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Contribution of RND-type efflux pumps in reduced susceptibility to biocides in *Acinetobacter baumannii*

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Herr Dr. Paul G. Higgins
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Die in dieser Arbeit angegebenen Experimente sind nach entsprechender Anleitung durch Herrn Dr. Kai Lucaßen und Herrn Dr. Paul G. Higgins von mir selbst ausgeführt und ausgewertet worden.

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Abbreviations

BZK	Benzalkonium chloride
CHX	Chlorhexidine digluconate
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPI	Efflux pump inhibitor
ETH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GP	Glucoprotamin
LPS	Lipopolysaccharide
MFP	Membrane fusion protein
MHK	Minimale Hemmkonzentration
MIC	Minimal inhibitory concentration
NMP	1-(1-Napthylmethyl)-piperazine
OMF	Outer membrane factor
OCT	Octenidine dihydrochloride
PaβN	Phenylalanine-arginine β-naphthylamide
QAC	Quaternary ammonium compound
RNA	Ribonucleic acid
TRI	Triclosan
WHO	World Health Organization

Zusammenfassung in deutscher Sprache

Das gramnegative Bakterium *Acinetobacter baumannii* kann Resistenzen gegen alle gängigen Antibiotika entwickeln und ist ein wichtiger nosokomialer Krankheitserreger. *A. baumannii* verursacht vor allem Infektionen bei immungeschwächten oder schwerkranken Patienten und ist bekannt für Ausbrüche auf Intensivstationen. Zu den wichtigsten klinischen Krankheitsbildern gehören Blutstrominfektionen, Lungenentzündungen, Harnwegsinfektionen und Wundinfektionen. Infektionen mit multiresistenten Stämmen schränken die Behandlungsmöglichkeiten teilweise stark ein und stellen eine große klinische Herausforderung dar.

A. baumannii besitzt außerdem die Fähigkeit, über lange Zeit in medizinischen Einrichtungen zu persistieren. Die Persistenz auf Oberflächen in der Krankenhausumgebung begünstigt Weiterverbreitung sowie Ausbrüche des Erregers. Ein weiterer wichtiger Übertragungsweg sind auch kontaminierte Hände des Krankenhauspersonals. Eine wirksame Dekontamination mit Bioziden wie Flächendesinfektionsmitteln und Handantiseptika ist deshalb unerlässlich, um einer Übertragung des Krankheitserregers vorzubeugen. In zahlreichen Bakterienspezies, darunter auch *A. baumannii*, wurde jedoch neben Antibiotikaresistenzen auch eine verminderte Empfindlichkeit gegenüber Bioziden beschrieben.

Verschiedene intrinsische und erworbene Resistenzmechanismen tragen zu einer verminderten antimikrobiellen Empfindlichkeit in *A. baumannii* bei. Dazu gehören auch bakterielle Effluxpumpen, wobei Effluxpumpen vom RND-Typ („resistance-nodulation cell-division“) in gramnegativen Spezies von besonderer klinischer Bedeutung sind. RND-Effluxpumpen bestehen aus drei Untereinheiten, welche sich über innere Zellmembran, Zellwand und äußere Zellmembran erstrecken. Diese Effluxpumpen können eine große Bandbreite an unterschiedlichen Substraten, einschließlich Antibiotika und Bioziden, aktiv aus der Zelle entfernen.

Die Expression der RND-Effluxpumpen AdeABC und AdeIJK bei *A. baumannii* unterliegt der Kontrolle durch Regulatorgene. Das Regulatorsystem *adeRS* ist ein transkriptioneller Aktivator vom Operon *adeABC*, während das Regulatorgen *adeN* ein transkriptioneller Repressor von *adeIJK* ist. Bei *A. baumannii* wurde festgestellt, dass AdeABC und AdeIJK die minimale Hemmkonzentration (MHK) für die Biozide Benzalkoniumchlorid und Chlorhexidindigluconat erhöhen können. Der Einfluss von Effluxpumpen auf die Abtötungskinetik von Bioziden wurde jedoch noch nicht näher untersucht.

Ziel der vorliegenden Stude war es, diese Forschungslücke zu schließen und die Auswirkung von RND-Effluxpumpen nicht nur auf die Empfindlichkeit gegenüber in Krankenhäusern häufig genutzten Bioziden, sondern auch auf das zeitabhängige Überleben von *A. baumannii* unter Biozid-Exposition zu untersuchen.

Dazu untersuchten wir Laborstämme und klinische *A. baumannii* Isolate, die aufgrund von Efflux-hemmenden oder Efflux-fördernden Mutationen der Regulatorgene *adeRS* und *adeN* unterschiedliche Expressionsniveaus der RND-Effluxpumpen AdeABC bzw. AdeIJK aufweisen. Diese Regulatormutationen haben in früheren Studien zu einer veränderten Empfindlichkeit gegenüber verschiedenen Antibiotika geführt. Ein weiterer Aspekt unserer Studie sind Untersuchungen, ob diese Mutationen zusätzlich zu den beschriebenen Veränderungen der Antibiotikaempfindlichkeit auch einen Einfluss auf die Biozidempfindlichkeit und auf das Überleben der Organismen unter Biozidexposition haben.

Als Laborstämme verwendeten wir den Referenzstamm *A. baumannii* ATCC 19606, die Knockout-Mutante *A. baumannii* ATCC 19606 Δ*adeRS* mit fehlender Expression von *adeABC*, und die Knockout-Mutante *A. baumannii* ATCC 19606 Δ*adeN* mit erhöhter Expression von *adeIJK*. Daneben verwendeten wir klinische Isolatpaare, die isogene Mutanten von Effluxpumpen-Regulatorgenen darstellen. Das klinische Isolat MB-5 weist eine Mutation in *adeS* auf, die zu erhöhter *adeABC*-Expression im Vergleich zum Elternstamm MB-2 führt. In einem anderen Isolatenpaar weist das klinische Isolat MB-273 eine Mutation in *adeN* auf, die zu erhöhter *adeIJK*-Expression im Vergleich zum Elternstamm MB-271 führt.

In der vorliegenden Arbeit wurde die Empfindlichkeit der Stämme mittels Bestimmung der MHK im Mikrodilutionsverfahren untersucht sowie das Überleben in einem Keimabtötungskinetik-Versuch bestimmt. Hierzu wurden die im klinischen Alltag häufig eingesetzten Biozide Chlorhexidindigluconat, Benzalkoniumchlorid, Ethanol, Glucoprotamin, Octenidindihydrochlorid und Triclosan verwendet. Dabei zeigte sich, dass die Variation der Expressionsniveaus der Effluxpumpen mehr Einfluss auf die Keimabtötungskinetik als auf die Biozidempfindlichkeit hatte.

Hinsichtlich der Effluxpumpe AdeABC führte die Überexpression von *adeABC* im klinischen Isolat MB-5 lediglich zu einer zweifachen Erhöhung der MHK von Glucoprotamin und Triclosan. Die Keimabtötungskinetik zeigte jedoch zusätzlich eine verminderte Keimabtötung für Benzalkoniumchlorid, Chlorhexidindigluconat und Octenidindihydrochlorid. Die fehlende Expression von *adeABC* im Laborstamm 19606 Δ*adeRS* wiederum führte zu einer Verminderung der MHK von Benzalkoniumchlorid, Chlorhexidindigluconat und Glucoprotamin. Die Keimabtötungskinetik von diesem Stamm bestätigte eine erhöhte Keimabtötung für diese Biozide zu allen gemessenen Zeitpunkten. In der Keimabtötungskinetik zeigte sich ferner eine erhöhte Keimabtötung für Octenidindihydrochlorid zu frühen Zeitpunkten, was in der MHK-Messung wegen einer einzelnen Messung nach 20 h nicht gezeigt werden konnte.

Unsere Studie bestätigt demzufolge, dass Benzalkoniumchlorid und Chlorhexidindigluconat Substrate von AdeABC sind. Im Vergleich zu anderen Studien war der Einfluss auf die MHK allerdings geringer ausgeprägt, während sich ein deutlicherer Einfluss in der veränderten

Abtötungskinetik zeigte. Ferner zeigt unsere Studie, dass auch Glucoprotamin und Octenidindihydrochlorid - abhängig vom Bakterienstamm - Substrate von AdeABC sind.
Was die Effluxpumpe AdeIJK anbelangt, beeinflusste die Überexpression von *adeIJK* im klinischen Isolat MB-273 weder die Keimabtötungskinetik noch die Empfindlichkeitstestung für die von uns getesteten Biozide. Anders präsentierte sich die Situation im Laborstamm 19606 Δ*adeN*, wo die Überexpression von *adeIJK* zwar keinen Einfluss auf die MHK hatte, aber eine verminderten Keimabtötung zu früheren Zeitpunkten für die Biozide Benzalkoniumchlorid, Chlorhexidindigluconat und Octenidindihydrochlorid zur Folge hatte. Dies deutet darauf hin, dass AdeIJK in Abhängigkeit vom Bakterienstamm dazu beiträgt, dass *A. baumannii* die Exposition gegenüber diesen Bioziden besser überleben kann.
Für Triclosan zeigten die Resultate in der Keimabtötungskinetik eine höhere Variabilität, allerdings konnten wir keinen größeren Einfluss vom Expressionsniveau der Effluxpumpen auf das Überleben von *A. baumannii* unter Triclosan-Exposition feststellen. Was Ethanol anbelangt, hatte das Expressionsniveau der Effluxpumpen keinen merklichen Einfluss auf die bakterielle Empfindlichkeit und das bakterielle Überleben.
Die Biozidkonzentrationen, die wir im Keimabtötungskinetik-Versuch einsetzten, lagen zumeist deutlich unter den für den klinischen Gebrauch empfohlenen Konzentrationen. Es ist jedoch wahrscheinlich, dass solche niedrigeren Biozidkonzentrationen auch im klinischen Alltag häufiger auftreten, z.B. wenn Biozidprodukte durch Anwendung auf nassen Oberflächen verdünnt werden. Biozide werden ferner in niedrigen Konzentrationen als Konservierungsstoffe in zahlreichen Medizinprodukten sowie in Produkten für den alltäglichen Gebrauch eingesetzt. Durch den extensiven Einsatz von Bioziden konnten außerdem niedrige Konzentrationen oder Restkonzentrationen, etwa von Benzalkoniumchlorid, auch in Abwassersystemen von Krankenhäusern und in zahlreichen natürlichen Umgebungen nachgewiesen werden. Bakterien sind also sowohl in klinischen als auch in natürlichen Umgebungen häufiger niedrigen Biozidkonzentrationen ausgesetzt.
Unsere Daten zeigen insgesamt, dass RND-Effluxpumpen das Überleben von *A. baumannii* unter Biozidexposition verbessern und so auch die Persistenz von Effluxpumpen-exprimierenden Stämmen begünstigen können. Dies unterstreicht ferner die Wichtigkeit von sachgemäßer Biozidanwendung in den empfohlenen Konzentrationen, um die Persistenz von *A. baumannii* in der Krankenhausumgebung zu verhindern.

1. Abstract

Acinetobacter baumannii is an important Gram-negative nosocomial pathogen, known to develop resistance against all known antibiotics. The pathogen especially affects immunocompromised or critically ill patients and is infamous for causing outbreaks in intensive care units. Clinical manifestations include bacteremia, pneumonia, urinary tract infections or wound infections. Infections with multi-drug resistant strains limit the treatment options and pose a major clinical challenge. Importantly, *A. baumannii* shows a propensity to persist in the clinical environment. Persistence on hospital environmental surfaces facilitates cross-transmission and outbreaks of *A. baumannii*. Transmission further occurs via the hands of health care workers. To prevent the transmission of the pathogen, efficacious decontamination with biocides such as surface disinfectants and hand antiseptics is essential. However, in addition to antibiotic resistance, decreased susceptibility to biocides has been described in numerous bacterial species, including *A. baumannii*.

Reduced antimicrobial susceptibility in *A. baumannii* is mediated by different innate and acquired resistance mechanisms, including bacterial efflux pumps. Efflux pumps of the resistance-nodulation cell-division (RND) family are of major clinical relevance in Gram-negative bacteria. RND-type efflux pumps are composed of tripartite compounds that span the bacterial envelope. They actively extrude a wide range of unrelated compounds, including different antibiotics and biocides, from the bacterial cytoplasm or periplasm to the outside of the cell. In *A. baumannii*, the expression of RND efflux pumps AdeABC and AdeIJK is tightly regulated. The regulatory system *adeRS* acts as a transcriptional activator of *adeABC*, and *adeN* is a transcriptional repressor of *adeIJK*. AdeABC and AdeIJK have been found to cause increased minimal inhibitory concentrations (MICs) for the biocides benzalkonium chloride and chlorhexidine digluconate. However, the role of efflux on killing kinetics of biocides has not been thoroughly assessed.

To address this gap, this study investigated the impact of differential RND efflux pump expression not only on the susceptibility but also on the time-dependent survival of *A. baumannii* to biocides commonly used in the clinical setting.

To this end, we used laboratory and clinical *A. baumannii* strains with differential expression levels of either efflux pump AdeABC or AdeIJK due to mutations in their corresponding efflux pump regulator genes *adeRS* and *adeN*. These mutations have been shown to affect the susceptibility to various antibiotics in previous studies. A further characteristic of this study is that we investigated the impact of these mutations on biocide susceptibility and survival in addition to the previously reported antibiotic susceptibility changes. We used the laboratory reference strain *A. baumannii* ATCC 19606, its knockout mutant *A. baumannii* ATCC 19606 Δ*adeRS* with lack of *adeABC* expression, and knockout mutant *A. baumannii* ATCC 19606

Δ*adeN* with increased *adeIJK* expression. As clinical strains, we used isolate pairs that are isogenic mutants of efflux pump regulator genes. Clinical isolate MB-5 has a mutation in *adeS* leading to increased *adeABC* expression compared to parental strain MB-2. In another isolate pair, MB-273 has a mutation in *adeN* leading to increased *adeIJK* expression compared to parental strain MB-271. We measured the strains' susceptibility via MIC determination in broth microdilution and their survival in time-kill assays following exposure to different hospital disinfectants and antiseptics, i.e., benzalkonium chloride, chlorhexidine digluconate, ethanol, glucoprotamin, octenidine dihydrochloride, and triclosan.

The impact of efflux was more pronounced in killing kinetic assays than in susceptibility testing. Regarding efflux pump AdeABC, overexpression in the clinical isolate MB-5 merely caused a 2-fold MIC increase for glucoprotamin and triclosan. The killing kinetics additionally showed reduced killing by benzalkonium chloride, chlorhexidine digluconate and octenidine dihydrochloride. On the other hand, lack of *adeABC* expression in the laboratory knockout mutant 19606 Δ*adeRS* caused an MIC reduction for benzalkonium chloride, chlorhexidine digluconate and glucoprotamin. The killing kinetics of this strain confirmed increased killing by these biocides at all time points tested (0.5, 1, 3, 24 h). Killing kinetics further revealed increased killing by octenidine dihydrochloride at earlier time points, which the MIC measurement was unable to show due to a single measurement after 20 h. Our study thus confirms benzalkonium chloride and chlorhexidine digluconate as substrates of AdeABC, although the impact on MIC levels is lower than in previous studies but could more clearly be shown using bacterial time-kill studies. Our study further indicates that glucoprotamin and octenidine dihydrochloride are strain-dependent substrates of AdeABC. Regarding AdeIJK, the overexpression of the efflux pump in the clinical isolate did not have an impact on the killing kinetics nor on the susceptibility testing for any of the biocides tested. On the other hand, overexpression of AdeIJK in the laboratory knockout mutant 19606 Δ*adeN* did not cause any MIC changes but led to reduced killing at earlier time points when exposed to benzalkonium chloride, chlorhexidine digluconate and octenidine dihydrochloride. This indicates that AdeIJK can contribute to increased survival to these biocides in a strain-dependent manner.

For triclosan, the results in the time-kill assay showed a higher variability and may therefore not be conclusive. We did however not observe a major impact of efflux pump expression on bacterial survival following triclosan exposure. Regarding ethanol, efflux pump expression levels did not notably influence bacterial susceptibility or survival.

The biocide concentrations tested in the time-kill assay were considerably below the concentrations recommended for routine use in the clinical setting. However, low biocide concentrations are likely to occur in practice, for example when biocidal products are diluted due to application on wet surfaces or wet skin. Biocides are also used at low concentrations as preservatives in many clinical applications and consumer products. Low or residual

concentrations of biocides such as benzalkonium chloride are further found in hospital sewage water and in natural environments due to extensive biocide usage. Bacteria are thus commonly exposed to low biocide concentrations both in the clinical setting and in natural environments. Our data indicate that RND-type efflux pumps can contribute to improved survival of *A. baumannii* when exposed to hospital antiseptics and disinfectants and thus may favour the persistence of efflux-expressing *A. baumannii*. These data highlight the importance of appropriate biocide use at recommended concentrations to prevent the persistence of *A. baumannii* in the hospital environment.

2. Introduction

2.1. The genus *Acinetobacter*

The genus *Acinetobacter* belongs to the family of *Moraxellaceae* within the order of *Gammaproteobacteria* and is defined as a Gram-negative, strictly aerobic, nonfermenting, nonfastidious, nonmotile coccobacillus.[1,2] It was first described in 1911 by Beijerinck, who isolated the organism from soil and named it *Micrococcus calco-aceticus*.[3] In 1954, Brisou and Prévot introduced the current genus denomination *Acinetobacter* (ancient greek *akinetos*: nonmotile) to discriminate between nonmotile and motile organisms within the genus *Achromobacter*.[4] The genus *Acinetobacter* was officially acknowledged in 1971 after publication of an extensive phenotypical analysis by Baumann et al.[5,6] In 1986, 12 different species within the genus *Acinetobacter* were identified by Bouvet and Grimont by DNA-DNA-hybridisation studies.[7] Currently, 75 distinct species within the genus have been described.[8] Possibilities to discriminate between the species by classic phenotypic methods are limited,[2] such as for species within the *A. calcoaceticus* – *A. baumannii* complex, which comprises both pathogenic species (e.g., *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. dijkshoorniae*, *A. seifertii*) and non-pathogenic species (*A. calcoaceticus*).[9,10] Molecular methods for species identification among isolates of the genus *Acinetobacter* are therefore used in research and clinical settings, such as molecular DNA fingerprinting techniques,[11] matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS),[12] or polymerase chain reaction (PCR)-based methods.[13] *Acinetobacter* spp. are considered ubiquitous as they can be isolated from almost all soil and surface water samples and are also found on living organisms such as vegetables and animals.[14-16] Within a cardiology ward in the university hospital of Cologne, *Acinetobacter* spp. were isolated from skin and mucosa samples among 75% of the patients, whereas the colonisation rate was 43% within the healthy population, and the carriage rate of *A. baumannii* was below 1%.[17]

2.2. *Acinetobacter baumannii*

A. baumannii is the clinically most relevant pathogen within the genus *Acinetobacter*.[2] Over the last four decades, *A. baumannii* has emerged as a concerning nosocomial pathogen that may cause prolonged outbreaks in healthcare facilities on a global scale. *A. baumannii* is a member of the ESKAPE group, which comprises six multi-drug resistant bacterial pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) that are major causes of nosocomial infections.[18] *A. baumannii* shows a propensity to cause outbreaks within intensive care units, preferably affecting critically ill or immunocompromised patients.[2] Frequent clinical manifestations of *A. baumannii* infections consist of ventilator-associated pneumonia,

bloodstream infections, wound and soft tissues infections, meningitis, and urinary tract infections.[2]

A. baumannii possesses a multitude of innate and acquired resistance determinants and frequently shows a resistance profile against numerous antibiotics, such as carbapenems and other β-lactams, fluoroquinolones, aminoglycosides, chloramphenicol and tetracyclines.[2] The emergence of multidrug-resistant strains poses a real challenge for the treatment of *A. baumannii* infections, and even pandrug-resistant *A. baumannii* isolates have been reported.[19,20] Multidrug resistance is defined as non-susceptibility to at least one agent in a minimum of three antimicrobial classes, while pandrug resistance corresponds to non-susceptibility to all agents in all antimicrobial classes.[21] As carbapenems are among the first-line treatment options against *A. baumannii* infections, the increasing resistance rates to carbapenems are of particular concern.[22] Shortage of treatment options confers carbapenem-resistant *A. baumannii* critical priority in the World Health Organization (WHO) priority pathogens list for research and development of new antibiotics.[23]

Other factors that contribute to the persistence of *A. baumannii* in the hospital environment are its prolonged desiccation tolerance, as clinical strains can survive up to four weeks on dry surfaces,[24] and its ability to form biofilms on biotic and abiotic surfaces.[25] *A. baumannii* biofilms are more difficult to eradicate than planktonic bacteria and can form on medical devices such as central venous catheters, ventilation tubes, orthopaedic devices or prosthetic heart valves.[26] Contaminated hospital equipment and patient-near surfaces can contribute to the epidemic spread of the pathogen as they can serve as additional reservoirs alongside humans as primary reservoirs. This highlights that an effective environmental decontamination is an important measure to prevent and control outbreaks of *A. baumannii*.[2,27]

2.3. *A. baumannii* antimicrobial resistance mechanisms

Multidrug resistance profile of *A. baumannii* is caused by innate resistance mechanisms, by mutations in the existing genome, and by the capacity of rapidly acquiring new genetic material carrying resistance determinants.[2,28]

The acquisition of resistance mechanisms by horizontal gene transfer can give rise to veritable resistance islands, i.e., variable genomic regions that contain clusters of different antimicrobial resistance genes, but also resistance determinants against biocides and heavy metals.[29,30] Mobile genetic elements that contribute to antimicrobial resistance via horizontal gene transfer in *A. baumannii* include plasmids, integrons, transposons and insertion sequences.[30,31] Insertion sequences can mobilise resistance genes and activate downstream genes by affecting promoter activity.[32,33] Mobile genetic elements in *A. baumannii* that are relevant for biocide tolerance are further described in section 2.6.3.

