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Master Thesis

Intrinsic bacterial defence mechanisms against biocides in Gram-negative bacteria

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Declaration of Authorship

I hereby declare that I am the sole author of this work. No assistance other than that which is permitted has been used. Ideas and quotes taken directly or indirectly from other sources are identified as such. This written work has not yet been submitted in any part.

Vienna, _____ _____

Signature

Dedication

Coming so far in life to graduate from university was never a certain thing for me. This achievement involved over the years a lot of people. Even throughout the whole period of a master thesis from scratch to finishing the manuscript you get in contact with a lot of people. I will try to mention everyone who had an impact on my path. Let's start with my family:

I would like to thank my father, stepmother and my grandparents for their support. Even through hard times of failure and my decision at the age of 24 to start studying for real they never stopped supporting me.

At university I would like to say thank you to:

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Abstract

Food safety measures guarantee safe and healthy foods for the world population and is globally seen as one of the most important topics. Even though food safety has developed substantially over the last decades due to the introduction of standards and hygiene concepts, most currently there are new challenges to be mastered. Due to wrong- and misuse of bactericidal substances bacteria might develop tolerance and resistance mechanism against these substances. The use of widely applied disinfectants like quaternary ammonium compounds (QACs) in sub-lethal concentration led to the formation of persistent microbes, which can resist the general cleaning and disinfection process in food plants. Especially Gram-negative bacteria possess an additional outer membrane with therein-anchored lipopolysaccharides (LPS), which act as an additional defence barrier and presents an opportunity for adaptation. A possible and promising alternative to QACs as disinfectants are ionic liquids (ILs), however so far in detail nothing is known about the influence of LPS on the efficacy of ILs.

The aims of this thesis were to find out, how different variations of LPS influence the efficacy of QACs and ILs, as alternative antimicrobial substances. Additionally, the formation of tolerances and resistances of bacteria against antibiotics and ILs after exposure of bacteria to sub-lethal concentrations of ILs over a long time period was investigated. In the course of this thesis QACs and ILs showed similar results and that LPS have a major influence on the efficacy of both substance classes. The efficacy did not increase with shorter LPS length but rather a formation of two levels with a breaking point in the outer core region of the LPS was observed. The IL [$C_{10,10}$MIM][Cl] was proven as a new promising disinfectant for further analysis and potential application. This IL was effective in the lowest concentrations and additionally its efficacy was not influenced by LPS. The long-term evolution experiment with exposure to sub-lethal concentrations did not show any formation of tolerances or resistance mechanisms against disinfectants or antibiotics. For future experiments the mode of action of the IL [$C_{10,10}$MIM][Cl] should be investigated and the long term evolution experiment repeated with a different approach of daily increasing sub-lethal stress.

Zusammenfassung

Lebensmittelsicherheit garantiert sichere Lebensmittel für die Bevölkerung und stellt global gesehen eines der wichtigsten Themen dar. Auch wenn sich in den letzten Jahrzehnten viel getan hat und die Lebensmittelsicherheit dank der Einführung von Standards und Hygienekonzepten auf einem hohen Niveau ist, gibt es aktuell neue Herausforderungen zu bewältigen. Durch die falsche Anwendung und Missbrauch von antimikrobiellen Substanzen werden bei Bakterien Toleranzen und Resistenzmechanismen gegen diese ausgebildet. Der Einsatz von üblichen Desinfektionsmitteln wie quartäre Ammoniumverbindungen (QACs) in sub-letalen Konzentrationen führt zur Bildung von persistenten Keimen, welche dem normalen Reinigungs- und Desinfektionsprozess in lebensmittelerzeugenden Betrieben widerstehen können. Speziell Gram-negative Bakterien verfügen mit ihrer äußeren Membran und den darin verankerten Lipopolysacchariden (LPS) über eine zusätzliche Abwehrinstanz und die Möglichkeit zur Anpassung. Eine mögliche und vielversprechende Alternative zu QACs als Desinfektionsmittel stellen ionische Flüssigkeiten (ILs) dar, jedoch ist aktuell im Detail bezüglich des Einflusses von LPS auf die Wirksamkeit nichts bekannt.

Die Ziele dieser Arbeit waren es herauszufinden, wie sich verschiedenste Varianten von LPS auf die Wirksamkeit von QACs auswirken und ob ILs als Alternative zu QACs dieselben oder andere Ergebnisse zeigen würden. Zusätzlich wurde untersucht, wie sich die Ausbildung von Toleranzen und Resistenzen von Bakterien gegenüber Antibiotika und ILs entwickelt nachdem die Bakterien über einen längeren Zeitraum sub-letalen Konzentration von ILs ausgesetzt worden sind. Im Zuge dieser Arbeit konnte gezeigt werden, dass QACs und ILs vergleichbare Ergebnisse liefern. Des Weiteren wurde aufgezeigt, dass LPS einen maßgeblichen Einfluss auf die Wirksamkeit von QACs und ILs haben. Die Wirksamkeit nimmt jedoch nicht linear mit abnehmender LPS Länge zu, sondern es kommt zur Ausbildung von zwei Plateaus mit einer Sprungstelle, welche im äußeren Kernbereich des LPS liegt. Als vielversprechende neue Substanz für die Zukunft als Desinfektionsmittel hat sich die IL $[C_{10,10}MIM][Cl]$ erwiesen. Diese IL war schon in niedrigster Konzentration wirksam und konnte ebenfalls zeigen, dass LPS in allen Abstufungen keinen Einfluss auf die Wirksamkeit hatten. Im Zuge des Langzeitexperimentes mit der dauerhaften Exposition von Bakterien zu sub-letalen Konzentrationen konnte keine Ausbildung von Toleranzen oder Resistenzen gegenüber Desinfektionsmittel oder Antibiotika beobachtet werden. Für zukünftige Experimente wäre es interessant den Wirkmechanismus der IL $[C_{10,10}MIM][Cl]$ zu erforschen und das Evolutionsexperiment in einem neuen Ansatz mit täglich steigendem sub-letalen Stress zu wiederholen.

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List of abbreviations

BHQ1	Black Hole Quencher 1
CFU	Colony-forming unit
CGSC	Coli Genetic Stock Center
ddH_2O	deionised water
dNTP	Nucleoside triphosphate
E. coli:	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAM	6-Carboxyfluorescein
HACCP	Hazard analysis and critical control points
HGT	Horizontal gene transfer
ILs	Ionic liquids
Kdo	Keto-deoxyoctulosonate
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharides
MIC	Minimum inhibitory concentration
N/A	Not applicable
NPN	N-Phenylnaphthalen-1-amine
OD_{600}	Optical density, measured at 600 nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QACs	Quaternary ammonium compounds
qPCR	Quantitative polymerase chain reaction
SAR	Structure-activity relationship
TSA+Y	Tryptone soy agar plus yeast extract
TSB+Y	Tryptone soy broth plus yeast extract

1 Introduction

1.1 Food safety in general

Food safety is an omnipresent topic which is of high importance for the food industry, economy, public health and especially for the customer. Food safety represents healthy food supply and stands therefore for ensuring that foods are physically, chemically and microbiologically clean (Özay and Özyildirim, 2017). In the last decades, consumption of locally produced foods has changed. Nowadays in our globalized world people consume foods that have their origin all over the world. The implementation of food safety systems based on the hazard analysis and critical control point (HACCP) concept made foods nowadays safer than ever before. Globally seen it is still a long way to go, over two million people especially in developing countries are dying because of unsafe food products and food diseases every year (Boddie and Kun, 2014; Weinroth et al., 2018). Due to varying knowledge of food safety among consumers and some food related incidents and outbreaks worldwide the confidence of consumers in food safety has begun to fluctuate (Jevšnik et al., 2008). As a result and to counter these issues, in 2002 the European Union founded the European Food Safety Authority (EFSA) (Grunert, 2005). The main tasks of the EFSA are to evaluate risks regarding food safety and to provide independent scientific expertise in terms of food- and feed safety. This risk analysis serves as a scientific basis for the European Commission for the development of new control measures and delivers an important contribution for consumerism (Heppner, 2004).

Food hazards can be of microbial, chemical or physical nature. Most occurring hazards in foods in the European Union 2016 were pathogenic microorganisms, allergens, foreign bodies, heavy metals and mycotoxins. All categories of foods and products are concerned. The most crucial ones are product categories like fish and fish products, meat and meat products as well as cereals and bakery products. Mentioned before, the biggest hazard of all were pathogenic microorganisms which consist mainly of the Gram-negative bacteria *Salmonella spp.*, *Escherichia coli* and *Campylobacter spp.*, the Gram-positive bacteria *Listeria monocytogenes* and Norovirus (European Union, 2017).

In the last decades antibiotics have been widely used to treat and prevent diseases in animals, disinfectants have been used for rinsing food plants and slaughterhouses and as well for the cleaning of equipment. Due to these applications antibiotics contribute to animal growth and disinfectants for food sanitation and a good hygienic state in food plants. However, through abuse, misuse and the general widespread application of antibiotics and disinfectants in recent years more bacteria with increasing resistance to these substances

have been isolated. Certain antibiotics might lead to a resistance against themselves, against chemically related substances (cross-resistance) and/or against unrelated substances (co-resistance). Up to now investigations have shown that antibiotic as well as disinfectant resistance is caused by changes of targeted genes, membrane permeability barriers, efflux pump systems and horizontal gene transfer as a result from long-time adaption, mutation and selective pressure (Zhou et al., 2017).

Nowadays there are many challenges for food safety in form of huge food plants, globalization and resistances to antimicrobial substances this master thesis is hopefully a contribution to get a better understanding for bacterial defence mechanisms and their influence on the efficacy of disinfectants and their adaptation to bactericidal substances.

1.2 Food related pathogenic bacteria used in this work

1.2.1 Differences between Gram-negative and Gram-positive bacteria

With Gram staining most bacteria can be easily divided into two groups, Gram-negative and Gram-positive bacteria. This method is mainly used for clinical diagnostic purposes and as well for the detection of bacteria in environmental samples. During the staining procedure bacterial samples get dyed with crystal violet. Iodine solution is added which forms with crystal violet an insoluble complex. Afterwards the bacterial cells get decolourized with alcohol, only Gram-positive bacteria keep the purple colour while Gram-negative bacteria lose it (Budin et al., 2012).

This behaviour can be explained by the structural differences between these two classes. Most Gram-negative bacteria are surrounded by an additional lipid bilayer, the so-called outer membrane. This outer membrane is in many ways different from the inner cell membrane. On the outside of the outer membrane there is attached a high amount of Lipopolysaccharides (LPS), that are characteristic for each bacterial species and act as an endotoxin. The outer membrane also contains transmembrane proteins, so-called porines. Through porines smaller molecules can diffuse into the periplasmatic space, while larger ones are blocked. On the inside of the outer membrane, lipoproteins connect the outer membrane with the thin peptidoglycan cell wall (Fritsche, 2016).

Gram-positive bacteria possess no outer membrane but instead have got more layers of cross-linked peptidoglycan, which are responsible for the Gram staining. They also possess teichoic and wall teichoic acids, which are responsible for various functions of Gram-positive bacteria like adhesion to host cells, maintaining the cell shape and antibiotic resistance (Brown et al., 2013). A comparison of Gram-negative and Gram-positive bacteria is shown in Figure 1.

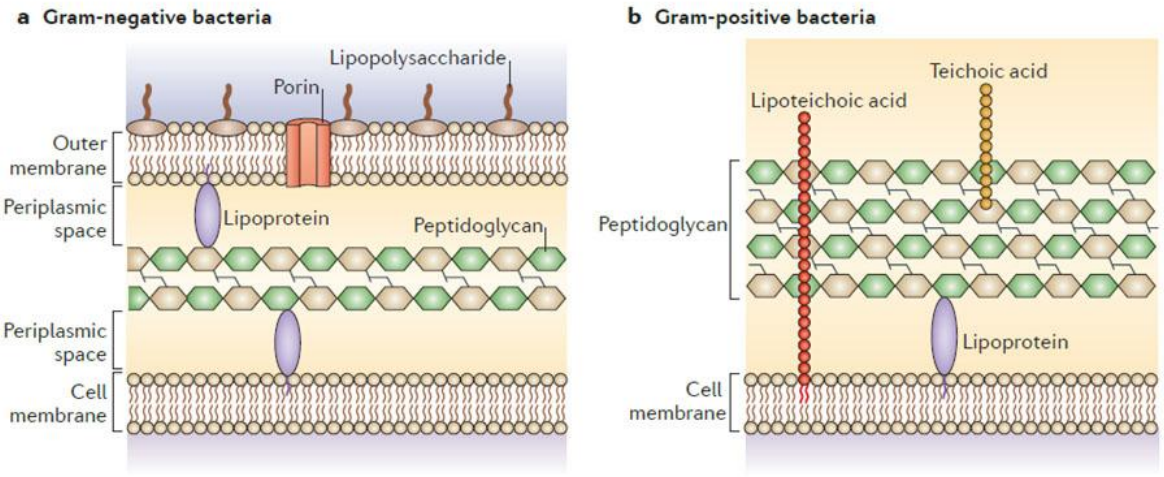

Figure 1: Comparison of Gram-negative and Gram-positive cell wall structure (Brown et al., 2015)

1.2.2 Escherichia coli

E. coli is a Gram-negative facultative anaerobic bacterium that can be found in the intestines of humans and numerous animals. It can be divided into two groups, pathogenic and non-pathogenic *E. coli*. Pathogenic *E. coli* are categorized as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC). Symptoms of an infection with these pathogenic bacteria can be diarrheal disease, urinary tract infection, sepsis and meningitis (Choi et al., 2017). *E. coli* can grow between a pH-value of 5–9 and is also able to survive for a short duration at a pH-value of 2. Due to these characteristics it is possible for *E. coli* to pass through the acid conditions of the stomach and colonize the intestinal tract. It is one of the most studied microorganisms and used in biotechnology industry for the production of heterologous proteins. Disorders caused by infections with pathogenic *E. coli* most often occur after the consumption of contaminated food and water during travel in developing countries and is the leading cause of traveller's diarrhea (Baker et al., 2016).

1.2.3 Listeria monocytogenes

L. monocytogenes is a Gram-positive facultative anaerobic bacterium that can be found naturally in the terrestrial environment, livestock manure, fresh and salt water, decomposing plants and thus in many foods which are associated with these habitats. Although the bacterial genus *Listeria* comprises of many species, outbreaks of listeriosis in humans are nearly exclusively reported for the species *L. monocytogenes (Jami et al., 2014)*. *L. monocytogenes* has the ability to grow under harsh conditions and can resist high concentrations of heavy metals and salts, low pH-values, low temperatures and as well a low a_w-values. The majority of listeriosis outbreaks have been linked to the consumption of contaminated food, mainly dairy products, ready-to-eat deli meats and produce. In healthy

humans listeriosis causes usually a self-limiting gastroenteritis whereas in immunocompromised individuals an invasive and systemic infection can occur which may lead to meningitis, encephalitis and septicaemia with a high mortality rate of 25–30 %. Listeriosis is also very harmful for pregnant individuals and can lead to stillbirth, abortion or septicaemia of the baby (Rychli et al., 2014).

1.3 Quaternary ammonium compounds as commonly used disinfectants

Quaternary ammonium compounds (QACs) are a class of antimicrobials that came into widespread use after the end of World War II. Nowadays they are widely used in the food and healthcare industry as well in consumer products with the purpose of cleaning, sanitizing and disinfecting surfaces. Their main advantages are low toxicity and ability to be formulated for specific applications or targeted organisms. QACs belong to the class of cationic detergents. The cationic part consists of a nitrogen atom with four attached groups. These four groups can be of very different nature and give the substance their specific properties. For example, side-chains with alkyl groups with a varying length from C_{12} to C_{16} have shown the highest antimicrobial activity. The anionic part usually consists of a chloride or bromide. Both parts interact together and form a QAC salt (Gerba, 2015). The general structure of QACs is shown in Figure 2.

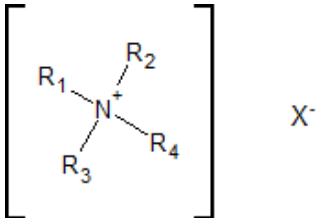

Figure 2: General structure of QACs

The main mode of action of QACs in bacteria is to disrupt the physical and ionic stability of the cell membrane. Because of their extensive use and inappropriate application, bacteria are exposed to a wide range of QAC concentrations. These concentration levels can be non-inhibitory, sub-inhibitory and over-inhibitory. Especially if bacteria are exposed to sub-inhibitory concentrations, these environments become selective and induce adaptation processes. This can also lead to a co-resistance to other antimicrobial substances like antibiotics. Resistance to QACs involves many different genes and is achieved by the over expression of efflux pumps, the acquisition of QAC-specific efflux pumps, modifications of the outer and cell membrane and through the alteration of porins. These developments have shown, that QACs could not be as effective as desired (Jaglic and Cervinkova, 2012; Zou et al., 2014; Tezel and Pavlostathis, 2015). Recently, a relatively new substance class with a

high tune- and designability, so called ionic liquids (ILs), has been suggested as a new class of promising disinfectants.

1.4 Ionic liquids as promising new disinfectants

Ionic liquids (ILs) are salts that consist of combinations of organic cations and anions, which are in the liquid aggregate state below 100°C or even at room temperature (Saadeh et al., 2009; Messali et al., 2013). ILs came to the forefront as a “green” alternative for Volatile organic compounds (VOCs). VOCs are widely used in many different chemical industries as solvents. Because of their toxic and hazardous nature, they can cause environmental problems and consequently their use is often restricted or even permitted. Ionic liquids seem to be a promising, suitable and cleaner alternative to these kind of substances due to their low vapour pressure even at higher temperatures.

Beside these unique properties, ILs possess further very special physicochemical properties such as non-flammability, good solvent properties, good chemical and thermal stability and many others. Theoretically there are 10^{18} possible combinations of different cations and anions, making ILs highly tuneable and designable for any specific purpose. Although ILs are considered as “green” solvents, there still remain many questions about IL-safety and toxicity (Patel and Lee, 2012; Weyhing-Zerrer et al., 2017). Already known is, that the following factors are responsible for the modulation of IL-toxicity: (I) the length of an alkyl side-chain of the cation, (II) nature of functional groups in the side-chain of the cation, (III) nature of the cation, (IV) nature of the anion and (V) interaction of anion and cation (Egorova and Ananikov, 2014). The concept of ILs and some typical cations and anions used in ILs is shown in Figure 3 and Figure 4.

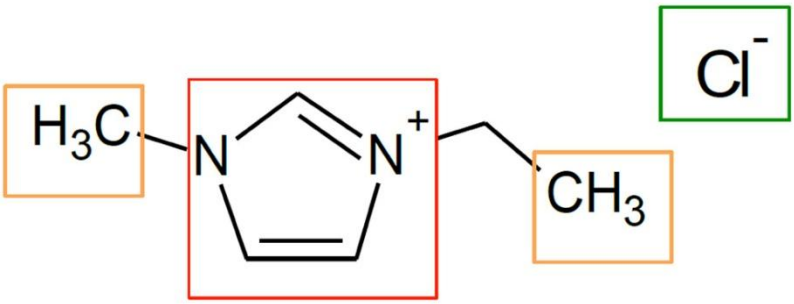

Figure 3: Concept of ILs shown exemplary on the structure of 1-Ethyl-3-methylimidazolium chloride. Red framed area: cationic core, orange framed area: variable side chains, green framed area: variable anion. Figure created by Tobias Gundolf

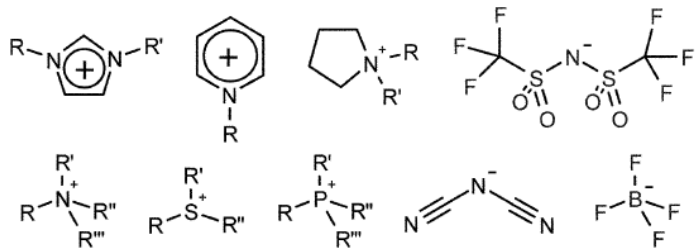

Figure 4: Typical cations and anions used in ILs (Wishart, 2009)

Due to their high tune- and designability, various beneficial properties of ILs have been utilized for a wide field of applications. They are used in chemical synthesis and catalysis, electrochemistry, biomass conversion and fuel production, liquid crystal development, metal extraction, drug delivery, biotransformation, biotechnology and many other fields (Patel and Lee, 2012; Egorova et al., 2017). Furthermore regarding food safety, ILs seem to be a promising class of novel biocides and disinfectants against bacteria and viruses (Hodyna et al., 2018; Sommer et al., 2018).

1.5 Adaptation of bacteria to bactericidal stress

Antibiotics are antimicrobial substances that have been produced by bacteria and fungi for millions of years. During the last 70 years humans have produced large amounts of antibiotic substances and used them widely worldwide for medicinal and agricultural purposes. Due to this use and naturally antibiotic synthesis and release, gradients of antibiotics are generated in the human body and the wider environment. Thereby, bacteria are exposed to concentrations that are higher and lower than the minimum inhibitory concentration (MIC). Treated humans, livestock, crops, aquacultures and the wider environment are closely connected to each other, which leads to a cycling of antibiotics and bacteria between these two. This cycle leads to the exposure of bacteria to sub-lethal antibiotic concentrations which can show their effects at least on three different levels:

- They act as selectors of resistance by enriching pre-existing resistant bacteria and selecting bacteria with *de novo* resistances.
- They act as generators of genetic and phenotypic variability by increasing the rate of adaptive evolution, which includes resistance development.
- They act as signalling molecules, thus influencing different physiological activities like virulence, biofilm formation and gene expression (Andersson and Hughes, 2014).

Further, through the intensive use and as well misuse of antibiotics human pathogens have developed resistances against antibiotics. These resistance mechanisms can occur mainly in two ways: Intrinsic resistance through mutation of genes and acquired resistance by horizontal gene transfer (HGT) between individual cells or across different species (Martinez,

2009). The primary methods behind HGT are conjugation (transfer of plasmids), transformation (uptake of DNA) and transduction (bacteriophages act as transporter for genetic information). Because many antibiotic resistance genes are located on mobile elements like plasmids, integrons and transposons conjugation is considered as the main method of HGT. Other environmental stresses besides antibiotics such as metals, detergents, nanomaterials and even ILs can cause the transmission of antibiotic resistance genes by HGT (Q. Wang et al., 2015).

Regarding QACs and ILs, the exposure to sub-lethal concentrations over a period of time can lead to more resistant and persistent pathogens. Bacterial defence mechanisms like efflux pumps, which are effective against QACs, have been shown to be effective against ILs as well (Mester et al., 2015). Other bacterial defence mechanisms are shown in Figure 5.

