Spring 2024

Investigating the Development of Alcohol Tolerance in the ESKAPE Pathogens

Hannah Durkee
hd909348@wcupa.edu

Investigating the Development of Alcohol Tolerance in the ESKAPE Pathogens

A Thesis Presented to the Faculty of the

Department of Biology

West Chester University

West Chester, Pennsylvania

In Partial Fulfillment of the Requirements for

the Degree of Master of Science in Biology

By

Hannah Durkee

May 2024

Dedication

I dedicate this thesis to my parents, Caroline and Steve Durkee. Your unwavering support has been an immense contributor to my success and how far I have come in my academic journey. I would like to dedicate this to you to express my gratitude for always believing in me and encouraging me to believe in myself.

Acknowledgements

I am extremely grateful for all of the faculty, members of the Buskirk lab, and peers who have helped me along this journey. The completion of this thesis would not have been possible without the guidance and support of my advisor, Dr. Sean Buskirk. I sincerely appreciate the patience, expertise, and advice that he has provided me with throughout this study. I am also thankful for the encouragement and guidance from the members of my thesis committee, Dr. Jessica Sullivan-Brown and Dr. Manuela Ramalho Sanchez. Lastly, I would like to extend my gratitude to the College of Sciences and Mathematics for funding this research through the CSM Graduate Research Award and Research in Mathematics and the Sciences (RIMS) Award.

Abstract

Antiseptics and disinfectants are used globally to kill or inhibit the growth of bacteria to prevent infection. Considering the well-documented development of antibiotic resistance, the potential for evolved tolerance to alcohol-based antimicrobial agents raises concern. The ESKAPE bacterial pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*) are a major cause of healthcare-associated mortality and present a threat to public health due to the progression of multi-drug resistance. We conducted an experiment to determine if the ESKAPE pathogens develop an increased tolerance to ethanol upon repeated exposure by selecting for survivors after a two- to four-log reduction in cells. After a 20-day cycle of ethanol exposure, we used whole genome sequencing to identify arisen mutations and analyze the genes and mechanisms that potentially contribute to ethanol tolerance. We discovered that the Gram-positive species, *E. faecium* and *S. aureus,* survived at higher ethanol concentrations and developed increased ethanol tolerance after repeated daily exposure. Ethanol tolerance was not observed in the Gram-negative species *K. pneumoniae* and *P. aeruginosa*. Genetic sequencing indicated that mutations in genes involved in peptidoglycan synthesis, two-component regulatory systems, and response to environmental stress are likely involved in adaptation to ethanol exposure.

Table of Contents

Chapter 1: Background

The ESKAPE Pathogens

The ESKAPE pathogens are an acronym for a group of six pathogenic species of bacteria including: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.* Infections caused by the ESKAPE pathogens are primarily nosocomial, meaning they are healthcare-acquired infections (Jadimurthy et al., 2022). They are responsible for over 40% of infections in the intensive care units of hospitals and can be fatal for immunocompromised or seriously ill patients. Some methods of transmission include contact, contaminated equipment, and improper sterilization (Jadimurthy et al., 2022; Motiwala et al., 2022). In 2017, in response growing antibiotic resistance, the ESKAPE pathogens were compiled and designated as the highest "priority status" according to the World Health Organization (WHO), emphasizing an urgent need for alternative treatments (De Oliveira et al., 2022). The WHO defines three levels of priority when referring to the urgency of antibiotic development: critical, high, and medium priority (Mancuso et al., 2021; Mulani et al., 2019). *E. faecium* and *S. aureus*, the two Gram-positive bacteria, are listed as high priority. The remaining four Gram-negative species are listed as critical priority (Mancuso et al., 2021).

A major problem surrounding these pathogens is their global threat of antimicrobial resistance. Antimicrobial resistance (AMR) occurs when drugs that were previously effective in treating infections are no longer effective in killing the microorganism (Mancuso et al., 2021). A major contributor to AMR is the overuse and misuse of antibiotics, resulting in the acceleration of resistance (Mancuso et al., 2021). Mobile genetic elements and the inevitability of genetic mutation has led to the development of antibiotic resistance against fluoroquinolones,

macrolides, ß-lactams, lipopeptides, and other antibiotics used as the last line of defense (De Oliveira et al., 2022). Through horizontal gene transfer, these pathogens can pass on AMR genes, making it more difficult for antibiotics to effectively kill the targeted bacteria (Mulani et al., 2019). This evolving concern could result in the inability for these infections to be cured, putting public health at risk. The ESKAPE pathogens are particularly a challenge because of their high capability of developing drug resistance mechanisms (Masoud et al., 2022). Resistance to antibiotics raises concern because of overlapping adaptive mechanisms that can increase virulence potential and alcohol tolerance (Yeung et al., 2022).

Because bacteria acquire resistance through several complex processes, antibiotics are grouped based off mechanisms of action or a specific target (Mancuso et al., 2021). The main mechanisms of resistance used by the ESKAPE pathogens include modification of bacterial target site, drug inactivation, the overexpression of efflux pumps, and biofilm formation (Denissen et al., 2021; Mulani et al., 2019). Some bacteria have acquired the ability to produce enzymes that modify or inactivate antibiotics that render the drug ineffective. For example, carbapenemases are enzymes produced by cells, leading to infectivity and resistance in beta-lactam antibiotics (Paczosa & Mecsas, 2016). Bacteria can make modifications to the target site of the antibiotic by making structural changes to the cell wall or other drug target sites to prevent binding and avoid detection. Alterations in the presence of efflux pumps and protein channels also improve antibiotic resistance by decreasing drug entry or accumulation in the cell. Efflux pumps can excrete the drug out of the cell, lowering its concentration therefore decreasing its efficacy. A major concern is the formation of biofilms which enclose bacterial communities in an extracellular matrix, acting as a biochemical shield. This protective barrier drastically contributes to reducing antibiotic susceptibility (De Oliveira et al., 2020; Santajit & Indrawattana, 2016).

Antibiotic resistance and alcohol tolerance are related in the context of bacterial adaptation, raising concern based on the prevalence of antibiotic resistance demonstrated among the ESKAPE pathogens. When bacteria develop resistance to antibiotics, the adaptive mechanisms acquired may confer tolerance to other environmental stressors, like alcohol. Dependent on the mechanisms of adaptation, bacteria that develop resistance to antibiotics may inadvertently contribute to ethanol tolerance (Yeung et al., 2022). In the following sections, we will delve deeper into the characteristics, resistance mechanisms, and clinical significance of each ESKAPE pathogen.

Enterococcus faecium

Enterococcus faecium is a Gram-positive coccus with some strains being a leading cause of nosocomial infections that can cause an array of harmful illnesses in humans. Non-pathogenic strains can be beneficial and may reside in food, plants, and gastrointestinal tracts of animals. The non-pathogenic strains can be used in fermentation of food and alcohol due to their ability to produce lactic acid (Kim & Marco, 2014). The pathogenic strains of *E. faecium* can cause severe infections in immunocompromised patients including urinary tract infections, bacteremia, endocarditis, and meningitis. The severity and multitude of infections that pathogenic strains of *E. faecium* cause become problematic because of growing resistance to antibiotics. More than half of the pathogenic strains that have been isolated exhibit resistance to ampicillin, penicillin, and vancomycin, three commonly used antibiotics. A majority of hospital acquired infections are due to vancomycin and ampicillin-resistant strains of *E. faecium* (Higuita & Huycke, 2014). The extent of antibiotic resistance demonstrated by *E. faecium* causes concern by potentially contributing to adaptive mechanisms involved in alcohol tolerance. A study by Pidot et al. (2018)

showed evidence of alcohol tolerance in *E. faecium*, where clinical isolates after 2010 were more tolerant to alcohol in comparison to isolates from before 2010. This study provides confirmation that increased tolerance to alcohol in bacteria can occur over a period of time. The increasing resistance of *E. faecium* is becoming a global concern and new methods of combatting AMR are essential to ensure that infections will remain successfully treated (Priyamvada et al., 2022).

