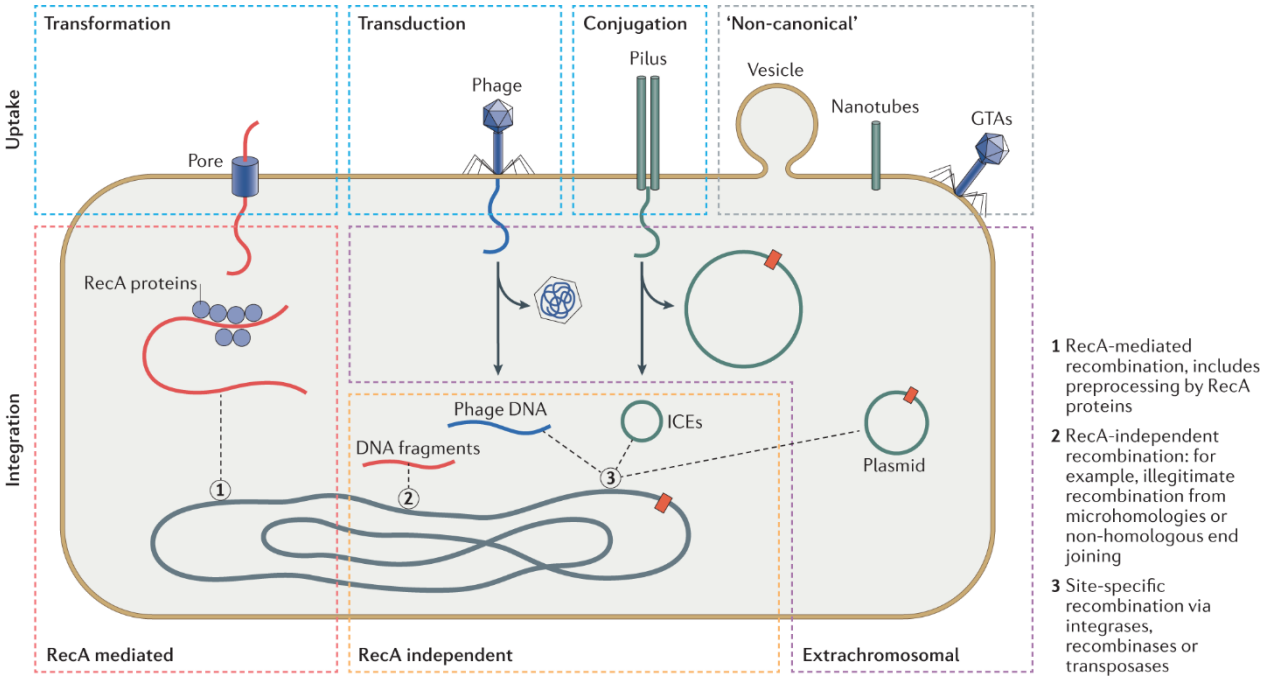

Figure 2. Mechanisms of horizontal gene transfer in bacteria taken from [102]

Antibiotic resistance

There are various molecular mechanisms being used by microorganisms to survive and evade killing by antimicrobials which will be discussed further in detail for antibiotics and biocides. Some of the major mechanisms for antibiotic resistances are i) lowering the intracellular antibiotic concentration, by decreased uptake or increased efflux via efflux pumps, ii) the antibiotic target site can be altered and iii) the antibiotic itself can be inactivated via enzymes which degrade or modify the antibiotic (Fig. 1) [11,103].

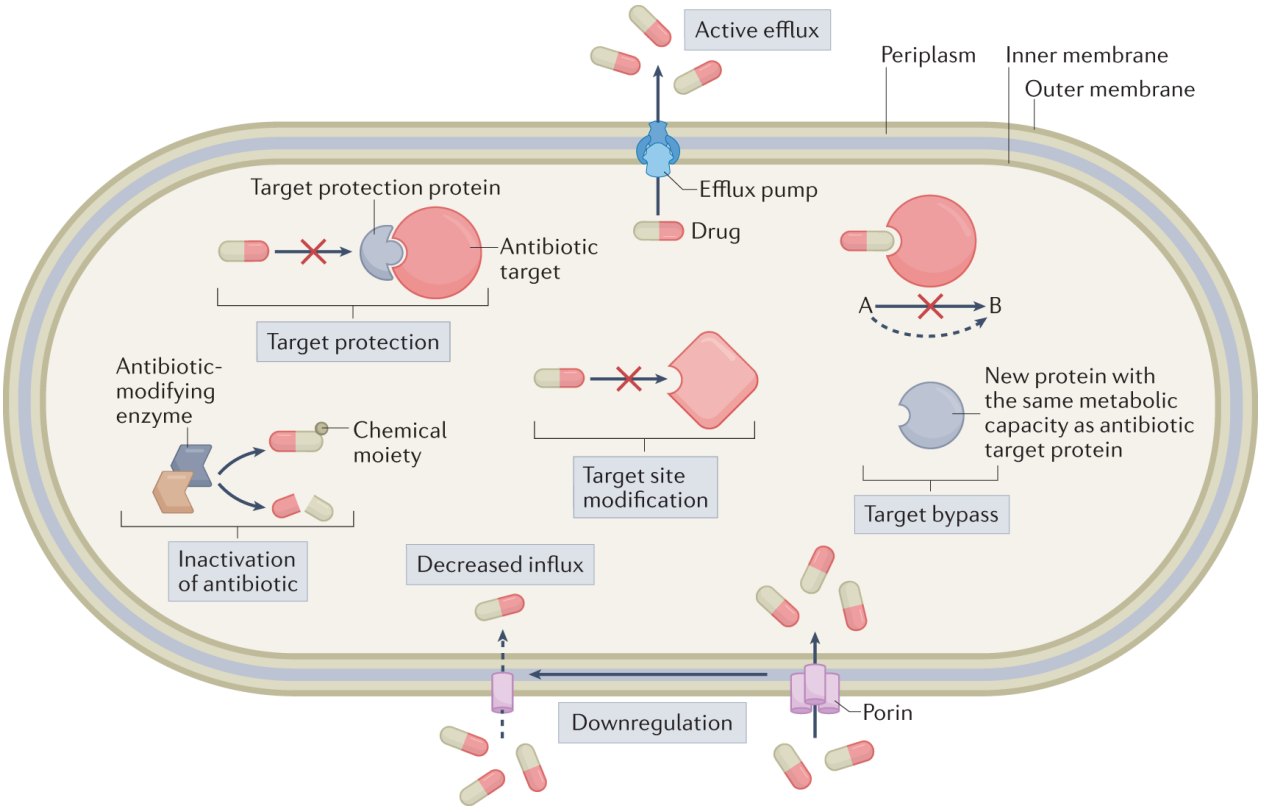

Figure 3. Major molecular mechanisms of bacteria conferring antibiotic resistance taken from [103]

Antibiotic resistance mechanisms can be based on genetic changes but also based on the expression of specific genes, which is also the basis for phenotypic variability as observed in persister subpopulations, tolerant subpopulations and tolerant strains [98,104,105]. Resistance to one antimicrobial compound can result in resistance to another compound; a phenomenon termed cross-resistance [106]. There are biocides resistance mechanisms to biocides that confer cross-resistance to antibiotics [107,108].

Biocide resistance

Some of the major mechanisms for biocide resistance are i) lowering the intracellular biocide concentration, by decreased uptake or increased efflux via efflux pumps, ii) the biocide target site can be altered, and the detoxification of the biocide, e.g. by degradation (Fig. 2).

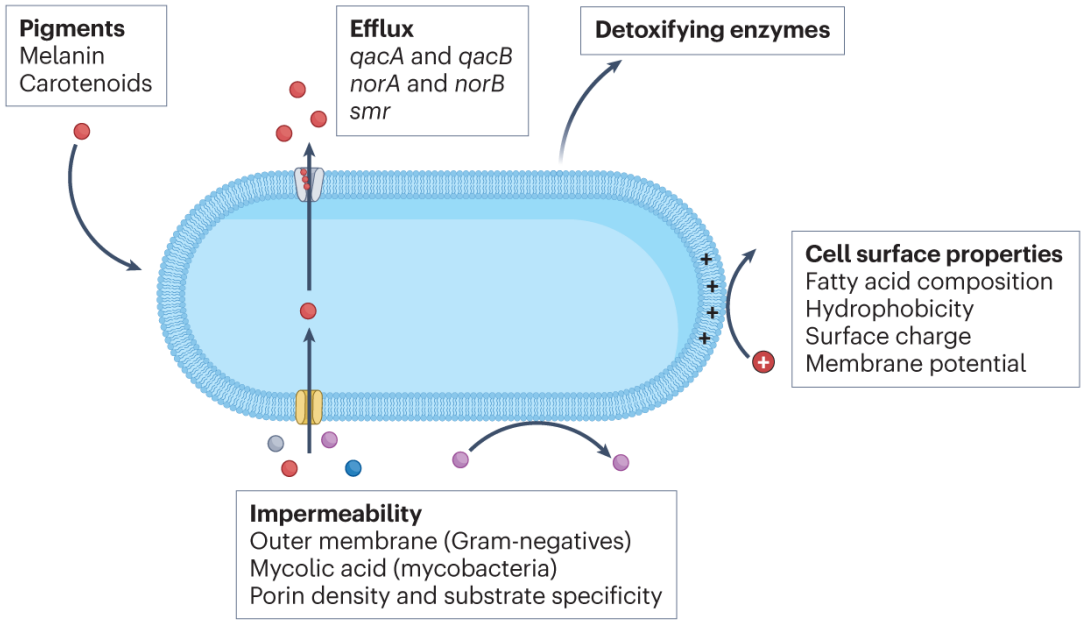

Figure 4. Major molecular mechanisms of bacteria conferring biocide resistance or tolerance taken from [109]

Increased efflux is a mechanism conferring resistance or tolerance to biocide [110,111] and is a frequently encountered resistance or tolerance mechanism in isolates from various environments such as clinical, veterinary, wastewater and production settings [112–114]. Efflux as resistance mechanism might be selected with a prolonged biocide exposure [115]. Efflux is decreasing the antimicrobial concentration within the microorganism. Another mechanism conferring decreased intracellular concentrations phenomenon is the decreased uptake of biocide [116]. Both efflux and influx are mechanisms that contribute to reduced susceptibility and are associated with the cell envelope. Other mechanisms associated with the cell envelope conferring biocide tolerance or resistance are change in cell wall charge [117–119] or composition [120–122]. Another mechanism that can confer decreased biocide susceptibility is biofilm formation [123], whereby the structure or density of the biofilm can affect the antimicrobial efficacy [124]. The mechanism of target modification or antimicrobial degradation, prevalent mechanism in antibiotic resistance, is rarely encountered with biocides except in some specific cases [22,54,125]. There are other resistance mechanisms, which are regulatory based such as the bacterial stress response and regulation [126,127] or such as phenotypical variability seen in the mechanisms of tolerance or persistence [54,128].

Consequence

Antimicrobial substances, such as antibiotics and biocides, are used in various application and settings and thus emission of those substances into the environment occurs. In these different environments those antimicrobial substances can affect microbial communities, which in turn might respond by adaption mechanisms which also underlies mechanisms of resistance [7,45]. Those stress adaption mechanisms may confer resistance to the specific antimicrobial its exposed to if it confer fitness advantages and might also confer cross-resistance mechanisms to other antimicrobial substances [48]. This was already shown in isolates from various environmental settings [49]. As the consumption of antibiotics and biocides are increasing, the claim that antimicrobial resistance being a leading cause of death by 2050 [3] becomes more plausible, which is also supported by studies assessing the global health threat if antimicrobial resistance [1,3,5,129].

Thus, further research on contributors to antimicrobial resistance is necessary especially for antimicrobial substances being used in outdoor environments, such as material preservatives. It is unknown if those biocides, which are introduced into various environments can affect evolutionary processes. This thesis aims to assess the effect of those biocides being used and present in various environments on the mechanisms underlying resistance.

Aims and scope of the thesis

The aim of this thesis is to investigate the effect of biocides on processes underlying microbial resistance evolution. Therefore, it was investigated if biocides affect resistance evolution, acquisition and spread. In addition, it was assessed how different selection pressures imposed by biocides affect resistance development and selection.

To achieve this, the effect of biocides on fundamental mechanisms relevant for resistance evolution, acquisition and spread, namely the mutation rate and horizontal gene transfer (HGT) processes, were investigated. The mutation rate gives the frequency of spontaneously occurring mutations and is one of the crucial parameters for resistance evolution. HGT includes different mechanisms, e.g. conjugation of plasmids between two cells and transformation of free DNA. These processes are related to the acquisition of mobile genetic elements, which is often linked to the spread and transmission of antimicrobial resistance in the environment.

The specific objectives of this thesis are:

1) To determine the effect of biocides on the mutation rate in the model bacteria *Escherichia coli*, *Bacillus subtilis* and *Acinetobacter baylyi*.
2) To determine the effects of biocides on the conjugation rate in the model bacterium *E. coli*.
3) To determine the effects of biocides on the transformation rate in the model bacterium *A. baylyi*.
4) To study the effect of the selection regime of a biocide on evolved phenotypic and genotypic resistance mechanisms.

The experiments were performed with biocides that have different chemical properties and that confer varying modes of action. Different model bacteria were selected to comprehensively investigate the effects of biocides on resistance mechanisms. To assess the effect of biocide on those different mechanisms laboratory experiments were carefully selected and if necessary adjusted.

Chapter 2: Effect of biocides on mutation rates and HGT

Abstract

Antimicrobial resistance (AMR) is a global health problem with the environment being an important compartment for the evolution and transmission of AMR. Previous studies showed that *de-novo* mutagenesis and horizontal gene transfer (HGT) by conjugation or transformation – important processes underlying resistance evolution and spread – are affected by antibiotics, metals and pesticides. However, natural microbial communities are also frequently exposed to biocides used as material preservatives, but it is unknown if these substances induce mutagenesis and HGT. Here, we show that active substances used in material preservatives can increase rates of mutation and conjugation in a species- and substance-dependent manner, while rates of transformation are not increased. The bisbiguanide chlorhexidine digluconate, the quaternary ammonium compound didecyldimethylammonium chloride, the metal copper, the pyrethroid-insecticide permethrin, and the azole-fungicide propiconazole increase mutation rates in *Escherichia coli,* whereas no increases were identified for *Bacillus subtilis* and *Acinetobacter baylyi.* Benzalkonium chloride, chlorhexidine and permethrin increased conjugation in *E. coli*. Moreover, our results show a connection between the RpoS-mediated general stress and the RecA-linked SOS response with increased rates of mutation and conjugation, but not for all biocides. Taken together, our data show the importance of assessing the contribution of material preservatives on AMR evolution and spread.

Introduction

Biocides are defined as products intended to destroy, deter, render harmless, or exert a controlling effect on harmful organisms by any means other than mere physical or mechanical action [47]. This definition by the EU biocidal product regulation is also used in a related sense outside the EU. Biocidal products mostly contain active chemical substances and are used for disinfection, material preservation or pest control in clinical, household or environmental settings [107]. Therefore, the risk of biocides for human health and the environment is assessed during their authorization. Biocidal active substances used in products for material preservation protect materials against biological decay. Consequently, material preservatives are directly released into the environment during their application or passively leach from materials during outdoor service life [70]. Material preservatives commonly contain bactericides, fungicides and/or insecticides, all of which may exert detrimental effects to humans and the environment [130]. While the fate and the eco-toxicological impact of material preservatives are assessed by standardized procedures, their effects on environmental microbial community function and composition are neglected in this process due to a limited knowledge base. However, it can be expected that material preservatives exert such effects because they are designed to be biologically active [131].

One way by which antimicrobials affect natural microbial communities is by promoting the evolution and transmission of antimicrobial resistance (AMR) [132,133]. The environment has been identified as a key reservoir for the evolution and spread of antimicrobial resistance [2]. The transmission of AMR from environmental bacteria to human pathogens is expected to be an important route for the occurrence of resistance in clinical settings, where it can result in treatment failures [107]. In fact, the WHO has identified AMR as one of the most pressing current and future public health problems [129]. There is a consensus in the political and scientific community that the issue of AMR needs to be addressed with a One-Health approach, including the clinical, veterinary and environmental dimensions of AMR [3]. Materials treated with biocides represent an important link between all these three compartments and therefore play a key role in the One-Health framework. In 2009, the European Chemicals Agency (ECHA) estimated that 0.13 Tg material preservatives are used in the EU alone [134]; compared to ca. 0.2 Tg antibiotics globally [135]. This suggests that material preservatives can serve as an equally important driver of AMR in the environment as compared to antibiotics.

A central process in AMR evolution in the environment is the acquisition of *de-novo* mutations in bacteria due to random mutational events in DNA [136]. It has been shown that the molecular processes involved in establishing DNA mutations are induced by stressful conditions [137,138]. Bacteria encounter stressful conditions in the environment upon exposure to

antimicrobial compounds such as biocides, antibiotics, and antimicrobial peptides. For example, antibiotics like ampicillin and ciprofloxacin have been shown to elevate bacterial mutation rates [139,140], whereas some antimicrobial peptides and other antimicrobials do not [141,142]. These studies reported that the RpoS-mediated general stress response and the SOS response are linked to the underlying molecular processes of mutagenesis [137,138]. In turn, it has been shown that some active substances used as disinfectants or antiseptics affect the bacterial stress response [143] and the SOS response [144]. It remains, however, unclear whether active substances used in material preservatives have a similar effect. This is of importance because, as opposed to disinfectants, these substances are in many cases in direct contact with the environment through materials used in human infrastructure.

A central process for AMR transmission in the environment is horizontal gene transfer (HGT) [136], which can also be affected by environmental stressors [145]. HGT is the transfer of mobile genetic elements like plasmids, transposons and integrons [146] via conjugation, transformation or transduction, whereby conjugation is the most dominant process for AMR transmission in the environment [132]. Conjugation is the transfer of a plasmid from a donor to a recipient bacterial cell from the same or another species also depending on the host range of the transferred plasmid [147,148]. Transformation is the uptake of extracellular DNA into a bacterial cell, followed by integration into the genome and functional expression [132], or followed by autonomous replication and functional expression in the case of plasmids acquired via natural competence [149,150]. Disinfectants have been reported to enhance conjugation by upregulating the SOS response [151,152] even at low concentrations [153]. Furthermore, disinfectants in water treatment have been reported to enhance transformation also by inducing the SOS response [154,155].

In this study, we hypothesize that active substances used in material preservatives increase de-novo mutations and HGT rates by affecting or triggering bacterial stress response pathways. The experiments were performed with the following active substances: chlorhexidine digluconate (CHX), benzalkonium chloride (BAC), didecyldimethylammonium chloride (DDAC), copper, 3-Iodo-2-propynyl N-butylcarbamate (IPBC), permethrin and propiconazole. The selected biocides were used since they are commonly used as material preservatives and can be detected in the environment (Table 1). CHX is not used as a material preservative, but it has a similar mode of action as BAC and DDAC and is used as a coating for antimicrobial surfaces, as preservative in cosmetic products and antiseptic [156,157]. We determined the mutation rates for the model microorganisms *Acinetobacter baylyi*, *Bacillus subtilis* and *Escherichia coli*. *E. coli* was selected because the effects of environmental conditions on mutation rates have already been studied in this species [158–161]. *A. baylyi* and *B. subtilis* were selected because they serve as model organisms originating from the

environment. Both species can be found in diverse environments, including soils, which can be exposed to material preservatives of infrastructure components [162–165]. Furthermore, we determined the effect of the selected active substances on the conjugational transfer rate of the broad host range plasmid pKJK5 between *E. coli* donor and recipient strains, as well as on the transformation frequency with a reporter rescue setup in *A. baylyi*. Both species were chosen because they serve as model organisms to study the effects of environmental conditions on conjugation [144,166,167] and transformation [154,168,169], respectively. The results show that mutation and conjugation rates are affected in a species and/or substance-dependent manner. Lastly, we demonstrate the substance-depend induction of the bacterial stress response by using *rpoS*::ß-gal (RpoS-mediated stress response) and *recA*::GFP (SOS response) in *E. coli* reporter strains, indicating that not all effects on mutation and conjugation can be explained by the induction of these well-known genes.