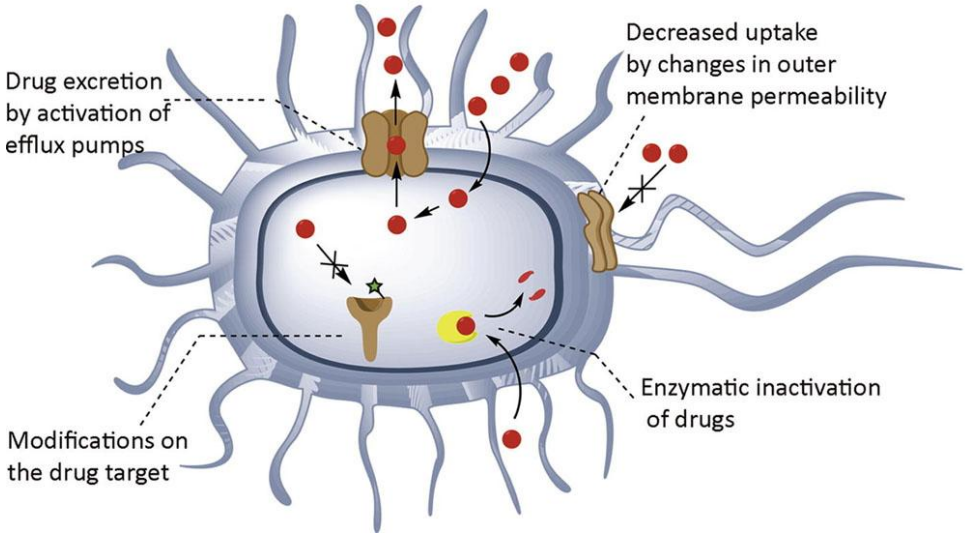

Figure 5: Mechanisms of bacterial resistance to antibiotics (González-Bello, 2017)

In this work, the main focus will be on the outer membrane and especially LPS.

1.6 Lipopolysaccharides as major component of the outer membrane of Gram-negative bacteria

About the influence of LPS on the efficacy of bactericidal substances is in detail currently little known. Thus, LPS were chosen as ideal study object for this master thesis to close this knowledge gap. LPS can be found in the outer leaflet of the outer membrane of most Gram-negative bacteria and act as a permeability barrier. It consists in general of three major parts: lipid A, core oligosaccharide and O-antigen. The O-antigen and parts of the core oligosaccharide are not essential for the growth of *E. coli*, thus strains possess different lengths and structures of LPS (Delcour, 2009). Some Gram-negative bacteria like Campylobacter only possess a light version of the LPS, the so called lipooligosaccharides (LOS). LOS differs from LPS in the way that the O-antigen is missing and these bacteria only

possess the lipid A and core oligosaccharide (Godschalk et al., 2007). The general structure of LPS is shown in Figure 6.

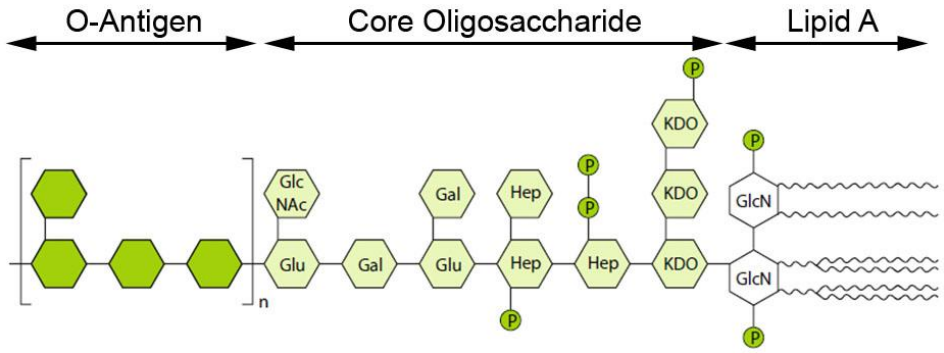

Figure 6: General structure of LPS. Modified figure, originally from Fritsche (2016)

1.6.1 Lipid A

In *E. coli* K-12 lipid A consists of a glucosamine disaccharide backbone which is on four positions acylated with 3-hydroxymyristic acid residues. The hydroxyl groups of the fatty acids linked to the non-reducing sugar residue are further acylated, resulting in characteristic so called “piggyback” structures. Both ends of the disaccharide backbone are typically phosphorylated, giving lipid A an overall negative charge (Nikaido, 2003). The structure of lipid A is mostly invariant and thereby well conserved over all LPS forming bacteria. It acts as an amphiphilic anchor in the outer membrane, giving the whole LPS molecule hold in the outer leaflet (Ferguson et al., 2000). Due to the nature of its structure, lipid A is the hydrophobic part of the LPS (Raetz et al., 2007). Of the whole LPS molecule, lipid A is the only part that acts as an endotoxin and is recognized by the innate immune system. LPS recognition is carried out by the so-called toll like receptor 4/myeloid differentiation protein 2/cluster of differentiation antigen 14 (TLR4/MD2/CD14) complex, a mammalian LPS receptor that is present in many different cell types. The detection of lipid A in the immune system is very sensitive and robust that a bloodstream infection with LPS producing bacteria causes an endotoxic shock, leading to severe health problem for humans (Miller et al., 2005). The general structure of *E. coli* K-12 lipid A is shown in Figure 7.

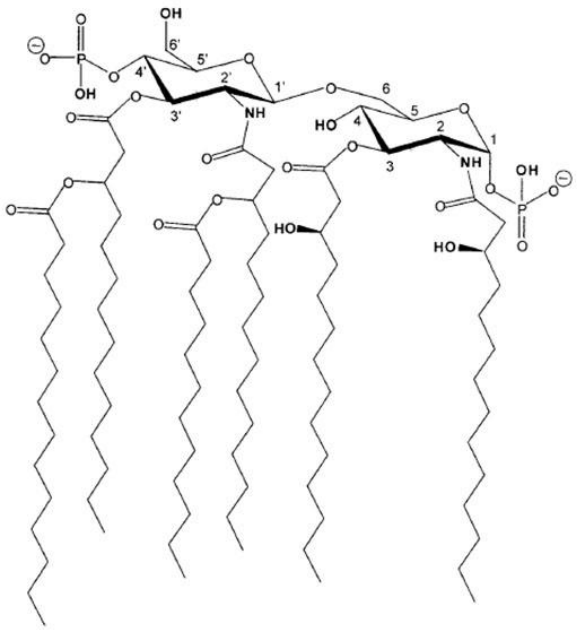

Figure 7: General structure of *E. coli* K-12 lipid A (Nikaido, 2003)

1.6.2 Core region

After lipid A the core region is the second part of the LPS molecule. The core region can be divided into two sub regions, the inner core and the outer core. The inner core is covalently bound to lipid A and consists of unusual sugars like keto-deoxyoctulosonate (Kdo) and heptose. The inner core plays an important role for bacterial viability and is therefore well conserved among all LPS forming bacteria. More variable is the outer core, which usually consists of hexose sugars like glucose and galactose (Zanoni et al., 2012). Some heptose residues of the inner core carry phosphoryl substituents that play an essential role for membrane stability. These phosphate containing groups are negatively charged and cross-linked with divalent cations. This relationship allows neighboured LPS molecules to interact with each other and overcome repulsion effects that would otherwise occur (Yethon et al., 2000). The missing of these phosphate groups in the inner core results in less membrane protection and therefore causes hypersensitivity to antimicrobial substances (Yethon et al., 1998; Wang et al., 2015).

1.6.3 O-Antigen

The O-antigen, also called O-polysaccharide or O-specific chain is the third major part of the LPS molecule. It is a polysaccharide that is covalently linked to the core region and consists of two to seven monosaccharide residues that form a subunit which can be repeated from one up to 25 times (Kabanov and Prokhorenko, 2010). It is a highly variable region with many different variations even in the same species of bacteria. The O-antigen plays a crucial role in pathogenic bacteria by helping them to colonize effectively the host tissue and

protecting them from complement mediated killing of the host immune system (Hagelueken et al., 2014; Blažková et al., 2015).

1.6.4 Variable length of lipopolysaccharides

LPS forming bacteria do not always express the full length of the LPS. For bacteria like *E. coli* the minimum required LPS length for viability is considered to be lipid A and two Kdo residues. This minimum length is only formed under optimal growth conditions (Klein et al., 2013). Strains that possess a LPS up to length of the inner core are called semi-rough (Re-LPS), strains with a LPS up to the length of the whole region are called rough (Ra-LPS) (Pupo et al., 2013). Bacterial strains that form a LPS to a length up to one O-antigen repetition are called semi-rough (Sr-LPS), while strains with a LPS length with several O-antigen repetitions are called smooth (S-LPS) (Sadovskaya et al., 2000). The classification in smooth and rough LPS can be explained from the observation of colonies on agar plates. Bacteria with smooth LPS form a smooth colony, while bacteria with rough LPS form a rough colony (Petter, 1993).

1.6.5 Lipopolysaccharide modifications and contribution to resistance

LPS provides an intrinsic resistance against lipophilic substances and thereby help Gram-negative bacteria to be much more resistant against such substances than Gram-positive bacteria (Yamasaki et al., 2013). Especially the hydrophilic core region plays a major role and acts as a barrier to these substances but only if the full core length is expressed (Delcour, 2009). Strains with a truncated core (deep-rough phenotype) showed a hyper-permeable outer membrane and an increased susceptibility against antibiotics like erythromycin, novobiocin and rifampicin (Page, 2012).

As described in the previous chapters, LPS in general are negatively charged through added phosphate groups in the lipid A and core region domain. To reduce negative effects from these charges, numerous bacteria add positive structure elements to their LPS or substitute negative charged structural elements. Commonly used for addition reactions are cationic sugars such as aminoarabinose and they are often added to phosphate residues in the lipid A domain. Phosphoethanolamine is another component that can be added/substituted in the lipid A or inner core domain of the LPS to reduce the overall negative charge of the LPS molecule. These changes result in less repulsion effects of neighbouring LPS molecules and in a more packed LPS layer. Strains which possess these LPS modifications show a way lower susceptibility to cationic agents such as polymyxin B (Delcour, 2009; Band and Weiss, 2015).

Until now the influence of LPS variations on the efficacy of QACs and ILs has not been investigated in detail. Thus, LPS were selected for this master thesis to get a deeper understanding in this matter.

1.7 Aim of this master thesis

Even though food safety has well developed, and today's foods are safer as never before, foodborne pathogens still remain as a threat for human health. Especially the over- and misuse of bactericidal substances has led to tolerant, resistant and persistent strains making common used disinfectants ineffective. Especially Gram-negative bacteria with their outer membrane and the LPS anchored within possess more tools for the defence against bactericidal substances than Gram-positive bacteria. According to the current state in research this master thesis aims to answer the following questions:

- How do different variations of LPS influence the efficacy of QACs?
- Can ILs be a suitable alternative to QACs and therefore be a proper enhancement? The need of more and other operating disinfectants in food industry is apparent and ILs could be a solution for problems with tolerant and persistent strains due to their highly tuneable structure.
- Do ILs show the same results as QACs? Due to some structural similarities between these two substance classes, some QACs can be considered ILs and *vice versa*.
- What influence has the LPS on the efficacy of ILs? Currently there is in detail not much known about the contribution of LPS towards the efficacy of ILs on Gram-negative bacteria.
- The exposure of bacteria to sub-lethal concentrations of disinfectants is a general problem that can often result in food plants due to the wrong use of disinfectants. In form of an evolution experiment the influence of sub-lethal concentrations of ILs on bacteria was investigated.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

During this master thesis the following chemicals were used:

Aceton	SIGMA-ALDRICH, St. Louis, Missouri, United States
Boric acid	SIGMA-ALDRICH, St. Louis, Missouri, United States
Chelex 100 Resin	BIO-RAD, Hercules, California, United States
EDTA	SIGMA-ALDRICH, St. Louis, Missouri, United States
EDTA Na_2 $2H_2O$	SIGMA-ALDRICH, St. Louis, Missouri, United States
Ethanol	VWR-CHEMICALS, Radnor, Pennsylvania, United States
Glycerol 86–89 %	SIGMA-ALDRICH, St. Louis, Missouri, United States
HCl 37 %	FISHER SCIENTIFIC, Pittsburgh, Pennsylvania, United States
KCl	MERCK, Darmstadt, Germany
KH_2PO_4	SIGMA-ALDRICH, St. Louis, Missouri, United States
Lysozyme	CARL ROTH, Karlsruhe, Germany
Methanol	MERCK, Darmstadt, Germany
Na_2HPO_4	MERCK, Darmstadt, Germany
NaCl	MERCK, Darmstadt, Germany
N-Phenylnaphthalen-1-amine	MERCK, Darmstadt, Germany
NaOH	CARL ROTH, Karlsruhe, Germany
TRIS	MERCK, Darmstadt, Germany
Triton-X Urea Xylene	FISHER SCIENTIFIC, Pittsburgh, Pennsylvania, United States

2.1.2 Antibiotics

During this master thesis the following antibiotics were used:

Ampicillin anhydrous	SIGMA-ALDRICH, St. Louis, Missouri, United States
Chloramphenicol	SIGMA-ALDRICH, St. Louis, Missouri, United States
Ciprofloxacin	SIGMA-ALDRICH, St. Louis, Missouri, United States
Clindamycin	OXOID, Hamsphire, United Kingdom
Erythromycin	OXOID, Hamsphire, United Kingdom
Gentamycin sulfate salt hydrate	SIGMA-ALDRICH, St. Louis, Missouri, United States

Kanamycin sulfate	SIGMA-ALDRICH, St. Louis, Missouri, United States
Levofloxacin	SIGMA-ALDRICH, St. Louis, Missouri, United States
Nalidixic acid	SIGMA-ALDRICH, St. Louis, Missouri, United States

2.1.3 Quaternary ammonium compounds

The used QACs during this master thesis are listed in Table 1.

Table 1: All used QACs

Abbreviation	Full Name	Manufacturer
BC	Benzalkonium chloride	SIGMA-ALDRICH, St. Louis, Missouri, United States
BE	Benzethonium chloride	SIGMA-ALDRICH, St. Louis, Missouri, United States
CP	Cetylpyridinium chloride monohydrate	APPLICHEM, Darmstadt, Germany
CTAB	Cetyltrimethylammonium bromide	APPLICHEM, Darmstadt, Germany
DB	Domiphen bromide	SIGMA-ALDRICH, St. Louis, Missouri, United States

2.1.4 Ionic liquids

2.1.4.1 Purchasable ionic liquids

ILs that were obtained from companies are listed in Table 2.

Table 2: Purchased ILs

Abbreviation	Full Name	Manufacturer
[$C_{10,10}$MIM][Cl]	1,3-Didecyl-2-methylimidazolium chloride	IOLITEC, Heilbronn, Germany
[C_{12}MIM][Cl]	1-Dodecyl-3-methylimidazolium chloride	IOLITEC, Heilbronn, Germany
[C_{14}MIM][Cl]	1-Methyl-3-tetradecylimidazolium chloride	IOLITEC, Heilbronn, Germany
[TIBMP][Tosylate]	Triisobutylmethylphosphonium tosylate	IOLITEC, Heilbronn, Germany
221PG	N/A	IOLILYTE, Heilbronn, Germany
C1EG	N/A	IOLITEC, Heilbronn, Germany
T2EG	N/A	IOLILYTE, Heilbronn, Germany

2.1.4.2 Synthesized ionic liquids

The following ILs, with exception of [$TMC_{16}A$][Cl] which was synthesized by myself, were synthesized at our laboratory by members of the CD-MOMICO work group according to the method described in chapter 2.2.1. All synthesized ILs are listed in Table 3.

Table 3: Synthesized ILs

Abbreviation	Full Name
[BMIM][FAP]	1-Butyl-3-methylimidazolium tris(pentafluoroethyl)trifluorophosphate
[C_{10}MIM][Cl]	1-Decyl-3-methylimidazolium chloride
[C_{16}MIM][Cl]	1-Hexadecyl-3-methylimidazolium chloride
[DODMA][Cl]	Dioctyldimethylimidazolium chloride
[EDMOA][FAP]	Ethyldimethyl-(2-methyloxyethyl)ammonium tris(pentafluoroethyl)trifluorophosphate
[EMIM][FAP]	1-Ethyl-3-methylimidazolium tris(pentafluorethyl)trifluorophosphate
[$TMC_{10}A$][Cl]	Decyltrimethylammonium chloride
[$TMC_{16}A$][Cl]	Hexadecyltrimethylammonium chloride
[TOMA][Cl]	Trioctylmethylammonium chloride
[TOMP][Cl]	Trioctylmethylphoshonium chloride
[TOP][Br]	Tetraoctylphosphonium bromide

2.1.5 General structure of quaternary ammonium compounds and ionic liquids

One of the used QACs and some ILs are exemplary shown for a better understanding of the concept and high tunability of ILs. Structures of all used substances can be found in the attachment section. Exemplary structures of QACs and ILs are shown in figure 8 to 11.

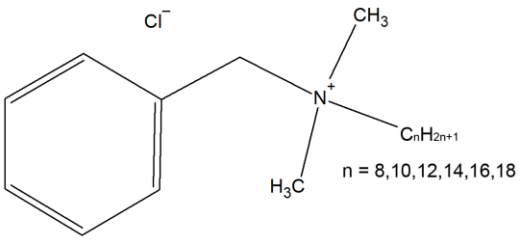

Figure 8: Exemplary structure of a QAC substance (Benzalkonium chloride)

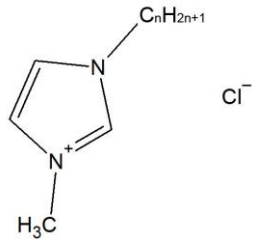

Figure 9: Exemplary structure of an imidazolium-based IL with one side-chain

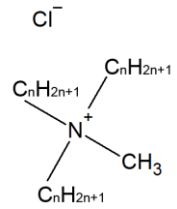

Figure 10: Exemplary structure of an ammonium-based IL with more than one side-chain

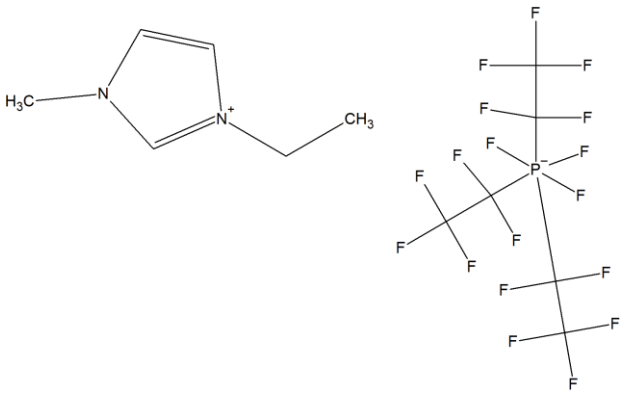

Figure 11: Exemplary structure of an imidazoilum-based IL with an active anion

2.1.6 Buffers

2.1.6.1 Phosphate buffered saline (PBS)

For a 1 M concentrated buffer 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were solved in 800 mL distilled water. Then the pH-value was adjusted to the desired pH-value with HCl and the volume filled up to 1 L. The buffer was afterwards autoclaved at 1 bar overpressure at 121°C for 15 minutes.

2.1.6.2 TRIS-HCl buffer

For a 2 M concentrated buffer 24.2 g of TRIS were solved in 70 mL of deionised water. Then the pH-value got adjusted to 7.0 with HCl and the volume filled up to 100 mL. The buffer was afterwards autoclaved at 1 bar overpressure at 121°C for 15 minutes.

2.1.6.3 Sample loading buffer

35 mg of bromophenol blue (SIGMA-ALDRICH, St. Louis, Missouri, United States) are mixed with 16 mL ≥99 % glycerol (SIGMA-ALDRICH, St. Louis, Missouri, United States) and 35 mL sterile ddH_2O.

2.1.6.4 TRIS-borate-EDTA buffer (TBE)

For a 1 M concentrated TBE buffer 10.8 g of TRIS, 5.5 g boric acid and 0.7 g of EDTA Na_2 $2H_2O$ were weighed in and solved in 900 mL ddH_2O. The pH-value of was adjusted with HCl to 8.0, with ddH_2O filled up to the 1 L mark and autoclaved at 1 bar overpressure at 121°C for 15 minutes.

2.1.7 Growth media

2.1.7.1 Tryptone soy agar + yeast (TSA+Y)

For one litre of TSA+Y 40 g of tryptic soy agar (MERCK, Darmstadt, Germany) and 6 g of yeast extract (OXOID, Hampshire, United Kingdom) were solved in one litre of deionised water and then autoclaved at 1 bar overpressure at 121°C for 15 minutes. After cooling down to 55°C in a water bath approximately 15 mL were poured into an empty petri dish. The filled petri dishes were left untouched at room temperature until the medium became solid.

2.1.7.2 Tryptone soy broth + yeast (TSB+Y)

For one litre of TSB+Y 36 g of listeria enrichment broth base (OXOID, Hampshire, United Kingdom) were solved in one litre of deionised water and then autoclaved at 1 bar overpressure at 121°C for 15 minutes.

2.1.7.3 Kanamycin plates

For the kanamycin resistance test one litre of TSA+Y was prepared as previously mentioned. Before the pouring of the medium into the empty petri dishes the TSA+Y medium was cooled down to hand temperature and then 30 mg of kanamycin sulphate were added. The medium was mixed thoroughly and then 15 mL of the medium were poured into each petri dish. The filled petri dishes were left untouched at room temperature until the medium became solid.

2.1.8 Chelex for DNA extraction

2.5 g Chelex were weighed in a 100 mL flask and mixed with 95 mL ddH_2O. Afterwards, 2.5 mL of 0.01 M TRIS-HCl buffer with a pH of 7 was added and the solution autoclaved at 1 bar overpressure at 121°C for 15 minutes.

2.1.9 *Escherichia coli* wild-type and mutants

The *E. coli* K-12 wild-type and the other single-gene deletion mutants which originate from the wild-type were ordered from the Coli Genetic Stock Center (CGSC) at Yale University, New Haven, Connecticut, USA. While the wild-type is not genetically modified, in the mutants

the targeted genes are replaced with a kanamycin resistance cassette (Baba et al., 2006). An overview of all ordered strains is listed in Table 4.

Table 4: Overview of all ordered single-gene deletion mutants from the CGSC

Mutant	Deleted gene	Encoded enzyme	Sequence length [bp]
JW3597	*waaL*	O-antigen ligase	1638
JW3601	*waaR*	α-1,2-Glucosyltransferase	1321
JW3602	*waaI*	α-1,3-Glucosyltransferase	1020
JW3606	*waaG*	α-1,3-Glucosyltransferase	1125
JW3595	*waaF*	Heptosyltransferase II	1047
JW3596	*waaC*	Heptosyltransferase I	960
JW3603	*waaB*	α-1,6-Galactosyltransferase	1080
JW3600	*waaY*	Core heptose(II) kinase	699
JW3605	*waaP*	Core heptose(I) kinase	798

2.1.9.1 Supposed LPS structures of the *E. coli* strains

All deleted genes encode for proteins which play a major role in LPS biosynthesis. Deletion of these genes resulted in LPS length mutants with a shrinking LPS length (JW3597, JW3601, JW3602, JW3606, JW3595 and JW3596) and LPS side-chain mutants (JW3603, JW3600 and JW3605) which possess a full LPS length but lack some side-chains.