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive coccus that can naturally occur on the human body and in the nasal cavity and perineum (Priyamvada et al., 2022). When present in these areas, it does not normally cause infection, however infection may occur if it enters the blood stream or spreads to internal tissues (Taylor & Unakal, 2022). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a commonly known infection that occurs most frequently in health-care settings. The treatment of *S. aureus* often depends on the drug resistance of the strain that is causing infection. Penicillin or vancomycin may be used, but sometimes the addition of alternative therapy is necessary to get rid of an infection. *S. aureus* can cause mild to life threatening infections, not limited to endocarditis, bacteremia, urinary tract infections, meningitis, pneumonia, and toxic shock syndrome (Taylor & Unakal, 2022). Infections are highly contagious and have shown to develop resistance rapidly to both old and new antibiotics. In only three years, strains of *S. aureus* developed resistance to penicillin and this pattern is threatening for those at risk for infection (Mancuso et al., 2021). Understanding the mechanisms of antibiotic resistance and alcohol tolerance in *S. aureus* and other ESKAPE pathogens is imperative for developing effective therapeutic strategies to ensure better patient outcomes.

Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative, capsule forming, bacillus bacterium that can be found in the outside environment, on medical devices, and in human mucosal surfaces (Paczosa & Mecsas, 2016). *K. pneumoniae* infections have a lack of treatment options which results in a high morbidity and mortality rate (Priyamvada et al., 2022). The most commonly affected group of individuals comprises immunocompromised individuals, although there has been a recent increase in susceptibility among healthy individuals (Paczosa & Mecsas, 2016). Infections that cause pneumonia can either be hospital acquired or, more rarely, community acquired. The mortality rate is alarmingly high, ranging from 50% to 100% among individuals suffering from septicemia, diabetes, and alcoholism (Ashurst & Dawson, 2023). This extreme mortality rate threatens the lives of immunocompromised individuals and remains alarmingly high even with sufficient treatment (Ashurst & Dawson, 2023). Along with pneumonia, *K. pneumoniae* can also cause septicemia, urinary tract infections, bacteremia, and meningitis. The most threatening mechanism of resistance is the expression of carbapenemases, an enzyme that can inactivate carbapenem antibiotics, resulting in resistance to almost all antibiotics on the market (Paczosa & Mecsas, 2016). Carbapenem antibiotics represent one of the last lines of defense against Gram-negative pathogens, yet some strains of *K. pneumoniae* have already shown carbapenem resistance (Mancuso et al., 2021). Due to its intrinsic resistance to ampicillin, expression of carbapenemases, and lack of effective treatments, *K. pneumoniae* has become a major global concern, especially because of its high mortality rate (Priyamvada et al., 2022).

Acinetobacter baumannii

Acinetobacter baumannii is a Gram-negative bacillus opportunistic pathogen, primarily associated with nosocomial infections. Infections can occur in the general public although this is less common. In the environment, *A. baumannii* can be found in soils and bodies of water. It has historically been prevalent in deserts where troops are deployed and is a major infection for injured soldiers, especially in Iraq (Howard et al., 2012). When causing an infection, it specifically targets moist tissues in the body such as mucous membranes and wounds (Howard et al., 2012). Common hospital acquired infections include urinary tract infections, meningitis, pneumonia, and bacteremia (Priyamvada et al., 2022). More recently, infections in the central nervous system, skin, and bone have been an arising concern (Peleg et al., 2008). The bacterium is capable of producing enzymes that degrade beta-lactam antibiotics therfore combination treatments with carbapenems have been most effective in treating infections (Mancuso et al., 2021). A majority of *A. baumannii* strains express multi-drug resistance which is a concern due to high morbidity and mortality rates of the infection (Saeed et al., 2022). In the last 15 years, there has been documentation of strains resistant to all known antibiotics due to its remarkable capability of acquiring genetic elements conferring resistance (Peleg et al., 2008). Urgent and concerted efforts are required from the healthcare community to develop effective treatment strategies posed by this pathogen. These threats reinforce the need for investigation of the potential threat of alcohol tolerance to ensure that methods of disinfection will remain effective.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacillus pathogen that can cause infections in healthy and immunocompromised individuals. In the environment, *P. aeruginosa* can be found in freshwater habitats although infections can also be acquired from swimming pools and

hot tubs (Wilson & Pandey, 2022). In healthcare settings, it is common for immunocompromised individuals to become infected because of invasive devices and in those with illnesses (Wilson & Pandey, 2022). This opportunistic pathogen can cause infections in the eyes, blood, urethra, respiratory tract, central nervous system, and skin (Feng et al., 2022 & Iglewsky, 1996). *P. aeruginosa* is one of the many multi-drug resistant (MDR) pathogens that causes 7.1% of health care acquired infections in the United States and is a global threat to human health (Qin et al., 2022). It is the third most common Gram-negative pathogen that causes infections of the bloodstream in clinical settings (Mancuso et al., 2021). *P. aeruginosa* poses a serious threat to patients with cancer, cystic fibrosis, and burns who are faced with a 50% mortality rate (Iglewsky, 1996). Acetic acid has been an effective agent in disinfectants and antiseptics in treating some infections that do not respond to treatment although drug resistance remains to be a concerning issue (Feng et al., 2022).

Enterobacter cloacae

Enterobacter cloacae is a Gram-negative, anaerobic bacillus species found naturally in plants, soil, and humans. While pathogenic strains of *E. cloacae* can cause disease in plants, several other strains can promote growth. In humans, it is an opportunistic pathogen that most commonly infects hospitalized individuals who are immunocompromised (Lui et al., 2013). It can cause a broad range of infections, including urinary tract infections, meningitis, osteomyelitis, sepsis, pneumonia, and lower respiratory infections (Ren et al., 2010). Within the *Enterobacter* genus exists an *Enterobacter cloacae* complex (ECC) consisting of six pathogenic strains that are clinically significant (Paauw et al., 2008). Over time, *E. cloacae* is becoming more genetically diverse, resulting in an emergence of MDR strains that carry several antibiotic

resistance genes. It is intrinsically resistant to penicillin and first and second generation cephalosporins, a class of ß-lactam antibiotics (Liu et al., 2021). In recent studies, *E. cloacae* strains have demonstrated resistance to colistin and carbapenems, two last-resort antibiotics used to treat Gram-negative infections. Efflux pumps play a role in antibiotic resistance as well as genes encoding for ß-lactamase and carbapenemases (Chen et al., 2021). Due to the increasing spread of antibiotic resistance genes, *E. cloacae* infections are becoming a global concern in maintaining effective treatment strategies.