Table 1. Relevant information on the selected active substances used in material preservatives

Antimicrobial (abbreviation) [chemical class]	**Mode of action***	**Target organism***	**Environmental concentration range* (min-max) water [µg L^{-1}] soil/sediment [µg kg^{-1}]**	**Concentrations used in the assays [µg L^{-1}]#**
Benzalkonium chloride (BAC) [QAC]	Perturbation of cell membrane [156]	Bacteria, viruses, fungi protozoans [170]	Water • groundwater 0.2 [171] • river 0.1-1.9 [172] • waste 49 [173] Sediment $3{\cdot}10^3$-$3.6{\cdot}10^6$ dw [172]	• Mutation rate *Ec* 30, *Bs* 1.2, *Ab* 240 • Conjugation *Ec* 300 • Transformation *Ab* $6{\cdot}10^3$
Didecyldimethyl-ammonium-chlorid (DDAC) [QAC]	Perturbation of cell membrane [156]	Bacteria, fungi and algae [174], insects [175]	Water • river 0.15-1.5 [172] • waste 61 [172] Sediment 0.5-$2.7{\cdot}10^3$ dw [172]	• Mutation rate *Ec* 20, *Bs* 0.2, *Ab* 20 • Conjugation *Ec* 200 • Transformation *Ab* 500
Chlorhexidine digluconate (CHX) [bisbiguanide]	Perturbation of cell membrane [156]	Bacteria and fungi [176]	Water • waste 4.47 [177]	• Mutation rate *Ec* 12, *Bs* 1.2, *Ab* 2 • Conjugation *Ec* 600 • Transformation *Ab* 10
Copper [Metal]	Generation of reactive oxygen species (ROS), oxidization of sulfhydryl groups, degradation of Fe-S cluster [178]	Bacteria, fungi and insects [179]	Water • river 0.012 [180] • sea 3 [181] Soil $43.3{\cdot}10^3$ [182]	• Mutation rate *Ec* 25, *Bs* 4, *Ab* $1{\cdot}10^3$ • Conjugation *Ec* 250 • Transformation *Ab* $25{\cdot}10^3$
3-Iodo-2-propynyl N-butylcarbamate (IPBC) [Carbamate]	Acetylcholinesterase inhibitor, hypothesized iodine toxicity [183]	Fungi and insects [71], herbs and as antimicrobial [184]	Water • river 0.005 [185] • waste 0.02 [185] Sediment 190 – 490 dw [186] Soil <$25{\cdot}10^6$ (detection limit) [186]	• Mutation rate *Ec* $2{\cdot}10^3$, *Bs* 400, *Ab* $1{\cdot}10^3$ • Conjugation *Ec* $100{\cdot}10^3$ • Transformation *Ab* $250{\cdot}10^3$
Permethrin [Pyrethroid]	Modulates the neural transmission in insects [78]	Insects [78]	Permethrin Water • river 0.006 – 0.02 [187] Sediment 20 [75] Pyrethroids Water • river 0.00005-$13{\cdot}10^3$ [80] • sea 0.0047-16.2 [80] • waste 0.013-1.25 [80] Sediment 0.08-$60{\cdot}10^3$ [80] Soil 0.07-$1.2{\cdot}10^3$ [80]	• Mutation rate *Ec* $10{\cdot}10^3$, *Bs* 400, *Ab* $2{\cdot}10^3$ • Conjugation *Ec* $100{\cdot}10^3$ • Transformation *Ab* $500{\cdot}10^3$
Propiconazole [azole]	Ergosterol-biosynthesis inhibitor [188]	Fungi [188]	Azoles Water 0.003 – 8.65 [189] Soil 1.1 – $2.6{\cdot}10^3$ [189] Propiconazole Water • waste 0.001 – 0.04 [76][187] • lake 0.0007 – 0.0019 [76] Soil 10 – $50{\cdot}10^6$ [190]	• Mutation rate *Ec* $10{\cdot}10^3$, *Bs* 400, *Ab* $2{\cdot}10^3$ • Conjugation *Ec* $100{\cdot}10^3$ • Transformation *Ab* $500{\cdot}10^3$

* references are shown in square brackets

Ec – E. coli; Bs – B. subtilis; Ab – A. baylyi; dw – dry weight

Materials and methods

Bacterial strains and cultivation conditions

A. baylyi ADP1/BD413 (ATCC 33305) was derived from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ; DSM 24193), *B. subtilis* 3610 (NCIB3610) was derived from the Bacillus Genetic Stock Center (BGSC with the Strain ID 3A1) and *E. coli* K12 MG1655 was a generous gift from Prof. Rupert Mutzel (Freie Universitaet Berlin). The reporter strains *E. coli* K12 MG1655 *rpoS*::ß-gal and *E. coli* K12 MG1655 *recA*::GFP have been described previously [141]. For the conjugation assay, the donor strain *E. coli* K12 MG1655 *lacI^q-pLpp-mCherry-Km^R* containing pKJK5::*gfpmut3b-TMP^R* was a generous gift from Dr. Uli Klümper (University of Exeter) [191] and the recipient strain *E. coli* K12 *Rif^R* (MIC_{Rif} ≥100 µg mL^{-1} rifampicin) was a spontaneous mutant from the *E. coli* K12 MG1655 ancestor selected on a rifampicin plate (200 µg mL^{-1} rifampicin). For the transformation assay, the strain *E. coli* K12 MG1655 containing pCLT *rbcL-Prrn-aadA* and the recipient strain *A. baylyi* BD413 *aadA*::*gfp* were generous gifts from Peying Hong (King Abdullah University), Daniele G. Daffonchio (King Abdullah University) and Elena Crotti (University of Milan).

All strains were cultivated in liquid defined media as follows: *E. coli* K12 MG1655 in defined M9G medium composed of M9 salts, minerals, trace elements and glucose as source of carbon and energy (42.2 mM Na_2HPO_4, 22 mM KH_2PO_4, 11.3 mM $(NH_4)_2SO_4$, 8.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 20 mM glucose, 0.0025 mM $FeCl_3$, 0.00495 mM $ZnCl_2$, 0.0021 mM $CoCl_2$, 0.002 mM Na_2MoO_4, 0.0003 mM $CaCl_2$, 0.0025 mM $CuCl_2$, 0.002 mM H_3BO_3), *A. baylyi* ADP1/BD413 in MA (31 mM Na_2HPO_4, 25 mM KH_2PO_4, 18 mM NH_4Cl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 0.041 mM nitrilotriacetic acid, 25 mM glucose, 0.0003 mM $CrCl_3$, 0.0003 mM $CoCl_2$, 0.0003 mM $CuCl_2$, 0.0003 mM $NiCl_2$, 0.0003 mM Na_2MoO_4, 0.0003 mM Na_2SeO_3, 0.0003 mM $FeCl_3$, 0.0003 mM H_3BO_3, 0.0001 mM $ZnCl_2$, 0.0001 mM $MnCl_2$), and *B. subtilis* 3610 in CSE (2.94 mM KH_2PO_4, 7.65 mM K_2HPO_4, 2.49 mM $(NH_4)_2SO_4$, 0.062 mM $MgSO_4$, 0.000837 mM $MnSO_4$, 0.024 mM tryptophan, 1.02 mM ammonium ferric citrate, 5.44 mM potassium glutamate, 10.2 mM di-sodium succinate, 2.775 mM glucose). Cultures were incubated at 37°C and shaken at 220 rpm. Lysogeny broth (LB) Lennox (0.16 mM yeast extract, 1.41 mM tryptone, 0.86 mM NaCl and 0.45 mM agar bacteriology grade) agar plates were used as solid media and incubated at 37°C unless otherwise indicated. The biocides benzalkonium chloride [BAC] (PO 12063), didecyldimethylammonium chloride [DDAC] (PO 34466), copper as $CuSO_4 \cdot 5H_2O$ (PO PHR1477), 3-Iodo-2-propynyl N-butylcarbamate [IPBC] (PO PHR1354), permethrin (PO 45614), propiconazole (PO 45642), bromoacetic acid [BAA] (PO 79-08-3) and the antibiotics ciprofloxacin (PO 17850-25G-F) and trimethoprim (PO PHR1056) were purchased from Sigma Aldrich (Merck KgaA, Darmstadt, Germany). Rifampicin (PO A2220) was purchased from AppliChem (AppliChem GmbH, Darmstadt, Germany) and chlorhexidine

digluconate [CHX] (PO 02191360-CF) was purchased from MP Biomedicals (MP Biomedicals Germany GmbH, Eschwege, Germany).

Antimicrobial susceptibility determination, growth rates, and cell killing

The minimum inhibitory concentration (MIC) was determined with the broth microdilution method according to Andrews (2001) [192] with slight modifications. Briefly, 200 µL total volume per well were used and the inoculum was adjusted to the number of cells used in the different assays (10^3 cfu mL^{-1} in mutation rate assay; 10^7 cfu mL^{-1} in conjugation rate assay; 10^9 cfu mL^{-1} in transformation rate assay). The cell densities of the inoculum were adjusted because of apparent effects of the cell density on the MIC. MICs of biocides (BAC, DDAC, CHX, copper, IPBC, permethrin and propiconazole) and antibiotics (ciprofloxacin and trimethoprim) were assessed after 24 h at 37°C and were defined as the MIC being the lowest concentration that inhibits the growth in liquid culture. Growth rates in the presence or absence of biocides or antibiotics were measured in defined media for *E. coli* in M9G medium, for *A. baylyi* in MA and for *B. subtilis* in CSE in 96-well polypropylene microtiter plates in a volume of 200 µL using a plate reader (Biotek Epoch 2 microplate reader) with the settings (37°C, fast orbital shaking, 5 min measuring interval). The growth rates were calculated from the increase in OD_{600} over time by inferring the time derivative using Gaussian processes implemented as published code [193] in Python 3.6. Cell killing of biocides and antibiotics after 4 h of exposure was assessed under the conditions of the mutation rate assay (see section 2.3) with the same pre-culture conditions, exposure medium and temperature, the antimicrobial concentrations shown in Table 1, and an initial cell inoculum of 10^3 cfu mL^{-1}. Cell killing was estimated by spot plating dilutions of the cell suspension at the start (t_0) and after 4 h (t_{4h}) of incubation on LB Lennox Agar at 37°C for 24 to 48 h and counting the colony forming units.

Effect of biocides on mutation rates

Mutation rates were determined with the Luria-Delbrück fluctuation assay [194]. Strains were revived from freezer stocks in 5 mL M9G (*E. coli*), MA (*A. baylyi*) and CSE (*B. subtilis*) medium and grown as overnight culture at 37°C. As a preconditioning step, cultures of the revived strains were diluted to 10^3 cfu mL^{-1} in 5 mL defined media and allowed to grow into stationary phase for 24-26 h. The preconditioned cultures were used to inoculate 54 independent cultures with $5 \cdot 10^3$ cfu mL^{-1} in the presence and absence of different antibiotics or biocides at non-lethal concentrations (Table 1, Table S1, Fig. S2) in 200 µL defined media and incubated until stationary phase for 24-26 h. Five to six independent cultures were diluted and spotted on LB Lennox agar plates to determine the final total number of cells per culture (N_t). The complete volume of the remaining 48 independent cultures was plated undiluted on LB Lennox agar plates supplemented with rifampicin to determine the mutant number per culture. All plates were counted after 24 h incubation at 37°C. The rifampicin concentrations used for mutant

selective media were selected based on literature values and prior experiments that showed a suitable number of spontaneous mutants (50 µg mL^{-1} for *B. subtilis* [195], 100 µg mL^{-1} for *A. baylyi* [196] and *E. coli* [139]). Rifampicin concentrations differed between strains due to varying baseline susceptibilities and the magnitude for rifampicin resistance emerging in spontaneous mutants. The absence of pre-existing mutants in the pre-cultures was confirmed by testing the pre-cultures on rifampicin selective media.

The data was analyzed using the program rSalvador in the R environment under the Mandelbrot-Koch model [197]. We calculated the maximum likelihood estimates of the expected number of rifampicin-resistant mutants per culture (m) and co-estimated the fitness parameter (*w*) for emerging rifampicin-resistant mutants relative to the wildtype using the newton.joint.MK function. The confidence intervals were estimated with the functions confint.profile.m and confint.profile.w, respectively. In 3 out of 30 cases (*B. subtilis* TMP, BAC and DDAC) the newton.joint.MK function could not estimate *w*. In these cases, the function mutestim in the program flan was used [198]. For statistical comparison between the treatments, the likelihood ratio distribution method implemented in the LRT.MK function was used, including the co-estimated values of *w* and the ratio between individual estimates of N_t for treatments with antibiotics/biocides to their respective controls without antibiotics/biocides. The genomic mutation rate μ_g (mutations per genome per replication) was calculated according to

$$\mu_g = \frac{m \cdot G}{N_t \cdot L} \qquad \text{equation 1}$$

, where G denotes the genome size (in base pairs per genome), N_t is roughly equivalent to the number of replication events during the experiment, and L denotes the size of the genomic locus (in base pairs), on which a mutation can confer resistance to rifampicin [199]. G and L are constant within each bacterial strain and their values are shown in Table S4. The values for N_t are shown in Fig. S1.

Effect of biocides on conjugation

A conjugation rate assay was performed with the donor strain *E. coli* MG1655::-*lacI*^{q}-*pLpp-mCherry-Km*^{R} harboring the plasmid pKJK5::*gfpmut3b-TMP*^{R} and the recipient *E.coli* MG1655 Rif^R. The conjugation rate assay was based on the experimental design as described previously [200], allowing the determination of the actual conjugation rate, which is – as opposed to the widely used conjugation frequency – largely independent of a range of experimental conditions (e.g. cell density, donor/recipient ratios). In short, early exponential phase (OD_{600} = 0.1) cultures of the donor and recipient strains were mixed in equal parts, biocides were added at subinhibitory concentrations (see Table 1 and Table S2) and further incubated for 2 h at 37°C and 1,200 rpm shaking. The growth rates of the various treatments

were calculated using the respective OD measurements from t_0 and t_{2h}. The concentration of total, donor, recipient and transconjugant cells were sampled at the beginning of the incubation (t_0) and after 2 h (t_{2h}) and determined by plating different dilutions on LB agar (total), LB agar supplemented with 30 µg mL^{-1} trimethoprim (selective for the donor strain), LB agar supplemented with 100 µg mL^{-1} rifampicin (selective for the recipient strain), and LB agar supplemented with rifampicin and trimethoprim (selective for the transconjugant strain). The plasmid transfer rate, γ (mL $cell^{-1}$ h^{-1}) was calculated as follows,

$$\gamma = \varphi \ln(1 + \frac{T}{R} \times \frac{N}{D}) \times \frac{1}{[N - N_0]} \qquad \text{equation 2}$$

, where φ denotes the growth rate (h^{-1}), N and N_0 denote the initial and final cell density, D denotes the donor density, R denotes the recipient density, and T denotes the transconjugant density (all in cfu mL^{-1}). Outliers in the dataset of the plasmid transfer rates for each treatment were determined with the Grubbs-test at p=0.05 in OriginLab (Origin 2019 Version 9.6.0.172). Five of 86 data points (~6 %) were removed as outliers. The fold-change of the plasmid transfer rates of each replicate treatment was calculated to its respective control without biocide performed on the same day. Statistical significance was tested for replicate treatments using the One-sample t-test against 1 at p=0.05 in OriginLab.

Effect of biocides on transformation

The transformation assay was performed using the recipient strain *A. baylyi* BD413 *aadA::gfp* with a reporter rescue cassette setup and donor DNA [201]. *aadA* encodes for an aminoglycoside adenylyltransferase conferring spectinomycin resistance and *gfp* encodes for green fluorescent protein conferring fluorescence. The donor DNA contains the P*rrn* promotor for the induction of *aadA::gfp* expression with flanking regions that are homologous to the promoterless upstream region of *aadA::gfp* in the genome of the recipient strain. Thus, spectinomycin resistance and fluorescence will be expressed upon successful uptake of donor DNA and recombination into its genome, converting a recipient cell into a transformat cell [201]. The transformation assay was based on the experimental design as described previously [154]. In short, an overnight culture is centrifuged at 4°C and 2,362 × g, washed twice in PBS, and adjusted via OD_{600} to a cell concentration of 1×10^{10} cfu mL^{-1}. Next, 100 µL of the adjusted cell suspension was added to 900 µL MA containing the donor DNA and biocides at subinhibitory concentrations (see Table 1 and Table S3), reaching a final cell density of 1×10^{9} cfu mL^{-1} in the assay. The donor DNA was amplified from the cloning vector pCLT hosted in *E. coli* K12 MG1655 using the following primer pair pCLT fwd 5´ CTG CTA AAA ACT ACG GTA GAG C 3´ and pCLT rev 5´ CCA GGA TCT CTA GAT TAT TTG C 3´ and was added at a concentration of 2 µg mL^{-1}. The concentration of total recipient and transformant cells was sampled after 24 h and determined by plating different dilutions on LB agar (total cell number)

and LB agar supplemented with 100 µg mL^{-1} spectinomycin (selective for the transformant). The water disinfection byproduct bromoacetic acid (BAA) was reported to significantly increase transformation frequencies with the same donor and recipient setup [154] and thus served as a positive control. Addition of MA medium instead of donor DNA was used as negative control. The transformation frequency was calculated by dividing the number of transformants over the total number of cells. Outliers in the dataset of the transformation frequencies for each treatment were determined with the Grubbs-test at p=0.05 in OriginLab. One out of 36 data points (~3%) was removed as outlier. The fold-change of the transformation frequencies of each replicate treatment was calculated to its respective control without biocide performed on the same day. Statistical significance was tested for replicate treatments using the One-sample t-test against 1 at p=0.05 in OriginLab.

rpoS and *recA* reporter assay

The soft-agarose transcription fusion reporter assay for *rpoS* and *recA* expression was performed as described in [141] with the modification of using minimal medium M9G instead of Mueller-Hilton medium. Briefly, mid-exponential phase (OD600~0.5) cultures of *E. coli* K12 MG1655 *rpoS*::ß-gal and *E. coli* K12 MG1655 *recA*::GFP were mixed in equal part with M9G medium supplemented with 0.6% agar and poured onto M9G medium agar plates (25 mL, agar height = 7 mm). Filter discs (Whatman antibiotic assay discs 6 mm) loaded with biocides were placed onto the plates. Discs were loaded with the following amounts: ciprofloxacin 10 µg, trimethoprim 10 µg, BAC 40 µg, DDAC 20 µg, CHX 400 µg, copper 100 µg, IPBC 100 µg, permethrin 40 µg, propiconazole 40 µg. The antibiotics ciprofloxacin and trimethoprim were used as positive controls, and water was used as a negative control. Biocides were used in amounts generating similar inhibition zones except for IPBC, permethrin and propiconazole, which did not create inhibition zones. The plates were incubated for 12 h at 37°C and images were taken with an office scanner (Epson Perfection V370 Photo Scanner) for the *rpoS*::ß-gal reporter strain and UV light transilluminator (ChemiDoc XRS Bio Rad) for the *recA*::GFP reporter strain. The intensity profiles of each image were analyzed with Image J (plotting the intensity profile) to determine the induction of the genes relative to the control (no biocide). To this end, the height of the intensity peak – typically emerging around the inhibition zone – compared to the background level further away from the inhibition zone was calculated. The fold-change intensity differences for the expression of *rpoS*::ß-gal and *recA*::GFP reporter strains of each replicate treatment to its corresponding negative control were compared using the One-sample t-test against 1 with p=0.05 in OriginLab.

