Ministry of Environment of Denmark
Environmental Protection Agency

The role of quaternary ammonium compounds and their degradation products in selection of bacterial resistance

Pesticide Research no. 238
June 2026

Publisher: The Danish Environmental Protection Agency

Authors: Skive, Bolette[1]; Mongelli, Andrea[2]; Bester, Kai[2]; Ingmer, Hanne[1]

[1]Section Food Safety and Zoonoses, Department of Veterinary and Animal Sciences,
Faculty of Health and Medical Sciences, University of Copenhagen
[2]Dept for Environmental Science, Center for Advanced Water Purification, Aarhus University

ISBN: 978-87-7564-097-3

Contents

Sammenfatning

Biocider anvendes overalt i samfundet til desinfektion og i rengøringsprodukter. Ved brug kan de aktive stoffer i biocider ende i affald, spildevand, eller i jorden, hvor de kan være til stede enten som intakte forbindelser eller som nedbrydningsprodukter. Disse nedbrydningsprodukter vil være et resultatet af miljøforhold som påvirker omsætningen af biocider såsom UV, temperatur, pH eller vil være metabolitter opstået i omsætningen medieret af bakterier. I lyset af at der findes miljøreservoirer, hvor biocider og deres metabolitter kan være til stede i længere perioder, er et centralt spørgsmål, hvorvidt nedbrydningsprodukter af biocider stadig har en bakterie-dræbende effekt eller vil selektere for bakterier, der har nedsat biocid-følsomhed.
I dette projekt har vi fokusseret på nedbrydningsprodukter af kvaternære ammonium forbindelse (QAC) af denne klasse er der to aktivstoffer reguleret i biociddirektivet: 1) Alkyl (C12-16) dimethylbenzyl ammonium chloride (ADBAC/BKC (C12-16)) og 2) DDAC (Didecyldimethylammonium chlorid). Vores fokus var på nedbrydningsprodukter af BAC 12 som er denne ingredienz med højeste koncentrationer i 1).
I dette projekt har vi undersøgt nedbrydningen af den kvaternære ammonium forbindelse (QAC) benzalkoniumchlorid, BAC-12 under miljøforhold og analyseret, hvorledes transformationsprodukter af BAC-12 påvirker to bakterielle patogener, nemlig *Staphylococcus aureus* og *Staphylococcus epidermidis*, der bærer *qacA*-genet, som koder for QAC tolerance. Vi var i stand til at isolere fire transformationsprodukter og derudover indkøbe yderligere otte produkter til test. Med disse transformationsprodukter viste vi, at mens de oprindelige BAC-12 og DDAC (Didecyldimethylammonium chlorid) generelt selekterede for bakterier, der bærer *qacA* sammenlignet med celler, som ikke bærer genet, var vi ikke i stand til at demonstrere selektion af *qacA*-holdige celler som følge af transformationsprodukterne. Yderligere viste vi, at stafylokokkerne er i stand til at metabolisere BAC-12, uanset om cellerne bærer *qacA* eller ej. Der er flere begrænsninger i vores undersøgelse. For det første blev kun en enkelt koncentration af transformations-produkterne undersøgt, og vi oplevede variabilitet i vores analyser. Desuden kan de undersøgte bakteriers metaboliske aktivitet have været påvirket af bestemmelserne af de minimale hæmmende koncentrationer. Fremtidige undersøgelser bør derfor rettes mod udvikling af forbedrede assays med højere kapacitet til vurdering af QAC-transformations produkters betydning for selektion af *qacA*-holdige celler, måske endda under miljøforhold, og mod mere detaljerede undersøgelser hvorledes bakteriel metabolisk aktivitet påvirker omsætningen af QAC'er og dermed selektion af biocid-tolerance gener såsom *qacA*.

Resume

Biocides are widely used in society as disinfectants and in cleaning products. Upon use, the active components of biocides may end up in sewage, wastewater or in the soil where they can be present either as parent compounds or degradation products. These degradation products result from exposure to environmental conditions e.g. UV, temperature, pH etc, or be metabolites in the turnover mediated by bacteria. Given that there are environmental reservoirs where biocides and their metabolites may be present for extended periods of time a key question is if degradation products of biocides still are able to kill bacteria or select bacteria that have decreased biocide susceptibility.

In this project we focussed on the degradation products of quarternary ammonium compounds (QAC). Two of these compounds are registered in the biocidal products directive (Commission Regulation (EU) No 1119/2014): 1) Alkyl (C12-16) dimethylbenzyl ammonium chloride (ADBAC/BKC (C12-16)) og 2) DDAC (Didecyldimethylammonium chlorid). Our focus was on degradation products of BAC 12 which is the main constituent of Alkyl (C12-16) dimethylbenzyl ammonium chloride (ADBAC/BKC (C12-16).

In this project we have examined the degradation of the quaternary ammonium compound (QAC) benzalkonium chloride, benzalkonium with an alkyl chain of 12 C atoms (BAC-12,) in environmental conditions and investigated how transformation products of BAC-12 impact two bacterial pathogens namely *Staphylococcus aureus* and *Staphylococcus epidermidis*, carrying the *qacA* gene encoding tolerance to QACs. We were able to isolate four major transformation products and in addition purchase eight additional transformation products for testing. Our results show that while BAC-12 and DDAC (Didecyldimethylammonium chloride) mostly showed strong selection for the bacteria carrying *qacA* compared to cells not carrying the gene, we were unable to demonstrate selection of *qacA* containing cells by the transformation products. Further we show that Staphylococci may be able to metabolize BAC-12 irrespectively of whether cells carry qacA or not. There are several limitations in our study. Firstly, only a single concentration of the transformation products was assessed, and we experienced variability in our competition assays. Furthermore, the metabolic properties of examined bacteria could have been affected by the determinations of minimal inhibitory concentrations. Thus, future studies should be directed at developing improved and high throughput assays for assessment of QAC transformation products on selection of *qacA* containing cells, perhaps even under environmental conditions, and at depth assessments of the impact of bacterial metabolic activity on the turnover of QACs and in selecting biocide tolerance genes such as *qacA*.

1. Introduction

1.1 Background

Quaternary ammonium compounds (QACs, i.e., any compound that contains a quarternary ammonium group) are cations with the general structure $[NR_4]^+$, where R can be an Alkyl, Aryl or other organic group (Moss et al., 1995). Often these compounds such as benzalkonium compounds are used in mixtures containing the same structure with different alkyl chain lengths. A prominent active ingredient in the biocidal products directive is Alkyl (C12-16) dimethylbenzyl ammonium chloride (ADBAC/BKC (C12-16)) – with CAS.-no. 68424-85-1.
In Figure 1, the most prominent single compound BAC-12 of this active ingredient is shown..
QACs are most often used in the form of a salts and have a wide variety of applications, both industrial and domestic. The synthesis of these compounds via the quaternisation process (The treatment of a tertiary amine with RX and ROH to produce the salt NR_4X) has been long documented, with origins in the 1950s (Doub et al., 1954).

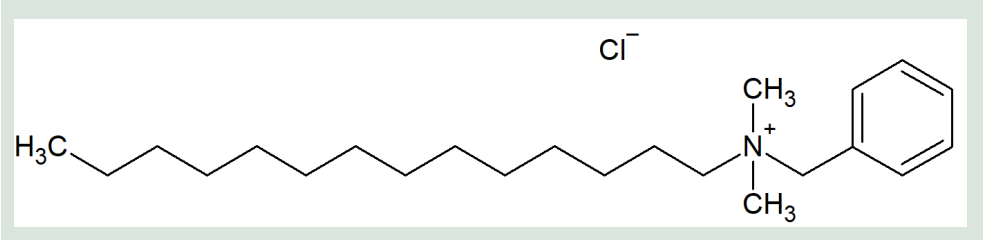


FIGURE 1. Benzyl dimethyl dodecyl ammonium chloride (BAC-12)

QACs are classified into several subclasses based on their chemical structure and alkyl chain length, including BACs (benzylalkyldimethyl ammonium compounds) and DDACs ((Didecyldimethylammonium chlorid) or more generally, dialkyldimethyl ammonium compounds). Each subclass is associated with distinct chemical properties and applications, ranging from disinfectants and preservatives to surfactants and biocides (Arnold et al., 2023) .
The use of QACs started considerably before research began into the impact of them on the environment (Li at al., 2009). Since then, the usefulness of these compounds has resulted in widespread use with numerous possibilities for entry into water systems including from domestic applications and runoff from industrial processes where QACs are being used for desinfection (Zhang et al., 2015; Paijnes et al., 2021). Subsequently QACs have been found to have negative effects on the health of ecosystems, being particularly toxic to various fish species (De Leo et al., 2020). Furthermore, there have been tentative links to the uses of QAC disinfectant and an increase in birth defects in laboratory mice (Maher et al. 2008). Aside from the effects on animals, another concern is the potential for QACs to select for antibiotic resistant bacteria (Hegstad et al., 2010). It is worth noting that often the same QAC compounds are subject to different EU regulation depending on the use, further obscuring the source of such compounds in the environment (**Commission Regulation (EU) No 1119/2014**).

1.1.1 Degradation of benzalkonium chloride

When used, BACs eventually enter sewage systems, wastewater, or soil, where they may persist as either parent compounds or degradation products. The term “degradation products” is used in this report synonymously with “transformation products”, thus, in the text they do not refer to different concepts. An overview of BAC-12 transformation products as used in this project wis shown in appendix 1.2. These degradation products form due to environmental factors such as UV exposure, , or as biotransformation products (metabolites) produced by bacterial

activity. Consequently, environmental reservoirs can act as long-term repositories for biocides and their metabolites, depending on their respective half-lives (Zhang et al., 2015).
The degradation of BACs in wastewater treatment plants is critical to prevent their environmental spread. Studies have shown high removal rates of BACs (over 99%) (Östman et al., 2018, Mongelli et al., 2024), primarily through sorption to sludge, with a portion potentially biodegraded (Ismail et al., 2010). However, BACs widespread use has raised concerns about its environmental impact and the development of microbial resistance (Hansen et al., 2007; Piddock et al., 2006; Oggioni et al., 2013; Maillard et al., 2022 and 2024) BAC biodegradation has been observed in single-strain catabolic experiments, but it can be inhibited at high concentrations (e.g., 100 mg/L BAC-12) (Khan et al., 2015, Khan et al., 2017). Initial degradation involves cleavage of the C-alkyl-N bond, forming benzyldimethyl amine (BDMA) and alkanaldehyde, which are further metabolized into simpler compounds like CO_2 and ammonia (Ertekin et al., 2017; Khan et al., 2015, Patrauchan et al., 2003; Tezel et al., 2011; Tezel et al., 2012; van Ginkel et al., 2004).
Pseudomonas species, such as *Pseudomonas aeruginosa* and Pseudomonas sp., have been extensively studied for their ability to degrade BAC and other QACs. The degradation process typically involves the following steps:

- Initial Uptake and Adaptation: Pseudomonas strains can adapt to high concentrations of BAC through physiological changes, such as the upregulation of efflux pumps and modifications in membrane charge to reduce BAC uptake (Kim et al., 2018) (Oh et al., 2014).

- Enzymatic Degradation: The primary step in BAC degradation is the dealkylation of the quaternary ammonium group, which is catalyzed by specific enzymes. For example, amine oxidase enzymes have been identified as critical for initiating BAC degradation, converting BAC into less toxic intermediates (Oh et al., 2014).

- Metabolic Pathways: The degradation intermediates are further processed through metabolic pathways, such as the tricarboxylic acid cycle, to produce carbon dioxide and water. This step is often facilitated by cooperative interactions among microbial communities, even in single-strain experiments (Ertekin et al., 2016) (Oh et al., 2014).

Research using Moving Bed Biofilm Reactors (MBBRs) with pure bacterial cultures (e.g., *Pseudomonas* sp.) has demonstrated effective BAC removal but lacked detailed metabolite pathways (Fortunato et al., 2019). Compared to conventional activated sludge (CAS) systems, MBBRs allow easier metabolite analysis due to biomass retention in biofilms (Li et al., 2023; Barwal et al., 2014 and 2017).
More recently, a deep study was conducted on BAC-12 and BAC-14 degradation in micro-MBBR experiment with a spontaneous biofilm developed from activate sludge inoculation. Parents compounds were fully degraded, and 33 metabolites were identified following the ω/α/β-pathway and the ω/β-pathway oxidation (Larsson et al. 2024).

1.1.2 Staphyloccocal tolerance towards QACs

One of the key determinants of tolerance to QACs in *S. aureus* is the *qacA* gene (Waasenaar et al., 2013). This gene encodes a multidrug efflux pump that confers reduced susceptibility to a variety of cationic antimicrobial compounds including chlorhexidine**.** The *qacA* gene is a genetic determinant that primarily resides on plasmids, although it has also been found integrated into the bacterial chromosome (Waasenaar et al., 2015). The presence of *qacA* on mobile genetic elements like plasmids is a critical factor in its dissemination among bacterial populations (LaBreck et al., 2020). Plasmids can be transferred between bacteria through horizontal gene transfer mechanisms such as conjugation, facilitating the rapid spread of resistance genes across different strains and even species. This mobility likely contributes significantly to

the increasing prevalence of antiseptic resistance in various environments (LaBreck et al., 2020).

The *qacA* gene encodes the QacA protein, a 514 amino acid multidrug efflux pump that belongs to the Major Facilitator Superfamily (MFS) which utilizes the proton motive force across the bacterial inner membrane to drive the export of substrates (Majumder et al., 2023). The predicted structure of the QacA protein features 14 transmembrane segments that span the bacterial cell membrane, forming a channel through which various compounds can be transported out of the cell. Specific regions within this structure, such as transmembrane helix segment and its flanking loop, as well as the extracellular helical hairpin loop are crucial for the structural and functional integrity of QacA, playing roles in substrate binding and transport (Majumder et al., 2023). Interestingly, the closely related QacB protein differs from QacA by only a single amino acid at residue and this seemingly minor change significantly impacts their substrate specificity, with QacB exhibiting reduced resistance to divalent cations (Mayer et al., 2001).

In addition to *qacA* and *qacB* that belong to the MFS efflux pump family, several other genes are designated as qac's namely *qacC*, *qacG*, *qacH* and *qacJ* that all belong to the small multidrug resistance family (SMR) (Waasenaar et al., 2015). Although these genes encode products with the potential to efflux QACs, *qacA* is by far the most prevalent (Mayer et al., 2001).

1.1.2.1 *qacA* and cross resistance

The *qacA* gene is commonly found on various plasmids within *S. aureus*, including the well-characterized pSK1 plasmid family (Meyer et al. 2001). The presence of *qacA* on conjugative plasmids provides direct evidence for its horizontal transfer between *Staphylococcus* strains and even to other genera (LaBreck et al., 2018). Furthermore, the *qacA* gene is often co-located with other antibiotic resistance genes on the same mobile genetic elements, contributing to the emergence of multidrug-resistant strains (Meyer et al., 2001). This physical linkage means that selection pressure from the use of either antiseptics or antibiotics can inadvertently select for resistance to both classes of compounds.

Another mode of cross-resistance involves efflux mediated by the *qacA* efflux pump. The efflux pump displays a remarkably broad substrate specificity, capable of recognizing and transporting a diverse range of antimicrobial compounds including the antibiotic, ethidium bromide and other DNA intercalating dyes as well as mono- and divalent cations (Brown and Skurray, 2001). This broad specificity means that bacteria expressing QacA can exhibit resistance to multiple different types of disinfectants and antiseptics commonly used in various healthcare and community settings.

1.1.2.2 Regulation of *qacA* gene expression by QacR

Regulation of *qacA* gene expression is achieved via the transacting repressor protein, QacR. QacR belongs to the TetR family of transcriptional repressor proteins, which all possess a helix-turn-helix DNA-binding domain at their N-terminal ends, and have highly divergent C-termini postulated to be involved in the binding of inducing compounds (Brown and Skurray, 2001). QacR specifically binds to an inverted repeat, IR1, which has been identified as the qacA operator region, and overlaps the identified promoter sequence for *qacA*. QacR has been shown to interact directly with a number of structurally dissimilar compounds and is activated by a conformational change that in some strains is constitutively in the "on" position accomplished via mutations (Takeuchi et al., 2019).

1.2 Project objectives

1) To examine if the degradation products of BAC-12 and low concentrations of BAC-12 select for bacteria carrying *qacA*
2) To determine if the metabolization of BAC-12 by environmental bacteria such as *Pseudomonas* lowers the concentration of BAC-12 and thus lowers the antimicrobial activity allowing for growth of pathogenic bacteria and selects for bacteria carrying *qacA*.
3) To determine if pathogenic bacteria carrying *qacA* or antibiotic resistance genes metabolize BAC-12.

1.3 Structure of report

Each work package will have its own section. Within these sections each experiment is described with material and methods and results. Each section will be summed up in a short discussion. In the end of the report an overall discussion will put the findings into scientific context and the report will end with a conclusion section as well as a perspective and limitations section.

2. Benzalkonium chloride degradation products

2.1 Degradation of BAC-12

The aim of this WP was to make metabolites of benzalkonium chlorides available for testing. To this end two strategies where followed:

a) Known metabolites that were commercially available were purchased.
b) Selected known compounds that where not commercially available, were produced by a bio-synthetical approach.

Nine known metabolites of BAC-12 were purchased from Sigma-Aldrich (St. Louis. MO. USA) (Appendix 1.2). Stock solutions were prepared in methanol (LiChrosolv gradient grade for liquid chromatography; Merck, Darmstadt, Germany) in DSMO (Sigma-Aldrich (St. Louis. MO. USA) and sterile water to test solubility.

Since previous degradation studies had revealed that microbial communities cleave the C-alkyl-N bond, forming metabolites like benzyldimethylamine (BDMA) and alkanaldehydes, which are further broken down (Larsson et al., 2024 Ertekin et al., 2017; Tezel et al., 2011), we selected four compounds not commercially available (Appendix 1.2) and produced them via MBBR incubation.

2.1.1 Materials and methods

2.1.1.1 MBBR based facility for biosynthesis

Due to multiple projects within the department of Enviromental Science of Aarhus University using MBBR, it was concluded that one lab reactor would be dedicated to this purpose (Figure 2 and Figure 3). The laboratory setup of the MBBR reactors closely mirrors the configuration of full-scale MBBRs used in wastewater treatment plants, maintaining comparable inflow and outflow rates as well as aeration conditions, which ensure consistent hydraulic retention times, substrate availability, and oxygen supply for microbial activity.

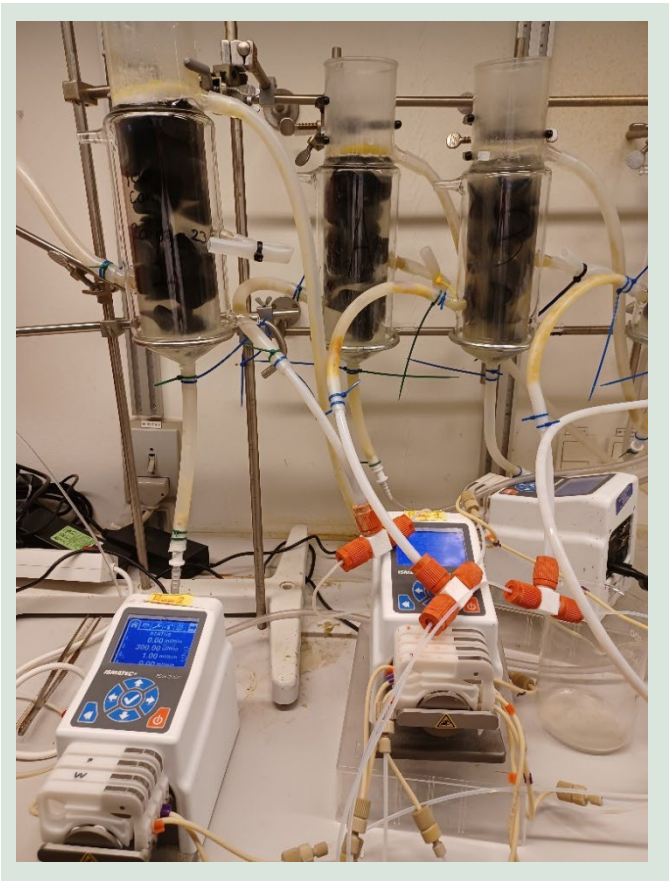


FIGURE 2. MBBR based facility for growing biofilms

Similar to the wastewater treatment plant set up, “feed materials” (Artificial wastewater solution and tap water) are pumped into the reactor at a constant rate. This then stimulates the growth of biomass on the carriers, moved around in the solution by the aeration originating from the bottom of the tank. The feed is also pumped in near the bottom to increase the retention time within the reactor before it leaves as excess via the waste tube at the top.

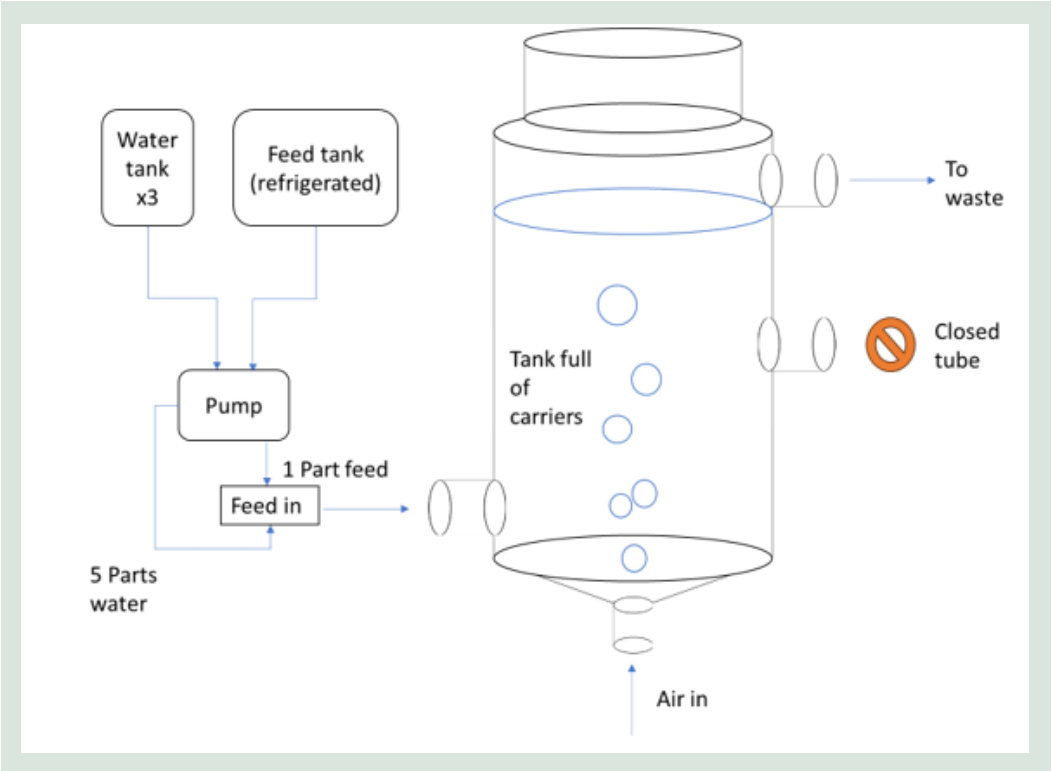


FIGURE 3. Diagram of the reserve reactors pictured in Figure 2.

Artificial wastewater for this project was prepared in a 10L tank with 100g of white sugar, 10g of yeast extract, 10g of ammonium bicarbonate and 1g of cellulose dissolved in 10L of deionised water. Other feed ratios have been successfully used in other projects within the department, particularly varying the nitrogen amount, but it should be noted that increasing cellulose content may lead to excess biomass and therefore a higher risk of the system getting blocked and overflowing. Before this mixture is pumped into the reactor, it is diluted with tap water at a T-piece connection for the PTFE tubing, using a Masterflex 4-channel peristatic pump to independently control both flow rates to achieve the preferred dilution of 1:5 and a total flow rate of 0.6 ml/min. The reason for using tap water to further simulate the conditions present in a wastewater treatment plant, with the feed mix only using deionized water to ensure all of the components stayed in solution.

FIGURE 4. AnoxKaldnes™ Z-MBBR MBBR Carriers.

These reactors will act as a reserve of carriers with an active biomass on. The carriers used (pictured in Figure 4) were selected as they have ample surface area for biomass to grow on and are easy to observe to see how much biomass they contained. Each reactor has 100 carriers in at all times, so if some are removed, new ones are used to replace them immediately. This gives the experiments described later in this report a safety net in case the conditions become too toxic for the bacterial cultures in the system,
The incubation stage was performed in a closed system in order to maximize the recovery of BAC-12 metabolites and to prevent their loss through the drainage pipe. This setup also ensured that no toxic compounds were released into the sewer system

2.1.1.2 Biosynthesis reactors

Based on Larsson et al., 2024, we scale up the production of BAC-12 transformation products to 1 L bioreactors (Figure 5).

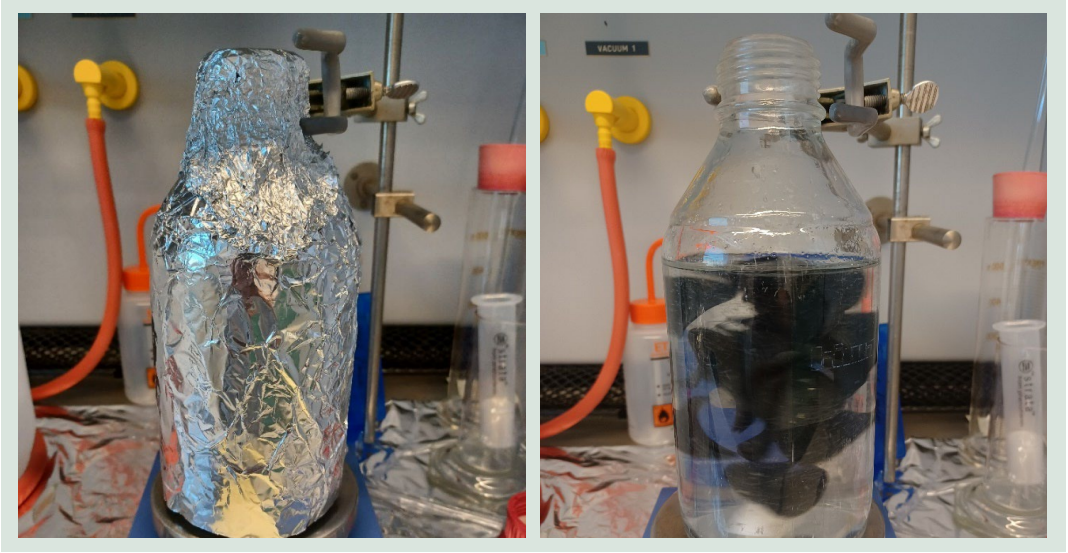


FIGURE 5. Biosynthesis reactors without and with foil covering respectively.