Gram-positive and Gram-negative Bacterial Cell Envelope

The bacterial cell envelope, the defining boundary between the cell and its environment, is an essential component to bacteria cells providing structure and acting as a protective barrier from its surroundings (Rohde, 2019). Crucial life processes take place at the cell envelope including nutrient transport, respiration, toxin secretion, and signaling. Being single celled organisms, bacteria can be exposed to hostile environments and therefore have evolved a complex barrier for protection. While shielding the cell from harmful surroundings, the cell envelope allows selective passage of nutrients from the exterior and waste products from the interior. This selective barrier facilitating the movement of substances in and out of the cell helps maintain osmotic pressure and cell integrity (Silhavy et al., 2015). Due to its crucial role in the survival and function of bacterial cells, the cell envelope is the most common target for antibiotics.

In bacteria, cell membranes are comprised of an amphipathic phospholipid bilayer, made up of a polar head and hydrophobic tails. The cell membrane achieves a selectively permeable barrier by use of proteins. Peptidoglycan, also called murein, is an essential structure that

provides stability and acts as an exoskeleton. It is composed of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) disaccharides that are cross linked together (Rohde, 2019). Several antibiotics target the peptidoglycan layer in bacteria cells, hindering the effectivity in Gram-negative bacteria due to the presence of an outer membrane barrier, in which Gram-positive bacteria lack (Heesterbeek et al., 2019). A major classifying factor in bacteria is the identification of a Gram-positive or Gram-negative cell wall via Gram stain. Gram-positive bacteria lack an outer membrane but have a thick layer of peptidoglycan, whereas Gram-negative bacteria possess an additional outer membrane with a thinner peptidoglycan layer (Figure 9).

The Gram-negative cell envelope is made up of both an inner membrane and an outer membrane. The defined space in between these two membranes is called the periplasm where the peptidoglycan cell wall resides (Eberlein et al., 2018). Because it determines the cells shape, exposure to enzymes or antibiotics targeting this structure ultimately leads to cell lysis (Silhavy et al., 2010). The outer cell membrane is a unique structure to Gram-negative bacteria, comprised of lipopolysaccharides (LPS), proteins, and porins. LPS are glycolipids that provide additional rigidity and prevent polar and non-polar compounds from entering the cell (Saha et al., 2021). The presence of LPS in Gram-negative bacteria is a major indicator of infection, being responsible for endotoxic shock which leads to septicemia, infection of the bloodstream (Silhavy et al., 2010). Additional outer membrane proteins allow the cell to attain selective permeability and provide further stability. The outer membrane itself is impermeable however the presence of proteins and porins allow molecules to undergo passive diffusion (Eberlein et al., 2018). The inner membrane on the other hand, is comprised of phospholipids and integral proteins that take part in energy production, lipid biosynthesis, and protein secretion (Silhavy et al., 2010). The cell wall in Gram-negative bacteria, consisting of the outer membrane and peptidoglycan layer, is

rather thin (<10 nm) due to its thin layer of peptidoglycan in comparison to Gram-positive bacteria (Mai-Prochnow et al., 2016).

The most notable differences in Gram-positive bacteria are the absence of an outer cell membrane and a thicker layer of peptidoglycan surrounding the cytoplasmic membrane, between 30 and 100 nm thick (Rhode, 2019). Because they lack the outer membrane, their thick layer of peptidoglycan assists in stability and withstanding turgor pressure. Embedded into the peptidoglycan cell wall are teichoic acids and lipoteichoic acids which make up about 60% of the Gram-positive cell wall. Many extracellular proteins reside in or around the peptidoglycan wall as opposed to the outer membrane in Gram-negative bacteria. Partly due to their lack of an outer membrane, Gram-positive bacteria have demonstrated greater susceptibility to antibiotics than that of Gram-negative (Silhavy et al., 2010). The differences in the cell envelope give Gram-negative and Gram-positive bacteria different properties, especially in how they respond to external stressors such as alcohol and antibiotics (Mai-Prochnow et al., 2016).

Alcohol Tolerance in Bacteria

Alcohol-based antiseptics such as ethanol and isopropyl alcohol are commonly used in hand sanitizers in both clinical and community settings. Since the COVID-19 pandemic, the use of alcohol-based hand sanitizers has increased at a rapid rate (Yeung et al., 2022). For over 40 years, the bacterial stress response to alcohol has been studied, primarily focusing on its effects on the bacterial cell membrane (Horinouchi et al., 2018). Stress responses occur when an organism is exposed to environmental stressors, such as alcohol, through changes of gene expression and physiology (Sun & Zhou, 2017). Exposure to alcohol causes alterations in membrane fluidity that induce conformational changes in membrane proteins and alter the

expression of essential proteins. The damage inflicted on the cell membrane can lead to ion leakage and energy loss of the cell (Horinouchi et al., 2018). Over time and at high enough concentrations, these disruptions in the cell membrane can ultimately lead to the death or inhibition of bacterial growth. While many disinfectants are reliable, the effectiveness of alcohol as a disinfectant varies depending on the species of bacteria and the concentration of alcohol being used (Pidot et al., 2018). The issue that arises is the potential of the bacteria causing healthcare-associated infections gaining tolerance to alcohol (Yeung et al., 2022).

Alcohol tolerance in bacteria is the ability to survive in the presence of alcohol that would otherwise be lethal. This adaptive response has been identified in a variety of pathogenic bacteria including *Bacillus cereus, Listeria monocytogenes,* and *Salmonella spp* (He et al., 2022). Bacteria with higher alcohol tolerances have adaptive mechanisms that allow them to survive and maintain cellular integrity. For example, gene mutations involving carbohydrate metabolism and biofilm formation have been found to protect cells from being killed when exposed to alcohol (Yeung et al., 2022). Current research on alcohol tolerance is currently focused on improving tolerance for industrial purposes therefore information on alcohol tolerance in the ESKAPE pathogens is limited.

In one study comparing clinical *E. faecium* isolates between 1998 and 2015, researchers found that the most recent isolates presented a 10-fold increase in alcohol tolerance compared to isolates prior to 2010. In this study, a mouse gut colonization model was used to assess the differences in alcohol sensitivity of 139 isolates of *E. faecium*. Broth cultures from each isolate were spread across the mouse cage floor followed by exposure to 70% isopropyl alcohol. The mouse gut contents were analyzed to determine the presence of *E. faecium* colonization in the gut. Increased gut colonization showed that isolates from later years were more tolerant to

ethanol. Genes involved in carbohydrate metabolism were mutated in the alcohol tolerant *E. faecium* isolates (Pidot et al., 2018). By lacking the experimental evolution aspect of the study, numerous factors could have contributed to increased alcohol tolerance. The exact causes of alcohol tolerance cannot be determined without removing additional environmental factors however these findings provide insight into the rate of real-world alcohol tolerance in clinical settings. The evidence of adaptation to alcohol exposure presents a concern for the widespread use of alcohol-based antimicrobials, possibly leading to alcohol-tolerant bacteria in the future.

The most prevalent research on alcohol tolerance focuses primarily on industrial ethanol fermentation because of the benefits to the public. Ethanol tolerance is essential for lactic acid bacteria when being used commercially to produce alcohol products (Liu et al., 2019). Many of these studies focus on the production of ethanol by *Saccharomyces cerevisiae* and its stress response as a result. Because of its production of beer and wine, the effects of alcohol exposure to the cellular membrane have been well studied, but rather for the purpose of developing mechanisms to increase ethanol tolerance (Horinouchi et al., 2018).