LPS structure determination is a highly demanding task which requires deep knowledge in the field of NMR spectroscopy, mass spectroscopy and chromatography. Due to lack of this knowledge, limited time of performing a master thesis and having no access to required devices the LPS structure of the *E. coli* K-12 wild-type was obtained from literature (Zhang et al., 2006) and after some conflicting structures in different publications confirmed by the LPS structure experts Prof. Kosma (University of Natural Resources and Life Sciences, Vienna, Austria) and Prof. Holst (Research Center Borstel, Borstel, Germany). Even though after figuring out the most probable LPS structure of the *E. coli* K-12 wild-type strain with LPS structure experts the presence of an O-antigen remained unclear. The absence or presence of the O-antigen is discussed in the discussion part of this master thesis. The supposed LPS structures for the wild-type and all mutants are shown in Figure 12.

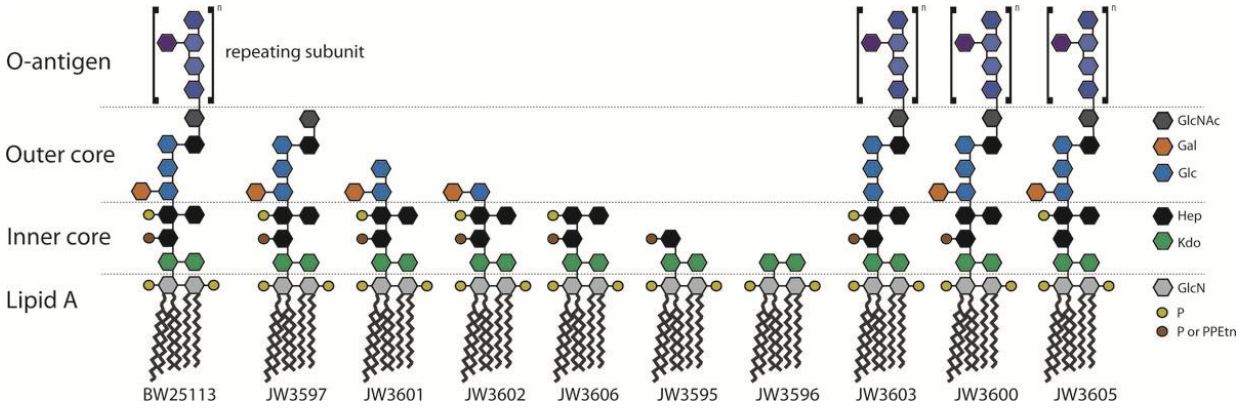

Figure 12: Overview of the different LPS variations of the ordered *E. coli* strains. Edited version, originally created by Patrick Mester

2.2 Chemical methods

2.2.1 Ionic liquid synthesis

2.2.1.1 Principle

Synthesis of non-commercially available ILs was performed according to the Carbonate Based Ionic Liquid Synthesis (CBILS) route. In this approach a cationic IL precursor is turned with a Brønsted acid with a pK_a < 9 into a full IL. The IL precursor and the acid have to be mixed in equimolar ratios to ensure that the reaction works properly and from a theoretical point of view there should be no by-products generated (Kalb et al., 2016). The CBILS route is shown in Figure 13.

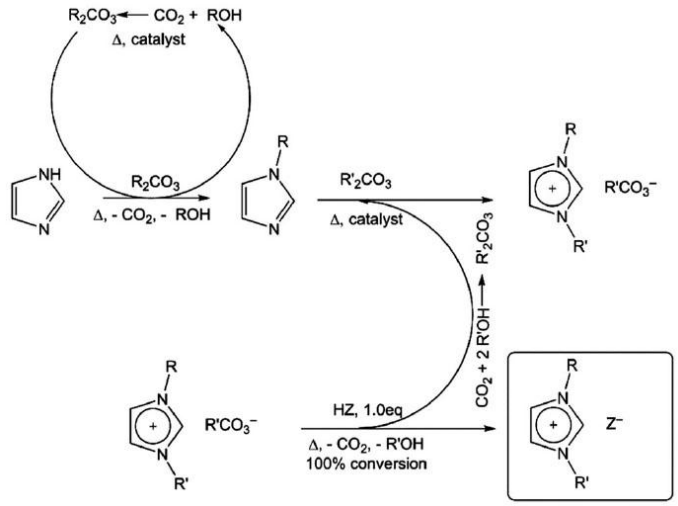

Figure 13: CBILS route (Kalb et al., 2016)

2.2.1.2 Materials

IL precursor

Acid

HCl 37 %

ddH_2O

Beaker glass 100 mL

SpeedVac	FISHER SCIENTIFIC SPD121P, Pittsburgh, Pennsylvania, United States
Vacuum pump	EDWARDS LS90PR, Burgess Hill, England

2.2.1.3 Execution

The IL synthesis is explained on the example [$TMC_{16}A$][Cl]. The IL precursor for this IL is [$TMC_{16}A$][Methylcarbonate]. The precursor has a stated purity of 52.9 %, the density was assumed as 1 kg/L. Thus, one litre of the precursor contains 529 g of [$TMC_{16}A$][Methylcarbonate]. The molecular weight is 359.6 g/mol. Through the following formula the mole in one litre precursor was calculated, resulting in the precursor concentration:

$$\mathrm{n} = \frac{\mathrm{m}}{\mathrm{M}} = \frac{529\,\mathrm{g}}{359.6\,\mathrm{g/mol}} = 1.47\,\mathrm{mol} \;\rightarrow\; \mathrm{c} = 1.47\,\mathrm{mol/L}$$

2 mL of the precursor were pipetted into a beaker glass and then mixed with 2.94 mL of 1M HCl which got diluted from 37 % HCl with ddH_2O to achieve equimolar ratios. The reaction takes place when CO_2 is released, to ensure the reaction was complete the beaker glass was stored overnight under a laboratory hood. On the next day CO_2 emission had stopped and the homogenic solution was transferred into 2 mL tubes. To remove remaining solvents, the tubes were put into the SpeedVac device and the ambient pressure reduced to 50 µbar. After two hours of waiting time ambient pressure was restored and the IL was ready for use.

2.3 Molecular biological methods

2.3.1 Polymerase chain reaction (PCR)

2.3.1.1 Principle

Analysis of specific nucleotide sequences can be achieved by PCR. During repeated cycles at different temperatures the DNA strand gets denatured, two primers (oligonucleotides) anneal to their complementary sequences and the DNA polymerase is responsible for the extension of the primers. This results after the second cycle in a specific exponential amplification of the desired DNA sequence (Saiki et al., 1988).

2.3.1.2 DNA extraction

During this master thesis there were two methods used for DNA extraction. The Nucleospin method takes a longer time for DNA extraction and provides smaller amounts of DNA, but the DNA is in return very pure. On the other hand, the Chelex DNA extraction method is very fast and effortless but delivers a DNA with a lower purity grade.

2.3.1.2.1 Nucleospin

The Nucleospin DNA extraction was carried out with an extraction kit from MACHEREY-NAGEL (Düren, Germany) and according to the following steps:

- Prepare an overnight culture of desired bacteria in TSB+Y
- Centrifuge 1 mL of overnight culture for 5 min at 5000 rcf and discard the supernatant
- Solve the pellet in 180 µL prelysis buffer (20 mM TRIS-HCl pH 7, 2 mM EDTA, 1 % Triton X-100 and 20 mg/mL lysozyme)
- Incubate for 30–60 minutes at 37°C and 750 rpm in a thermo-block
- Add 25 µL of proteinase K solution and incubate overnight at 56°C in a thermo-block
- Add 200 µL of solution B3, vortex and incubate 10 minutes at 70°C
- Add 210 µL of ethanol 96 % and vortex
- Load samples on the column
- Add 500 µL of solution BW
- Centrifuge for 2 minutes at 11000 rpm
- Discard flow-through
- Add 600 µL of solution B5
- Centrifuge for 2 minutes at 11000 rpm
- Discard flow-through and take a new collecting vessel
- Centrifuge for 1 minute at 11000 rpm
- Add 50 µL 70°C pre-warmed ddH_2O and incubate for 1 minute at room temperature
- Centrifuge for 1 minute at 11000 rpm
- Repeat the last two steps once
- Use flow-through for PCR

2.3.1.2.2 Chelex

With an inoculation loop some colonies of desired bacteria were picked from an agar plate and solved in 100 µL TRIS-HCl pH 7 buffer. Then 400 µL of Chelex were added and after vortexing put on the thermo-block at 100°C for 10 minutes. Afterwards the whole solution was centrifuged 5 seconds at 15000 rcf and the supernatant carefully separated for further PCR analysis.

2.3.1.3 Primer design

The sequences of the deleted genes were obtained from the gen database of the National Center for Biotechnology Information (NCBI). These gene sequences were then used in the Primer Blast tool from NCBI to get suggestions for suitable primer pairs. Afterwards for all PCR approaches primer were chosen where the melting temperature T_m is around 60°C, the GC-content between 50–60 %, the product length between 150 and 300 bp and where no repetitions of four or more nucleotides of the same type occur. The primer sequences were also put into the NCBI database to ensure that the sequences occur exclusively in *E. coli* and are not present in other bacteria. An overview of all used primers is listed in Table 5.

Table 5: Overview of all used primers for the single-gene deletion verification PCR

Mutant	Deleted gene	Gene length [bp]	Product length [bp]	Primer Type	Primer sequence	Start	Stop	Length [bp]	GC content [%]	T_m [°C]
JW3597	*waaL*	1638	229	Forward	GCAACCCTACTCCTGTTCCC	646	665	20	60.0	60.04
				Reverse	ACATTGCCAGTCTTGCACCT	874	855	20	50.0	60.18
JW3601	*waaR*	1321	236	Forward	TTGCCTATGGTGTCGATGCT	89	108	20	50.0	59.46
				Reverse	TGCTCTTGACCAGACCTGTG	324	305	20	55.0	59.61
JW3602	*waaI*	1020	260	Forward	TAATGGTGATCGACTGCGCT	276	295	20	50.0	59.54
				Reverse	TAGCAATTCCCGCAACACCT	535	516	20	50.0	59.96
JW3606	*waaG*	1125	185	Forward	CGCCATTATGCCGCATTTGA	364	383	20	50.0	59.97
				Reverse	CGGCTGTTTGGGATTTGCTC	548	529	20	55.0	60.11
JW3595	*waaF*	1047	244	Forward	AAAGAAGCCTGGCCGCTAAT	373	392	20	50.0	60.03
				Reverse	CCAGCTCCGCATAGTGGTAG	616	597	20	60.0	59.97
JW3596	*waaC*	960	172	Forward	ATGCTGGCGAATATGCCGTA	527	546	20	50.0	59.97
				Reverse	AATCCTTCCGCCAGTCGTTT	698	679	20	50.0	59.96
JW3603	*waaB*	1080	227	Forward	TGGGTGATGGCTCCGATTTC	640	659	20	55.0	60.11
				Reverse	GGAGACACAATCGGCACTGA	879	860	20	55.0	60.04
JW3600	*waaY*	699	229	Forward	CAACATGGCATGGTTTCTGGC	456	476	21	52.4	60.67
				Reverse	TCCCTTTCAAACGCCGCAT	685	667	19	52.4	60.60
JW3605	*waaP*	798	241	Forward	TGATCGCACGTGTAGCAACT	152	171	20	50.0	60.04
				Reverse	TCTCTTTGCGTCAGGCCAAT	392	373	20	50.0	59.96

2.3.1.4 Materials

Primers 5µM	EUROFINS SCIENTIFIC, Brussels, Belgium
$MgCl_2$ 50 mM	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
dNTPs	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
PCR buffer 10x	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
Platinum Taq polymerase	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
DEPC-treated H_2O	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
Thermocycler	BIO-RAD T100, Hercules, California, United States

2.3.1.5 Execution of polymerase chain reaction

The PCR master mix for one reaction was mixed according to the protocol listed in Table 6.

Table 6: Protocol of PCR master mix for one reaction

	Volume [µL]
H_2O	13.55
PCR buffer 10x	2.50
MgCl2	0.75
Primer forward	1.00
Primer reverse	1.00
dNTPs	1.00
Taq polymerase	0.20
Template	5.00
Total	25.00

The first try with a standard PCR temperature profile led to unspecific bands on the agarose gel. Therefore, a PCR approach with a gradient temperature during the annealing phase from 56°C to 63°C was performed with the strains BW25113 and JW3597 to determine an optimal annealing temperature for further PCR approaches. The gradient PCR showed, that between this temperature range every temperature led to no unspecific bands and is therefore suitable. An annealing temperature of 59°C was chosen, leading to the following PCR temperature profile shown in Table 7.

Table 7: PCR temperature profile

Lid Heating	105°C		
Polymerase Activation	94°C	2 min	
Denaturation	94°C	15 s	
Annealing	59°C	30 s	30 x
Elongation	72°C	45 s	
Final elongation	72°C	5 min	
Storage	12°C	∞	

2.3.2 Agarose gel electrophoresis

2.3.2.1 Principle

With agarose gel electrophoresis it is possible to separate a mixture of DNA segments according to their size. Smaller segments move faster through the gel, while bigger segments are held back and therefore move slower. At the same time a DNA ladder with fragments of known size is running with the samples, providing a pattern to estimate the size of DNA fragments from the samples (Southern, 1975).

2.3.2.2 Materials

Analytical balance	SARTORIUS AC121S, Göttingen, Deutschland
Erlenmeyer flask 250 mL	
Agarose	VWR-CHEMICALS, Radnor, Pennsylvania, United States
TBE buffer 1x	
Microwave	SILVA-SCHNEIDER, Anif, Austria
Peq Green DNA dye	VWR-CHEMICALS, Radnor, Pennsylvania, United States
Gel caster, try and comb for 20 pockets	BIO-RAD, Hercules, California, United States
1 kb ladder	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
Sample loading buffer	
Electrophoresis chamber	BIO-RAD, Hercules, California, United States
Power supply	BIO-RAD, Hercules, California, United States
Gel documentation system	BIO-RAD Geldoc 2000, Hercules, California, United States
Software	BIO-RAD Quantity One 4.3.1, Hercules, California, United States

2.3.2.3 Execution

For the separation of the PCR products a 1.5 % agarose gel was prepared. Therefore 1.5 g of agarose were weighed in an Erlenmeyer flask and mixed with 100 mL of TBE buffer 1x. The flask was out into the microwave and heated until the agarose was complete dissolved in the buffer. Afterwards, 2 µL of Peq Green were added and the hot solution cooled down under running tap water. The solution was poured into the already prepared gelcaster tray with comb and waited, until the gel has reached room temperature and became solid. In the meantime, the electrophoresis chamber was filled with TBE buffer 1x.

The comb from the gelcaster was carefully removed and the tray put into the electrophoresis chamber. The power supply was adjusted to 120 V and 30 minutes and the run started.

After the run the power supply was turned off, the tray from the chamber removed and put into the gel documentation system. There the tray was aligned for the camera and a picture with UV light taken.

2.3.3 Real-time quantitative polymerase chain reaction (qPCR)

2.3.3.1 Principle

A qPCR approach with a hydrolysis probe for the *E. coli* species verification was used. The difference between this qPCR approach and the previous mentioned PCR is, that in addition to the primer pair a fluorescent dye marked probe is used. This probe is an oligonucleotide, marked at the 5'-end with a reporter dye (FAM) and at the 3'-end with a quencher (BHQ1). As long as the fluorescent reporter is bound on the probe and therefore located nearly to the quencher, all light emissions are absorbed by the quencher. If the probe binds in the correct location on the DNA strand, through the 5'-3' exonuclease activity of Taq polymerase the probe gets depleted into nucleotides, leading to the state where reporter and quencher are no longer located closely to each other. The reporter can now exhibit fluorescence without hindrance. This approach has the advantage compared to a common PCR that the gain of fluorescent can be viewed in real-time after each cycle, with the use of standards making it easy for quantification and a higher specificity through the additional binding of the probe (Heid et al., 1996).

This qPCR approach targets the *sfmD* gene, which encodes for a putative outer membrane protein and has 100 % exclusiveness in *E. coli* (Kaclíková et al., 2005).

2.3.3.2 Primers and hydrolysis probe

The primers and probe for *E. coli* verification were used according to Kaclíková et al. (2005) and are listed in Table 8.

Table 8: Used primers and probe for qPCR

Primers and Probe	Sequence	Length [bp]
ert2-Forward	5'-ACTGGAATACTTCGGATTCAGATACGT-3'	27
ert2-Reverse	5'-ATCCCTACAGATTCATTCCACGAAA-3'	25
ert2-Probe	5'-**FAM**-CAGCAGCTGGGTTGGCATCAGTTATTCG-**BHQ1**-3'	28

Primers and probe were already available in our working group and were not designed and ordered by myself.

2.3.3.3 Materials

Primers 5 µM

Probe 5 µM

$MgCl_2$ 50 mM	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
dNTPs	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
PCR buffer 10x	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
Platinum Taq polymerase	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
DEPC-treated H_2O	THERMO FISHER SCIENTIFIC, Waltham, Massachusetts, United States
Thermocycler	STRATAGENE MX3000P, San Diego, California, United States
Software	STRATAGENE MxPro 4.1, San Diego, California, United States

2.3.3.4 Execution

The PCR master mix for one reaction was mixed according to the protocol listed in Table 9.

Table 9: Protocol for the qPCR master mix for one reaction

	Volume [µL]
H_2O	10.55
PCR buffer 10x	2.50
MgCl2	1.75
Primer forward	1.50
Primer reverse	1.50
Probe	1.00
dNTPs	1.00
Taq polymerase	0.20
Template	5.00
Total	25.00

The used temperature profile is shown in Table 10.

Table 10: Temperature profile used for qPCR approach

Polymerase Activation	94°C	2 min	
Denaturation	94°C	15 sec	50x
Annealing and elongation	60°C	1 min	

2.4 Microbiological methods

2.4.1 Recording of growth curves

2.4.1.1 Principle

Through recording growth curves of bacteria it is possible to compare trends and calculate important parameters e.g. the doubling time from different strains. In order to record growth curves the optical density at 600 nm of a bacterial suspension is measured in regular time intervals and plotted in a diagram (Mytilinaios et al., 2012). Calculation of the doubling time is explained in chapter 2.4.1.4.

2.4.1.2 Materials

Desired bacteria on TSA+Y plates

TSB+Y

Semi-micro cuvettes 10x10x45 GREINER BIO-ONE, Kremsmünster, Austria

Photometer	HEWLETT PACKARD 8452A, Palo Alto, California, United States
Software	AGILENT TECHNOLOGIES, Santa Clara, California, United States
Microtiter plate 96 well	THERMO SCIENTIFIC, Waltham, Massachusetts, United States
Microplate reader	TECAN infinite F200, Männedorf, Switzerland
Software	TECAN i-control 1.5, Männedorf, Switzerland

2.4.1.3 Execution

One colony of desired bacteria was picked from a TSA+Y plate, solved in 1 mL of TSB and incubated at 37°C for 24 h. The next day, 100 µL from this overnight culture were mixed with 900 µL of TSB+Y and incubated at 37°C for 3 h to ensure, that the bacteria were in the exponential growth phase. A volume of 150 µL TSB+Y was provided in every well and 50 µL of bacterial solution added, resulting in 200 µL volume in each well. The bacterial solution was prepared in advance based on the assumption, that an OD_{600} from the 3 h culture of 0.6 equals a bacterial concentration of 10^8 CFU/mL. The 3 h culture was diluted to a concentration which contained $5*10^5$ bacterial cells in 50 µL inoculum. After inoculation, the microtiter plate was given into a microplate reader at 37°C and the OD_{600} every hour measured. For each strain to obtain a representative mean the growth curve was recorded 4 times.

2.4.1.4 Calculation of doubling time

After recording the growth curve, the first three data points in the exponential growth phase in the linear growth range were used for calculating the doubling time. Based on these points the trend line was calculated and the obtained formula transformed to the variable x. Then the points of time from OD 0.25 and 0.50 were calculated and the difference between these two determined, resulting in the doubling time of the desired strain (Mytilinaios et al., 2012). An example of the doubling time calculation is shown in Figure 14.

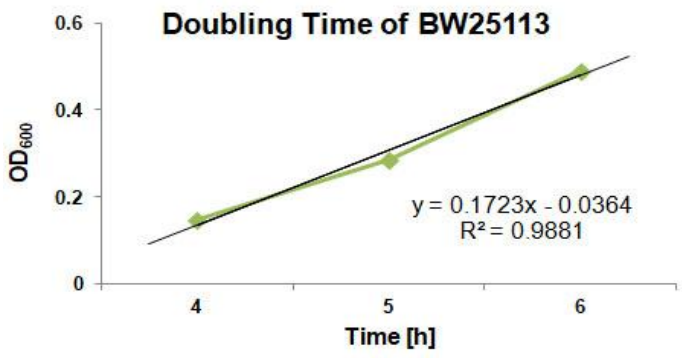

Figure 14: Example for the calculation of the doubling time from the *E. coli* wild-type strain BW25113

2.4.2 Determination of the minimum inhibitory concentration

2.4.2.1 Principle

The MIC is the concentration of a bactericidal substance, at which no visible growth of bacteria can be observed. For MIC determination a serial dilution of the desired substance in growing medium is prepared and then every dilution inoculated with the same amount of bacteria. After incubation at certain conditions each dilution is checked visibly for turbidity, indicating bacterial growth. The MIC is the first dilution of the series where no bacterial growth was observable (Knapp et al., 2015).

2.4.2.2 Materials

Desired bacteria on TSA+Y plates
TSB+Y
Desired bactericidal substances

Semi-micro cuvettes 10x10x45	GREINER BIO-ONE, Kremsmünster, Austria
Photometer	HEWLETT PACKARD 8452A, Palo Alto, California, United States
Software	AGILENT TECHNOLOGIES, Santa Clara, California, United States
Multichannel pipette	EFLAB Titertek 50-200 µL, Helsinki, Finland
Microtiter plate 96 well	THERMO SCIENTIFIC, Waltham, Massachusetts, United States
Microplate reader	TECAN infinite F200, Männedorf, Switzerland
Software	TECAN i-control 1.5, Männedorf, Switzerland

2.4.2.3 Execution

The MIC determination was carried out in 96 well microtiter plates and performed at least three times on different days. A two-fold serial dilution of the desired substance was created in TSB+Y and 150 µL provided in advance in the microtiter plate in each well. The bacterial solution was prepared in the same way as described in chapter 2.4.1.3. and 50 µL containing $5*10^5$ CFU added to each dilution series, resulting in 200 µL total volume per well. Before incubation the optical density was measured at 610 nm in a microplate reader and afterwards the plates were incubated at 37°C for 24 h. The next day the plates were measured again and the difference calculated between these two absorptions. The MIC was then determined as the concentration where the difference in absorption was below 0.1 and the well showed no visible bacterial growth. The upper limit for the MIC determination was set to 5000 mg/L, all MIC values above this limit were considered as not effective. The experiment was performed at least three times on different days. Based on these values the mean value and the confidence interval with a significance level of $\alpha=0.05$ was calculated.