Results and Discussion

Effect of material preservatives on mutation rates

We investigated whether biocides used as material preservatives affect mutation rates to assess the impact of those material preservatives on the evolvability of microorganisms in the environment. We found that biocides affect the mutation rates in a substance and species-dependent manner (Fig. 1). To this end, we grew bacteria in the presence of biocides and antibiotics (including ciprofloxacin and trimethoprim as controls), plated these cultures on rifampicin selective plates and counted spontaneously occurring rifampicin resistant mutants. The selected antibiotics were used because they were reported to increase mutation rates in *E. coli* [139,141]. The selected antibiotic and biocide concentrations were well below the minimum inhibitory concentration (Table S1) and did neither cause significant effects on growth rates (Fig. S1) nor induced measurable cell death at the inoculum size in the mutation rate assay of 10^3 cfu mL^{-1} (Fig. S2). Therefore, the concentrations are not expected to lead to artifacts in the estimation of the mutations rates, which have been shown recently to be biased by concentrations that affect growth and induce cell death [202].

First, we checked the robustness of our results by comparing the baseline mutation rates in media without antimicrobials to those reported in the literature. The obtained baseline mutation rates μ_g (in mutations per genome per replication) for *E.* coli (3.51×10^{-4}), *A. baylyi* (3.41×10^{-4}) and *B. subtilis* (4.97×10^{-3}) are in a similar range to those reported previously (8.7×10^{-4} for *E. coli*; 1.67×10^{-5} for *A. baylyi*; 2×10^{-2} for *B. subtilis*) [203–205]. The difference between our data and the literature could be explained by differences in the cultivation conditions; e.g. we used minimal media instead of full media. It was shown that nutrient availability can affect the mutation rate in *E. coli* and that *E. coli* cultures with intermediate nutrient availability have the lowest mutation rates [206]. Obtaining comparable mutation rates to the literature shows that our approach accurately quantifies mutation rates and is thus suited to determine the effects of biocides on mutation rates.

Next, we checked whether our approach repeats known effects of the antibiotics ciprofloxacin and trimethoprim on spontaneous mutation rates. Our data showed no increase in the mean number of rifampicin resistant mutants for any of the tested microorganisms exposed to both antibiotics (Fig. 1), which have previously been reported to increase mutation rates in *E. coli* [141,143,207]. The reason for this discrepancy might be related to the low antibiotic concentrations in our assay and the deviations in assay design. We grew cells at low concentrations of ciprofloxacin (0.6 µg L^{-1}) and trimethoprim (200 µg L^{-1}) to accumulate rifampicin resistance mutations in the presence of both antibiotics (Table 1), while other reports pre-incubated cells with high concentrations (60 µg L^{-1}& 2,000 µg L^{-1}, respectively) and then grew the cells without the antibiotics before selective plating [141]. Our data, therefore, suggest

that the effects of ciprofloxacin and trimethoprim on mutation rates reported previously may not occur around low, environmentally relevant concentrations (0.03 µg L^{-1} & 0.7 µg L^{-1}, respectively) [208].

Next, we investigated the effect of biocides on mutation rates. For *E. coli*, the mutation rates were significantly increased upon exposure to CHX (3.5 fold), DDAC (2.7 fold), copper (2.2 fold), permethrin (2.7 fold) and propiconazole (2.8 fold) (Fig. 1B). For *A. baylyi*, IPBC and for *B. subtilis*, permethrin significantly lowered the mutation rate by approximately halving it in comparison to the control. We experimentally confirmed that the significant increases in mutation rate observed for CHX, DDAC permethrin and propiconazole in *E. coli* were not affected by strong fitness differences between the wildtype and the emerging rifampicin-resistant mutants (Fig. S4). The experimental data showed that copper significantly reduced rifampicin-resistant mutant fitness relative the wildtype. These fitness differences could bias the estimation of differences in mutation rates. However, the co-estimation of mutant fitness (*w*) from the empirical mutant distributions (Fig. S3) showed a fitness decrease of rifampicin-resistant mutants relative to the wildtype in copper (mean w = 0.93) consistent with the experimental data (0.86). Thus, the apparent reduced mutant fitness was part of the statistical comparison of the effects of copper on the mutation rate. The opposing effects of permethrin on the mutation rates of *B. subtilis* and *E. coli* might be based on the different stress-response systems induced in these species, which then leads to opposing downstream effects on stress-induced mutagenesis [209,210]. Taken together, our results show that the effects of biocides on mutation rates are not only substance-dependent but also differ across microbial genera.

An especially noteworthy result was the increase of the mutation rate in *E. coli* in the presence of DDAC and CHX. The effects for DDAC and CHX were apparent at concentrations (20 and 12 µg L^{-1}, respectively) that have been shown to occur in wastewater (61 and 4.47 µg L^{-1}) (Table 1) and that are close to their eco-toxicological predicted no effect concentration for fresh water (max. 6.3 and 1 µg L^{-1}) [172]. Wastewater is known to comprise diverse populations of *Enterobacteriaceae*, including *E. coli* and a high diversity of antibiotics at high concentrations [211]. Therefore, wastewater is thought to be a hotspot for antibiotic resistance evolution and spread [212]. Thus, this result suggests that DDAC and CHX in wastewater may facilitate the evolution of antibiotic resistance in wastewater by increasing mutation rates in *Enterobacteriaceae*.

The observed increase of the mutation rate of *E. coli* when exposed to copper was consistent with previous reports [213,214]. For propiconazole, our results also agree with previous results that showed a mutagenic potential for *E. coli* in mouse liver cells, where it increased mutation rates by 2-fold as compared to the increase observed by us (2.8 fold) [215]. The effects

observed on the mutation rates of *E. coli* for permethrin and propiconazole are surprising because both are not used to target bacteria and consequently do not affect bacterial growth [216,217] (Table 1). Thus, our approach led us to determine the effect on mutation rates at concentrations far above those measured in the environment (8,000 & ~1,000 fold higher than in water, respectively), which is different to the assays with BAC, DDAC, CHX and copper (Table 1). However, such high concentrations might be experienced by bacteria on surfaces of materials preserved with biocidal products devoid of bactericidal substances, which is common in wood preservatives. Therefore, our results suggest that bacteria could have increased mutation rates when residing on preserved material surfaces that only contain fungicides (propiconazole) and insecticides (permethrin).

Taken together, our results show that material preservatives affect mutation rates of bacteria at subinhibitory concentrations in a compound and species-dependent manner. This effect can be exerted by compounds for which bacteria are not the target organisms (e.g. permethrin and propiconazole), but also by compounds that target bacteria directly at their low environmentally relevant concentrations (e.g. CHX and DDAC). Thus, our findings highlight the risk of material preservatives to contribute to the evolution of antimicrobial resistance in the environment by increasing the mutation rates of bacteria. This could increase the pollution of the environment with antimicrobial resistance genes/traits and increase the probability of the transmission of resistant strains from the environment to humans [218,219].

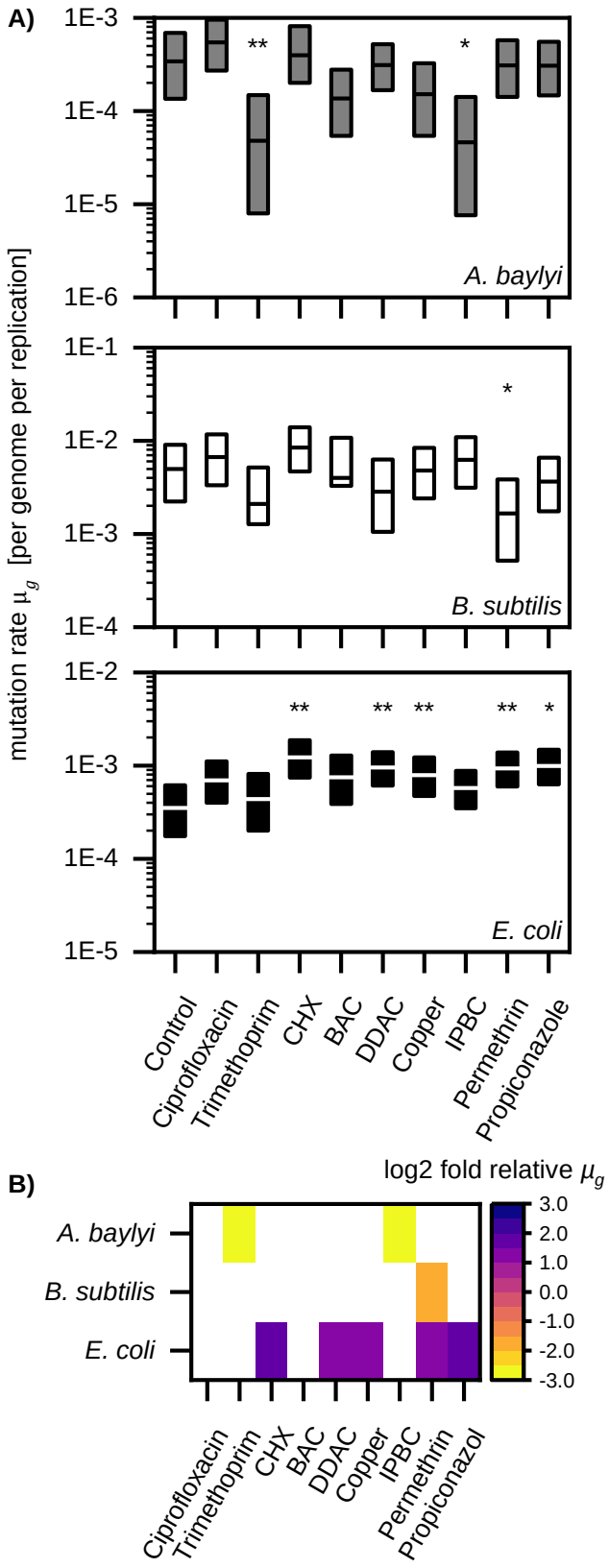
A)
mutation rate μ_g [per genome per replication]
1E-3
1E-4
1E-5
1E-6
A. baylyi
1E-1
1E-2
1E-3
1E-4
B. subtilis
1E-2
1E-3
1E-4
1E-5
E. coli
Control
Ciprofloxacin
Trimethoprim
CHX
BAC
DDAC
Copper
IPBC
Permethrin
Propiconazole
B)
log2 fold relative μ_g
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
A. baylyi
B. subtilis
E. coli
Ciprofloxacin
Trimethoprim
CHX
BAC
DDAC
Copper
IPBC
Permethrin
Propiconazol

Fig. 1. Material preservatives affect mutation rates.

A) Mutation rate μ_g for *A. baylyi*, *B. subtilis* and *E. coli* grown in the presence of subinhibitory concentrations (see Table 1) of antibiotics and biocides, as determined by a fluctuation assay on rifampicin-loaded selective plates. The bars represent the 95% confidence interval around the mean of μ_g shown with horizontal lines as calculated by rSalvador (N=48). Statistical significance of differences between control and treatments was assessed using a likelihood ratio test under the Mandelbrot-Koch model (*$p \leq 0.05$; **$p \leq 0.005$). Note the different y-axis scaling. **B)** Heatmap of significantly affected mutation rates μ_g. The color key shows the log2 transformed relative differences in μ_g for the different treatments to the control, obtained by dividing μ_g of a respective treatment by μ_g of the control followed by a log2 transformation. For example, the value 0 on the scale means no difference between treatment relative to the control ($\log_2(1)=0$) and a value of 1 means a doubling of the mutation rate relative to the control ($\log_2(2)=1$). White areas represent treatments with non-significant differences.

Effect of biocides on conjugation

The effect of biocides used as material preservatives on the conjugational transfer of a broad-host-range plasmid was investigated to assess whether this is a potentially relevant process in the environment. The results show that the microbicides BAC and CHX as well as the insecticide permethrin increase conjugation rates in *E. coli* (Fig. 2). To this end, we determined the plasmid transfer rate of the broad-host-range plasmid pKJK5 between *E. coli* donor and recipient cells [220]. The plasmid transfer rate was calculated based on the growth rate, the number of donor, recipient and transconjugant cells, and the total cell number [200]. This approach excludes artifacts introduced by experimental parameters that affect conjugation rates estimated by simply determining the transconjugant frequency. Such sensitive experimental parameters include, e.g. the initial donor-recipient ratios or growth of the transconjugant during the experiment [144,166,200].

Our results showed that DDAC, copper, IPBC and propiconazole did not significantly increase HGT, while CHX and permethrin showed a significant 4-fold and BAC a significant 2-fold increase (Fig. 2). Our findings for CHX and BAC are consistent with the literature [56,153]. The reported concentrations of BAC and CHX in wastewater are one and two orders of magnitude lower than those used in our assay (see Table 1). However, wastewater from buildings with high use of disinfectants (hospitals and animal staples) may release concentrations that are in the range of those used in our assay [221,222]. The substances may therefore pose a high risk for HGT linked to antibiotic resistant strains released from hospitals and animal staples.

In the literature, contrasting results have been reported for copper: either an increase in the conjugational transfer frequency (e.g. of the pCM184-Cm plasmid) by copper ions [223] or copper nanoparticles at low environmentally relevant concentrations [166], or a decrease in the conjugational transfer frequency (e.g. of the pJP4 and pADP1 plasmids) over a range of copper and copper nanoparticle concentrations (10,000, 20,000, 50,000, 100,000 µg L^{-1}) [224]. The disagreement of our results with these studies could be due to different experimental

conditions, copper concentrations (we used 250 µg L^{-1}), strains and plasmids used or the difference in experimental approaches (rate vs. frequency determination) [200,220,225].

Biocides that do not target nor inhibit bacteria were used at higher concentrations than those that inhibit bacteria. Of those biocides, permethrin led to a significant, ~4-fold increase in the conjugation rate (Fig. 2). The data on permethrin concentrations in the environment is scarce, but pyrethroids are present at concentrations in river water just one order of magnitude lower than concentrations used in our conjugation assay (Table 1). Additionally, pyrethroid insecticides are used extensively in various environments to control pests, and their biodegradation is slow, leading to an accumulation of pyrethroids and subsequent exposure of bacteria in soil [226]. Moreover, bacteria residing on surfaces of materials preserved with biocidal products that exclusively target fungi and insects will be exposed to permethrin. Previous studies demonstrated that surfaces treated with antifouling paints are hotspots for HGT [227]. Together with our data, this suggests that pyrethroids, and especially permethrin, enhance HGT and that the interface of preserved materials with the environment maybe be a hotspot for HGT. In turn, increased HGT could promote the exchange of antibiotic resistance genes – such as those harbored on the environmental pKJK5 plasmid used in our work [228] – in e.g. soil, which is known to be a large reservoir for a diverse microbial community with a shared resistome [229]. This should be considered, because humans might be exposed to these material-environment interfaces as they typically live nearby infrastructures that are protected with biocides.

Taken together, our results show that the material preservatives permethrin and BAC as well as the antiseptic CHX increase HGT via conjugation in bacteria at concentrations that might be encountered in the natural environment. Our findings highlight the risk of material preservatives contributing to the spread of resistance in environmental bacteria.

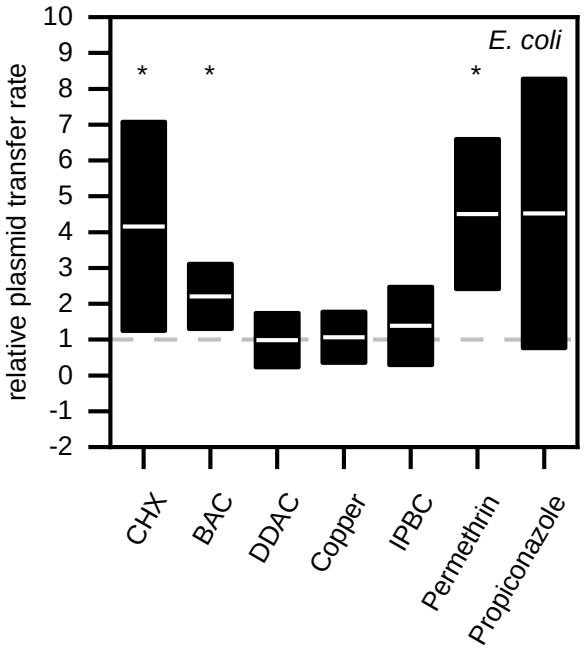

Fig. 2 Material preservatives affect horizontal gene transfer via conjugation in *E. coli*. Plasmid transfer rate of *E. coli* treated with subinhibitory concentrations (see Table 1) of biocides relative to untreated cells. The plasmid transfer rate was determined by a conjugation rate assay using *E. coli lacI^q-pLpp-mCherry-Km^R* + pKJK5*::gfpmut3b-TMP^R* as donor and *E. coli Rif^R* as recipient. The bars represent the 95% confidence interval around the mean shown as horizontal lines (N=6-18). Statistical significance of the treatment was assessed using a One-sample t-test (*$p \leq 0.05$).

Effect of biocides on transformation

The effect of biocides used as material preservatives on HGT via transformation was investigated to assess whether this is a potentially relevant process in the environment. To this end, we determined the transformation frequency of naked DNA into the naturally competent model organism *A. baylyi* using a previously developed reporter strain [230] (Fig. 3). The exogenous DNA harbored the promotor for the reporter gene cassette present in the reporter strain [201]**.** The water disinfection byproduct BAA was used as a positive control in our setup, since it was reported that BAA increases transformation [154]. The data showed increased transformation frequencies in individual replicates in response to BAA (Fig. 3). However, the overall effect of BAA was not statistically significant due to a high variance among replicates. Evaluation of the data before and after incubation showed that the cell density did not significantly increase during the assay for any of the biocides (Fig. S5). However, the cell number in incubations with BAA, BAC and copper decreased slightly which could have confounded the calculated frequencies if transformants and donors are killed at different rates.

The transformation frequencies did not increase under the effect of any of the investigated biocides at subinhibitory concentrations but decreased upon exposure to IPBC and copper (Fig. 3). We expected that copper increases transformation because it has been reported to induce the SOS response linked to *recA* activation [166], which is a molecular link to increased

transformation in *A. baylyi* [154]. However, natural transformation is a process which can become costly under stressful conditions [231]. Thus, biocide concentrations close to the MIC might turn the benefit of transformation into a cost. The lower transformation frequency might be explained by copper and iodine reacting with the exogenous donor DNA [232,233] and thus lowering the available or transformable DNA concentration in the assay. Taken together, our data show that material preservatives at subinhibitory concentrations do not increase HGT via transformation in *A. baylyi*. Our findings suggest that the risk of the tested material preservatives contributing to the spread of resistance via natural transformation in bacteria in the environment is low.

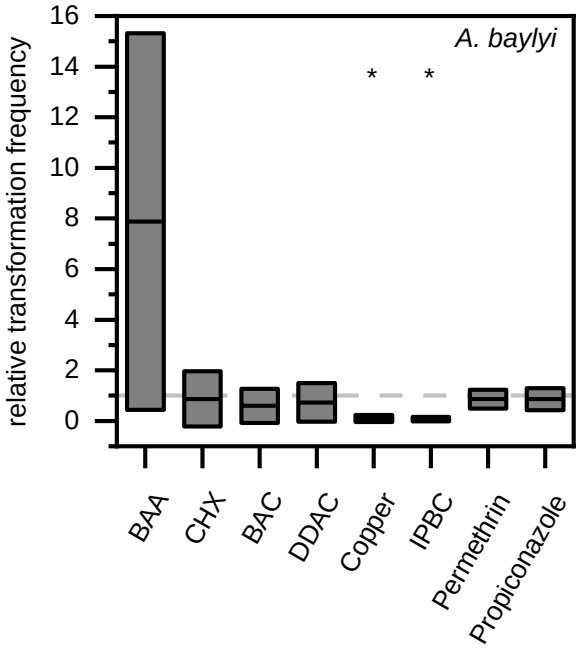

Fig. 3 Material preservatives do not increase horizontal gene transfer via transformation in *A. baylyi*. Fold-change of transformation frequency of *A. baylyi* treated with subinhibitory concentrations (see Table 1) of biocides relative to untreated cells. The transformation frequency was determined by a transformation assay using an *A. baylyi* reporter strain and donor DNA. The bars represent the 95% confidence interval around the mean shown as horizontal line (N=3-6). Statistical significance of the treatment was assessed using the One-sample t-test ($p \leq 0.05$).