The Dynamic Process of Bacterial Evolution

Experimental evolution is a dynamic process involving experimental populations where changes in genotype and phenotype can be observed in a controlled environment (Barrick & Lenski, 2013). In many evolutionary experiments, genetic changes are monitored due to a selective pressure imposed by different environmental conditions such as temperature, nutrient availability, or competition (Kawecki et al., 2012). Experimental evolution can be applied to several areas of biology. In 1988, Richard Lenski began investigating microbial evolution by initiating the Long-Term Evolution Experiment (LTEE). The experiment began with 12

populations of *E. coli* from the same ancestral strain, which have been evolving for over 30 years (Maddamsetti, 2021). The goal was to assess the divergence and adaptations among these independent populations (Lenski et al., 1991). With the introduction of genetics in evolution experiments, his work provided insight into genes and pathways that arise due to adaptation (Jagdish & Nguyen Ba, 2022).

With recent advances in DNA sequencing, the scope of experimental evolution has expanded by introducing the genetic component that allows researchers to identify mutations that arise either naturally or due to a selective pressure. For example, mutations can be identified that confer drug resistance which can aid in the development of drugs that target the genes conferring resistance (Kawecki, et al., 2012). With DNA sequencing, bacteria are suitable for the study of evolution due to their rapid generation time since mutation rates occur frequently (Steenackers et al., 2016). Bacterial survival is reliant upon their ability to adapt to stressful conditions, and when stronger selective pressures are applied, adaptive mutations can arise even in a few days (Kawecki et al., 2012; Ramijan et al., 2018). Another advantage in studying evolution in bacteria is their viability after being frozen to create a stock that can be preserved for later usage. This allows for the comparison of evolved and ancestral populations, as well as testing whether an evolutionary outcome can be replicated from different timepoints in an experiment (Barrick & Lenski, 2013).

Understanding Alcohol Tolerance through Previous Research

Previous work by a West Chester University graduate student, Vaughn Perveiler, investigated the development of alcohol tolerance in evolved populations of *S. aureus* in the thesis titled, “Investigating the development of alcohol tolerance by the opportunistic pathogen

Staphylococcus aureus" (Perveiler, 2022). In the study, experimental evolution was used to determine if *S. aureus* was capable of developing ethanol tolerance upon repeated exposure. Specifically, four strains of *S. aureus* each founded three independent populations (for a total of twelve populations). Each population was exposed to ethanol for 15 seconds each day for 21 consecutive days. After repeated ethanol exposure, it was found that all four strains developed a statistically significant increase in ethanol tolerance by the end of the experiment. To identify the mechanisms of ethanol tolerance that developed, genomic DNA was extracted from the evolved populations and sequenced. In comparison to the ancestors, multiple mutations were detected in each evolved population. There were several genes mutated in multiple populations, which was compelling evidence that the mutated genes were responsible for ethanol tolerance. In addition, many mutations were detected within genes involved in cell wall homeostasis (detected in all four strains), and based on the literature, these mutations are likely causing a thickening of the Gram-positive cell wall as a mechanism to protect itself from damage (Perveiler, 2022).

Research Objectives

Given the results of the previous study, we plan to use the same approach to investigate the development of ethanol tolerance in a variety of bacteria. Specifically, we chose a group of six pathogenic bacteria collectively referred to as the ESKAPE pathogens. This group of pathogens was chosen because of their frequent role in healthcare associated infections and the alarming emergence of multi-drug resistance. They are a highly virulent group of bacteria and are associated with the highest mortality rate of nosocomial infections (Mulani et al., 2019). Because of their virulent nature and ability to rapidly develop new mechanisms of resistance,

investigating the possibility of ethanol tolerance would allow us to take proactive measures and expand our knowledge on the widespread use of alcohol-based antiseptics.

The ESKAPE pathogens include both Gram-positive (*Staphylococcus aureus*, *Enterococcus faecium*) and Gram-negative pathogens (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*). Since Gram-positive and Gram-negative bacteria differ in their cell wall structure, and cell wall homeostasis was the mechanism of ethanol tolerance by *S. aureus*, we hypothesized that Gram-positive and Gram-negative bacteria differ in their ability to develop ethanol tolerance and potential mechanisms of ethanol tolerance. Since *E. faecium* is Gram-positive (like *S. aureus*), we expect it to develop ethanol tolerance through mechanisms similar to those previously described for *S. aureus*. On the other hand, existing knowledge on ethanol tolerance in Gram-negative bacteria is limited. Since the Gram-negative cell wall's thickness may be constrained by the presence of an outer membrane, Gram-negative bacteria may not be able to develop ethanol tolerance to the same extent as Gram-positive bacteria. Alternatively, Gram-negative bacteria may develop ethanol tolerance through different mechanisms than Gram-positive bacteria. Here we expand on the earlier work in *S. aureus* (Perveiler, 2022) by following the same approach to investigate mechanisms of tolerance among the ESKAPE pathogens.

Our first goal was to determine the ethanol concentrations killing between 99% and 99.99% (2- to 4-log reduction) of cells for each of the ESKAPE pathogens. Doing so will select for cells that may already have genes conferring alcohol tolerance. Once the ethanol concentrations were determined, an evolutionary approach was applied, and each species were exposed to its corresponding ethanol concentrations for 20 consecutive days. After the populations have evolved, we performed an assay to compare the growth of the ancestral and

evolved populations to determine if ethanol tolerance has occurred in each species. We used whole genome sequencing to identify mutations associated with ethanol tolerance that arose. By identifying genes involved in ethanol tolerance, we have gained insight into cellular processes and pathways that may be targeted for therapeutic interventions if ethanol tolerance becomes a greater issue in the future.

Chapter 2: Methods

Bacterial Strains

All bacterial isolates used in this study were procured from American Type Culture Collection (ATCC). Upon receipt from ATCC, each species was streaked on Tryptic Soy Agar (TSA) and a single colony was transferred into Tryptic Soy Broth (TSB) in triplicate to create broth cultures. Throughout the experiments, bacterial cultures were grown in 5 ml TSB and incubated at 37ºC with continuous shaking (225 rpm) for approximately 24 hours. To ensure long term preservation, frozen stocks were prepared by transferring 600 µL of overnight cultures into cryovials containing 400µl of 50% glycerol and placed at -80ºC for future use.

Ethanol Sensitivity Assay of Ancestral Strains

To assess the sensitivity of all six ESKAPE strains to ethanol, we manually quantified the number of bacteria present before and after a 15 second ethanol exposure. To quantify bacteria, we performed a serial dilution with quantitative plate counts and used this data to determine the number of bacteria before and after ethanol exposure.

To quantify the number of cells before ethanol exposure, an overnight culture of bacteria was serially diluted in phosphate buffered saline (PBS) and spread across the surface of TSA plates using sterile glass beads. Specifically, the 10^{-5} and 10^{-6} dilutions were plated in duplicate to ensure the accuracy of pre-exposure counts. The plates were incubated at 37ºC, and the number of colony forming units (CFUs) were counted after 24 hours. The number of bacteria per milliliter of overnight culture was determined using Equation 1 in the Appendix. These steps were repeated for each of the six ESKAPE pathogens.