In this master thesis own intern quality standards from the CD-MOMICO laboratory were used and therefore the growing media mentioned in EUCAST guidelines for determination of MIC values, Mueller-Hinton broth and agar, were replaced with TSB+Y and TSA+Y.

2.4.3 Disk diffusion test

2.4.3.1 Principle

The disk diffusion test is a simple method to determine bacterial susceptibility to bactericidal substances. In this test a bacterial suspension is spread out with a tip on a solid universal medium. Afterwards, a paper disc with a certain amount of a bactericidal substance is put on the agar plate and incubated at 37°C for 24 h. Then the diameter of the zone of growth inhibition is measured and the results compared with other samples or standards published by national/international committees (Jorgensen and Ferraro, 2009).

2.4.3.2 Materials

Overnight culture of desired bacteria in TSB+Y	
TSB+Y	
TSA+Y plates	
Semi-micro cuvettes 10x10x45	GREINER BIO-ONE, Kremsmünster, Austria
Spectrophotometer	HEWLETT-PACKARD 8452A, Palo Alto, California, United States
Photometer-Software	AGILENT TECHNOLOGIES ChemStation 10.01, Santa Clara, California, United States
Sterile cotton swabs	
Desired antibiotics	
Paper discs	BECTON DICKINSON, Franklin Lakes, New Jersey, United States
Tweezers	

2.4.3.3 Execution

100 µL of the overnight culture were mixed with 900 µL of TSB+Y and incubated at 37°C for 3 h. Then, the OD_{600} of this 3 h culture was determined and the OD_{600} of this bacterial suspension adjusted to 0.6. Afterwards a cotton swab was put into the prepared suspension and spread out on TSA+Y plates. For all antibiotics stock solutions were prepared and a certain amount of each antibiotic pipetted on an empty paper disc. The paper discs were put with the aid of tweezers on the TSA+Y plates and incubated at 37°C for 24 h. After incubation the diameter of the zone of growth inhibition was measured with a ruler. The experiment was performed at least three times on different days.

In this master thesis own intern quality standards from the CD-MOMICO laboratory were used and therefore the growing media mentioned in EUCAST guidelines for disk diffusion testing, Mueller-Hinton broth and agar, were replaced with TSB+Y and TSA+Y.

2.4.4 Cell surface hydrophobicity assay

2.4.4.1 Principle

The determination of cell surface hydrophobicity is a method to obtain more information about the surface properties of bacteria (Gómez Zavaglia et al., 2002). The principle behind this method originates from the distribution of nonpolar residues on the surface of bacteria. When there are more non-polar residues on the surface of a bacterium, the bacterial affinity for a nonpolar solvent is increased. An aqueous bacterial suspension is mixed with xylene, thus forming two distinct phases. Depending on the presence of nonpolar residues on the outer membrane, bacteria can adhere to the surface of the xylene droplets. The cell surface hydrophobicity is then defined through the difference in turbidity of the aqueous phase, measured before and after the addition of xylene (Bibiloni et al., 2001).

2.4.4.2 Materials

10 mL of overnight culture of desired bacteria in TSB, incubated at 37°C	
Vortex	IKA Vortex Genius 3; Staufen, Germany
Centrifuge	EPPENDORF 5810R, Hamburg, Germany
Glass cuvettes 10 mm 3500 µL	HELLMA ANALYTICS, Müllheim, Germany
Spectrophotometer	HEWLETT-PACKARD 8452A, Palo Alto, California, United States
Photometer-Software	AGILENT TECHNOLOGIES ChemStation 10.01, Santa Clara, California, United States
PBS (50 mM, pH 7.2)	
Xylene	FISHER SCIENTIFIC, Pittsburgh, Pennsylvania, United States

2.4.4.3 Execution

The cell surface hydrophobicity assay was carried out similar to the assay published from Wang et al. (2015). The overnight culture was centrifuged at 4000 rpm for 5 minutes and the supernatant discarded. The pellet was washed twice with 2 mL PBS and after the washing procedure solved in 1 mL PBS. From this bacterial suspension a 1:10 dilution was created and 0.5 mL mixed with 3 mL PBS in a glass cuvette, resulting in total in a 1:70 dilution of the overnight culture. Then the OD_{600} was determined, sole PBS was used as blank. Based on

this value, 5.5 mL of the bacterial suspension in PBS with an OD_{600} of 0.5 was made. 0.5 mL of this suspension were put together with 3 mL PBS and the OD_{600} was measured (A_0). Afterwards 1 mL of xylene was added, vortexed for 2 minutes and then incubated for 1 h at room temperature. After incubation the upper organic solvent phase was thoroughly removed and the OD_{600} from the lower aqueous phase measured (A_1). The cell surface hydrophobicity was then calculated according to the following formula:

$$Hydrophobicity\ H\ \% = \frac{(A_0 - A_1)}{A_0} * 100$$

The execution of the cell surface hydrophobicity assay with the transformation of the two phases is shown in Figure 15.

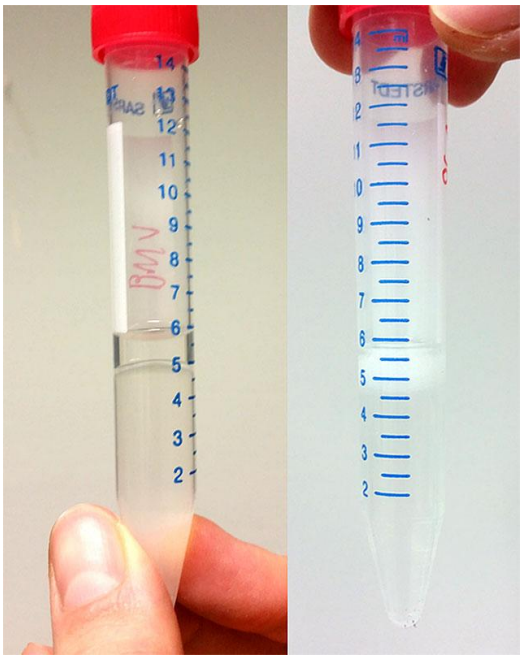

Figure 15: Execution of the cell surface hydrophobicity assay, showing the transformation of the two phases. The left part showing the lower phase with an OD adjusted bacterial suspension with added xylene phase on top before vortexing and incubating, the right part showing both phases after vortexing and 1 h of incubation time with soaked up bacterial cells in the upper xylene phase

The experiment was performed three times on three days each, equalling in nine measurements totally. Based on these values the mean value and the confidence interval with a significance level of α=0.05 was calculated.

2.4.5 Membrane permeability assay

2.4.5.1 Principle

An intact outer membrane from Gram-negative bacteria is a permeability barrier for many detergents, dyes, bile acids and hydrophobic substances like N-Phenylnaphthalen-1-amine

(NPN). In aqueous environment, NPN shows a weak fluorescence, while in phospholipid environments the fluorescence is highly increased. An intact outer membrane is a good barrier due to the presence of LPS but once damaged this circumstance allows NPN to diffuse into the phospholipid layer, resulting in an increased fluorescence (Helander and Mattila-Sandholm, 2000).

2.4.5.2 Materials

10 mL of overnight culture of desired bacteria in TSB, incubated at 37°C	
Vortex	IKA Vortex Genius 3; Staufen, Germany
Centrifuge	EPPENDORF 5810R, Hamburg, Germany
Glass cuvettes	HELLMA ANALYTICS 10 mm 3500 µL, Müllheim, Germany
Fluorescence spectrophotometer	MOLECULAR DEVICES SpectraMax M3, San José, California, United States
Photometer-Software	MOLECULAR DEVICES SoftMax Pro 6.4, San José, California, United States
PBS (20 mM pH 7.4)	
NPN	MERCK, Darmstadt, Germany
Aceton	SIGMA-ALDRICH, St. Louis, Missouri, United States

2.4.5.3 Execution

The membrane permeability assay was executed similar to the assay published from Wang et al. (2015). The bacterial overnight culture was centrifuged at 4000 rpm for 5 minutes and the supernatant discarded. During the washing procedure, a NPN stock solution in acetone was made and based on this stock a 1 mM dilution in PBS was prepared. Afterwards the pellet was solved in 2 mL of PBS and the OD_{600} got determined. Then the OD of the bacterial suspension was adjusted to 0.5 in the same buffer. 1.92 mL of this suspension was pipetted into a glass cuvette and the emission measured at 420 nm with an excitation of 350 nm (A_0). Afterwards 80 µL of the 1 mM NPN solution were added and the cuvette mixed thoroughly by inversion. The emission was immediately measured at the same conditions before the NPN addition (A_1). As blank the PBS buffer was used and treated like a usual sample. The membrane uptake factor was calculated according to the following formulas:

$$NPN\ uptake\ factor = \frac{A_{1,sample} - A_{0,sample}}{A_{1,PBS} - A_{0,PBS}}$$

The experiment was performed three times on three days each, equalling in nine measurements totally. Based on these values the mean value and the confidence interval with a significance level of $\alpha=0.05$ was calculated.

2.5 Evolution experiment

2.5.1 Principle

Antibiotic resistances of bacteria is nowadays a well-known problem and did put the focus on biocidal substances e.g. disinfectants as promoters of these. To force evolution an evolutionary pressure can be applied by step-wise exposure to gradually increasing concentrations of a biocidal substance. Thus, these sub-MIC concentrations can create stable resistances (Thomas et al., 2000). In the evolution experiment carried out in this thesis this approach was slightly adjusted and instead of increasing concentrations the concentrations were held on a constant level.

2.5.2 First approach

2.5.2.1 Used microorganisms

For the first approach of the evolution experiment a set of various LPS and efflux mutants of *E. coli* and *L. monocytogenes* from foodborne outbreaks was used. A list of all used bacterial strains for the first approach of the evolution experiment is listed in Table 11.

Table 11: List of all used bacterial strains for the first evolution experiment approach

Bacteria	Strain	Specialty
E. coli	BW25113	Wild-type
	JW3596	*ΔwaaC*, LPS length mutant
	JW3597	*ΔwaaL*, LPS length mutant
	JW3605	*ΔwaaP*, LPS side-chain mutant
	JW3606	*ΔwaaG*, LPS length mutant
	JW0451-2	*ΔacrB*, RND efflux pump mutant
	JW1052-1	*ΔmdtH*, MFS efflux pump mutant
L. monocytogenes	EGDe	Laboratory strain
	6179	+Transposon 6188
	ScotA	Foodborne outbreak strain
	QC1	Foodborne outbreak strain
	MFPt1	Persistent food plant strain

All *E. coli* strains were ordered from the CGSC from Yale University (New Haven, Connecticut, United States), the *L. monocytogenes* strains were obtained from the strain collection of the Institute of Milk Hygiene of the Veterinarian University of Vienna (Vienna, Austria).

2.5.2.2 Used ionic liquids

To examine the difference of different structural classes, ILs with different active cations and with one to three side-chains were used. All used ILs for the first approach of the evolution experiment are listed in Table 12.

Table 12: Used ILs for the first evolution experiment approach

IL	Specialty
$[TMC_{16}A][Cl]$	Ammonium based cation, one side-chain
$[C_{10,10}MIM][Cl]$	Imidazolium based cation, two side-chains
[TOMP][Cl]	Phosphonium based cation, three side-chains

All chemical structures can be found in the attachment.

2.5.2.3 Used concentrations

From previous MIC screenings, the MIC for the selected strains and ILs was known and used for choosing three concentrations. Two sub-MIC concentrations were chosen representing around one half and one fourth of the MIC and one sub-inhibitory concentration. The sub-inhibitory concentration was selected from 24 h growth-curves, recorded from the MIC screening where the curves showed a time delay in the beginning of the microbial growth. An overview of all concentrations of ILs used for the bacteria is listed in Table 13 for all *E. coli* strains and in Table 14 for all *L. monocytogenes* strains.

Table 13: Overview of all concentrations used for the *E. coli* strains in [mg/L]

		E. coli strains						
IL	Concentration	BW25113	JW3596	JW3597	JW3605	JW3606	JW0451-2	JW1052-1
[TOMP][Cl]	Sub-MIC 1	2.0	1.0	2.0	1.0	1.0	1.0	1.0
	Sub-MIC 2	1.0	0.5	1.0	0.5	0.5	0.5	0.5
	Sub-Inhib.	4.0	1.5	4.0	1.5	2.5	2.5	2.5
$[C_{10,10}MIM][Cl]$	Sub-MIC 1	0.6	0.6	0.6	0.6	0.6	0.6	0.6
	Sub-MIC 2	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	Sub-Inhib.	2.0	1.0	2.0	1.0	1.0	1.0	1.0
$[TMC_{16}A][Cl]$	Sub-MIC 1	6.0	0.8	6.0	0.8	0.8	0.8	0.8
	Sub-MIC 2	3.0	0.4	3.0	0.4	0.4	0.4	0.4
	Sub-Inhib.	7.0	1.5	7.0	3.0	3.0	3.0	3.0

Table 14: Overview of all concentrations used for the *L. monocytogenes* strains in [mg/L]

		L. monocytogenes strains				
IL	Concentration	EGDe	6179	ScotA	QC1	MFPt1
[TOMP][Cl]	Sub-MIC 1	0.6	0.6	0.6	0.6	0.6
	Sub-MIC 2	0.3	0.3	0.3	0.3	0.3
	Sub-Inhib.	1.3	1.3	1.0	1.0	1.0
[$C_{10,10}$MIM][Cl]	Sub-MIC 1	0.6	0.6	0.6	0.6	0.6
	Sub-MIC 2	0.3	0.3	0.3	0.3	0.3
	Sub-Inhib.	1.0	1.0	1.0	1.0	1.0
[TMC_{16}A][Cl]	Sub-MIC 1	1.5	1.5	1.5	1.5	1.5
	Sub-MIC 2	0.8	0.8	0.8	0.8	0.8
	Sub-Inhib.	3.0	3.0	3.0	3.0	3.0

2.5.2.4 Materials

Bacterial strains

ILs

ddH_2O

TSB+Y

TSA+Y plates

Inoculation loops 1 µL	SARSTEDT, Nümbrecht, Germany
Analytical balance	SARTORIUS TE214S, Göttingen, Deutschland
Eppendorf tube 2 mL	EPPENDORF, Hamburg, Germany
Glycerol 30 %	
Cryogenic vials 2 mL	BIOLOGIX, Jinan, China
Laminar flow cabinet	EHRET V-190, Tulln, Austria

2.5.2.5 Execution

Stock solutions of all ILs in ddH_2O were created and the desired concentration adjusted in TSB+Y media. The finished IL-TSB+Y mixture was autoclaved at 121°C and 1 bar overpressure for 15 minutes. After cooling down, a stock of 1 mL of each concentration in Eppendorf tubes was generated, lasting for 10 days of the experiment and were stored in the cooling room at 4°C.

The bacterial strains were spread out from a stock on TSA+Y medium, incubated at 37°C for 24 h. After that, a colony was picked with an inoculation loop from the plates and 1 mL of TSB+Y medium inoculated and incubated at 37°C for 24 h. 1 µL of this overnight culture was then used for inoculating the different concentration lines of each bacterial strain. After 24 h at 37°C if growth was visible, 1 µL of each concentration line was put into a new Eppendorf tube with the same concentration and incubated again at the same conditions as before. The

inoculation process was performed under a laminar flow cabinet with turned on ventilation. Every 10 days 1 µL of the culture was crossed out on TSA+Y medium as quality control and a glycerol stock was generated. The TSA+Y plates were incubated at 37°C for 24 h, in case of *L. monocytogenes* at 37°C for 48 h. For the stock, 500 µL of the bacterial suspension was mixed with 250 µL 30 % sterile glycerol in a cryogenic vial and stored at -80°C. If at some point of the experiment no microbial growth was visible, the concentration line was still continued and during the quality control on TSA+Y medium checked, if the bacteria have died or if they were just inhibited. In case of no growth on TSA+Y the respective concentration was no longer continued. The whole experiment was supposed to last for 40 days and the experimental schema is shown in Figure 16.

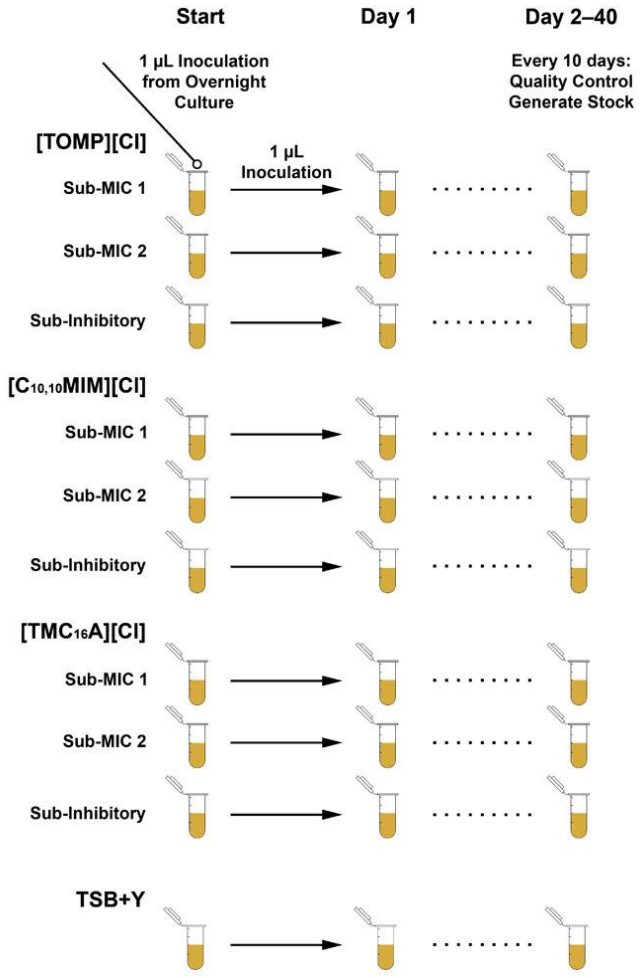

Figure 16: Schema of the evolution experiment approach for one bacterial strain

2.6 Statistics

In this master thesis a minimum of statistical tools was used to show the deviation of microbiological experiments. The following statistical values were used:

Arithmetic average

$$\bar{x} = \frac{1}{n} * \sum_{i=1}^{n} x_i$$

x_i: Measurement
n: Number of measurements

Standard deviation

$$s = \sqrt{\frac{1}{n-1} * \sum_{i=1}^{n} (x_i - \bar{x})^2}$$

$\bar{x}$: Arithmetic average
x_i: Measurement
n: Number of measurements

Confidence interval

Since the number of measurements was always far lower than 30, instead of the z-factor the t-factor was used for the calculation of the confidence interval.

Lower/Upper limit = $\bar{x} \pm t * \frac{s}{\sqrt{n}}$

$\bar{x}$: Arithmetic average
s: Standard deviation
n: Number of measurements
t: Factor from Student's t-distribution. Used t-values ($1 - \frac{\alpha}{2}, \alpha = 0.05$; degrees of freedom df = n-1) can be found in the attachment.

3 Results

3.1 Lipopolysaccharides and influence on the efficacy of disinfectants

Before the main part of this master thesis was carried out the ordered strains from the CGSC had to undergo several verifications. These verifications involved molecular biological verifications to proof that each strain belongs to the species *E. coli* and the respective gene is truthfully missing. Since a single-gene deletion may also impair the growth or resistibility of a strain several microbiological verifications were carried out. These included the recording of 24 h growth curves, calculation of doubling times and stress tests with substances that are known for their ability to be membrane permeable. For a better understanding of the influence of the LPS variations on the hydrophobic character of the outer membrane and the membrane permeability the cell surface hydrophobicity and the membrane permeability assay were performed. The results of these verifications were afterwards taken into consideration for the interpretation and discussion of the results of the MIC screening from the QACs and ILs.

3.1.1 Strain characterization

3.1.1.1 Molecular biological verifications

The qPCR verification confirmed that all mutants and the wild-type obtained from the CGSC were *E. coli*. Exemplary results for the qPCR verification are shown in Figure 17.

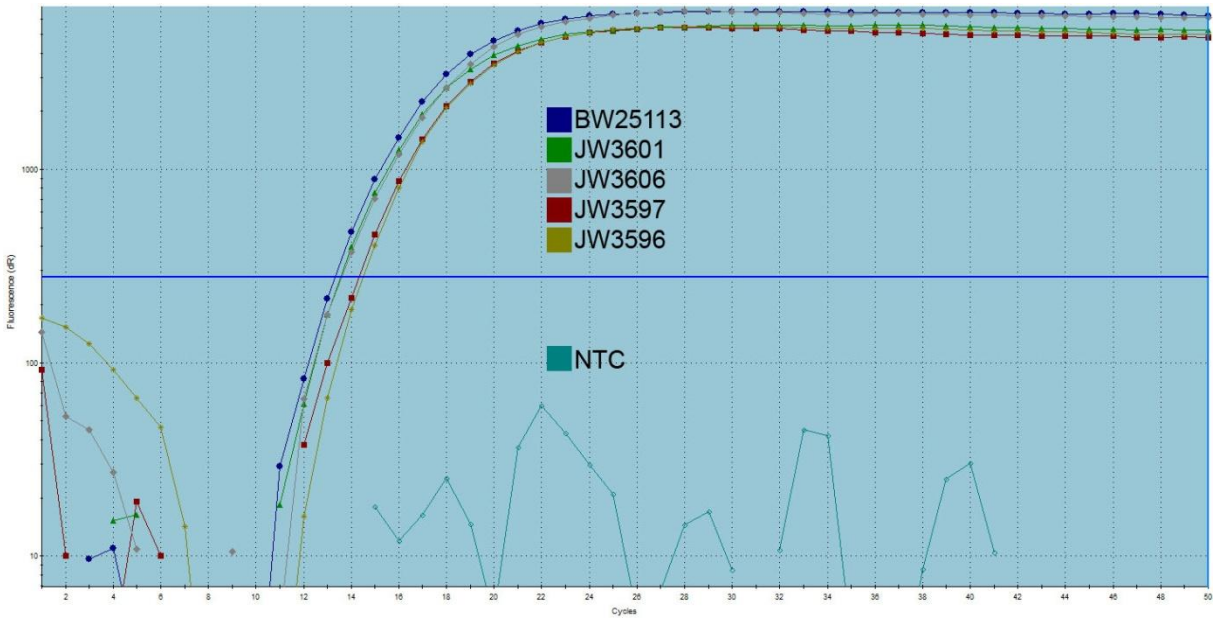

Figure 17: Amplification plot of the qPCR *E. coli* verification, showing exemplary the results of the *E. coli* strains for the wild-type, a selection of mutant strains and as comparison the negative template control (NTC)

The PCR single-gene deletion verification results showed, that for all mutants, except the strain JW3595, the single-gene deletion could be confirmed. Exemplary results for the single-gene deletion are shown in Figure 18. At first, I used a standard PCR protocol that did not

lead to satisfying results and showed unspecific bands in the agarose gel. After checking the recommendations from the manufacturer of the Taq polymerase, reading some online PCR guides and performing a gradient PCR I adapted the PCR template (different annealing temperature, lowering primer and $MgCl_2$ concentrations) and thus could avoid the creation of unspecific bands for most bacterial strains.