Effect of biocides on the bacterial stress response

The effect of biocides used as material preservatives on the bacterial RpoS-mediated general stress response and SOS response was investigated to assess whether their induction by material preservatives can mechanistically explain the observed effects. To this end, we measured the effect of biocides on gene expression of *rpoS* and *recA* with *E. coli* reporter strains [141]. Antibiotics and biocides were used in a concentration gradient from lethal via subinhibitory concentrations to full absence, generating an inhibition zone surrounded by an induction zone in a soft-agarose transcription fusion reporter assay (Fig. 4) [141]. The bacterial stress response and the SOS response were significantly induced for the positive control

ciprofloxacin [141,234] and for most of the biocides; except for propiconazole and permethrin (Fig. 4). The absence of induction by propiconazole and permethrin is consistent with the observation that both do not show antibacterial activity in *E. coli* (Tables S1 & S2) [82,83,235]. For IPBC, only the RpoS-mediated general stress response was induced, but not the RecA response. Moreover, our results are in agreement with the literature, showing that in *E. coli* BAC and copper induce the RpoS-mediated general stress response and copper induces in addition the SOS response [166,223,236,237].

Previous reports showed an effect of the RpoS-mediated general stress response [137,138,238,239] and the SOS response on the mutation rate [139,140,240]. Therefore, the induction of these pathways might explain the increased mutation rates in response to those biocides that showed increases in *rpoS* or *recA* induction along increases in mutation rates (CHX, DDAC, and copper). However, comparing the effect of biocides on *rpoS* and *recA* (Fig. 4) with the effect on the mutation rates (Fig. 1 and 5A) shows that induction of these pathways is neither necessary nor sufficient to explain increases in mutation rates. CHX, DDAC, copper, permethrin and propiconazole were increasing mutation rates in *E. coli*, but only CHX, DDAC and copper showed increased induction in at least one stress response pathway. In contrast, BAC induced *rpoS* and *recA* but did not increase the mutation rate (Fig. 5A). There are four explanations for these observations: (i) the concentrations in the mutation rate assay were below those of BAC that induced *rpoS* and *recA*; (ii) permethrin and propiconazole induce alternative pathways that affect mutagenesis, (iii) while rifampicin resistance as the chosen marker is mainly mediated by point mutations [241], RpoS and RecA might mainly mediate other types of mutations (deletions and insertions), and (iv) the amount of permethrin and propiconazole in the disc used in the reporter assay was not sufficient to establish a concentration in the agar that equals the high concentration used in the mutation rate assay. Previous studies support option (ii) observing a direct DNA damaging effect of permethrin in mice [242] and preliminary indications for such effects by propiconazole [215,243]. Option (iii) is unlikely because studies on the mutational spectrum affected by downstream effectors of RecA and RpoS important for stress-induced mutagenesis (e.g. DinB, UmuDC and MutS) do not suggest a tendency for insertions/deletions over point mutations [244–246]. The last option (iv) is unlikely as can be shown by a simple calculation. Using the amount of active substance loaded in the filter disc and assuming a radius of 1 mm of detectable, induced cells around the 6 mm disc, we calculated the concentration of active substance in a cylinder of agar (height = 7 mm) below and around the disc. This calculation shows that propiconazole and permethrin were deployed at a concentration of ~150,000 µg L^{-1}, which is well above the concentration in the mutation rate assay (Table 1; 10,000 µg L^{-1}). Taken together, our results show a link

between the RpoS-mediated general stress and the SOS response with increased mutation rates, but not for all biocides.

Comparing the results of the induction of *rpoS* and *recA* to the effects of biocides on HGT rates revealed a similar trend as those observed for the effects on the mutation rates (Fig. 5B). The effect of biocides on the induction of both stress pathways is neither necessary nor sufficient to explain increases in conjugation rates. BAC, CHX and permethrin were increasing the conjugation rate, but only BAC and CHX induced the stress response pathways. RecA, the trigger protein of the SOS response, has been shown to be involved in conjugation and transformation [154,247,248]. Indeed, previous reports showed that copper induces both stress responses and also increases conjugation rates [166,223]. However, induction of *recA* by copper and DDAC was not related to increased conjugation and transformation rates in our experiments (Fig. 2 & 3). This effect might be explained by the up to 2.5 fold lower copper concentrations used by us or by the different mating partner and transferable plasmid used in our conjugation assays as compared to those reported in the literature [166,223].

The observed differences between the induction of *rpoS* and *recA* to the effects of the biocides on mutagenesis, conjugation and transformation might be explained by the biocide concentrations used in those assays. In the soft agarose assay the reporter strains are exposed to a range of biocide concentrations, whereas the effects on the rates of mutation and HGT were measured at a single subinhibitory concentration. However, our results showed that many material preservatives induce the RpoS-mediated general stress and the SOS response, indicating a risk to affect rates of mutation and HGT at least at some concentrations. This might be important because pollutants have been shown to establish concentration gradients in the environment and thus microorganisms are likely exposed to such gradients [249,250]. Our data therefore raises the question on how realistic environmental concentrations are related to the induction of different stress response pathways and how this in turn affects rates of mutation and HGT. Moreover, our work raises the question as to whether material preservatives that are non-toxic to bacteria induce known mutational pathways other than those mediated by RpoS and RecA [251–254] or currently unknown pathways that affect rates of mutation and HGT. The work presented here provides the basis for future investigations, in which careful selection of environmentally relevant biocide concentrations should be performed and transcriptomic responses should be measured.

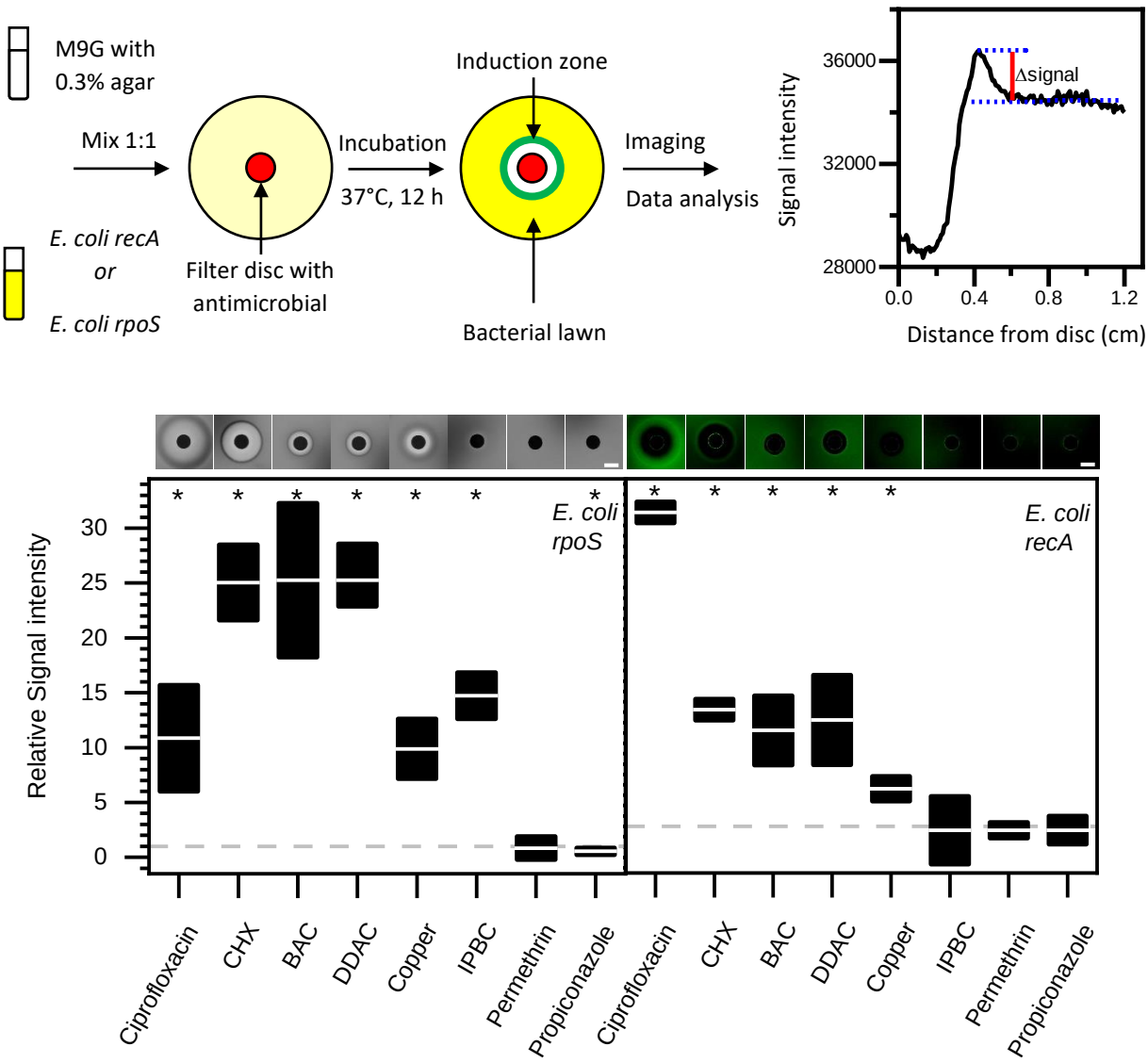

Fig. 4. Material preservatives affect bacterial RpoS-mediated general stress and RecA-linked SOS responses. A) Schematic of the reporter fusion assay. Imaging of plates was done with an office scanner (transmission) for *rpoS*::ßgal and with a UV transilluminator (fluorescence) for *recA*::GFP. A representative example for the analysis of the image data is shown for one replicate of *recA* expression in the presence of CHX. **B)** The induction of the bacterial stress response when exposed to antibiotics and biocides for *rpoS* and a component of the SOS response (*recA*) in *E. coli* reporter strains. The bars represent the 95% confidence interval around the mean shown as horizontal grey dotted line. Statistical significance of the treatment was assessed using the One-sample t-test ($p \leq 0.05$). The pictures above the graph show representative images for the corresponding treatments. The scale bar is 6 mm.

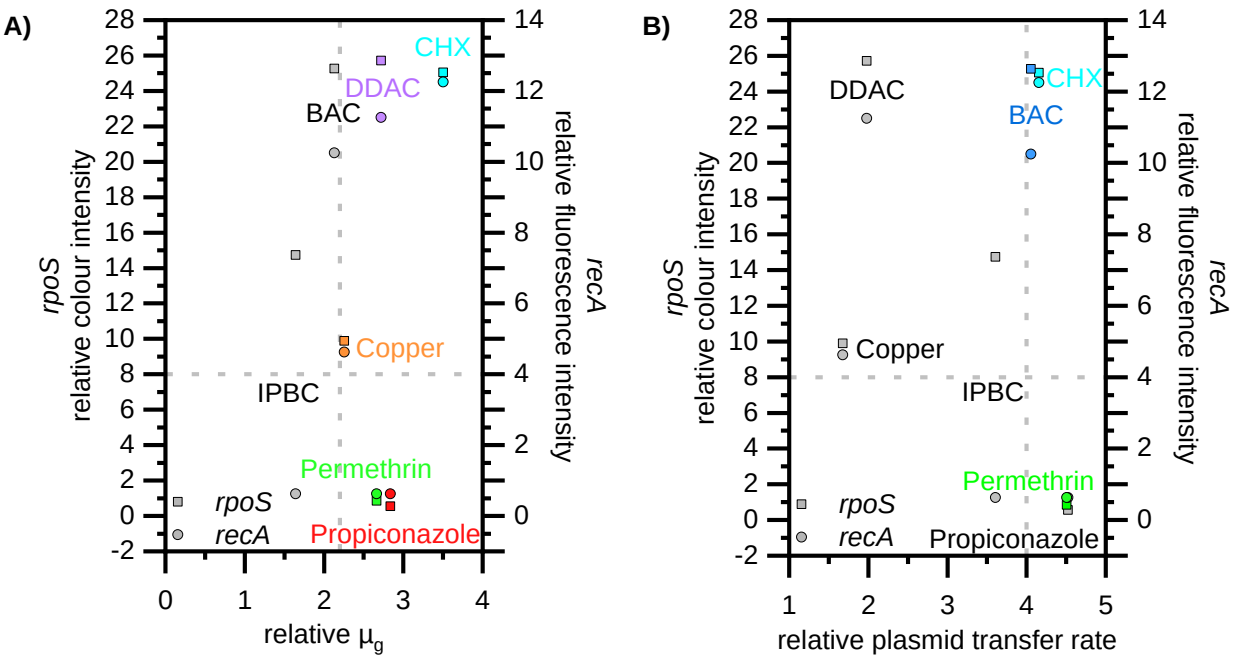

Fig. 5. Relationship between effects of biocides on the relative rates of mutation or plasmid transfer with their effect on *rpoS* and *recA* induction.

The effect of biocides on the induction of the bacterial RpoS-mediated general stress response (*rpoS,* squares) and the RecA-linked SOS response (*recA,* circles) (taken from Fig. 4) compared to **A)** the relative mutation rate μ_g (mutations per genome per replication) (taken from Fig. 1B) and **B)** the relative plasmid transfer rate (taken from Fig. 2). The response of *rpoS* and *recA* have been measured with *E. coli* reporter strains in a soft agar diffusion assay. The data shows the mean values of each parameter. Biocides that show significant ($p \leq 0.05$) effects on mutation or plasmid transfer rates are displayed with colored symbols, while biocides with non-significant effects are shown in grey. The dotted lines are a guide to the eye to distinguish the four possible outcomes (e.g. upper right: effect on rate linked to induction of *rpoS*/*recA*). Note that the plasmid transfer rate data of propiconazole is shown right of the dotted line, even though its rate is not significantly increased.

Conclusions

Material preservatives can increase rates of mutation and HGT of bacteria. Antibacterial biocides can induce their effect at environmentally relevant concentrations, while fungicidal or insecticidal biocides can induce their effects at high concentrations at the material-environment interface. The effects are moderate; ca. 2 to 4-fold for mutation rates and 4-fold for conjugation rates. Future studies need to assess how these effects contribute to the evolution and spread of antibiotic resistance in nature at their environmentally relevant concentrations.

Supplementary information

Mutation rate assay – MICs and biocide concentrations

The concentrations of the different biocides used in the mutation rate assay were chosen to be below the minimum inhibitory concentration (MIC) and to not induce measurable cell death at the initial cell densities of the mutation rate assay (i.e. 10^3 cfu mL^{-1}). The resulting concentrations may thereby not cause any bias in the estimation of the mutations rates [202]. A stepwise approach was chosen to derive these concentrations for each combination of test strain and active substance. First, the MIC was determined with an inoculum of 10^3 cfu mL^{-1} (Table S1). Then, we tested for the absence of initial killing at or below 1/50 MIC for *A. baylyi* and *B. subtilis* and 1/100 MIC for *E. coli* using an inoculum of 10^3 cfu mL^{-1} (Fig. S2). The MIC for the non-antibacterial active substances (i.e. permethrin, propiconazole and IPBC) was for some strains above the solubility maximum (~1×10^6 µg L^{-1} in the different culture media at room temperature for all 3 substances). In such cases (as indicated in Table S1), the concentrations used to test for the absence of initial killing were chosen relative to the solubility maximum and tested to be at or below 1/100 MIC.

Table S1. MICs and their relation to biocide concentrations chosen for the mutation rate assay

		E. coli	
Biocide	**MIC (µg L^{-1})***	**Concentration in mutation rat assay (µg L^{-1})**	**relation of assay concentration to MIC**
BAC	$3 \cdot 10^3$	30	1/100 MIC
DDAC	$2 \cdot 10^3$	20	1/100 MIC
CHX	$6 \cdot 10^3$	12	1/500 MIC
Copper	$2.5 \cdot 10^3$	25	1/100 MIC
IPBC	$1 \cdot 10^{6\#}$	$2 \cdot 10^3$	1/500 MIC
Permethrin	$1 \cdot 10^{6\#}$	$10 \cdot 10^3$	1/100 MIC
Propiconazole	$1 \cdot 10^{6\#}$	$10 \cdot 10^3$	1/100 MIC
Ciprofloxacin	300	0.6	1/500 MIC
Trimethoprim	$20 \cdot 10^3$	200	1/100 MIC
		B. subtilis	
BAC	$3 \cdot 10^3$	1.2	1/2500 MIC
DDAC	50	0.2	1/250 MIC
CHX	$6 \cdot 10^3$	12	1/500 MIC
Copper	$2 \cdot 10^3$	4	1/500 MIC
IPBC	$250 \cdot 10^3$	400	1/500 MIC
Permethrin	$100 \cdot 10^3$	400	1/250 MIC
Propiconazole	$100 \cdot 10^3$	400	1/250 MIC
Ciprofloxacin	$2 \cdot 10^3$	8	1/250 MIC
Trimethoprim	$5 \cdot 10^3$	4	1/1250 MIC
		A. baylyi	
BAC	$12 \cdot 10^3$	240	1/50 MIC
DDAC	$1 \cdot 10^3$	20	1/50 MIC
CHX	100	2	1/50 MIC
Copper	$50 \cdot 10^3$	$1 \cdot 10^3$	1/50 MIC
IPBC	$500 \cdot 10^3$	$1 \cdot 10^3$	1/500 MIC
Permethrin	$1 \cdot 10^{6\#}$	$2 \cdot 10^3$	1/500 MIC
Propiconazole	$1 \cdot 10^{6\#}$	$2 \cdot 10^3$	1/500 MIC
Ciprofloxacin	$1.8 \cdot 10^3$	36	1/50 MIC
Trimethoprim	$800 \cdot 10^3$	$16 \cdot 10^3$	1/50 MIC

* at starting cell density of 10^3 cfu mL^{-1}

MIC above solubility maximum of $1 \cdot 10^6$ µg L^{-1}

Conjugation rate assay – MICs and biocide concentrations

The concentrations of the different biocides used in the conjugation rate assay were chosen to be below the MIC at the initial cell densities of the assay (i.e. 10^7 cfu mL^{-1}) (Table S2). The concentrations were chosen to be 1/10 MIC determined at 10^7 cfu mL^{-1}. The growth data obtained during the conjugation rate assay showed no signs of growth inhibition or cell death. The MIC for the non-antibacterial active substances (i.e. permethrin, propiconazole and IPBC) was above the solubility maximum (~$1{\cdot}10^6$ µg L^{-1} in the different culture media at room temperature for all 3 substances). Therefore, the concentrations used in the conjugation rate assay were chosen relative to the solubility maximum instead of the MIC.