The closed reactor was set up using a 1L screw cap bottle on a stirrer plate, with a total of 50 carriers taken (approximately) equally between all 4 reactors with the hope that the more diverse the bacterial system was, the better the range of metabolites would be, and the more resistant the system would be to toxins). A magnetic stir bar was inserted along with 600ml of tap water, and the top of the bottle was capped with aluminium foil instead of a screw lid to promote some (albeit limited) aeration of the system. Instead of having a drainage tube, the system would be drained manually, with excess water being poured down the sink in the case of a finished feed cycle or extracted/ collected as chemical waste after a BAC-12 incubation cycle.

2.1.2 Results

After the adaptation time of 1 month the carriers in the reserve reactors developed a thick and homogeneous biofilm (Figure 6).

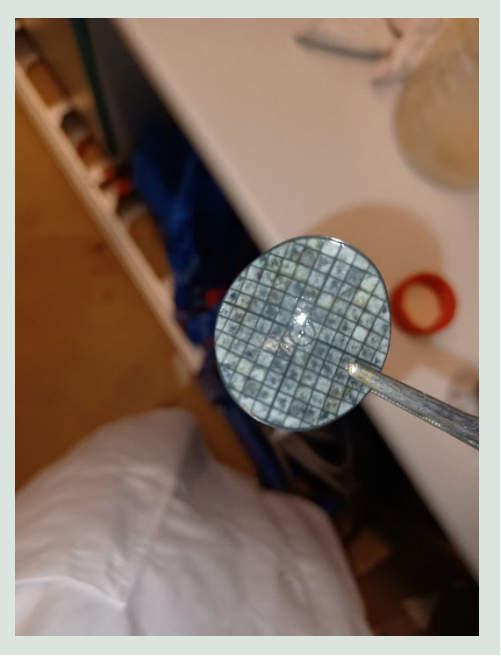


FIGURE 6. Biofilm growth on the virgin carriers.

Preliminary experiments indicate a good production of BAC-12 degradation products after 25-48 h from the start of the incubation (Figure 7).

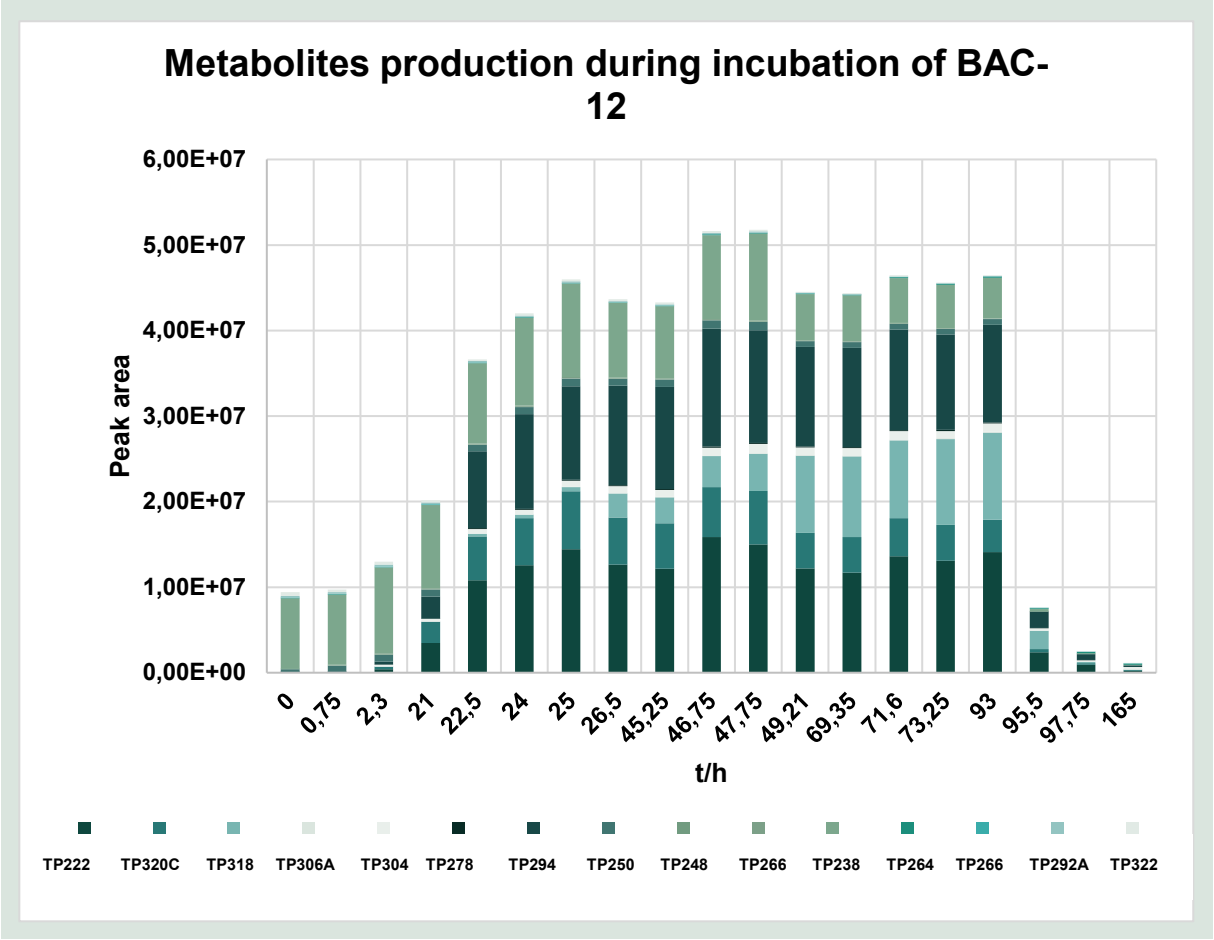


FIGURE 7. Plot of the metabolites production. Each bar represents the sum of the peak area of the BAC-1 degradation products during the time of the incubation.

By examining the time course of peak areas, we identified a suitable incubation time between ~25 and 45 hours, as all metabolites were present in this interval and most showed their highest abundance in this interval. A harvest time of 44 hours was selected, as it fell within this range and was convenient to integrate with the regular lab work schedule..

2.2 Up-concentrations and HPLC-isolation

2.2.1 Methods

Solid Phase Extraction

Solid phase extraction was performed on the 600 ml samples gained from incubation cycles, using a Phenomenex Gigatube Strata x 33um polymeric RP 5g/60mlnon-polar cartridge. The method for extraction for this was based off previous methods used in the department and consisted of:

1) Wash twice with an amount of methanol equal to the volume of the solid phase (the solid phase is nominally hydrophobic so methanol must be used before water can be)
2) Wash twice with 1 solid phase volume (SPV) of water (this allows re-equilibration so the metabolites can be caught in the solid phase).
3) Pass the entire sample through the cartridge in ~50 ml portions.
4) Wash once with 2 SPV of water (this helps remove any remaining metabolites from biomass caught at the top of the solid phase).

5) Elute metabolites into a new container using 50 ml of methanol with 5% formic acid (formic acid had been shown to better extract QACs out of the same solid phase during previous studies within the department).

It should be noted that the volume of the collected samples vary with the abundance of biomass in each sample. Samples with more biomass had less eluted volume due to blockages in the filter, so the samples had a volume between 40 and 50 ml (Figure 8).

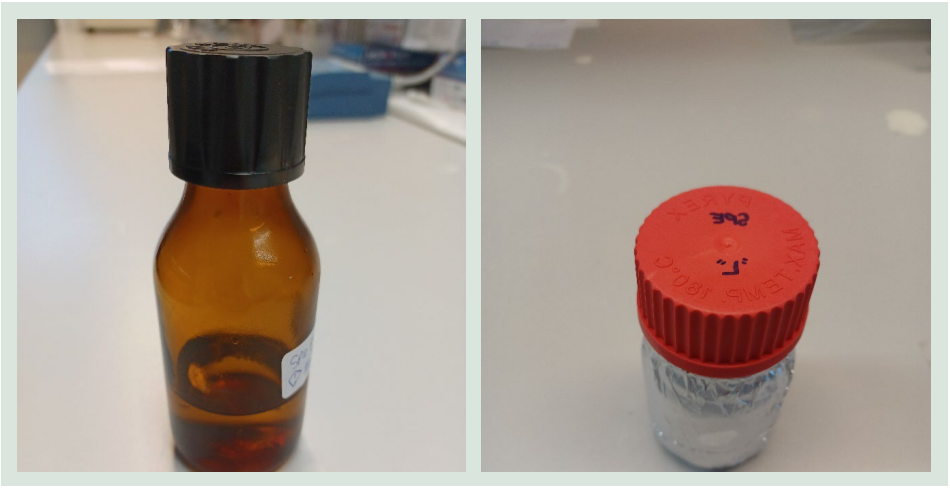


FIGURE 8. Solid Phase extraction (SPE) samples in brown glass bottle and 100ml screw cap with foil covering respectively.

The practical set up for this process initially involved draining the waste from the sample into a beaker via gravity, but this was quickly abandoned after the realisation that this would cause the extraction to require 12 hours of (intermittent) attention, which although possible, was highly inefficient. Instead, for the collection of the waste, a vacuum was set up as laid out in the figure below:

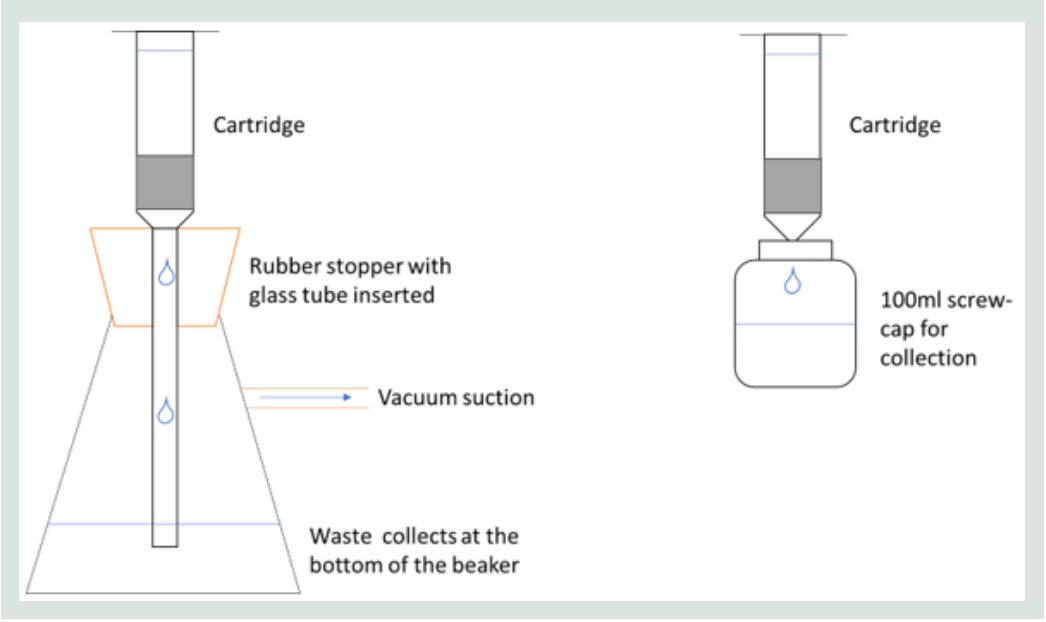


FIGURE 9. Set up for SPE, sample processing (left) and sample elution (right).

Due to the larger scale of this solid phase extraction (SPE) compared to the other SPE approachess used for purely analytical purposes, a set-up usually used for a Buchner funnel was

used, with the graduated bottom of the cartridge allowing a tight seal between it and the rubber stopper once under vacuum. The glass tube inserted into the stopper serves two purposes here: to stop liquid entering the vacuum system, and to minimise the possible contact of the rubber stopper with the system, although the syringe- like tip at the bottom of the cartridge was positioned such that it was not touching either. After the waste was collected in the vacuum flask, it was swapped out for a 100ml screw cap bottle (as above) and allowed to drain via gravity. As this happened both the collection bottle and cartridge were covered in aluminium foil to reduce contamination.

FIGURE 10. An example of the cartridge type used for SP

Sample Concentration

Samples were concentrated using a rotary evaporator. The evaporation process was performed under reduced pressure at a controlled water bath temperature of 45°C, ensuring the efficient removal of the solvent without degrading the target compounds. The concentrated samples were then transferred to glass vials and stored at -20°C until further analysis.

HPLC-isolation

The isolation of the BAC-12 degradation products was made on a HPLC-UV equipped with a fraction collector ((Dionex Ultimate 3000). The chromatographic separation was performed with a Synergy Polar-RP 80 Å (150 X 2 mm; Phenomenex Torrance, CA, USA). The HPLC gradient consisted of two mobile phases: Water (A) and Methanol (B) (Supelco, HPLC grade). The flow was set at 0.350 ml/min and the following gradient was used: 0-5 min 0% B; from 4 to 18 min 100% B with a linear ramp gradient; from 18 to 22.5 min 100 % B: from 22.5 to 23 min 0% B with a linear ramp gradient and from 23 to 28 min 0% B. The volume of injection was 50 µl. The collection of the fraction consisted of six 4-minute fractions, collected over the 25-minute run with ~30 seconds at the start and the end not collected.

2.2.2 Results

The base of the first metabolite detection method was built from the QAC detection method, keeping the chromatography settings the same as it allowed easy comparison of metabolite retention times to the parent compound (BAC-12). Further detection parameters were then added for the 19 searched for metabolites, according to Larsson et al., 2024.
Whereas the original QAC detection method provided a good basis for the analysis of the metabolites, the ability to separate such substances was found to be somewhat limited.

Many metabolites effectively co-elute, most likely due to their similar structures. Peak clusters that have more than 2 signals indicateco-elution of several metabolites. The aim of the metabolite separation method is therefore not to isolate all peaks but to identify several metabolites than can be isolated with the resources available in the project.
With our chromatographic technique we were able to isolate 4 transformation products (TPs), i.e, TP250, TP278, TP320C and TP 318 (Figure 11). (compare appendix 1.2 and 1.3

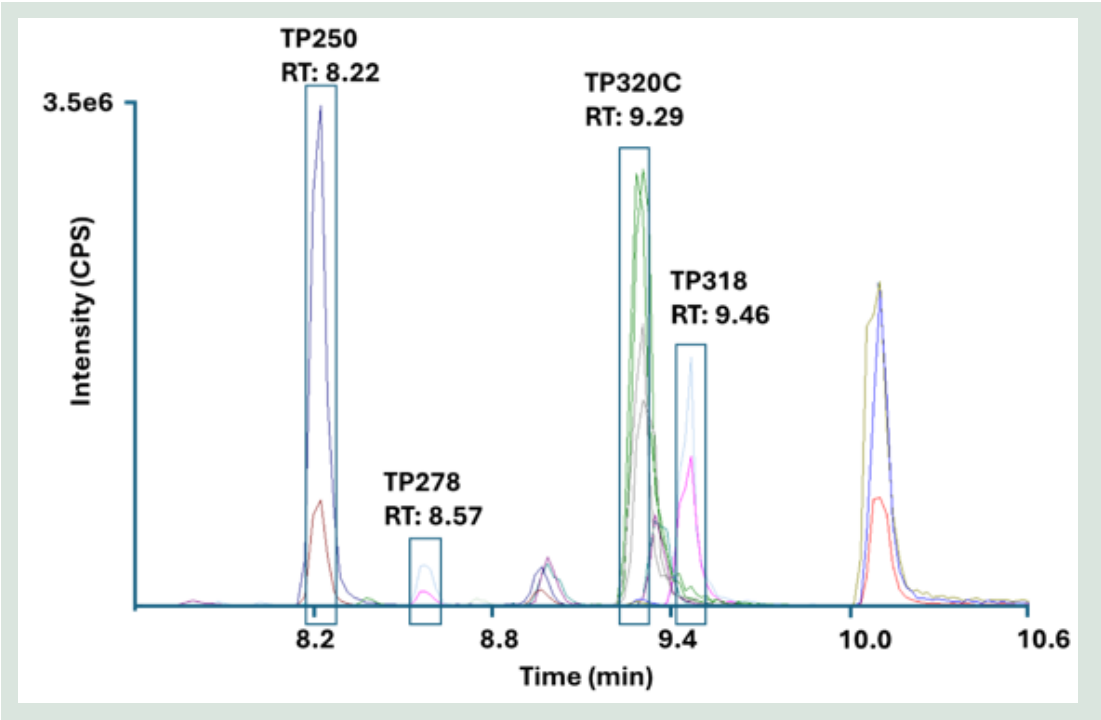
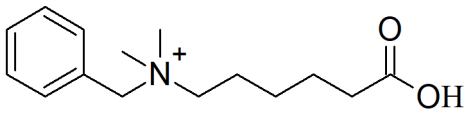
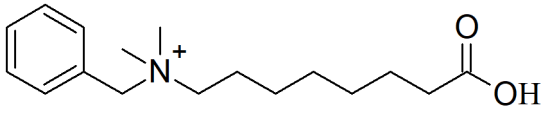
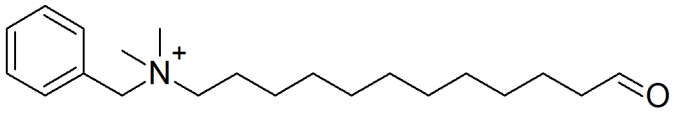
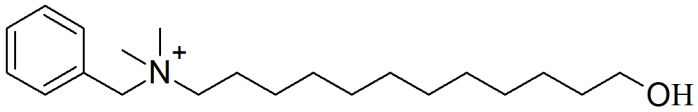


FIGURE 11. Chromatogram for the reversed phase chromatographic separation of TPs produced by BAC-12 metabolization in MBBR experiments. The four TPs highlighted are the ones that we have been able to isolate.

In order to verify the quality of the fractionation method and identify which BAC-12 transformation products have been successfully isolated, an High Resolution Mass Spectrometry non-target analysis method was used (Larsson et al., 2024) on the faction collected (Table 1).

TABLE 1. Name and molecular formula of the BAC-12 transformation products (TPs) isolated.

Compound	Molecular formula	Quantity produced (mg)
TP250		14.00
TP278		35.00
TP318		11.75
TP320C		8.75

From the analysis of the collected fractions, it is evident that four BAC-12 transformation products were successfully isolated. The isolated TPs suggest that the catabolic pathway involves ω oxidation followed by α- and β- -oxidation (Larsson et al., 2024) (Appendix 1.4). To obtain sufficient quantities of these TPs for toxicological tests, multiple cycles of HPLC were applied to the incubation-derived samples, yielding amounts ranging from 8.75 to 35 mg of TPs.

3. Staphylococcal strain collection and selection

3.1 Screening for *qac* gene containing strains

To screen for the presence of *qacA* and *smr/qacC* genes several stain collections and genome resources were analyzed. The collections included 54 clinical *Staphylococcus epidermidis (S. epidermidis)* strains from Hvidovre Hospital that had not been whole genome sequenced (Skovgaard et al. 2013), and 14 whole genome sequenced methicillin resistant *Staphylococcus aureus* (MRSA) from Statens Serum Institut (SSI) (Mikkelsen et al., 2023) were screened by polymerase chain reaction (PCR) to determine the presence or absence of plasmid carried *qac*-type genes. Included as controls for the PCR were 3 *Staphylococcus aureus* (*S. aureus*) strains from the Japan Collection of Microbes (JCM) with accession numbers JCM 16554 (*smr/qacC* positive strain); JCM 16555 (*qacA* positive strain); JCM 16556 (*qacB* positive strain) and one *smr* positive strain of unknown origin, HI3549. An overview of strains relevant to this report is shown in appendix 1.1.

In addition, genome sequences of MRSA strains and methicillin susceptible, MSSA strains from the Department of Clinical Microbiology, Hvidovre Hospital were screened for the presence of *qacA* genes. These sequences had been obtained on a MiSeq machine (Illumina) with a minimum mean depth of coverage of 30x.

.

3.1.1 Material and methods

Plasmid extraction

The *S. epidermidis* and *S. aureus* strains were streaked from -80°C onto tryptic soy agar (TSA) plates and grown overnight (ON) at 37°C. Liquid cultures were prepared by inoculating 2-3 colonies into 5 ml trypic soy broth (TSB) and grown ON at 37°C with 180 rpm shaking. Plasmid extractions were performed with GeneJET Plasmid Miniprep Kit (Thermo Fisher) according to manufactures instructions including a pre-incubation step for 30 min in a heating block 37°C, 180 rounds per minute (repm) shaking with lysostaphin at a final concentration of 1.5 µg/ml. DNA contents were measured on a spectrophotometer **(**NanoDrop, Thermo Scientific**)** giving an average of 29 µg/µl [CI 95%: 19 – 39].

PCR for *qacA* and *smr*

For *S. aureus* the template was 1-2 µl from the respective plasmid extraction into each PCR mixture with a final volume of 25 µl, containing 1 µl of each primer (stock conc. 10 µM), 12 µl 2x master mix (DreamTaq Green PCR Master Mix (2X), Thermo Fisher) and the rest nuclease-free water (Thermo Fisher). A pilot study showed that the PCR detection was possible as a multiplex PCR analysing for *qacA* and *smr* simultaneously. The primers used were as described by El Sayed Zaki et al (2018).

The PCR analysis of *S. epidermidis* strains was performed in the following cycles: Initially 95°C for 2 min x 1; then 40 cycles (denaturation at 94°C for 30 s, annealing at 53°C for 60 s, and extension at 70°C for 60 s), final 72°C for 10 min x1, while for *S. aureus* the following cycles were used: Initially 95°C for 3 min x1; then 30 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s). The primers used were as described by Noguchi et al. (2006) and Ribič et al., (2017). PCR products were analysed by agarose gel electrophoresis and strains carrying the target genes were included as a control.

Bioinformatic analysis and sequencing of plasmids

As *qacA* genes are likely to be found on plasmids, genome sequences were analyzed by PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/). Thus, genome sequence contigs were searched for the presence of both the *qacA* gene and a plasmid replicon. Taking this approach the search was narrowed down to 29 sequences where *qacA* gene was in the same contig as PlasmidFinder found to be a plasmid. To validate these findings, eight of the *S. aureus* strains identified in the initial screening as well as two *qacA* positive *S. epidermidis* strains from our PCR screening were genome sequenced using Nanopore technology (Oxford Nanopore). The *S. aureus* nanopore data were assembled with flye and medaka (https://www.ridom.de/u/Flye_Assembler.html) and then merged with the Illumina reads using unicycler. The *S. epidermidis* samples were assembled with flye and medaka, as only Nanopore data were available. Abricate (https://bio.tools/ABRicate) was used together with PlasmidFinder database to look for plasmid replicons in the assembled plasmids. Abricate and Resfinder (http://genepi.food.dtu.dk/resfinder) database were used to investigate the presence of antibiotic resistance genes. pLannotate (https://www.plannotate3.com/) was used to annotate other genes present in the plasmids and make a graphic representation of them.

3.1.2 Results

PCR for *qac* genes

The screening for *qac* genes by PCR of the plasmid extracted from the 54 *S. epidermidis* strains showed that 42% had the *qacA* gene, 30% the *smr* gene and 4 strains had both *qacA* and *smr*. PCR was performed on 14 of the MRSA from SSI, and as predicted from the genome sequence only one strain carried the *qacA* gene.

Sequenced and assembled *qacA* plasmids

qacA was carried together with its regulatory gene *qacR* on large plasmids (27-45 kb). Several isolates contained multiple different plasmids with multiple antibiotic resistance genes and heavy metal resistance genes. Some plasmids carried transfer genes indicating that they may be able to move via conjugation. Based on these results a plasmid of 31076 bp present in *S. epidermidis* strain HI3487 (named HI3487qac) was selected for further studies together with the *S. epidermidis* strain HI3534 (named HI3534no-qac) that did not contain *qacA* and displayed similar growth characteristics as HI3587 (data not shown). The map of the plasmid from HI3487 is shown in Figure 12.

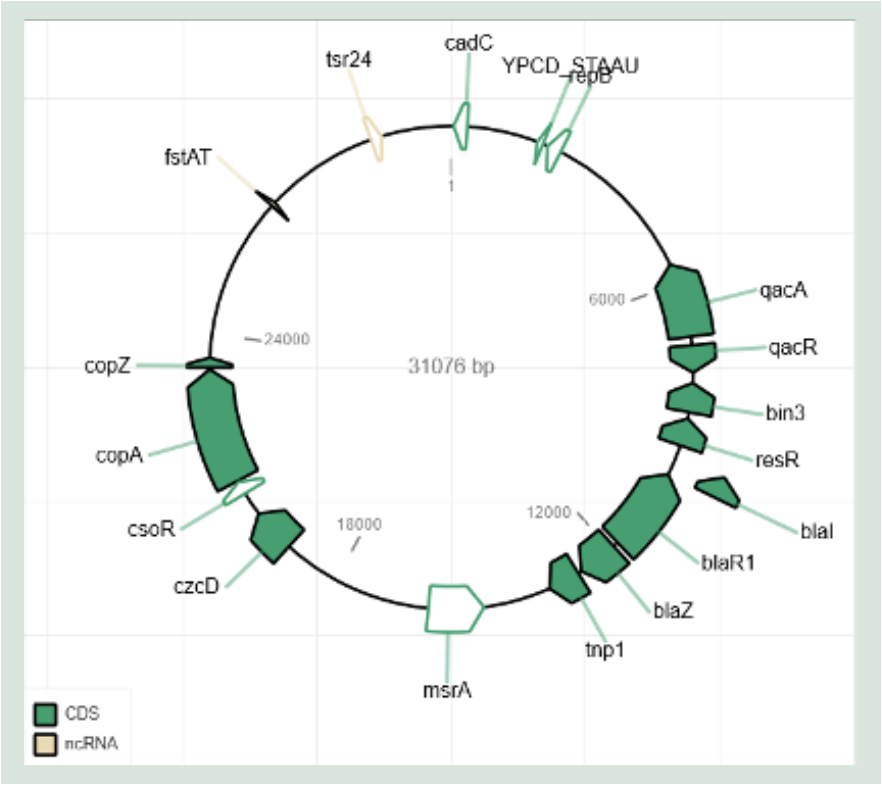


FIGURE 12. Plasmid map of the *qacA-qacR* containing plasmid in *S. epidermidis* strain HI3587. Highlighted in the map are genes for copper resistance (*cop*), cadmium resistance (*cad*) and a β-lactamase encoding β-lactam resistance (*blaZ*).