Antibiotics act via disruption of different bacterial biosynthesis pathways such as cell wall, DNA, RNA or protein synthesis by targeting specific bacterial enzymes.[34] For example, beta-lactams inhibit cell wall synthesis via interacting with penicillin binding proteins, quinolones inhibit DNA replication by inhibiting the bacterial DNA gyrase and topoisomerase, and aminoglycosides inhibit protein biosynthesis via targeting the 30S subunit of bacterial ribosomes.[34] The mode of action of antibiotics can either be bacteriostatic, when bacterial growth is inhibited, or bactericidal, when bacterial cell death is caused.[34]

Resistance against every class of antibiotics has been reported in *A. baumannii*.[19] Antibiotic resistance mechanisms in *A. baumannii* include enzymatic degradation of antimicrobials, target site modification, decreased membrane permeability, and active efflux.

Enzymatic degradation of beta-lactam antibiotics, including carbapenems, by beta-lactamases forms the main resistance mechanism to this antibiotic class in *A. baumannii*.[22] Oxacillinases belong to Ambler class D beta-lactamases and are carbapenem-hydrolysing enzymes that play a major role in conferring carbapenem resistance in *A. baumannii*, such as the acquired oxacillinases OXA-23-like (the most frequent one), OXA-40-like, OXA-51-like, OXA-58-like, OXA-143-like, and OXA-235-like.[22,35] Aminoglycoside-modifying enzymes, such as acetyltransferases, nucleotidyltransferases and phosphotransferases are widely present among MDR *A. baumannii* and often encoded on integrons.[36]

Target site modifications also play an important role in antibiotic resistance in *A. baumannii*. Ribosomal RNA methylation confers high-level aminoglycoside resistance by preventing binding to the ribosomal target site.[37] Quinolone resistance is mediated by modifications in bacterial DNA gyrase or topoisomerase IV which impair antibiotic binding,[38] and modifications in the expression of penicillin-binding proteins can contribute to beta-lactam resistance.[39]

While degradation enzymes and modifications of the target site affect specific antibiotic classes or single compounds, other mechanisms such as decreased outer membrane permeability and efflux overexpression can simultaneously confer resistance to multiple antimicrobial classes.[40] Size and composition of outer membrane porin channels affect the diffusion of substances, including antimicrobials, across the outer membrane.[40] *Acinetobacter* spp. intrinsically possess a relatively low outer membrane permeability (less than 5% in comparison to *E. coli*) as a result of a small number of small-sized outer membrane porins.[41] Decreased expression or loss of the porins CarO or OprD in *A. baumannii* is further associated with carbapenem resistance via reduced membrane permeability for these compounds.[42]

Efflux pumps notably contribute to multidrug resistance in *A. baumannii*, as they can actively extrude a wide number of structurally unrelated antimicrobial classes, including antibiotics such as aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, tetracyclines and tigecycline, and also different dyes and biocides.[43-46] Our work will focus on efflux pumps

as mediators of biocide tolerance in *A. baumannii*. Bacterial tolerance mechanisms to biocides are described more in detail in section 2.6.

2.4. Bacterial efflux pumps

2.4.1. Role and structure

Bacterial efflux pumps are chromosomally encoded or plasmid-borne components of the cell membrane ubiquitous in Gram-negative bacteria, where they were first described in 1993.[47] They actively extrude metabolic end products and deleterious substances from intra- to extracellular space and contribute to cell survival in a harmful environment by maintaining cell homeostasis.[47-49] In addition, efflux pumps have been shown to play a role in bacterial stress responses, fitness and virulence.[47] This indicates that efflux pumps achieve ancient physiological functions and have been essential components of bacterial cells even before the appearance of strong antibiotic selection pressure in the last decades.[47,49] Efflux pump substrates include structurally diverse compounds such as bile salts, solvents, dyes, detergents, as well as biocides and antibiotics.[49] From a clinical perspective, bacterial efflux pumps play an important role in antimicrobial resistance, as they enable bacteria to survive high concentrations of diverse antimicrobial agents. Moreover, efflux pumps can interact in a synergistic way with other resistance mechanisms to confer higher resistance levels.[47]

According to their structure and their primary energy source, bacterial efflux pumps can be classified into distinct families.[47] The major families of bacterial efflux pumps are the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the more recently described proteobacterial antimicrobial compound efflux (PACE) family, and the resistance nodulation-cell division (RND) family [Figure 1].[47,50]

The ABC transporter superfamily uses free energy from ATP hydrolysis to catalyse efflux, whereas the other transporter families utilize proton motive force as energy source and function thus as proton/drug antiporters or secondary transporters.[47,51] RND efflux pumps form tripartite complexes that span the inner membrane, the periplasm and the outer cell membrane and extrude compounds directly to the outside of the cell.[47] Some efflux pumps from the ABC, MATE and MFS superfamilies are organised in a similar manner. Other efflux pumps, such as most MFS and SMR pumps, consist of a single transporter located in the inner, cytoplasmic membrane, which extrudes compounds from cytoplasm to the periplasmic space, where lipophilic molecules can passively diffuse back to the cytoplasm via the inner membrane.[47] This makes single-compound pumps less effective than tripartite pumps. Their efficacy can however be increased by interplay with RND pumps to extrude substrates from the periplasm to the outside of the cell.[47,52]

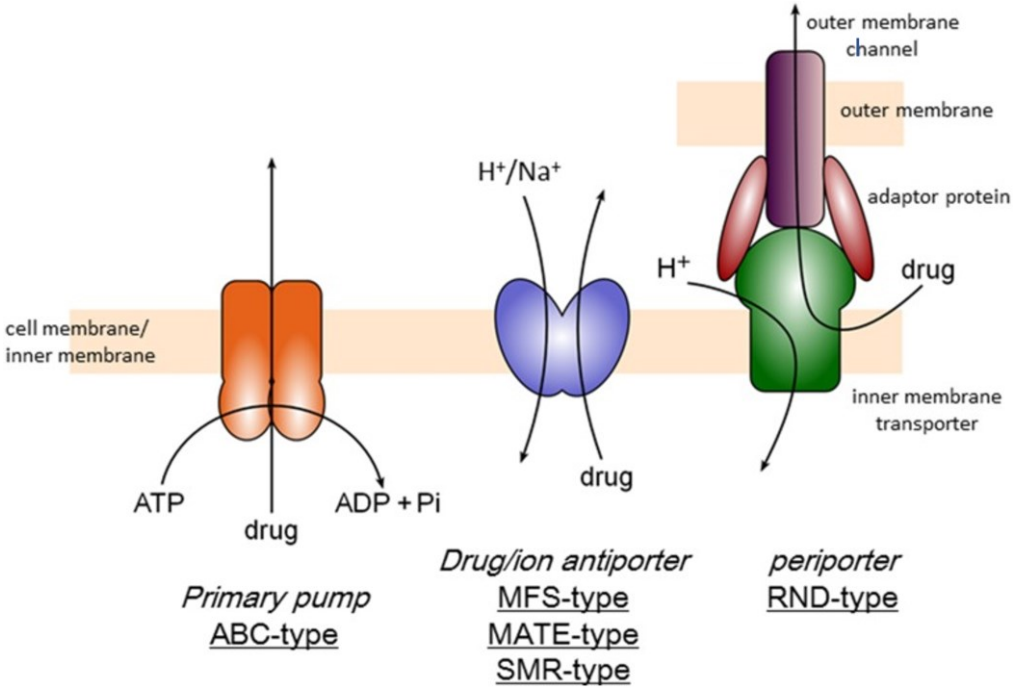

Figure 1. Classification and schematic overview of efflux transporters in Gram-negative bacteria. Figure taken from Yamaguchi et al, Frontiers in Microbiology.[53]

2.4.2. RND-type efflux pumps in *A. baumannii*

RND-type efflux pumps are among the clinically most relevant efflux superfamily in Gram-negative bacteria and show the broadest range of substrates, including multiple antibiotics, biocides, detergents, bile salts and dyes.[49] RND-type efflux pumps form a tripartite complex composed of an inner membrane transporter responsible for substrate recognition and transport of substrates from cytoplasm to the periplasmic space,[54,55] an outer membrane factor (OMF) that provides a channel for the substrate to cross the outer membrane,[56] and an adaptor protein resp. membrane fusion protein (MFP) enabling coupling of reactions separated in the two different membranes [Figure 1].[57,58] The pumps extrude substrates from the cytoplasm or periplasm directly to the extracellular environment, efficiently lowering the intracellular concentrations of toxic compounds. This feature explains the contribution of tripartite pumps to multidrug resistance, as extruded drug compounds must re-enter the cell via the poorly permeable bacterial outer membrane.[47,48]

In *A. baumannii*, three main RND-type efflux pumps, AdeABC (Acinetobacter drug efflux), AdeFGH and AdeIJK, have been characterised so far. AdeABC was the first tripartite RND efflux pump described in *A. baumannii,* in which the *adeABC* operon encodes the MFP AdeA, the inner membrane transporter AdeB and the OMF AdeC.[43] AdeABC does not account for intrinsic resistance in *A. baumannii*.[59] However, upon overexpression, AdeABC extrudes antimicrobials of various classes such as β-lactams, aminoglycosides, fluoroquinolones,

tetracyclines, tigecycline, macrolides-lincosamides and chloramphenicol, thus contributing to multidrug resistance.[43,59] Contribution of AdeABC to carbapenem resistance is controversial. While carbapenems have been shown to be substrates of AdeABC, some studies suggest that carbapenem efflux by AdeABC causes decreased carbapenem susceptibility in synergy with carbapenemases,[59,60] whereas others report that AdeABC efflux does not confer increased resistance to carbapenems.[61,62] AdeABC has further been shown to efflux biocides such as chlorhexidine digluconate and benzalkonium chloride.[63]

AdeIJK is the major constitutively expressed RND pump in *A. baumannii*.[45] The MFP, the inner membrane transporter and the OMF are encoded by *adeI*, *adeJ* and *adeK*, respectively. AdeIJK contributes to intrinsic resistance in *A. baumannii* and effluxes many antimicrobial classes, overlapping with the AdeABC spectrum, including β-lactams, chloramphenicol, fluoroquinolones, erythromycin, lincosamides, tetracyclines and tigecycline, and has also been suggested to extrude the biocides chlorhexidine digluconate and benzalkonium chloride.[45,63]

The third RND-type efflux pump in *A. baumannii*, AdeFGH, was described in 2010. The MFP AdeF, the transporter AdeG and the OMP AdeH are encoded by the *adeFGH* operon.[44] AdeFGH can extrude chloramphenicol, fluoroquinolones, trimethoprim, cotrimoxazole, tetracyclines and tigecycline, and has been suggested to contribute to resistance upon overexpression, although at a minor level than the other RND efflux pumps.[44,64] On the other hand, there are also reports about downregulation of AdeFGH upon exposure to meropenem or tigecycline.[64,65]

RND-type efflux pumps in Gram-negative bacteria are tightly regulated.[66] In *A. baumannii*, expression of efflux pump AdeABC is regulated by the two-component regulatory system AdeRS. AdeS corresponds to a sensor kinase which detects environmental changes and activates AdeR, the transcriptional response regulator of the efflux pump AdeABC.[67] The *adeRS* complex lies directly upstream of the *adeABC* operon and acts as trancriptional activator, as the inactivation of *adeRS* leads to lack of AdeABC expression and to increased antimicrobial susceptibility.[67,68] In *A. baumannii* ATCC 19606, deletion of *adeRS* inhibits *adeB* expression and leads to increased susceptibility to aminoglycosides, macrolides, fluoroquinolones, chloramphenicol, rifampicin, tetracyclines and glycylcyclines.[69] AdeIJK is regulated by the TetR transcriptional repressor AdeN.[70] AdeFGH is regulated by the LysR-type transcriptional regulator AdeL.[44]

Mutations in efflux pump regulatory pathways can induce efflux pump overexpression and decreased antimicrobial susceptibility.[66] In *A. baumannii*, overexpression of efflux pump transporter gene *adeB* can result from disruption by insertion sequences and from distinct amino acid substitution mutations in *adeS* or in *adeR*, subsequently leading to antimicrobial resistance.[71-74] In particular, insertion of IS*Aba1* in *adeS* has repeatedly been reported to cause *adeB* overexpression and reduce tigecycline susceptibility.[75,76] Concerning the efflux pump

AdeIJK, disruption of *adeN* by IS*Aba1* or by IS*Aba125* leads to increased *adeJ* expression and reduced susceptibility to tigecycline in clinical isolates.[73,76] In *A. baumannii* ATCC 19606, deletion of *adeN* provokes overexpression of AdeIJK and decreased susceptibility to various antibiotics.[77] In AdeFGH, mutations in *adeL* have been suggested to lead to efflux pump overexpression and reduced antibiotic susceptibility under in vitro conditions, but not in a study involving clinical strains.[44,71]

2.5. Biocides

2.5.1. Terminology

According to the Biocides Directive (98/8/EC) of the European Parliament and Council, biocidal products are intended to "destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means".[78] This definition is very broad and includes fungicides, pesticides, herbicides and rodenticides, along with preservatives, disinfectants and antiseptics. In this work and in most cited publications, the term 'biocide' refers to chemical compounds that kill or inhibit the growth of microorganisms but does not include antibiotics, and comprises disinfectants, antiseptics, and preservatives. While disinfectants are substances used to kill microorganisms on inanimate surfaces, antiseptics are used to reduce microorganisms on living organisms, such as on human skin and mucous tissues and wounds, whereas preservatives are added to consumer products to prevent the growth of microorganisms.[78] In contrast, antibiotics are used to control the growth or kill microorganisms in humans or animals, although in the clinical context, the term 'antibiotic' is employed for substances that tackle bacterial infections specifically.[78,79] In our work, such as in other works dealing with biocides as opposed to antibiotics, the term 'antibiotic' comprises both "naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria".[80] The term 'antimicrobial' is used as umbrella term comprising both biocides and antibiotics.[78]

2.5.2. Mechanisms of action of biocides

Biocidal mechanisms of action are diverse and often involve different target sites within one pathogen.[80,81] Different mechanisms usually act synergistically, the biocidal effect being determined by the relevance of target sites for the pathogen's survival, and the overall damage inflicted to the cell.[81] Contrary to lethal effects reached by higher concentrations of biocides, lower concentrations can cause an inhibitory effect on pathogens which might translate in a reversible or sublethal damage of the cells.[82,83] Biocidal action likely represents a sequence of cell surface interaction, cell penetration, and interaction with intracellular target sites. This rather unspecific mode of action is in contrast with that of many antibiotics, which often focus on one specific cell target.[80]

Several biocides can act on the cell wall and on the bacterial outer membrane in Gram-negative bacteria. For example, glutaraldehyde induces cross-linking of proteins in the cell envelope, whereas permeabilising agents increase the permeability of the outer membrane in Gram-negative bacteria by releasing lipopolysaccharides (LPS).[82]

Biocides can damage the bacterial inner cytoplasmic membrane via membrane disruption with leakage or coagulation of intracellular components, via inhibiting the proton-motive force of the membrane, or via targeting membrane-associated enzymes.[82] Further modes of action of biocides on an intracellular level include cross-linking of proteins within the cell, interaction with or oxidisation of thiol groups, oxidative damage to amino acids and DNA, inhibition of DNA and RNA synthesis, DNA strand break, and interaction with ribosomes.[80,82] Autolytic processes triggered by accumulation of cell damage also contribute to the biocidal effect.[82,83] Certain biocides, such as triclosan, have been reported to act on specific enzymatic target sites when used at inhibitory concentrations, contrary to the more general damage inflicted by higher, lethal concentrations.[82]

Mechanisms of actions of specific biocides tested in this study will be described more in detail in the following sections. Further, antimicrobial activity of biocides is dependent on various factors such as pH, temperature, presence of organic material, test method, biocide concentration, contact time, and formulation effects.[80,84] Factors related to the microorganisms such as the type and number of pathogens, or biofilm formation, also affect the efficacy of biocidal action.[84]

2.5.3. Biocide use and clinical relevance

Biocides have been used since antiquity for wound care or for preservation, and are nowadays essential decontamination tools in a multitude of applications and environments.[80,83,85] In the clinical setting, biocides are employed in hand hygiene as hand washes or rubs, for wound antisepsis in the form of irrigation solutions or impregnated on wound dressings, for pre-operative skin antisepsis, as well as for disinfection of surfaces and medical instruments. Biocides are further used for eradication of multidrug-resistant bacterial strains in healthcare personnel and patients.[85] Rigorous application of disinfection and antisepsis measures, in particular compliance with hand hygiene protocols, is a major determinant to prevent nosocomial transmission, hospital-acquired infections, and reduces patient morbidity and mortality.[86,87] Biocides play also an important role in eradicating *A. baumannii* from hospital environments.[2]

Besides their clinical use, biocidal products are also widely used in healthcare, food, water, and manufacturing industries.[81] They are essential for preservation of foodstuff and pharmaceutical products.[82] When used as preservatives, biocides are generally present in lower concentrations then when used for antisepsis or disinfection. Biocide use has been

increasing over the last decades and broadened to a larger number of applications, partly driven by an increase in antibiotic-resistant bacteria, and by a rising public awareness of hygiene and of microbial contamination.[82] In particular, biocides can be found in a growing number of consumer products, including soaps, mouthwashes, or lotions.[83]

Biocide use has further seen a sharp increase since the beginning of the SARS-CoV-2 pandemic in 2020. In 2020, global sales in surface disinfectants increased by 30% compared to the previous year, to reach a total of US$ 4.5 billion.[88] Hand hygiene has been identified as a crucial factor for infection control during the pandemic, and alcohol-based hand sanitisers have been recommended by the WHO to prevent the spread of SARS-CoV-2.[89] Hand hygiene measures have since been widely established in public and private settings.