Table S2. MICs and their relation to biocide concentrations chosen for the conjugation rate assay

	E. coli		
Biocide	**MIC (µg L^{-1})***	**Concentration in conjugation rate assay (µg L^{-1})**	**relation of assay concentration to MIC**
BAC	$3{\cdot}10^3$	300	1/10 MIC
DDAC	$2{\cdot}10^3$	200	1/10 MIC
CHX	$6{\cdot}10^3$	600	1/10 MIC
Copper	$2.5{\cdot}10^3$	250	1/10 MIC
IPBC	$1{\cdot}10^{6\#}$	$100{\cdot}10^3$	1/10 MIC
Propiconazole	$1{\cdot}10^{6\#}$	$100{\cdot}10^3$	1/10 MIC
Permethrin	$1{\cdot}10^{6\#}$	$100{\cdot}10^3$	1/10 MIC

* at starting cell density of 10^7 cfu mL^{-1}

MIC above solubility maximum of $1{\cdot}10^6$ µg L^{-1}

Transformation rate assay – MICs and biocide concentrations

The concentrations of the different biocides used in the transformation rate assay were chosen to be below the MIC at the initial cell densities of the assay (i.e. 10^9 cfu mL^{-1}) (Table S3). The concentrations were chosen to range between ½ to 1/10 of the MIC determined at 10^9 cfu mL^{-1}. The concentration for BAA was chosen to correspond the concentration that was reported previously to stimulate transformation [154]. The MIC for the non-antibacterial active substances (i.e. permethrin, propiconazole and IPBC) was above the solubility maximum (~$1 \cdot 10^6$ µg L^{-1} in the different culture media at room temperature for all 3 substances). Therefore, the concentrations used in the transformation rate assay were chosen relative to the solubility maximum instead of the MIC.

Table S3. MICs and their relation to biocide concentrations chosen for the transformation rate assay

		A. baylyi	
Biocide	**MIC (µg L^{-1})***	**Concentration in transformation rate assay (µg L^{-1})**	**relation of assay concentration to MIC**
BAA	n.d.	$27.84 \cdot 10^3$	-
BAC	$12 \cdot 10^3$	$6 \cdot 10^3$	½ MIC
CHX	$1 \cdot 10^3$	500	½ MIC
DDAC	100	10	1/10 MIC
Copper	$50 \cdot 10^3$	$25 \cdot 10^3$	½ MIC
IPBC	$500 \cdot 10^3$	$250 \cdot 10^3$	½ MIC
Propiconazole	$1 \cdot 10^{6\#}$	$500 \cdot 10^3$	½ MIC
Permethrin	$1 \cdot 10^{6\#}$	$500 \cdot 10^3$	½ MIC

n.d. not determined

* at starting cell density of 10^9 cfu mL^{-1}

MIC above solubility maximum of $1 \cdot 10^6$ µg L^{-1}

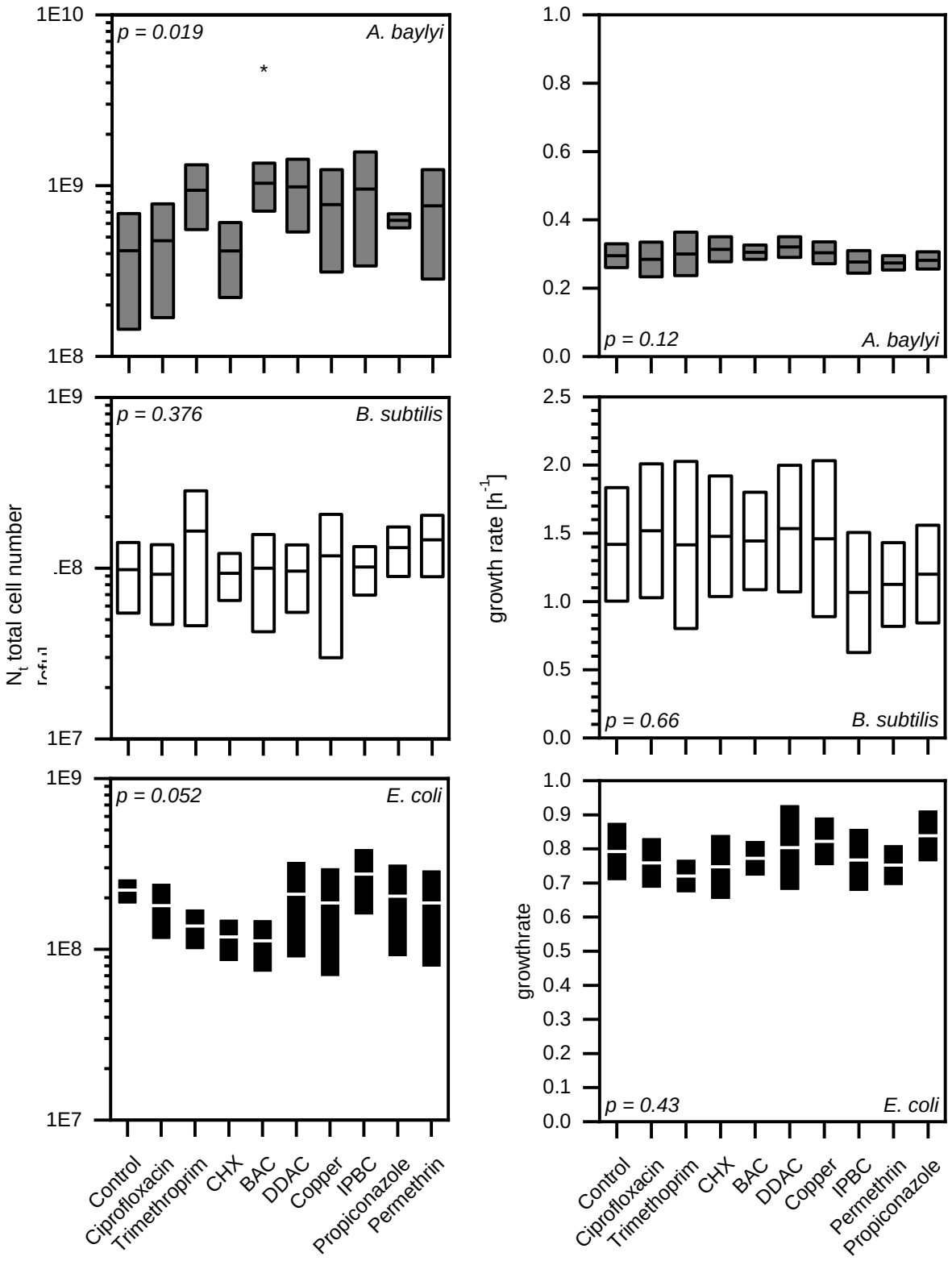

Fig. S1. The effect of biocides on the final total cell number (N_t) and the growth rate in the mutation rate assay. The final total cell number in 200 µL cell suspension of *A. baylyi*, *B. subtilis* and *E. coli* (left panel) was measured at the end of the mutation rate assay. The growth rates (right panel) were measured in M9G at the same antibiotic/biocide concentrations as were used in the mutation rate assay (Tables 1 and S1). The bars represent the 95% confidence interval around the mean as shown with horizontal lines (N_{Nt} = 5 to 6 and $N_{growth\ rate}$=7). Statistical significance of differences between control and treatments was assessed using a one-way-ANOVA with

a Dunnett post hoc test (Prism 5, Graphpad Software) and significant differences at $p \leq 0.05$ were marked with an asterisk (*A. baylyi* control vs BAC). P-values of the one-way-ANOVA are shown in each sub-panel.

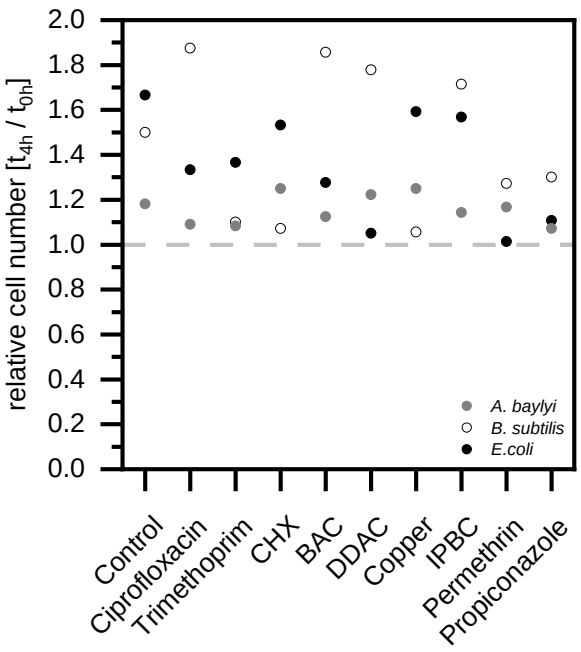

Fig. S2. Effect of biocides on cell death during mutation rate assay.

Relative cell number after 4 h of incubation as divided by the initial cell number for *A. baylyi*, *B. subtilis* and *E. coli*. The cells were inoculated in M9G medium at 10^3 cfu mL^{-1} as used in the mutation rate assay and sampled before and after 4 h exposure to biocides or antibiotics at concentrations shown in Table S1. Each circle represents the result of a single incubation (N=1). The analysis showed that neither antibiotics nor biocides lead to relevant killing at the concentrations used in the mutation rate assay after 4 h.

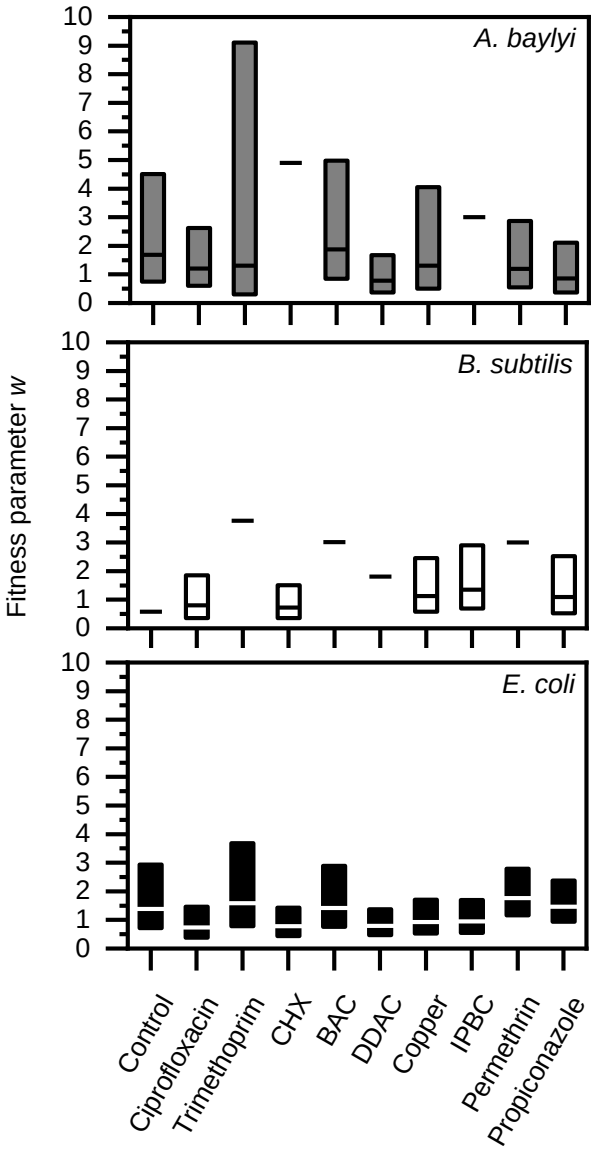

Fig. S3. Estimation of fitness (*w*) of rifampicin resistant mutants that emerged in the mutation rate assay relative to the wildtype.

Fitness of rifampicin-resistant mutants relative to the wildtype in the presence of biocides/antibiotics was estimated using the newton.joint.MK function in rSalvador or mutestim in flan (for *B. subtilis* TMP, BAC, DDAC). The bars represent the 95% confidence interval around the mean of *w* as shown with horizontal lines. rSalvador and flan were not able to determine the confidence intervals for all treatments. In these cases, only the mean of *w* is shown with a horizontal line.

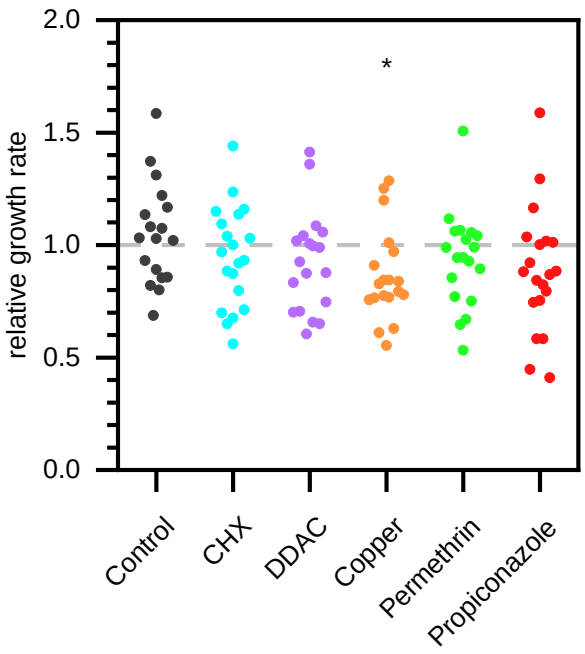

Fig. S4. Fitness of rifampicin-resistant *E. coli* mutants relative to the wildtype in the presence and absence of biocides.

The growth rate [h^{-1}] of isolated rifampicin resistant *E. coli* mutants (N=20) was determined in the presence and absence of biocides, which showed significant increases in mutation rates (Fig. 1B). The growth rates were measured in M9G at the same antibiotic/biocide concentrations as were used in the mutation rate assay (Tables 1 and S1). Symbols show the growth rate of each mutant relative to the mean growth rate of the wildtype in the same condition (Fig. S1). Statistical significance of the treatment to 1 was assessed using a One-sample t-test (*$p \leq 0.05$). The results show that copper significantly reduces the mutant fitness relative to the wildtype.

Table S4. Parameters for the calculation of the genomic mutation rate (μ_g)*

Strain	G (bp per genome)#	L (bp)§
E. coli	4,608,319 [255]	69 [256]
B. subtilis	4,214,598 [257]	8 [205]
A. baylyi	3,598,621 [258]	17 [196]

* references are shown in brackets next to each value

genome size

§ size of the genomic locus, on which a mutation can confer resistance to rifampicin

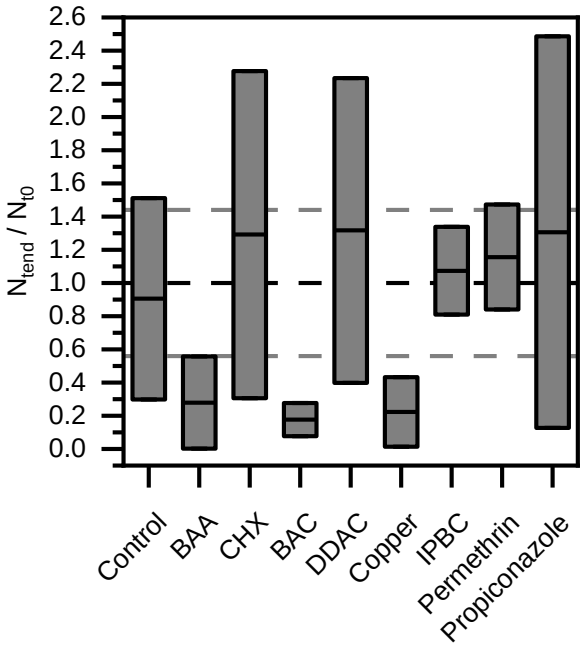

Fig. S5. Effect of biocides on the final cell number in the transformation assay.

The final cell number of *A. baylyi* at the end ($N_{t(end)}$) of the transformation assay relative to the cell number adjusted by optical density (OD_{600}) at the start ($N_{t(0)}$) of the transformation assay. The bars represent the 95% confidence interval around the mean as shown with horizontal lines (N=3-6). The black dashed horizontal line denotes the level (1) at which $N_{t(end)}$ does not change relative to $N_{t(0)}$. The grey dashed horizontal lines represent the 95% confidence interval of the accuracy with which $N_{t(0)}$ was adjusted.

Chapter 3: Effect of biocides selection pressures on evolution of resistance

Abstract

Biocides are used as disinfectants and material preservatives to control bacteria across various applications. Bacterial resistance to biocides has commonly been studied using adaptive laboratory evolution (ALE) experiments with growth at subinhibitory concentrations linked to serial subculturing over many generations. Furthermore, it has been shown recently that *E. coli* adapts to repeated lethal stress imposed by the biocide benzalkonium chloride (BAC) by increased survival (i.e. tolerance) and not by evolving the ability to grow at increased concentrations (i.e. resistance). Here, we investigate the contributions of evolution for tolerance as opposed to resistance in ALE experiments with *E. coli* exposed to subinhibitory BAC concentrations. We find that BAC concentrations, which are potentially selective in common ALE experiments (i.e. concentrations close to the half maximal effective concentration; EC50), show initial killing (~40%) before the population resumes growth. This indicates that cells face a twofold selection pressure; one for increased survival and one for increased growth in the presence of BAC. To disentangle the effects of both selective pressures, we conducted two ALE experiments: (i) one with initial killing and continued stress at the EC50 during the growth phase and (ii) another with initial killing and no stress during the growth phase. Phenotypic and genotypic characterization of the evolved populations revealed differences between the two ALE experiments and treatment-specific adaptations. Both evolutionary treatments led to a reduction of lag time, likely driven by the initial killing phase present in both treatments. However, growth at higher BAC concentrations was only selected when BAC was present during the growth phase. Genotypic characterization by whole genome sequencing of the evolved populations revealed parallelism in mutated genes among replicate populations for treatments with higher selection pressure and distinct differences across treatments. Treatments with higher selection pressures selected for BAC treatment specific mutations, such as membrane associated (*lpxM* and *lpxL*) genes or stress response related genes (*rssB*, *hslO* and *tufA*), while the lowest selection pressure selected for mutations in genes for regulation (*cyaA*) and cellular structure (flagella *fliJ*). Our findings underpins that the design of an ALE experiment to assess the potential for biocide resistance evolution should match the selective pressure imposed by the experiment to the conditions for which resistance risks should be assessed. While selection for survival may be appropriate to assess resistance risks emerging during use of the biocide at lethal concentrations, selection for growth in the presence of low concentrations may rather reflect selective effects imposed by diluted biocides in the environment. Thus, our work highlights the importance of careful experimental design to assess the risk of biocide resistance mechanisms in diverse applications in specific settings.

Introduction

Antimicrobial resistance is currently a global burden and will likely become more severe in the future [1,6]. While there is an ongoing increase of antimicrobial resistance there is very little progress accomplished in the development and admission of new antimicrobial compounds [259–261]. Therefore, the underlying mechanisms and causes for selection, evolution and spread of antimicrobial resistance must be further investigated and if possible, prevented.

Biocides are used to control bacteria across various applications [4,107]. This widespread usage of biocides is reflected in the presence of biocide residues in the environment [70]. Biocides are clear contributors to the burden of antimicrobial resistance [45,262–265]. A supporting example is the treatment failure of human pathogenic fungi with azole-based drugs in clinical settings. This treatment failure is partly related to the use of azole-based pesticides or biocides, which contribute to the evolution and spread of azole resistant fungi in the environment [266–270].

Benzalkonium chloride (BAC) is a quaternary ammonium compound used in a wide range of applications, e.g. as an antiseptic, a disinfectant, or as material preservative [54] This explains the occurrence of BAC residues various matrices like food, wastewater, soils and sediments [86,131,271]. Further on, it was shown that low BAC concentrations which also occur in the environment can increase the spread of antibiotic resistance via horizontal gene transfer via transformation [272,273] or conjugation [264]. Thus, the prolonged exposure of microorganisms to BAC can promote and contribute to the antimicrobial resistance problem [262,274,275]. Therefore, BAC resistance evolution and the effect on further resistance mechanisms need to be investigated.