Following the pre-exposure serial dilutions and plate counts, 1 mL of the same overnight culture was transferred to a microcentrifuge tube and centrifuged for 1 minute at maximum speed (15,000 rpm) to form a cell pellet. After aspirating and discarding the supernatant, the cell pellets were resuspended in ethanol for 15 seconds. Initially, we tested each bacterial species against a broad range of ethanol concentrations (30%, 40%, 50%, and 60%). We repeated the assay using a more precise range of ethanol concentrations depending on the lethal concentration from the previous assays in 2.5% intervals. We plated 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions for each ethanol concentration and calculated the number of bacteria after ethanol exposure using Equation 1. Finally, the percentage of bacteria survival was determined using the number of bacteria post-exposure and the number of bacteria pre-exposure with Equation 2 in the Appendix. R Studio was used to visualize the ethanol sensitivity of each bacteria species utilizing the ggplot2 package (Wickham, 2016). By plotting survival as a function of ethanol concentration, we could identify an ethanol concentration for use in our evolution experiment. Based on this data, we identified an ethanol concentration that results in a 2- to 4-log reduction in cell count for each species, to be used in the evolution experiment.

Evolution Experiment

After establishing the target ethanol concentrations for each species, we initiated the bacterial evolution process. For a series of 20 days, each species was exposed to the previously determined ethanol concentrations. Based on the data from the Ethanol Sensitivity Assays, *E. faecium* and *S. aureus* were exposed to 50% ethanol, *A. baumannii* and *E. cloacae* to 40%, and *K. pneumoniae* and *P. aeruginosa* to 35%. We performed the exposures at approximately the same time each day to allow for 24 hours for population expansion.

First, clonal stocks (the 'ancestors') were inoculated and incubated overnight. The following day, the cultures were divided to form three replicate populations for each species that were then processed independently. For each replicate population, a 1 ml aliquot was transferred into a microcentrifuge tube and centrifuged for 1 minute at maximum speed (15,000 rpm) to form a pellet. After aspirating and discarding the supernatant, the pellet was resuspended with 1 ml of the corresponding ethanol concentration for 15 seconds. After 15 seconds, 100 µl was immediately transferred into 5 ml of TSB and incubated overnight. This cycle was repeated for 20 consecutive days across all six ESKAPE pathogens. Every third day, the overnight cultures were streaked onto TSA plates to examine for visual evidence of contamination. Additionally, 600 µl of overnight cultures were transferred to a cryovial along with 400µl of 50% glycerol and placed at -80ºC to preserve a frozen stock. If contamination occurred, the freezer stocks enabled us to backtrack to the most recent stock, eliminating the need to restart the experiment from the beginning.

Ethanol Sensitivity Assay of Evolved Populations and Ancestral Strains

To assess the ethanol sensitivity of the evolved populations, we repeated the pre-and post-exposure serial dilutions using the same ethanol concentrations determined for the evolution experiment. To improve the efficiency and repeatability of the assays, we automated the process using a liquid handling robot (OpenTrons 2, OT-2) (Figure 11). Using an in-house protocol, the robot assisted in PBS distribution, ethanol exposure, and serial dilutions using a 96-well plate. To begin the assay, each bacterium was inoculated and incubated overnight before distributing 200 µl of culture into wells of the 96-well plate. The cells were pelleted using the Beckman Coulter Avanti® J-E centrifuge at 4,000 rpm for 10 minutes. The robot performed ethanol

exposures and serial dilutions, and the cells were plated by hand from the 96-well plate. From the wells, 100 µl was transferred to TSA plates from the 10^{-1} – 10^{-6} dilutions. Equations 1 and 2 were used to quantify the number of bacteria per milliliter and survival rates. Assays were performed in triplicate for each ancestor and its three corresponding evolved populations to compare survival. Microsoft Excel was used to generate scatter plots of the evolved and ancestral bacteria survival upon ethanol exposure. To determine significance in bacteria survival between evolved and ancestral populations, one-way ANOVA with post hoc Tukey's HSD were used.

DNA Extractions and Whole Genome Sequencing

Genomic DNA was isolated according to the Qiagen DNAeasy Blood and Tissue Kit protocol. DNA was extracted from each ancestral strain (n=6) and the corresponding evolved populations (n=18). Extractions were performed as recommended in the manual. An additional pre-treatment (enzymatic lysis buffer containing lysozyme) was required to digest the thick cell wall of the gram-positive bacteria.

After extracting the DNA from each of the 24 isolates, DNA purity and concentration were tested using a Nanodrop spectrophotometer. The samples were sent for whole genome sequencing at SeqCenter (Pittsburgh, PA) and SeqCoast (Portsmouth, NH). For each sample, over 400 Mbp of genomic data was provided, which was sufficient for ample coverage across each species' genome (Table 3). To analyze the whole genome sequence data, we used a bioinformatics tool called *breseq* to identify mutations in comparison to the ATCC reference genomes (Deatherage & Barrick, 2014). We ran the *breseq* program with the –p argument for population analysis to reveal mutations that were detected at less than 100% in each population. The *breseq* output included a list of suspected mutations that were compared to the ancestor and

reference genomes. After receiving the output from *breseq,* each predicted mutation was confirmed manually by viewing reads using Integrated Genome Viewer (Robinson et al., 2011). This allowed us to identify with confidence, the mutations that arose in the evolved populations over the course of the evolution experiment.

Chapter 3: Results

The goal of this study was to determine if an increase in alcohol tolerance occurs in the six ESKAPE pathogens after 20-days of repeated ethanol exposure. We hypothesized that an increase in alcohol tolerance will occur due to selection imposed by multiple rounds of alcohol exposure. Based on previous work (Perveiler, 2022), we predict that adaptation will proceed through mutations in genes involved in cell wall biosynthesis. However, we expect to observe a distinction between genes mutated in Gram-negative versus Gram-positive species due to the inherent differences in the cell wall structure.

Gram classification impacts ethanol sensitivity

First, we identified and acquired type strains corresponding to each of the ESKAPE species (Table 1). Then we determined the ethanol susceptibility of each strain by conducting alcohol sensitivity assays, in which overnight cultures were exposed to varying ethanol concentrations (ranging from 30-60%) for 15 seconds. Bacterial survival was determined using quantitative plate counts, where the number of bacteria after ethanol exposure was compared to the number of bacteria present in the culture before exposure. In general, the Gram-negative bacteria (*K. pneumoniae, A. baumannii, P. aeruginosa,* and *E. cloacae*) were more sensitive to ethanol compared to the Gram-positive species (*E. faecium* and *S. aureus*). Specifically, the viability of Gram-positive bacteria remained near 100% at ethanol concentrations through 45%, followed by a drastic decrease in survival when exposed to 50% ethanol (Figure 2). Meanwhile, a reduction in viability of the Gram-negative species occurred at much lower ethanol concentrations, between 35% and 40% (Figure 2). The differences in ethanol susceptibility

between Gram-positive and Gram-negative bacteria revealed that Gram-positive bacteria already have intrinsic or adaptive mechanisms for surviving ethanol exposure.