While on the one hand enhanced disinfection and antiseptic measures remain an essential tool to contain infections and to interrupt the spread of contagious pathogens, the increasing use of biocides also raises concerns about possible detrimental effects.[90] Extensive or inappropriate biocide application may lead to new issues and challenges such as increased tolerance to biocidal products or development of cross-resistance to antibiotics.[83] Further, negative ecological implications such as contamination of ground water and ecosystems may occur.[90]

Biocidal agents frequently used in the healthcare sector include biguanides (chlorhexidine, polyhexanide), quaternary ammonium compounds (QACs, such as benzalkonium chloride or cetrimide), phenols (including the bisphenolic agent triclosan), alcohols (ethanol, isopropanol), aldehydes (glutaraldehyde, formaldehyde), metallic salts (silver compounds, mercury compounds), halogen-releasing agents (chlorine compounds such as sodium hypochlorite, or iodine compounds such as povidone-iodine), peroxygens (hydrogen peroxide), diamidines (propamidine), octenidine dihydrochloride, and glucoprotamin, among others.[78,80,91]

In this study, we tested the biocidal compounds benzalkonium chloride, chlorhexidine digluconate, ethanol, glucoprotamin™, octenidine dihydrochloride, and triclosan. Their properties and usage are further described in the following sections.

2.5.3.1. Benzalkonium chloride

Benzalkonium chloride (BZK) belongs to the chemical class of QACs and shows broad-spectrum activity against bacteria, viruses, and fungi.[92] Products containing BZK were introduced into the market in 1935 and have since been on the rise.[92] Nowadays, BZK is used as surface disinfectant in clinical, industrial, agricultural and consumer settings, and as a common antimicrobial preservative agent in ophthalmic, nasal, and otic solutions. It is further included in numerous cosmetic and personal hygiene products, in laundry detergents, and in products for maintenance of water sites such as pools, ponds, and fountains.[92,93] BZK-based hand sanitisers have been proposed as alternatives to alcohol-based formulations in the face

of supply shortages during the SARS-CoV-2 pandemic.[94] They have a considerably longer residual antibacterial effect than alcohols and are also less skin-irritating.[95] However, as exposure to low-level BZK can induce bacterial tolerance to BZK and cross-resistance to different antibiotic classes, BZK-based or BZK-supplemented hand sanitisers, skin antiseptics or surface disinfectants are not recommended or are even banned by European authorities.[92]
In-use concentrations of BZK range from lower concentrations of 100–200 mg/L when used as preservative in ophthalmic solutions, to >500 mg/L when used for surface disinfection.[93,96] BZK consists of a mixture of alkyl dimethyl benzyl ammonium chloride compounds with differences in the length of their alkyl chain, which ranges from C_8 to C_{18}.[96]
Like other QACs, BZK is a cationic surface-active and membrane-active agent. Mechanisms of action involve penetration and disruption of the cell wall and disorganisation of the cell membrane, causing leakage of intracellular material. The compound also acts on intracellular targets, as it leads to degradation of nucleic acids and proteins.[97] As BZK within ophthalmic solutions can provoke ocular adverse effects such as dry eye and ocular inflammation in a dose- and time-dependent way, it has been suggested that BZK-free ophthalmic solutions should be preferred.[98]
BZK has a toxic effect on the aquatic environment and on aquatic organisms and animals in general.[92,99] However, due to its widespread use, BZK residues have been detected in wastewater effluents of hospitals, and in ground water and soil samples.[92,100]

2.5.3.2. Chlorhexidine digluconate

Chlorhexidine is a cationic bisbiguanide antimicrobial agent extensively used in both clinical and consumer settings. Chlorhexidine is used for surface disinfection and for skin and wound antisepsis, and can be found in wipes, hand washes, wound dressings and wound irrigation solutions.[101] To prevent central venous catheter-associated infections, some catheter models are impregnated with chlorhexidine, and skin antisepsis of the insertion site with chlorhexidine is also performed.[102,103] The biocidal agent is further used for skin decolonisation of patients carrying methicillin-resistant *Staphylococcus aureus* (MRSA).[104] Chlorhexidine is used as preservative agent in ophthalmic products,[80] and one of the most frequently used compounds in mouth washes.[101] The compound adheres on oral tissue and is gradually released, leading to prolonged antimicrobial activity.[105,106]
Chlorhexidine is commonly used in one of its salt forms, especially as chlorhexidine digluconate (CHX), due to better water solubility.[107] Its in-use concentrations for antisepsis use range from 1000–40,000 mg/L,[107] while lower concentrations are applied when chlorhexidine is used as a preservative agent.[101] CHX acts against a wide range of Gram-negative and Gram-positive bacteria. At lower concentrations, it induces cytoplasmic membrane rupture and leakage of intracellular components, and at higher concentrations or longer exposure times, it

causes precipitation of intracytoplasmic proteins.[80] Combination with alcohol enhances the antimicrobial efficacy of CHX.[103] However, CHX has cytotoxic effects on various body cells, including gingival fibroblasts and epithelial cells.[105]

2.5.3.3. Ethanol

Ethanol (ETH), isopropanol and n-propanol are the alcohols that are commonly used as biocides. They are effective against a broad range of bacteria and fungi. ETH is more virucidal than isopropanol, while isopropanol has a slightly broader antibacterial activity.[108] Alcohols are used in many products for antisepsis of intact skin or for surface disinfection, at concentrations of 60–95 vol%, as the best antimicrobial activity lies within this range.[78,109] Alcohols are the antimicrobial compounds most used in hand antisepsis, notably in hand rubs, gels, and foams.[78,108] They combine a high antimicrobial efficacy with further advantages such as wide availability, relatively low cost, ease of use, time-effectiveness and less skin irritation compared to traditional handwashing with soap, which increases health worker compliance with hygiene protocols and explains their popularity in bed-side applications and for routine hand hygiene.[109,110] Alcohols are used at lower concentrations as preservative agents or in combination with other biocides to potentiate their antimicrobial effect. As they evaporate quickly, alcohols do not have a noticeable residual effect, which may lead to slow bacterial regrowth after application. Alcohol-based antimicrobial products can contain other biocides that show a longer residual activity such as chlorhexidine, octenidine or triclosan, or excipients that reduce the evaporation time.[80,110]

Although the mode of action of ETH is not fully understood, it is thought to be related to damage in cell membrane and cell wall, decrease of cross-membrane proton gradient, and protein denaturation.[80,111] At low concentrations, in *E. coli*, ETH negatively affects transcription and translation processes, causes cell hypoxia, and leads to decreases in ATP production and in biosynthesis.[112,113]

2.5.3.4. Glucoprotamin

Glucoprotamin (GP) was introduced as a novel disinfectant in the early 1990s. It is an amine derivative and the reaction product of L-glutamic acid and cocopropylene-1,3-diamine.[114] As an amine derivative, GP is thought to act in an unspecific way via disruption and disorganization of cell membranes.[115,116] Glucoprotamin™ is the active compound of different commercial biocidal products, such as Incidin™ Plus, which was used in this study.[117] This product is commonly used for surface disinfection, at concentrations ranging from 0.25 vol% for limited virucidal action, or 0.5 vol% for effective action against most bacteria and yeasts, to 3 vol% for mycobactericidal action. Other studies validated the efficacy of GP against bacteria, mycobacteria, fungi and viruses, although the antiviral activity against surface-dried viruses is limited.[114,115,118,119] GP is also used as disinfectant for medical instruments.[120] GP has good

ecotoxicological properties and shows less toxicity compared to aldehyde-based disinfectants, and compared to QACs, no biocide residues are left on surfaces after exposure.[115]

2.5.3.5. Octenidine dihydrochloride

Octenidine dihydrochloride (OCT) is a bispyridine cationic biocidal compound that was introduced in the 1980s and is nowadays a widely established antiseptic in a variety of clinical settings.[121,122] OCT is used for skin antisepsis of premature newborns, before skin-penetrating procedures, for antisepsis of mucous membranes before surgical procedures and in mouth washes, and for wound antisepsis as a wound irrigation solution or in wound dressings.[122,123]

Product formulations contain between 500–1000 mg/L OCT.[85] The OCT molecule is formed by two cationic aminopyrimidines, linked and flanked by hydrophobic hydrocarbon groups, which provides OCT an amphipathic character.[121] Like CHX, to which it presents structural similarities, OCT causes bacterial cell membrane disruption, leading to leakage and coagulation of intracellular material.[124]

OCT acts effectively against Gram-positive and Gram-negative bacteria, yeasts and fungi, and is able to disrupt and clear bacterial oral plaques and wound biofilms.[122] OCT has been successfully used in MRSA decontamination regimes, and possibly represents an alternative in face of reported resistance to the classical MRSA eradication treatment with CHX and mupirocin.[125]

At the same time, OCT presents a lower cell toxicity compared to other antiseptics.[105,126] OCT has also been shown to have anti-inflammatory properties, to promote skin wound healing, and to have a longer residual and therefore infection-preventive effect than other biocides such as BZK.[127,128]

2.5.3.6. Triclosan

Triclosan (TRI) belongs to the chemical class of bisphenols, which consist of interconnected hydroxy-halogenated derivatives of two phenolic groups.[80] TRI is a biocide with antimicrobial activity against bacteria, including bacterial biofilms, and fungi.[129]

TRI is one of the most commercialised antimicrobial compounds. It is used nowadays in non-alcohol based hand rubs, in medical products including catheters or sutures, in a large variety of personal care products such as toothpaste or deodorants, in household items such as cutting boards or textiles, or in food storage containers.[129,130] Concentrations of TRI range from 1000–3000 mg/L when used as a preservative in cosmetic products, to up to 20,000 mg/L in antiseptics.[131,132]

At low concentrations, TRI has an inhibitory effect on bacteria by targeting a specific enzyme, the enoyl-acyl carrier protein reductase FabI, which is a key enzyme in the fatty acids synthesis pathway in bacteria.[133] At higher, lethal concentrations, TRI provokes membrane disruption and leakage of intracellular components.[82]

Due to its widespread use, TRI is ubiquitously detected in different environmental compartments.[134] TRI can be found in sewage treatment plants, where it is only partially eliminated, and further released into aquatic and natural environments. TRI can further be detected in human body fluids.[135]

The ubiquity of TRI raises concerns about its toxicity, as it has toxic effects against aquatic organisms at near-environmental concentrations,[134] the propensity to accumulate in fatty tissues because of its hydrophobicity, and endocrine disruptor effects.[129]

Another alarming fact about the ubiquity of TRI is its potential to induce resistance to antibiotics by selecting for genetic mutations, such as mutations causing overexpression of efflux pump and beta-lactamase genes, and downregulation of membrane permeability genes.[136]

2.6. Decreased biocide susceptibility

2.6.1. Definitions

The terms ‘resistance to biocides’ on one hand, and ‘tolerance to biocides’ or ‘decreased biocide susceptibility’ on the other hand, have different meanings and are not to be confounded.

Regarding antibiotics, a bacterium is termed resistant to an antibiotic when the MIC of the antibiotic is above a clinical resistance breakpoint, e.g. that set by EUCAST, which signifies that the likelihood of therapeutic failure is high even when exposure to the antibiotic compound is increased.[137] Unlike for antibiotics, there are no comparable breakpoints for biocide resistance.[138]

Currently, resistance to a biocidal agent is commonly defined as failure of bacterial inactivation by an in-use concentration of the biocidal agent.[85,139] ‘Increased tolerance’ to a biocidal agent is equivalent to ‘reduced susceptibility’ and is defined as MIC increase compared to those typical of the species, i.e. wildtype.[85] In the present work, we use these definitions. Bacterial strains might thus display decreased susceptibility to a biocide without being clinically resistant.

Adaptation is characterised by an increase in MIC through selective pressure via exposure to sublethal concentrations or step-wise increasing concentrations of a certain biocide.[83,116] There happens to be a lack of differentiation in the literature between stable and unstable adaptation, i.e., it is not always tested if the MIC increase persists after removal of biocide exposure or if the MIC shifts down again.[116]

2.6.2. Occurrence of decreased biocide susceptibility

Biocides usually act via a combination of different mechanisms and often have more general cell targets as opposed to the specific targets of antibiotic compounds. Clinically relevant bacterial tolerance to biocides is therefore less likely to develop than antibiotic resistance.[81]

However, decreased biocide susceptibility has been described in many environmental and clinical bacterial strains against numerous biocidal compounds routinely used in clinical, industrial and consumer settings. Decreased biocide susceptibility has been suggested to arise from widespread usage of biocides within the last decades.[81,85] The increased use of biocides during the SARS-CoV-2 pandemic has reinforced these concerns.[90]

For example, contemporary *K. pneumoniae* isolates show higher CHX MICs than older isolates from the pre-chlorhexidine era.[140] A similar situation presents with modern clinical isolates of *S. epidermidis* showing increased TRI tolerance.[141] Similarly, *E. faecium* isolates recovered several years after the systematic introduction of alcohol-based hand rubs in an Australian hospital have been suggested to be more tolerant to isopropanol than earlier isolates.[142]

Increased tolerance in terms of MIC increase has been reported for BZK and CHX in many species including *K. pneumoniae, E. coli, Serratia marcescens, S. aureus, Stenotrophomonas maltophilia* and *A. baumannii,*[85,143,144] for the cationic biocide OCT in *K. pneumoniae*, MRSA, *P. aeruginosa* and *Proteus mirabilis*,[101,145-147] for polyhexanide in *Enterococcus faecalis* and *S. aureus*,[85] and for silver in *Enterobacter cloacae*, *E. coli* and *K. pneumoniae*.[85] Decreased TRI susceptibility has been reported on numerous occasions in species such as *E. coli*, *P. aeruginosa*, *S. aureus*, *Salmonella enterica*, *Campylobacter jejuni* and *A. baumannii*.[46,49,59] In another study, clinical carbapenem-resistant *K. pneumoniae* isolates showed higher ETH MICs than a standard laboratory strain.[148] Tolerance to other agents includes iodophor tolerance in *S. aureus* and tolerance to oxidising agents in *E. coli* and *Bacillus subtilis*.[149-151]

In most cases, increased tolerance levels of bacterial isolates are still lower than the recommended working concentrations of biocides. As such, frequently used clinical biocides including propanol, CHX and TRI were efficacious against *A. baumannii* when used at appropriate concentrations and exposure times in a study from 2010.[152] However, there are reports about isolates of formerly susceptible bacterial species reaching biocidal tolerance levels above the in-use concentrations, thus becoming resistant. For example, after in-vitro adaptation to BZK, various bacterial species including *P. aeruginosa* and *Enterobacter* spp. can show BZK MICs above in-use antiseptic concentrations.[143] Biocide resistance can not only be induced under laboratory conditions, but also occur in the clinical setting. Tissue dispensers with tissues for surface disinfection based on BZK or GP contained a high number of *Achromobacter* spp. strains resistant to in-use concentrations of the biocidal product.[153] Similarly, a QAC-based hospital surface disinfectant was contaminated with resistant isolates of *S. marcescens* and *Achromobacter xylosoxidans*.[154] Contamination of biocidal products with biocide-resistant bacteria can cause outbreaks of healthcare-associated infections such as bloodstream infection or septic arthritis.[143] This was the case for isolates of *B. cepacia, P. aeruginosa* or *Achromobacter* spp. isolated from BZK-based biocidal products.[143] In Brazil, a clone of *Mycobacterium massiliense* resistant to a 2% glutaraldehyde working solution used

for surgical instrument disinfection caused an epidemic of postsurgical infections.[155] Further, isolates of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* isolated from hand soap dispensers containing CHX tolerated working concentrations of 10,000 mg/L of CHX.[156]

2.6.3. Biocide tolerance: intrinsic or acquired

As for antibiotics, tolerance to biocides can be generally classified as intrinsic or acquired.[90] Intrinsic tolerance is chromosomally encoded and naturally expressed.[90] Differences in intrinsic biocide susceptibility between bacterial species often rely on differential permeability barriers and can be ranked with the modified Spaulding classification.[157] Bacterial endospores, which are simplified forms of the bacteria with a specialized coating protecting them against harsh external conditions,[158] present the lowest biocide susceptibility, followed by mycobacteria and vegetative Gram-negative bacteria. The corresponding permeability barrier is the coat of bacterial spores, the cell wall of mycobacteria, and the outer membrane and the lipopolysaccharide (LPS) layer in Gram-negative bacteria. Vegetative Gram-positive bacteria generally have a higher biocide susceptibility, although exceptions exist within bacterial groups and for specific biocidal compounds.[81,159] Adaptation to selective pressure of a biocide or acquisition of tolerance determinants can also modify the classification.[81]

Increased tolerance to biocides can develop through changes in gene expression without underlying genotypic changes, such as in bacterial stress response, or through modifications of the bacterial genome. The latter corresponds to acquired tolerance, which results either from mutations in existing genes or from acquisition of new genetic material via horizontal gene transfer of biocide-tolerance genes.[46,90,160]

As the genetic and transcriptomic adaptations to antimicrobial exposure require the deployment of additional cellular resources, they generally come with a fitness cost measured by a reduced bacterial growth rate. Therefore, a downshift to pre-exposure susceptibility levels can be beneficial for cell fitness and survival once the biocide exposure has ended.[83,161]

Exposure to sublethal biocide concentrations induces a bacterial stress response, which leads to transient or permanent changes in bacterial gene expression and aims to reduce the negative impact of the biocide on the cell. It aims in particular at reducing biocide concentration inside the cell, and at allowing the cell to repair injuries inflicted by the biocide.[81,83] Stress response also favours beneficial mutations.[162] Stress response mediates the bacterial adaptation to biocide exposure and can thus lead to increased biocide tolerance.[83]

As bacterial stress responses are tightly regulated, biocide stress often induces changes in regulatory pathways.[162] Biocides may directly interact with regulator proteins or act by modifying the expression of regulator genes, affecting in both scenarios the downstream gene expression.[162] For example, TRI can bind to TetR-like repressor SmeT of multidrug efflux pump SmeDEF in *S. maltophilia*, and cause pump overexpression and reduced antimicrobial

susceptibility.[163] Oxidising agents can interact with major regulators, such as SoxRS in *E. coli*, leading to overexpression of multiple biocidal tolerance mechanisms.[162]

Biocide exposure has been shown to decrease bacterial growth,[83,164] which has been attributed to direct growth-limiting effects of the biocidal compound on the bacterial cell on one hand.[81] On the other hand, growth decrease has also been suggested to be a result of stress response, reflecting the induction of bacterial tolerance mechanisms, and also allowing damage repair.[83] As such, growth reduction has been suggested as an indirect bacterial tolerance mechanism, as antimicrobials typically act on growing cells with higher metabolic turnover.[83,162]

Acquired biocide tolerance in bacteria can be a result of cellular genetic mutations.[83] In particular, stress response in bacteria induces an increase in the bacterial mutation rate via a rise in double-stranded DNA breaks followed by error-prone repair mechanisms.[162] This leads to random mutations, but also selects for mutations that give the cell a survival benefit during biocide exposure, such as mutations leading to constitutive expression or overexpression of biocide tolerance genes.[162,165] In *E. coli*, exposure to low doses of ETH selects for mutations that modify bacterial stress response pathways to increase ETH tolerance.[113]

Biocide exposure can select for mutations in global transcriptional regulator genes, such as *ramR* in *S. enterica,* or for mutations in specific regulatory genes, such as efflux regulator gene *nfxB* in *P. aeruginosa*, and induce expression of bacterial tolerance mechanisms including efflux.[166-168] Mutations in the target gene of TRI, *fabI*, have been reported in *S. enterica* isolates with increased TRI tolerance after exposure to the biocide.[167] Bacteria also tend to accumulate compensatory mutations that attenuate the fitness cost of adaptive changes favouring biocide tolerance.[81,169]

Acquired biocide tolerance can further emerge via horizontal gene transfer. Horizontal gene transfer can lead to the acquisition of new genetic material with biocide-tolerance determinants in bacteria and contributes to intra- and interspecies spread of decreased biocide susceptibility.[160]

Widely spread mobile biocide tolerance elements are the so-called '*qac'* genes, the quaternary ammonium compounds tolerance genes.[46] They encode various efflux pumps that confer tolerance to different QACs, including BZK, across species, and are the main determinants of QAC tolerance in *S. aureus*.[46,170] The *qac* genes are often encoded on integrons and on plasmids.[171] Integrons often contain antimicrobial resistance genes, and are able to move within or between DNA molecules.[31,160] Integrons can be transferred onto plasmids, which are circular or linear extrachromosomal replicons able to move between bacteria via conjugation.[90,160,172] The transfer of integrons containing both antibiotic resistance and biocide tolerance genes can thus confer reduced susceptibility to both classes of antimicrobials.