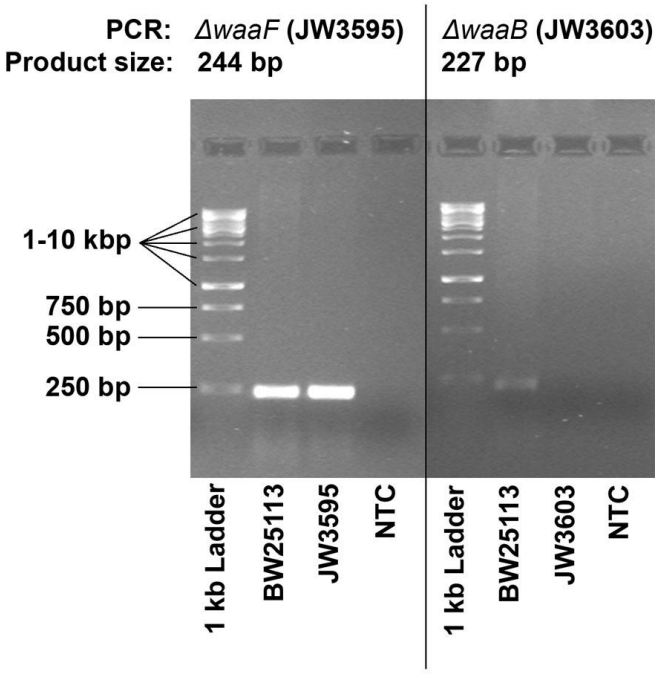

Figure 18: Results of the single gene deletion verification of respective *E. coli* strains JW3595 and JW3603

Additionally all mutants grew on kanamycin plates, showing that single-gene deletion was carried out and the targeted genes were replaced with a kanamycin resistance.

3.1.1.2 24 h growth curves, doubling times and stress tests

To investigate possible impairments of the mutants due to the single-gene deletion 24 h growth curves were recorded, the doubling time was calculated and the MIC was determined for substances, where a modified LPS should have no effect on their efficacy.

The 24 h growth curves of the mutant strains showed no major deviations compared to the growth curve of the wild-type strain BW25113. The optical densities at the end of the 24 h recording period were not taken into consideration due to the fact that some strains tend to aggregate at later time points on the bottom of a well leading to false optical density values.

The results of the doubling time calculation are listed in Table 15.

Table 15: Doubling times of all used *E. coli* strains and parameters from the linear trend line (y=k*x+d) used for the calculation

Strain	k	d	R^2	Doubling time [min]	Mean value [min]
BW25113	0.1508	0.1453	0.9575	99	97
	0.1343	0.134	0.9441	112	
	0.1585	0.2075	0.9658	95	
	0.1787	0.1023	0.9878	84	
JW3597	0.1471	0.2612	0.889	102	105
	0.141	0.2571	0.9137	106	
	0.1336	0.2551	0.8143	112	
	0.1494	0.2506	0.9233	100	
JW3601	0.153	0.1047	0.9652	98	104
	0.1363	0.1395	0.9338	110	
	0.1515	0.131	0.961	99	
	0.1396	0.1086	0.9573	107	
JW3602	0.1009	0.2534	0.8745	149	151
	0.0824	0.2186	0.7755	182	
	0.1108	0.2552	0.9022	135	
	0.1081	0.1961	0.9055	139	
JW3606	0.1493	0.1765	0.9295	100	98
	0.1645	0.1677	0.954	91	
	0.1395	0.1942	0.8885	108	
	0.1598	0.1226	0.9405	94	
JW3595	0.1627	0.1036	0.9757	92	89
	0.162	0.066	0.9912	93	
	0.1531	0.0862	0.9878	98	
	0.2059	0.0368	0.9925	73	
JW3596	0.1627	0.0227	0.9998	92	90
	0.1646	0.0194	0.9997	91	
	0.1647	0.0242	0.999	91	
	0.1728	0.0046	0.9997	87	
JW3603	0.1814	0.0519	0.9881	83	79
	0.1938	0.0509	0.9869	77	
	0.2062	0.0525	0.9864	73	
	0.1808	0.0358	0.9958	83	
JW3600	0.2088	0.1149	0.9997	72	70
	0.2177	0.0336	0.9994	69	

	0.2081	0.0628	0.9966	72	
	0.2203	0.0271	0.9997	68	
JW3605	0.1808	0.0511	0.9819	83	83
	0.1759	0.0791	0.9871	85	
	0.1799	0.1149	0.9922	83	
	0.1849	0.0669	0.9919	81	

The LPS length mutant strain JW3602 showed with 151 min a way longer doubling time than the wild-type BW25113 with 97 min. The side-chain mutant strain JW3600 showed the fastest doubling time with 70 min. In general, the experiment showed that the doubling times of LPS side-chain mutants were faster than for all other strains. The other strains showed no abnormal fluctuation from the doubling time of the wild-type BW25113.

The MIC screening with ethanol (starting concentration 72 % v/v), KH_2PO_4 (starting concentration 13.5 % w/v), NaCl (starting concentration 22.5 % w/v), urea (starting concentration 4 mol/L) in a microtiter plate with a two-folded dilution series showed no difference between the strains. Also the stress test in TSB+Y with adjusted pH values ranging from acidic (pH 3) to alkaline values (pH10) showed no differences within the strains. The results of the stress tests are listed in Table 16.

Table 16: Overview of the results for the stress tests of all *E. coli* strains showing the mean MIC values with confidence interval (α=0.05), the number of measurements and the lower and upper limit in [mg/L]

	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
Ethanol [%]	4.5 ± 0	4.5 ± 0	4.5 ± 0	4.5 ± 0	4.5 ± 0	4.5 ± 0	3.8 ± 1.3	4.5 ± 0	4.5 ± 0	3.8 ± 1.3
	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (2.3/4.5)	n = 3; (4.5/4.5)	n = 3; (4.5/4.5)	n = 3; (2.3/4.5)
KH_2PO_4 [%]	13.5 ± 0	13.5 ± 0	13.5 ± 0	13.5 ± 0	13,5 ± 0	13.5 ± 0	13.5 ± 0	13.5 ± 0	13.5 ± 0	13.5 ± 0
	n = 3; (13.5/13.5)	n = 3; (13.5/13.5)	n = 3; (13.5/13.5)	n = 3; (13.5/13.5)	n = 3; (13.5/13.5)	n = 3; (13.5/13.5)	n = 3; (13,5/13,5)	n = 3; (13.5/13.5)	n = 3; (13,5/13,5)	n = 3; (13.5/13.5)
NaCl [%]	11.3 ± 0	11.3 ± 0	11.3 ± 0	11,3 ± 0	11,3 ± 0	11.3 ± 0	11.3 ± 0	11.3 ± 0	11.3 ± 0	11.3 ± 0
	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)	n = 3; (11.3/11.3)
Urea [mol/L]	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)
pH-value (alkaline)	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0
	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3
pH-value (acidic)	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
	n = 3; (4,0/4,0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)	n = 3; (4.0/4.0)

3.1.1.3 Cell surface hydrophobicity assay

The results of the cell surface hydrophobicity assay showed an increasing hydrophobicity from the strain with the longest LPS (BW25113) to the strain with the shortest LPS (JW3596). The LPS side-chain mutants JW3603 and JW3605 showed high hydrophobicity values like strains with a shorter LPS. The LPS side-chain mutant JW3600 showed a different value than the other two side-chain mutants, having a hydrophobicity value as low as the wild-type BW25113. The results of the cell surface hydrophobicity assay are listed in Table 17.

Table 17: Results of the cell surface hydrophobicity assay of all *E. coli* strains measured at 600 nm, stated in [%]

	Measurement	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
Approach 1	1	41.3	61.6	63.3	81.9	91.1	60.7	92.4	71.8	55.8	85.8
	2	36.8	58.0	71.9	82.2	93.2	58.3	92.3	84.3	45.0	86.2
	3	38.0	58.7	69.8	84.9	90.9	61.9	88.5	79.0	44.2	82.4
Approach 2	4	48.3	54.4	52.2	49.8	92.5	37.4	90.5	81.5	38.2	66.7
	5	38.3	39.3	46.0	64.2	87.2	30.0	84.4	81.6	36.0	66.0
	6	40.3	44.2	38.4	62.5	89.0	39.3	96.6	76.6	36.6	62.6
Approach 3	7	49.9	46.2	31.2	55.5	65.6	40.9	73.7	55.8	39.2	35.8
	8	49.5	43.3	28.8	57.8	65.2	35.4	57.9	53.4	46.3	52.5
	9	55.6	42.6	38.3	53.8	67.3	37.4	62.1	71.1	15.3	52.6
	Mean value:	44.2	49.8	48.9	65.9	82.5	44.6	82.0	72.8	39.6	65.6
	Confidence interval (α=0.05):	5.1	6.4	12.6	10.5	9.6	9.4	10.9	8.6	8.5	13.2

3.1.1.4 Membrane permeability assay

The results of the membrane permeability assay showed a similar trend like the cell surface hydrophobicity assay. Starting at the wild-type BW25113 with a full length LPS the membrane permeability is slightly decreasing for the LPS outer core mutants JW3597, JW3601 and JW3602, possessing a slightly reduced LPS. The LPS inner core mutants JW3606 and JW3596 with a shorter LPS showed increased membrane permeability compared to the previous mentioned strains. Viewed overall, the membrane permeability is increasing from the longest LPS length to the shortest. The LPS side-chain mutants JW3603 and JW3600 showed a membrane permeability as low as the wild-type BW25113. The LPS side-chain mutant JW3605 showed increased membrane permeability, similar to the level of LPS inner core mutant strains. The results of the membrane permeability assay are listed in Table 18.

Table 18: Results of the membrane permeability assay of all *E. coli* strains. Excited at 350 nm, measured at 450 nm and stated as NPN uptake factor

	Measurement	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
Approach 1	1	1.8	0.8	1.1	1.3	3.0	1.4	3.6	1.3	0.8	2.7
	2	1.3	0.8	0.8	1.4	2.8	1.2	3.2	1.3	1.2	2.7
	3	1.4	1.3	0.8	1.2	2.6	1.3	3.3	1.0	1.0	2.0
Approach 2	4	0.8	0.9	1.0	0.8	2.0	1.4	1.9	1.3	1.1	1.9
	5	0.8	1.0	1.0	0.9	1.7	1.3	2.0	1.4	1.1	1.6
	6	1.1	0.8	1.1	1.1	1.6	1.2	2.4	1.4	1.0	1.5
Approach 3	7	1.2	1.2	1.1	1.2	2.1	1.3	2.5	1.5	1.3	1.7
	8	1.2	1.2	1.1	1.2	1.8	1.0	2.2	1.2	1.1	1.4
	9	1.4	1.1	1.0	1.0	1.9	1.1	2.3	1.3	1.1	1.5
	Mean value:	1.2	1.0	1.0	1.1	2.2	1.3	2.6	1.3	1.1	1.9
	Confidence interval (α=0.05):	0.2	0.2	0.1	0.1	0.4	0.1	0.5	0.1	0.1	0.4

3.1.2 Disc diffusion test

A disc diffusion test with six different antibiotics was performed as a confirmation and thereby carried out with a selection of *E. coli* strains. The antibiotics ampicillin, ciprofloxacin and getamicin showed no major difference between the wild-type and the LPS length- and side-chain mutants. Nalidixic acid, clindamycin and erythromycin showed a trend to higher inhibition zones with decreasing LPS length. LPS side-chain mutants that possess a full length LPS had a similar susceptibility as LPS mutants with shorter LPS. The results of the disc diffusion test are listed in Table 19.

Table 19: Results of the disc diffusion test with several antibiotics of the *E. coli* strains, showing the mean value of the inhibition zone [mm], the number of measurements (n) and the lower and upper limit

	Ampicillin 10 µg/disc	Ciprofloxacin 5 µg/disc	Clindamicin 2 µg/disc	Erythromycin 15 µg/disc	Gentamicin 10 µg/disc	Nalidixic acid 10 µg/disc
BW25113	23	32	resistant	16	22	11
	n = 3; (18.7-26.3)	n = 3; (23.6-41.1)		n = 3; (10.3-21.5)	n = 3; (19.6-23.6)	n = 3; (9.4-12.8)
JW3597	23	32	resistant	16	22	11
	n = 3; (17.2-29.4)	n = 3; (23.8-40.7)		n = 3; (9.8-21.5)	n = 3; (18.3-26.5)	n = 3; (7.1-14.7]
JW3606	21	35	13	21	23	18
	n = 3; (19.8-22.5)	n = 3; (21.1-48.9)	n = 3; (7.6-18.4)	n = 3; (12.3-29.5)	n = 3; (19.3-27.2)	n = 3; (15.8-21.1)
JW3596	22	34	16	24	23	20
	n = 3; (19.9-21.9)	n = 3; (23.3 - 44.9)	n = 3; (10.6-21.2)	n = 3; (19.3-29.5)	n = 3; (19.1-27.4)	n = 3; (19.0-21.5)

3.1.3 Investigation of the influence of lipopolysaccharides on the efficacy of disinfectants

To investigate the influence of different LPS variations from the *E. coli* strains on the efficacy of QACs and ILs a MIC screening with five different QACs and 18 different ILs that consisted of several different IL-classes was carried out. For further understanding of the influence of LPS variations on the susceptibility to bactericidal substances the MIC of a selection of *E. coli* strains from six different antibiotics was determined.

3.1.3.1 Quaternary ammonium compounds

The group of QACs comprising of five different substances showed the same pattern for all strains. The wild-type BW25113 and the LPS length mutants JW3597 and JW3601 had a similar susceptibility, forming a plateau. The other LPS length mutants from mutant JW3602 to JW3596 also showed a similar susceptibility, forming a second plateau. The three LPS side-chain mutants JW3603, JW3600 and JW3605 showed an increased susceptibility, similar to the strains from the second plateau. The results of the MIC screening are listed in Table 20.

Table 20: Results of the MIC screening of all *E. coli* strains for QACs, showing the mean value with confidence interval (α=0.05), number of measurements (n) and upper/lower limit in [mg/L]

QAC	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
Benzalkonium chloride	24.2 ± 4.9	21.7 ± 5.8	21.7 ± 5.8	6.7 ± 0.7	10.2 ± 6.3	6.7 ± 0.7	8.3 ± 5.6	6.7 ± 0.7	10.2 ± 6.3	10.2 ± 6.3
	n = 6; (15.0/30.0)	n = 3; (15.0/25.0)	n = 3; (15.0/25.0)	n = 3; (6.3/7.5)	n = 3; (3.1/15.0)	n = 3; (6.3/7.5)	n = 6; (3.1/15.0)	n = 3; (6.3/7.5)	n = 3; (3.1/15.0)	n = 3; (3.1/15.0)
Benzethonium chloride	20.0 ± 7.6	13,3 ± 1,4	13.3 ± 1.4	6.7 ± 0.7	4.6 ± 2.5	6.7 ± 0.7	5.0 ± 1.9	6.7 ± 0.7	6.7 ± 0.7	7.7 ± 4.7
	n = 6; (12.5/30.0)	n = 3; (12.5/15.0)	n = 3; (12.5/15.0)	n = 3; (6.3/7.5)	n = 3; (3.1/7.5)	n = 3; (6.3/7.5)	n = 6; (3.1/7.5)	n = 3; (6.3/7.5)	n = 3; (6.3/7.5)	n = 3; (3.1/12.5)
Cetylpyridinium chloride monohydrate	7.1 ± 0.7	7.1 ± 0.7	7.1 ± 0.7	3.5 ± 0.4	2.9 ± 1.0	3.5 ± 0.4	2.3 ± 0.7	3.5 ± 0.4	5.8 ± 1.9	3.5 ± 0.4
	n = 3; (6.3/7.5)	n = 3; (6.3/7.5)	n = 3; (6.3/7.5)	n = 3; (3.1/3.8)	n = 3; (1.9/3.8)	n = 3; (3.1/3.8)	n = 3; (1.9/3.1)	n = 3; (3.1/3.8)	n = 3; (3.8/7.5)	n = 3; (3.1/3.8)
Cetyltrimethylammonium bromide	13.3 ± 1.4	13.3 ± 1.4	10.8 ± 2.9	6.7 ± 0.7	4.6 ± 2.5	6.7 ± 0.7	5.6 ± 2.3	6.7 ± 0.7	6.7 ± 0.7	5.6 ± 2.3
	n = 3; (12.5/15.0)	n = 3; (12.5/15.0)	n = 3; (7.5/12.5)	n = 3; (6.3/7.5)	n = 3; (3.1/7.5)	n = 3; (6.3/7.5)	n = 3; (3.1/7.5)	n = 3; (6.3/7.5)	n = 3; (6.3/7.5)	n = 3; (3.1/7.5)
Domiphen bromide	10.8 ± 2.6	10.8 ± 2.9	10.8 ± 2.9	3.3 ± 0.4	2.2 ± 0.8	3.3 ± 0.4	1.7 ± 0.2	3.3 ± 0.4	5.4 ± 1.4	2.2 ± 0.8
	n = 6; (7.5/12.5)	n = 3; (7.5/12.5)	n = 3; (7.5/12.5)	n = 3; (3.1/3.8)	n = 3; (1.6/3.1)	n = 3; (3.1/3.8)	n = 5; (1.6/1.9)	n = 3; (3.1/3.8)	n = 3; (3.8/6.3)	n = 3; (1.6/3.1)

3.1.3.2 Ionic liquids with one side-chain

ILs with one side-chain with a high efficacy showed the same pattern like QACs. In this group of ILs two exceptions occurred, [C_{10}MIM][Cl] and [TMC_{10}A][Cl]. These two ILs had a way lower toxicity to the strains than the other ILs with one side-chain and did not show the two plateaus with the breaking point. The results of the MIC screening are listed in Table 21.

Table 21: Results of the MIC screening of all *E. coli* strains for the ILs with one side-chain, showing the mean value with confidence interval (α=0.05), number of measurements (n) and upper/lower limit in [mg/L]

IL	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
[C_{10MIM}][Cl]	207,1 ± 92,1 n = 7; (125.0/400.0)	175.0 ± 43.3 n = 3; (125.0/200.0)	141.7 ± 52.0 n = 3; (100.0/200.0)	108.3 ± 14.4 n = 3; (100.0/125.0)	70.8 ± 26.0 n = 3; (50.0/100.0)	108.3 ± 14.4 n = 3; (100.0/125.0)	119.6 ± 60.3 n = 7; (50.0/200.0)	108.3 ± 14.4 n = 3; (100.0/125.0)	141.7 ± 52.0 n = 3; (100.0/200.0)	87.5 ± 21.7 n = 3; (62.5/100.0)
[C_{12MIM}][Cl]	47.3 ± 7.4 n = 10; (37.5/60.0)	45.4 ± 9.4 n = 6; (37.5/60.0)	53.3 ± 5.8 n = 3; (50.0/60.0)	26.7 ± 2.9 n = 3; (25.0/30.0)	11.4 ± 2.4 n = 6; (9.4/15.0)	21.7 ± 5.8 n = 3; (15.0/25.0)	10.1 ± 9.7 n = 10; (6.3/37.5)	21.7 ± 5.8 n = 3; (15.0/25.0)	26.7 ± 2.9 n = 3; (25.0/30.0)	11.4 ± 2.4 n = 6; (9.4/15.0)
[C_{14MIM}][Cl]	14.9 ± 3.8 n = 10; (9.4/20.0)	15.5 ± 4.1 n = 7; (10.0/20.0)	13.3 ± 1.4 n = 3; (12.5/15.0)	6.7 ± 0.7 n = 3; (6.3/7.5)	3.3 ± 0.9 n = 6; (2.5/5.0)	6.7 ± 0.7 n = 3; (6.3/7.5)	2.2 ± 0.7 n = 6; (1.6/3.1)	6.7 ± 0.7 n = 3; (6.3/7.5)	13.3 ± 1.4 n = 3; (12.5/15.0)	4.6 ± 2.8 n = 6; (2.5/10.0)
[C_{16MIM}][Cl]	7.7 ± 1.9 n = 9; (7.7/1.9)	8.2 ± 2.0 n = 6; (8.2/2.0)	8.3 ± 1.4 n = 3; (8.3/1.4)	5.4 ± 1.9 n = 3; (5.4/1.9)	3.0 ± 0.7 n = 5; (3.0/0.7)	5.4 ± 1.9 n = 3; (5.4/1.9)	3.5 ± 1.1 n = 8; (3.5/1.1)	5.4 ± 1.9 n = 3; (5.4/1.9)	6.7 ± 1.4 n = 3; (6.7/1.4)	3.5 ± 1.0 n = 5; (3.5/1.0)
[TMC_{10}A][Cl]	117.9 ± 31.3 n = 7; (50.0/150.0)	133.3 ± 14.4 n = 3; (125.0/150.0)	108.3 ± 28.9 n = 3; (75.0/125.0)	66.7 ± 7.2 n = 3; (62.5/75.0)	91.7 ± 50.5 n = 3; (62.5/150.0)	66.7 ± 7.2 n = 3; (62.5/75.0)	83.0 ± 35.1 n = 7; (31.3/125.0)	66.7 ± 7.2 n = 3; (62.5/75.0)	87.5 ± 33.1 n = 3; (62.5/125.0)	112.5 ± 45.1 n = 3; (62.5/150.0)
[TMC_{16}A][Cl]	13.1 ± 1.2 n = 8; (12.5/15.0)	13.0 ± 1.1 n = 5; (12.5/15.0)	10.8 ± 2.9 n = 3; (7.5/12.5)	5.4 ± 1.4 n = 3; (3.8/6.3)	3.3 ± 0.3 n = 5; (3.1/3.8)	6.7 ± 0.7 n = 3; (6.3/7.5)	2.8 ± 0.6 n = 7; (1.9/3.1)	6.7 ± 0.7 n = 3; (6.3/7.5)	6.7 ± 0.7 n = 3; (6.3/7.5)	3.9 ± 1.4 n = 5; (3.1/6.3)

3.1.3.3 Ionic liquids with two or more side-chains

The group of ILs with two side-chains, consisting of the ILs [DODMA][Cl] and [$C_{10,10}$MIM][Cl], showed no uniform results. While [DODMA][Cl] showed the same pattern like QACs, the result for [$C_{10,10}$MIM][Cl] was different. This IL had a high efficacy on bacteria and due to this property the dilution series had to be adapted from a two-folded to a dilution series in 0.5 mg/L steps. Despite the increased resolution [$C_{10,10}$MIM][Cl] did show a different pattern than QACs. All bacterial strains had nearly the same MIC value.

The group of ILs with three side-chains, represented by [TOMA][Cl] and [TOMP][Cl] showed no uniform results. While [TOMA][Cl] showed the same pattern as QACs, [TOMP][Cl] showed a different pattern without the formation of plateaus or breaking point.