In the next phase of the project (the evolution experiment), we exposed each bacteria species to ethanol to determine if repeated exposure leads to the development of ethanol tolerance. Therefore, it is necessary to identify an appropriate ethanol concentration which will impose a strong selective pressure, but not kill all of the bacteria. As a general guideline, we wanted to identify an ethanol concentration that causes between a two- and four- log reduction in survival (99% - 99.99%). Based on our data (Figure 2), 50% was identified as an appropriate ethanol concentration for the Gram-positive pathogens, *E. faecium* and *S. aureus.* For the Gram-negative pathogens, lower concentrations were necessary, 40% for *A. baumannii* and *E. cloacae,* and 35% for *K. pneumoniae* and *P. aeruginosa.*

Repeated exposure to ethanol selects for evolved bacterial populations

Next, we designed and conducted a 20-cycle evolution experiment consisting of daily ethanol exposure followed by rescue of survivors in broth. To begin, each species (ancestor) was divided into three independent cultures, which were maintained throughout the experiments as replicate populations. Every three days, a portion of each population was stocked for future analysis and to serve as a backup in case of contamination. The populations founded from the Gram-positive species (*S. aureus* and *E. faecium*) were successfully evolved without issues arising. Unfortunately, contamination was pervasive for many of the gram-negative species, mostly affecting the *E. cloacae, A. baumannii,* and *K. pneumoniae* populations. By plating evolving populations onto Mannitol Salt Agar (MSA) plates (which select for *Staphylococcus* species due to a high salt concentration), we detected contamination in several of the gram-

negative populations. When contamination of an evolving population was detected, we revived the most recent freezer stock that exhibited no signs of contamination. Due to unresolved contamination, evolution of *E. cloacae* and *A. baumannii* was discontinued and subsequent analysis of these species was not performed. Upon completion of the 20-day regimen, evolved populations were revived for phenotypic and genotypic analysis.

Elevated ethanol tolerance is most prominent in Gram-positive bacteria

To determine if repeated ethanol exposure caused an increase in ethanol tolerance, the ancestral and evolved populations were exposed to the appropriate concentration of ethanol. However, to achieve the replication necessary for comparison between evolved populations and their corresponding ancestor, alcohol sensitivity assays were automated using a liquid handling robot, which introduced some deviation from evolution experiment that was conducted manually (see Methods for a full description). Survival following exposure was determined by quantitative plate counts. Ethanol tolerance was assessed through two related metrics: total viable cells following exposure and percent survival. A one-way ANOVA with post hoc Tukey's HSD was utilized to determine whether a significant difference in growth had occurred in the evolved populations compared to their respective ancestors. The statistical tests demonstrated any significant differences in cell survival between the ancestors and each evolved population, and which comparisons are significant.

The evolved populations of *E. faecium* generally exceeded the growth of the ancestor after exposure to 50% ethanol. An increase in cell growth was demonstrated in evolved populations 1 (* $p < 0.05$) and 2 (** $p < 0.01$) in comparison to cell survival of the ancestor (Figure 4). The ancestor and population 3 showed a similar average in number of survivors and

percent survival therefore no statistical difference was observed. An increase in average percent survival is demonstrated in evolved populations 1 (64.5%) and 2 (103.4%) however statistical significance was only observed within population 2 (** $p < 0.01$) in comparison to the ancestor (19.3%) (Table 5).

In *S. aureus,* an increase of growth was observed in the evolved populations with growth being detected in 10^{-6} dilutions whereas growth was only detected in the ancestral population in the 10^{-4} dilution. While the cell growth following ethanol exposure in the evolved populations generally exceeded that of the ancestor, statistical significance was observed only in population 2 (** $p < 0.01$) (Figure 5a). The ancestral populations exhibited the lowest average percent of cell survival (0.95%) compared to evolved population 1 (12.6%), evolved population 2 (35.4%), and evolved population 3 (20.1%) (Figure 5b). Notably, statistical significance was observed in evolved population 2 (** $p < 0.01$) and evolved population 3 (* $p < 0.05$).

K. pneumoniae demonstrated an increased number of surviving cells following 35% ethanol exposure however no differences were observed in percent survival. In evolved populations 1 and 2, a significant increase in growth was observed (** $p < 0.001$) in comparison to the ancestor (Figure 6a). Compared to the ancestor, a decrease in percent survival was observed in evolved population 3. However, no statistical significance in percent survival was observed in evolved populations 1 (75.0%), 2 (73.5%), or 3 (35.4%) compared to the ancestor (63.8%) (Figure 6b).

In all three *P. aeruginosa* evolved populations, no significant differences were observed in comparison to the ancestor. Growth between evolved and ancestral populations remained consistent overall, with evolved population 3 showing slightly higher survival than the remaining populations (Figure 7).

Genes mutated across evolved populations suggest mechanisms of ethanol tolerance

To identify the molecular mechanisms responsible for ethanol tolerance, we sequenced the genomes of each evolved population as well as the ancestral strains. In particular, we were interested in identifying mutations that arose during the evolution experiment, and therefore were present in the evolved populations but absent from the ancestors. Genomic DNA was extracted using the Qiagen DNAeasy Blood and Tissue Kit. The DNA concentrations and purities were assessed using a Nanodrop spectrophotometer with the isolated DNA (Table 4). The 260/280 and 260/230 ratios in Table 4 provides information about the presence of contaminants in a DNA sample by measuring the absorbance at these wavelengths. A ratio of 1.8 for 260/280 indicates that the sample is relatively free of contaminants, whereas a ratio below 1.8 indicates that contaminants are present. Similarly, an ideal 260/230 ratio can range from 2.0 – 2.2, with a lower number being evidence of contamination. These contaminants could be residual components of the growth media, reagents used during extraction, or other organic compounds. While all species exhibited minimal protein contamination based on the 260/280 ratio, evidence of some contamination was observed based on the 260/230 ratio across all species, apart from *E. faecium*. The 260/230 ratios are fairly consistent within the species, suggesting that a lower ratio may result from inherent differences in cell physiology between the species. While DNA concentration varied, sometimes drastically, the values exceeded the minimum quantity required for Illumina whole genome sequencing.

Raw sequence data was processed using *breseq* to compare the genomes of the ancestral and evolved populations and identify evolved mutations. Average coverage across each genome ranged from 90.6x to 676.1x (Table 3), which was sufficient for detecting mutations present at

frequencies as low as 5% in each population. Recommendations for adequate sequence coverage range from 30x to 50x (Lander & Waterman, 1988). The average genome coverages in our data surpass the recommendations therefore discovery of variants can be made with a higher degree of confidence. For all *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* populations, the percentage of mapped reads exceeded 95%, indicating strong alignment of sequence data to the reference genome and minimal DNA contamination (Table 2). In contrast, poor read alignment was detected for the *A. baumannii* and *E. cloacae* species, verifying our previous concerns of pervasive contamination for these populations. Indeed, alignment to the *E. faecium* reference genome resulted in much better alignment. As a result, we continued with analysis of the *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* species.

Whole genome sequencing revealed the presence of mutations in every evolved population, with the exception of a single of population of *P. aeruginosa* (Figure 8). An average of 7 and 9 mutations were detected in the *E. faecium* and *S. aureus* populations, respectively. All *E. faecium* and *S. aureus* populations contained at least one fixed mutation (present in all cells within the population). Fewer mutations were detected in the *K. pneumoniae* and *P. aeruginosa* populations, with the former averaging 2 mutations and the latter averaging only 1. Only a single fixed mutation was detected across the *K. pneumoniae* populations, while none were detected across the *P. aeruginosa* populations.