Adaptive laboratory evolution experiments (ALE) are commonly used to better understand the risks and mechanisms of antimicrobial resistance evolution [276,277]. Hereby, a serial transfer of microorganisms in the presence of certain antimicrobials is used to study the population dynamics and molecular mechanisms of adaption [117,276,278]. However, the precise selective pressure exerted during those ALE experiments and its consequent impact on adaption mechanisms often remain unexplored.

Furthermore, various factors can affect the selective force of antimicrobial compounds, such as biocides. These include the physical stability of biocides [279], the specific growth conditions of microorganisms, such as the growth media and culturing vessels used [280], as well as the interactions between the antimicrobial and microorganisms, along with their population size [281,282]. These factors collectively contribute to the uncertainty of selection pressure and population dynamics during ALE experiments. The phenomenon of the microbial population size affecting the efficacy of an antimicrobial is termed inoculum effect [283]. This

inoculum effect has been observed with a range of antimicrobials, including antibiotics [284–286], biocides [287–289] and antimicrobial peptides [290]. Specifically, the inoculum effect of quaternary ammonium compounds, such as benzalkonium chloride (BAC), could affect ALE experiments. Since BAC causes an initial decrease of a fraction of the population while leaving the rest of the population potentially unaffected [52,287]. Consequently, this could lead to the selection of diverse survival mechanisms, such as tolerance or persistence.

Microbial survival mechanisms, such as resistance, tolerance and persistence can be distinguished by determining the lethal concentration and the time required to reduce specific portions of the population [291]. While a susceptible population is killed upon exposure to higher concentrations of an antimicrobial compounds, resistant populations manage to survive and grow at lethal concentrations for susceptible populations. Tolerant populations can survive a transient exposure to lethal stress by slower growth or longer lag phases [291,292]. Likewise, a persistent subpopulation can survive a transient exposure to lethal stress while the majority of the population is killed and this mechanism can be either time or- dose dependent [291]. Additionally, tolerance can mask persistence [293] and thus to further study one of those mechanisms individually the experimental conditions need to be carefully considered.

These survival mechanisms exhibit different fitness trade-offs [293] and synergistic interactions between those survival mechanisms can occur [98]. Moreover, selection for increased survival in form of persistence can be achieved rapidly [117,294]. Nonetheless those survival mechanisms promote the evolution of antibiotic resistance by increasing survival and mutation rates [128] and promote the spread of antibiotic resistance [295,296].

The aim of this study is to disentangle the effects of different selection pressures being present in serial transfer evolution experiments. In serial transfer evolution experiments with antimicrobials a fully grown culture is repeatedly exposed to a subinhibitory concentration of an antimicrobial. The culture is diluted and grown in the presence of the antimicrobial. It is assumed that the main selective driver is for growth in the presence of the antimicrobial stress during the growth phase. However, our initial data showed that subinhibitory concentrations of BAC are lethal to a part of the population in the lag phase and thereby may exert selection for survival. In our serial transfer evolution experiment setup (Fig. 1), we have two treatments that aim to disentangle both selective drivers: i) one treatment to select for survival and growth with BAC being present in the lag and growth phase (SG), and ii) a second treatment to select for survival only with BAC being present in the lag phase and removed before the growth phase (S). The phenotypic and genotypic analysis of the evolved lines of the two treatments was performed to provide insights into the adaptations that are distinct for survival as opposed to those for survival and growth. Differences between treatments indicated that selection for

growth is not the only selective pressure in serial transfer evolution experiments with BAC. The two treatments used in our serial subculture evolution experiment emphasize the opposing mechanisms of a stressor during serial transfer evolution experiments selecting for either tolerance or resistance.

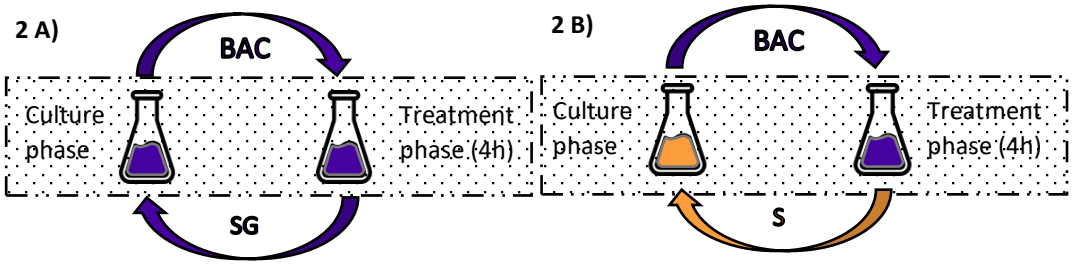

Figure 1. Serial subculture evolution experiment to assess the effect of benzalkonium chloride (BAC). (A) Selection for survival and growth (SG) and **(B)** the selection for survival (S). Each of the two evolutionary treatments consisted of 12 parallel populations of which 6 populations were exposed to BAC according to the evolutionary treatment and 6 additional populations served as controls treated without BAC exposure, but otherwise being propagated exactly in the same way as the BAC-treated populations. BAC was present during the lag and the growth phase in the selection for survival and growth (SG) treatment, whereas BAC was removed after 4 h of exposure at the end of the lag phase (before the growth phase) in the selection for survival (S) treatment.

Material and Methods

Bacterial strain, chemicals, media, and cultivation conditions

Escherichia coli K12 MG1655 was a generous gift from Prof. Rupert Mutzel (Freie Universität Berlin) and cultivated in liquid defined media M9G composed of M9 salts, minerals, trace elements and glucose as source of carbon and energy (42.2 mM Na_2HPO_4, 22 mM KH_2PO_4, 11.3 mM $(NH_4)_2SO_4$, 8.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 20 mM glucose, 0.0025 mM $FeCl_3$, 0.00495 mM $ZnCl_2$, 0.0021 mM $CoCl_2$, 0.002 mM Na_2MoO_4, 0.0003 mM $CaCl_2$, 0.0025 mM $CuCl_2$, 0.002 mM H_3BO_3) and incubated overnight at 220rpm and 37°C. When cultivated on solid media, Lysogeny broth (LB) Lennox (0.16 mM yeast extract, 1.41 mM tryptone, 0.86 mM NaCl and 0.45 mM agar bacteriology grade) agar plates were used and incubated overnight at 37°C. Growth parameters of *E. coli*, such as growth rate and lag time were determined using a plate reader (Biotek Epoch 2 microplate reader) set to 37°C, fast orbital shaking, and a 5 min measuring interval. The lag times and growth rates were calculated from the increase in OD_{600} over time by inferring the time derivative using Gaussian processes implemented as published code (Swain et al., 2016) in Python 3.6. The biocide benzalkonium chloride [BAC] (PO 12060) was purchased from (Merck KGaA, Darmstadt, Germany). The antibiotics colistin (PO 537340), gentamicin (PO 412367), ciprofloxacin (PO 412310), and ampicillin (PO 412252) were purchased as E-test stripes from Biomérieux (bioMérieux Deutschland GmbH, Nürtingen, Germany).

Antimicrobial susceptibility determination

Susceptibility to BAC was determined with the broth microdilution method using 200 µL total volume per well [192]. The minimum inhibition concentration (MIC) was defined as the lowest concentration that inhibits the growth in liquid cultures. The EC50 of BAC was determined by fitting the growth rate data at various BAC concentrations to a sigmoidal fit using a dose-response curve with variable Hill slope given by parameter "p" and the Levenberg Marquardt iterative algorithm in OriginLab (Origin 2021 Version 9.8.0.200).

The effect of the selected subinhibitory BAC concentration on the viable cell number of the *E. coli* ancestor and the evolved *E. coli* populations was assessed by performing growth curves, which were analyzed by plating and counting of colony forming units at certain time points. *E. coli* overnight cultures were adjusted to 10^6 cfu mL^{-1}, incubated without and with 4 µg mL^{-1} BAC at 37°C and 1300 rpm in a tabletop incubator (Thermoshaker, Starlab). A sub-sample of the cell suspension was retrieved before the addition of BAC and at hourly intervals until 4 h or after 2 h only. The sub-sample of the cell suspension was diluted appropriately, plated on LB agar, the plates were incubated at 37°C for 24 h, and the colony forming units were counted.

Susceptibility to antibiotics was assessed using E-test stripes following the EUCAST disk diffusion guidelines [297] with slight modifications. Instead of MH2 agar plates, LB agar plates were used and incubated at 37°C. The *E. coli* ancestor was used as a reference point to the evolved *E. coli* populations with the two distinct BAC treatments.

Serial subculture evolution experiment

Daily serial subculture evolution treatments with an M9G pre-adapted *E. coli* K12 MG1655 strain [117] were carried out over 15 days, resulting in approximately 150 generations. Each of the two evolutionary treatments (Fig. 1), the selection for survival only and the selection for survival and growth treatment, had each six biological replicates treated with BAC and six corresponding controls treated without BAC, resulting in total 24 evolutionary populations. In both evolutionary treatments, BAC was present at sub-MIC concentrations for 4 h, which includes the lag phase and the begin of the exponential phase of the *E. coli* ancestor. After 4 h of BAC exposure, the BAC-containing M9G media is replaced by BAC-free M9G media in the selection for survival only treatment, whereas the BAC-containing M9G media remained in the selection for survival and growth treatment.

All cultures were adjusted to 3×10^6 cfu/mL fresh M9G media prior to the treatment. Cells in both treatments were exposed to 4 µg/mL BAC. Cells in the corresponding controls were exposed with M9G medium only. The treatments and controls had a final volume of 520 µL. The cells suspensions were incubated in a tabletop shaker (Thermoshaker, Starlab) at 37°C with 12000 rpm for 4 h and subsequently centrifuged for 5 min at 8000 rpm before the media

was changed to fresh M9G medium without BAC in the selection for survival treatment and their corresponding controls. The selection of survival and growth treatments were also centrifuged but the cell pellet was immediately resuspended in the supernatant (BAC-containing M9G medium). Cultures of both treatments and the corresponding controls were incubated at 37°C and shaken in a tabletop (Thermoshaker, Starlab) at 1200 rpm. Daily freezer stocks in 20 % glycerol were prepared and stored at -80°C. The evolved populations from the different treatments and controls from the last day were further used for phenotypic and genotypic characterization.

Genotype analysis of the evolved populations

Chromosomal DNA of the populations in 4 of 6 biological replicates of each treatment and the corresponding controls was isolated from glycerol freezer stocks using the peqGOLD bacterial DNA kit (PO 13-3450-01). Genome sequencing was performed by Eurofins Genomics (Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany) on a Illumina system with 150 bp paired-end reads and ~300-fold coverage. Quality of the reads was assessed using the fastqc tool (Wingett & Andrews, 2018) showing that no further trimming was necessary. The analysis of mutations was performed using the breseq pipeline (Deatherage & Barrick, 2014) and the *E. coli* K12 MG1655 reference genome (NCBI RefSeq NC_000913.3). Intra- and intergenic mutations were removed from the list of identified mutations of each BAC-treated population based on the following two criteria: (i) mutations that were present below a threshold of 5 % of the total reads, and (ii) intra- and intergenic mutations that were also present in the corresponding controls. The mutated genes were clustered into categorys by MultiFun Terms found on BioCyc (https://biocyc.org/).

Results

Subinhibitory BAC concentrations are lethal for a fraction of the population and delay initial growth

The aim of the study is to understand the selection drivers during serial transfer evolution experiments with BAC. Common serial transfer evolution experiments operate with subinhibitory concentrations of antimicrobials to ensure growth and simultaneously maintain a selection pressure. Therefore, the susceptibility of *E. coli* to BAC was tested determining the dose response curve and time kill kinetics. Dose-response analysis showed that BAC has a narrow concentration range (between 2.5 and 5 µg mL^{-1}) at which it affects the growth rate (Fig. 2 A). The IC50, the concentration at which the growth rate is lowered to 50%, is estimated to be 4.36 µg mL^{-1} BAC. The MIC of BAC of the *E. coli* ancestor was determined to be 5 µg mL^{-1}. Exposing *E. coli* to 4 µg mL^{-1} BAC showed that the initial growth was delayed as compared to unexposed cells (Fig. 2 B). The observed delay of growth was based on the significant reduction of 40% viable cells after 2 h in the presence of subinhibitory BAC concentrations (Fig. 2 C). This data suggests that serial transfer evolution experiments at subinhibitory concentrations may exert two types of selection pressures: selection for faster growth and enhanced survival at subinhibitory BAC concentrations.

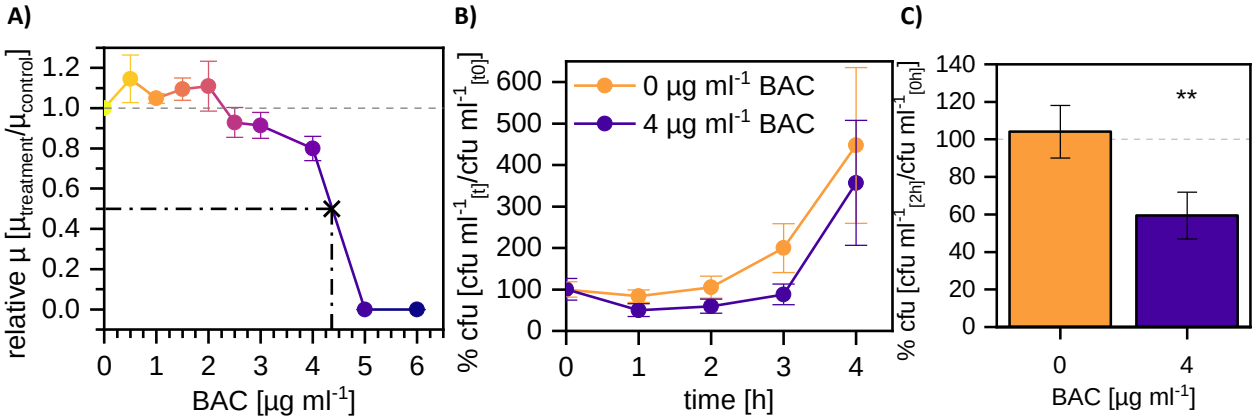

Figure 2. Benzalkonium chloride (BAC) susceptibility of the *E. coli* ancestor.

A) Dose-response curve for the growth rate of *E. coli* as a function of BAC concentration (color gradient from yellow to purple). The measured growth rate shown as dots with standard deviation (N=4) was calculated relative to the growth rate without exposure to BAC. The IC_{50} is 4.36 µg mL^{-1} BAC and is shown as a cross and dotted line. **B)** Initial growth curve of *E. coli* with (purple line) and without (orange line) exposure to BAC shown as the increase of colony forming units over time relative to the initial inoculum shown as dots with standard deviation (N=5 to 6). **C)** The surviving fraction of *E. coli* after 2 h exposure with and without 4 µg mL^{-1} BAC. Statistical significance was assessed using the one sample t-test (** $p<0.005$).

Phenotypes evolved in the presence of BAC are linked to selection pressures

To test whether subinhibitory BAC concentrations differentially affect evolution for survival or for survival and growth, we performed a serial transfer evolution experiment with two treatments (Fig. 1) and 14 transfers at a subinhibitory BAC concentration (4 µg mL^{-1}). Phenotypic adaptions were assessed at the end of the evolution experiment by determining survival after 2 h in the presence of BAC, the growth rate, the lag time and the MIC for BAC of the evolved populations (Fig. 3).

The data showed no reduction in cell number upon BAC exposure for single evolved lines, while the ancestor showed an average survival of 60 %; the relative survival after 2 h BAC exposure was 100 % for 3 of 6 lines of the survival treatment (S), 6 of 6 lines for the corresponding control (Ctrl S), 2 of 6 lines for the survival and growth treatment (SG) and 3 of 6 lines for the corresponding control (Ctrl SG). However, this differences could not be substantiated with a statistical test because the technical variance was only assessed for cfu dterminations of the ancestor. Moreover, the statistical analysis between the evolutionary treatments and the corresponding controls showed there was no significant difference in the surviving fraction (Mann-Whitney $p>0.05$; Fig. 3A). This indicates that mutations that are not specific to BAC exposure confer increased survival in single evolutionary lines and that selection for survival is generally a weak driver for differences between both evolutionary treatments (S and SG) in our experiment.

Furthermore, the data showed that the evolutionary treatments affected the growth rate (ANOVA $p<0.001$; Fig. 3A). However, statistical analysis showed a significantly different growth rate only in part (~58 %) of the replicate evolutionary lines of the treatments and their controls as compared to the ancestor. Additionally, the evolutionary treatments were not significantly different from their respective controls (Mann-Whitney $p<0.05$). The occurrence of significantly different growth rates in the presence of BAC in treatment and control populations in comparison to the ancestor suggests that those adaptations are underpinned by unspecific adaptation to the general conditions of the evolution experiment rather than to the specific treatments.

In addition, the data showed that the evolutionary treatments evolved shorter lag times as compared to the ancestor (ANOVA $p<0.001$; Fig. 3B). Five of six of the evolved lines in the evolutionary treatment for survival only (S) and survival and growth (SG) treatment had significantly shorter lag times as compared to the ancestor (Bonferoni post hoc test $p<0.05$). While the ancestor had a lag time of 8.7 h in the presence of BAC, the evolved treatment lines that were significantly different exhibited lag times around 7.2 to 7.8 h. The lines that evolved with exposure to BAC had significantly shorter lag times in comparison to their corresponding

controls without BAC (Mann-Whitney $p<0.05$). This shows that the presence of BAC during evolution was required to select for shorter lag times. Those shorter lag times might be explained by higher growth, increased survival or earlier onset of growth. However, the first two mechanisms were not significant across all replicates in our data, suggesting that earlier onset of growth is overall the strongest driver of shorter lag times.

Moreover, the data showed that the populations in the treatment for selection for survival and growth acquired the ability to grow in the presence of higher BAC concentrations (Mann-Whitney $p<0.01$; Fig. 3D). While the populations selected for growth and survival had a 1.1-to-1.6-fold increase in MIC relative to the ancestor, the populations selected for survival only had a 0.9 to 1.1 fold increase in MIC. This suggest that evolution of growth at increased BAC concentrations requires selection for growth in the presence of BAC. Selection for survival only is not sufficient to evolve growth at increased BAC concentrations.

Taken together, the phenotypic analysis showed that the differences between the evolutionary treatments in response to BAC are linked with the applied selection pressure. The evolutionary treatments with selection for survival only and for survival and growth selected for a shorter lag time. While selection for survival and growth was required to select for growth at increased BAC concentrations. However, the observed effects on the phenotype are relatively weak which might be based on the low selection pressure applied and the short duration of the evolution experiment.