In the IL group with four side-chains only the IL C1EG was effective and showed the same pattern as QACs. The other ILs 221PG, T2EG, [TIBMP][Tosylate] and [TOP][Br] had a MIC of >5000 mg/L, thus showing according to the defined upper MIC limit that these are not effective against *E. coli*. Therefore, for the ILs 221PG and T2EG not all strains were screened because strains with full length LPS, shortest LPS and an example of the side-chain mutants showed in the pre-screening no susceptibility to these ILs. The results of the MIC screening are listed in Table 22.

Table 22: Results of the MIC screening of all *E. coli* strains for the ILs with two or more side-chains, showing the mean value with confidence interval (α=0.05), number of measurements (n) and upper/lower limit in [mg/L]

	IL	BW25113	JW3597	JW3601	JW3602	JW3606	JW3595	JW3596	JW3603	JW3600	JW3605
ILs with two side-chains	[DODMA][Cl]	104.2 ± 24.6	91.7 ± 14.4	91.7 ± 14.4	45.8 ± 7.2	22.9 ± 3.6	45.8 ± 7.2	20.8 ± 5.1	45.8 ± 7.2	58.3 ± 14.4	22.9 ± 3.6
		n = 6; (75,0/150,0)	n = 3; (75.0/100.0)	n = 3; (75.0/100.0)	n = 3; (37.5/50.0)	n = 3; (18.8/25.0)	n = 3; (37.5/50.0)	n = 6; (12.5/25.0)	n = 3; (37.5/50.0)	n = 3; (50.0/75.0)	n = 3; (18.8/25.0)
	[$C_{10,10}$MIM][Cl]	1.7 ± 0.3	1.7 ± 0.3	1.7 ± 0.3	1.5 ± 0.0	1.0 ± 0.0	1.5 ± 0.0	1.0 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.2 ± 0.3
		n = 3; (1.5/2.0)	n = 3; (1.5/2.0)	n = 3; (1.5/2.0)	n = 3; (1.5/1.5)	n = 3; (1.0/1.0)	n = 3; (1.5/1.5)	n = 3; (1.0/1.0)	n = 3; (1.5/1.5)	n = 3; (1.5/1.5)	n = 3; (1.0/1.5)
ILs with three side-chains	[TOMP][Cl]	6.8 ± 2.7	5.6 ± 1.5	6.7 ± 0.7	5.4 ± 1.4	2.8 ± 0.8	5.4 ± 1.4	2.2 ± 1.7	5.4 ± 1.4	6.7 ± 0.7	2.8 ± 0.8
		n = 8; (4.0/12.5)	n = 5; (4.0/7.5)	n = 3; (6.3/7.5)	n = 3; (3.8/6.3)	n = 5; (2.0/3.8)	n = 3; (3.8/6.3)	n = 8; (1.0/6.3)	n = 3; (3.8/6.3)	n = 3; (6.3/7.5)	n = 5; (2.0/3.8)
	[TOMA][Cl]	6.8 ± 0.7	6.9 ± 0.7	7.1 ± 0.7	4.6 ± 1.4	2.5 ± 0.7	3.5 ± 0.4	1.7 ± 0.7	4.6 ± 1.4	5.8 ± 1.9	1.7 ± 0.2
		n = 7; (6.3/7.5)	n = 4; (6.3/7.5)	n = 3; (6.3/7.5)	n = 3; (3.8/6.3)	n = 4; (1.9/3.1)	n = 3; (3.1/3.8)	n = 7; (0.9/3.1)	n = 3; (3.8/6.3)	n = 3; (3.8/7.5)	n = 4; (1.6/1.9)
ILs with four side-chains	[TOP][Br]	>5000	>5000	>5000	>5000	>5000	>5000	2.083.3 ± 721.7	>5000	>5000	>5000
		n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3; (1.250.0/2.500.0)	n = 3	n = 3	n = 3
	C1EG	168.8 ± 25.9	140.0 ± 22.4	133.3 ± 28.9	41.7 ± 7.2	42.5 ± 19.0	41.7 ± 7.2	39.8 ± 30.8	41.7 ± 7.2	66.7 ± 14.4	42.5 ± 19.0
		n = 8; (150.0/200.0)	n = 5; (100.0/150.0)	n = 3; (100.0/150.0)	n = 3; (37.5/50.0)	n = 5; (25.0/75.0)	n = 3; (37.5/50.0)	n = 8; (18.8/100.0)	n = 3; (37.5/50.0)	n = 3; (50.0/75.0)	n = 5; (25.0/75.0)
	221PG	>5000	>5000	-	-	>5000	-	>5000	-	-	>5000
		n = 2	n = 2	-	-	n = 2	-	n = 2	-	-	n = 2
	T2EG	>5000	>5000	-	-	>5000	-	>5000	-	-	>5000
		n = 2	n = 2	-	-	n = 2	-	n = 2	-	-	n = 2
	[TIBMP][Tosylate]	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
		n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3

3.1.3.4 Ionic liquids with an active anion

The three ILs [BMIM][FAP], [EMIM][FAP] and [EDMOA][FAP] showed a MIC >5000 mg/L and therefore were not effective against *E. coli*.

3.2 Evolution experiment

The first attempt of the experiment had to be aborted already after ten days due to problems with cross-contaminations. The quality control on TSA+Y showed that especially the lines of *L. monocytogenes* were heavily contaminated by other bacteria, presumably *E. coli* from other lines. The evolution experiment was aborted and the whole experiment redesigned for a second approach.

3.2.1 Viable strains after the second attempt of the experiment

This experiment produced a lot of strains. To reduce the amount of strains to a selection of interesting strains, the growth curve trend, doubling time and the MIC were used for comparison. After the selection of interesting strains a MIC screening with disinfectants and antibiotics was performed to see if adaptations of the bacteria to bactericidal stress did occur.

At the end of the evolution experiment all strains that were still viable after 40 days of continuous incubation and demonstrated to be a pure culture on a streaked plate were kept. Strains that showed contaminations in the quality control or did not grow due to too much bactericidal pressure were discarded. The remaining strains are listed in Table 23.

Table 23: Remaining bacterial strains after the second approach of evolution experiment

Bacteria	Strain	Remaining strains	TSB+Y control
E. coli	BW25113	45 of 45	1 of 1
	JW3596	26 of 45	1 of 1
	JW0451-2	41 of 45	1 of 1
L. monocytogenes	6179	25 of 45	1 of 1
	QC1	17 of 45	0 of 1

3.2.2 Selection of interesting strains

In total, 158 strains were obtained from the 40 days evolution experiment. To separate interesting strains for further experiments, for all strains a 24 h growth curves in TSB+Y were recorded and furthermore the doubling times were determined. The MIC was also determined for all strains, which were exposed to [TOMP][Cl], [$C_{10,10}$MIM][Cl] and [TMC_{16}A][Cl]. All measured values were compared with the control strains, which were incubated only in TSB+Y without any bactericidal substances and checked for deviations. If the growth curve trend showed deviations, the doubling time was unusually longer or the MIC

was higher as the corresponding TSB+Y control strain the strains were selected for further MIC screening and disc diffusion test. All selected strains are listed in Table 24.

Table 24: Selected strains with deviations from the TSB+Y control for further testing

Bacteria	Strain code	Incubated in	Growth curve trend	Doubling time	MIC BC	MIC [TOMP][Cl]	MIC [$C_{10,10}$MIM][Cl]	MIC [TMC_{16}A][Cl]
BW25113	B43	[TMC_{16}A][Cl] 7.0 mg/L		X				
JW3596	C9	[TOMP][Cl] 0.5 mg/L	X	X	X			
	C11	[TOMP][Cl] 1.5 mg/L				X	X	X
	C17	[$C_{10,10}$MIM][Cl] 0.6 mg/L	X	X				
0451-2	D12	[TOMP][Cl] 2.5 mg/L		X	X			
	D27	[$C_{10,10}$MIM][Cl] 1.0 mg/L		X	X			
	D32	[TMC_{16}A][Cl] 0.8 mg/L		X	X			
6179	S3	[TOMP][Cl] 0.6 mg/L	X	X				

From the *L. monocytogenes* line QC1 no strains were selected due to a contamination of the 40 days TSB+Y control. In total 12 strains were selected for further test, eight strains which were incubated in TSB+Y and an IL and four from the TSB+Y controls (B46 for BW25113, C46 for JW3596, D46 for 0451-2 and S46 for 6179).

3.2.3 Minimum inhibitory concentration

The MIC was determined in 96 wells microtiter plates in two-folded dilution series on three different days for the QAC benzalkonium chloride and the ILs [TOMP][Cl], [TMC_{16}A][Cl] and [$C_{10,10}$MIM][Cl].

For all substances the results showed no differences between the controls grown in TSB+Y and most of the strains grown in TSB+Y and the respective ionic liquid. The only exceptions were the strains C11 and D12. Strain C11 showed for all substances a way higher MIC than the other C-strains like the wild-type control strain B46. All results of the MIC screening are listed in Table 25.

Table 25: Results of the MIC screening of selected bacterial strains after the evolution experiment, showing the mean value with confidence interval (α=0.05), number of repetitions (n) and lower/upper limit in [mg/L]

	B46	B43	C46	C9	C11	C17	D46	D12	D27	D32	S46	S3
Benzalkonium chloride	25.0 ± 0.0	25.0 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	25.0 ± 0.0	3.1 ± 0.0	2.1 ± 0.9	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	6.3 ± 0.0	6.3 ± 0.0
	n = 3; (25.0/25.0)	n = 3; (25.0/25.0)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (25.0/25.0)	n = 3; (3.1/3.1)	n = 3; (1.6/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (6.3/6.3)	n = 3; (6.3/6.3)
[TMC_{16}A][Cl]	12.5 ± 0.0	12.5 ± 0.0	2.1 ± 0.9	1.6 ± 0.0	12.5 ± 0.0	1.6 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0
	n = 3; (12.5/12.5)	n = 3; (12.5/12.5)	n = 3; (1.6/3.1)	n = 3; (1.6/1.6)	n = 3; (12.5/12.5)	n = 3; (1.6/1.6)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)	n = 3; (3.1/3.1)
[$C_{10,10}$MIM][Cl]	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
	n = 3; (2.0/2.0)	n = 3; (2.0/2.5)	n = 3; (1.0/1.0)	n = 3; (1.0/1.0)	n = 3; (1.5/2.0)	n = 3; (1.0/1.0)	n = 3; (1.5/1.5)	n = 3; (1.5/1.5)	n = 3; (1.5/1.5)	n = 3; (1.5/1.5)	n = 3; (1.0/1.0)	n = 3; (1.0/1.5)
[TOMP][Cl]	6.3 ± 2.2	7.5 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	7.5 ± 0.0	1.9 ± 0.0	3.8 ± 0.0	7.5 ± 0.0	3.8 ± 0.0	3.8 ± 0.0	1.9 ± 0.0	1.9 ± 0.0
	n = 3; (3.8/7.5)	n = 3; (7.5/7.5)	n = 3; (1.9/1.9)	n = 3; (1.9/1.9)	n = 3; (7.5/7.5)	n = 3; (1.9/1.9)	n = 3; (3.8/3.8)	n = 3; (7.5/7.5)	n = 3; (3.8/3.8)	n = 3; (3.8/3.8)	n = 3; (1.9/1.9)	n = 3; (1.9/1.9)

3.2.4 Disc diffusion test

The disc diffusion test with 10 µg of the antibiotics ampicillin, chloramphenicol, ciprofloxacin, gentamicin, levofloxacin and nalidixic acid was performed with all selected strains three times on different days.

For all antibiotics the results showed no differences between the control strains grown in TSB+Y and most of the strains grown in TSB+Y and the respective ionic liquid. The only exception was strain C11, showing lower inhibition zones like the wild-type control strain B46. After the MIC determination this was the second time this strain showed unusual results, making this strain a possible candidate for a contamination. All results of the disc diffusion test are listed in Table 26.

Table 26: Results of the disc diffusion test of selected strains, showing the mean value with confidence interval (α=0.05), number of repetitions (n) and lower/upper limit in [mm] inhibition zone

	B46	B43	C46	C9	C11	C17	D46	D12	D27	D32	S46	S3
Ampicillin 10 µg	17.3 ± 3.1	15.3 ± 2.3	18.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	22.0 ± 1.7	22.0 ± 1.7	20.7 ± 1.2	21.3 ± 0.6	36.3 ± 1.5	36.3 ± 0.6
	n = 3; (14.0/20.0)	n = 3; (14.0/18.0)	n = 3; (17.0/19.0)	n = 3; (18.0/20.0)	n = 3; (18.0/20.0)	n = 3; (18.0/20.0)	n = 3; (21.0/24.0)	n = 3; (21.0/24.0)	n = 3; (20.0/22.0)	n = 3; (21.0/22.0)	n = 3; (35.0/38.0)	n = 3; (36.0/37.0)
Chloramphenicol 10 µg	16.7 ± 1.5	9.0 ± 2.6	25.7 ± 0.6	29.0 ± 1.0	16.3 ± 1.5	26.0 ± 0.0	30.0 ± 0.0	29.0 ± 1.0	29.3 ± 0.6	29.0 ± 0.0	23.7 ± 1.2	22.7 ± 1.2
	n = 3; (15.0/18.0)	n = 3; (7.0/12.0)	n = 3; (25.0/26.0)	n = 3; (28.0/30.0)	n = 3; (15.0/18.0)	n = 3; (26.0/26.0)	n = 3; (30.0/30.0)	n = 3; (28.0/30.0)	n = 3; (29.0/30.0)	n = 3; (29.0/29.0)	n = 3; (23.0/25.0)	n = 3; (22.0/24.0)
Ciprofloxacin 10 µg	28.0 ± 0.0	26.7 ± 1.5	28.7 ± 0.6	32.3 ± 2.5	27.7 ± 1.5	30.3 ± 1.5	32.3 ± 0.6	32.7 ± 1.5	32.3 ± 0.6	32.3 ± 0.6	21.3 ± 0.6	23.0 ± 1.0
	n = 3; (28.0/28.0)	n = 3; (25.0/28.0)	n = 3; (28.0/29.0)	n = 3; (30.0/35.0)	n = 3; (26.0/29.0)	n = 3; (29.0/32.0)	n = 3; (32.0/33.0)	n = 3; (31.0/34.0)	n = 3; (32.0/33.0)	n = 3; (32.0/33.0)	n = 3; (21.0/22.0)	n = 3; (22.0/24.0)
Gentamicin 10 µg	20.3 ± 2.5	18.3 ± 1.5	19.0 ± 3.0	16.7 ± 1.5	18.0 ± 0.0	18.3 ± 0.6	21.3 ± 5.5	18.3 ± 4.5	19.0 ± 4.6	21.3 ± 7.1	25.0 ± 1.0	23.3 ± 0.6
	n = 3; (18.0/23.0)	n = 3; (17.0/20.0)	n = 3; (16.0/22.0)	n = 3; (15.0/18.0)	n = 3; (18.0/18.0)	n = 3; (18.0/19.0)	n = 3; (16.0/27.0)	n = 3; (14.0/23.0)	n = 3; (15.0/24.0)	n = 3; (15.0/29.0)	n = 3; (24.0/26.0)	n = 3; (23.0/24.0)
Levofloxacin 10 µg	31.7 ± 0.6	30.7 ± 0.6	36.3 ± 1.2	38.3 ± 0.6	32.0 ± 1.0	36.0 ± 0.0	37.0 ± 1.0	37.0 ± 0.0	37.3 ± 0.6	36.7 ± 1.2	28.0 ± 0.0	27.7 ± 2.1
	n = 3; (31.0/32.0)	n = 3; (30.0/31.0)	n = 3; (35.0/37.0)	n = 3; (38.0/39.0)	n = 3; (31.0/33.0)	n = 3; (36.0/36.0)	n = 3; (36.0/38.0)	n = 3; (37.0/37.0)	n = 3; (37.0/38.0)	n = 3; (36.0/38.0)	n = 3; (28.0/28.0)	n = 3; (26.0/30.0)
Nalidixic acid 10 µg	13.0 ± 1.0	9.3 ± 2.5	24.3 ± 1.2	24.7 ± 2.5	13.7 ± 2.5	25.0 ± 1.0	20.7 ± 1.2	22.0 ± 2.0	21.7 ± 1.2	21.7 ± 1.5	0.0 ± 0.0	0.0 ± 0.0
	n = 3; (12.0/14.0)	n = 3; (7.0/12.0)	n = 3; (23.0/25.0)	n = 3; (22.0/27.0)	n = 3; (11.0/16.0)	n = 3; (24.0/26.0)	n = 3; (20.0/22.0)	n = 3; (20.0/24.0)	n = 3; (21.0/23.0)	n = 3; (20.0/23.0)	n = 3; (0.0/0.0)	n = 3; (0.0/0.0)

4 Discussion

The first part of the discussion is dedicated to the main part of this master thesis and is regarding to the influence of LPS on the efficacy of QACs and ILs. At first the results of the strain characterization in all facets will be discussed and afterwards the results of the MIC screening.

The second part of the discussion is dedicated to the evolution experiment. The execution of the respective evolution approaches will be discussed and consequently also the results of the MIC screening will be elucidated.

4.1 Lipopolysaccharide and ionic liquid efficacy

4.1.1 Strain characterization

To make sure, that all ordered strains from the CGSC were *E. coli* with their respective gene missing, molecular biological verifications by PCR and qPCR were carried out.

To further assure that the mutants were not impaired by their single-gene deletion, microbiological verifications were made prior to the MIC screening. Therefore, the kanamycin resistance of the mutant strains was tested, the doubling time calculated, the growth curve recorded and a stress test to non membrane permeable substances performed.

To get further knowledge on how modifications in the LPS structure of the mutant strains influence the properties of the outer membrane, a cell surface hydrophobicity assay and a membrane permeability assay were carried out.

4.1.1.1 Molecular biological verifications

The qPCR verification targeting the *smfD* gene, having 100 % exclusivity in *E. coli,* showed that all strains were *E. coli* bacteria. The qPCR protocol is shown in chapter 2.3.3.2.

The wild-type strain BW25113 showed in every PCR approach a specific band at the respective base pair length. These results confirmed that the wild-type possesses all tested LPS synthesis genes. For the LPS mutant strains all single-gene deletions could be confirmed except for the strain JW3595. This strain showed in the PCR targeting the *waaF* gene a specific PCR product at 244 bp which should not occur. The PCR protocol is shown in chapter 2.3.1.5.

To make sure a copy of the gene does not exist in the genome of *E. coli* K-12 the whole genome was downloaded from the NCBI database and checked for another binding site of the primer pair. In the genome no other binding site could be found, leading to the following possible conclusions:

- The PCR I designed for the gene *waaF* was deficient. Other primers should be designed and tested. To get more clues what went wrong the PCR product could be sent to a sequencing laboratory.
- The mutant strain JW3595 from the CGSC is another mutant and was wrongly sent to us as JW3595 strain. Since the strain possesses the kanamycin resistance it is highly probable that single-gene deletion was performed for this strain but possibly another gene than the *waaF* gene was deleted. The strain should be ordered and examined with PCR again.

Due to the restricted period of time during my master thesis there was no time left to perform any actions to solve this problem. Therefore, all results from the strain JW3595 of this master thesis will be excluded from the discussion.

All other strains did meet the requirements and could be verified correctly. Therefore, further microbiological verifications were carried out. A summary of the molecular biological verification results are listed in Table 27.

Table 27: Overview of the results of the molecular biological verifications for the *E. coli* strains

Strain	*E. coli verification*	Kanamycin resistance	Deleted gene	Deletion verification
BW25113	yes	no	-	-
JW3597	yes	yes	*waaL*	yes
JW3601	yes	yes	*waaR*	yes
JW3602	yes	yes	*waaI*	yes
JW3606	yes	yes	*waaG*	yes
JW3595	yes	yes	*waaF*	**no**
JW3596	yes	yes	*waaC*	yes
JW3603	yes	yes	*waaB*	yes
JW3600	yes	yes	*waaY*	yes
JW3605	yes	yes	*waaP*	yes

4.1.1.2 Microbiological verifications

All strains from the CGSC except the wild-type BW25113 grew on TSA+Y agar with 30 µg/mL kanamycin added. This fact serves as additional evidence that the single-gene deletion was carried out successfully.

Further, from all strains a growth curve trend was recorded and the doubling time calculated. The susceptibility of all strains to membrane permeable substances was also investigated. An overview of these results is listed in Table 28.

Table 28: Overview of the results of the microbiological verifications of the bacterial strains, showing the differences to the wild-type strain BW25113

Bacterial strains	Doubling time	Growth curve	Stress test
JW3597	no	no	no
JW3601	no	no	no
JW3602	(yes)	no	no
JW3606	no	no	no
JW3596	no	no	no
JW3603	no	no	no
JW3600	no	no	no
JW3605	no	no	no

The growth curve trends of all strains compared to the wild-type strain BW25113 showed no deviations. Some strains reached a higher end OD_{600} value than others, but due to the fact that some strains precipitate at the ground of the microtiter plate wells this value was not rated. The doubling times of the mutant strains were all near the doubling time of 97 min of the wild-type BW25113, except the strain JW3602 with a doubling time of 151 min. Possible explanations for this deviations could be that the deletion of the gene *waaI* was leading to a slower growth or that colonies of this strain were more likely to precipitation which did lead to wrong measured OD values that caused through the calculation a slower doubling time. In general, all three LPS side-chain mutants seemed to have a slightly faster doubling time than the wild-type strain.

The MIC screening with the membrane permeable substances ethanol, KH_2PO_4, NaCl, urea and TSB+Y with different pH values showed no difference between the mutant strains and the wild-type. Due to these results all mutants were viewed as not impaired from the single-gene deletion.

Even after the molecular biological and microbiological verifications the question about the absence or presence of the O-antigen in the wild-type BW25113 and the LPS side-chain mutants remained unsolved. Usually the *E. coli* K-12 type does not possess an O-antigen but in the publication from (Nakao et al., 2012) an O-antigen was mentioned and the wild-type strain BW25113 possessed the gene *waaL* which encodes for the O-antigen ligase. I also tried to get some more information about the LPS structure of the wild-type from the CGSC, but they never answered my requests. A method to check the LPS structures for an O-antigen and compare all LPS structures of the strains to each other would have been a SDS-PAGE. At first the LPS has to be extracted with a suitable method (e.g. hot phenol water extraction) and then purified from nucleic acids and capsular polysaccharides through precipitation or enzymatic degradation. Afterwards the LPS extracts are separated in a 12 %

SDS-PAGE and made visible with silver staining (Castro et al., 2010). Due to the limited period of time in this master thesis this verification could not be performed.

Overall, after the microbiological verifications the strains did comply with the requirements of a non-impairment. The strains did show nearly the same doubling time, no noticeable deviances in the growth trend comparison and showed the same susceptibility to membrane permeating substances. Therefore, all further experiments of this master thesis were carried out.