Most mutations detected in the evolved populations are found in distinct genes (see Tables 7-10). However, there were several genes that were mutated in multiple different populations (Table 6), indicating with high probability that they are adaptive and contribute to the development of ethanol tolerance. For *E. faecium*, one gene (*atpC*) was mutated in all three populations, while another gene (*sasA_3*) was mutated in two populations. In addition, identical

intergenic mutations were detected in two *E. faecium* populations. For *S. aureus*, one gene (*rsbV*) was mutated in all three populations, while two others, *stp* and HHPGJKJE_01811 (encoding a hypothetical protein), were mutated in two populations each. In *K. pneumoniae*, a single gene (*nhaR*) was mutated in two populations. Meanwhile, identical intergenic mutations were detected in two *P. aeruginosa* populations.

Chapter 4: Discussion

Alcohol-based disinfectants are relied upon in both healthcare settings and the general population as a prevention method for the spread of microorganisms and transmission of illnesses. As antibiotic resistance continues to rise, it raises concern for the potential for bacteria to become tolerant to commonly used disinfectants. To ensure that the spread of infections can be prevented and controlled, it is imperative to investigate the potential risks of repeated ethanol exposure to pathogens and to inform healthcare workers and the public of the possible implications if not properly used. In this study, we tested the susceptibility of the ESKAPE pathogens to ethanol and repeatedly exposed them to concentrations that kill in excess of 99% of cells in the population. Next, we examined the growth of bacteria of the ancestral and evolved populations to determine if the evolved populations had developed better survival capabilities following ethanol exposure. Finally, we identified mutations contributing to ethanol tolerance by sequencing the genomes of the ancestral and evolved populations.

Gram-positive bacteria can better tolerate the effects of ethanol exposure

After first determining the concentrations of ethanol that resulted in a two- to four-log reduction in cells, we discovered that the Gram-positive ESKAPE pathogens survived at a higher concentration of ethanol than the Gram-negative species. To our knowledge, this data has not yet been documented. The Gram-positive species were able to survive in ethanol concentrations up to 50% whereas the Gram-negative species could only survive after exposure to 35%-40% ethanol. From this data we can conclude that, within the ESKAPE pathogens, Gram-positive bacteria can better tolerate ethanol compared to the Gram-negative pathogens. A contributing factor to this outcome would likely be related to the differences in cell wall structure that allow

Gram-positive bacteria to survive in higher ethanol concentrations. This suggests that Gram-positive bacteria already possess cellular mechanisms that contribute to increased alcohol tolerance (Yeung et al., 2022).

Another indicator that Gram-positive bacteria are less susceptible to ethanol was demonstrated by recurring contamination of Gram-negative populations by Gram-positive bacteria. Due to the Gram-positive ESKAPE pathogens tolerating higher ethanol concentrations, they managed to contaminate Gram-negative cultures at several points during the experiment. Analyzation of the *A. baumannii* and *E. cloacae* whole genome sequencing indicated that contamination by *E. faecium* occurred and went undetected. Because *E. faecium* can survive at a higher ethanol concentration, it was able to outgrow the Gram-negative populations being exposed to only 40% ethanol. As a result, contamination can quickly turnover the number of cells to be dominated by the Gam-positive species.

Gram-positive bacteria raise concern due to the threat of increasing alcohol tolerance

One of our main objectives was to determine if ESKAPE pathogens can develop tolerance to ethanol while also comparing differences among Gram-positive and Gram-negative species. In previous studies, repeated exposure to disinfectants has shown that bacteria can develop adaptive mechanisms to enhance survival (Forman et al., 2016). However, limited research is available comparing ethanol tolerance in Gram-negative and Gram-positive bacteria Based on previous research, we expected to observe an increase in ethanol tolerance with mechanisms of tolerance differing between Gram-negative and Gram-positive species. In this study, it was evident that the Gram-positive species have greater access to adaptive mutations

than the Gram-negative species based on the extent of ethanol tolerance following 20-days of repeated ethanol exposure.

Evidence that *E. faecium* is capable of developing ethanol tolerance was previously demonstrated by Pidot et al., (2018) comparing isolates between 1997 and 2015. The results from our research show similar evidence after repeated ethanol exposure. The increase in bacteria survival in two evolved populations show strong evidence that *E. faecium* has the ability to develop alcohol tolerance. When cells are exposed to an environmental stressor (ethanol) and kill a majority of the population, the remaining cells likely possess an adaptive advantage allowing for a better chance in survival (Merlo et al., 2020). The repeated transfer of this small portion of the population that survives among generations selects for cells that possess adaptive mutations. Over time, more beneficial mutations accumulate which ultimately leads to increased ethanol tolerance.

In *S. aureus,* there was a 26-fold increase in percent survival on average among the three evolved populations (Figure 5b). In previous research, similar data was collected showing that repeated ethanol exposure to *S.aureus* resulted in a significant increase in alcohol tolerance across four different strains (Perveiler, 2022). This is strong evidence that *S. aureus* is capable of becoming less susceptible to ethanol upon repeated exposure.

Within the evolved populations of *K. pneumoniae,* greater survival was observed in evolved populations however there were no changes in percent survival compared to the ancestor. Determining the number of survivors is only providing the number of bacteria in 1ml of culture both before and after ethanol exposure, independently. While calculating the percent survival, the pre-exposed cell growth is also factored in. This provides a more accurate interpretation of the effects on ethanol on survival by showing what percentage of the original

population survived the ethanol exposure. Having no differences in percent survival indicates that the increased number of survivors is not necessarily attributed to increase in ethanol tolerance. In all but one replicate of one population, the pre-exposure growth of the evolved populations was greater than that of the ancestors. This could mean that *K. pneumoniae* is becoming better at growing in the media, but not necessarily better at surviving ethanol exposure. With this contrast in results, a clear trend in evolved ethanol tolerance cannot be concluded. Additional research would be required by looking deeper into how the pre-exposure growth in the evolved populations differs from that of the ancestor while including more replicates to validate the outcome.

The evolved populations of *P. aeruginosa* demonstrated no evidence of becoming more tolerant to ethanol. Throughout the evolutionary process, *P. aeruginosa* struggled to survive in many instances in which no growth occurred following incubation. Having the lowest number of mutations present in multiple populations (Table 6), the lack of *P. aeruginosa* to develop tolerance to ethanol was anticipated. From this we can conclude *P. aeruginosa* has a disadvantage in adapting to repeated ethanol exposure. Increased tolerance to ethanol is less likely to occur, however evolution over a longer time period would be necessary to confirm the ability (or lack thereof) to become more ethanol tolerant.

Adaptive mechanisms conferring alcohol tolerance vary in function

The simple presence of a mutation does not indicate that it contributes to the observed phenotype (ethanol tolerance). For example, at least some mutations are expected to be hitchhikers, which increase in frequency not because they themselves are adaptive but instead because they occur on the background of an adaptive mutation (Maynard & Haigh, 2007).

However, it is extremely unlikely that a single gene would be mutated in multiple independent populations, given the randomness at which mutations occur within a genome. Therefore, any genes mutated in more than one population are likely adaptive and contribute to the observed phenotype.

The genomic sequencing data of the ancestral and evolved populations demonstrated that a greater number of mutations had occurred in the Gram-positive species than the Gram-negative (Table 6). All mutations listed were present in more than one population. The independent evolution of these mutations is a compelling indication that the genes involved could be contributing to the effects of repeated ethanol exposure.