3.2 Construction of isogenic strain pairs

3.2.1 Material and methods

3.2.1.1 Short description of strains and methods

To create an isogenic pair of strains with near identical genetic background but differing in the presence or absence of *qacA*, several approaches were taken.

The first approach to create an isogenic strain pair in *S. aureus* was done by transformation to transfer the plasmid/plasmids from the SSI strain collection, into the *S. aureus* lab strain 8325-4, which is cured of all phages and plasmids, and is widely used as a prottypic *S.aureus* strain.

The second approach was to move a *qacA* carrying plasmid into *S. aureus* laboratory strains by phage transduction from either 8 *S. aureus* strains from Hvidovre hospital with the *S. aureus* phages Φ11 and ΦNM1 or from two *qacA* positive *S. epidermidis* strains (3486 and 3487) using the four *S. epidermidis* phages ΦIPLA6, ΦIPLA7, ϕPH15 and ϕCNPH82. As phage transduction efficacy is enhanced if the transducing phage is integrated in the genome of the strains from which DNA is to be transduced, the transducing phages were first attempted to be integrated in the strains carrying the *qacA* plasmids.

Thirdly we cloned the *qacA* gene and the *qacR* regulator into the pRAB12-*lacZ* by substituting *lacZ* with a *qacA-qacR* fragment in *Escherichia coli* K-12 strain IM08B. Plasmid DNA was retrieved from transformants and transformed into the clinically relevant *S. aureus* strain JE2 to obtain JE2 containing the gene of interest (JE2qac). As. Control, the reporter plasmid pRAB12-*lacZ* was transformed into the JE2 strain as well (JE2lacZ). This resulted in a strain pair with one strain carrying pRAB12-*lacZ* and the other pRAB12-*qacA/qacR* in strain JE2.

3.2.1.2 Detailed method descriptions

Electroporation-mediated transformation of wildtype *S. aureus* plasmid

Electrocompetent cells of the *S. aureus* lab strain 8325-4 was prepared according to previously described method by Monk and Steiner (2021) and the transformation was performed according to same reference with small modifications, in short. Thawed electrocompetent cells were centrifuged at 5,000 x g for 1 min. Supernatant discarded and cells resuspended in 50 µl of 10% glycerol including 500 mM sucrose. Purified plasmid from the MRSA 109868 (5 µl) was added to the cells and transferred to 0.1 cm electroporation cuvette (Biorad or MBP). Pulsed 21 kV/cm, 100 Ω and 25 uF. Time constant 2.0-2.4 ms. Immediately 1 ml of TSB including 500 mM sucrose was added, mixed gently by pipetting and transferred to a Eppendorf tube. Incubated at 37°C for 1-1.5 h and 100 µl plated out on TSB agar with selective antibiotic erythromycin 10 µg/ml. The remaining cells were concentrated (7000g for 5 min), resuspended in 100 µl TSB including 500 mM sucrose and plated on TSB agar with erythromycin 10 µg/ml and incubated overnight at 37 °C. As negative control, an aliquot of competent cells without plasmid DNA added was treated similarly.

Phage infection, induction and transduction

The receptor *S. aureus* and *S. epidermidis* strains mention in 3.2.1.1 were grown over night in TSB and diluted 1/50 with fresh TSB and grow to 1.4 $OD_{540.}$ $CaCl_2$ was then added to final conc. 4.4 mM. Phage lysates from appropriate phages mentioned in section 3.2.1.1 were obtained from our collection and 100 µl from serial dilutions was transferred to individual tubes to 1 ml recipient culture, incubated at 37°C for 20 min, and then added 3 ml TSB with 0.4% TSB agar (TSA) per tube and quickly plated out on TSA plates containing 17 mM sodium citrate. Incubated over night for 24 h at 37°C.

Two of the *S. aureus* strains (MS59, M7088) that showed positive phage infection with Φ11 were checked for integration of the phage by PCR detecting the *Sa5* gene. In short bacteria from the center of the plaques of the phage infection were re-streaked onto TSA plates. Two colonies per strain were picked into Eppendorf tubes with 50 µl MilliQ water and 5 µl lysostaphine and incubated for 30 min at 37°C followed by 10 min at 99°C to extract DNA.

These DNA templates were added 1-2 µl from each respective extraction into each PCR mixture with a final volume of 25 µl, containing 1 µl of each primer (stock conc. 10 µM), 12 µl 2x master mix (DreamTaq Green PCR Master Mix (2X), Thermo Fisher) and the rest nuclease-free water (Thermo Fisher). The PCR was performed in the following cycles: Initially 95°C for 5 min x 1; then 35 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s), final 72°C for 10 min x1. PCR products were kept at 4°C until they were run on agarose gel electrophoresis to show whether a PCR product of the expected size was present. The primer set for the Φ11 *Sa5* gene was: Sa5-F (AAAGATGCCAAACTAGCTG) and Sa5-R (CTTGTGGTTTTGTTCTGG) (product length, 375 bp). Positive control was phage Φ11 DNA and negative controls were the same *S. aureus* strains that had not been infected, and a reaction with water added instead of DNA template. Phage induction was performed to excise the integrated Φ11 and potentially package the *qacA* containing plasmid that via transduction could be transferred to a new cell. From cells carrying the *qacA* plasmid and the integrated phage Φ11, the phage was induced by mitomycin C (final concentration: 2 µg/ml; Stock concentration 1 mg/ml) at 32ºC until lysis of the cells (3-4 hr).

Cloning in know vector plasmid

To clone the *qacR-qacA* genes into a vector that replicates both in *S. aureus* and *E. coli*, the plasmid pRAB12-lacZ (Helle et al., 2011) was restricted with the enzymes *EcoRII* and *Pst1* to remove the *lacZ* gene that was replaced by the *qacR-qacA* genes obtained by PCR from strain MS59. After ligation, the plasmids were transformed into *E. coli* IM08B and selected on LB plates containing 100 ug/ml ampicillin. Transformants were screened by PCR for the presence of *qacA*/R. Plasmids from clones containing *qacA/R* were purified and transformed into electrocompetent JE2 and 4220 together with the empty vector.

.

3.2.2 Results

Despite major efforts to transfer *qacA* containing plasmids from the *S. aureus* strain collection we failed to obtain transconjugants both by transformation and conjugation. Also we were unable to move the plasmid by transduction presumably because the plasmids were too large to be carried between strains by the transducing phage. However, we did succeed to clone the *qacA-qacR* gene pair into the plasmid vector pRAB12-*lacZ* by substituting the *lacZ* gene with the *qacA-qacR* genes. Subsequently the resulting plasmid was designated pRAB12-*qacA* and was transformed into *S. aureus* JE2 together with the original plasmid pRAB12-*lacZ* that served as a control forming the strains JE2(qacA) and JE2(lacZ).

4. BAC12 and degradation products in selection of *qac* containing Staphylococci

4.1 Minimal inhibitory concentration (MIC) and sub-inhibitory MIC

To be able to answer the project objective 1 namely whether: "The degradation products of BAC and low concentrations of BAC select for bacteria carrying *qac* genes" we aimed to determine the minimal inhibitory concentration (MIC) and subinhibitory MIC (sub-MIC) as defined below for the strains in the strain collection to later be able to co-culture these strains under sub-MIC concentrations of QACs to investigate if these conditions would *qac*-containing strains an advantage over the non-*qac*-containing strains.

4.1.1 Material and methods

The strains selected for the analysis were the *S. aureus* strain pair JE2(qac) and JE2(lacZ) carrying the *qacR-qacA* genes and *lacZ*, respectively. For *S. epidermidis,* the strains investigated were the HI3487qac and HI3534no-qac.

Susceptibility testing

The strains were grown overnight in TSB and diluted down to an optical density (OD) at 600 nm of 0.001, which corresponded to approximately $1x10^6$ colony forming units (CFU)/ml. The inoculum was further diluted a factor 2 when added to media containing the compound of interest and thus had the final inoculum size recommended for MIC testing (https://clsi.org/). The compounds were diluted in TSB from stock. For BAC-12 the concentrations tested were 2-fold dilutions from 8 mg/l to 0.125 mg/l. The transformation products were tested in 2-fold dilutions in concentrations from 8000 mg/l to 125 mg/l. All compounds were tested in technical duplicates and some in biological duplicates as well. Similarly, the susceptibility of HI3487qac and HI3534no-qac to antibiotics was tested for tetracycline (TET), gentamycin (GEN) and erythromycin (ERY).

The experiments were performed in a BioScreen plate reader using 100 well plates with a final volume of 300 µl/well. The incubation was at 37°C degrees with continuous shaking at medium amplitude and optical density was read every 10 min for 24 h.

4.1.2 Results

The sub-MIC (sub-MIC) was defined as the highest concentration of the compound that allowed growth of the strain at a similar level as the growth observed when no compound was present (control) as shown in Figure 13.

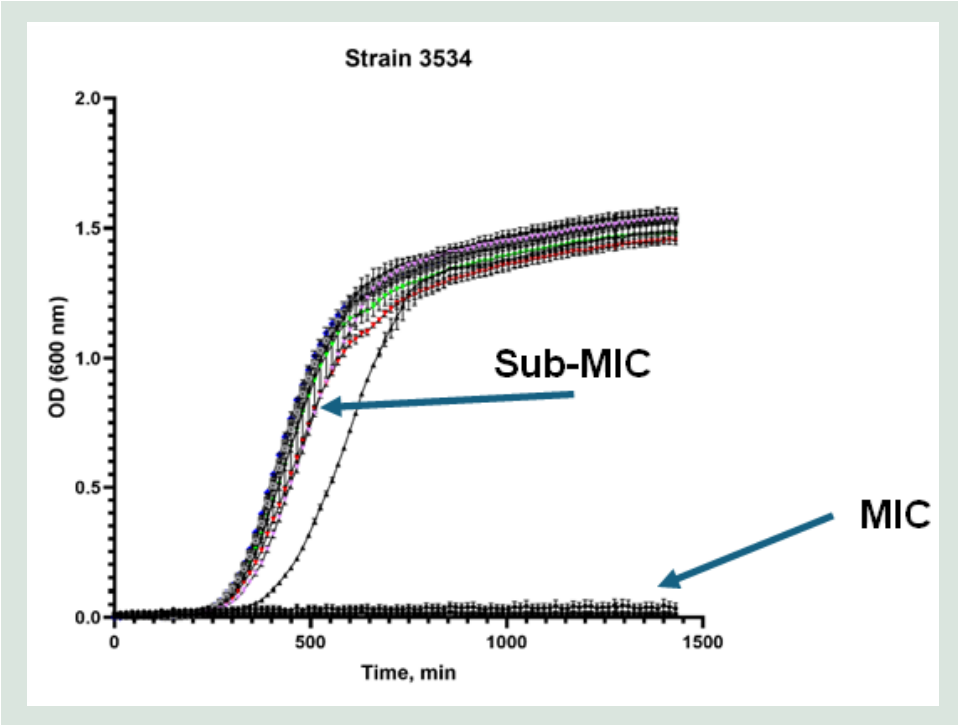


FIGURE 13. Definition of Sub-MIC and MIC concentrations based on growth in Bioscreen plate reader of strain HI3534no-qac.

The two strain pairs *S. aureus* (JE2qac and JE2lacZ) and *S. epidermidis*, HI3487(qac) and HI3534(no-qac) were the ones that were carried further to the co-culture experiments; thus those are the only for which MIC's and sub-MIC's were investigated for all compounds and thus reported. Importantly, the strain pair HI3487qac and HI3534no-qac differed in susceptibility to antibiotics making it possible to select HI3487(qac) and count the number of cells on agar plates with gentamycin while HI3534(no-qac) could enumerated on agar plates with erythromycin (Table 2).

TABLE 2. Antibiotic susceptibilities of strains HI3487qac and HI3534no-qac.

Species	ID	qacA	qacC (smr)	BAC-12 µg/ml	TET µg/ml	GEN µg/ml	ERY µg/ml
S. epidermidis HI3487	**+ qac**	***Yes***	***Yes***	8	2	**>32**	4-8
S. epidermidis HI3534	**- qac**	No	No	1-2	< 0.75	1	**>180**

The susceptibilities towards the parent compounds BAC-12 and DDAC are shown in Table 3 below. Interestingly, the *qacA*-containing strains were clearly less susceptible towards the parent compounds compared to the non-*qac* strains, with a 4-fold to 16-fold difference, which confirms the important role of the *qacA* gene for bacteria exposed to quaternary ammonium compounds. The result is especially interesting for the *S. aureus* strain pair, as this pair is genetically isogenic only differing in the presence or absence of *qacA-qacR* or *lacZ* gene, respectively.

TABLE 3. MIC and sub-MIC concentration (mg/l) of the parent compound BAC-12 and DDAC towards the *S. aureus* (JE2qac and JE2lacZ) and *S. epidermidis*, 3487qac and 3534no-qac strain pairs.

Conc. in mg/L	*S. epidermidis* qac (3487)	*S. epidermidis* no-qac (3534)	*S. aureus* qac (JE2qac)	*S. aureus* no-qac (JE2lacZ)
BAC-12 MIC	**8**	**2**	**16 - 8**	**4 - 0.5**
BAC-12 sub-MIC	2	0.25	1	1 - 0.25
DDAC MIC	**8 - 2**	**4 - 1**	**2 - 1**	**2 - 1**
DDAC sub-MIC	2 - 0.5	1 - 0.25	1	0.5 – 0.25

The susceptibilities towards the degradation compounds are shown in Table 4 below. The differences between the MIC and sub-MIC were similar to what was seen for the parent compounds, but the differences between the qac and no-qac strains were less pronounced and in general showed around a 2-fold difference. For some of the compounds the MIC could not be established as the compounds could not be added at higher concentrations because this would result in concentrations of the organic solvent DMSO > 4%, which in our pilot studies was found to affect the staphylococcal growth negatively. The compound QR3 was challenging to get into suspension and was generally in two phases. The compound was vigorously shaken before it was utilized, but for the JE2lacZ results it is likely that the mixing was not effective and thus, the data for this compound is less reliable.

TABLE 4. MIC and sub-MIC concentration (mg/l) of the degradation compounds of BAC-12 towards the *S. aureus* (JE2qac and JE2lacZ) and *S. epidermidis* (3487qac and 3534no-qac) strain pairs.

Conc. in mg/L	*S. epidermidis* qac (3487)	*S. epidermidis* no-qac (3534)	*S. aureus* qac (JE2qac)	*S. aureus* no-qac (JE2lacZ)
QR1 MIC	**4000-8000**	**4000**	**8000**	**4000**
QR1 sub-MIC	1000	500	500	250
QR2 MIC	**4000**	**4000**	**>8000**	**>8000**
QR2 sub-MIC	500-1000	500	1000	1000
QR3 MIC	**1000**	**500**	**2000**	**>8000**
QR3 sub-MIC	125	125	250	>8000
QR4 MIC	**1000**	**1000**	**2000**	**>8000**
QR4 sub-MIC	125-250	125-250	250	500
QR5 MIC	**8000**	**4000**	**8000**	**4000**
QR5 sub-MIC	500	250	500	500
QR6 MIC	**>8000**	**>8000**	**>8000**	**>8000**
QR6 sub-MIC	4000	2000	4000	4000
QR7 MIC	**>8000**	**>8000**	**>8000**	**>8000**
QR7 sub-MIC	2000	2000-4000	8000	8000
QR9 MIC	**>8000**	**>8000**	**>8000**	**>8000**
QR9 sub-MIC	2000	1000	1000	1000
TP_250 MIC	**2000**	**2000**	**2000**	**2000**
TP_250 sub-MIC	250	250	64	64
TP_278 MIC	**1000**	**1000**	**1000**	**1000**
TP_278 sub-MIC	125	125	125	125
TP_318 MIC	**>500**	**>500**	**>500**	**>500**
TP_318 sub-MIC	125	125	64	125
TP_320 MIC	**400**	**400**	**400**	**400**
TP_320 sub-MIC	100	50	50	100

4.2 Co-culture of strain pairs

4.2.1 Material and methods

The two strain pairs *S. epidermidis* (3487qac and 3534no-qac) and *S. aureus* (JE2qac and JE2lacZ) were used in the co-culture studies.

4.2.1.1 Co-culture protocol

Stains were recovered from the -80°C degrees stocks on TSA or TSA with chloramphenicol (CAM) 10 µg/ml to retain the *qac* of *lacZ* plasmid in the JE2 strain. Grown ON at 37°C degrees. Biological triplicates of colony suspensions were made in 0.9% sterile NaCl and OD (600 nm) measured and adjusted in TSB or TSB+CAM 10 µg/ml respectively to OD 0.001. To determine the actual CFU/ml an aliquot of each inoculum was diluted and plated on TSA or TSA with CAM, grown ON at 37°C degrees and colonies counted. The strain pairs were then mixed 1:1 and 100 µl inoculated into appropriate wells of a 96 well plate that had been prepared with TSB or TSB+CAM 10 µg/ml and 2 x sub-MIC concentration of the compound to be tested. Total volume per well 200 µl. Controls were the strain pairs in biological triplicates and

at least technical triplicates without exposure to compounds to evaluate the competition between the strains without potential selective pressure. In addition, each strain was grown to confirm growth and ensure that an appropriate sub-MIC had been chosen. Negative controls were media alone or media including the compound. The plates were covered with parafilm® and incubated ON at 37°C degrees with 180 rpm shaking.
After 1 day of exposure an aliquot per well were diluted 10^{-3} and used to inoculate a new 96 well plate that had been prepared similarly with TSB or TSB+CAM 10 µg/ml and 2 x sub-MIC concentration of the compound to be tested, covered with parafilm® and incubated ON at 37°C with 180 rpm shaking. This procedure was repeated once more the following day. Thus, the strain pairs were exposed in total 3 days to the compounds.

To assess the distribution of the strains after co-culture and exposure to the compounds the following analyses were performed: For the *S. epidermidis* (3487qac and 3534no-qac the strains were distinguished based on differences in antibiotic resistance, where 3487(qac) was resistant to gentamicin (GEN) and could be selected for on TSA with GEN 10 µg/ml and 3534(no-qac) was resistant to erythromycin (ERM) and could be selected for on TSA with ERM 150 µg/ml. From the 96 well co-culture plates an aliquot from each well was diluted sufficiently to get single colonies when plated on TSA and grown ON at 37°C degrees. Forty-five single colonies from this non-selective plate were picked randomly onto both of the selective plates (GEN and ERM) and incubated ON at 37°C degrees. Depending on the strain colonies would grow on either GEN or ERM. The plating was done after 1 day of co-culture and again after 3 days of co-culture.
For the *S. aureus* (JE2qac and JE2lacZ), the strains were distinguished based on the plasmids carrying either *lacZ* encoding a β-galactosidase or *qacR-qacA* that does not encode β-galactosidase. The expression of the *lacZ* gene on the pRAB12lacZ plasmid is tetracycline-inducible and was thus induced by plating on TSA with CAM containing a final concentration of 850 ng/ml anhydrotetracycline hydrochloride (AHT) (Thermo Scientific Chemicals, CAS # 13803-65-1). The plates further contained 200 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Thermo Scientific X-Gal), the substrate for the *lacZ* encoded β-galactosidase providing an insoluble blue colour product to colonies expressing lacZ while those expressing *qacR-qacA* were white. From the 96 well co-culture plates an aliquot from each well was diluted to get single colonies when plated on TSA+CAM+AHT+X-gal and grown ON at 37°C degrees in the dark, as AHT is light sensitive. The plates were kept 1-2 days in the dark at 4°C degrees, as this intensified the blue colour and made it easier to distinguish the colonies. The plating was done after 1 day of co-culture and again after 3 days of co-culture.

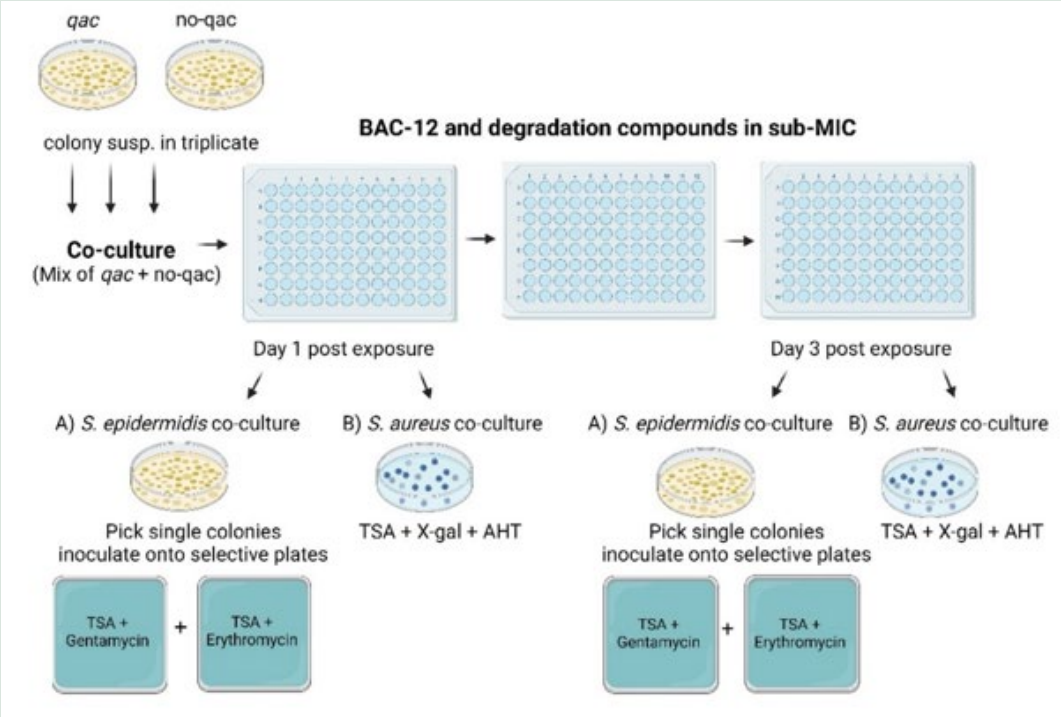


FIGURE 14. Methodology for co-culturing the *qac* and no-qac strains.
Colonies from each strain were suspended, adjusted to similar optical densities and then mix 1:1. These co-culture mixes were inoculated into 96 well plates containing sub-MIC concentration of the compounds to be tested for possible selection. After 1 day of incubation and exposure the co-culture was diluted and inoculated into a new 96 well plate containing sub-MIC concentration of the compounds and this was repeated after 2 days of exposure. After 1 day of exposure an aliquot was further A) plated on TSA to be further selected on antibiotic selective plates the following day or B) plated on TSA with AHT and X-gal to select by counting white/blue colonies. This was repeated after 3 days of exposure. This allowed for separation of the *qac* and no-qac strains after the co-culture.

The discrete data from counting each of the strains was formatted into percentages and corrected for any imbalance from the initial inoculum, which should ideally have the two strains in the pair 1:1, but the data from the CFU/ml count of the inoculum showed small imbalances (for *S. epidermidis* 2 to 11%; for *S. aureus* 2 to 16%), which were thus accounted for. From this data the ratios between the *qac*/no-qac strain were calculated:

$$\textbf{Ratio} = \frac{\%\ \boldsymbol{qac}}{\%\ \mathbf{no-qac}}$$

Ratio > 1 = more *qac* than no-qac bacteria present in the co-culture.
Ratio < 1 = the no-qac strain the most numerous in the co-culture.

To be able to calculate ratios the number 0 could not be allowed. Thus, in cases of 0% this number was changed to 1%.

4.2.2 Results

4.2.2.1 *S. epidermidis* co-culture of *qac* and no-qac strain

All exposure to compounds were at the sub-MIC of the most susceptible strain (table 3 and 4). The exposure was done in biological triplicates. The controls without exposure to compound were in biological triplicates and at least technical triplicates.

The co-culture of the *S. epidermidis* (3487qac and 3534no-qac) strain pair when exposed to sub-MIC of the parent compounds BAC12 at 25 mg/L and DDAC at 50 mg/L showed a clear and significant selection of the *qac* strain after 3 days of co-culture, where the no-qac strain had been completely outcompeted (Figure 15A). Interestingly, this selection does not appear after 1 day of co-culture at the sub-MIC concentration, but after 3 days of co-culture the *qacA* containing strain showed a clear advantage in competition with the strain without *qacA*. This could be due to upregulation of genes associated with increased tolerance e.g. the *qacA* gene.