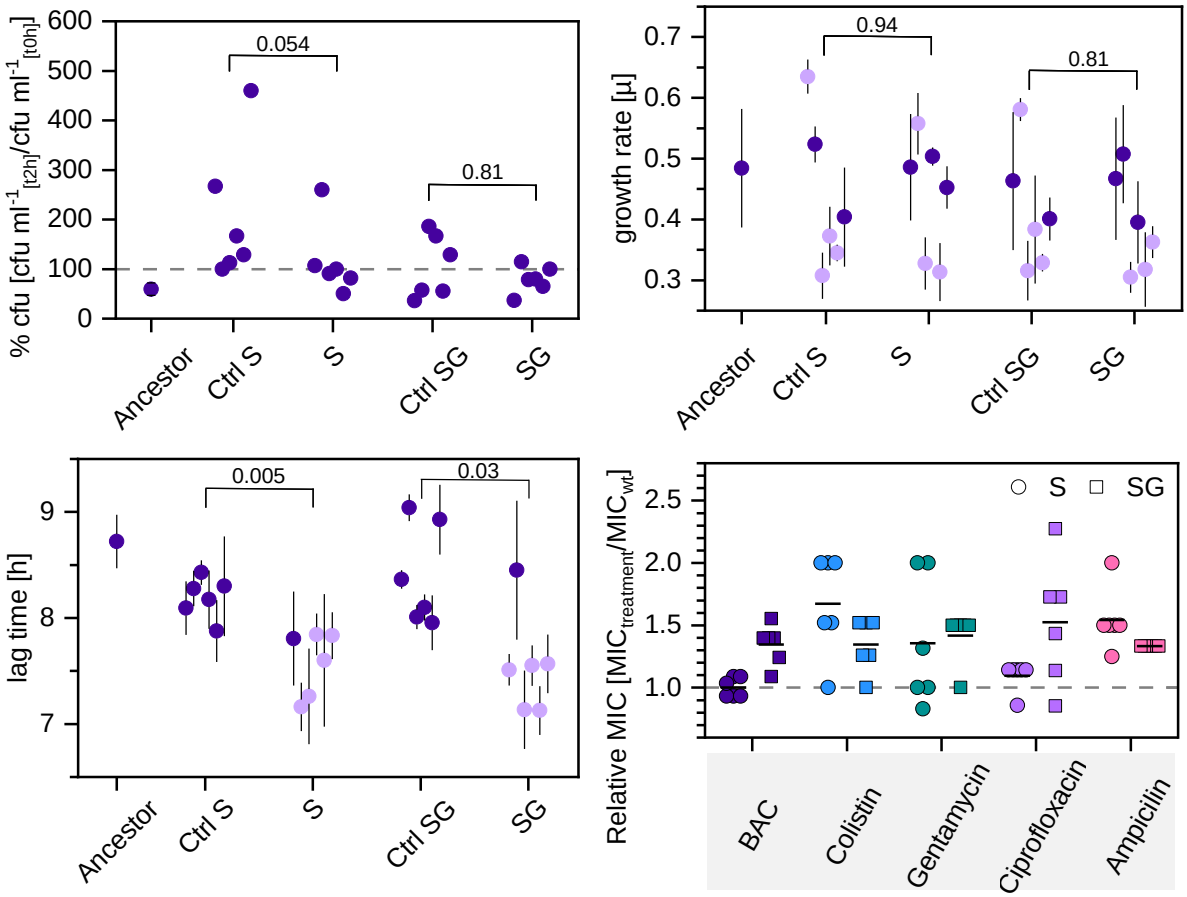

Figure 3. Phenotypic changes of *E. coli* populations evolved for increased survival (S) or survival and growth (SG), their corresponding controls (Ctrl) and the ancestor in the presence of benzalkonium chloride (BAC). A) Surviving cell fraction after 2 h exposure with 4 µg mL^{-1} BAC relative to the begin of the incubation. **B)** Growth rates and **C)** lag times at 4 µg mL^{-1} BAC exposure. **D)** Susceptibility for BAC and antibiotic. The two different evolutionary BAC treatments are indicated by circles for the survival only and squares for the survival and growth treatment. The various tested antimicrobials can be distinguished by colors (dark blue = BAC, light blue = colistin, green = gentamycin, purple = ciprofloxacin, pink = ampicillin). Each data point represents one evolutionary lineage. The error bars in A) for the ancestor only, and in B) and C) for all datapoints indicate the standard deviation around the determined mean of replicate survival (N=6) and growth assays (N=12), respectively. Means of single evolved lineages shown in light purple color are significantly different to the ancestor (One way ANOVA $p<0.001$, Bonferroni post hoc test $p<0.005$). If the values are shown in dark purple, there was no significant difference to the ancestor. Significance between the evolutionary treatments and their corresponding controls is assessed with the Mann-Whitney-Test. P-values are indicated above the brackets.

Consequences of BAC evolution on antibiotic susceptibility

Evolutionary adaptations to BAC are known to lead to decreased susceptibility to antibiotics. Therefore, we investigated whether the distinct exposure to BAC in the two different evolutionary treatments differentially affected antibiotic susceptibility. To this end, we measured susceptibility of a range of selected antibiotics that have different mode of actions. The data shows that there is overall a decreased antibiotic susceptibility of populations coming

from both evolutionary treatments (Fig. 3D). For ampicillin, we determined a significant 1.3-fold MIC increase for the survival and growth treatment (SG) and 1.5-fold MIC increase for the survival only treatment (S). For colistin and gentamicin, we observed a heterogeneous adaptation regarding antibiotic susceptibility that was largely independent of evolutionary treatments. Specifically, about half of the populations exhibited a 1.5-to 2-fold MIC increase, while 6 of 24 populations did not show a change in MIC relative to the ancestor. For ciprofloxacin, there is no increase in MIC for populations exposed to the survival only treatment (S). In contrast, populations exposed to the survival and growth treatment (SG) had a 1.5-fold MIC increase relative to the ancestor. Taken together, there was a low effect of the BAC evolutionary treatments on the antibiotic susceptibility and weak parallelism for specific antibiotics. In addition, the data shows that adaptation of antibiotic susceptibility during BAC exposure occurs upon selection for survival only as well as for survival and growth; this observation is contingent on the type of antibiotic. The opposing effects of adaptation to ciprofloxacin indicate that selection drivers (growth or survival) during BAC exposure can affect adaptation to antibiotics.

Evolved genotypes are linked to BAC-mediated selection pressure

Next, we asked whether the differences in BAC-mediated selection pressure and associated phenotypes is underpinned by differences in evolved genotypes. To this end, genotypic adaptions were assessed at the end of the evolution experiment by sequencing the replicate populations of both evolved lines (S and SG) and their corresponding controls with a coverage of 300-fold (Fig. 4). In addition, we incorporated an additional, already published dataset into the analysis, in which replicate lines were evolved over 14 treatment cycles that consisted of exposure to a lethal BAC concentration (~9 fold MIC) for 15 min followed by growth in the absence of BAC [117]. This treatment was shown to select for persistence or increased tolerance and thus was designated as selection for persistence (P). The same ancestor *E. coli* strain was used for all evolution experiments (S, SG and P). Sequence analysis was conducted by setting a threshold of 5 % occurrence of mutations in the total reads of each sample. The evolved controls showed 19 mutations in the selection for survival experiment, 29 mutations in the selection for survival and growth experiment and 12 mutations in the selection for persistence experiment. Those mutations in intragenic regions or in genes were subtracted from the mutations in the evolutionary lines treated with BAC. Thus, only BAC and treatment specific mutations were further compared by categorizing mutations into intra- and intergenic (Fig. S2), clustering mutated genes according to treatment (Fig. 4), and by categorizing mutated genes using the Biocyc GO terms (main and subcategory) (Fig. S3).

The analysis showed that the total number of inter- and intragenic mutations is highest in the treatment selecting for persistence with 44 mutations, followed by 24 mutations in the

treatment selecting for survival and lowest in the treatment selecting for survival and growth with 16 mutations. The degree of fixation of mutations in specific genes or promoter regions was highest in the treatment selecting for persistence (5 of 6 lines had 100% fixation for at least one gene). The treatment selecting for survival and growth had intermediate fixation of mutations (1 of 4 lines had 100% fixation for at least one gene) and the treatment selecting for survival only had the lowest fixation of mutations (the highest fixation of 38 % of one gene was reached by 1 of 4 lines) (Fig. 4). This suggests that the duration of the evolution experiment was limiting the occurrence and selection of well adapted genotypes. Overall, there are more intergenic than intragenic mutations in the treatment selecting for persistence (P), while for the other treatments (S and SG) more intragenic than intergenic mutations are present (Fig. S2). Most of the intergenic mutations occurred in the promoter regions across treatments (S=80%, SG=100%, P=82%). This indicates that adaptation to BAC was driven by adaptation of gene expression.

The mutated genes that fixed in 5 % of the total population were clustered according to treatment. This analysis showed that a distinct set of mutations in specific genes or promoter regions were associated to each evolutionary treatment (Fig. 4). In the selection for persistence treatment, the mutated genes with the highest frequency and parallelism are associated with the membrane (*lpxM* and *lpxL*) and the stress response (*rssB*). The selection for survival treatment showed low parallelism with only two replicate lines having a mutation in the same gene (*yfdL*). In addition to mutations in yfdL, mutated genes were associated with regulation (*cyaA*) and cellular structure (flagella *fliJ*). In the selection for survival and growth treatment, the mutated genes with the highest parallelism are associated with the stress response (*hslO* and *tufA*).

Next, we clustered mutated genes and promoter regions according to treatment and functional categories (Fig. S3). This analysis showed that mutations in the treatment for survival and growth and the treatment for survival do not occur in shared functional categories. In contrast, the treatment for persistence shared mutations in functional categories with both evolutionary treatments (S and SG). The treatment selecting for survival and growth and the treatment selecting for persistence shared mutations in genes or promotor regions which are associated to metabolism (metabolism of other compounds), cell processes (adaptations) and information transfer (protein related). The treatment selecting for survival and the treatment selecting for persistence shared mutations in the functional categories in regulation (type of regulation) and metabolism (synthesis of macromolecules).

Taken together, there are differences between the treatments regarding the number of mutations, the level of fixation, the parallelism between replicate lines, and the identity of mutated genes and their functional categories. This indicates that each of the three evolutionary treatments is associated to a different selection pressure leading to different evolved adaptations.

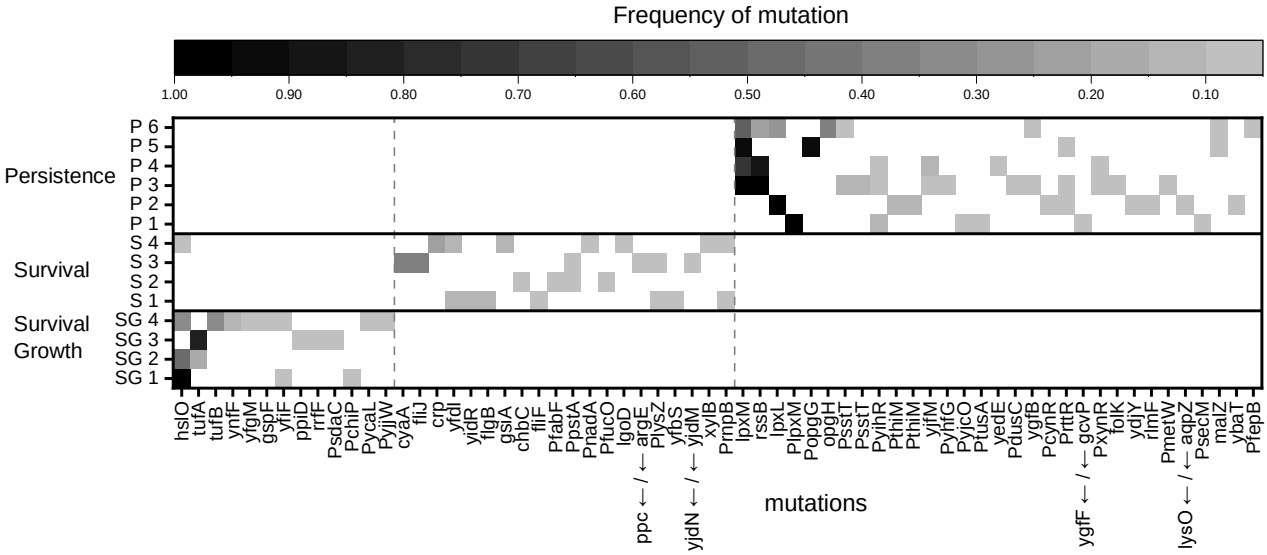

Figure 4. Mutated genes and promotor regions in response to different evolutionary treatments with exposure to benzalkonium chloride.

Heatmap of the mutated genes, promotor regions (genes and promotor, depicted with P, with 5 % cut off) and mutations in intergenic regions (both genes divided by / surrounding the intergenic region, depicted with arrows indicating location, with 5% cut off) in the evolved *E. coli* lines clustered to the treatments: selection for survival and growth (SG), selection for survival only (S), and selection for persistence (P). The intensity scale depicts the frequency of mutations in the specific loci.

Discussion

We determined the effects of different selection regimes in serial transfer evolution experiments using subinhibitory BAC concentrations with two distinct treatments i) selection for survival only and ii) selection for survival and growth. The used subinhibitory BAC concentration reduced the growth rate of the *E. coli* ancestor and caused initial killing upon exposure and thus its presence exerts a selection pressure for survival and growth. We hypothesized that the distinct selective pressures in both treatments select for distinct adaptation mechanisms.

The characterization of the evolved phenotypes and genotypes showed treatment-specific adaptations. Distinct phenotypic adaptations were apparent in lag times and growth at increased BAC or antibiotic concentrations (Fig. 3). Treatment-specific genotypic adaptations were apparent in clustering of mutated genes according to the selection pressure (Fig. 4). Initial killing by BAC in both experimental treatments selected for shorter lag times most likely by earlier onset of growth. In contrast, the presence of BAC during the growth phase was required to select for growth at increased BAC concentrations (Fig. 3D). In addition, changes in susceptibility to a range of antibiotics were treatment specific (Fig. 3D). Overall, the determined phenotypes and genotypes showed signs of relatively weak adaptations and adaptations towards the general experimental conditions unspecific to BAC. BAC unspecific adaptations were apparent because the evolved growth rates (Fig. 3B) and survival fractions (Fig. 3A) of the controls were comparable to the evolutionary lines exposed to BAC. Weak adaptation to BAC was visible as relatively small differences in susceptibility to BAC or antibiotics and as a low degree of fixation and parallelism of mutated genes. This indicates that adaptive laboratory evolution experiments with subinhibitory BAC concentrations exert a low selection pressure and thus require a high number of generations to establish treatment specific adaptations that are fixed within the population. Therefore, we argue that standard serial transfer evolution experiments are not an ideal approach to select for growth at high BAC concentrations. One reason for low selection pressures is the narrow selective window of BAC which does not allow to dynamically fine tune the exposure to concentrations that decrease the growth rate but does not lead to full growth inhibition. In turn, selecting for growth at increased concentrations may be facilitated by experimental evolution approaches that allow to continuously adjust the selection pressure by increasing exposure concentrations in morbidostat continuous culture systems or on gradient plates [92,95,96]. These approaches would also allow to select solely on growth, a treatment with a selection pressure that could not be established with our approach.

To our knowledge, this is the first attempt to disentangle the distinct selection pressures on survival and growth of an antimicrobial in serial transfer evolution experiments. Our data shows

that during serial transfer evolution experiments these different selection pressures are present. This observation could be also valid for other antimicrobials such as biocides and antibiotics. Experimental evolution with antibiotics has shown that tolerance is a stepping stone for the evolution of resistance [292,294,298]. Such tolerance may be selected by increased survival or shortened lag time during the initial exposure phase with subinhibitory concentrations. Stress response during the initial exposure phase is known to be mediated by heterogeneity and thus this heterogeneity serves as basis for evolution of increased survival [117,294]. While we observed heterogenous killing in the ancestor (Fig. 2C) under our experimental conditions, we did not investigate the basis of this heterogeneity in detail. However, since heterogeneity has been shown to be involved in evolution of tolerance to BAC [117], it is likely that it also plays a role under our experimental conditions.

An apparent phenotypic adaptation was that evolved lines showed reduced lag times (Fig. 3C). One possible consequence of the evolved shorter lag times might be the increase of the biomass to BAC ratio to counteract BAC toxicity. Decreasing toxicity by increasing growth rates have been shown to dilute antibiotics inside single cells, enhancing phenotypic tolerance [299]. An alternative explanation for the evolution of shorter lag time is that surviving cells evolve mechanisms that counteract accumulation of cellular damage, allowing them to resume growth earlier than non-evolved cells. Those mechanisms have been shown previously to be related to increased efflux [112,300,301] or reduced uptake by increasing outer membrane charge [117]. In addition, increasing chaperon activity could minimize cellular damage. Accordingly, our data showed in the treatment selecting for survival and growth mutations in the chaperon-related genes *hslO* and *tufA*. The gene *hslO* encodes the redox-regulated molecular chaperone Hsp33 [302] and its activity is regulated by redox state upon oxidative stress conditions [303]. The gene *tufA* is a subunit of a transcription factor, which rescues the activity of oxidative stress sensitive *hslO* knock outs in *Vibrio cholerae* [304]. Similarly, the treatment selecting for survival only had a loss of function mutation in *cyaA*, which has been reported to be involved in pan-tolerance to antibiotics and disinfectants by reducing ROS formation [305]. The gene *cyaA* encodes for adenylate cyclase, which synthesizes the second messenger 3',5'-cyclic adenosine monophosphate (cAMP) being involved in regulation of sugar metabolism. A mutation in *cyaA* decreases ROS in cells residing in the lag phase reducing damage of biomass [305]. Thus, a mutation in *cyaA* may evolve, because it protects cells in the survival only treatment that do not grow in the presence of BAC. In contrast, chaperones like *hslO* enable constant removal of damaged biomass to allow growth in the presence of BAC, explaining its occurrence in the survival and growth treatment. In summary, the evolved phenotypes and genotypes allow to hypothesize that the two distinct treatments led to the evolution of distinct mechanism to cope with BAC induced redox stress. If BAC is present

during the growth phase, it selects for active redox regulation. In contrast, the accumulation of redox species is avoided if BAC is present in the lag phase only.

The findings of this study have implications for the design and interpretation of adaptive laboratory evolution experiments that aim to assess potential adaptive mechanisms upon exposure to antimicrobials. The design of the evolution experiment needs to be aligned to the expected selection pressure exerted by the antimicrobial on the microorganisms. For example, if one aims to determine adaptive mechanisms to a lethal dose of a disinfectant, one should expose cells to a lethal dose [117]. An evolution experiment with sublethal concentrations might not be suitable to determine specific adaptive mechanisms to lethal doses because our experiment showed that growth in the presence of the disinfectant selected for distinct mechanisms. Such growth in the presence of an active substance is unexpected during the process of disinfection. In contrast, if one aims to determine adaptive mechanisms to a diluted disinfectant in the environment, one should determine whether the relevant environmental concentration reduces the growth rate or leads to partial killing. If the relevant environmental concentration does not induce partial killing, the concentrations in the evolution experiment should be adjusted to a non-lethal dose because our experiment showed that evolution for survival selects for distinct adaptive mechanisms. In conclusion, our work highlights the importance of fine tuning the selective pressures present in adaptive laboratory evolution experiments to the relevant exposure scenarios.

Supplementary information

Experimental conditions

There are different BAC concentrations present in the dataset used in the genotypical analysis of the *E. coli* evolved lines. The evolutionary treatment conducted in this serial transfer evolution experiment had two treatments with subinhibitory BAC concentrations, either selecting for survival and growth (SG) or survival only (S). In both treatments BAC is present at subinhibitory concentrations during the treatment phase and only in the SG treatment a prolonged exposure to subinhibitory BAC concentrations is given. In contrast, in the serial transfer evolution experiment conducted in another experiment [117] in our lab BAC was applied at lethal concentrations in the treatment phase and very low subinhibitory BAC concentrations were present during the growth phase.

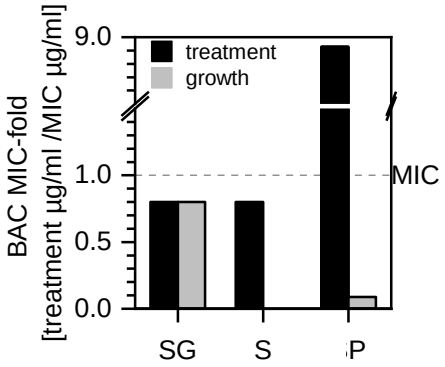

Fig S1 BAC exposure during the treatment and growth phase in the evolution experiments

Relative concentrations of BAC applied in the evolutionary treatments during the treatment and growth phase. Hereby the treatment concentration divided by the minimum inhibitory concentration (MIC) measured with the *E. coli* ancestor. The dotted line is at the level of the MIC of the *E. coli* ancestor.