Regarding inter-species transfer, the conjugative transfer of a plasmid harbouring a *qacA* gene from *S. aureus* to *E. coli* increased CHX tolerance in *E. coli*.[173] Mobile genetic elements also

contribute to the spread of an additional *fabI* allele, conferring TRI tolerance via target duplication in *S. aureus*.[174]

2.6.4. Cell envelope alterations

Modifications in the cell envelope, i.e., cell wall and cytosolic cell membrane in Gram-positive bacteria, and outer cell membrane, cell wall and inner cell membrane in Gram-negative bacteria, can contribute to enhanced biocide tolerance.[90,175] As the cell envelope usually presents the first point of contact between the biocide and the cell, alterations can lead to reduced biocide penetration of the cell and prevent or reduce further cell damage.[175]

Indeed, the composition and permeability of the bacterial cell envelope also account for intrinsic bacterial tolerance to biocides.[83] Alterations in the LPS layer further have the potential to increase tolerance to membrane-active agents, as has been suggested for CHX in *Pseudomonas stutzeri*.[176]

Changes in the outer membrane's net negative charge have been suggested as a tolerance mechanism to cationic biocides. A lesser negative membrane potential may reduce the electrostatic adsorption of the positively charged biocidal compounds.[175] For example, adaptation to BZK in *P. aeruginosa* induced mutations that reduce the outer membrane's net negative charge.[175] Reduced negative cell surface charge has also been suggested to contribute to BZK tolerance in a *Pseudomonas fluorescens* strain.[177]

As additional tolerance mechanism, increased hydrophobicity of cell surface might render it more difficult for cationic biocides to penetrate the cell envelope and has been associated with increased BZK tolerance in *S. enterica*,[178] and with increased CHX tolerance in *P. stutzeri*.[179]

Changes in the fatty acid or phospholipid composition of the bacterial membrane are further cell envelope modifications that can confer increased biocide tolerance.[83] In *P. aeruginosa*, adaptation to QACs was associated with specific changes in the membrane's fatty acid composition.[180] In a recent study, high-level tolerance to the antiseptic OCT in *P. aeruginosa* has been associated with changes in the phospholipid biosynthesis pathway causing modifications in the phospholipid composition of the plasma membrane.[181] Changes in fatty acid and phospholipid content have also been associated with TRI tolerance in *S. aureus*.[182]

Modifications in membrane protein composition, and in particular in bacterial porins, can also affect bacterial tolerance.[83] Reduced porin expression reduces the permeability of the bacterial cell membrane to hydrophilic compounds, and has been associated with reduced susceptibility to BZK in *S. enterica* serovar Typhimurium, *P. aeruginosa* and *E. coli*.[83,175,183,184] In *A. baumannii*, adaptation to BZK and to a TRI-containing biocidal product was associated with decreased expression of the porin-coding genes *ompA* and *carO*.[185] Adaptation to CHX involved decreased expression levels of *carO* in one out of two clinical *A. baumannii* isolates.[185]

In general, porin loss may often act in combination with other tolerance mechanisms, such as increased efflux, to confer bacterial tolerance to a biocide.

2.6.5. Enzymatic degradation

Some bacterial species are able to produce specific enzymes that degrade biocides and render them less toxic for the bacterial cell.[83] The presence of the antioxidant enzymes catalase and superoxide dismutase in *E. coli* can decrease the bacterial susceptibility to the oxidising agents hydrogen peroxide and superoxide.[186] An isolate of *E. cloacae* that showed tolerance to high concentrations of parabens, which are antimicrobial compounds used as preservatives in a wide range of products, was found to be able to degrade parabens.[187]

Moreover, TRI degradation has been reported in TRI-tolerant *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. *denitrificans.*[188] QACs, including BZK, can be degraded by bacterial species from the genera *Pseudomonas, Xanthomonas,* and *Aeromonas*.[189]

It has been noted that sole enzymatic detoxification of biocides is rather unlikely to confer tolerance to higher concentrations of biocides, as in general, the function of the degradation enzymes will also be negatively affected by the biocide.[83] Degradation enzymes rather work in synergy with other bacterial tolerance mechanisms to enable bacteria to survive biocide exposure.[83]

2.6.6. Target site modification

As most biocides have various, unspecific target sites within the bacterial cell, it is generally unlikely that bacteria achieve biocide tolerance via a specific target modification, in contrast to antibiotic resistance.[90]

TRI presents an exception to this general consideration. Notably, at low concentrations, TRI also has a specific bacterial target site in contrast to the general mode of action of most biocides. TRI tolerance can be mediated by target site mutations or increased expression of the target site. In *P. aeruginosa*, tolerance to TRI has been associated with mutations in *fabI*, the specific target gene of TRI coding for an enoyl-acyl carrier reductase involved in the fatty acid biosynthesis.[190] Mutations in *fabI* or overexpression of *fabI* have also been shown to mediate TRI tolerance in other species such as *E. coli*,[191] *S. aureus*,[192] and *A. baumannii*.[193]

Moreover, tolerance to TRI in clinical isolates of *S. aureus* was mediated by an additional *sh-fabI* allele acquired via horizontal gene transfer from *Staphylococcus haemolyticus*. This indicates that triclosan may select for and drive the spread of mobile genetic elements.[174]

2.6.7. Biofilm formation

Biofilm formation in bacteria can notably reduce the efficacy of antimicrobial compounds, and in particular the antibacterial efficacy of biocides.[194] Biofilms consist of single- or multispecies communities of microorganisms that are attached to a surface and enclosed in a self-secreted exopolysaccharide matrix.[194] Growth in an organised biofilm community provides bacteria with

an ecologically equilibrated environment that can enable them to withstand hostile conditions such as nutrient deprivation, desiccation, or biocide exposure.[195] Compared to the planktonic mode of growth, bacteria in biofilms can be 10–1000 times less susceptible to antimicrobial agents.[194] The exopolymer matrix in biofilms acts as a permeability barrier which limits the diffusion of biocides to the inner layers and inactivates the biocidal compounds. Phenotypic adaptation of the biofilm cells to different stress factors such as nutrient and oxygen gradients or biocide exposure further involves different gene expression patterns, which can lead to upregulation of genes encoding biocide tolerance mechanisms such as efflux pumps.[196] Notably, the organization within a biofilm also favours the exchange of resistance determinants between cells via horizontal gene transfer, as has been shown for *A. baumannii*, which has a high propensity to form biofilms.[26]

Biofilm formation has been reported to render bacteria resistant to biocides at recommended in-use concentrations. For example, a *S. enterica* biofilm could not be eradicated by working concentrations of sodium hypochlorite, sodium hydroxide, or BZK.[197] CHX-based medical wound dressings and CHX solutions at working concentrations had limited efficacy against a multispecies-biofilm containing *K. pneumoniae* and *P. aeruginosa* cells,[198] and CHX also showed reduced efficacy in eliminating cells in *A. baumannii* biofilms compared to the efficacy against planktonic cells.[199] In another study, a TRI-based sanitiser could only partly reduce biofilms of *Listeria* spp., whereas ETH-based and QAC-based sanitisers were even less effective.[200]

Increased tolerance or resistance of biofilms to biocides is of high clinical relevance as it can prevent an effective decontamination. Indeed, bacterial biofilms, and in particular *A. baumannii* biofilms, can form on medical devices and implants and colonise human tissues, where they can cause severe, difficult-to-treat infections.[26,195]

2.6.8. Efflux of biocides

Active efflux is an important mechanism for bacteria to increase their survival to biocide exposure, and often acts in interplay with other tolerance mechanisms, such as decreased outer membrane permeability.[83] In some studies, efflux alone has been suggested to cause high-level tolerance, such as tolerance to working concentrations of TRI.[83,201]

Some efflux-related biocide tolerance gene-products are acquired by bacteria via mobile genetic elements, such as the *qac* genes. Other efflux-related biocide tolerance-encoding genes in Gram-negative bacteria are in general chromosomally encoded.[46]

Efflux pump expression has frequently been shown to be induced by subinhibitory concentrations of biocides.[83] Overexpression is often mediated via differential expression of efflux pump regulatory genes or via direct interaction of stress factors, including biocides, with a regulatory protein,[73,163] which can lead to either de-repression or activation of the actual efflux

pump gene expression.[202,203] Repressors that have been associated with biocide tolerance include TetR-like repressors such as SmvR in various Gram-negative bacteria, QacR in *S. aureus*, or AdeN in *A. baumannii*, which regulate the efflux pumps SmvA, QacA, and AdeIJK, respectively.[145,202,204] Efflux overexpression leading to decreased biocide susceptibility can also be governed by changes in global gene regulators such as MarA and SoxS.[205,206] The induction of efflux pumps can confer tolerance to the specific biocide that the bacterial strains were exposed to, but may also confer increased tolerance to other biocides and antibiotics that are substrates of the same efflux pump.[83,207] The potential of biocides to cause cross-resistance to antibiotics via efflux pump induction is further discussed in section 5.2.

Homologous efflux pumps can be encoded across different bacterial species and confer biocide tolerance. Efflux pumps from all the characterised efflux families have been described to contribute to biocide tolerance in bacteria.[46]

Efflux pumps from the RND family have been associated with decreased biocide susceptibility in various bacterial species. In *P. aeruginosa*, constitutionally expressed RND efflux pump MexAB-OprM has been shown to be responsible for resistance to working concentrations of TRI (>1000 mg/L).[201] RND pump MexCD-OprJ also mediates TRI and CHX tolerance upon overexpression, which is induced by mutations in its regulatory gene *nfxB*.[208,209] TRI is also a substrate of RND efflux pumps MexEF-OprN and MexJK-OpmH in *P. aeruginosa*.[201,208] RND efflux pump AcrAB-TolC has been found to extrude biocidal compounds such as CHX, QACs and TRI in *E. coli* and *K. pneumoniae*,[210,211] as well as TRI in *S. enterica* serovar Typhimurium.[212] RND pump SmeDEF in *S. maltophilia* has also been shown to extrude TRI and cause resistance to various antibiotics upon overexpression. The overexpression of the pump is induced when TRI binds to its repressor protein SmeT.[163] Similarly, RND pump SdeXY contributes to reduced BZK, CHX and TRI susceptibility in *S. marcescens*.[213]

In *A. baumannii*, RND efflux pump AdeABC has been found to mediate CHX and BZK tolerance.[63] In another study, adaptation to CHX or BZK led to increased expression of the efflux pump AdeB and SMR-family efflux pump AbeS in *A. baumannii* clinical isolates.[185] The RND pump AdeIJK was overexpressed upon triclosan exposure via deletions in its Tet-R like repressor gene *adeN* and was suggested to contribute to TRI tolerance in *A. baumannii*.[204] AdeIJK has also been associated with reduced susceptibility to BZK and CHX in *A. baumannii*.[63] Additionally, AbuO, an outer membrane protein homologous to TolC, which constitutes an RND efflux pump in *E. coli*, contributes to reduced BZK, CHX, and TRI susceptibility in *A. baumannii*.[214]

MFS pumps can also mediate biocide tolerance. Plasmid-encoded efflux pumps QacA and QacB from the MFS family extrude QACs in *S. aureus* and *E. faecalis*.[46] QacA has additionally been shown to extrude divalent cations such as CHX, whereas QacB extrudes monovalent cations.[46,215]

Overexpression of MFS efflux pumps NorA and NorB has also been associated with increased BZK and CHX tolerance in *S. aureus*.[46] The MFS family efflux pump SmvA contributes to reduced susceptibility to cationic biocides across several Gram-negative species.[145] Overexpression of SmvA, mediated by mutations in its TetR repressor gene *smvR* or by a loss of the regulator gene, induced increased tolerance to CHX in strains of *K. pneumoniae* and other *Enterobacterales* spp.,[145,207] and also mediated tolerance to other cationic biocides such as QACs in *K. pneumoniae* and in *S. enterica*,[145,216] and to OCT in *P. aeruginosa*.[181,217] Adaptation to OCT in *K. pneumoniae* resulted from mutations in key residues of SmvA, which caused a stronger interaction between SmvA and the OCT molecule.[145] The MFS pump KpnGH also contributes to reduced susceptibility to BZK, CHX and TRI in *K. pneumoniae*.[218]

The MFS transporter MdtM has been reported to play a role in QAC tolerance in *E. coli*.[219] It has been suggested that the increased tolerance is partly due to an interplay of the cytoplasmic membrane transporter MdtM with the RND efflux system AcrAB-TolC.[219] TolC-independent MFS pumps that contribute to BZK tolerance in *E. coli* are EmrD and MdfA/CmlA/CmlB/Cmr transporters.[220] In *A. baumannii*, MFS efflux pump AmvA contributes to CHX and QAC tolerance.[221]

Efflux pumps of the SMR family contribute to reduced biocide susceptibility in a wide number of pathogens. Various integron- or plasmid-encoded 'qac' pumps mediating decreased susceptibility to QACs belong to the SMR family. In Gram-positive bacteria, QacC/D, QacEΔ1, QacG, QacH, and QacJ are encoded in *S. aureus*,[46] and QacEΔ1 in *E. faecalis.*[222] In Gram-negative bacteria, the SMR efflux pumps QacE, QacEΔ1, QacF and QacG can be found across many species, including *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*.[46,148,223]

Other SMR efflux transporters associated with tolerance to QACs are EmrE and SugE in various Enterobacterales including *E. coli*,[220] while KpnEF is involved in tolerance to BZK, CHX, and TRI in *K. pneumoniae*.[224] In *A. baumannii*, SMR transporter AbeS has been associated with reduced susceptibility to BZK and CHX.[144]

Regarding the MATE family, overexpression of the MATE efflux pump MepA in *S. aureus* was caused by mutations inactivating its repressor gene *mepR* and has been suggested to decrease bacterial susceptibility to CHX and QACs.[225] Other MATE transporters that contribute to decreased susceptibility to QACs including BZK are PmpM in *P. aeruginosa* and NorM in *Neisseria* spp.[46] MATE transporter AbeM extrudes QACs and TRI in *A. baumannii*.[226]

Regarding biocide tolerance mediated by the ATP-driven ABC family of efflux pumps, EfrAB has been associated with CHX and TRI tolerance in Gram-positive bacteria, including *E. faecalis* and *Staphylococcus* spp.[46,227]

Other efflux families related to biocide tolerance include the transporter protein AceI (Acinetobacter chlorhexidine efflux) from the proteobacterial antimicrobial compound efflux (PACE) family, which specifically extrudes CHX in *A. baumannii*.[161] AceI homologs in other

proteobacterial lineages, such as *Burkholderia cenocepacia*, *P. aeruginosa*, or *Vibrio parahaemolyticus,* also play a role in CHX and QAC tolerance.[50]

Further, CepA (Chlorhexidine Efflux Protein A), an efflux pump of the Cation Diffusor Facilitators (CDF) superfamily, which usually extrude zinc and heavy metal ions, contributes to CHX tolerance in *K. pneumoniae*.[220]

2.7. Aim of the work

Investigating the mechanisms of increased biocide tolerance in *A. baumannii* and the contribution of efflux in particular is of clinical relevance, because effective decontamination with biocides is necessary to prevent the spread of the pathogen in the hospital environment. The aim of this work was to investigate the impact of differential expression of RND-type efflux pumps AdeABC and AdeIJK mediated by mutations in their corresponding regulator genes on susceptibility and on survival of *A. baumannii* to biocide exposure. We investigated the commonly used biocides benzalkonium chloride (BZK), chlorhexidine digluconate (CHX), ethanol (ETH), glucoprotamin (GP), octenidine dihydrochloride (OCT) and triclosan (TRI). While previous studies have found that RND-type efflux pumps in *A. baumannii* can cause MIC increases for some of the biocides tested, the role of efflux in killing kinetics has not been well investigated. Our work aimed at closing this gap via studying killing kinetics upon biocide exposure in addition to biocide susceptibility.

3. Publication

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Contribution of RND-Type Efflux Pumps in Reduced Susceptibility to Biocides in *Acinetobacter baumannii*

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Abstract: Bacterial efflux pumps are among the key mechanisms of resistance against antibiotics and biocides. We investigated whether differential expression levels of the RND-type efflux pumps AdeABC and AdeIJK impacted the susceptibility to commonly used biocides in multidrug-resistant *Acinetobacter baumannii*. Susceptibility testing and time–kill assays of defined laboratory and clinical *A. baumannii* strains with different levels of efflux pump expression were performed after exposure to the biocides benzalkonium chloride, chlorhexidine digluconate, ethanol, glucoprotamin, octenidine dihydrochloride, and triclosan. While the impact of efflux pump expression on susceptibility to the biocides was limited, noticeable differences were found in kill curves, where AdeABC expression correlated with greater survival after exposure to benzalkonium chloride, chlorhexidine digluconate, glucoprotamin, and octenidine dihydrochloride. AdeABC expression levels did not impact kill kinetics with ethanol nor triclosan. In conclusion, these data indicate that the overexpression of the RND-type efflux pumps AdeABC and AdeIJK contributes to the survival of *A. baumannii* when exposed to residual concentrations of biocides.

Keywords: efflux; biocide; disinfectant; RND-type efflux pump; *Acinetobacter baumannii*

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1. Introduction

Acinetobacter baumannii is a Gram-negative pathogen that frequently shows resistance against numerous antimicrobial classes, including carbapenems and other β-lactam agents, aminoglycosides, chloramphenicol, fluoroquinolones, and tetracyclines [1]. This poses a real challenge for the treatment of *A. baumannii* infections in the clinical setting, where the pathogen has a propensity to severely affect critically ill or immunocompromised patients, causing ventilator-associated pneumonia, bloodstream infections, meningitis, urinary tract infections, or wound and soft tissue infections [1]. Current treatment options favour antibiotic combination therapy including ampicillin–sulbactam, carbapenems, polymyxins, or tigecycline [2]. However, pan-drug-resistant *A. baumannii* strains have been reported and carbapenem-resistant *A. baumannii* is considered as priority 1 ("critical") in the WHO priority pathogens list for research and development of new antibiotics [3]. Carbapenem resistance can be mediated by carbapenem-hydrolysing beta-lactamases, polymyxin resistance by lipopolysaccharide modifications, and tigecycline resistance by active efflux [4]. In addition to antibiotic resistance, decreased susceptibility to biocides has been reported in *A. baumannii* and other bacterial species [5,6]. Biocides are essential decontamination tools used in the clinical setting to prevent hospital-acquired infections [7]. Commonly used biocides include the quaternary ammonium compound (QAC) benzalkonium chloride, the bisbiguanide agent chlorhexidine, ethanol, glucoprotamin, the cationic compound octenidine dihydrochloride, and the bisphenolic agent triclosan. They are used as antiseptics

for human skin, wounds, and mucous membranes, as surface disinfectants, and they are also components of numerous consumer products such as mouthwashes, lotions, and soaps [7–9].