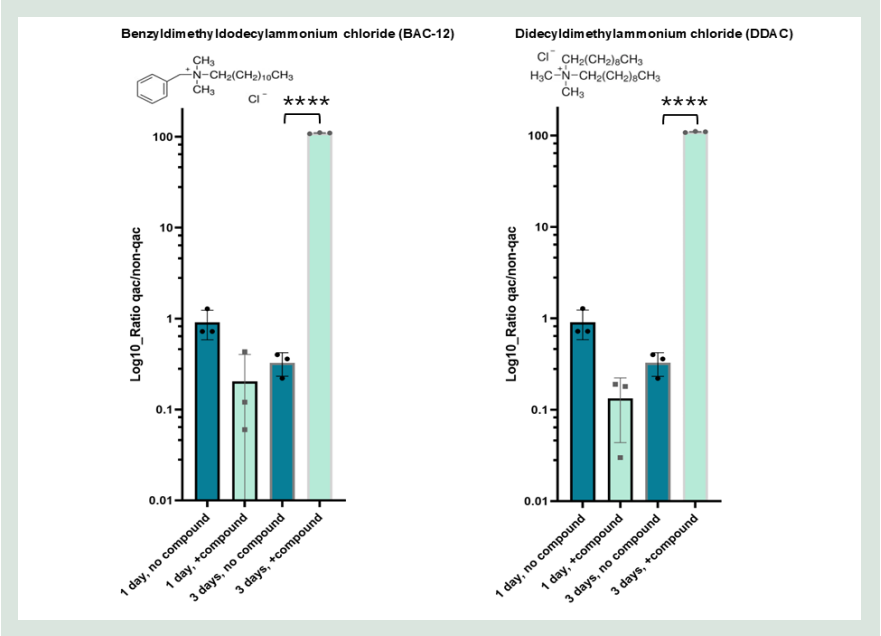


FIGURE 15A. Co-culture of *S. epidermidis qac/no-qac*. The barplots show the ratios between the strains after 1 day and 3 days of co-culture with the dark green bars representing controls with no treatment and light green bars representing exposure to sub-MIC conc. of respective compound. Left graph exposure to BAC-12; right graph exposure to DDAC. **** = $p < 0.0001$.

The co-culture of the *S. epidermidis* (3487qac and 3534no-qac) strain pair exposed to sub-MIC of the commercially available degradations products gave varied response (Figure 15B). For the QR1 there was a tendency towards selection of the *qacA* containing strain after 3 days of exposure (3 days, + compound: mean 5.5; SD 6.3). However, there were large variations in the response and thus no significant differences could be detected.

The exposure to sub-MIC of QR2 resulted in a significant ($p = 0.01$) difference between the ratios of the treated versus non-treated group after 3 days of co-culture in favour of the *qacA* containing strain, even though the ratio of 3487qac and 3534no-qac was not large (3 days, + compound: mean 1.3; SD 0.3).

The exposure to sub-MIC of QR3 and QR4 seemed to favour the 3534no-qac strain. After 3 days of co-culture there was a significant difference in ratios between the non-treated control and the treated groups. Both compound QR3 and QR4 lacks the benzene ring, which may change the properties of the compounds compared to the parent compound.

Exposure to sub-MIC of QR6, QR7 and QR9 showed no significant selection for either of the strains. QR5 was not tested as there was a shortage of compound.

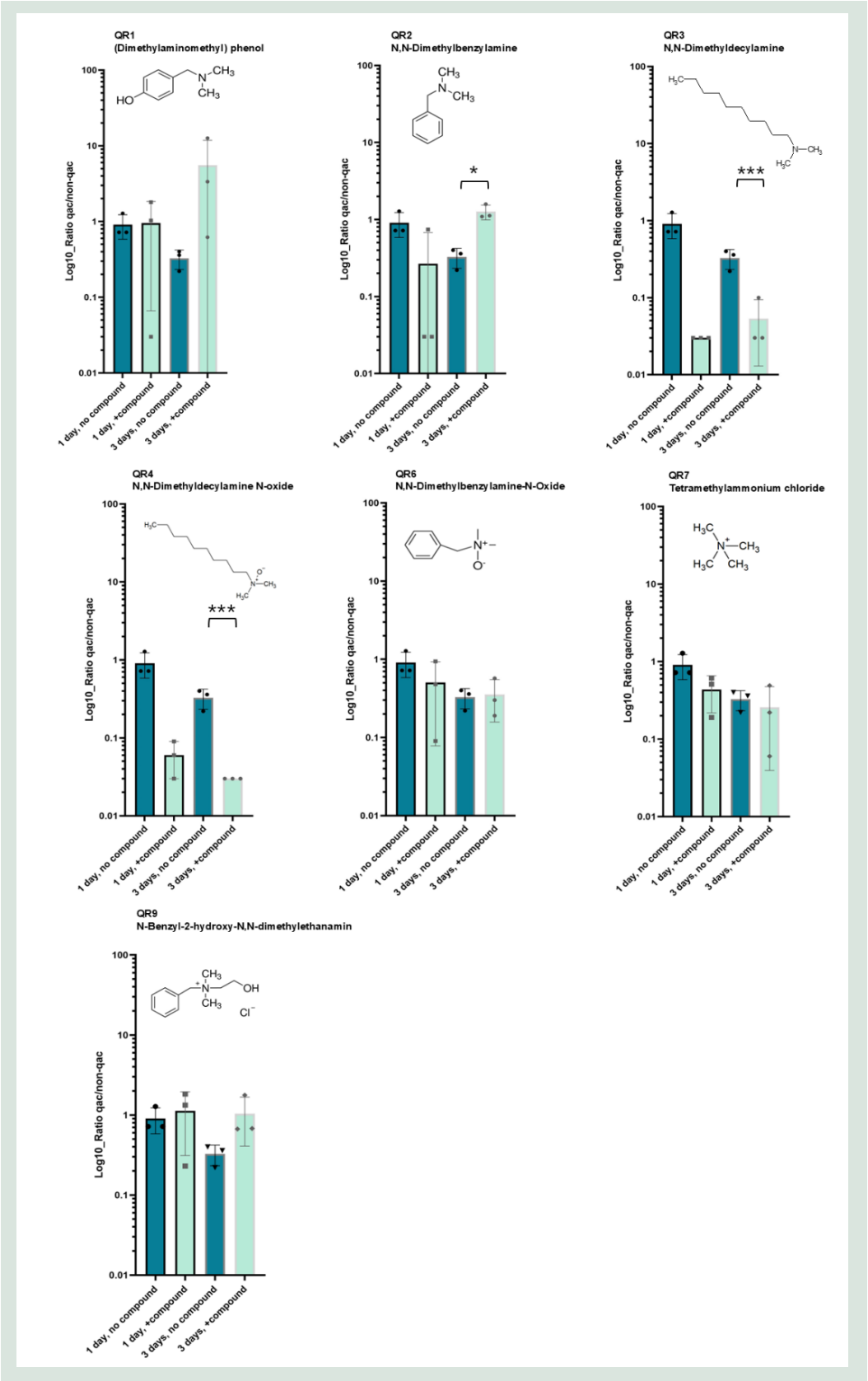


Figure 15B. Co-culture of *S. epidermidis* qac/no-qac. The barplots show the ratios between the strains after 1 day and 3 days of co-culture with the dark green bars representing control with no treatment and light green bars representing exposure to the respective

compound. Graphs from left to right in reading direction exposure to QR1; QR2; QR3;QR4; QR6; QR7; QR9.

For the experiment with exposure to the transformation products isolated from the degradation of BAC12 (Figure 15C) the *qac* strain was for some reason favoured slightly after 1 day of co-culture (1 day, no compound: mean 3.7; SD 0.9). For the TP278 and TP318 this resulted in significant difference between the non-treated control and the treated groups, with the treated group being less favourable for the *qac* strain, thus not likely to select for *qac*.
The TP320C could potentially give some selection for the *qac*, as there was significant difference between the control and the treatment ratios.

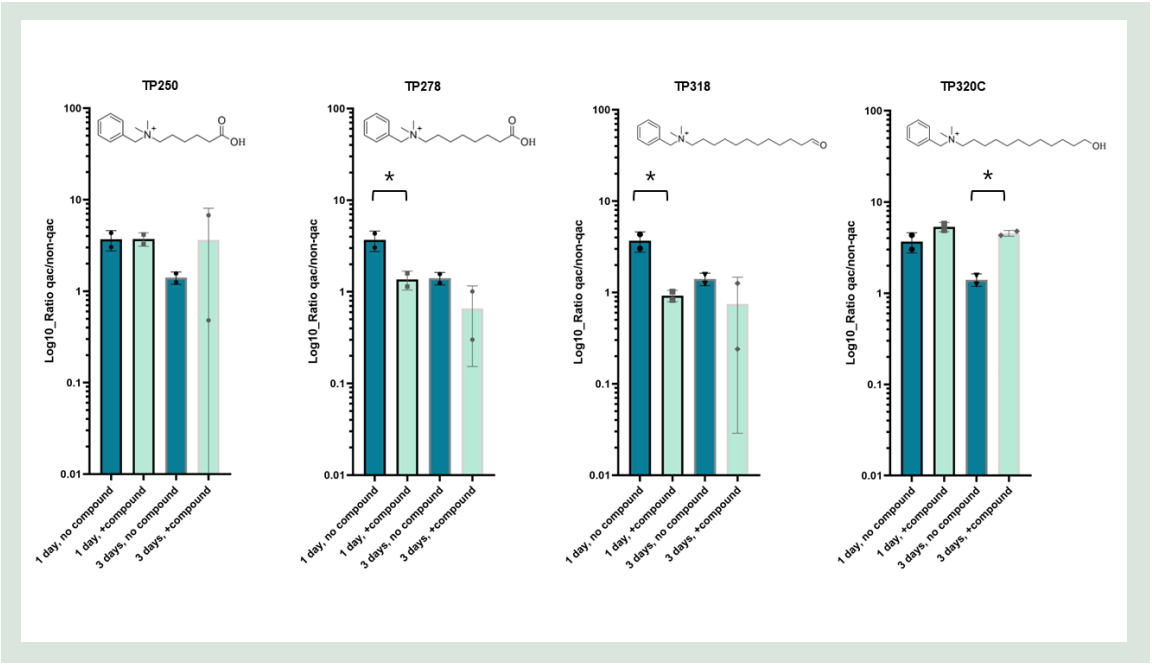


FIGURE 15C. Co-culture of *S. epidermidis* qac/no-qac. The barplots show the ratios between the strains after 1 day and 3 days of co-culture with the dark green bars representing control with no treatment and light green bars representing exposure to the respective compound. Graphs from left to right in reading direction exposure to sub-MIC of transformation products TP250; TP278; TP318; TP320C.

4.2.2.2 Co-cultures of *S. aureus* JE2qac and JE2lacZ

All exposures to compounds were at the sub-MIC of the most susceptible strain as shown in Table 3 and 4. The exposures were performed in biological triplicates. The controls without exposure to compound were in biological triplicates and at least technical triplicates.
The co-culture of the *S. aureus* (JE2qac and JE2lacZ) strain pair when exposed to sub-MIC of the parent compounds BAC-12 at 0.25 mg/l and DDAC at 0.50 mg/l showed no selction for the BAC-12, whereas a significant selection of the *qac* strain after 1 and 3 days of co-culture was seen for DDAC, where the strain without *qacA* was completely outcompeted (Figure 15D).

The lack of selection for the *qacA* containing strain when exposed to BAC-12 could be due to the sub-inhibitory concentration chosen. The sub-MIC for the JE2lacZ had an interval of 1-0.25 mg/L, whereas the sub-MIC for the JE2qac was 1 mg/L. The lower range of 0.25 mg/l was chosen as the sub-MIC and at this low concentration the JE2qac strain did not have any apparent growth advantage. It could be interesting to investigate the effect of 0.5 and 1 mg/L of BAC-12, which would still be within the sub-MIC of JE2lacZ. However, time restraints did not allow this.

The sub-MIC concentration of DDAC for the JE2lacZ strain was in the range 0.5-0.25 mg/L, whereas the sub-MIC for JE2qac was 1 mg/L. Here the sub-MIC 0.5 mg/L was chosen, that resulted in a clear selection of the JE2qac strain, which differs from the JE2lacZ strain only by the presence of the *qacR-qacA* genes instead of *lacZ*. At this higher sub-MIC range, the *qacA* gene thus seemed to be the explanation for the advantage of the strain in competition with the JE2lacZ strain.

It is worth noting that the ratio of the JE2qac strain to the JE2lacZ strain was oftentimes > 1 meaning that the JE2qac strain appeared to compete better even in the absence of QACs or QAC transformation produces. Here it should be mentioned that blue colouring of the colonies expressing *lacZ* were not trivial to detect. First, the JE2lacZ strain grew with smaller colonies, thus the co-culture had to be plated at sufficiently low concentration to allow true single colonies on the selective plate to promote *lacZ* expression and not have them masked by JE2qac colonies. To enhance LacZ accumulation and colouring of colonies, the plates were kept 1-2 days refrigerated and in the dark. These procedures allowed the JE2lacZ and JE2qac strains to be distinguished. However, it cannot be completely ruled out that JE2lacZ has been underestimated on some plates.

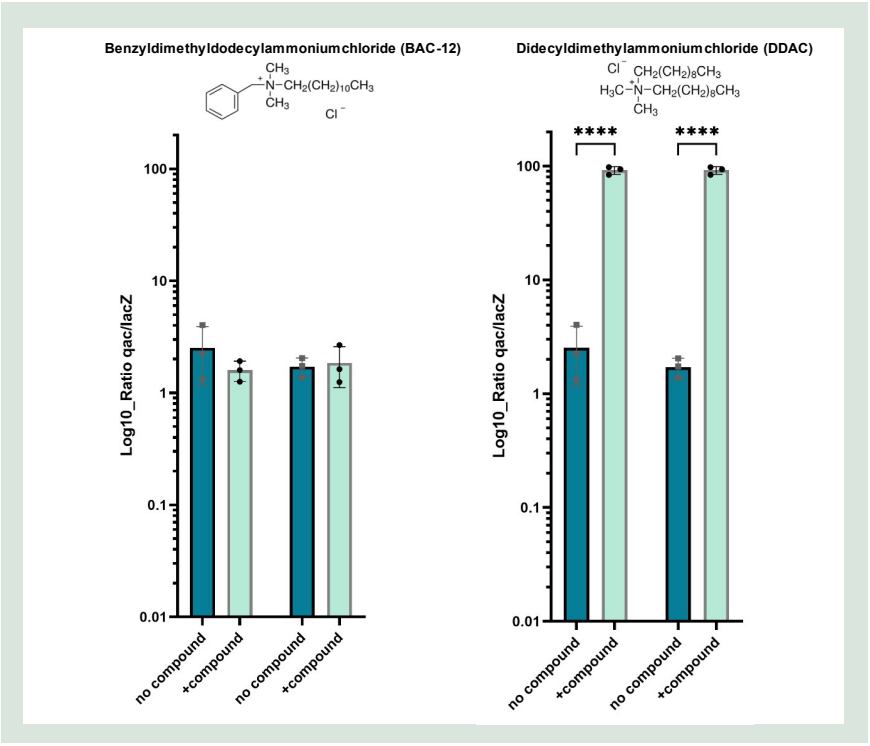


Figure 15D. Co-culture of *S. aureus* JE2qac/JE2lacZ. The barplots show the ratios between the strains after 1 day (first two bars) and 3 days (last two bars) of co-culture with the dark green bars representing control with no treatment and light green bars representing exposure to the respective compound.

The co-culture of the *S. aureus* (JE2qac and JE2lacZ) strain pair exposed to sub-inhibitory concentrations of the commercially available degradations products gave varied response (Figure 15E).

For the compounds QR1 and QR2 there were no significant difference in ratios between the strains when comparing with and without exposure to the degradation product.

The exposure to sub-MIC of compound QR3 and QR4 seemed to favour the JE2qac strain, however the variation between the triplicates were so large that the effect was not significant. QR3 was generally hard to handle, as it was not completely soluble, as described earlier, and thus, the concentration applied may have varied substantially influencing the outcome of the assay.

For the exposure to compound QR4 a substantial variation between triplicates was noted, which currently cannot be explained. Furthermore, exposure to sub-inhibitory concentrations of compound QR5, QR6, QR7 and QR9 resulted in no significant selection for either of the strains.
To summarise the commercial degradation productions did not seem to select for the JE2qac strain at sub-MIC concentrations.

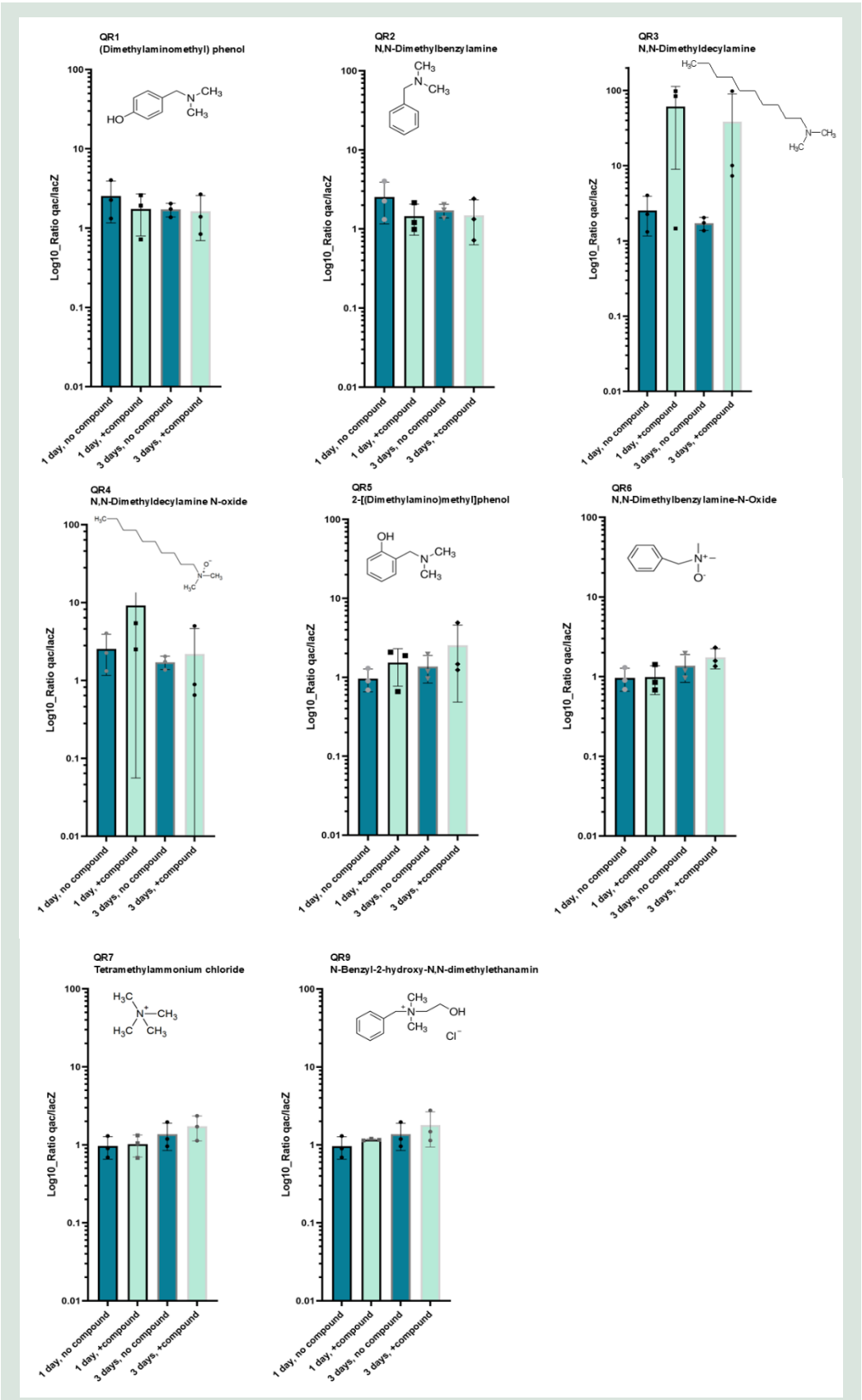


FIGURE 15E. Co-culture of *S. aureus* JE2qac/JE2lacZ. The barplots show the ratios between the strains after 1 day and 3 days of co-culture with the dark green bars representing control with no treatment and light green bars representing exposure to the respective compound.

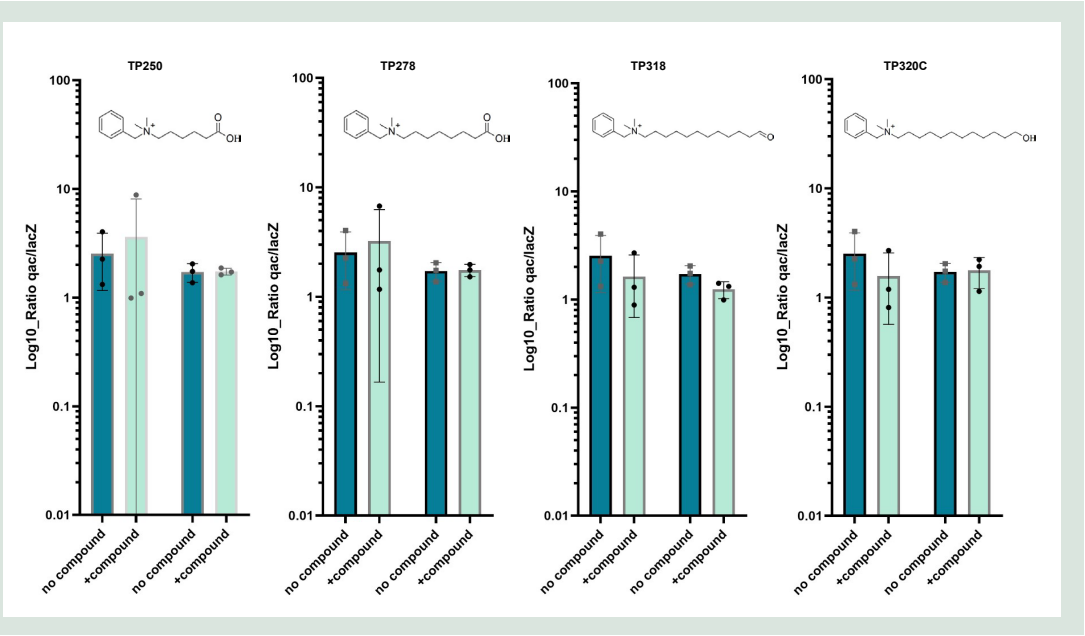


FIGURE 15F. Co-culture of *S. aureus* JE2qac/JE2lacZ. The barplots show the ratios between the strains after 1 day (first two bars) and 3 days (last two bars) of co-culture with the dark green bars representing control with no treatment and light green bars representing exposure to the respective compound. Graphs from left to right in reading direction exposure to transformation products TP250; TP278; TP318; TP320C.

4.3 Discussion

In summary, we observed selection of the *qacA* containing strains in the presence of the parent compounds BAC-12 and DDAC but did not observe selection by any of the degradation products. A challenge in these assays is selection the concentration at which the compounds should be tested. When attempting to determine the minimal inhibitory concentration for the transformation products we were unable to do so for some reflecting that the compounds had completely lost their antimicrobial activity. However, for other degradation products we were able to determine a MIC reflecting that the transformation products retained a slight antimicrobial activity with a MIC substantially higher than the parent compounds. Given the laborious assay used for assessment of the competition between *qacA* containing and non-*qacA* cells we only selected one concentration (the sub-MIC) for the competition experiments and the results only provide limited information about the selective power of the degradation products.

5. Impact of biological BAC degradation on *qac* containing Staphylococci

5.1 Degradation of BAC-12 by *Pseudomonas nitroreducens*

Biodegradation of QACs has been described in section 1.1.1 . On one hand the degradation of the parent compounds is crucial to reduce the environmental impact they can have, but on the other hand it is important also to investigate the impact of the degradation compounds (transformation products). Microbial consortia capable of degrading BACs have been enriched from various environmental sources, including sewage, activated sludge, soil, and marine sediments. These communities are often dominated by species from the genera *Pseudomonas* and *Achromobacter* (Tezel & Pavlostathis, 2015). *Pseudomonas nitroreducens* has been identified as a key BAC degrader, often dominating microbial communities in BAC-contaminated environments. This species exhibits adaptive responses to BAC exposure, potentially enhancing its biodegradation capabilities over time (Oh et al., 2014).
The degradation pathway of BAC typically involves enzymatic cleavage of the C-alkyl-N bond, producing various intermediates:

- Primary degradation products: benzyldimethylamine (BDMA), benzylmethylamine, benzylamine, and benzaldehyde (Sakarya et al. 2021), as well BAC-12 tranformation products formed via α-, β- and ω-oxidation (Larrson et al., 2024)

- End product: benzoic acid, which can be further metabolized via central metabolic pathways (Patrauchan & Oriel, 2003).

The degradation product BDMA has a half maximal effective concentration (EC_{50}) value approximately 500 times higher than BAC, indicating a substantial reduction in toxicity following initial biodegradation steps (Oh et al., 2014). Overall, BAC degradation significantly lowers its environmental toxicity, reducing its impact on higher organisms such as daphnia, crayfish, and fish. However, the continuous presence of BAC in aquatic and terrestrial environments exerts selective pressure on microbial populations, leading to the development of BAC-resistant strains. Prolonged BAC exposure alters bacterial gene expression, affecting traits such as motility, biofilm formation, and overall fitness. The potential for cross-resistance to antibiotics due to BAC exposure highlights the ecological risks associated with the widespread use of QACs (Tezel & Pavlostathis, 2015). Understanding the genetic and biochemical pathways involved in BAC degradation can inform the development of bioremediation strategies to mitigate BACs pollution in various environments.

5.1.1 Material and Methods

Four *Pseudomonas nitroreducens* (*P. nitroreducens*) strains were purchased from the Culture Collection University of Gothenburg (ccug.se).
Optimal growth conditions were established regarding growth media and temperature. A temperature of 30°C with shaking (180 rpm) and a large surface for aeration gave the best results. TSB and LB were both suitable media, but TSB was chosen, as that is the preferred media for Staphylococci.

MIC and sub-MIC determination

The MIC and sub-MIC towards BAC-12 was found for each strain by growing them in bioscreen plates with continuous shaking and measuring every 15 min in a plate reader for 24 h, as described in section 4.1.1 except for the temperature, which was set to 30°C.

Biodegradation of BAC-12 by *Pseudonomas*

Pilot studies were conducted to establish a protocol for biodegradation of BAC-12 by *P. nitroreducens*. Different media compositions, incubation periods and BAC-12 concentrations were tested (data not shown), which led to the following protocol partially based on a method previously described (Oh et al, 2014).