Genotypical analysis

The genotypical analysis showed differences in the number of mutations occurring in genes or in intergenic regions. Further on these occurring mutations can be clustered for specific functions using BIOCYC GO Terms.

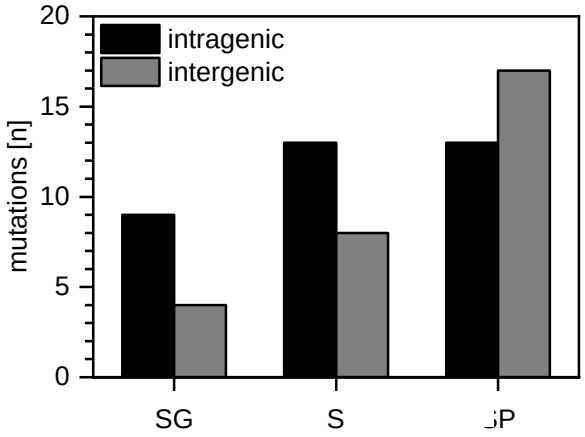

Fig S2 Number of mutations in the intra- or intergenic area for the various evolutionary treatments.

Number of mutated loci (intragenic and intergenic) with a 5% frequency cut off.

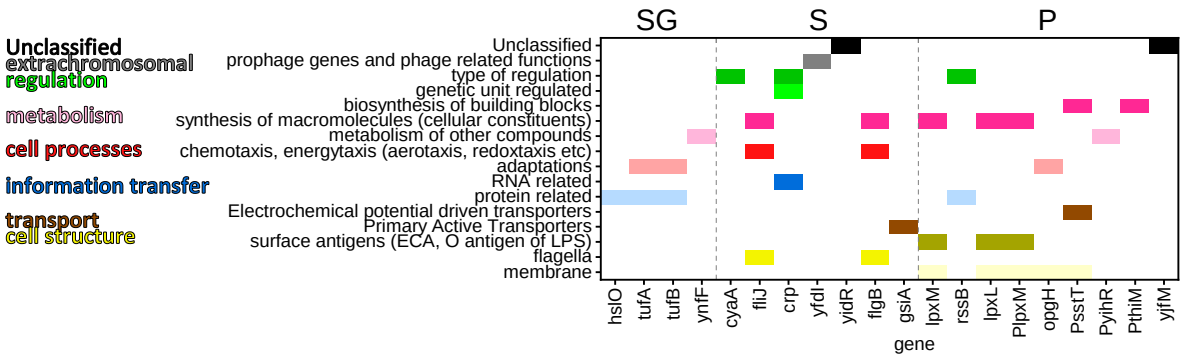

Fig S3 Mutations in the evolved populations with various BAC treatments clustered by functions.

Mutations occurring in the various treatment with a 10% frequency cut off were clustered and categorized using BIOCYC GO Terms (main and sub category). Hereby a colour code has been applied based on the main category. The dotted lines divides the different evolutionary treatments.

Chapter 4: Discussion and concluding remarks

This thesis mainly focuses on the effect of biocides on mechanisms of antimicrobial resistance evolution. Hereby a range of biocides used in various applications has been selected and used in several experiments e.g., chlorhexidine digluconate which is used as disinfectant in clinical settings [49] or permethrin which is used as wood preservative or insecticide in outside environments [306]. The selection of these biocides was based on their representation of diverse chemical classes with distinct chemical properties and their usage in diverse environments, in which biocides may come into contact with natural microbial communities. Additionally, factors such as the ECHA admission period for usage, their frequency of application and their presence in the environment as contaminants was considered. Our goal was to evaluate the impact of the selected biocides on evolutionary resistance mechanisms through laboratory experiments using different bacterial species: i) *E. coli* as model laboratory organism, ii) *A. baylyi* and iii) *B. subtilis* as model soil microorganisms. By using *E. coli* as model organism the observed effects can be explained using existing literature. Furthermore, the relevance of observed effects is enhanced by comparing the observations of *E. coli* with other microorganisms that naturally occur in the environment, such as soil model microorganisms.

In this thesis the evolutionary resistance mechanism of *de novo* mutation, horizontal gene transfer via conjugation and transformation, and the impact of the selection pressure were studied. The studied mechanisms are important processes in resistance evolution and thus might contribute to the global health threat of antimicrobial resistance. Therefore, it is essential to assess the effect of biocides on the various mechanisms of resistance evolution.

In chapter 2, the selected biocides were used at subinhibitory concentrations in a fluctuation assay for mutation rates, in a conjugation assay for plasmid transfer rates and in a transformation assay for transformation frequency. Notably, some biocides affected the tested evolutionary resistance mechanisms in a species and substance-dependent manner e.g., subinhibitory DDAC concentrations increase mutation rates (Chapter 2 Fig. 1) and subinhibitory BAC concentration increase the relative plasmid transfer rate in E. coli (Chapter 2 Fig. 2).

In chapter 3, the effect of the BAC selection pressure on the mechanisms of biocide resistance evolution was investigated. To this end, serial subculture evolution experiments were conducted applying two distinct subinhibitory BAC treatments; one which selects for survival only by applying BAC only during an initial survival phase and second treatment for survival and growth by applying BAC in an initial survival phase with continued BAC presence during the growth phase. At the end of the evolution experiment, the evolved phenotypes and

genotypes showed treatment specific adaptions (Chapter 3 Fig. 2 and Fig. 3). Specifically, the evolved genotypes undermined that the survival and growth treatment selected for adaptations to survive and grow in the presence of BAC, while the survival only treatment selected for adaptations to only survive a transient BAC stress.

In summary, we could show in experiments conducted in this thesis that biocides can influence various mechanism of resistance evolution in some of the selected microorganisms. However, it is crucial to acknowledge that the evolved resistance mechanism might be specific and based on the exerted selection pressure. In the following section, the potential mechanisms underlying these observed effects, the relevance of the findings for the environment and human health, as well as implications and future research will be discussed.

Currently, several questions remain on the specific mechanisms underlying the observed effects of the various biocides on the evolutionary mechanisms. Some biocides that affected the mutation rate and HGT activated the expression of the gene encoding for the general stress regulator *rpoS* and the RecA-linked SOS response, providing a plausible explanation for the observed effects. However, this explanation did not account for all the used biocides that affected the mutation rate and HGT, suggesting the involvement of other mechanisms. BAC selected in the serial transfer evolution experiment for selection regime specific adaptations, and the selected and fixed mutations can be broadly classified as related to stress regulation in E. coli. Consequently, the observed effects of biocides on resistance evolution mechanisms in the various experiments can be partially attributed to stress response and regulatory mechanisms. The literature also highlights the role of stress response mechanisms since it was shown that the bacterial stress response and regulation serves as a basis for survival and adaptation [126,127]. In this thesis, stress regulatory response mechanisms were predominantly observed, in contrast to other studies that have reported biocide adaptation through various mechanisms such as increased efflux or decreased influx, membrane change or biofilm formation [109]. A variety of microbial response mechanisms to biocides can be expected since biocide often act unspecific at multiple target sites [61] in contrast to antibiotics which typically have a primary target site [307].

The variety of response mechanisms to biocides are undermined by our experimental results. Despite using biocides with similar mode of actions, obtained different results indicating either affecting or not affecting resistance mechanisms. For instance, the surface-active cationic antimicrobials chlorhexidine digluconate (CHX), a bisguanide, as well as BAC and DDAC, which are QACs, have been used. CHX and BAC were affecting mutation rate and HGT via conjugation, while DDAC did not affect either of these mechanisms. While QACs in general share a similar mode of action by interacting with microbial membranes, the antimicrobial

efficacy of QACs differ based on their chemical structure parameters such as composition and alkyl chain length [48,53]. The structural and thus antimicrobial efficacy difference might explained the observed effect, however it is more likely that the observed effects of the biocides might be unrelated to the observed effect on mutation rates and HGT mechanisms.

These observed effects suggest that there are various microbial response mechanisms to biocides and thus the specific biocide impact and the specific response mechanisms might be difficult to predict.

Furthermore, not only the type and composition of a biocide can affect microbial resistance mechanisms but also their concentration and dynamics associated with the concentration may affect the development of resistance. An illustrative example for such concentration dynamics is the inoculum effect of antimicrobials, which describes the effect that the efficacy of an antimicrobial is affected by the cell density or population size [286]. This effect is observed in a range of antimicrobials such as antimicrobial peptides [290], antibiotics [284,286] and biocides [287]. The inoculum effect might serve as a basis for mechanisms of resistance evolution since it can cause a heterogeneity in killing and thus a variety of survival mechanisms can arise [308]. Additionally, the applied concentration of a biocide is crucial, as illustrated by the concentration threshold observed in biocides such as DDAC, for which lower concentrations exhibit a bacteriostatic effect, while higher concentrations exhibit a bactericidal effect [309]. Furthermore, an exposure of a microbial population to a non-lethal concentration might enable the survival at higher concentrations. Such effects were reported for BAC. On the one hand, the exposure to low BAC concentration led to less microbial damage and increased survival at higher BAC concentrations [310]. On the other hand, exposure to subinhibitory concentrations led to the evolution of stable cross-resistance to antibiotics [311,312]. Similar effects were observed with antibiotics at environmentally relevant subinhibitory concentrations, at which antibiotic resistance evolved [313]. Thus, this highlights the potential of low or subinhibitory antimicrobial concentrations to drive resistance evolution in microorganisms.

Such concentration dependent effects may occur in various environments and settings, where a range of antimicrobials in broad concentration range might be present. These concentrations vary based on the diverse applications of biocides and diverse entry pathways of biocides into the environment including direct emission and indirectly via waters coming from sewage treatment [75,314]. High antimicrobial concentrations can be found in clinical settings [222] or urban constructions [315], while low antimicrobial concentrations are for example associated with waste water treatment plant effluents [316].

There are numerous settings where biocides can be introduced into the environment and subsequently get into contact with natural microbial communities. One example of such setting is wood-based constructions i.e. fences in the outdoor environment, which are constantly exposed to various stresses such as weathering and biodegradation. Material preservatives are applied to protect these exposed materials against decay. However, during application and through weathering, runoff events can introduce the active substances into the environment and thus expose natural microbial communities [70]. These microbial communities are now exposed to a range of concentrations of the active substances. Depending on the active substance used in the material preservative, these substances might have different chemical properties thus affecting the substance lifetime in the soil, mobility, and interaction with microorganisms. Our research demonstrated that active substances being used in material preservatives can affect microbial mechanisms underlying resistance evolution. Thus, these active substances being present in this environment might facilitate the spread and evolution of resistance.

Another example of a setting is a wastewater treatment plant. A critical environment, where a range of antimicrobial substances at varying concentrations meet a diverse and fluctuating microbial community. These wastewater treatment plants receive various inflows, with hospital sewage often containing high levels of antibiotic and biocide residues and microorganisms that were already exposed to these antimicrobials [222,317]. Thus, in the waste water treatment plant antimicrobials such as antibiotics and biocides are present together with various microorganisms and also resistance genes [212]. It was already shown in the literature that waste water treatment plants are hotspots for the selection and transmission of antimicrobial resistance [318,319]. Additionally, the results of this thesis also shows that biocides at concentrations that occur in waste water treatment plants affect various mechanisms underlying mechanisms of resistance evolution. Thus, especially in those environments where a range of concentration of antimicrobials are present together with various microorganism, a range of antimicrobial response mechanisms might be selected, thus leading to a variety of resistance mechanisms being selected and facilitated. [320,321]

There are numerous other examples in distinct settings that demonstrate that there is an effect of antimicrobial substances being introduced into the environment having consequences on microbial communities and potential implications for human health in the context of resistance mechanisms [322]. Those risks are particularly pronounced in settings where selection pressures by antimicrobials and a diversity of microorganisms might be present, such as clinical settings or wastewater treatment plants.

It has been predicted that by 2050 antimicrobial resistance might be one of the major contributors to annual deaths [1,3,323]. Thus, contributors to increases in antimicrobial resistance have to be identified and monitored, and stewardship programs need to be implemented [6,314]; especially in settings where antimicrobial usage and antimicrobial resistance or antimicrobial resistant microorganisms come directly into contact with humans such as clinical, community or public facilities [324]. Humans are already exposed to antimicrobial resistance genes in the environment [325], increasing the risk of infection with antimicrobial resistant microorganisms [326,327]. Furthermore, humans can be exposed to disinfectant resistant microorganisms when contamination of disinfectants occurs [328]. Thus, disinfectants can be an additional source of infections and outbreaks in humans [329,330]. Those healthcare associated infections and outbreaks causes deaths and increased health care cost [331].

A cruical step forward is the continued investigation of associated risks with antimicrobial usage. However, it is essential to always consider and reflect on any possible limitations. Our study had certain experimental limitations associated to biocide concentrations. We could only use a narrow concentration windows and thus our results are limited with respect to the broad range of concentrations observed in the environment. The selected concentrations in this thesis can be found for some biocides in certain settings in the environment but might not always display the real environmental conditions as microorganisms are exposed to a broader range of concentration and thus, a range of responses might occur in the environment. Furthermore, when very low concentrations are used in an experimental setup there might be no effects observed immediately or effects of the experimental procedure might mask the responses imposed by the low concentrations. Similarly, for very high concentrations used in experiments it might be hard to disentangle the effect of the tested antimicrobial on the microorganism when there are lethal effects and thus, a range of survival mechanisms are triggered. Nonetheless, our experiments demonstrates that selection pressure is a key element when it comes to adaptation and the mechanisms underlying resistance. While high selection pressure might select for specific resistance mechanisms, low selection pressure can select for various evolutionary pathways. In our studies, we found that there are distinct adaptations when selection was focused solely on survival compared to selection focused on both survival and growth. The selection for survival only can be encountered in environments where microorganisms need to withstand a lethal stress for a limited time which can be found in various environments such as in clinical environments during disinfection or batch treatment in life stock industry.

Future studies should be carefully designed by considering that various factors might affect the observed effects and outcomes. For instance, it was already shown that the effect of

biocides might differ based on the selected culture medium and culturing vessel used [280]. Additionally, future studies with experimental designs more representative of environmental conditions, using environmental isolates or natural microbial communities will provide additional data to generalize the findings presented in this thesis and enable a comprehensive risk assessment of the processes by which biocides affect antimicrobial resistance evolution.

Overall, biocides have the potential to impact human health, particularly in connection to the threat of antimicrobial resistance. Consequently, biocides should be used only when necessary, in a strictly controlled and limited manner. This highlights the need for efforts to mitigate the risk associated with antimicrobial substances, antimicrobial resistance genes and antimicrobial resistant microorganisms for both human and environmental safety. Therefore, monitoring human exposure to antimicrobials, antimicrobial emission into the environment and implementing appropriate stewardship programs is crucial. Simultaneously, public awareness and understanding should be actively promoted to encourage the sustainable use of consumer products containing biocides [332]. It is hoped that this dissertation has provided a better understanding of the effects of biocides on processes underlying resistance evolution, with the overall goal of aiding the establishment of more sustainable and safe use of antimicrobial substances.

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List of publications and author contributions

This thesis is based on the following manuscripts:

Chapter 2: Effect of biocides on mutation rates and HGT is based on the manuscript

Schmidt, S. B., Rodriguez-Rojas, A., Rolff, J., & Schreiber, F. (2022). Biocides used as material preservatives modify rates of *de novo* mutation and horizontal gene transfer in bacteria. Journal of Hazardous Materials, 437, 129280. DOI: 10.1016/j.jhazmat.2022.129280

Author contribution: Selina Schmidt: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Review & Editing, Visualization; **Alexandro Rodríguez-Rojas:** Resources, Writing – Review & Editing; **Jens Rolff**: Supervision, Writing – Review & Editing; **Frank Schreiber**: Conceptualization, Methodology, Writing – Original Draft, Review & Editing, Supervision, Project administration, Funding acquisition.

I performed all experimental work, data analysis, data visualization reported in this manuscript including growth assays, antimicrobial susceptibility testing, mutation rate assays, conjugation rate assay, transformation rate assays and reporter gene assays. The manuscript was written together with Frank Schreiber.

Chapter 3: Effect of biocides selection pressures on evolution of resistance is based on the draft manuscript

Schmidt, S. B., Täschner, T., Nordholt, N., & Schreiber, F. (2022). Selection for survival and for growth in adaptive laboratory evolution experiments with benzalkonium chloride. In preparation for Evolutionary Applications

Author contribution: Selina Schmidt: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Review & Editing, Visualization; **Tom Täschner**: Formal analysis, Investigation, Writing - Review & Editing; **Niclas Nordholt**: Resources, Methodology, Software, Writing - Review & Editing; **Frank Schreiber**: Conceptualization, Formal analysis, Writing - Original Draft, Review & Editing, Supervision, Project administration, Funding acquisition.

I supervised the experimental work of the M.Sc. thesis of Tom Täschner (Title: Phenotypic and evolutionary adaption of bacteria to biocide stress, 2022; Biotechnology, Berlin University of Applied Sciences and Technology) related to conducting the evolution experiment and initial phenotypic testing of the ancestor and the evolved populations. I performed further phenotypic growth assays, antimicrobial susceptibility testing, population genomic sequencing, sequence data analysis, integrative data analysis, and data visualization. The manuscript was written together with Frank Schreiber

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Curriculum Vitae

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Education

Promotion - Graduate program "Biodiversity, Evolution & Ecology" **Mechanisms of biocide resistance evolution,** Supervision: Dr. Frank Schreiber, Prof. Jens Rolff, Prof. Angus Buckling, Prof. Martin Ackermann Federal institute for material research and testing and Free University Berlin	2023
Master of Science – Biology **Biocide impact on the persister phenotype of E. coli,** Master thesis, Specialization in Microbiology, Supervision: Dr. Frank Schreiber, Prof. Rupert Mutzel, Federal institute for material research and testing and Free University Berlin	2017
Bachelor of Science – Biology **Characterization of Schizophyllum commune thin 1 mutants**, Bachelor thesis, Specialization in Microbiology, Supervision: Dr. Sophia Wirth, Prof. Erika Kothe Institute for microbial communication, FSU Jena	2015
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Conferences and presentations

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World microbe forum online poster presentation "Biocides Used as Material Preservatives Modify Rates of De Novo Mutation And Horizontal Gene Transfer in Bacteria"	2021
4th Evo Eco PhD Meeting in Wittenberg talk same title as ISME17	2020
DRS Doktorandensymposium 2019 in Berlin poster presentation same title as ISME17	2019
FEMS 2019 (8th Congress of European Microbiologists) **in Glasgow** poster presentation same title as ISME17	2019
EDAR 2019 (5th International Symposium on the Environmental Dimension of Antibiotic Resistance), **in Hong Kong** poster presentation same title as ISME17	2019
ISME17 (17th International Symposium on Microbial Ecology) **in Leipzig** poster presentation "Antimicrobial resistance in soil microbes mediated by resistance evolution and horizontal gene transfer (HGT)"	2018
BAM PhD Day in Berlin poster presentation "Priming in soil microbial communities mediated by biocide-induced horizontal gene-transfer"	2018
International CRC 973 Symposium in Berlin poster presentation "Priming in soil microbial communities mediated by biocide-induced horizontal gene-transfer"	2018

Publications and publications in preparation

Selection for survival and for growth in adaptive laboratory evolution experiments with benzalkonium chloride	2023
Schmidt, S. B., Rodríguez-Rojas, A., Rolff, J., & Schreiber, F. (2022). Biocides used as material preservatives modify rates of de novo mutation and horizontal gene transfer in bacteria. Journal of Hazardous Materials, 437, 129280.	2022
Nordholt, N., Kanaris, O., Schmidt, S. B., & Schreiber, F. (2021). Persistence against benzalkonium chloride promotes rapid evolution of tolerance during periodic disinfection. Nature Communications, 12(1), 6792.	2021