Concerns have been raised that the widespread use of biocides may lead to reduced biocide susceptibility and cross-resistance to antibiotics, thus facilitating the selection and spread of multi-drug-resistant (MDR) pathogens [5]. Furthermore, inadequate cleaning or exposure to subinhibitory concentrations of biocides might lead to the persistence of *A. baumannii* in the clinical setting [10,11]. Bacterial efflux pumps are among the key mechanisms in reduced antibiotic and biocide susceptibility [5,12]. Broad substrate efflux pumps can extrude a wide number of unrelated antibiotics as well as biocides out of bacterial cells, contributing to the MDR phenotype [12]. Among Gram-negative bacteria, the most clinically relevant efflux pumps belong to the resistance-nodulation cell-division (RND) family [13]. These tripartite pumps are tightly regulated, and mutations in their regulators can lead to efflux pump overexpression [14]. In *A. baumannii,* the RND pumps AdeABC and AdeIJK play a role in conferring multi-drug resistance, including resistance to aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, and tigecycline [1,15–17], and have been associated with the efflux of biocides such as benzalkonium chloride and chlorhexidine in studies involving knockout mutants of the efflux transporters AdeB or AdeJ [18], and with triclosan efflux in a clinical correlation study of reduced triclosan susceptibility and efflux overexpression [19]. AdeABC is regulated by the two-component regulatory system AdeRS [20], while AdeIJK is regulated by the TetR-like repressor AdeN [21]. We have previously shown that mutational hotspots in these regulators affect the expression of the corresponding RND efflux pumps, leading to changes in antimicrobial susceptibility [17,22,23]. However, while susceptibility to biocides has been investigated before, the impact of efflux on killing kinetics of biocides has not been well studied. Therefore, the aim of this study was to investigate the impact of RND efflux pumps in *A. baumannii* on their survival when exposed to different concentrations of commonly used biocides.

2. Results

2.1. Susceptibility Testing

The MIC results of the tested biocides for laboratory and clinical *A. baumannii* strains with efflux pump regulator mutations and differential efflux pump expression levels are summarised in Table 1.

The knockout of *adeRS* resulting in the lack of *adeB* expression in *A. baumannii* ATCC 19606 led to a 4-fold MIC reduction for chlorhexidine digluconate and 2-fold MIC reductions for the biocides glucoprotamin and octenidine dihydrochloride compared to the wildtype parent. In isolate pair 1, *adeS* mutant MB-5, which overexpresses *adeB*, showed a 2-fold increase in MIC for glucoprotamin and triclosan compared to MB-2. MB-43, an *adeS* mutant from isolate pair 2 that shows increased AdeABC efflux, showed a 2-fold MIC increase for chlorhexidine digluconate, whereas in isolate pair 5, no changes in biocide MICs were observed in the *adeR* mutant strain overexpressing *adeB*.

Laboratory and clinical strains with mutations in *adeN* and associated *adeIJK* overexpression, i.e., laboratory strain ATCC 19606 Δ*adeN* and clinical strains MB-273 and MB-1044 in isolate pairs 3 and 4, respectively, did not show any differences in biocide MICs compared to wildtype.

Table 1. Distribution of biocide MICs determined by microbroth dilution.

	***A. baumannii* Strain**	**Biocide MIC (mg/L)**					
		BZK	**CHX**	**ETH**	**GP**	**OCT**	**TRI**
	ATCC 19606 wt	16	16	197,500 *	17	4	1
	ATCC 19606 Δ*adeRS*	16	4	197,500	8.5	2	1
	ATCC 19606 Δ*adeN*	16	16	197,500	17	4	1
Isolate pair 1	MB-2	16	16	197,500	17	4	0.5
	MB-5 (IS*Aba1* disrupts *adeS)*	16	16	197,500	34	4	1
Isolate pair 2	MB-7	16	16	n.d.	17	4	4
	MB-43 (IS*Aba1* disrupts *adeS*)	16	32	n.d.	17	4	4
Isolate pair 3	MB-271	16	16	197,500	34	4	4
	MB-273 (IS*Aba1* disrupts *adeN*)	16	16	197,500	34	4	4
Isolate pair 4	MB-131	32	16	n.d.	17	4	4
	MB-1044 (IS*Aba125* disrupts *adeN*)	32	16	n.d.	17	4	4
Isolate pair 5	Isolate F	16	16	n.d.	17	4	4
	Isolate G (mutation in *adeR*)	16	16	n.d.	17	4	4

BZK: benzalkonium chloride; CHX: chlorhexidine digluconate; ETH: ethanol; GP: glucoprotamin; OCT: octenidine dihydrochloride; TRI: triclosan; n.d.: not determined. * corresponds to 25 vol%.

2.2. Time–Kill Assay

Time–kill assays were performed with *A. baumannii* ATCC 19606 wt, *A. baumannii* ATCC 19606 Δ*adeRS*, *A. baumannii* ATCC 19606 Δ*adeN*, and *A. baumannii* isolate pairs 1 and 3 (isolate pairs with the highest expression level for the respective efflux pump in the isogenic mutant) to assess bacterial survival in relation to the exposure time. Biocide concentrations with the biggest difference in killing for strains with differential *adeABC* expression are shown in Figure 1. Additional concentrations are shown in Supplementary Data Figures S1 and S2.

A. baumannii ATCC 19606 Δ*adeRS* showed increased killing when grown in the presence of benzalkonium chloride, chlorhexidine digluconate, glucoprotamin, and octenidine dihydrochloride compared to the wildtype parent (Figure 1 and Figure S1).

Exposure to benzalkonium chloride at 8 mg/L caused >3-log CFU reduction at all time points in *A. baumannii* ATCC 19606 Δ*adeRS*, while the wildtype showed a ~1-log CFU reduction until 3 h, and substantial regrowth by 24 h. Grown in 8 mg/L of chlorhexidine digluconate, *A. baumannii* ATCC 19606 Δ*adeRS* showed a >3-log reduction in CFU count after 1 h and thereafter, while *A. baumannii* ATCC 19606 wt showed a <1-log CFU reduction after 1 h, and regrowth after 3 h (Figure 1). *A. baumannii* ATCC 19606 Δ*adeRS* also showed increased killing at 4 mg/L of chlorhexidine digluconate (Figure S1). The killing rate of *A. baumannii* ATCC 19606 Δ*adeRS* was higher than the wildtype in 8.5 mg/L glucoprotamin, showing ~4-log reduction at 1 h and beyond (Figure 1), and in 4.2 mg/L glucoprotamin during the first 3 h (Figure S1). *A. baumannii* ATCC 19606 Δ*adeRS* exhibited more killing than wildtype during the first 1 h of exposure to 0.5 mg/L octenidine dihydrochloride (Figure 1).

Considering the range of the results, the *adeRS* knockout showed similar kill kinetics compared to wildtype upon exposure to ethanol. At higher concentrations of the tested biocides, ≥3-log CFU reductions were observed in both strains but killing rates of the *adeRS* knockout strain were higher after 0.5 h for chlorhexidine digluconate and glucoprotamin (Figure S1).

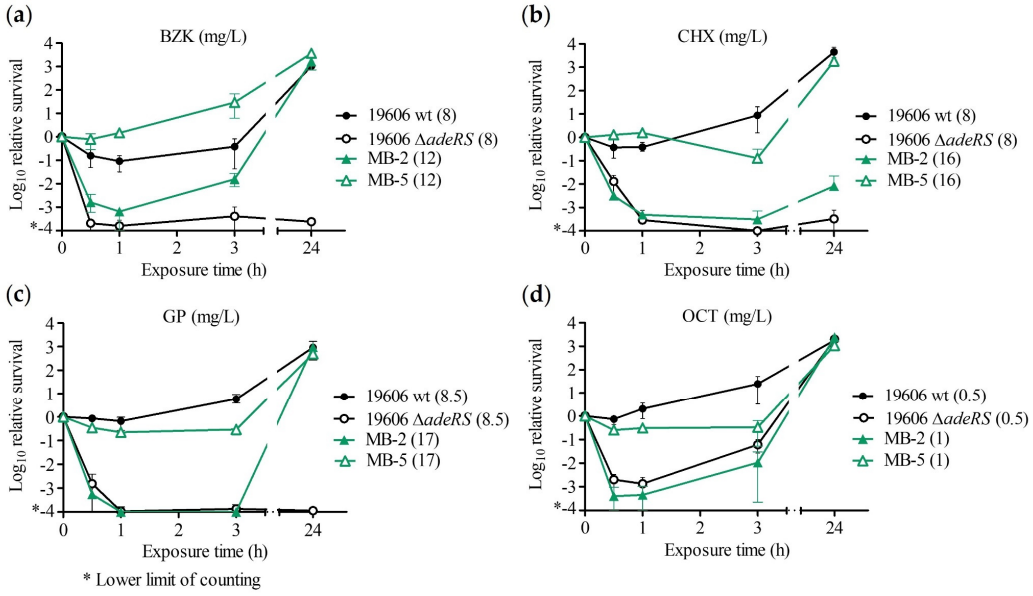

Figure 1. Impact of *adeABC* expression for laboratory strains *A. baumannii* ATCC 19606 and *A. baumannii* 19606 Δ*adeRS* (*adeABC* not expressed) and for clinical strains *A. baumannii* MB-2 and *A. baumannii* MB-5 (*adeABC* overexpressed) in the time–kill assay. Time–kill curves of (**a**) benzalkonium chloride (BZK), (**b**) chlorhexidine digluconate (CHX), (**c**) glucoprotamin (GP), and (**d**) octenidine dihydrochloride (OCT). Tested concentrations (mg/L) written in parentheses. Error bars represent the range from three independent experiments.

In isolate pair 1, the strain MB-5 with *adeABC* overexpression exhibited less killing with benzalkonium chloride, chlorhexidine digluconate, glucoprotamin, and octenidine dihydrochloride than the parental strain MB-2 (Figure 1 and Figure S2). During the first 3 h of exposure to chlorhexidine digluconate at 16 mg/L, *A. baumannii* MB-5 showed up to >3-log-fold less killing compared to MB-2 (Figure 1). Similar results were seen with exposure to benzalkonium chloride at 12 mg/L, chlorhexidine digluconate at 8 mg/L, and glucoprotamin at 12 mg/L and at 8.5 mg/L (Figure S2), although both strains showed substantial regrowth at 24 h. MB-2 also showed more initial killing with exposure to 1 mg/L of octenidine (Figure 1). For other concentrations of biocides, including ethanol, little or no (<1 log-fold) differences were observed (Figure S2).

Biocide concentrations with the biggest difference in killing for strains with *adeIJK* overexpression are shown in Figure 2. Additional concentrations are shown in Supplementary Data Figures S3 and S4.

Compared to the wildtype, *A. baumannii* ATCC 19606 Δ*adeN* showed less killing upon exposure to 12 mg/L of benzalkonium chloride, and during the first 3 h of exposure to 8 mg/L of benzalkonium chloride and to 16 mg/L of chlorhexidine digluconate. Further, *A. baumannii* ATCC 19606 Δ*adeN* showed slightly less killing (~1-log-fold difference) with 17 mg/L of glucoprotamin during the first 0.5 h, as well as less killing with 1 mg/L of octenidine, and 98,750 mg/L of ethanol at 3 h ($\leq$1-log CFU reduction vs. $\geq$3-log reduction in wt). When exposed to other concentrations of these biocides, *A. baumannii* ATCC 19606 Δ*adeN* showed similar killing kinetics to *A. baumannii* ATCC 19606 wt (<1-log-fold difference) (Figure S3).

In *A. baumannii* isolate pair 3, the *adeIJK*-overexpressing strain MB-273 showed earlier regrowth (after 3 h) compared to MB-271 when exposed to benzalkonium chloride at 16 mg/L (Figure S4). For other concentrations and biocides tested, survival rates between MB-273 and MB-271 were comparable (Figure 2 and Figure S4).

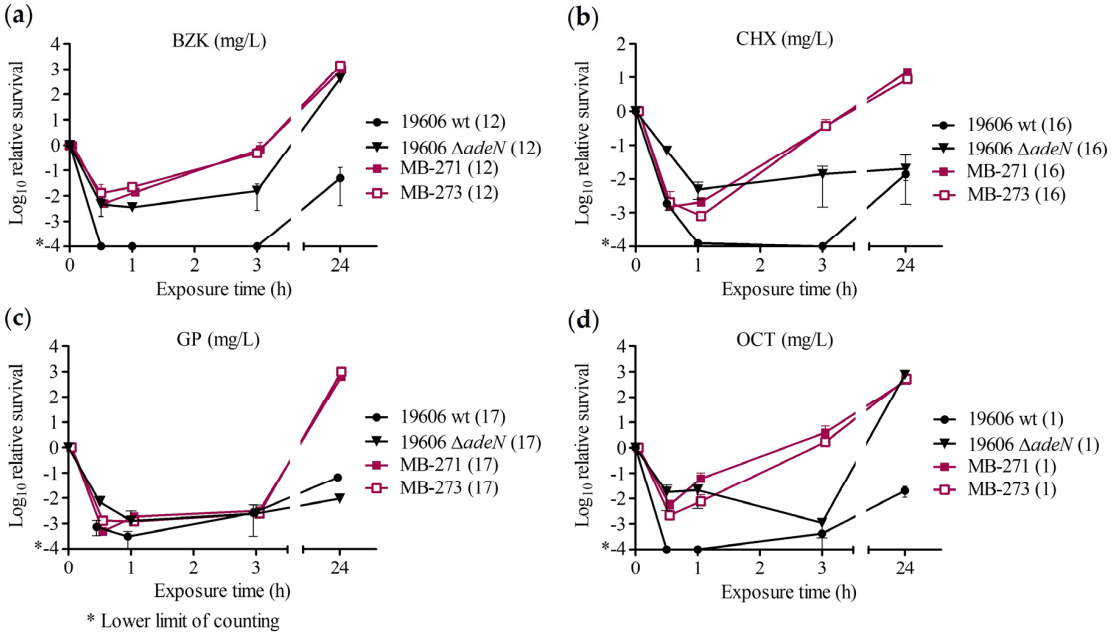

Figure 2. Impact of *adeIJK* overexpression for laboratory strains *A. baumannii* ATCC 19606 and *A. baumannii* 19606 Δ*adeN* (*adeIJK* overexpressed), and clinical strains *A. baumannii* MB-271 and *A. baumannii* MB-273 (*adeIJK* overexpressed) in the time–kill assay. Time–kill curves of (**a**) benzalkonium chloride (BZK), (**b**) chlorhexidine digluconate (CHX), (**c**) glucoprotamin (GP), and (**d**) octenidine dihydrochloride (OCT). Tested concentrations (mg/L) written in parentheses. Error bars represent the range from three independent experiments.

2.3. Dose–Response Assay

Dose–response assays were performed with *A. baumannii* ATCC 19606 wt, Δ*adeRS*, Δ*adeN*, and *A. baumannii* isolate pairs 1 and 3 to assess the contribution of RND pumps to triclosan susceptibility (Figure S5). Triclosan was bacteriostatic up to a concentration of 8 mg/L and at higher concentrations reduced the number of viable cells. Based on these results, the concentrations for the time–kill assays with triclosan were chosen (Figure S6). When exposed to triclosan at 16 and 32 mg/L, *A. baumannii* MB-2 was killed slightly more rapidly than MB-5, and *A. baumannii* ATCC 19606 Δ*adeN* showed slightly decreased killing during the first 1 h, within the limits of reproducibility owing to variability in survival rates. *A. baumannii* MB-273 exhibited less killing than MB-271 after 30 min exposure to triclosan at 32 mg/L. For the other isolate pairs and concentrations tested, killing rates were similar.

3. Discussion

Reduced susceptibility of pathogens to biocides has repeatedly been reported and may impede efficacious decontamination in the hospital environment [5,24]. In *A. baumannii* and other Gram-negative bacteria, increased tolerance to biocides defined as an increase in MICs above those typical for a species is mediated by efflux pumps such as the chromosomally encoded RND pumps, or by plasmid-encoded pumps from the small multi-drug resistance (SMR) superfamily, also referred to as 'qac' pumps as they extrude quaternary ammonium compounds (QACs) [18,25,26]. Broad-substrate RND efflux pumps can extrude both antibiotics and biocides from bacterial cells, raising concerns whether they can confer cross-resistance to different antimicrobial agents [12]. In *A. baumannii*, RND efflux pumps have been shown to cause decreased susceptibility to various antimicrobials [17,27].

Bacterial strains were chosen to include both *A. baumannii* laboratory reference strains and efflux regulator knockout strains, which showed differences in susceptibility to various antibiotics depending on their efflux pump expression level [28,29], and clinical isolates, i.e., isolate pairs 1–5, that were previously shown to efflux antimicrobials through overexpressed RND efflux pumps [17,30]. We sought to determine if these pumps are also capable

of reducing susceptibility to the commonly used biocides included in this study. We demonstrated that the expression of AdeABC can affect the susceptibility to commonly used biocides such as chlorhexidine digluconate, glucoprotamin, and octenidine dihydrochloride in a strain-dependent manner when tested using broth microdilution. Furthermore, differential efflux pump expression levels were seen to cause differences in kill kinetics although MICs may not be altered.

We found that in addition to previously reported increased resistance to various antimicrobials, clinical *A. baumannii* isolates overexpressing AdeABC exhibited decreased killing when exposed to benzalkonium chloride, chlorhexidine digluconate, glucoprotamin, and octenidine dihydrochloride in time–kill assays. Laboratory reference and knockout *A. baumannii* strains served to verify these results, as they represent a targeted manipulation of efflux pump regulatory genes.

Our results confirm that the quaternary ammonium compound benzalkonium chloride and the biguanide chlorhexidine are substrates of efflux pump AdeABC in *A. baumannii*, as was found by Rajamohan et al., in a study from 2010 [18], and in a recent study involving *A. baumannii* ATCC 19606 and efflux pump mutants [31]. Unlike in the latter study, where knockout of *adeB* in *A. baumannii* ATCC 19606 provoked a 4-fold decrease in benzalkonium chloride MIC, in our study, *adeABC* expression levels did not affect MICs, but impacted on benzalkonium chloride kill curves. Alongside RND pumps, chlorhexidine susceptibility in *A. baumannii* is also mediated by efflux pump AmvA from the major facilitator superfamily (MFS) [32], and by the specific chlorhexidine-efflux pump AceI from the proteobacterial antimicrobial compound efflux (PACE) family [31,33]. RND-type efflux pumps further contribute to reduced chlorhexidine and QAC susceptibility in *Pseudomonas aeruginosa* [34,35], *Escherichia coli* [36], and *Serratia marcescens* [37].

Our results indicate that AdeABC can extrude the disinfectant glucoprotamin and the cationic antiseptic octenidine in a strain-dependent manner. Increased tolerance to octenidine was also found to be mediated by efflux in *P. aeruginosa* and *Klebsiella pneumoniae*, where the efflux system SmvA (MFS superfamily) extrudes octenidine [38,39]. To our knowledge, no previous studies regarding the impact of efflux on glucoprotamin susceptibility have been carried out in *A. baumannii*.

The efflux pump AdeIJK has been reported to efflux benzalkonium chloride and chlorhexidine [18], which may add to the predominant impact of AdeABC [31]. However, in our study, the overexpression of AdeIJK in clinical strains did not impact on their susceptibility to these biocides. Nevertheless, while we did not observe any impact on the MIC, the Δ*adeN* laboratory mutant of *A. baumannii* ATCC 19606 exhibited decreased killing at early time points in time–kill assays with benzalkonium chloride and chlorhexidine digluconate, indicating that AdeIJK expression can confer a survival advantage over strains that do not express the pump, although this observation appears to be strain-dependent.