P. nitroreducens strain (54619) was streaked from -80°C onto a TSA plate containing 32 µg/ml BAC-12 and grown 20-24 h at 30°C. Three Erlenmeyer flasks with TSB and 32 µg/ml BAC-12 were inoculated with 2-3 colonies each and incubated ON 30°C, shaking 180 rpm. A positive control without BAC-12 was included.

The ON cultures were spun down for 7 min at 8000 g, supernatant decanted and cells washed with 10 ml of filter sterilized tap water. This process was repeated twice.

New Erlenmeyer flasks were prepared in triplicate containing 40 ml of either:

a. TSB with 48 mg/l BAC-12
b. TSB with 8 mg/l BAC-12
c. 1:10 TSB and TW with 48 mg/l BAC-12
d. 1:10 TSB and TW with 8 mg/l BAC-12

Positive controls were flasks with media and no BAC-12.

Flasks were inoculated with washed *P. nitroreducens* at a final OD 0.01.

Negative controls were one of each of the above-mentioned combinations with no bacteria added.

Incubation for 5 days at 30°C, shaking 180 rpm, in dark to avoid photo degradation. Samples taken at time: 0 h; 2 h; 19 h; 25 h; 48 h; 120 h. Samples were spun down for 7 min at 10.000 g. Supernatants were pipetted into 1.5ml HPLC glass vials (Mikrolab, Aarhus) and stored at 4°C. At the final time point the pelleted *P. nitroreducens* were stored as well at -20°C.

5.1.2 Results

The BAC-12 MIC towards the *P. nitroreducens* strains were between 128 – 256 mg/L and the sub-MIC were between 4 – 8 mg/L. Detailed information on the strains in appendix 1.1.
One strain (54619) was chosen for the biodegradation. The strain had a sub-MIC at 8 mg/L, where the growth was similar to the positive control for that same strain, however, the strain did grow in conc. between 16 - 128 mg/L.

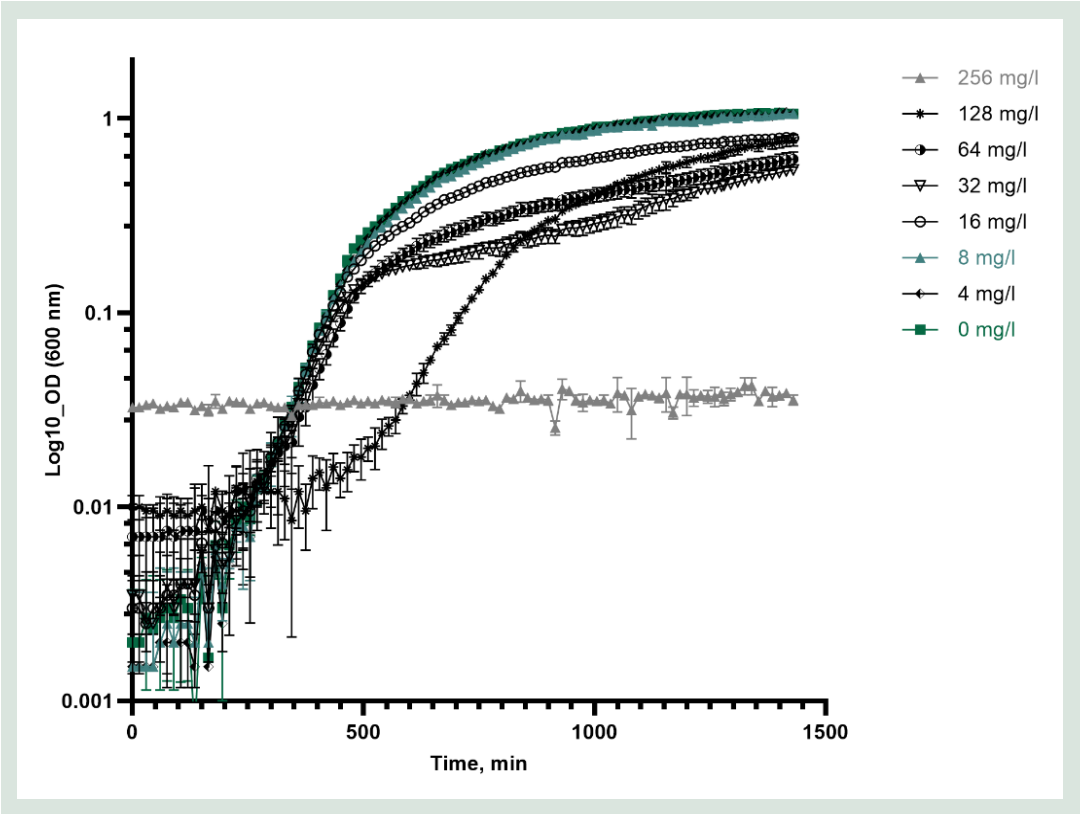


FIGURE 16. Growth curves for *Pseudomonas nitroreducens* (strain 54619) with BAC-12. The control grown without BAC-12 in dark green squares (0 mg/L). The sub-MIC in green triangles (8mg/l). MIC in light grey triangles (256 mg/L).

The degradation experiment showed difference between the two media concerning the produced biomass. The full TSB media allowed for more growth of *P. nitroreducens* and thus more biomass compared to the 1:10 TSB and tap water (TW). The reason for including the diluted growth medium in the experiments was to mimic the environmental conditions with lower amounts of nutrients available. Further, the lower nutrient availability could potentially force *P. nitroreducens* to use more of the BAC-12 as a carbon source for growth.

5.2 MIC for Staphylococci exposed to the supernatant from the degradation experiment

5.2.1 Material and Methods

Broth Microdilution test for MIC determination

To provide an initial assessment of how the microbial turnover of BAC by *P. nitroreducens* impacts Staphylococci that carry *qacA*, the supernatants from the biodegradation were applied to our strain sets described in 3.2 and 3.3.
The *S. epidermidis* strains HI3487qac and 3534no-qac and the *S. aureus* JE2qac and JE2lacZ were streaked onto TSA and TSA plus CAM 10 µg/ml respectively and grown over night at 37°C.

The pH of the spent supernatants were tested and found slightly alkaline (8 – 8.8) and were thus adjusted with HCl to reach a pH close to the normal pH of TSB (6.7-7).
96 well plates were prepared with a 2-fold dilution range from the supernatants taken from the TSB and 48 µg/ml BAC-12 and 1:10 TSB and TW and 48 µg/ml BAC-12. To give a finer range of concentrations a 2-fold dilution series was prepared from supernatants initially diluted 1/3, thus the final concentrations tested were 24; 16; 12; 8; 6; 4; 3; 2; 1; 0.5 g/L. The dilutions were performed in spent supernatant from the positive controls, to evaluate the possible effect that spent supernatant from *P. nitroreducens* could have on the Staphylococci.
The negative controls containing media and BAC-12 but no bacteria were tested against Staphylococci as well, to determine BAC-12 degradation unrelated to *P. nitroreducens* e.g. due to incubation, aeration and/or components in the media.
Further controls were spent supernatant spiked with fresh non-degraded BAC-12, and sterile MilliQ water likewise spiked with fresh non-degraded BAC-12 in the same dilution range. Each of the triplicates from the degradation experiment were tested in technical duplicates.
Inoculums of the Staphylococci were prepared in 2 times concentrated TSB to a final OD of 0.001, which corresponds to approx. 5 x 10^5 CFU/ml. The final volume was 150 µl/well. Plates were sealed with parafilm and incubated at 37°C with 180 rpm shaking for 16 - 20 h. Results were read macroscopically as growth or no-growth for a given strain and concentration. First well with no growth defined the MIC value.

5.2.2 Results

The spent supernatant from the degradation experiments (section 5.1.1.) supposedly contain both degraded and non-degraded BAC-12. Therefore the MIC will indicate the concentration that was present in the media at the beginning of the degradation experiment, thus if an increase in MIC is seen compared to what was obtained with non-degraded BAC-12 this likely reflects degradation or inactivation of BAC-12 has taken place.
As seen in Table 5 the results from the MIC testing of the Staphylococci when exposed to the spent supernatant indicate that degradation of BAC-12 had taken place. The degradation performed in TSB media resulted in three out of four strains growing in the 24 mg/L wells, and the non-qac *S. epidermidis* had a MIC of 16 mg/L. Thus, an at least 4-fold up to 16-fold increase in MIC makes it likely that part of the BAC-12 had been degraded by the *P. nitroreducens* (Table 5). Unfortunately, the degradation experiment could not be performed at higher concentrations of BAC-12, as the *P. nitroreducens* would not grow well at these concentrations.
The negative control was BAC-12 was incubated in the media without adding *P. nitroreducens* resulted in an increased MIC of the Staphylococci as well indicating that some degree of degradation occurred independently of the *P. nitroreducens* e.g. due to aeration of the BAC-12.
The spent supernatant to which BAC-12 had been added resulted in an increased MIC of the Staphylococci as well. This could be due to components present in the spent supernatant such as enzymes secreted by the *P. nitroreducens*, but remains to be determined.

TABLE 5. Apparent MIC's (mg/L) of the Staphylococci exposed to the spent supernatants from the degradation experiments done in full TSB media. The supernatants will contain both degraded and non-degraded BAC-12 and thus MIC indicate the concentration present in the media in the beginning of the degradation experiment. No confidence interval reported, as the triplicates all gave the same results, and the controls were performed in singlets or duplicates for comparison within the experiment.

Tested	MIC *S. epidermidis* qac (3487)	MIC *S. epidermidis* non-qac (3534)	MIC *S. aureus* qac (JE2qac)	MIC *S. aureus* non-qac (JE2lacZ)
Spent Supernatant TSB with BAC-12	**>24**	**16**	**>24**	**>24**
NC incubated BAC-12	12	3	16	4
Spent Supernatant TSB spiked with BAC-12	12	2	16	3
BAC-12 in sterile water	**6**	**1**	**8**	**2**

The results from the MIC testing of the Staphylococci when exposed to the spent supernatant from the diluted media containing 1:10 TSB and tap water resulted in only an up to 2-fold increase in MIC (Table 6). This suggests that the degradation by *P. nitroreducens* was less pronounced under nutrient deprived conditions.
The negative control was BAC-12 was incubated in the media without adding *P. nitroreducens* resulted in no or only slight increased MIC of the Staphylococci indicating that degradation independently of the *P. nitroreducens* was likely less pronounced in the diluted media.
The spent supernatant spiked with fresh non-degraded BAC-12 resulted in no or only slightly increased MIC. The non-degraded BAC-12 in sterile water is reported with a MIC 3-4 mg/L as the test was done in duplicate and gave these results.

TABLE 6. Apparent MIC's (mg/L) of the Staphylococci exposed to the spent supernatants from the degradation experiments done in 1:10 TSB and TW media. No confidence interval reported, as the triplicates all gave same results, and the negative controls (NC) were performed in singlets or duplicates for comparison within the experiment.

Tested	MIC *S. epidermidis* qac (3487)	MIC *S. epidermidis* non-qac (3534)	MIC *S. aureus* qac (JE2qac)	MIC *S. aureus* non-qac (JE2lacZ)
Spent Supernatant 1:10 TSB and TW with BAC-12	**12**	**2**	**16**	**4**
NC incubated BAC-12	8	2	12	4
Spent Supernatant 1:10 TSB and TW spiked with BAC-12	6	2	12	2
BAC-12 in sterile water	**6**	**1**	**12**	**3-4**

5.3 Analysis of the supernatants for BAC 12 transformation products using HPLC-MS

5.3.1 Material and Methods

Metabolic identification was performed using a SCIEX ExionLC 2.0+ UHPLC system (AB SCIEX, Framingham, MA, USA) for chromatographic separation and an API 6600 quadrupole/time-of-flight (Q-TOF) mass analyzer (AB SCIEX, Framingham, MA, USA) for detection. Chromatographic separation utilized a reversed-phase (RP) system with an Acquity UPLC BEH Shield RP-18 column (1.7 µm, 2.1 × 30 mm; Waters, Milford, MA, USA) maintained at 30°C and an injection volume of 10 µL. The HPLC gradient employed two mobile phases: Chromasolv water with 0.1% formic acid (Phase A) and Chromasolv methanol with 0.1% formic acid (Phase B), both from Honywell (Riedel-de Haën GmbH, Seelze, Germany). The flow rate was set at 0.3 mL/min, with the gradient programmed as follows: 0–1 min, 0% B; 1–9 min, linear ramp to 80% B; 9–11 min, linear ramp to 100% B; and 11–16 min, 100% B maintained.

Metabolite identification was conducted supported by MarkerView (Version 1.3.1: 2017) and MetabolitePilot (Version 2.0.4; 2018) as well as ChemSketch Software (Advanced Chemistry Development, Inc, 2021) with additional analysis of chromatograms in SciexOS (Version 1.6.1; 2019). Peak identification in MarkerView was set to identification within a period from 1 to 12 min; retention time tolerance was put to 0.1 min and mass tolerance was set to 20 ppm.

5.3.2 Results

Figure 17 shows the results for BAC-12 concentrations in samples during incubation with *P. nitroreducens* under different conditions, including varying initial BAC-12 concentrations and media (full TSB, 1:10 waster:TSB, as mentioned above in 5.1.1). In general, no clear degradation of BAC-12 was observed. Only in the incubation with 1:10 TSB and TW at an initial concentration of 48 mg/L BAC-12 did a slow decline of BAC-12 appear during the test. However, a similar trend was also observed in the control experiment (same conditions but without bacteria), suggesting that this decline is not biologically driven.

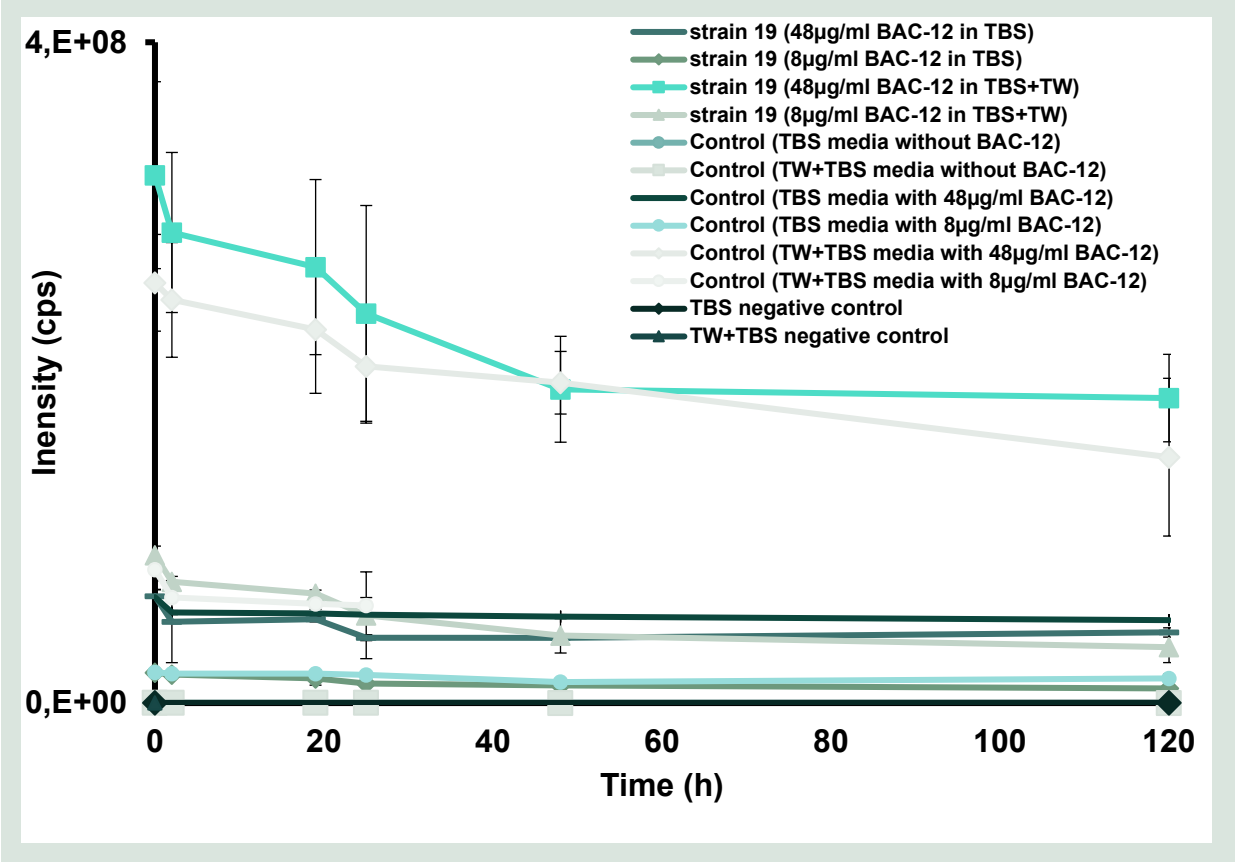


FIGURE 17. Degradation of BAC-12 by *Pseudomonas nitroreducence* (strain 54619).

5.4 Discussion

Chemical analysis of the supernatants indicated that BAC-12 was not effectively degraded in any of the tested media. Furthermore, no transformation products (TPs) of BAC-12 were detected. These findings suggest that, despite differences in biomass production, *P. nitroreducens* does not appear to utilize BAC-12 as a primary carbon source under the conditions tested. The observed biomass increase in full TSB medium is likely due to the presence of other available nutrients rather than BAC-12 degradation. This outcome highlights the need for further investigation into the metabolic pathways of *P. nitroreducens* and its potential capacity (or inability) to degrade BAC-12 under different environmental or nutritional conditions.

6. Metabolization and/or extrusion of BAC by Staphylococci

6.1 Degradation experiment the Staphyloccal strain pairs

6.1.1 Material and Methods

The Staphylococcal strain pairs described in 3.2 and 3.3, which were *S. epidermidis* strain HI3487qac and HI3534no-qac and the *S. aureus* strains JE2qac and JE2lacZ, were used in these degradation and extrusion experiments.

MIC and sub-MIC determination

The MIC and sub-MIC towards BAC-12 is described in section 4.1.1. However, the optimal sub-MIC concentration under the conditions of the degradation experiment were established in pilot studies and shown in Tabel 7 below.

TABLE 7. Sub-MIC concentration of BAC-12 allowing for growth of the Staphylococcal strains.

Strain	Sub-MIC BAC-12 mg/l
S. epidermidis qac	2
S. epidermidis non-qac	0.5
JE2qac	4
JE2non-qac	1

Biodegradation and/or extrusion of BAC-12 by *Staphylococci*

The *S. epidermidis* strains HI3487qac and HI3534no-qac and the *S. aureus* strains JE2qac and JE2lacZ were streaked onto TSA and TSA plus CAM 10 µg/ml respectively and grown ON at 37°C.

The following day 2-3 colonies were inoculated into 5 ml of TSB and TSB plus CAM 10 µg/ml respectively including the sub-MIC concentrations of BAC-12 (Table 7) and grown 20-24 h at 37°C, shaking 180 rpm. A positive control without BAC-12 was included.

ON cultures were spun down for 7 min at 8000 g, supernatant decanted and cells washed with 1.8 ml of sterile 0.9% NaCl water. This process was repeated twice.

New Erlenmeyer flasks were prepared in triplicate containing 16 ml of TSB containing the BAC-12 concentrations corresponding to the MIC determined in Table 7 namely:

a. TSB with 2 µg/ml BAC-12
b. TSB with 0.5 µg/ml BAC-12
c. TSB with 4 µg/ml BAC-12 and CAM 10 µg/ml
d. TSB with 1 µg/ml BAC-12 and CAM 10 µg/ml

Positive controls were flasks inoculated with the respective strains in media without BAC-12. Flasks were inoculated with washed Staphylococcal strains at a final OD 0.01 in flasks containing the sub-MIC concentration of BAC-12 that had been determined for the given strain (Table 7).

Negative controls were one of each of the above-mentioned combinations (a-d) with no bacteria added, and one with media only.
Incubation for 5 days at 37°C, shaking 180 rpm, in dark to avoid photo degradation. Samples taken at time: 0 h; 21 h; 27 h; 96 h; 120 h. Samples were spun down for 7 min at 10.000 g. Supernatants were pipetted into glass vials (company) and stored at 4°C. The pelleted Staphylococci were stored at -20°C at all time points except time zero.

6.1.2 Results

The sub-MIC concentrations chosen allowed for good growth of the strains, with dense cultures after 21 h. However, at the 96 h and 120 h the cultures became less dense due to lysis of dead cell. The cultures had foul odour, and the pellets were smaller compared to the samples taken at 21 h and 27 h again supporting that cells were dead likely due to lack of nutrients. For the results regarding degradation and/or extrusion, see 6.2.2.

6.2 Analysing the supernatants for BAC 12 transformation products using HPLC-MS

6.2.1 Material and Methods

The same analytical method previously described in section 5.2.1 was used to analyse these supernatant samples.

6.3 Results

6.3.1 BAC-12 degradation

In the supernatants of the incubated Staphylococcus strains, significant degradation of BAC-12 was observed. This is clearly evidenced by the results presented in Figure 18 and Figure 19, which demonstrate a notable decrease in BAC-12 concentration over the course of the incubation period.

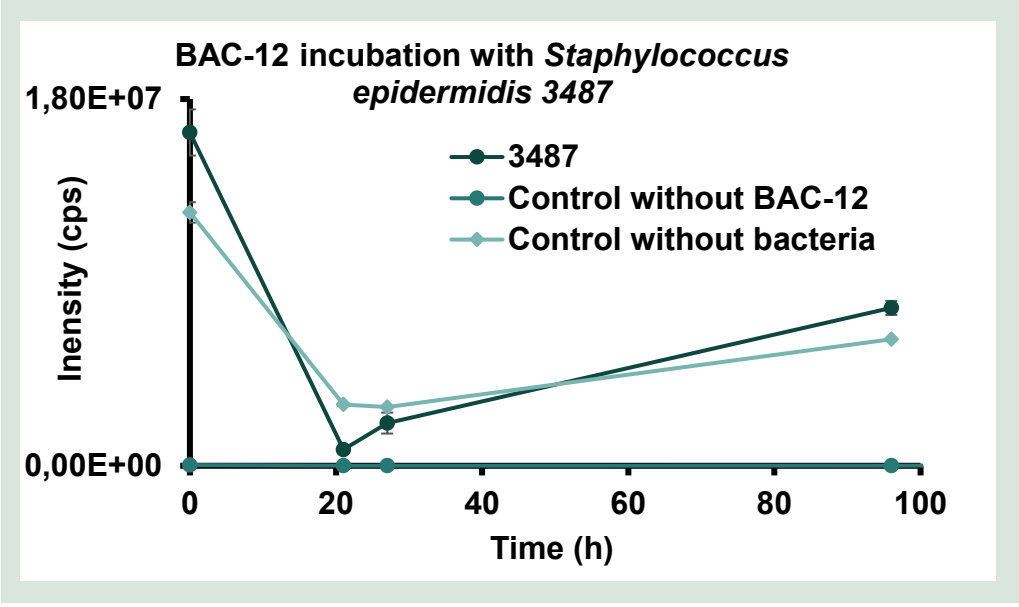


FIGURE 18. BAC-12 degradation with *Staphylococcus epidermidis* strain HI3487qac.

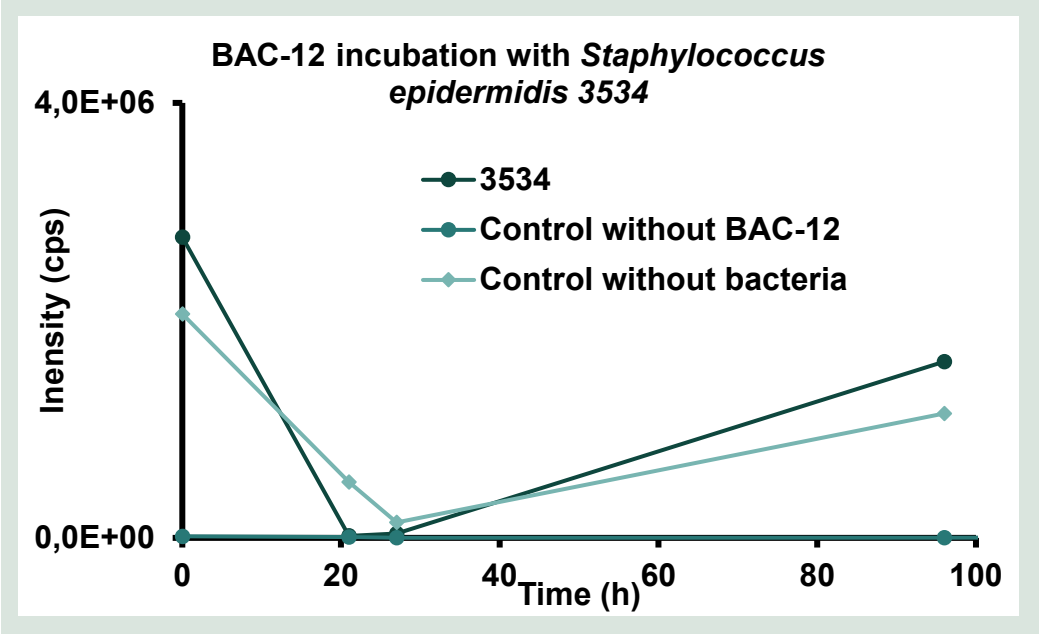
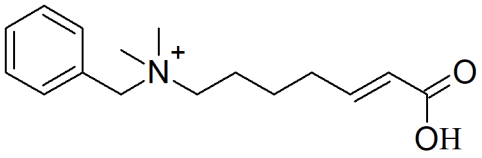
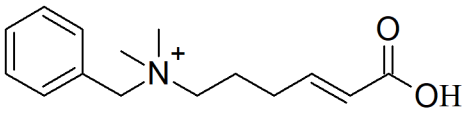
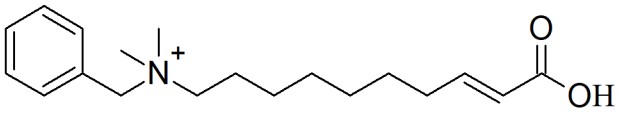
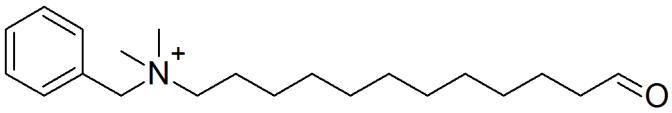


FIGURE 19. BAC-12 degradation with *Staphylococcus epidermidis* strain HI3534no-qac.