4.1.1.3 Cell surface hydrophobicity and membrane permeability

Prior to these experiments, the hypothesis was that the cell surface hydrophobicity and the membrane permeability, expressed through the NPN uptake factor, would increase linearly with a decreasing LPS length. The results of the cell surface hydrophobicity assay did accord with the hypothesis and confirmed the results from (Wang et al., 2015), which showed that with decreasing LPS outer core length the hydrophobicity of cells is increasing (Figure 19). With every sugar residue that was missing the non-polar character of the outer membrane was more apparent and the xylene pulled more cells in the upper phase resulting in a higher cell surface hydrophobicity for strains with a shortened LPS. These results were in accordance with my that bacterial strains with a shorter LPS will show higher hydrophobicity values. A breaking point, shown exemplary in Figure 20 of the benzalkonium chloride MIC screening between the mutant strains JW3601 and JW3602, did not occur. The results of the LPS side-chain mutants were slightly different from the results of the benzalkonium chloride MIC screening shown in Figure 20. From the three side-chain mutants the two strains JW3603 and JW3605 showed a high cell surface hydrophobicity as high as LPS mutant strains with a shorter LPS. These results accord with the effect described in the literature (Yethon et al., 2000) that the side-chains in the LPS structure are essential for the integrity of the LPS. If the side-chains are missing the LPS structures can't interact well enough with each other leading to repulsion and thus not being able to form a tight barrier against the environment. An exception was the third LPS side-chain mutant strain JW3600. This strain showed a cell surface hydrophobicity as low as the wild-type strain BW25113, leading to the assumption that the missing phosphate group of this strain does not result in repulsion and thus shield successfully against the non-polar character of the outer membrane.

The results of the membrane permeability assay showed also an increasing membrane permeability with a decreasing LPS length and therefore confirmed the results from (Wang et al., 2015), although the membrane permeability is not increasing linearly like the cell surface hydrophobicity. The mutant strains JW3597, JW3601 and JW3602 that possess LPS lengths belonging to the outer core region showed a slightly decreased membrane permeability than the wild-type strain BW25113. Further reducing the LPS length to inner core lengths

increased the membrane permeability strongly. Unlike the breaking point in the benzalkonium chloride MIC screening between the mutant strains JW3601 and JW3602 shown in Figure 20, the breaking point in the membrane permeability assay is shifted between the mutant strains JW3602 and JW3606. The highest membrane permeability showed the strain JW3596 with the shortest LPS length. These results accord partly with my hypothesis before the execution of the membrane permeability assay stating that membrane permeability will increase with decreasing LPS length. An explanation for these results could be that the uptake of the non-polar substance NPN from the membrane permeability assay is hindered by the polar sugar residues of the LPS. Only after these residues of the outer core are removed, a certain level of NPN uptake is no longer hindered as before. The two LPS side-chain mutant strains JW3603 and JW3600 showed a membrane permeability as low as the LPS wild-type strain BW25113. These results did not accord with my hypothesis before the execution of the membrane permeability assay stating that LPS side-chain mutant strains will have nearly the same membrane permeability as the LPS length mutant strains with a shorter LPS. These results also deviate from the results of the benzalkonium chloride MIC screening shown in Figure 20, where all LPS side-chain mutants were as susceptible as LPS mutants with a shortened LPS length. An explanation for these results could be that the missing galactose residue of the JW3603 strain and the missing phosphate residue of the JW3600 strain are not important for the LPS integrity towards the barrier function to non-polar substances like NPN. The third LPS side-chain mutant strain JW3605 showed an increased membrane permeability compared to the two other strains mentioned before. An explanation for this result could be that the missing phosphate/pyrophosphorylethanolamine residue is more important for the barrier function of non-polar substances like NPN than the two other residues previously mentioned.

The presence of the O-antigen in *E. coli* K-12 wild-type BW25113 remained still unknown due to the fact that for both examinations the mutant strain JW3597 didn’t differ that much from the wild-type BW25113. Maybe the O-antigen did not contribute a lot in both assays or the wild-type BW25113 and the LPS side-chain mutants did not possess an O-antigen. The results of the cell surface hydrophobicity and the membrane permeability assay are shown in Figure 19. The results in form of a table can be found in chapter 3.1.1.3 for the cell surface hydrophobicity assay and in chapter 3.1.1.4 for the membrane permeability assay.

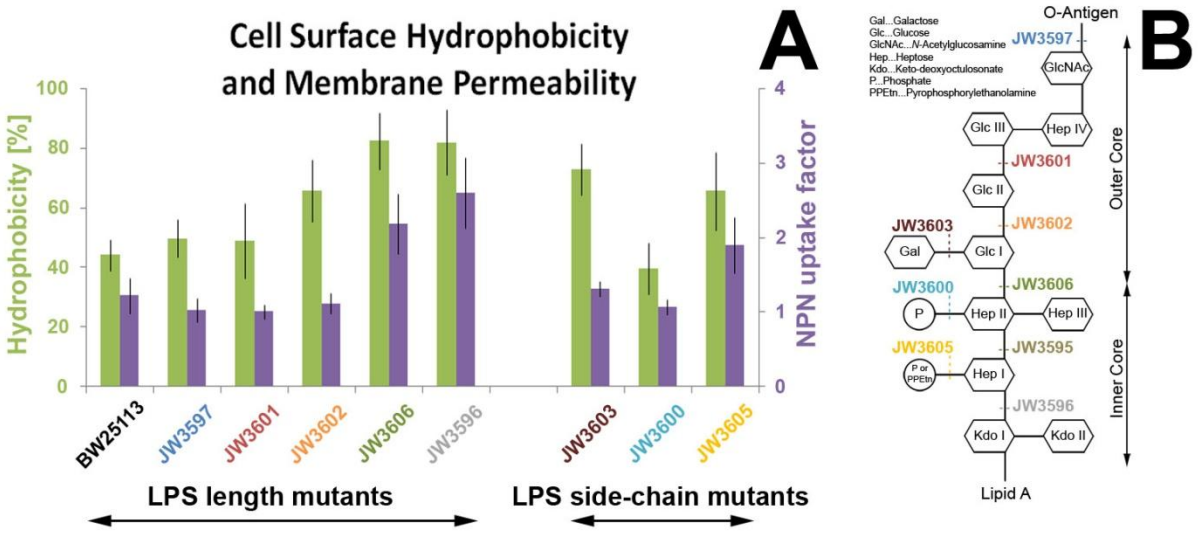

Figure 19: Comparison of the results of the *E. coli* strains of the cell surface hydrophobicity and membrane permeability assay (A) with the supposed LPS structures of the strains (B). Bars showing the mean MIC value and error bars showing the confidence interval (α=0.05)

4.1.2 Minimum inhibitory concentration

The main part of this master thesis was to investigate the influence of LPS on the efficacy of QACs and ILs. Since ILs are seen as a promising alternative to common QACs the results of both substance classes were compared to each other to see if ILs show the similar results like QACs. This was achieved through a MIC screening with 5 different QACs and 18 different ILs. To gather additional information of the influence of the LPS variations on the efficacy of another bactericidal substance class four strains were screened with 6 different antibiotics. The results of the MIC screening can be found in chapter 3.1.3.

4.1.2.1 Disc diffusion test

The results from the antibiotics ampicillin, ciprofloxacin and gentamicin did not show major differences between the strain with full LPS length or strains with shortened LPS lengths. For these antibiotics the LPS had no barrier function to overcome. The results of the remaining antibiotics clindamycin, erythromycin and nalidixic acid showed that with decreasing LPS length the strains got more susceptible, leading to similar results as QACs and ILs with the two plateaus and the breaking point. The results in form of a table are shown in chapter 3.1.2.

4.1.2.2 Quaternary ammonium compounds

The results of the MIC determination showed for all five QACs the formation of two levels. The three strains including the wild-type BW25113, JW3597 and JW3601 forming one higher level and all other strains a second lower one, showing a breaking point that lays in the outer core region of the LPS. These results do not accord with the hypothesis that the MIC will decrease with decreasing LPS length. In fact the length of the LPS played no role until the glucose residue Glc II was removed. After this LPS residue, a major decrease in the MIC

was observable. Further decrease in LPS length did not lead to further decreasing MIC. A possible explanation for this result could be that the glucose residue Glc II at the breaking point is essential for the integrity of the LPS in *E. coli* K-12. Is this structure element missing it is possible that the LPS molecules can't form a tight barrier due to repulsion effects of the LPS molecules among each other. Similar to the previous results it was not possible to prove the absence or presence of the O-antigen. Either the O-antigen did not contribute to the resistance against the QACs or the wild-type BW25113 and the LPS side-chain mutants did not possess it. The results of the LPS side-chain mutants JW3603, JW3600 and JW3605 showed that every single side-chain of the LPS structure is essential for the strains in terms of susceptibility against biocide substances. These results are according to the hypothesis that LPS side-chains are essential for LPS integrity. The trend was the same for all tested QACs, the results showed all the same pattern. A comparison of the results of the MIC determination with the supposed LPS structures of the strains is therefore shown for benzalkonium chloride in Figure 20 representing the results of the group of QACs. The results for each individual substance in form of a table are listed in chapter 3.1.3.1.

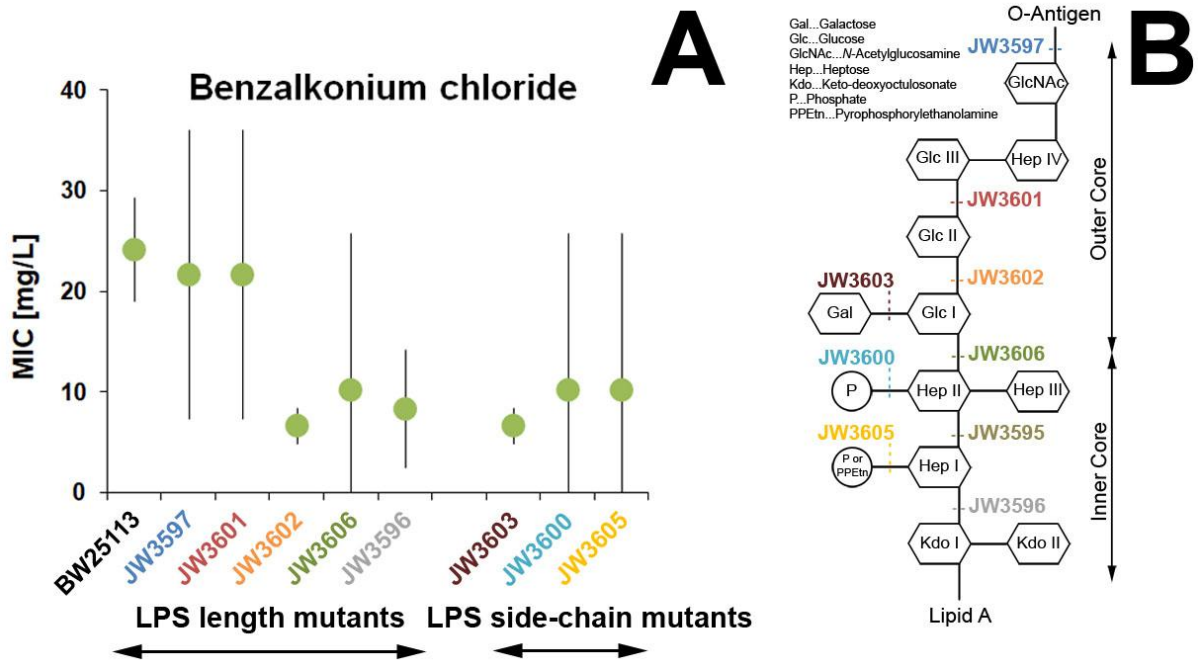

Figure 20: Comparison of the results of the *E. coli* strains of the MIC determination of benzalkonium chloride representing the QAC group (A) with the supposed LPS structures of the strains (B). Dots showing the mean MIC value and error bars showing the confidence interval (α=0.05)

4.1.2.3 Ionic liquids

4.1.2.3.1 Ionic liquids with one side-chain

Similar to the results obtained for QACs, the results of ILs with one side-chain did show the formation of two MIC plateaus with a distinct breaking point. Only in the case of [C_{10}MIM][Cl] and [TMC_{10}A][Cl], this breaking point was not as distinct which is probably due to their generally higher MIC value. Furthermore they did not show the known pattern with the two plateaus and the breaking point. The well-described cationic alkyl side-chain effect (Egorova and Ananikov, 2014) was confirmed. From the side-chain length C_{10} to C_{16} the results showed strongly decreasing MIC values, with C_{16} as most effective substance. Maybe if the side-chain length of the ILs reaches nearly the same length as the phospholipids in the outer membrane of the bacteria, these ILs could easier overcome this barrier or bind into the outer membrane and thus dramatically reduce the barrier function of the outer membrane. The results also showed that this effect is not dependent on the type of cation which was used, this effect occurred for both imidazolium based and ammonium based cations. Since the ILs with one side-chain showed similar results only two of them are exemplary shown in Figure 21. The results of all ILs in form of a table are listed in chapter 3.1.3.2.

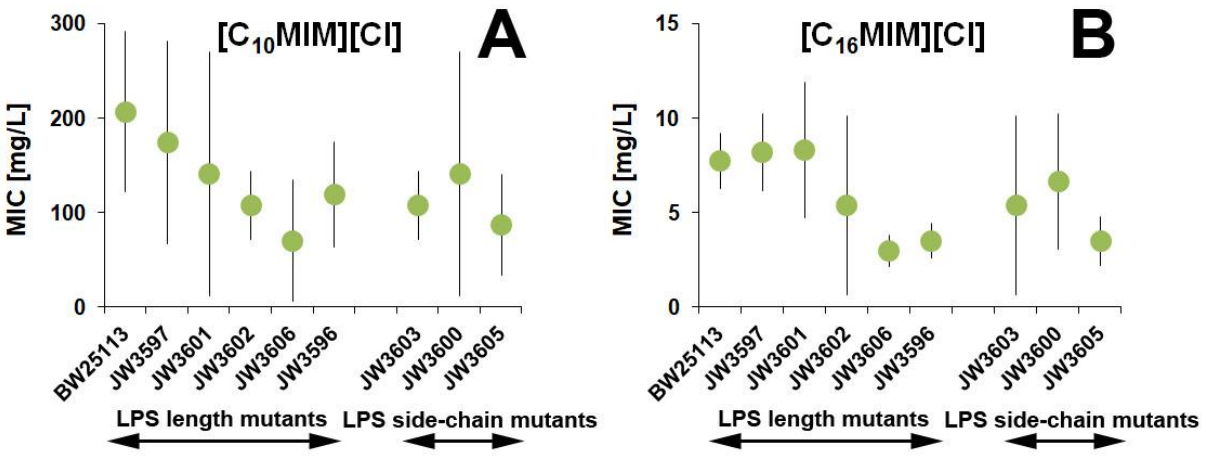

Figure 21: Comparison of the results of the MIC screening of the *E. coli* strains for ILs with one side-chain. [C_{10}MIM][Cl] represents the group of ILs with one side-chain with a lesser toxicity (A) and [C_{16}MIM][Cl] represents the group of ILs with one side-chain and a higher toxicity (B) to the *E. coli* strains. Dots showing the mean MIC value and error bars showing the confidence interval (α=0.05)

4.1.2.3.2 Ionic liquids with two or more side-chains

The results of the IL [DODMA][Cl] with two C_8 alkyl side-chains did show the same pattern as the QACs and the ILs with one side-chain. An interesting exception was the IL with two C_{10} side-chains [$C_{10,10}$MIM][Cl]. With the standard two-fold serial dilution, which was used for the whole MIC screening, all strains had the same MIC value. Even after the dilution series was performed in 0.5 mg/L steps and thus increasing the resolution of the MIC screening, the strains did not show differences in their susceptibility to [$C_{10,10}$MIM][Cl]. With a mean value of

around 1.8 mg/L for the MIC of the wild-type BW25113 this substance was the most effective bactericidal substance of the whole screening, making it an interesting substance for further research and a promising candidate for a new disinfectant. Most importantly, for this substance it did not matter if the strains possessed the full LPS length or the most shortened one. Since [$C_{10,10}$MIM][Cl] can overcome the LPS barrier easily, the mode of action of this substance should be a focus of further experiments.

The two ILs representing the group of ILs with three side-chains [TOMA][Cl] and [TOMP][Cl] showed similar results. All in all the results showed that ILs with three side-chains were highly effective against the strains confirming the SAR effect from the literature that ILs with more side-chains are more effective than ILs with a lesser number of side-chains. [$C_{10,10}$MIM][Cl] has only two side-chains and is a deviation from this rule, but in general this effect could be confirmed. A comparison of the MIC screening is shown in Figure 22. The results in form of a table are shown in chapter 3.1.3.3.

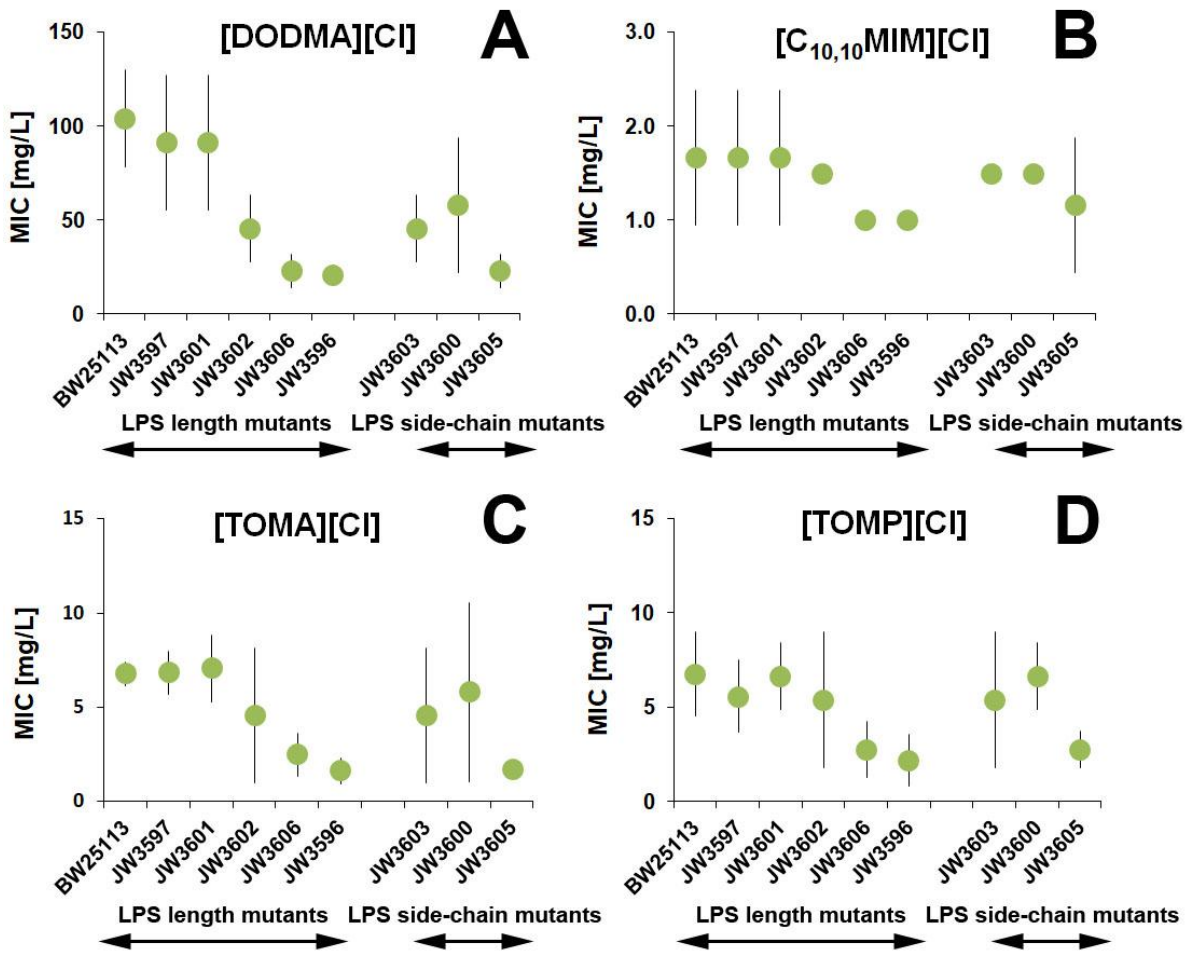

Figure 22: Comparison of the results of the MIC screening of the *E. coli* strains for ILs with two and three side-chains. [DODMA][Cl] (A) and [$C_{10,10}$MIM][Cl] (B) represent the group of ILs with two side-chains and [TOMA][Cl] (C) and [TOMP][Cl] represent the group of ILs with three side-chains. Dots showing the mean MIC value and error bars showing the confidence interval (α=0.05)

The ILs C1EG and [TOP][Br] represented the IL group with four side-chains. While the results of C1EG did show the same results as QACs, [TOP][Br] was not effective against the bacterial strains and poorly soluble in water. C1EG did also show a higher MIC as expected according to the SAR effect of the increasing effectiveness with more side-chains. Thereby the results showed that the SAR effect is only observable to a number of three side-chains. Four side-chains seem to be less or not effective against the bacterial strains representing Gram-negative bacteria. The results of the MIC screening for all ILs are shown in Figure 23.

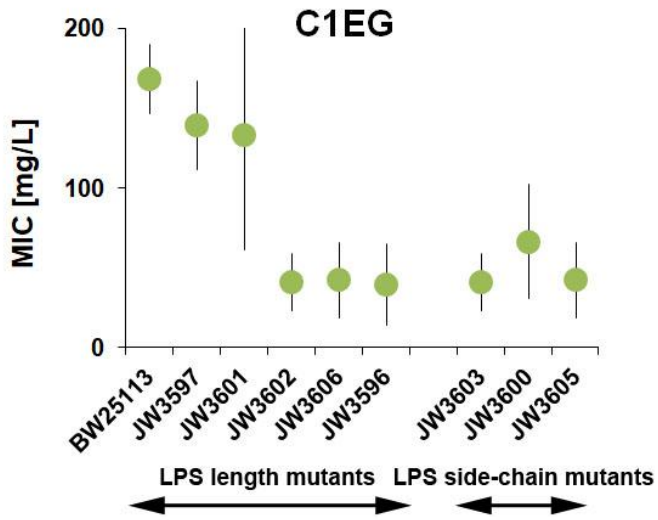

Figure 23: Results of the MIC screening of the *E. coli* strains for the IL with four side-chains C1EG. Dots showing the mean MIC value and error bars showing the confidence interval (α=0.05)

4.1.2.3.3 Ionic liquids with an active anion

The results of the three ILs [BMIM][FAP], [EMIM][FAP] and [EDMOA][FAP] showed that ILs with the active [FAP] anion were not effective against the tested bacterial strains. These ILs were also poorly soluble in water like the four side-chain IL [TOP][Br]. Overall, these results confirm that the LPS acts as a barrier to hydrophobic substances. The results in form of a table are shown in chapter 3.1.3.4.