Except for decreased killing at a single time point in 19606 Δ*adeN*, susceptibility or survival to ethanol was not impacted by differential expression of AdeABC nor AdeIJK in the strains tested in our study. In a study by Prieto et al., exposure to subinhibitory concentrations of ethanol could lead to overexpression of AdeABC in a strain-specific manner and activated the *adeABC* promoter region in *A. baumannii* ATCC 17978, but not in ATCC 19606 [40]. This may suggest that AdeABC can contribute strain-dependently to the development of ethanol tolerance. In the same study, adaptation to ethanol did not activate the promoter region of *adeIJK*. This is according to our findings, where AdeIJK did not notably mediate ethanol tolerance.

In *P. aeruginosa* and in *Stenotrophomonas maltophilia*, decreased susceptibility to triclosan has been shown to be mediated by RND-type efflux pumps [41,42]. In *A. baumannii*, efflux pumps that have been associated with decreased triclosan susceptibility are AdeIJK [43], AbeM (MATE superfamily) [44], and AdeABC, as a recent study found *A. baumannii* ATCC 19606 Δ*adeB* exhibiting a 4-fold MIC decrease [31]. Yu et al. further correlated decreased triclosan susceptibility with increased *adeB* expression in clinical *A. baumannii* strains [19]. In our study, in contrast, *adeN* knockout or mutant strains that overexpressed AdeIJK did not

show MIC differences compared to their corresponding parental strain. The only difference was a one-dilution MIC increase of triclosan in an isolate that overexpressed AdeABC.

The triclosan time–kill assays showed high variability, making it difficult to interpret the results. This unstable assay might be due to low triclosan solubility and precipitation effects at higher concentrations. Technical challenges due to precipitation of triclosan have also been reported by others [45,46]. In the dose–response assay though, all isolates showed similar curves at lower concentrations, and there was no big difference seen at higher concentrations despite a higher variability, indicating that efflux pump expression level had little effect on triclosan susceptibility in our assays.

Although the concentrations of biocides tested in the kill curve studies were considerably lower than recommended for routine use in the clinical setting, the results of this study suggest that efflux may play a role in the survival of *A. baumannii* when exposed to lower or residual concentrations of biocides. Other factors that can contribute to survival of *A. baumannii* to biocide exposure include biofilm formation, decreased outer membrane porin expression, and target site modification [47–49]. Residual concentrations may become relevant in practice when biocidal formulations are diluted, for example, through the application of hand antiseptics on wet hands, or when biocides such as benzalkonium chloride are used at lower concentrations, e.g., as preservatives in commercial products [50]. Residual concentrations of biocides are further found in different environments, such as low-level benzalkonium chloride in hospital wastewater [51]. These considerations enhance the significance of proper biocide use at recommended concentrations.

4. Materials and Methods

4.1. Bacterial Strains

A. baumannii strains used in the study are listed in Table 2. The *adeRS* and *adeN* knockouts in *A. baumannii* ATCC 19606 were obtained using markerless mutagenesis [52]. *adeRS* knockouts do not express *adeABC* [28], while the knockout of repressor gene *adeN* led to the overexpression of *adeIJK* [29]. Five sets of clinical *A. baumannii* isolate pairs were also included in this study [17,30]. The isolate pairs were defined as two *A. baumannii* isolates collected from the same patient at two different time points, that were shown to be clonally related but differed in their antimicrobial susceptibility to tigecycline owing to increased efflux.

Table 2. Bacterial strains used in the current study.

	***A. baumannii* Strain**		**Relevant Characteristics**	**Reference**
Laboratory strains	ATCC 19606		Reference strain	[53]
	ATCC 19606 Δ*adeRS*		*adeABC* not expressed	[28]
	ATCC 19606 Δ*adeN*		2.5-fold increase in *adeIJK* expression	[29]
Clinical strains	Isolate pair 1	MB-2	Wildtype	[17]
		MB-5	IS*Aba1* insertion in *adeS*, 45-fold increase in *adeABC* expression	[17]
	Isolate pair 2	MB-7	Wildtype	[17]
		MB-43	IS*Aba1* insertion in *adeS*, 35-fold increase in *adeABC* expression	[17]
	Isolate pair 3	MB-271	Wildtype	[17]
		MB-273	IS*Aba1* insertion in *adeN*, 6-fold increase in *adeIJK* expression	[17]
	Isolate pair 4	MB-131	Wildtype	[17]
		MB-1044	IS*Aba125* insertion in *adeN*, 2-fold increase in *adeIJK* expression	[17]
	Isolate pair 5	Isolate F	Wildtype	[30]
		Isolate G	Missense mutation in *adeR*, 7-fold increase in *adeABC* expression	[30]

4.2. Biocides

The biocides used in this study were benzalkonium chloride (Sigma-Aldrich, Steinheim, Germany), chlorhexidine digluconate (Molekula Ltd., Darlington, UK), ethanol (Merck, Darmstadt, Germany), glucoprotamin™ (Incidin™ Plus (26 wt% glucoprotamin), Ecolab, Monheim, Germany), octenidine dihydrochloride (AmBeed, Illinois, IL, USA), and triclosan (Molekula Ltd., Darlington, UK). The biocides were diluted in sterile distilled water, except for triclosan, which was diluted in a 1:1 ratio of acetone and phosphate-buffered saline due to its low water solubility.

4.3. Susceptibility Testing

Biocide MICs were determined using serial broth microdilution in cation-adjusted Mueller–Hinton broth (CAMHB) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [54]. Serial 2-fold dilutions were performed with benzalkonium chloride (range tested: 0.06–32 mg/L; recommended working concentrations: 100–200 mg/L when used as preservative, ≥500 mg/L for surface disinfection), chlorhexidine digluconate (range tested: 0.06–32 mg/L; working concentrations: 500–40,000 mg/L), ethanol (range tested: 770–395,000 mg/L, corresponding to 0.1–49.5 vol%; recommended working concentrations: 474,000–750,500 mg/L, corresponding to 60–95 vol%) [55], glucoprotamin (range tested: 0.26–135 mg/L, corresponding to 0.000098–0.05 vol% of Incidin™ Plus; recommended working concentrations: 1350–8100 mg/L of glucoprotamin, corresponding to 0.5–3 vol% of Incidin™ Plus), octenidine dihydrochloride (range tested: 0.03–16 mg/L; recommended working concentrations: 500–2000 mg/L), and triclosan (range tested: 0.016–8 mg/L; recommended working concentrations: 1000–20,000 mg/L). Experiments were repeated three times independently.

4.4. Kill Kinetics

A time–kill assay was performed with minor modifications according to the National Committee for Clinical Laboratory Standards guideline M26-A [56]. Briefly, 200 µL of an overnight culture of the bacterial strain were added to 10 mL of CAMHB and the bacteria were grown at 37 °C in a shaker-incubator until mid-log phase. Then, 50 µL of the log-phase culture were added to a tube containing 10 mL of CAMHB to achieve a final inoculum of approx. 5×10^5 CFU/mL. The equivalent of biocide needed to reach the desired concentration was added. Different biocide concentrations were tested; 0.25, 0.5, 1, 2 × MIC, except for triclosan, for which higher concentrations were needed to reduce growth. At t = 0, 0.5, 1, 3 and 24 h, a 0.5 mL aliquot of the respective biocide solution was removed, serially diluted in 0.9% NaCl, and 100 µL were plated onto Mueller–Hinton agar. After overnight incubation in air at 37 °C, CFU counts were performed. The lower limit of counting was set at 4 log-fold reduction of the initial number of bacterial cells, corresponding to approx. 5 CFU/plate or 50 CFU/mL. CFU/mL values were calculated, and relative survival plotted using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). All time–kill assay experiments were repeated three times independently, including growth and sterility controls.

4.5. Dose–Response Assay with Triclosan

Triclosan was added to tubes containing the bacterial inoculum as described above. The final triclosan concentrations ranged from 0.0625 to 128 mg/L. At t = 0 and 3 h, a 0.5 mL aliquot was removed and treated as described above to determine bacterial counts.

5. Conclusions

In conclusion, these data suggest that the expression of RND efflux pumps contributes to the survival of *A. baumannii* in the hospital setting despite the use of biocides which can ultimately kill them. The efflux pumps give the organism a window of opportunity to persist and be transferred to other surfaces. As *A. baumannii* is disseminated via the hands of healthcare workers and via contaminated surfaces and medical equipment [1],

effective decontamination through proper biocide use at recommended concentrations is particularly important to prevent the spread of the pathogen.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11111635/s1. Figure S1: Time–kill curves for *A. baumannii* ATCC 19606 wt and 19606 Δ*adeRS* (*adeABC* not expressed) with additional biocide concentrations: (**a**) benzalkonium chloride (BZK), (**b**) chlorhexidine digluconate (CHX), (**c**) glucoprotamin (GP), (**d**) ethanol (ETH), and (**e**) octenidine dihydrochloride (OCT). Error bars represent the range from three independent experiments; Figure S2: Time–kill curves for *A. baumannii* MB-2 and MB-5 (*adeABC* overexpressed) with remaining biocide concentrations: (**a**) BZK, (**b**) CHX, (**c**) GP, (**d**) ETH, and (**e**) OCT. Error bars represent the range from three independent experiments; Figure S3: Time–kill curves for *A. baumannii* ATCC 19606 wt and 19606 Δ*adeN* (*adeIJK* overexpressed) with additional biocide concentrations: (**a**) BZK, (**b**) CHX, (**c**) GP, (**d**) ETH, and (**e**) OCT. Error bars represent the range from three independent experiments; Figure S4: Time–kill curves for *A. baumannii* MB-271 and MB-273 (*adeIJK* overexpressed) with remaining biocide concentrations: (**a**) BZK, (**b**) CHX, (**c**) GP, (**d**) ETH, and (**e**) OCT. Error bars represent the range from three independent experiments; Figure S5: Dose response of *A. baumannii* regulatory system mutant strains to triclosan (TRI): (**a**) for *A. baumannii* ATCC 19606, *A. baumannii* ATCC 19606 Δ*adeRS* and *A. baumannii* ATCC 19606 Δ*adeN*, (**b**) for *A. baumannii* MB-2 and *A. baumannii* MB-5, and (**c**) for *A. baumannii* MB-271 and *A. baumannii* MB-273. Error bars represent the range from three independent experiments, Figure S6: Time–kill curves with different TRI concentrations: (**a**–**c**) for *A. baumannii* ATCC 19606, *A. baumannii* ATCC 19606 Δ*adeRS* and *A. baumannii* ATCC 19606 Δ*adeN*, (**d**–**f**) for *A. baumannii* MB-2 and *A. baumannii* MB-5, (**g**–**i**) for *A. baumannii* MB-271 and *A. baumannii* MB-273. Error bars represent the range from three independent experiments.

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4. Discussion

The Gram-negative bacterium *Acinetobacter baumannii* is an important nosocomial pathogen that primarily affects immunocompromised patients and is dreaded for causing outbreaks on intensive care units.[2] Clinical manifestations include bloodstream infections, pneumonia, urinary tract infections, meningitis, and wound infections, which are usually difficult to treat due to multidrug resistance.[2] Carbapenem-resistant *A. baumannii* is spreading globally, and pandrug-resistant isolates which are resistant to last resort antibiotics such as colistin or tigecycline have been reported.[19,20] Antimicrobial resistance in *A. baumannii* is caused by numerous innate and acquired resistance mechanisms, among which carbapenem-hydrolyzing oxacillinases and efflux pumps play a predominant role.[2] Furthermore, the spread of *A. baumannii* is facilitated by its propensity to persist in the hospital environment. Persistence is promoted by the pathogen's capacity to withstand hostile environmental conditions, e.g., desiccation and biocide exposure, via protective mechanisms such as biofilm formation.[2]

Biocides are essential tools for infection control in the clinical setting. For example, skin antisepsis and surgical instrument disinfection have allowed the establishment of modern aseptic surgery, regular application of hygienic hand rubs in healthcare personnel reduces the incidence of nosocomial infections, and surface disinfection inhibits the spread of pathogens via contaminated surfaces.[86,87,228] Regarding *A. baumannii*, biocides are also crucial for preventing nosocomial infections, as the pathogen usually propagates through contaminated medical equipment or via the hands of healthcare workers.[90]

However, reduced bacterial susceptibility to biocides has been described in numerous studies.[83,140,143] Reduced susceptibility was observed for many biocidal compounds and in various bacterial species, including *A. baumannii*.[83,85,185] On some occasions, bacteria that are resistant to in-use concentrations of biocides have even been reported.[143] This has raised many concerns, as efficacious decontamination in the hospital environment may be at stake, and biocide-resistant bacteria may lead to increased morbidity and mortality.

While in most studies, bacterial strains with biocide MICs above working concentrations were trained with increasing biocide concentrations under laboratory conditions, which might not represent a realistic clinical scenario, there are also examples of clinical strains of formerly susceptible bacteria showing in-situ resistance to in-use biocide concentrations.[154-156] For example, an *A. baumannii* isolate collected from a hand soap dispenser was resistant to working concentrations of 10,000 mg/L of CHX.[156]

Fortunately, in most cases, decreased bacterial biocide susceptibility manifests itself via an MIC increase that is still far below the in-use concentrations of biocides.[78] Wisplinghoff et al. showed in a study from 2010 that commonly used biocides tested at in-use concentrations and

with appropriate exposure times effectively killed sporadic and epidemic *A. baumannii* strains.[152]

Nevertheless, tolerance to low-level biocide concentrations becomes clinically relevant when biocides are used at lower concentrations than recommended.[139,229] Upon improper application of biocides, such as at diluted concentrations or with an insufficient exposure time, reduced susceptibility or increased survival to biocides may contribute to the persistence of *A. baumannii* in the hospital environment.

Bacteria possess different mechanisms to abide exposure to biocides. These include compound-specific mechanisms, such as enzymatic degradation or target site modification, as is the case with TRI.[83,90,188] More commonly, bacteria with decreased biocide susceptibility show unspecific tolerance mechanisms, such as alterations in the cell envelope, decrease of the cell permeability, or biofilm formation.[83,90,175,185,194]

Further, active efflux has been found to contribute substantially to decreased biocide susceptibility.[46] In the presence of biocides, efflux pumps can confer a competitive advantage to isolates overexpressing them.[185] All characterised families of efflux pumps have been associated with biocide tolerance.[46] Among these families, RND efflux pumps play a predominant role. RND pumps are particularly efficacious as they extrude biocides directly to the outside of the cell.[47] RND efflux has been reported to confer decreased susceptibility to biocides such as BZK, CHX, and TRI across many Gram-negative species including *P. aeruginosa*, *K. pneumoniae*, *E. coli* and *S. marcescens*.[46,208,211,213] The expression of the RND efflux pumps AdeABC and AdeIJK in *A. baumannii* is tightly regulated. AdeABC is regulated by the two-component regulatory system AdeRS, while AdeIJK is regulated by the TetR-like repressor AdeN.[67,70] Specific mutations in the regulator genes *adeRS* and *adeN* have been shown to affect the expression of their corresponding efflux pump operons and to have an impact on the susceptibility to various antibiotic classes.[69,74,76,230] AdeABC and AdeIJK have been found to contribute to reduced susceptibility to the biocides BZK and CHX in *A. baumannii*.[63] While previous studies have focused on MICs for assessing the impact of efflux on biocide susceptibility,[63,185] the role of efflux in survival of *A. baumannii* to biocide exposure over time has not been well investigated.

To address this gap, this study investigated the impact of differential RND efflux pump expression not only on the susceptibility of *A. baumannii* to the various biocides but also on the time-dependent survival of *A. baumannii* to biocides commonly used in the clinical setting. To better understand and counteract the emergence of decreased biocide susceptibility in *A. baumannii*, it is indeed of primary importance to elucidate the mechanisms that the pathogen employs to withstand biocide exposure.

The determination of time-dependent bacterial survival to biocide exposure provides valuable additional information on the role of efflux within the first hours of biocide exposure, compared to the sole MIC measurement after 20 h. Time-dependent survival also simulates a more realistic clinical scenario. Indeed, in the clinical setting, bacteria may be frequently exposed for a few hours to low biocide concentrations, such as during surface disinfection.

Therefore, we firstly assessed the role of RND efflux pumps on bacterial susceptibility to biocides via MIC determination in broth microdilution. Secondly, we performed a time-kill assay to assess the role of efflux pump expression on time-dependent bacterial survival, where we measured the number of viable cells after 0.5, 1, 3, and 24 h of biocide exposure.

It has been repeatedly pointed out that the use of laboratory reference strains does not necessarily reflect realistic clinical situations, as reference strains isolated decades ago may have different properties than current clinical isolates.[69,83,174] In our work, we therefore chose to include both clinical isolates and laboratory reference strains showing a differential expression of either efflux pump AdeABC or AdeIJK due to mutations in their efflux pump regulator genes, *adeRS* or *adeN*. These regulator mutations have affected the susceptibility to various antibiotics in previous studies. A further characteristic of this study is that we investigated the impact of these mutations on biocide susceptibility and survival in addition to the previously reported antibiotic susceptibility changes.

Clinical isolate pairs 1–5 were isogenic efflux pump regulator mutants that have been shown to extrude antimicrobials via efflux overexpression and represented real-life conditions. the laboratory knockout mutants of reference strain *A. baumannii* ATCC 19606 were obtained by a targeted knockout of the efflux pump regulatory system *adeRs* or the regulatory gene *adeN*, respectively. *A. baumannii* ATCC 19606 Δ*adeRS* has been shown to lack expression of *adeABC*, and *A. baumannii* ATCC 19606 Δ*adeN* has shown increased *adeIJK* expression.[69,77] The laboratory strains served to verify the results obtained from the clinical isolates.

We tested biocides that are commonly used in the clinical setting. These include benzalkonium chloride, chlorhexidine digluconate, ethanol, glucoprotamin, octenidine dihydrochloride, and triclosan. Regarding the surface disinfectant glucoprotamin and the antiseptic octenidine dihydrochloride, to our knowledge, there are no previous studies assessing the role of RND efflux on decreased susceptibility in *A. baumannii*. The effect of efflux pumps on bacterial susceptibility to these biocidal compounds is further discussed in sections 5.1. – 5.6.