The degradation of BAC-12 occurred rapidly during the first 24 hours of incubation. However, in the subsequent hours, an unexpected increase in BAC-12 concentrations was observed. This trend could potentially be explained by solvent evaporation during incubation at 37°C, which may have led to an up-concentration of BAC-12 in the samples. Another explanation could be that BAC-12 initially stored inside the cells have been released due to the lysis of dead cell as described in 6.1.2.

The confirmation of BAC-12 degradation is supported by the identification of four main transformation products: TP318, TP304, TP248 and TP262. The identification was made on m/z ppm error < 20 ppm and retention time and comparison with literature (Larsson et al. 2024); The 3 digit number after TP means the monoisotopic mass of the compound (Table 8).

TABLE 8. BAC-12 transformation products identified

Compound	Molecular formula
TP262	
TP248	
TP304	
TP318	

Since these transformation products are not commercially available, it was not possible to quantify them by. But comparing the area of the peak of the transformation products with the area of the peak of BAC-12 we are able to determine if the concentration of these compounds is increasing/decreasing during the incubation time.

6.3.2 Biodegradation products

6.3.2.1 Results for *Staphylococcus epidermidis* HI3487qac

Figure 20 to Figure 23 show the results regarding the formation of transformation products. It can be observed that the most prominently produced TPs are TP262 and TP248, followed by smaller quantities of TP304 and TP 18. Their production peaks around 10 hours of incubation, with the exception of TP318, which exhibits a significant increase in concentration at 120 hours of incubation.

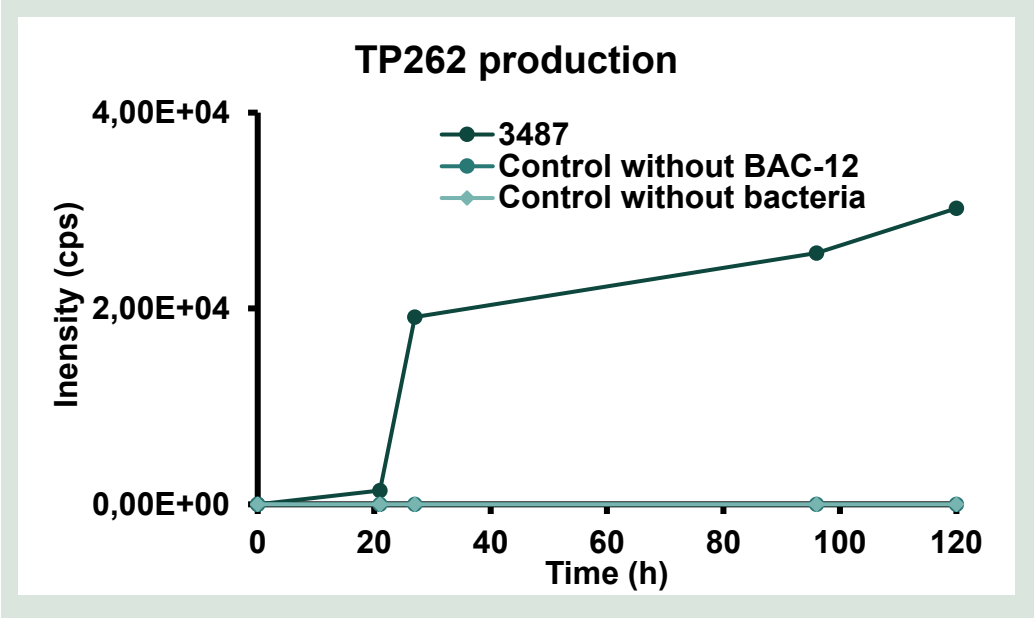


FIGURE 20. TP262 production with *Staphylococcus epidermidis* HI3487qac.

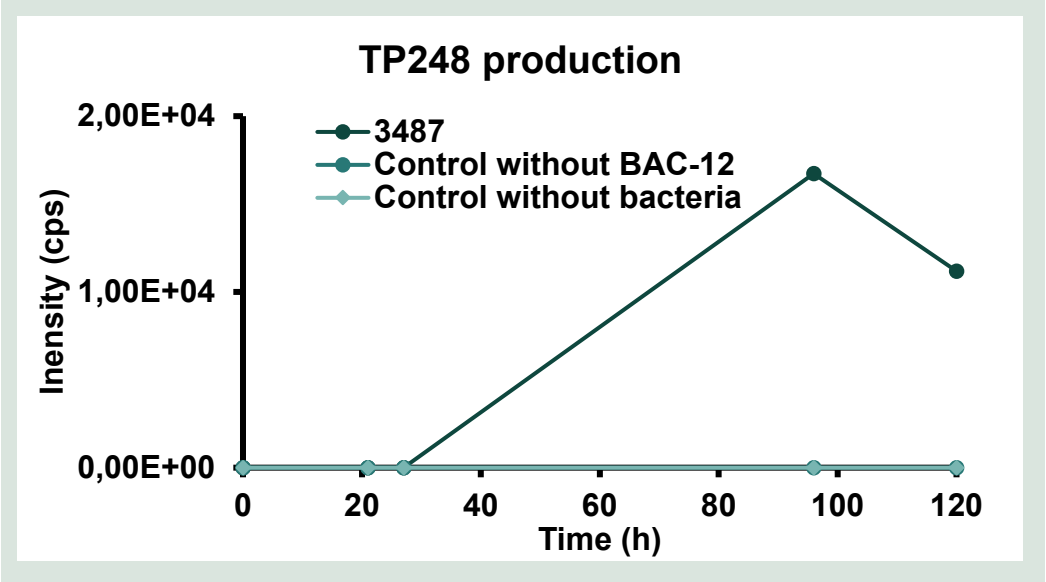


FIGURE 21. TP248 production with *Staphylococcus epidermidis* HI3487qac.

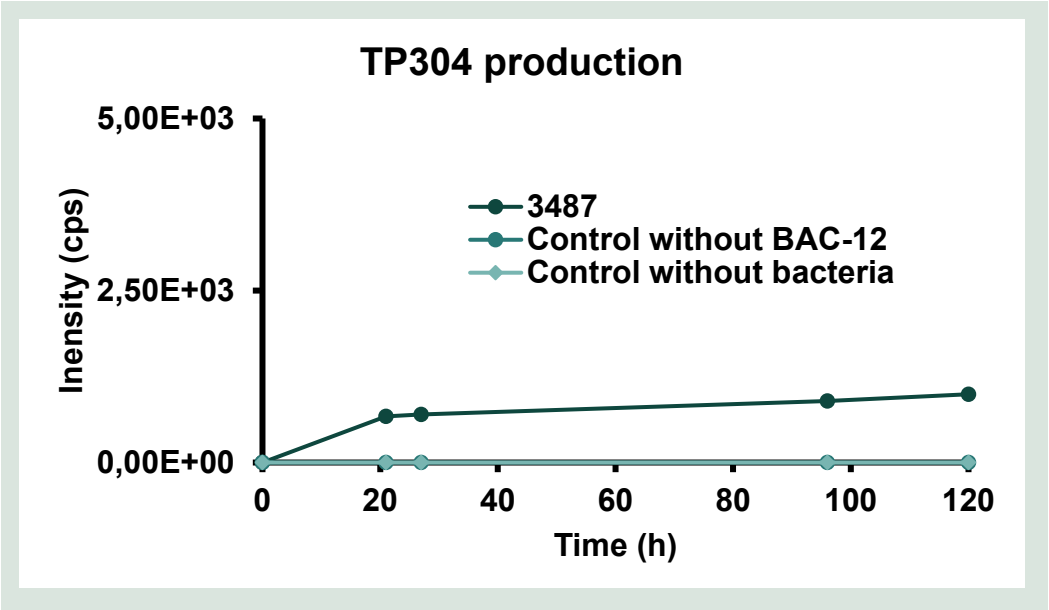


FIGURE 22. TP304 production with *Staphylococcus epidermidis* HI3487qac.

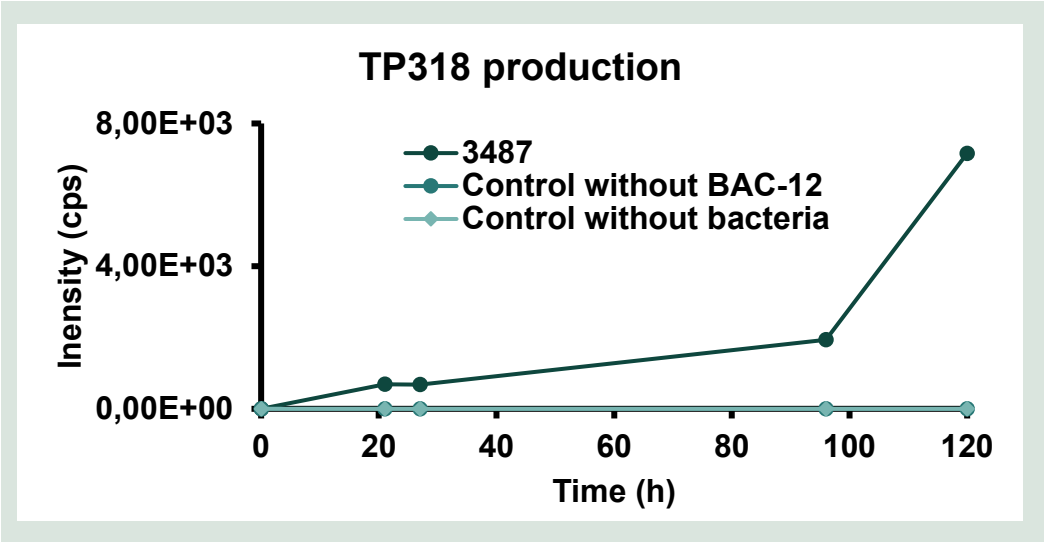


FIGURE 23. TP318 production with *Staphylococcus epidermidis* HI3487qac.

6.3.2.2 Results for *Staphylococcus epidermidis* HI3534no-qac.

Thus, the formation of transformation products with strain not containing *qacA* is different with respect to the strain containing *qacA*. The TP262 and TP318 are initially formed in the first 20 h and then and subsequently degraded during the incubation. Vice versa, TP 248 and TP304 are constantly formed with the maximum at the end of the incubation period (120h) (Figure 24 to Figure 27).

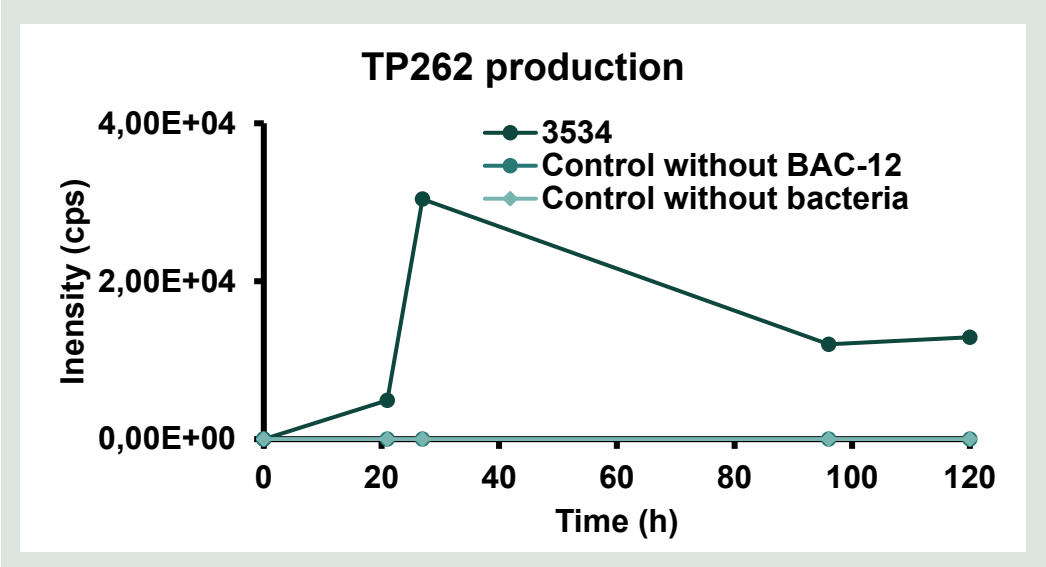


FIGURE 24. TP262 production with *Staphylococcus epidermidis* HI3534no-qac.

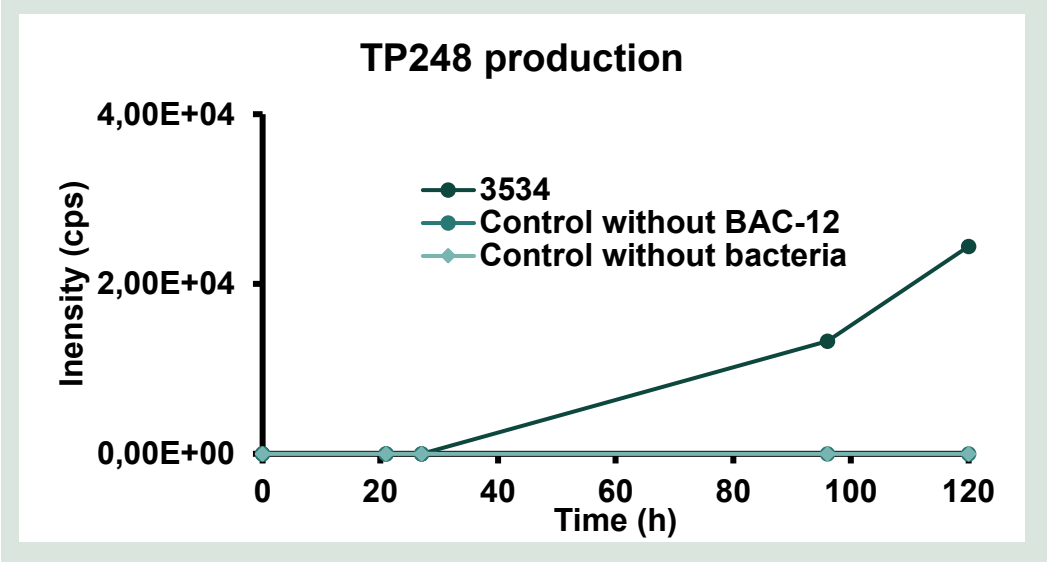


FIGURE 25. TP248 production with *Staphylococcus epidermidis* HI3534no-qac.

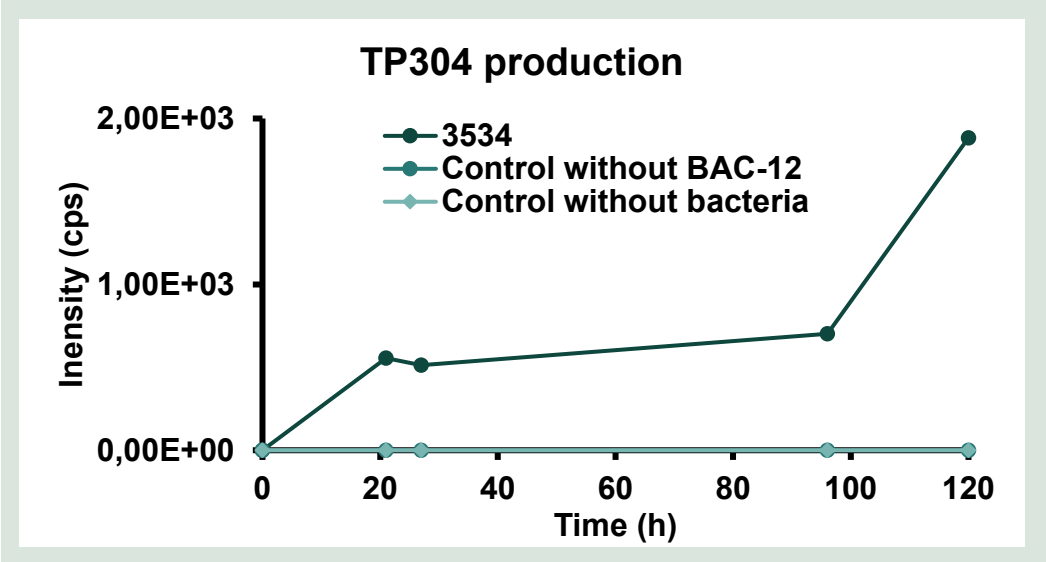


FIGURE 26. TP304 production with *Staphylococcus epidermidis* HI3534no-qac.

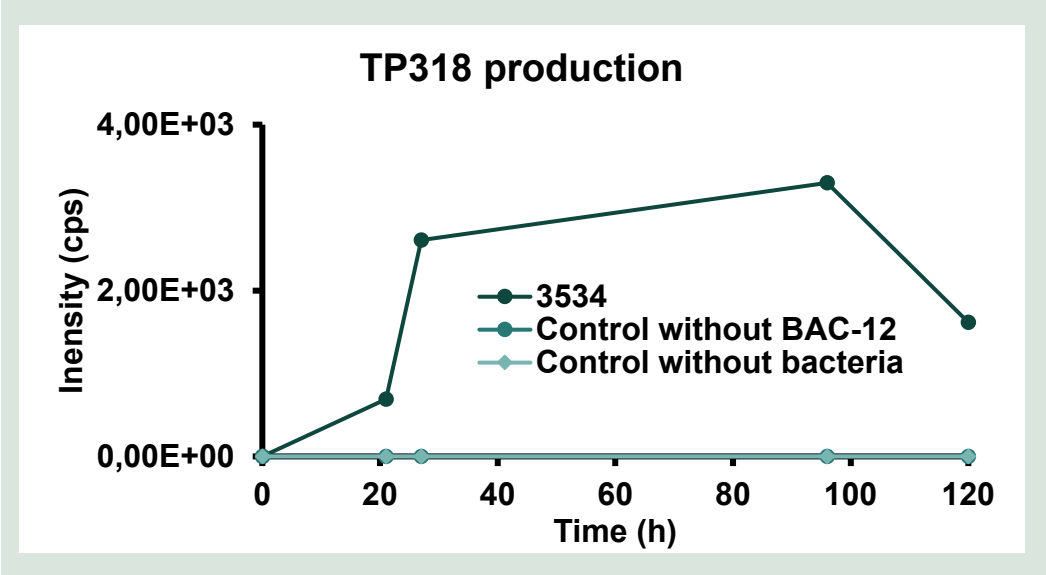


FIGURE 27. TP318 production with *Staphylococcus epidermidis* HI3534no-qac.

6.3.2.3 *Staphylococcus aureus* JE2qac and JE2lacZ

Analysis of the supernatants from the *S. aureus* JE2qac and JE2lacZ incubations indicate a BAC-12 degradation of 90-95% (Figure 28 and Figure 29). However, the production of transformation products is different between the two strains (Figure 30 to Figure 37). *S. aureus* JE2qac incubation resulted in substantial production of TPs, with the maximum reached between 100 and 120h and a small further degradation of TP262 and TP318. In contrast, *S. aureus* JE2lacZ incubation resulted in a very low concentration of all TPs monitored, indicating that other metabolic pathways or BAC-12 degradation mechanism are involved.

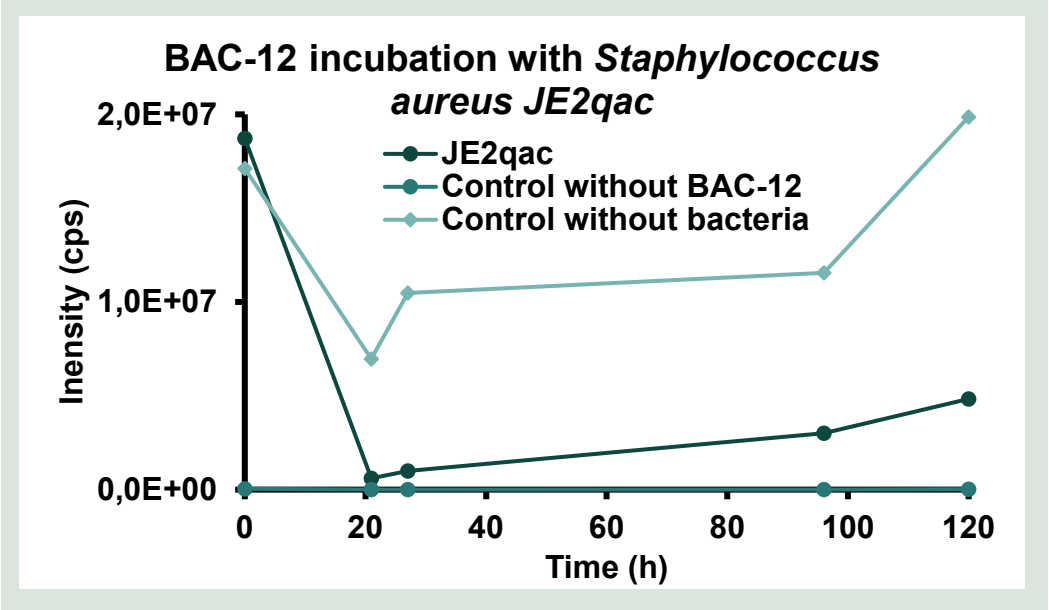


FIGURE 28. BAC-12 degradation with *Staphylococcus aureus* JE2qac.

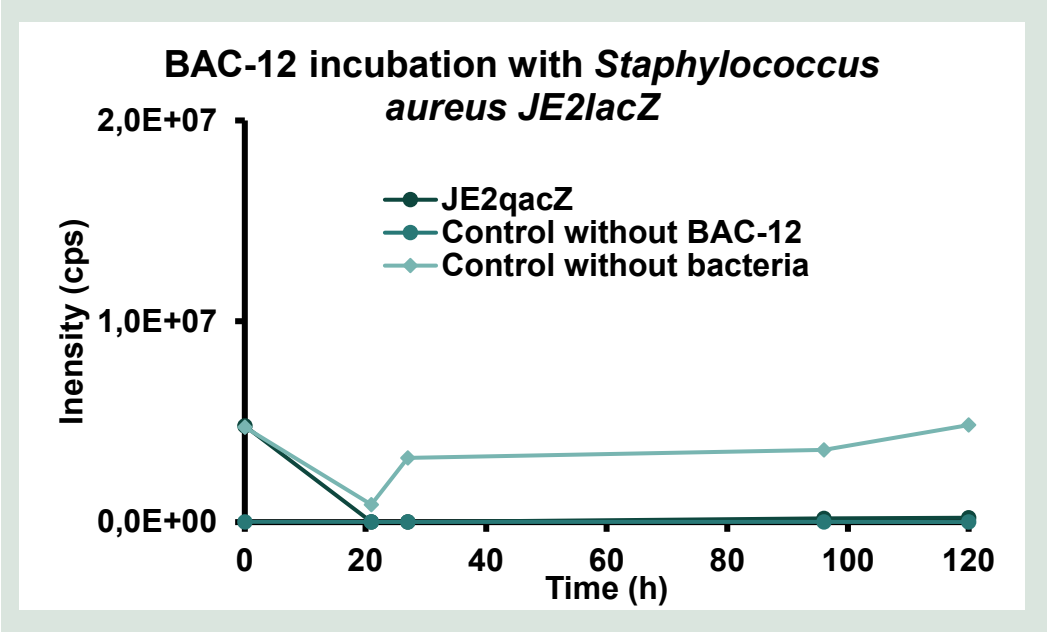


FIGURE 29. BAC-12 degradation with *Staphylococcus aureus* JE2lacZ.

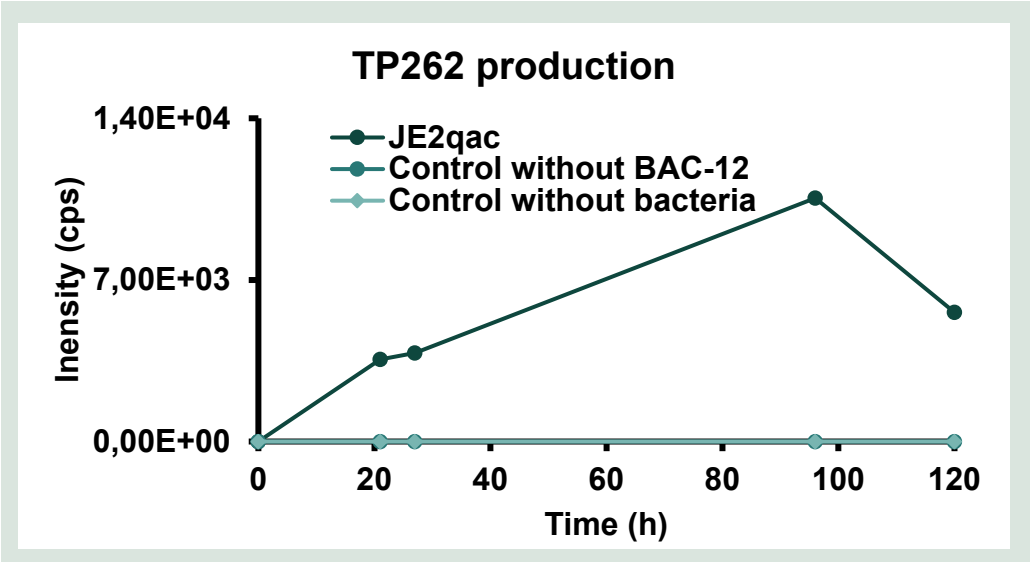


FIGURE 30. TP262 production with *Staphylococcus aureus* JE2qac.