4.2 Evolution experiment

The hypothesis at the beginning of the evolution experiment was that if bacteria are exposed long term to sub-lethal concentrations of ILs, they will acquire tolerance or resistance to these ILs and possibly also against other ILs and antibiotics. To proof this hypothesis different bacterial strains were exposed to different ILs and different sub-lethal concentrations.

To cover both blocks of gram staining, *E. coli* was chosen as representative for gram-negative and *L. monocytogenes* as representative for the group of gram-positive bacteria. Three different ILs were chosen to cover a wide range of IL classes. [$TMC_{16}A$][Cl] was

representing the class of ILs with one side-chain, [$C_{10,10}$MIM][Cl] the class of ILs with two side-chains and [TOMP][Cl] representing the class of ILs with three side-chains. The role of sub-inhibitory concentrations was earlier explained in chapter 1.5. Thus, three different sub-inhibitory concentrations of each IL were determined for each strain resulting in the concentration Sub-MIC 1 (approximately one half of the MIC), Sub-MIC 2 (approximately one quarter of the MIC) and Sub-inhib (concentration of the respective IL slightly under the MIC). For an easier labelling during the experiment, each bacterial strain was assigned with a letter. The letters for each strain are listed in chapter 4.2.1. Each strain that got through the experiment, was additionally to the letter a continuously number assigned. An overview of the surviving strains of the second approach that showed different results than the respective control strains and therefore were further tested is shown in Table 24.

4.2.1 Second approach

The first approach of the evolution experiment was aborted after ten days due to cross contaminations. For the second approach all working steps were reconsidered and some adoptions were made:

- The number of bacterial strains was reduced from twelve to five. The remaining *E. coli* strains were BW25113 (B strains), JW3596 (C strains) and 0451-2 (D strains). The remaining *L. monocytogenes* strains were 6179 (S strains) and QC1 (Q strains).
- The number of replicates for each concentration line was raised from one to five.
- All working steps from preparing the medium with the correct concentrations over to the inoculation and stock generating process were exclusively performed under the laminar flow cabinet with turned off ventilation.
- During the handling the *L. monocytogenes* strains were always inoculated first and after each species the gloves were changed. This measure should prevent the cross contamination of the slower growing *L. monocytogenes* strains with the faster growing *E. coli* strains.

All other conditions like used ILs, concentrations and length of the experiment were kept the same.

Even after reducing the number of different strains from twelve to five, increasing the number of replicates from one to five and separating the strains from each other in the inoculation process still cross-contaminations occurred, especially with the way slower growing *L. monocytogenes* lines of QC1 and 6179. In total only 26 of 46 and 17 of 46 of these approaches were pure cultures at the end of the experiment or could grow in TSB+Y and the respective IL concentrations. A similar result showed the *E. coli* strain JW3596 which possesses the shortest LPS length. This strain also grew slower, making him prone to cross-

contaminations. Only 27 of 46 approaches came through. The wild-type strain BW25113 and the efflux mutant strain 0451-2 had less problems with contaminations and bactericidal conditions, resulting in 46 of 46 and 42 of 46 pure and grown cultures at the end of the experiment. An overview of the strains that made it to the end of the second approach is listed in Table 23. The final selection of eight interesting strains for further experiments and the criteria in which they showed a deviating behaviour than the respective control strains is listed in Table 24.

4.2.2 Minimum inhibitory concentration screening and disc diffusion test

The MIC screening with benzalkonium chloride and the three ILs [TOMP][Cl], [$TMC_{16}A$][Cl] and [$C_{10,10}MIM$][Cl] of the eight selected interesting strains and their four respective control strains showed no differences between these groups. On the first look, the strain C11 looked promising with higher MIC values than all other C-strains. To confirm these results, a PCR targeting the *waaC* gene was carried out. After gel electrophoresis the C11 strain showed a specific band at 172 bp like the wild-type BW25113, which should not occur if C11 would have been the supposed JW3596 strain. In consideration of the deviant MIC results and the results of the PCR confirmation it is highly likely that C11 was a cross contamination with the wild-type BW25113. The only small deviation in the MIC screening from the control strains was observable for strain D12. This strain was incubated 40 days in TSB+Y and 2.5 mg [TOMP][Cl] and showed a twice as high MIC in [TOMP][Cl] than all other D-strains. A possible explanation for this effect is, that this strain adapted better to this IL than the others during the 40 day experiment. All other strains showed no difference for all substances compared to the respective control strain and other strains from the same type of bacteria. Overall the results showed that a 40 days exposure to these three different ILs in the same concentration did not lead to higher MIC values. Possible explanations could be that 40 days were not long enough or that evolution is a process that occurs randomly. It is also possible that the chosen concentrations of the ILs were not high enough to force the adaptation of the bacterial strains. For a next try the IL concentration should not be kept constant but rather be increased every day in small steps. The results in form of a table are shown in chapter 3.2.3.

The disc diffusion test with the antibiotics ampicillin, chloramphenicol, ciprofloxacin, gentamicin, levofloxacin and nalidixic acid showed for all strains no deviation from their respective control strain and from other strains of the same type of bacteria. These results strengthen the evidence, that the time period of this experiment was not long enough or that the IL concentration was not high enough to force the bacteria to adapt. The results in form of a table are shown in chapter 3.2.4.

Overall, the results from the evolution experiment of the selected strains from day 0 and day 40 did neither show any difference in the MIC values of the ILs they were incubated in nor in

the MIC values of different antibiotics. For the experiment a different approach as stated from (Andersson and Hughes, 2014), with constantly kept concentrations of ILs instead of daily increasing ones, was chosen. It is possible, that this change in the setting of the experiment did not put enough evolutionary pressure on the bacteria. The development of tolerances and resistances to bactericidal substances is currently a highly emerging topic and of crucial importance for food safety and thus for the health of consumers. The experiment should be repeated with daily increasing IL concentrations to put more selective pressure on the bacteria and the period of time maybe prolonged. This could lead to answers if bacteria exposed to sub-lethal concentrations of ILs do also acquire tolerances and resistances against these and other substances and if so, what adaptations did the bacteria use to achieve them. Understanding these mechanisms in detail could lead to the development of bactericidal substances that can overcome these resistance mechanisms and therefore increase their efficacy and food safety.

5 Conclusions and outlook

The initial idea behind the evolution experiment was to get a first clue if bacteria exposed to sub-lethal concentrations of ILs develop over time resistances to the respective IL they were exposed to, other ILs and antibiotics. Since the decision to keep the IL concentration constant and not increase it slightly every day did not turn out as expected, the bacterial strains were presumably not put enough evolutionary pressure to develop resistances. Overall, the execution of evolution experiments is challenging and these kind of experiments have not been performed with ILs.

Regarding LPS, this is the first study dealing in detail with the influence of LPS as effective barrier on the antimicrobial efficacy of ILs. The effects of ILs with an increasing cationic alkyl side-chain length (up to C_{16}) and the influence of the nature of the functional groups in the side-chain of the cation increasing the efficacy of ILs could be confirmed (Egorova and Ananikov, 2014). Also the function as barrier of the LPS against lipophilic substances (Yamasaki et al., 2013) was proofed in form of confirming the results of (Weyhing-Zerrer et al., 2017), showing that hydrophobic [FAP] anion based ILs are not effective against Gram-negative bacteria. The most promising and surprising IL in this work was [$C_{10,10}$MIM][Cl], an imidazolium based cation with two C_{10} side-chains and chloride as anion. This IL showed by far the lowest MIC values and overcame the LPS barrier in all variations with ease. However, the mode of action of this IL is currently unclear and should be investigated in the future. Besides [$C_{10,10}$MIM][Cl], the two ILs with three side-chains [TOMA][Cl] and [TOMP][Cl] showed as well a great efficacy on *E. coli* and future research should also involve these ILs. The results of the MIC screening of these three ILs for the wild-type strain showed a way lower MIC than the most commonly used QAC benzalkonium chloride, making them possible alternative disinfectants and revealing their potential for the future. Another interesting approach for further experiments would be the combination of the active IL cations, identified in this study, with divalent cation binding anions such as EDTA or citrate with the intention to further decreasing the MIC.

Additionally, the results of this master thesis revealed a in the literature never before mentioned breaking point, located in the outer core region of the LPS. This breaking point confirmed the importance of the core region (Delcour, 2009; Page, 2012) and showed that strains with a linear decreasing LPS length did not show a linear decreased MIC value. This one missing glucose residue in the LPS length from mutant JW3601 to JW3602 seemed to be very important for LPS integrity. An assumption is that this glucose residue is stabilizing adjacent flanking LPS molecules and after the removal repulsion effects due to a steric hindrance occurs, weakening LPS integrity and making the outer membrane targetable and

accessible. Furthermore, the results of the study have confirmed that LPS side-chains are from essential importance in regard to the resistance of *E. coli* (and presumably other Gram-negative bacteria) against microbials (Yethon et al., 1998; Wang et al., 2015). It didn't matter if the LPS side-chain mutants had a missing sugar residue or a missing phosphate residue, all LPS side-chain mutants were similar susceptible as the LPS mutant strain with the shortest LPS length despite possessing a full length LPS. The absence or presence of the O-antigen in the wild-type strain BW25113 could not be confirmed and therefore the contribution of the O-antigen remained unknown. Extraction of the LPS with further SDS-PAGE analysis and silver staining should deliver an answer to this open question.

The results obtained in this master thesis contributed to the publication "Influence of bacterial lipopolysaccharide modifications on the efficacy of antimicrobial ionic liquids" from (Gundolf et al.) and successfully published in (2018) in the Journal of Molecular Liquids, Volume 271 and was also presented at conferences and used for posters.

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7 List of figures

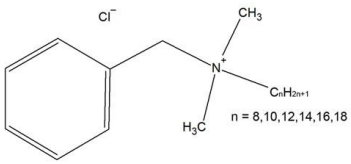
8 List of tables

9 Attachments

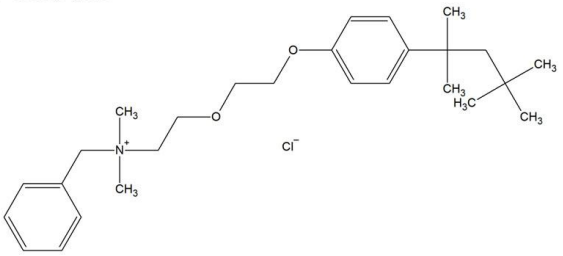
9.1 Chemical structures

9.1.1 Chemical structures of all used QACs

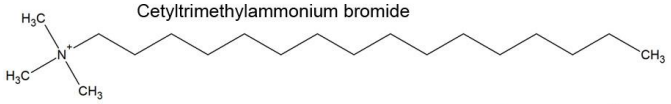
Benzalkonium chloride



Benzethonium chloride



Cetyltrimethylammonium bromide



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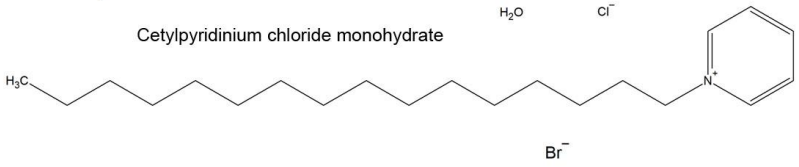
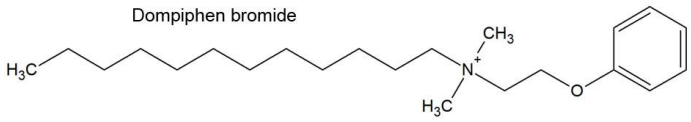
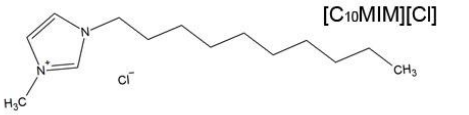


Dompiphen bromide

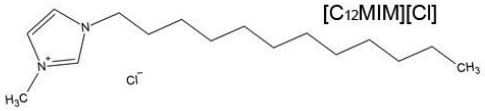


9.1.2 Chemical structures of all used ILs with one side-chain

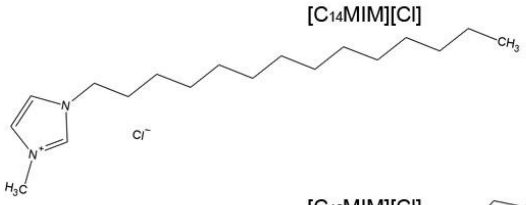
[C10MIM][Cl]



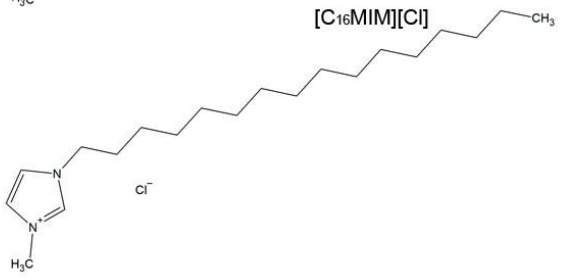
[C12MIM][Cl]



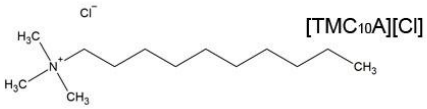
[C14MIM][Cl]



[C16MIM][Cl]



[TMC10A][Cl]



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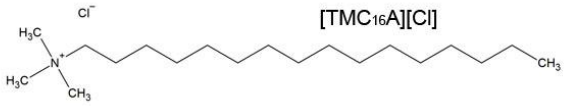
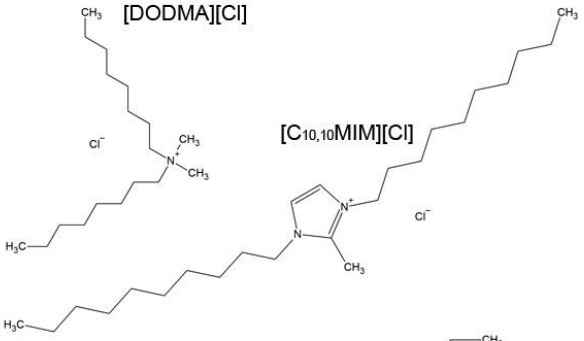


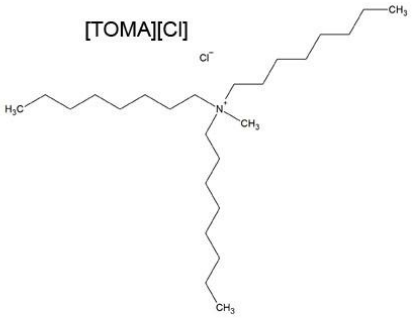
9.1.3 Chemical structures of all used ILs with two or more side-chains

[DODMA][Cl]

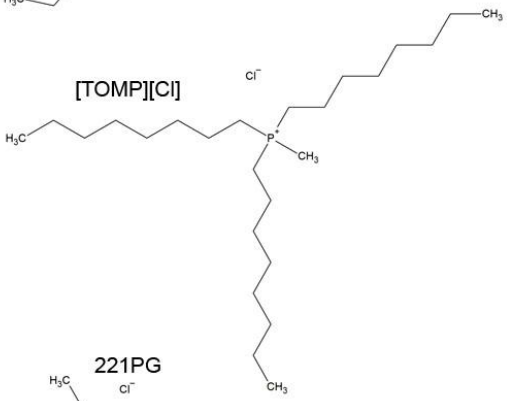


[$C_{10,10}$MIM][Cl]

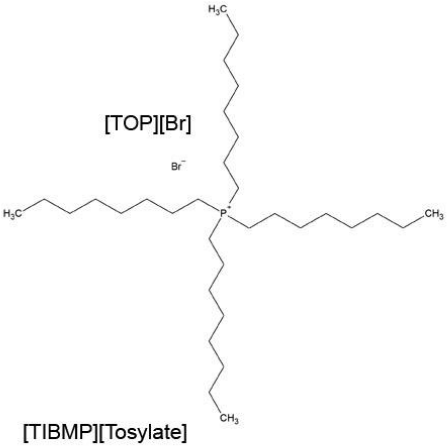
[TOMA][Cl]



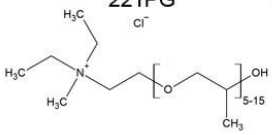
[TOMP][Cl]



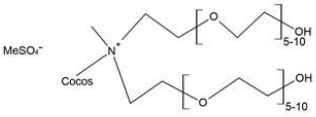
[TOP][Br]



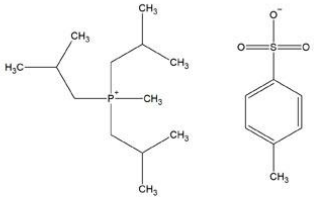
221PG



C1EG

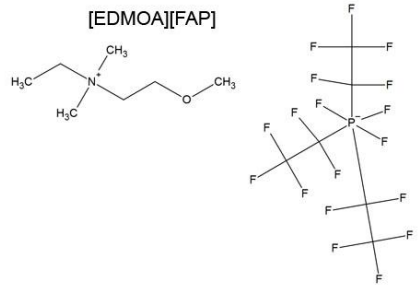


[TIBMP][Tosylate]

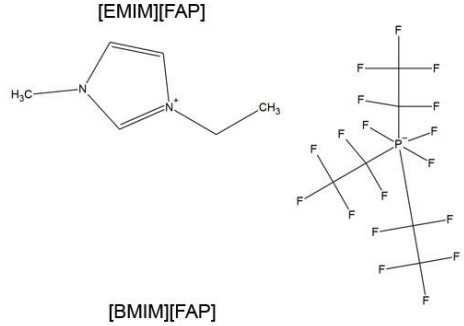


9.1.4 Chemical structures of all used ILs with an active anion

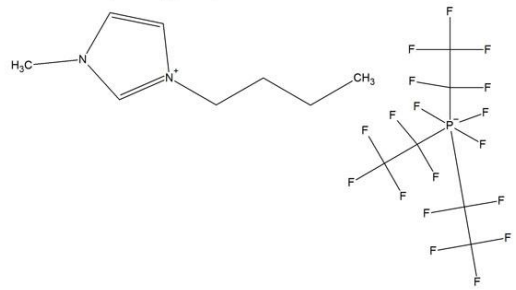
[EDMOA][FAP]



[EMIM][FAP]



[BMIM][FAP]



9.2 Tables of all results

9.3 t-factor

The t-value table was retrieved from the University of Cologne under http://eswf.uni-koeln.de/glossar/tvert.htm on 29.06.2018.

Table 29: t-value table for the calculation of the confidence interval

	Level of significance				
df	0,900	0,950	0,975	0,990	0,995
1	3,078	6,314	12,706	31,821	63,656
2	1,886	2,920	4,303	6,965	9,925
3	1,638	2,353	3,182	4,541	5,841
4	1,533	2,132	2,776	3,747	4,604
5	1,476	2,015	2,571	3,365	4,032
6	1,440	1,943	2,447	3,143	3,707
7	1,415	1,895	2,365	2,998	3,499
8	1,397	1,860	2,306	2,896	3,355
9	1,383	1,833	2,262	2,821	3,250
10	1,372	1,812	2,228	2,764	3,169
11	1,363	1,796	2,201	2,718	3,106
12	1,356	1,782	2,179	2,681	3,055
13	1,350	1,771	2,160	2,650	3,012
14	1,345	1,761	2,145	2,624	2,977
15	1,341	1,753	2,131	2,602	2,947
16	1,337	1,746	2,120	2,583	2,921
17	1,333	1,740	2,110	2,567	2,898
18	1,330	1,734	2,101	2,552	2,878
19	1,328	1,729	2,093	2,539	2,861
20	1,325	1,725	2,086	2,528	2,845

9.4 Published Publication

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Influence of bacterial lipopolysaccharide modifications on the efficacy of antimicrobial ionic liquids

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ABSTRACT

The increasing industrial use of ionic liquids (ILs) now demands accurate (eco)toxicological evaluations based on their structural composition. Although bacteria are major test organisms for this research, fundamental bacterial resistance mechanisms against antimicrobial ILs have been neglected. This deficiency could hamper interpretation of related experimental findings. Among the most important resistance mechanisms encountered in bacteria are cell membrane modifications, which can hinder the entry of antimicrobials such antibiotics or quaternary ammonium compounds (QACs). This study investigates the role of lipopolysaccharides (LPS), a major component of the outer leaflet of Gram-negative bacteria, and modifications of its length on the IL activities using three different single-gene deletion mutants of *E. coli* K-12. In total, 19 different ILs, covering various structural motifs as well as active pharmaceutical ILs (API-ILs) based on the intracellular operating antibiotic nalidixic acid were investigated. Results reveal that, while known structure-activity relationships (SARs), such as the side-chain effect, were relevant for all tested strains, decreasing the LPS length leads to a higher susceptibilities to antimicrobial ILs as well as API-ILs. These findings highlight the importance of considering membrane changes, such as LPS modification, as important bacterial defense mechanisms.

1. Introduction

Over the past few decades, ionic liquids (ILs), defined as organic salts with melting points below 100 °C [1], have attracted substantial attention from both academia and industry [2]. This has resulted from their unique physiochemical properties, including high thermal and chemical stabilities, high ionic conductivity, low flammability, low volatility or tuneable solubility [3,4]. Further, these properties can be modified by varying possible cation-anion combinations, making ILs particular interesting for numerous applications, such as chemical synthesis and catalysis [5,6], biomass conversion [7], electrochemistry [8], biotechnology [9] or fuel production and processing [10]. However, increasing industrial use necessitates thorough investigations of their hazard potential.

It has already been demonstrated that ILs can have significant (eco) toxicological impacts on algae, fungi, higher plants, mammalian cells as well as bacteria [11,12]. Of the available test systems, bacteria are most commonly employed for toxicity studies due to their short generation times and rapid growth [13,14]. Toxicity mechanisms have since been identified for bacteria, and these depend upon IL structural composition, such as the cation alkyl side-chain effect [15,16], the number of functional groups on the cationic side chains, intrinsic properties of the cation [17–19], chaotropicity of the anion [20], the presence of fluorinated anions [21] and others that have been extensively reviewed elsewhere [22,23]. While pronounced toxicological activity is disadvantageous in ILs used as solvents or catalysts, IL toxicity is expected for pharmaceutical and medical applications [24] requiring antibacterial/-fungal [25,26] or cytotoxic activities [27].

So called API-ILs facilitate the incorporation of active pharmaceutical ingredients (API) into an IL form [28]. Dual active API-ILs, for example, combine biologically active cations and anions creating an IL with synergistic, beneficial characteristics. An example of this is didecyldimethylammonium ibuprofen, where an antibacterial cation is combined with an anti-inflammatory anion [29]. A study by Ferraz et al. [30] showed that the API-IL [C_{16}Pyr][Amp] was more effective at inhibiting bacterial growth than the native [Na][Amp] and could even overcome bacterial resistance. In contrast, Mester et al. [31] demonstrated that API-ILs with the nalidixate anion had no synergistic effects compared to the nalidixate as antibiotic alone for both susceptible as well as resistant *Salmonella* strains. Bacterial resistance of this type is not only recognized for API-ILs. It could also be demonstrated that within a group of different Gram-positive and Gram-negative species, the toxicities of antimicrobial ILs were completely divergent, suggesting that intrinsic defense mechanisms might also play an essential role [32].

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