An additional consideration is that the exposure to the low biocide concentrations that we tested may have resulted in a bacterial stress response leading to overexpression of additional biocide tolerance mechanisms, such as, e.g., overexpression of (other) efflux pumps, or factors contributing to reduced membrane permeability.[83] Overexpression of other tolerance mechanisms during biocide exposure might have mitigated the effect of the divergent pump expression level in the isogenic mutants and may be another explanation for the minor

differences in MIC in the isogenic mutants overexpressing their respective efflux pumps. However, in studies where a biocide MIC increase was induced via exposure of bacteria to subinhibitory biocide concentrations, exposure times were generally longer than in our study.[143]

4.1. Benzalkonium chloride

Increased bacterial tolerance to the widely used QAC BZK has been reported on numerous occasions. Bacterial strains from over 50 species showed MIC increases after laboratory adaptation to BZK, some of them reaching MICs above BZK working concentrations (>1000 mg/L).[143] BZK-based biocidal products contaminated with resistant isolates have also caused hospital outbreaks in the past.[143]

Bacterial tolerance to BZK is mediated via various mechanisms, including reduction of outer membrane permeability, changes in fatty acid and phospholipid composition, enzymatic degradation, biofilm formation, and efflux.[189]

Different efflux pump families are involved.[92] A prominent example are the plasmid-encoded 'qac' efflux pumps, which were named after their ability for extruding QACs including BZK. In Gram-negative species including *A. baumannii,* the qac pumps QacE and QacEΔ1 from the SMR family are found.[223,231] Additional efflux pumps associated with BZK tolerance in *A. baumannii* include the SMR transporter AbeS and MFS efflux pump AmvA.[144,221]

RND-type efflux pumps, which were the focus of our work, have also been reported to extrude BZK,[92] such as MexAB-OprM and MexCD-OprJ in *P. aeruginosa,*[232] AcrAB-TolC in *E. coli* and in *K. pneumoniae,*[205,210,211] as well as plasmid-encoded RND pump OqxAB in *E. coli*.[233]

Our results confirm that the RND-type efflux pump AdeABC in *A. baumannii* can extrude BZK. This was first described in a study from 2010 by Rajamohan et al.[63] A recent study by Migliaccio et al. comparing efflux pump mutants of *A. baumannii* ATCC 19606 to the wild-type laboratory strain also confirmed the BZK efflux activity of AdeABC.[234] However, in our study, differential expression of *adeB* in the laboratory and clinical strain pair caused by mutations or disruption of the regulatory system *adeRS* did not impact the MICs of BZK. This is opposed to findings of the previously mentioned studies, where a 4-fold MIC decrease for BZK was observed in the *adeB* knockout mutants of the clinical strain *A. baumannii* AC0037, or of the laboratory strain *A. baumannii* ATCC 19606, respectively.[63,234] While we did not observe MIC changes, we found an impact of *adeB* expression on BZK kill curves both in laboratory and clinical strains, which made us infer that BZK is indeed a substrate of AdeABC, and that AdeABC can contribute to increased survival of *A. baumannii* to BZK exposure even if MICs remain unchanged.

Rajamohan et al. also reported that the efflux pump AdeIJK can extrude BZK.[63] They observed a 6-fold MIC decrease for BZK in the *adeJ* knockout strain of clinical isolate AC0037. In

contrast, Migliaccio et al. found no MIC decrease in the *adeJ* knockout mutant of *A. baumannii* ATCC 19606.[234] We only found a slight impact of AdeIJK on survival to BZK. In both the laboratory and the clinical strain pairs we tested, knockout or mutations in repressor gene *adeN* led to overexpression of efflux pump AdeIJK. In the clinical strains, this neither impacted the BZK MIC, nor the survival to BZK in the time kill assays. While the Δ*adeN* laboratory mutant of *A. baumannii* ATCC 19606 did not show decreased susceptibility to BZK by MIC, we however observed reduced killing at earlier time points in the kill curves. This indicates that expression of AdeIJK can be beneficial for the survival of *A. baumannii* to BZK exposure in a strain-dependent manner.

The missing impact of *adeJ* overexpression in the kill kinetics of the clinical isolate pair might also result from the fact that the increase in efflux pump expression was not as pronounced as in the clinical isolate pair with *adeB* overexpression (6-fold increase of *adeJ* expression in isolate pair 3, compared to 45-fold increase of *adeB* expression in isolate pair 1).

4.2. Chlorhexidine digluconate

CHX has numerous applications as a disinfectant, antiseptic, or preservative in both clinical and non-clinical settings.[101] However, since the introduction of CHX-containing products, bacterial tolerance to CHX has evolved.[140] Increased tolerance to CHX can be induced via laboratory adaptation,[85,140] and resistance to working concentrations of CHX (>10,000 mg/L) has been reported in clinical isolates of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*.[156]

Bacterial tolerance to CHX can develop via different mechanisms, such as decreased porin expression, changes in LPS composition, organisation in biofilms, and active efflux as a major tolerance determinant. [176,179,185,199]

Different families of efflux pumps contribute to decreased CHX susceptibility, such as the MFS efflux pump SmvA in *K. pneumoniae* and in *Enterobacteriaceae* spp., with SmvA overexpression being caused by loss of or by mutations in its TetR-like repressor gene *smvR.*[145,207] In *A. baumannii*, the specific chlorhexidine-efflux pump AceI from the PACE family contributes to CHX tolerance,[161,234] as well as MFS efflux pumps AmvA and AedF,[221,235] and SMR exporter AbeS.[144] RND-type efflux pumps involved in decreased CHX susceptibility across species include MexCD-OprJ in *P. aeruginosa*,[168,208,209] AcrAB-TolC in *E. coli* and in *K. pneumoniae*,[205,210,211] and SdeXY in the nosocomial pathogen *S. marcescens*.[213]

Overexpression of RND efflux pump AdeABC in *A. baumannii* can be induced by exposure to low-level concentrations of CHX, as was shown in *A. baumannii* ATCC 17978 [64,161] and in a set of 23 clinical *A. baumannii* isolates,[236] but not in *A. baumannii* ATCC 19606.[64] This is indicative for a strain-specific pump induction.

The RND-type efflux pump AdeABC in *A. baumannii* has been shown to extrude CHX and to confer decreased CHX susceptibility, as was first described by Rajamohan et al. in 2010.[63,234]

In their study, knockout of *adeB* in a clinical *A. baumannii* isolate led to an 8-fold MIC decrease for CHX. Furthermore, a reduced susceptibility to CHX was associated with *adeB* overexpression in a sample of 85 clinical *A. baumannii* isolates.[63] Knockout of *adeB* in *A. baumannii* ATCC 19606 also led to an 8-fold MIC decrease for CHX in a study by Migliaccio et al.,[234] compared to the 4-fold MIC decrease for CHX that we observed in ATCC 19606 Δ*adeRS,* a strain that also lacks *adeB* expression.

The results of our study confirm that CHX is a substrate of AdeABC and suggest that mutations in their regulatory system AdeRS can alter susceptibility and survival to CHX via efflux expression. The expression of *adeB* impacted on the CHX MIC for some strains, although the fold changes in MIC were smaller than in the previously mentioned studies. Indeed, the overexpression of *adeB* only led to a 2-fold MIC increase in one out of three clinical isolate pairs, indicating that CHX efflux mediated by AdeABC may be strain-specific. Further, isolate pair 5 that showed no MIC change only exhibited a 7-fold increase in *adeB* expression, while isolate pair 2 with the 2-fold MIC increase had a 35-fold increase in *adeB* expression. This might also partly explain the strain-dependent findings in the susceptibility testing. A study from 2017 by Lin et al. supports the strain-dependency hypothesis, as the authors did not find an association between reduced CHX susceptibility in clinical *A. baumannii* isolates and increased *adeB* expression.[236]

The impact of AdeABc expression in our work was more pronounced in the kill curves, where lack of pump expression correlated with increased killing, and overexpression led to decreased killing rates in the clinical isolate pair that had not shown MIC differences. This suggests that AdeABC can increase the survival to low concentrations of CHX especially at earlier time points, and not only after 20 h of contact, which is when the MIC is measured.

Efflux pump AdeIJK has also been reported to extrude CHX. Knockout of efflux transporter protein *adeJ* in clinical *A. baumannii* isolate Ab0037 induced a 2-fold MIC decrease for CHX in the aforementioned study by Rajamohan et al.[63] The authors also found that *adeJ* overexpression correlated with reduced CHX susceptibility in a sample of 85 clinical *A. baumannii* isolates.[63] On another note, in the study by Migliaccio et al., knockout of *adeJ* in ATCC 19606 did not produce an MIC change. Exposure to low concentrations of CHX however induced AdeIJK expression in *A. baumannii* ATCC 19606.[234] Conversely, low-level CHX significantly reduced the promotor activity of AdeIJK in *A. baumannii* ATCC 19606 in a study by Prieto et al.[64]

In our work, AdeIJK overexpression mediated by mutations in the regulator encoding gene *adeN* did not cause MIC changes for CHX in the clinical, nor in the laboratory isolates. Nevertheless, similar to our findings with BZK, the time-kill assay could show an impact of AdeIJK overexpression, as the laboratory knockout mutant *A. baumannii* ATCC 19606 Δ*adeN* had decreased killing rates at early time points with some CHX concentrations. This suggests

that the overexpression of AdeIJK can confer a survival advantage when exposed to CHX in a strain-dependent manner, as the clinical isolate pair overexpressing AdeIJK did not show differences in killing.

Our findings further support that AdeIJK may contribute, albeit in a strain-specific manner, to the predominant role of AdeABC in CHX efflux, as has been suggested before.[234]

4.3. Ethanol

Development of bacterial tolerance to alcohols such as ETH or isopropanol is of particular concern, as alcohol-containing biocidal products such as hand sanitisers are fundamental tools to prevent the transmission of infectious pathogens.[90]

Reports on increased tolerance to ETH are limited, and evidence for acquired bacterial resistance against alcohols at in-use concentrations has not been reported so far.[110] *E. faecium* isolates showed tolerance to the alcoholic antiseptic isopropanol at 23 vol% and were 10-fold more tolerant than strains isolated over a decade before.[142,237]

In *A. baumannii*, exposure to low concentrations (≤ 1 vol%) of ETH may lead to increased pathogenicity and biofilm formation.[238,239] According to Edwards et al., this is of particular concern, as the abundant use of alcohol-based hand sanitisers in clinical settings may promote the exposure of *A. baumannii* to low-level ETH due to an ETH “background level” and thus favour pathogenicity.[238]

Regarding the role of efflux in ETH tolerance in *A. baumannii*, differential expression of the efflux pumps AdeABC and AdeIJK did not have an impact on ETH susceptibility in our study, nor notably impacted on *A. baumannii* kill curves. Prieto et al. observed that exposure to subinhibitory concentrations of ETH could activate the AdeABC promoter region in *A. baumannii* in a strain-specific manner, indicating that the pump may contribute to ETH adaptation.[64] ETH caused AdeABC overexpression in *A. baumannii* ATCC 17978, but not in ATCC 19606, and decreased the expression of the *adeRS* promoter in ATCC 19606.[64] Our findings support these results in the sense that knockout of *adeRS* leading to no expression of *adeABC* did not affect ETH susceptibility in *A. baumannii* ATCC 19606. It might however be of interest to perform future assays with ETH and regulator mutants of laboratory strain *A. baumannii* ATCC 17978.

Regarding AdeIJK, Prieto et al. found that exposure to ETH downregulated the promoter expression of *adeIJK* in *A. baumannii* ATCC 17978 but did not significantly affect the *adeIJK* promoter activity in ATCC 19606, indicating that AdeIJK did not mediate the adaptation to ETH in these strains, which supports our findings.[64]

4.4. Glucoprotamin

Previous reports of bacterial tolerance to the disinfectant GP are scarce. A standard antibiotic-resistant strain of *P. aeruginosa* showed decreased GP susceptibility compared to an antibiotic-susceptible strain.[240] Gram-negative bacteria that formed biofilms on catheters were also in large part non-susceptible to disinfection with GP at working concentrations.[241] An outbreak of pseudobacteremia at the Heidelberg University Hospital was caused by a biofilm-forming strain of *A. xylosoxidans* that showed reduced killing by GP.[153,242] The *A. xylosoxidans* isolate originated from a contaminated reusable surface disinfection tissue dispenser containing a GP solution. The tissue dispenser had been incorrectly processed, which allowed *A. xylosoxidans* to adapt to GP and to persist on the dispenser.

In our work, differential expression of AdeABC due to regulator mutations affected susceptibility to GP (2-fold changes in MIC) in a strain-dependent manner. This was confirmed in an even more noticeable way in the time kill assays, where overexpression of AdeABC led to less killing. Our results thus indicate that GP is a substrate of AdeABC, and that overexpression of the pump can contribute to decreased GP susceptibility and to increased survival in *A. baumannii*. Overexpression of AdeIJK did not affect susceptibility to GP in the microbroth dilution, nor notably impact on the kill curves, indicating that AdeIJK does not extrude GP in the strains we tested. To our knowledge, efflux of GP has not previously been reported in *A. baumannii*.

4.5. Octenidine dihydrochloride

OCT is a widely used antiseptic compound which combines a high antibacterial efficacy with a low cell toxicity.[122] Because of these favourable characteristics, it is foreseeable that OCT will replace other antiseptic compounds with higher cytotoxicity.[122,126] The development of bacterial tolerance to OCT would therefore be especially concerning.

Exposure to increasing concentrations of OCT has led to decreased OCT susceptibility in MRSA and in various Gram-negative bacteria.[147,243,244] Shepherd et al. observed that some adapted strains of *P. aeruginosa* could withstand over 50% of the working concentration of an OCT-based biocidal product, and an unstable increase in OCT tolerance of *P. aeruginosa* was also observed in a simulated clinical setting with regular OCT exposure.[147]

In our study, deletion of *adeRS* causing lack of *adeB* expression led to a 2-fold MIC decrease in the laboratory strain *A. baumannii* ATCC 19606, but the *adeRS* disruption causing *adeB* overexpression did not affect OCT susceptibility in the microbroth dilution. As for the other biocides tested, the impact of AdeABC expression was more noticeable in the kill curves. Higher expression levels were associated with decreased killing during the initial time points in both laboratory and clinical isolates. Our findings thus indicate that AdeABC can extrude OCT up to a certain degree. OCT efflux by AdeABC may contribute to decreased killing of

A. baumannii at low concentrations of OCT, especially during the first hours of exposure. Regarding AdeIJK, the only impact observed was a slightly decreased initial killing in the laboratory strain ATCC 19606 Δ*adeN* that overexpressed *adeJ*. Further research with more *A. baumannii* strains is needed to assess the role of AdeIJK in OCT tolerance.

Efflux also mediated increased tolerance to OCT in other studies. In various Gram-negative bacteria, the efflux system SmvA from the MFS superfamily contributes to OCT tolerance. Low-level exposure to OCT can select for mutations in the TetR-like repressor gene *smvR.* These regulator mutations can cause SmvA overexpression and reduced OCT susceptibility in *P. aeruginosa* and *P. mirabilis*.[101,181,217] In *K. pneumoniae*, SmvA overexpression was mediated by direct mutations in the *smvA* gene rather than by functional loss of the regulator.[145]

On another note, Malanovic et al. suggested in a study from 2020 that due to the unspecific mode of action of OCT, which consists of physically induced cell envelope disruption, tolerance to OCT would be unlikely to arise.[124] The authors also noted that when used at low concentrations, there may not be enough OCT molecules to sufficiently act on all the bacterial cells, which may result in insufficient killing. They hypothesised that this may have led to incorrect assumptions of OCT tolerance development in some cases where exposure to low-level OCT concentrations was studied.[124] We can rule out that the use of low OCT concentrations biased the differences in killing observed in our work, as the isogenic strains we analysed were pairwise exposed to the same concentrations of OCT, and reproducible differences in the killing depending on AdeABC expression were achieved.

Tolerance to OCT can further be mediated by a combination of tolerance mechanisms, in which efflux may be an important first adaptation step. In a recent study, early tolerance to low-level OCT exposure in *P. aeruginosa* was associated with efflux, whereas tolerance to higher levels of OCT was achieved through additional mutations in phospholipid synthases leading to changes in plasma membrane composition.[181]

4.6. Triclosan

TRI is widely used as disinfectant or antiseptic in hospital and consumer products.[193] However, the widespread use of TRI favours tolerance development. Numerous reports describe decreased TRI susceptibility both after laboratory adaptation and in situ,[83,141,245] such as in clinical *A. baumannii* strains collected from Chinese hospitals.[193,246]

Low-level tolerance can arise via overexpression or modifications in the TRI target site and is often mediated by mutations in *fabI*, as has been reported for many species including *A. baumannii*.[46,141,193] Other tolerance mechanisms include decreased porin expression,[185,212] enzymatic degradation,[188] and, notably, bacterial efflux pumps, such as efflux pump AbeM from the MATE superfamily in *A. baumannii*.[226] Intrinsic high-level tolerance to TRI was attributed

to RND efflux pumps, such as MexAB-OprM in *P. aeruginosa* and AcrAB-TolC in *S. enterica*.[201,212] AcrAB-TolC also mediates TRI tolerance in *E. coli* and *K. pneumoniae*.[205,210] TRI can induce overexpression of RND efflux pumps, which can result in increased TRI MICs.[49] By binding to the regulator SmeT, TRI induces expression of SmeDEF and its efflux in *S. maltophilia*.[163]

In *A. baumannii*, RND efflux pumps AdeABC and AdeIJK have been associated with increased TRI tolerance.[204,234] In our study, efflux pump regulator mutations that affected the expression of AdeABC had limited impact on TRI susceptibility by broth microdilution. Overexpression of AdeABC produced a 2-fold MIC increase in one clinical isolate, while no MIC changes were observed in the other three isolate pairs with differential AdeABC expression. In particular, *A. baumannii* ATCC 19606 Δ*adeRS*, which did not express *adeB*, had the same MIC as *A. baumannii* ATCC 19606. This contrasts with the findings of a study by Migliaccio et al., in which *A. baumannii* ATCC 19606 Δ*adeB* exhibited a 4-fold MIC decrease.[234] The solvent used for the dilution of TRI was not specified by Migliaccio et al. They may have used a distinct TRI solvent, leading to a different TRI precipitation, which may explain the differences in the results. In another study by Yu et al., increased TRI tolerance in clinical *A. baumannii* isolates was also associated with overexpression of *adeB*.[246] Yu et al. also looked at the efflux pump regulatory system *adeRS*. They found that specific mutations in the regulator sensor kinase gene *adeS* led to increased *adeB* expression in clinical isolates with reduced TRI susceptibility. However, other mutations in *adeS* led to decreased *adeB* expression, both in isolates with increased and reduced TRI susceptibility. This further indicates that the impact of AdeABC on TRI susceptibility is strain-specific.

Regarding AdeIJK, TRI selected for strains overexpressing this pump in *A. baumannii* ATCC 17978 in a study by Fernando et al.[204] As in our study, AdeIJK overexpression was caused by a mutation in repressor gene *adeN*, in that case a 73-bp deletion in *adeN.* Besides inducing tolerance to various antibiotics, AdeIJK overexpression produced as well a 2-fold MIC increase for TRI in a *P. aeruginosa* strain lacking other native RND pumps, suggesting that AdeIJK might be responsible for low-level TRI tolerance.[204] In this regard, the *adeJ* deletion mutant from *A. baumannii* clinical strain BM4587 also showed a 4-fold decrease in TRI MIC, and the isogenic mutant overexpressing *adeJ* displayed a 2-fold MIC increase.[59] In our study however, AdeIJK overexpression caused by *adeN* knockout or *adeN* mutations did not produce MIC changes in any of the strains tested. Consistent with our findings, Yu et al. did not find a significant correlation between *adeJ* overexpression and increased TRI tolerance in clinical *A. baumannii* isolates.[246]

In our study, the time-kill assays with TRI showed a relatively high variability, which rendered it difficult to interpret the results. We conjecture that the time-kill assays were unstable because of low solubility of TRI, and additional precipitation effects at higher concentrations. Other

authors have also reported technical difficulties due to precipitation effects of TRI.[58,201] Due to low water solubility of TRI, we chose a different solvent for this biocide, i.e., a 1:1 solution of acetone and phosphate-buffered saline. We decided to avoid popular TRI solvents such as ETH to prevent biocidal effects by the solvent. Eventually time-kill assays for TRI did not provide us with reliable information about killing kinetics related to efflux expression at earlier time points. However, in the dose-response assay, all the isolates that we compared pairwise showed similar curves at lower concentrations. At higher concentrations, despite more variable results, no big differences were seen in the killing rates. This indicates that the expression level of efflux pumps had little impact on the TRI killing activity and, more generally, on TRI susceptibility in our assays.

Due to the partly heterogenous findings in different studies, the role of RND-type efflux pumps and their regulatory systems for TRI tolerance in *A. baumannii* should be further investigated.