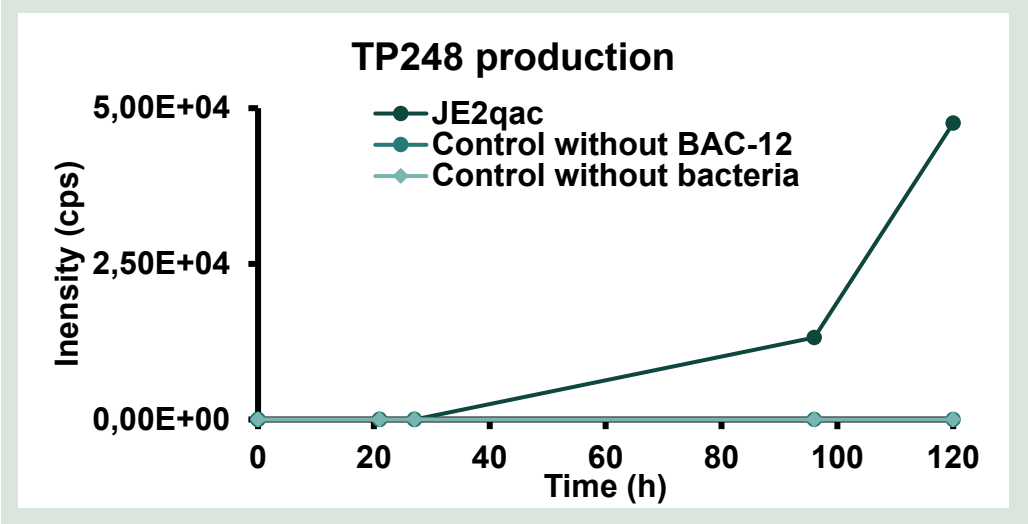


FIGURE 31. TP248 production with *Staphylococcus aureus* JE2qac.

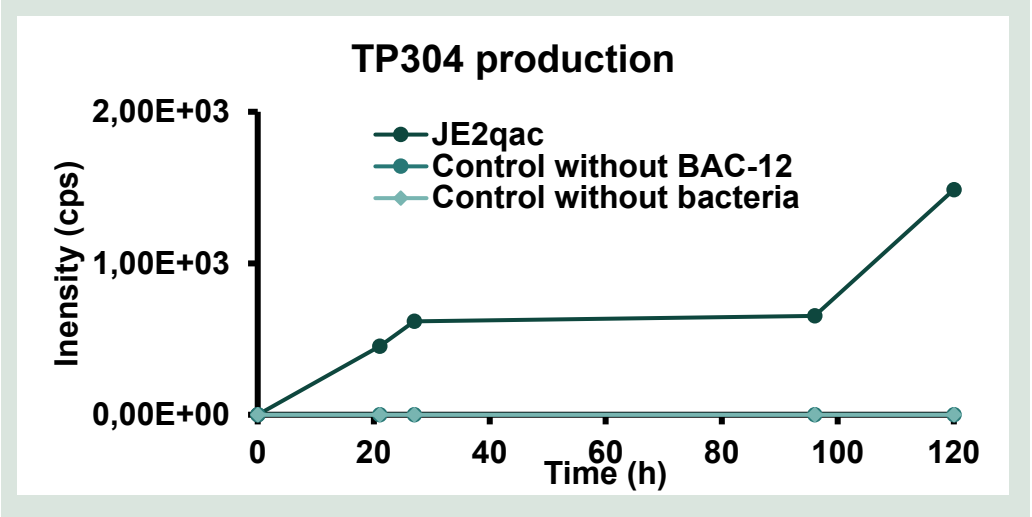


FIGURE 32. TP304 production with *Staphylococcus aureus* JE2qac.

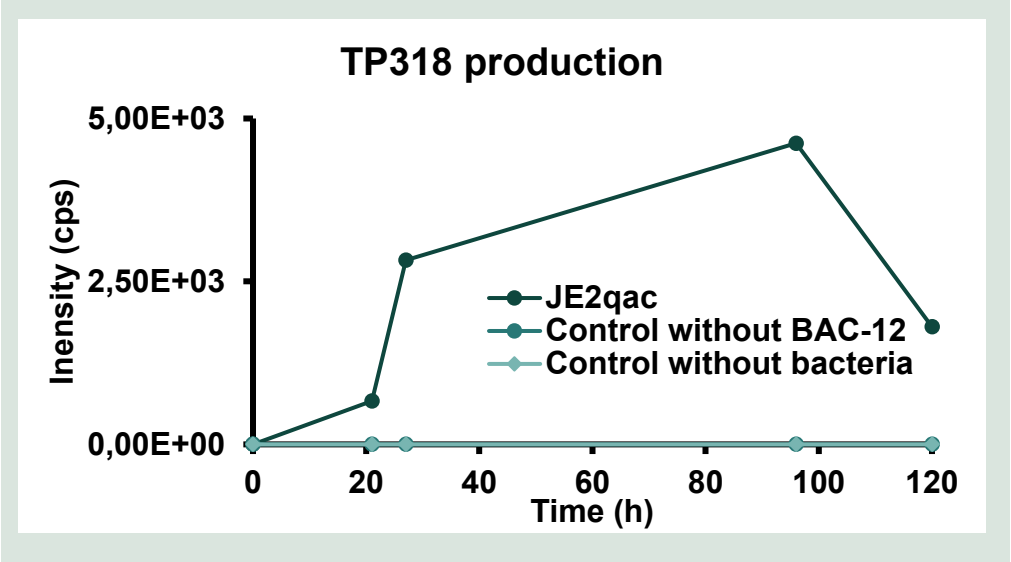


FIGURE 33. TP318 production with *Staphylococcus aureus* JE2qac.

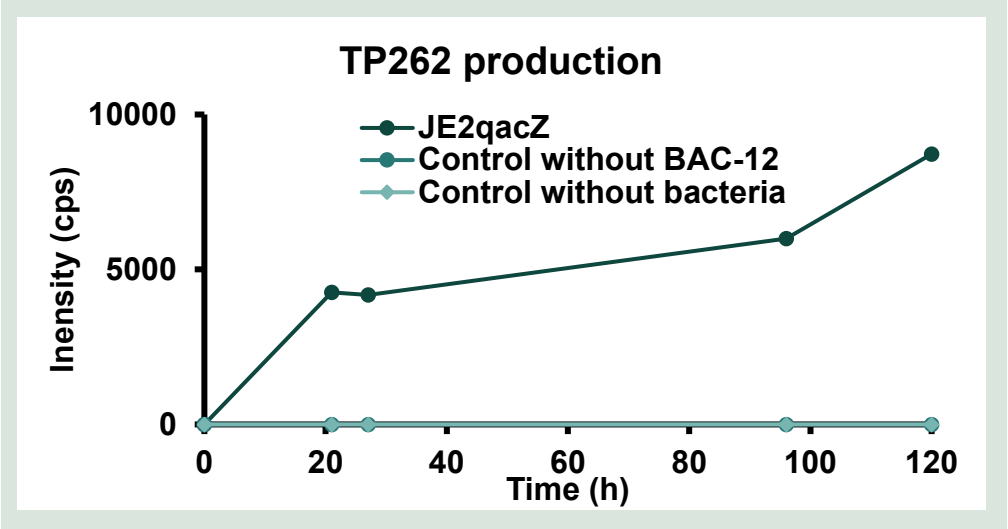


FIGURE 34. TP262 production with *Staphylococcus aureus* JE2lacZ.

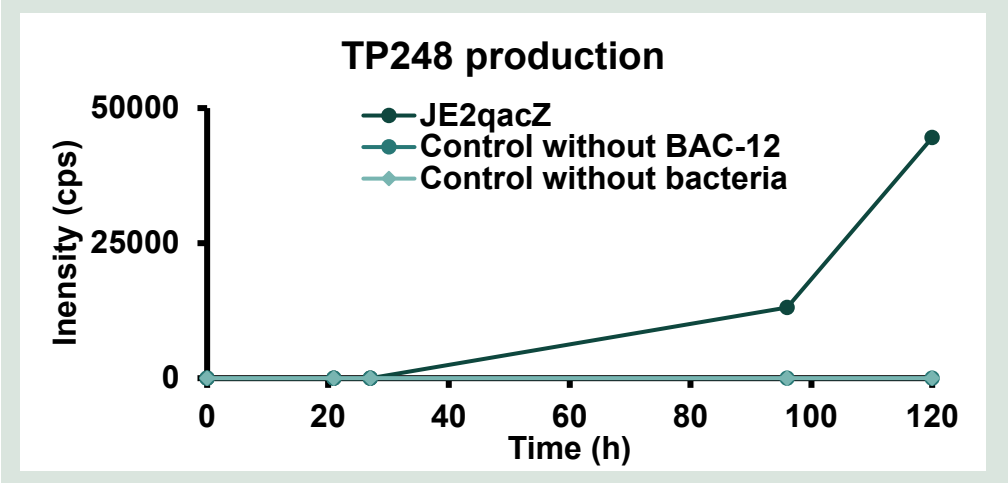


FIGURE 35. TP248 production with *Staphylococcus aureus* JE2lacZ.

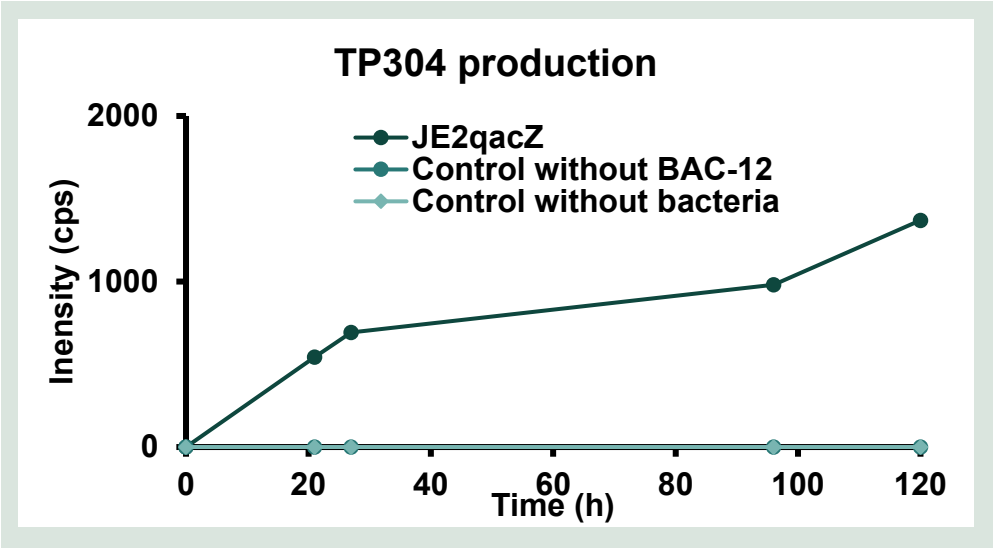


FIGURE 36. TP304 production with *Staphylococcus aureus* JE2lacZ.

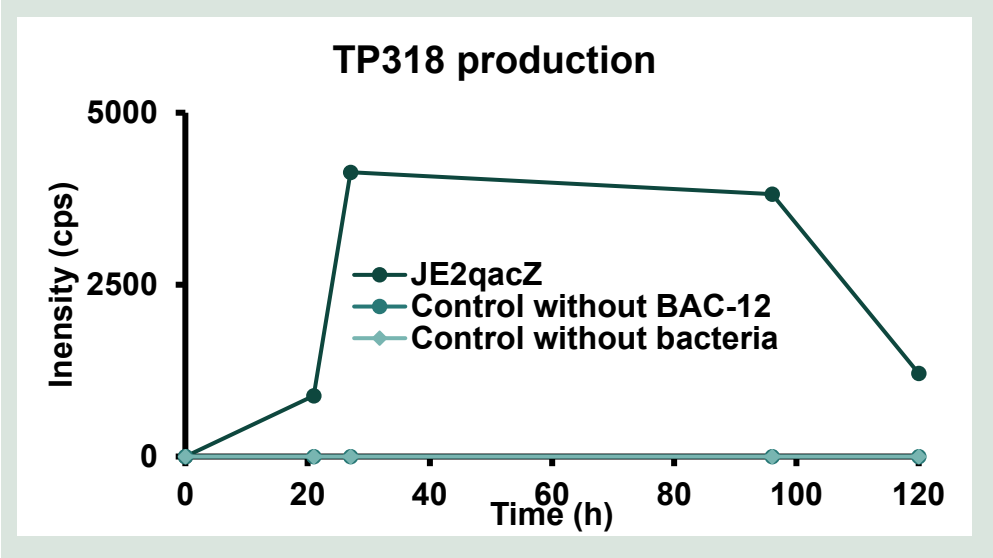


FIGURE 37. TP318 production with *Staphylococcus aureus* JE2lacZ.

6.4 Discussion

All the *Staphylococcus* strains tested were able to metabolize BAC-12, indicating that the ability to degrade this compound is not exclusive to resistant strains but is a shared metabolic trait among the strains examined. Notably, even the non-resistant strain was able to degrade BAC-12, which suggests that degradation can occur independently of specific resistance mechanisms. However, resistance in some strains is likely facilitated by a combination of different-mechanisms, primarily the action of efflux pumps that actively transport BAC-12 out of the cell, reducing its intracellular concentration. Additionally, these resistant strains appear to have enhanced metabolic pathways that enable them to efficiently break down BAC-12. This dual mechanism—efflux and metabolism—contributes to the strains' ability to resist and degrade BAC-12 more effectively compared to non-resistant strains (Table 9).

TABLE 9. Summarizing the observed differences in BAC-12 degradation capacity and TPs production across different bacterial strains. The symbols indicates a range from - (no degradation) to +++ (strong degradation).

Strain	qac genes	BAC-12 degrada-tion	TP262	TP248	TP304	TP318
Staphylococcus epidermidis 3487	qacA	++	++	++	+	+
Staphylococcus epidermidis 3534	none	++	++	++	+	+
Staphylococcus aureus JE2qac	qacA (qacA – cloned via pRAB12)	+++	++	++	++	++
Staphylococcus aureus JE2lacZ	pRAB12 –lacZ plasmid vector	+++	+	+	+	+
Pseudomonas nitrore-ducens strain 19	N/A	-	-	-	-	-

7. Overall discussion and conclusions

Quaternary ammonium compounds (QACs) are widely used in many parts of society and while they are largely predicted to be removed in sewage plants there is a risk that these compounds and their degradation products may be present in the environment. In this project we have examined the degradation of BAC-12 and assessed the transformation products as well as DDAC for their antimicrobial properties as well as for their ability to select for bacteria that harbour *qacA* involved in extruding QACs from bacterial cells by efflux.

The degradation of BAC is complex, and the separation of the BAC degradation products proved somewhat difficult, most likely due to co-elution of transformation products with similar structures. However, despite these challenges we were able to isolate 4 transformation products and confirm their structure, namely TP250, TP278, TP320 and TP318. In addition, we included several transformation products that were commercially available.

To examine the impact of BAC-12 transformation products on pathogenic bacteria we selected *S. aureus* and *S. epidermidis* as target bacteria. These bacteria are among the major causes of skin and soft tissue infections as well as a large number of invasive infections such as endocarditis, osteomyelitis and bacteraemia while at the same time as being transmitted in health care environments and causing nosocomial infections (Schilcher et al., 2020). Available to the project we had clinical collections of both *S. aureus* and *S. epidermidis* strains. Due to experimental challenges associated with the genetic manipulation of the clinical strains we ended up with two strain pairs representing a *qacA* positive and a *qacA* negative strain for both S*. epidermidis* and *S. aureus*. Such challenges are common for clinical strains (Jones et al., 2015). For *S. epidermidis* we decided on two strains that had similar growth properties and where one of the strains contained a >30 kb plasmid expression *qacA* and the *qacR* repressor needed to control *qacA* gene expression. In addition, the strains could be distinguished by their antibiotic resistance markers with the *qacA* positive strain (HI3487qac) being resistant to gentamycin and the *qacA* negative strain (HI3534no-qac) being resistant to erythromycin. These properties allowed the two strains to be co-cultured without any significant growth bias and subsequently allowed for the determination of the ratio of *qacA* positive cells relative to the *qacA* negative cells.

For *S. aureus* we created an isogenic strain pair by cloning the *qacA-qacR* genes into the plasmid pRAB12-*lacZ* by substituting the *lacZ* gene with the *qac* genes. In this strain pair we also confirmed that the plasmids themselves did not affect the growth of JE2, the *S. aureus* USA300 strain employed. For this strain pair we were able to differentiate the cells based on the *qacA* negative cells expressing β-galactosidase, the *lacZ* gene product that in the presence of the substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) results in blue colonies. While this in principle distinguished *qacA* positive cells from *qacA* negative cells we experienced that slightly too many cells on the agar plates interfered with the *lacZ* attributed colouring of the *qacA* negative colonies and this challenge may have been a source of variability in our assessments of competition between *S. aureus* cells carrying *qacA* genes and those that did not contain *qacA* as discussed below.
With our strain pairs we turned to examining susceptibility to BAC-12, DDAC and the transformation products. For this we used a traditional broth dilution assay for assessing the minimal inhibitory concentration (MIC) and in addition we defined a “sub-inhibitory” concentration as

the highest concentration of a compound that allowed growth similar a given strain in the absence of compound. When examining the antimicrobial properties of the parent compounds we found that the MIC for BAC-12 was greater for cells harbouring *qacA* for both *S. aureus* and *S. epidermidis* while the difference was less pronounced for DDAC. Importantly for all tested transformation products the MICs were between 200 and >1000 times greater than the values observed for the parent compound, BAC-12. Thus, we were not able to detect antimicrobial properties of any of the transformation products.

Subsequently we assessed how the parent compound and the transformation products affected the competition between strains with and without *qacA* when co-cultivated. Here we observed that for the *S. epidermidis* strain pair sub-MIC concentrations of both BAC-12 and DDAC provided strong selection of the strain harbouring the *qacA* gene where after three days of exposure the *qacA* containing strain had taken over the entire population. Similar results were observed for DDAC in *S. aureus* whereas a sub-MIC concentration of BAC-12 did not select the *qacA* containing strain during co-cultivation in this strain pair. This was somewhat surprising as we had noted a fourfold difference in MIC between the JE2qac compared to the JE2non-qac. However, we observed less difference in the sub-MIC concentrations between the two strains and this may be the reason that it was not translated into selection of the *qacA* containing strain. For the transformation products we also assessed the minimal inhibitory concentration and were for some of the products still able to detect antimicrobial activity although much greater concentrations than the parent compounds were needed. Based on these analyses we estimated a sub-MIC testing concentration and with that we observed either no or marginal selection of the *qacA* containing strains. In fact, for several of the transformation products (compound QR3, QR4, TP318 and TP320) we observed that the *qacA* containing strain was counter-selected by the compounds in the *S. epidermidis* strain pair. This observation is in parallel with what has been observed for antibiotics where some of the degradation products of tetracyclin counterselect strains carrying the resistance genes (Palmer et al., 2010). For the *S. aureus* strain pair were did not see selection or counterselection of the *qacA* during co-cultivation. However, for the *S. aureus* stains we experienced substantial variation between assays. Thus, what may appear as selection of *qacA* by QR3 was not statistically significant and more repetitions will be needed to assess whether there may be a selection. In conclusion based on these results we were not able to demonstrate selection of *qacA* containing *S. aureus* or *S. epidermidis* strains in the presence of BAC-12 degradation products. However a major caveat is that we were only able to assess competition at a single concentration of the transformation products and that the sub-MIC values selected for assessment were almost arbitrarily selected due to the minimal antimicrobial activity left in the transformation products.

Lastly, we examined biodegradation of BAC-12 either by *Pseudomonas nitroreducens* or by staphylococcal strains carrying the *qacA* gene. For *P. nitroreducens* it has previously been reported to degrade QACs however in our hands we were unable to detect any degradation of BAC-12 over a five-day period. The difference between our data and those reported may be that we conducted our experiments in diluted broth media where alternative carbon sources are available and thus, the metabolization of QACs is less pronounced. In contrast for all the staphylococcal strains tested we were able to observe BAC-12 being metabolized. These results were seen irrespectively of whether the strains carried the *qacA* gene or not indicating that the ability to degrade BAC-12 is not exclusive to resistant strains but is a shared metabolic trait among the strains examined. However, more detailed studies will be needed to determine the role of the *qacA* encoded efflux activity in ridding bacterial cells from metabolic degradation products of BAC-12. The dual mechanism—efflux and metabolism—of *qacA* expressing cells may contribute to the strains' ability to resist and degrade BAC-12 more effectively compared to non-resistant strains.

8. Perspectives and limitations

The study shows that the presence of *qacA* strongly influences the minimal inhibitory concentrations of both *S. aureus* and *S. epidermidis* to to the compounds BAC-12 and DDAC. Also, our data show that in the presence of subinhibitory concentrations of these compounds, there is a selection of cells ongoing favouring those carrying the *qacA* gene. This effect was particularly apparent for *S. epidermidis*. Further, our results indicate that at for those concentrations of BAC-12 transformation products that were tested, these degradation products do not select for strains that carry the *qacA* gene. However, this conclusion can only be drawn at a single concentration of the transformation products due to the inherent difficulties of defining a minimal inhibitory concentrations for the transformation products and the low throughput of the competition assay assessing the competition between *qacA*-containing and non-containing strains. Thus, our results only provide a snapshot of the potential effects of transformation products on this competition. For providing a more nuanced picture of the ability of BAC-12 transformation products to select for *qacA* containing cells it will be valuable with a high through-put assay that can cover a variety of concentrations and ideally also the impact of environmental factors. To this end we observed that Staphylococcal cells are themselves able to degrade BAC-12 and that they do so irrespectively of the presence of the *qacA* gene. This turnover could be affected by the growth rate of the strains but this was not examined in the presence study. It could be interesting to determine the impact of degradation products produced by *S. aureus* on the selection of *qacA*-containing cells. Further, our finding that *S. aureus* and potentially other pathogens are able to metabolize BAC-12 should be taken into considerations in future assessments of BAC-12 susceptibility testing.

9. References

Arnold, W. A.; Blum, A.; Branyan, J.; Bruton, T. A.; Carignan, C. C.; Cortopassi, G.; Datta, S.; DeWitt, J.; Doherty, A.-C.; Halden, R. U.; Harari, H.; Hartmann, E. M.; Hrubec, T. C.; Iyer, S.; Kwiatkowski, C. F.; LaPier, J.; Li, D.; Li, L.; Muñiz Ortiz, J. G.; Salamova, A.; Schettler, T.; Seguin, R. P.; Soehl, A.; Sutton, R.; Xu, L.; Zheng, G. Quaternary Ammonium Compounds: A Chemical Class of Emerging Concern. Environ. Sci. Technol. 2023, 57 (20), 7645–7665. https://doi.org/10.1021/acs.est.2c08244.

Barwal, A., Chaudhary, R., 2014. To study the performance of biocarriers in moving bed biofilm reactor (MBBR) technology and kinetics of biofilm for retrofitting the existing aerobic treatment systems: a review. Rev Environ Sci Biotechnol 13, 285–299. https://doi.org/10.1007/s11157-014-9333-7.

Barwal, A., Chaudhary, R., 2017. Optimization of operational parameters in moving bed biofilm reactor with low cost polystyrene biocarrier by the response surface method. Water Qual Res J 52 (1), 26–41. https://doi.org/10.2166/wqrjc.2017.026.

Bouteh, E.; Ahmadi, N.; Abbasi, M.; Torabian, A.; Van Loosdrecht, M. C. M.; Ducoste, J. Biodegradation of Organophosphorus Pesticides in Moving Bed Biofilm Reactors: Analysis of Microbial Community and Biodegradation Pathways. Journal of Hazardous Materials 2021, 408, 124950. https://doi.org/10.1016/j.jhazmat.2020.124950.

Brown MH, Skurray RA. Staphylococcal multidrug efflux protein QacA. J Mol Microbiol Biotechnol. 2001, 3(2):163-70.

Casas, M. E.; Chhetri, R. K.; Ooi, G.; Hansen, K. M. S.; Litty, K.; Christensson, M.; Kragelund, C.; Andersen, H. R.; Bester, K. Biodegradation of Pharmaceuticals in Hospital Wastewater by Staged Moving Bed Biofilm Reactors (MBBR). Water Research 2015, 83, 293–302. https://doi.org/10.1016/j.watres.2015.06.042.

COMMISSION REGULATION (EU) No 1119/•2014 - of 16 October 2014 - amending Annex III to Regulation (EC) No 396/•2005 of the European Parliament and of the Council as regards maximum residue levels for benzalkonium chloride and didecyldimethylammonium chloride in or on certain products -.

Daub, G. H.; Castle, R. N. THE SYNTHESIS OF SOME SUBSTITUTED BENZYL IODIDES1. J. Org. Chem. 1954, 19 (10), 1571–1574. https://doi.org/10.1021/jo01375a002.

DeLeo, P. C.; Huynh, C.; Pattanayek, M.; Schmid, K. C.; Pechacek, N. Assessment of Ecological Hazards and Environmental Fate of Disinfectant Quaternary Ammonium Compounds. Ecotoxicology and Environmental Safety 2020, 206, 111116. https://doi.org/10.1016/j.ecoenv.2020.111116.

di Biase, A., Kowalski, M. S., Devlin, T. R. & Oleszkiewicz, J. A. Moving bed biofilm reactor technology in municipal wastewater treatment: A review. J. Environ. Manage. 247, 849–866 (2019).

El Sayed Zaki M, Bastawy S, Montasser K. Molecular study of resistance of Staphylococcus aureus to antiseptic quaternary ammonium compounds. J Glob Antimicrob Resist. 2019, 17:94-97. doi: 10.1016/j.jgar.2018.11.022.

Ertekin, E., Konstantinidis, K.T., Tezel, U., 2017. A rieske-type oxygenase of pseudomonas sp. BIOMIG1 converts benzalkonium chlorides to benzyldimethyl amine. Environ Sci Technol 51, 175–181. https://doi.org/10.1021/acs.est.6b03705.

Ertekin, E.; Hatt, J. K.; Konstantinidis, K. T.; Tezel, U. Similar Microbial Consortia and Genes Are Involved in the Biodegradation of Benzalkonium Chlorides in Different Environments. Environ. Sci. Technol. 2016, 50 (8), 4304–4313. https://doi.org/10.1021/acs.est.5b05959.

Fey, P. D., Endres, J. L., Yajjala, V. K., Widhelm, T. J., Boissy, R. J., Bose, J. L., et al. (2013). A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* 4:e00537-12. doi: 10.1128/mBio.00537-12

Fortunato, M.S., Baroni, S., Gonzalez, A.J., Alvarez ´ Roncancio, J.D., Storino, A., Parise, C., et al., 2019. Biodegradation and detoxification of benzalkonium chloride in synthetic and industrial effluents in upflow biofilm aerobic reactors. Water, Air Soil Pollut 230, 79. https://doi.org/10.1007/s11270-019-4126-9.

Hansen, L. H.; Jensen, L. B.; Sørensen, H. I.; Sørensen, S. J. Substrate Specificity of the OqxAB Multidrug Resistance Pump in Escherichia Coli and Selected Enteric Bacteria. Journal of Antimicrobial Chemotherapy 2007, 60 (1), 145–147. https://doi.org/10.1093/jac/dkm167.

Hegstad, K. et al. Does the Wide Use of Quaternary Ammonium Compounds Enhance the Selection and Spread of Antimicrobial Resistance and Thus Threaten Our Health? Microb 2010, Drug Resist. 16, 91–104..

Helle L, Kull M, Mayer S, Marincola G, Zelder ME, Goerke C, Wolz C, Bertram R. Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. Microbiology (Reading). 2011, 157(Pt 12):3314-3323. doi: 10.1099/mic.0.052548-0.

Ismail, Z. Z.; Tezel, U.; Pavlostathis, S. G. Sorption of Quaternary Ammonium Compounds to Municipal Sludge. Water Research 2010, 44 (7), 2303–2313. https://doi.org/10.1016/j.watres.2009.12.029.

Jones MJ, Donegan NP, Mikheyeva IV, Cheung AL. Improving transformation of Staphylococcus aureus belonging to the CC1, CC5 and CC8 clonal complexes. PLoS One. 2015, 10(3):e0119487. doi: 10.1371/journal.pone.0119487.

Khan, A.H., Macfie, S.M., Ray, M.B., Sorption and leaching of benzalkonium chlorides in agricultural soils. J Environ Manag 2017, 196, 26–35. https://doi.org/10.1016/j.jenvman.2017.02.065.

Khan, A.H., Topp, E., Scott, A., Sumarah, M., Macfie, S.M., Ray, M.B., 2015. Biodegradation of benzalkonium chlorides singly and in mixtures by a Pseudomonas sp. Isolated from returned activated sludge. J Hazard Mater 299, 595–602. https://doi.org/10.1016/j.jhazmat.2015.07.073.

Kim, M.; Hatt, J. K.; Weigand, M. R.; Krishnan, R.; Pavlostathis, S. G.; Konstantinidis, K. T. Genomic and Transcriptomic Insights into How Bacteria Withstand High Concentrations of Benzalkonium Chloride Biocides. Appl Environ Microbiol 2018, 84 (12), e00197-18. https://doi.org/10.1128/AEM.00197-18.

LaBreck PT, Rice GK, Paskey AC, Elassal EM, Cer RZ, Law NN, Schlett CD, Bennett JW, Millar EV, Ellis MW, Hamilton T, Bishop-Lilly KA, Merrell DS. Conjugative Transfer of a Novel Staphylococcal Plasmid Encoding the Biocide Resistance Gene, qacA. Front Microbiol. 2018, 9:2664. doi: 10.3389/fmicb.2018.02664.

Larsson, Y.; Mongelli, A.; Kisielius, V.; Bester, K. Microbial Biofilm Metabolization of Benzalkonium Compounds (Benzyl Dimethyl Dodecyl Ammonium & Benzyl Dimethyl Tetradecyl Ammonium Chloride). Journal of Hazardous Materials 2024, 463, 132834. https://doi.org/10.1016/j.jhazmat.2023.132834.

Larsson, Y., Nicolausz, M., Kisielius, V., Møller, HB., Gosewinkel, **U., Bester, K**: Metabolic pathways for biotransformation of benzalkonium compounds in fungal- and bacteria-based biofilm reactors J. Haz Mat. 496, 139494, 2025, https://doi.org/10.1016/j.jhazmat.2025.139494

Leyva-Díaz, J. C.; Monteoliva-García, A.; Martín-Pascual, J.; Munio, M. M.; García-Mesa, J. J.; Poyatos, J. M. Moving Bed Biofilm Reactor as an Alternative Wastewater Treatment Process for Nutrient Removal and Recovery in the Circular Economy Model. Bioresource Technology 2020, 299, 122631. https://doi.org/10.1016/j.biortech.2019.122631.

Li, R., Liang, C., Svendsen, S.B., Kisielius, V., Bester, K., 2023. Sartan blood pressure regulators in classical and biofilm wastewater treatment – Concentrations and metabolism. Water Res 229, 119352. https://doi.org/10.1016/j.watres.2022.119352.

Li, R.; Kadrispahic, H.; Koustrup Jørgensen, M.; Brøndum Berg, S.; Thornberg, D.; Mielczarek, A. T.; Bester, K. Removal of Micropollutants in a Ceramic Membrane Bioreactor for the Post-Treatment of Municipal Wastewater. Chemical Engineering Journal 2022, 427, 131458. https://doi.org/10.1016/j.cej.2021.131458.

Li, X. & Brownawell, B. J. Analysis of Quaternary Ammonium Compounds in Estuarine Sediments by LC−ToF-MS: Very High Positive Mass Defects of Alkylamine Ions as Powerful Diagnostic Tools for Identification and Structural Elucidation. Anal. Chem. 81, 7926–7935 (2009).

Maillard, J.-Y. Impact of Benzalkonium Chloride, Benzethonium Chloride and Chloroxylenol on Bacterial Antimicrobial Resistance. Journal of Applied Microbiology 2022, 133 (6), 3322–3346. https://doi.org/10.1111/jam.15739.

Maillard, J.-Y.; Pascoe, M. Disinfectants and Antiseptics: Mechanisms of Action and Resistance. Nat Rev Microbiol 2024, 22 (1), 4–17. https://doi.org/10.1038/s41579-023-00958-3.

Maher, B. Lab disinfectant harms mouse fertility. Nature 453, 964–964 (2008).

Majumder P, Ahmed S, Ahuja P, Athreya A, Ranjan R, Penmatsa A. Cryo-EM structure of antibacterial efflux transporter QacA from Staphylococcus aureus reveals a novel extracellular loop with allosteric role. EMBO J. 2023, 42(16):e113418. doi: 10.15252/embj.2023113418.

Mayer S, Boos M, Beyer A, Fluit AC, Schmitz FJ. Distribution of the antiseptic resistance genes qacA, qacB and qacC in 497 methicillin-resistant and -susceptible European isolates of Staphylococcus aureus. J Antimicrob Chemother. 2001, 47(6):896-7.

Mikkelsen K, Bowring JZ, Ng YK, Svanberg Frisinger F, Maglegaard JK, Li Q, Sieber RN, Petersen A, Andersen PS, Rostøl JT, Høyland-Kroghsbo NM, Ingmer H. An Endogenous Staphylococcus aureus CRISPR-Cas System Limits Phage Proliferation and Is Efficiently Excised

from the Genome as Part of the SCCmec Cassette. Microbiol Spectr. 2023, 11(4):e0127723. doi: 10.1128/spectrum.01277-23.

Monk IR, Stinear TP. From cloning to mutant in 5 days: rapid allelic exchange in Staphylococcus aureus. Access Microbiol. 2021, 3(2):000193. doi: 10.1099/acmi.0.000193.

Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ. Complete Bypass of Restriction Systems for Major Staphylococcus aureus Lineages. mBio. 2015, 6(3):e00308-15. doi: 10.1128/mBio.00308-15.

Mongelli, A., Koning, J. T., Larsson, Y. J., Vollertsen, J., Stutz, M., & Bester, K. (2024). Emissions of quaternary alkylammonium compounds (QUAT-Fate) (ISBN: 978-87-7038-613-5). The Danish Environmental Protection Agency. https://www2.mst.dk/Udgiv/publications/2024/04/978-87-7038-613-5.pdf

Moss, G. P., Smith, P. a. S. & Tavernier, D. Glossary of class names of organic compounds and reactivity intermediates based on structure (IUPAC Recommendations 1995). Pure Appl. Chem. 67, 1307–1375 (1995).

Nair D, Memmi G, Hernandez D, Bard J, Beaume M, Gill S, Francois P, Cheung AL. Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. J Bacteriol. 2011, 193(9):2332-5. doi: 10.1128/JB.00027-11.

Noguchi N, Nakaminami H, Nishijima S, Kurokawa I, So H, Sasatsu M. Antimicrobial agent of susceptibilities and antiseptic resistance gene distribution among methicillin-resistant Staphylococcus aureus isolates from patients with impetigo and staphylococcal scalded skin syndrome. J Clin Microbiol. 2006, 44(6):2119-25. doi: 10.1128/JCM.02690-05.

Novick R. Properties of a cryptic high-frequency transducing phage in Staphylococcus aureus. Virology. 1967, 33(1):155-66. doi: 10.1016/0042-6822(67)90105-5.

Oggioni, M. R.; Furi, L.; Coelho, J. R.; Maillard, J.-Y.; Martínez, J. L. Recent Advances in the Potential Interconnection between Antimicrobial Resistance to Biocides and Antibiotics. Expert Review of Anti-infective Therapy 2013, 11 (4), 363–366. https://doi.org/10.1586/eri.13.16.

Oh, S.; Kurt, Z.; Tsementzi, D.; Weigand, M. R.; Kim, M.; Hatt, J. K.; Tandukar, M.; Pavlostathis, S. G.; Spain, J. C.; Konstantinidis, K. T. Microbial Community Degradation of Widely Used Quaternary Ammonium Disinfectants. Appl Environ Microbiol 2014, 80 (19), 5892–5900. https://doi.org/10.1128/AEM.01255-14.

Paijens, C.; Bressy, A.; Frère, B.; Tedoldi, D.; Mailler, R.; Rocher, V.; Neveu, P.; Moilleron, R. Urban Pathways of Biocides towards Surface Waters during Dry and Wet Weathers: Assessment at the Paris Conurbation Scale. Journal of Hazardous Materials 2021, 402, 123765. https://doi.org/10.1016/j.jhazmat.2020.123765.

Palmer AC, Angelino E, Kishony R. Chemical decay of an antibiotic inverts selection for resistance. Nat Chem Biol. 2010, 6(2):105-7. doi: 10.1038/nchembio.289.

Patrauchan, M.A., Oriel, P.J., 2003. Degradation of benzyldimethylalkylammonium chloride by Aeromonas hydrophila sp. K. J Appl Microbiol 94, 266–272. https://doi.org/10.1046/j.1365-2672.2003.01829.x.

Piddock, L. J. V. Multidrug-Resistance Efflux Pumps ? Not Just for Resistance. Nat Rev Microbiol 2006, 4 (8), 629–636. https://doi.org/10.1038/nrmicro1464.

Ribič U, Klančnik A, Jeršek B. Characterization of Staphylococcus epidermidis strains isolated from industrial cleanrooms under regular routine disinfection. J Appl Microbiol. 2017, 122(5):1186-1196. doi: 10.1111/jam.13424.

Sakarya, F. K.; Haznedaroglu, B. Z.; Tezel, U. Biological Removal of Benzalkonium Chlorides from Wastewater by Immobilized Cells of Pseudomonas Sp. BIOMIG1 in an up-Flow Packed Bed Reactor. Journal of Hazardous Materials 2021, 418, 126210. https://doi.org/10.1016/j.jhazmat.2021.126210.

Schilcher K, Horswill AR. Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies. Microbiol Mol Biol Rev. 2020, 84(3):e00026-19. doi: 10.1128/MMBR.00026-19.

Skovgaard S, Nielsen LN, Larsen MH, Skov RL, Ingmer H, Westh H. *Staphylococcus epidermidis* isolated in 1965 are more susceptible to triclosan than current isolates. PLoS One. 2013, 8(4):e62197. doi: 10.1371/journal.pone.0062197.

Takeuchi K, Imai M, Shimada I. Conformational equilibrium defines the variable induction of the multidrug-binding transcriptional repressor QacR. Proc Natl Acad Sci U S A. 2019, 116(40):19963-19972. doi: 10.1073/pnas.1906129116.

Tezel, U., Pavlostathis, S.G., 2011. Role of Quaternary Ammonium Compounds on Antimicrobial Resistance in the Environment. In: Keen, P.L., Montforts, M.H.M.M. (Eds.), Antimicrobial Resistance in the Environment. John Wiley & Sons Inc, pp. 349–387. https://doi.org/10.1002/9781118156247.ch20.

Tezel, U. & Pavlostathis, S. G. Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. Curr. Opin. Biotechnol. 33, 296–304 (2015).

Tezel, U., Tandukar, M., Martinez, R.J., Sobecky, P.A., Pavlostathis, S.G., 2012. Aerobic biotransformation of n -tetradecylbenzyldimethylammonium chloride by an enriched pseudomonas spp. community. Environ Sci Technol 46, 8714–8722. https://doi.org/10.1021/es300518c.

Tchobanoglous, G., Burton, F. L. (Franklin L., Stensel, H. D., & Metcalf & Eddy. Wastewater engineering : treatment and reuse. (Boston : McGraw-Hill, 2003).

van Ginkel, C.G. 2004. Biodegradation of Cationic Surfactants. In Handbook of Detergents Part B: Environmental Impact, 121, 523–549. Marcel Dekker.

Wassenaar TM, Ussery D, Nielsen LN, Ingmer H. Review and phylogenetic analysis of qac genes that reduce susceptibility to quaternary ammonium compounds in Staphylococcus species. Eur J Microbiol Immunol (Bp). 2015, 5(1):44-61. doi: 10.1556/EUJMI-D-14-00038.

Wessels S, Ingmer H. Modes of action of three disinfectant active substances: a review. Regul Toxicol Pharmacol. 2013, 67(3):456-67. doi: 10.1016/j.yrtph.2013.09.006.

Östman, M.; Lindberg, R. H.; Fick, J.; Björn, E.; Tysklind, M. Screening of Biocides, Metals and Antibiotics in Swedish Sewage Sludge and Wastewater. Water Research 2017, 115, 318–328. https://doi.org/10.1016/j.watres.2017.03.011.

Ødegaard, H., Rusten, B. & Westrum, T. A new moving bed biofilm reactor - applications and results. Water Sci. Technol. 29, 157–165 (1994).

Zhang, C.; Cui, F.; Zeng, G.; Jiang, M.; Yang, Z.; Yu, Z.; Zhu, M.; Shen, L. Quaternary Ammonium Compounds (QACs): A Review on Occurrence, Fate and Toxicity in the Environment. Science of The Total Environment 2015, 518–519, 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007.

Acknowledgements

Postdoc Ifigeneia Kyrkou from Hanne Ingmer's group for assembly and annotation of the *S. epidermidis* strain 3534 and for resistance gene search in both strain 3487 and 3534

Senior Scientist Martin Bojer from Hanne Ingmer's group for help with cloning in S. aureus JE2.

Lab. Tech Gitte Petersen for practical help with the labor intensive co-culture experiments.

Intern student Isaac Pearson for the production and isolation of the BAC-12 transformation product.

Asst. Prof. Janine Bowring from Hanne Ingmer's group for scientific sparing.

PhD Jose Alfredo Samaniego Castruita, bioinformatician at Department of Clinical Microbiology at Hvidovre Hospital for work done as described in section 3.1.1. Bioinformatic screening and sequencing of plasmids. Further thank you to Associate Professor Mette Damkjær Bartels and Professor Henrik T. Westh for giving us access to the strain collection at Hvidovre Hospital and to Clinical Senior Specialist Sarah Juel Paulsen for Nanopore sequencing of the selected *S. aureus* from their collection.

Thank you to Danish Environmental Protection Agency for funding this project.

Appendix 1.

Appendix 1.1

Strain list

IM08B is a mutant that allows plasmid DNA to bypass a conserved Staphylococcal type IV restriction system, which was identified as the major barrier to transformation with foreign DNA (Monk et. Al, 2015)

8325-4, *S. aureus* (Novick, 1967).

RN4220 *S. aureus,* restriction defective of 8325-4 (Nair et al., 2011)

JE2, *S. aureus,* CC8, USA300, plasmid cured (Fey et al., 2013)

JE2qac = *S. aureus* JE2 transformed with pRAB12-qacA (qacA substituting lacZ)

JE2lacZ = *S. aureus* JE2 transformed with pRAB12-lacZ

MS59, S. aureus strain containing *qacA-qacR* plasmid (Mikkelsen et al., 2023)

M7088, S. aureus strain lacking *qacA-qacR* (Mikkelsen et al., 2023)

HI3487 qac-containing, gentamycin resistant *S. epidermidis.* Other strain name: BD-62b 2/11-11 (Skovgaard et al., 2013)

HI3534 no-qac, erythromycin resistant *S. epidermidis.* Other strain name: Sissel Skovgaard (BD-45 19/9-11) (Skovgaard et al., 2013)

JCM 16554 *S. aureus, smr/qacC* positive strain, from DSMZ culture collection (https://bacdive.dsmz.de/strain/161809).

JCM 16555 *S. aureus, qacA* positive strain, from DSMZ culture collection (https://bacdive.dsmz.de/strain/161810).

JCM 16556 *S. aureus, qacB* positive strain, from DSMZ culture collection (https://bacdive.dsmz.de/strain/161811).

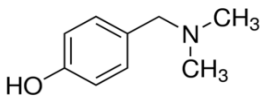
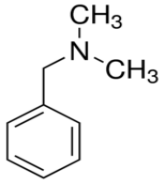
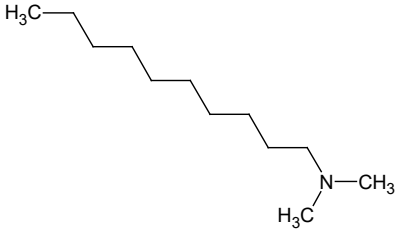
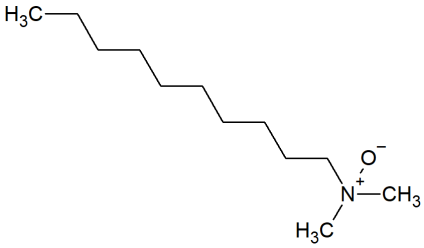
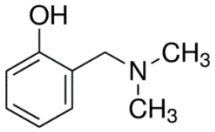
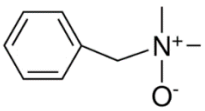
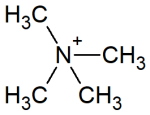
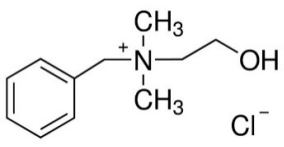
HI3549 *S. aureus*, *smr/qacC* positive. Other strain name: JCM1554 *smr* positiv 5/1-12 (Skovgaard et al., 2013).

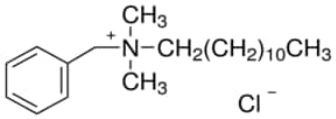
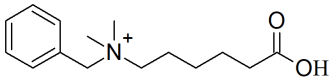
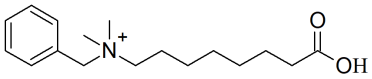
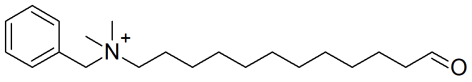
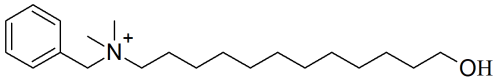
Pseudomonas nitroreducens (*P. nitroreducens*) strains were purchased from the Culture Collection University of Gothenburg (ccug.se).

P. nitroreducens 54619 strain collection from Gothenberg collection (https://www.ccug.se/strain?id=54619).

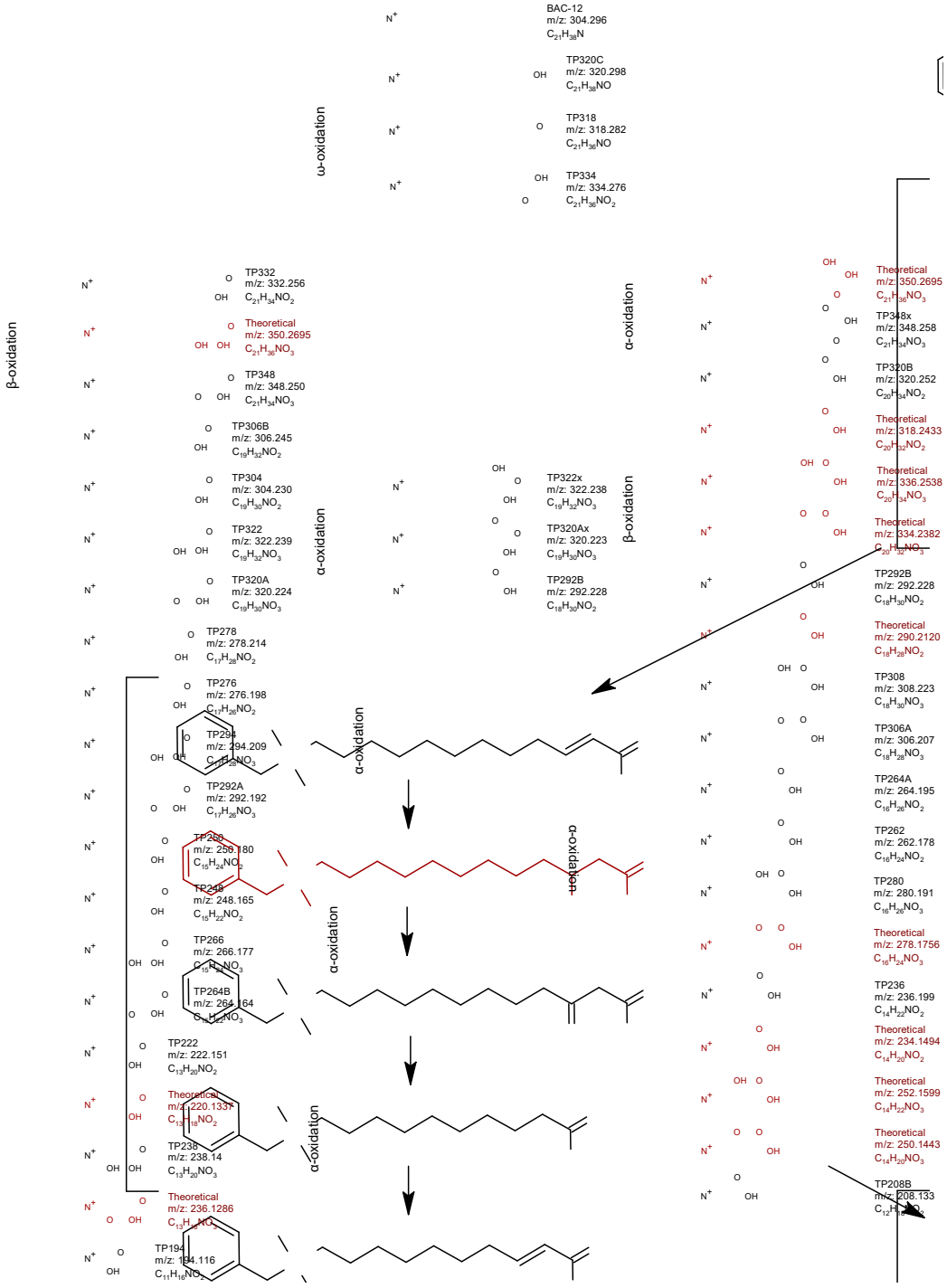
Plasmids
pRAB12-*lacZ* plasmid containing *lacZ* reporter gene (Helle et al., 2011).

Appendix 1.2

Compound ID	Chemical name	Chemical formula
TP151A	(Dimethylaminomethyl) phenol	
TP208B	N,N-Dimethylbenzylamine	
TP186	N,N-Dimethyldecylamine	
TP201	N,N-Dimethyldecylamine N-oxide	
TP151B	2-[(Dimethylamino)methyl]phenol	
TP151C	N,N-Dimethylbenzylamine-N-Oxide	
TP074	Tetramethylammonium chloride	
TP180	N-Benzyl-2-hydroxy-N,N-dimethylethanamin	

Compound ID	Chemical name	Chemical formula
BAC-12	Benzyldimethyldodecylammonium chloride (BAC-12)	
TP250	TP250	
TP278	TP278	
TP318	TP318	
TP320C	TP320C	

Appendix 1.3



Metabolites of BAC 12 formed by α, β and ω oxidation. All metabolites in black are detected by HPLC-HRMS using product ion spectra and thus reaching confidence level 2 or higher. The incubations were conducted by a heterogenous multispecies microbial biofilm community on Z200 carriers in micro MBBRs at 22 °C under aerobic conditions (from Larsson et al., 2024). Additionally several multihydroxylated and isomers of the compounds described in this paper have been revealed (Larsson et al., 2025)

The role of quaternary ammonium compounds and their degradation products in selection of bacterial resistance

Biocidal quaternary ammonium compounds (QACs) are extensively used as disinfectants and in cleaning products, and their active components may enter wastewater, or soil after use. In these environments, these biocides can persist either as intact compounds or as degradation products formed through exposure to UV light, temperature shifts, pH changes, or microbial metabolism. Therefore, it is important to understand whether degradation products retain antibacterial activity or can select for bacteria with reduced biocide susceptibility.
This project focused on degradation products of quaternary ammonium compounds (QACs), specifically benzalkonium chloride with a 12 carbon alkyl chain (BAC 12), the major constituent of ADBAC/BKC (C12 16). Two related QACs, ADBAC/BKC (C12 16) and DDAC, are registered under the EU Biocidal Products Regulation. The study examined how BAC 12 degrades under environmental conditions and whether its transformation products affect two bacterial pathogens, Staphylococcus aureus and Staphylococcus epidermidis, carrying qacA genes associated with QAC tolerance.
In the project four major BAC 12 transformation products were isolated while several other ones were available commercial. The project demonstrated that while BAC 12 and DDAC strongly selected for qacA positive bacteria, none of the tested degradation products produced similar selection pressure. Additionally, Staphylococci were able to metabolize BAC 12 regardless of the qacA status of the cells. Study limitations include testing only one concentration of each transformation product and variability in competition assays. Future work should develop improved, high throughput methods to assess how QAC transformation products influence selection of qacA containing bacteria under environmentally relevant conditions and explore the role of bacterial metabolism in QAC turnover and tolerance development.

The Danish Environmental
Protection Agency
Lerchesgade 35
DK - 5000 Odense C

www.mst.dk