4.7. Efflux as mediator of antibiotic cross-resistance

The term cross-resistance or cross-tolerance is used when a particular mechanism confers resistance or decreased susceptibility to several antimicrobial classes.[90,247] The presence of different genes whose products confer antimicrobial tolerance or resistance via different mechanisms is termed co-resistance.[90,247] Co-resistance occurs when different antimicrobial resistance genes spread via the same mobile genetic elements.[247] Regarding biocides, it has been suggested that increased biocide tolerance may also induce cross- and co-resistance to antibiotics.[83,90]

Numerous studies have shown how exposure to biocides can induce resistance to antibiotics under laboratory conditions. Exposure and adaptation to BZK led to increased antibiotic resistance in several species including *A. baumannii*, where it provoked increased gentamicin resistance.[96,248] Laboratory adaptation to CHX induced cross-resistance to ceftazidime and imipenem in *E. coli* and *K. pneumoniae*,[248] to colistin in *P. aeruginosa*,[249] and increased the MICs of sulbactam, ciprofloxacin and meropenem in a clinical *A. baumannii* isolate.[185]

There are several studies about associations between biocide exposure, decreased biocide susceptibility and increased antibiotic resistance in clinical and environmental strains isolated from in situ conditions.[90] For example, chlorination of drinking water was suggested to contribute to AMR selection, as chlorine tolerance correlated with decreased antibiotic susceptibility among several bacterial species isolated from chlorinated water.[250] Decreased BZK and TRI susceptibility was also positively associated with resistance to ampicillin and chloramphenicol resistance in *Salmonella* spp. isolated from hen egg shells.[251] However, these studies do not always find a causality of the association, and a common underlying tolerance mechanism is not always elucidated. Decreased biocide and antibiotic susceptibility may appear simultaneously, but be selected for by different factors, such as exposure to different

antimicrobial compounds.[90] This is especially likely in isolates from environments with a widespread use of biocides and antibiotics, such as hospitals or the food industry.

Furthermore, other studies did not find associations between reduced biocide susceptibility and antibiotic resistance. For example, Fernandez-Cuenca et al. did not find a significant association between increased BZK or CHX tolerance and increased antibiotic tolerance in 49 clinical *A. baumannii* isolates.[185] In TRI-tolerant mutants of *E. coli* and *K. pneumoniae*, susceptibility was decreased for some antibiotics, but increased for other antibiotic compounds.[206] Interestingly, *S. enterica* strains also showed increased susceptibility to various antibiotics following BZK, CHX and TRI exposure.[252] As a possible explanation, the authors of the previous studies argued that bacterial stress response to biocides induces a complex interplay of different pathway changes which might increase susceptibility to other antimicrobial compounds as a "collateral effect".[206] A fitness cost of adaptation to biocides has also been suggested to prevent the spread of cross-resistant strains.[138,206]

Cole et al. also showed that the domestic use of biocidal products based on BZK and TRI did not increase the prevalence of antibiotic-resistant bacteria in the homes or on the skin of users, compared to non-users.[253,254] In contrast to aforementioned studies, these results made the authors suggest that increased biocide use in consumer products does not necessarily promote the spread of AMR.[254]

Due to their broad substrate-specificity, bacterial efflux pumps are the tolerance mechanism with the most relevant role in the context of antibiotic cross-resistance.[81,83]

For example, in *E. coli*, adaptation to BZK induced cross-tolerance to CHX and cross-resistance to the antibiotic chloramphenicol via efflux.[255] In *Klebsiella pneumoniae*, adaptation to CHX via overexpression of efflux pump SmvA concomitantly led to colistin resistance in five of six isolates.[207] Efflux has also been suggested to mediate cross-tolerance to OCT and to ciprofloxacin and chloramphenicol in *Enterobacter* spp.[217] TRI exposure has repeatedly been associated with antibiotic resistance via induction of RND efflux pumps, such as in *E. coli*,[136] *S. maltophilia*,[163] *S. enterica*,[248] and *P. aeruginosa*.[208]

ETH and GP, the other biocidal compounds tested in our study, have to our knowledge not been associated with induction of antibiotic resistance.[248]

The laboratory knockout strain *A. baumannii* ATCC 19606 Δ*adeRS* was more susceptible to various antibiotics, such as amikacin, azithromycin and last-resort compounds meropenem and tigecycline in a previous study by Lucaßen et al.,[69] and showed increased susceptibility or increased killing to several biocides (BZK, CHX, GP, OCT) in our study. As the regulator mutation leading to lack of AdeABC expression is the only genetic difference between the two strains, it is likely that absence of AdeABC efflux caused 'cross-susceptibility' to biocides and antibiotics. In clinical isolate pair 1, insertion of IS*Aba1* in *adeS* causing AdeABC overexpression could also improve the survival to these biocides, and was found in a previous

study to cause increased resistance to antibiotics such as amikacin, chloramphenicol, erythromycin, gentamicin, levofloxacin and tigecycline,[76] which supports the cross-tolerance effect of AdeABC.

In a study by Fernando et al., laboratory adaptation to TRI in *A. baumannii* ATCC 17978 led to overexpression of AdeIJK, which caused cross-resistance to numerous antibiotics including piperacillin-tazobactam, doxycycline, moxifloxacin and tigecycline.[204] Regarding the *A. baumannii* strains used in our study, Gerson et al. found that the strain overexpressing AdeIJK in clinical isolate pair 3 due to an IS*Aba1* insertion in *adeN*, MB-273, showed decreased susceptibility to tigecycline, minocycline, ciprofloxacin and meropenem.[76] We did not however detect efflux-mediated cross-tolerance to biocides in this strain. Regarding the laboratory knockout strain *A. baumannii* ATCC 19606 Δ*adeN*, Gerson et al. showed that overexpression of AdeIJK caused a 2-fold MIC decrease for antibiotics such as ciprofloxacin, erythromycin, minocycline, rifampicin and tigecycline.[77] We found that AdeIJK overexpression in this strain led to increased survival in the time-kill curves of some biocides (BZK, CHX, OCT) although not causing biocide MIC changes, suggesting that AdeIJK may contribute in a strain-specific manner to a certain level of biocide and antibiotic cross-tolerance.

Our study thus confirms that increased survival to the biocides BZK, CHX, and OCT and cross-tolerance to antibiotic compounds can be mediated by efflux pumps, as has been suggested for these compounds in other studies mentioned above. The results of our study further suggest the potential of efflux pump AdeABC to confer cross-tolerance to GP and antibiotics. Efflux pumps AdeABC and AdeIJK were however not found to mediate cross-tolerance to TRI and antibiotics in our study, contrary to the role of efflux in cross-tolerance to TRI and antibiotics in other studies cited above.[204,208,248]

4.8. Residual biocide concentrations and the need for biocide stewardship

Most biocidal compounds in our study were tested at concentrations that were fairly below the in-use concentrations for antiseptic or disinfection ends, except for ETH, where the concentration tested (24.5 vol%) was less than a 3-fold dilution from the lower limit of in-use concentrations (60–95 vol%). As such, in time-kill assays, we tested concentrations around the MIC level, ranging from 0.125 x MIC to 4 x MIC, apart for TRI, where concentrations were higher (up to 64 mg/L) but still notably lower than in-use concentrations (>1000 mg/L). In-use concentrations have generally been recommended to exceed 100–1000 times the MIC.[256] This is comprehensible by the fact that according to testing procedures, in-use concentrations of disinfectants and antiseptics for use in human medicine have to cause a 5 log-fold CFU reduction (resp. 3 log-fold CFU reduction for hygienic hand wash products) of a bacterial inoculum within 1 or 5 minutes, or within 60 minutes for surface disinfectants.[257] In contrast, MICs correspond to the lowest biocidal concentration with absence of visible bacterial growth

after overnight incubation.[258] This corresponds to ≤3 log-fold CFU reduction (as the minimum bactericidal concentration (MBC) corresponds to a 3 log-fold reduction, and the MBC is equal or higher to the MIC) within 16–20 h, which can be achieved with a lower concentration of biocide. Bacterial strains might thus display decreased susceptibility to a biocide as indicated by MIC increases without being clinically resistant.

While it is reassuring that in-use concentrations of biocides are still effective, increased survival or tolerance to lower concentrations is also clinically relevant, as it can contribute to the spread of pathogens in environments with low-level biocide exposure, such as in clinical settings and natural environments. Common factors favouring the appearance of lower biocide concentrations in the hospital setting include inappropriate application of biocidal products, overdilution with water or other solvents, application on wet surfaces, or spreading a limited amount of biocide on a bigger surface, which reduces the effective amount of biocide per surface unit.[259] With regard to the latter, Gebel et al. showed that the application of a sufficient volume of isopropanol at 70 vol% is primordial to guarantee its biocidal efficacy, and incomplete killing of *E. faecium* in a previous study was attributed to an insufficient volume of the biocide.[237] In antiseptic mouth washes or antiseptic wound products, the biocidal compound can be diluted with saliva or serum, blood or pus, and the agent also interacts with organic material, which may reduce the effective concentration. Extensive biocide usage in the hospital setting can further favour the occurrence of low biocide “background concentrations”, as has been suggested by Edwards et al.[238]

Furthermore, biocidal compounds are used at lower concentrations in a multitude of applications other than those for proper disinfection or antisepsis ends, such as in preservatives. As a preservative in ophthalmic solutions, BZK can be used at concentrations as low as 100 mg/L, while the concentration of CHX in catheter maintenance solutions reaches 150 mg/L, which is less than 10-fold the concentrations we tested.[93,101] Biocides are also being extensively used at low concentrations in hundreds of consumer products, such as mattresses, curtains, cleaning devices, or cutting boards.[260] Chen et al. described that TRI is gradually released at low concentrations from some plastic and fabric materials, and that residual TRI concentrations remain on surfaces and are stable in the environment.[193]

As a result of their extensive use, residual concentrations of biocides can further be found in many natural environments**.** Residual concentrations of BZK have been detected in hospital wastewater effluents, ground water and soil samples.[92,100,261,262] In an analysis by the European Food Safety Authority from 2013, BZK was found in 3.5% of food samples.[263] TRI is also a ubiquitous compound, and low concentrations can be detected on a global scale in environments such as sewage treatment plants, rivers, lakes, sediments, soil, groundwater and drinking water,[134,264] and in living organisms, including seafood and humans, where residues have been found in samples of urine, breast milk, and serum.[129,135] Low

concentrations of CHX have also been detected in sewage treatment plants and in their effluent water, meaning that CHX is further released into aquatic environments.[265] Numerous other biocides, including ETH, have also been found in hospital wastewater effluents.[266]

Due to dilutive effects in the clinical setting and because of the presence of low biocide concentrations in many consumer products or as residues in natural environments, it is thus likely that bacteria encounter a wide range of biocide concentrations, including the lower concentrations we tested.

The presence of low-level or subinhibitory biocide concentrations can have problematic consequences. On the one hand, there is a toxicity issue, as low-level biocide concentrations can have detrimental effects on natural environments and living organisms. For example, BZK, CHX and TRI can have toxic effects on aquatic environments and aquatic organisms.[92,99,134,265]

Uptake of residual biocide concentrations may also have a certain toxicity potential in humans. For example, TRI has endocrine disruptor effects and was associated with negatively affecting the hypothalamic-pituitary-thyroid axis as well as with allergen sensitisation,[267,268] while CHX could interfere with sex hormone receptor pathways.[265]

On the other hand, low or residual concentrations of biocides can select for strains with reduced biocide susceptibility, and possibly also for decreased susceptibility to other antimicrobial compounds including antibiotics, as has been discussed in previous chapters.

Concerns have therefore been raised among scientists and policy makers that the extensive and increasing usage of biocidal products in healthcare, consumer products, sewage treatment, and food and agricultural industries may further aggravate the current global public health threat of antimicrobial resistance.[78] The increase in biocide use since the beginning of the SARS-CoV-2 pandemic has further enhanced these concerns. [90,101]

Because of these considerations, some authors have called for the establishment of an antiseptic stewardship initiative to counter biocide overuse.[85,269]

Stewardship of biocides implies that their use should be optimised and that they should only be applied when their benefit outweighs potential risks.[269] The authors especially emphasize that indiscriminate use of biocides in consumer products should be prevented, as there is often no proof for an additional health benefit of the biocidal agent, whereas there are risks for antimicrobial tolerance development. In the same regard, demands include that alcohol-based hand rubs and skin antiseptics shall not be supplemented with other biocidal compounds if they are not proven to confer additional health benefit.[269] For example, the supplementation of alcohol-based hand rubs with BZK, CHX, OCT, TRI, hydrogen peroxide, polyhexanide or peracetic acid did not lead to a better antimicrobial activity,[270] nor to proven health benefits, and is therefore not recommended by WHO guidelines.[271]

For surface disinfection, peracetic acid, hydrogen peroxide or sodium hypochlorite should be preferred over benzalkonium chloride due to lower selective pressure.

As a current example of biocide overuse, some have criticised the indiscriminate usage of surface disinfection for prevention of SARS-CoV-2 transmission, especially after it has been shown that transmission of the virus via contaminated surfaces in public settings is uncommon.[88,272,273] Reconsideration of common disinfection practices within and beyond the scope of the SARS-CoV-2 pandemic with a bigger emphasis on the antimicrobial stewardship perspective might be beneficial.

Further, correct application of biocides is necessary to achieve full biocidal efficacy and should be warranted at every usage. Application according to the manufacturer's instructions shall prevent the application of lower, ineffective concentrations. Important factors are adequate contact times and appropriate contact between the biocidal product and the target surface, prior cleaning of the concerned surface to remove organic dirt, and also the choice of an appropriate biocidal product, as some pathogens display intrinsic resistance to certain biocides.[139,229] The emergence of resistance to in-use biocide concentrations and contamination of biocidal products by contaminating bacteria was indeed mostly due to errors in the product application process, such as preparation with unsterile tap water, or insufficient processing of reusable tissue dispensers, which enabled bacterial adaptation and formation of biocide-resistant biofilms.[153,229] Indeed, improper application of biocides has also permitted the selection and spread of *A. baumannii* in the hospital setting.[274]

When applied correctly and at concentrations recommended for antisepsis or disinfection, approved biocidal products are still highly effective against bacteria, including *A. baumannii,* and remain an indispensable tool in preventing the emergence and dissemination of pathogens in clinical settings.[139]

4.9. Efflux pumps: target site for new antimicrobials?

Considering the contribution of efflux pumps to the spread of antimicrobial resistance, targeting the inhibition of efflux pumps in the development of much-needed new antimicrobial drugs seems a promising idea. Efflux pump inhibitors (EPIs), i.e., chemical compounds that inhibit the activity of efflux proteins, have been shown to restore susceptibility to different antibiotics in various in vitro experiments.[275] For example, in *A. baumannii*, phenylalanine-arginine β-naphthylamide (PAβN) increased susceptibility to rifampicin and clarithromycin, and 1-(1-napthylmethyl)-piperazine (NMP) increased susceptibility to linezolid, chloramphenicol and tetracycline.[276] EPIs are being studied with regard to serving as antimicrobial adjuvants, which could be co-administered with specific antimicrobial compounds to safeguard the antimicrobial efficacy of the latter and prevent the selection of resistant bacteria.[277] However, to date, no EPI has successfully passed clinical trials and been released on the market, although various synthetic and natural compounds show promising effectiveness in inhibiting efflux pumps.[277] This is mainly due to toxicity concerns.[277] Indeed, as efflux pumps confer important

physiological functions not only in bacteria, but also in mammalian cells, more general or broader EPIs can also show toxic effects on the host. NMP acts as a serotonin agonist, while PaβN causes renal toxicity.[275] Reserpine, the first identified plant-derived EPI, is neurotoxic.[277] Research is advancing though, and EPIs with more specific targets are being investigated.[277] Zimmermann et al. identified an approved drug, the tyrosine kinase inhibitor nilotinib, which worked in vitro as an inhibitor of the *S. aureus* efflux pump NorA, and decreased ciprofloxacin MIC 2- to 4-fold in a strain-dependent manner and at clinically achievable plasma concentrations.[278] However, promising results with EPIs in in vitro experiments cannot directly be transferred to in vivo conditions, and it is not yet feasible to co-administer EPIs with antibiotics as a therapeutic concept.

On the other hand, to prevent increased tolerance to biocides or even biocide resistance, adding EPIs to biocidal formulations might be a promising approach. Ethylenediaminetetraacetic acid (EDTA) has been proposed as a suitable EPI compound for these ends. In a study by Abriouel et al., subinhibitory concentrations of a biocidal product containing EDTA, lactic acid and hydrogen peroxide inhibited the expression of several multidrug efflux pumps in planktonic and biofilm cells.[279] This was attributed to the efflux-inhibiting effect of EDTA.[279] In another study, EDTA inhibited the expression of the MFS efflux pump EfrAB in *Enterococcus* spp. and therefore increased the bacterial susceptibility to CHX, TRI and to different antibiotics, which made the authors suggest to use EDTA as a food preservative.[227] In a study by Singkham-In et al., the natural compound resveratrol increased the susceptibility of carbapenem-resistant *A. baumannii* to CHX via inhibition of efflux pump AdeABC.[280]

EPIs have also been proposed to be employed as anti-biofilm agents, as they have been suggested to eradicate bacterial biofilms and consequently increase susceptibility to both antibiotics and biocides.[281]

As stated above, the uptake of EPIs within the host co-administered with an antibiotic formulation has a certain toxicity potential. However, the toxicity potential of EPIs may be less when they are added to biocides due to lower systemic absorption. While antibiotics are often administered systemically via oral or intravenous application, antiseptics are only used for topical application on skin, mucous membranes, and wounds, which lowers their systemic absorption. Toxicity of EPIs may be even less of a concern for surface disinfectants than for antiseptics, which are applied on human tissue. Regarding antiseptics, product formulations that impede the penetration of the EPI into human cells may reduce their toxicity.

Further, in order to decrease antimicrobial tolerance via efflux inhibition, it has been suggested that targeting specific efflux pump regulatory pathways governing efflux pump expression might present a potential alternative to compounds directly acting on the pumps.[275] This may allow the inhibition of specific bacterial efflux pumps without interfering with physiologically relevant efflux pumps within the host organism. AdeRS is a two-component regulatory system,

which have generally been proposed as promising bacterial drug targets.[282] In particular, AdeRS has been suggested as a target for new drugs against MDR *A. baumannii*.[283]

On that account, new findings about the interplay between regulatory systems, efflux pump expression and antimicrobial susceptibility are also relevant for the development of new antimicrobial agents. Our work focused on efflux pump regulatory systems, and we showed that targeting the *A. baumannii* efflux pump regulators *adeRS* and *adeN* displays a potential impact on survival to biocide exposure. These regulatory systems might therefore be potential targets to overcome RND-efflux mediated biocide tolerance.

4.10. Outlook

Reduced susceptibility to biocides is currently found in numerous bacterial isolates across species and may become an even more important clinical challenge in the future. To address this issue, a multifactorial approach is required.

On the one hand, more research is needed to gain a better understanding of biocide tolerance in bacteria. Further transcriptomic and proteomic analyses of the *A. baumannii* response to biocide exposure should be carried out to get a deeper insight into the induction of biocide tolerance mechanisms. This may eventually facilitate the development of compounds that are less prone to be affected by bacterial tolerance mechanisms such as efflux pumps, and for which increased bacterial survival would be less likely to occur. Efflux pump inhibitors may be promising targets in this regard and should be further investigated.

On the other hand, there is a need for raising awareness of the importance of appropriate biocide application to counteract the emergence of biocide tolerance and potential antibiotic cross-resistance. This includes using biocides at recommended concentrations and preventing overuse of biocides at low concentrations in consumer products. To this end, in analogy to antibiotic stewardship, biocide stewardship initiatives should be considered.

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