In the evolved populations of *E. faecium,* two genes were mutated in multiple populations (Table 6). Present in all three evolved populations, both a deletion and duplication occurred within the *atpC* gene, which encodes an ATP synthase epsilon chain. This gene product is part of a group of multi-subunit proteins and is known to be involved in the inhibition of ATP synthase in several bacterial species, but has not been well studied in *E, faecium* (Figure 10) (Sielaff et al., 2018). Because both a deletion and duplication mutation occurred within this gene, its role in alcohol tolerance is unclear. A possible explanation would be that it is a hitchhiker gene present in a region of the genome that expands and contracts. If any beneficial mutations occurred in this region of the genome, *atpC* could have tagged along solely by being located in the same region. In this case, it would be a neutral mutation that does not benefit or inhibit the bacterial population. Another mutation encoding for an adaptive-response sensory-kinase, *sasA,* was present in two populations at 100% frequency. The protein encoded by *sasA* is a member of a two-component regulatory system, sensing environmental stressors from outside the cell and sending a signal through the cell wall to a response regulator (Figure 10) (Dvornyk et al., 2004).

Research on its function in *E. faecium* is limited however it has been identified in a vancomycin resistance gene cluster (Xavier et al., 2021). The role of *sasA* in the *van* operon cluster is not fully understood. Being part of an antibiotic resistance gene cluster could indicate that similar adaptive mechanisms are involved, contributing to ethanol tolerance. This highlights the significance of antibiotic resistance and the potential for overlapping adaptive mechanisms or cross-resistance to occur. Another mutation was detected in two populations and occurred in the in the intergenic region upstream of two genes predicted to encode hypothetical proteins. Since the same identical mutation was detected in both populations, it is likely that the mutation was present at a low frequency in the ancestor rather than arise independently in both populations. The adaptive mechanisms that developed in *E. faecium* are not fully understood however sensing and responding to environmental changes play a role in adaptation.

In *S. aureus,* three mutations were identified in multiple evolved populations, suggesting that these mutations play a role in ethanol tolerance by developing independently (Table 6). One gene that has been previously identified as a contributor to ethanol tolerance was *stp,* a serine/threonine phosphatase gene (Figure 10). In an earlier study, this gene was prevalent in two different species of *S. aureus* (Perveiler, 2022). This gene is involved in the regulation of peptidoglycan synthesis, a major component of the cell wall (Liang et al., 2021). The observation that the *stp* gene was mutated in multiple different *S. aureus* strains and populations indicates that it is at least partially responsible for the increased ethanol tolerance demonstrated in Figure 5. The gene's role in peptidoglycan syntheses implies that mechanisms of ethanol tolerance can be related to the cell wall turnover. Another gene that was mutated in all three populations, *rsbV,* encodes an anti-sigma-B factor antagonist (Figure 10). In *S. aureus, rsbV* is involved in regulating transcription when an environmental stressor is present to aid in bacterial survival

(Durr-e-Shahwar et al., 2019). The increase in bacterial survival validates that the *rsbV* gene is involved in the evolution of *S. aureus* to adapt to ethanol exposure. The third mutated gene is predicted to be a hypothetical protein therefore the function and significance is unknown. Overall, based on the observed mutations and their functions, it can be concluded that mutations related to cell wall synthesis and gene expression can assist *S. aureus* in adapting to repeated ethanol exposure.

Within the evolved populations of *K. pneumoniae,* one mutation was observed in two evolved populations (Table 6). The mutation coded for a transcriptional activator protein, *nhaR,* in which regulates a group of regulatory proteins called the LysR family (Rahav-Manor et al., 1992; Toesca et al., 2001). It is responsible for positive regulation of *nhaA* which encodes for a sodium proton antiporter that is essential for growth at high sodium concentrations and high pH (Rahav-Manor et al., 1992). Within the LysR family, NhaR activates the expression of *osmC,* a gene involving response to environmental stressors (Figure 11). The expression of *osmC* is induced when bacteria cells are exposed to adverse conditions and is highly conserved among Gram-negative and Gram-positive bacteria. By regulating *nhaA* and *osmC* gene expression, the presence of *nhaR* aids in responding to environmental stressors (Toesca et al., 2001). Limited research on *nhaR* in *K. pneumoniae* is available although its function in other Gram-negative bacteria emphasize its significance in response to changes in the environment. Based on the percent survival of evolved *K. pneumoniae* (Figure 6b), there was no drastic increase in growth that would suggest major beneficial mutations against ethanol exposure.

Only one mutation was detected in *P. aeruginosa*, located in the intergenic region between the *pnp* gene, coding for a polyribonucleotide nucleotidyltransferase (PNPase), and the *rpsO* gene*,* coding for ribosomal protein S15. Specifically, the 71 bp deletion is located upstream

of *pnp* and downstream of *rpsO.* Since promoter and regulatory elements are often located upstream of genes, the mutation likely impacts the expression of *pnp.* Research has shown that in *P. aeruginosa,* PNPase is an essential gene that controls gene expression of type III and VI secretion systems and plays a role in response to environmental stressors in other bacteria (Figure 11) (Fan et al., 2019). Since *pnp* is involved in environmental stress response, the deletion may impact the cell's ability to cope with environmental stressors, like ethanol. Another study demonstrated that mutation of the *pnp* gene causes increased resistance to aminoglycoside antibiotics in *P. aeruginosa* (Fan et al., 2019). The association of mutation of the *pnp* gene and antibiotic resistance suggests that the mutation could be an overlapping adaptive mechanism in the presence of ethanol or certain antibiotics.

The prevalence of mutations and increased survival among the Gram-positive species provide evidence that Gram-positive bacteria have more potential in developing tolerance to ethanol. Mutations involving the cell wall were present in both *E. faecium* and *S. aureus* indicating that adaptations in the cell wall contribute to ethanol tolerance. Very few mutations were identified in the Gram-negative species and the evidence of ethanol tolerance was insufficient to conclude that repeated ethanol exposure influences survival. In contrast, Gram-negative bacteria have demonstrated greater resistance to antibiotics compared to Gram-positive bacteria (Breijyeh et al., 2020; Silhavy et al., 2010). This contradicts the ability of cross-resistance to occur between antimicrobial agents and antibiotics. For cross-resistance to occur, the environmental stressor must target the same cellular pathways (Colclough., 2019). Our findings indicate that the antibiotic resistance mechanisms in Gram-negative bacteria must be involved in different pathways than those that confer alcohol tolerance. This is likely due to the structural differences in the cell wall. The Gram-negative outer membrane acts as a barrier to

many antibiotics whereas Gram-positive bacteria lack the outer membrane but have a thicker layer of peptidoglycan exposed directly to the environment (Breijyeh et al., 2020). This suggests that mechanisms of alcohol tolerance may be more achievable in Gram-positive bacteria due to their thick peptidoglycan layer. It also emphasizes the role of the outer membrane in antibiotic resistance, which likely has a less significant role in alcohol tolerance.

Overall, mechanisms of ethanol tolerance vary by species but adaptations involving the cell wall and peptidoglycan synthesis could be a significant mechanism in Gram-positive bacteria. The mechanisms of action of tolerance to disinfectants are not fully understood because of their nonspecific nature, targeting a variety of different mechanisms (Rozman et al., 2022). To further understand the roles of the mutations that were described, gene knockout or overexpression of genes conferring tolerance could determine the severity of their impact on ethanol tolerance.

This research could be expanded by determining if increased ethanol tolerance contributes to increased antibiotic resistance or other environmental stressors. Repeating this study on a broader range of Gram-positive and Gram-negative pathogens would provide more insight into the differences in ethanol tolerance that were demonstrated to offer more substantial evidence in regard to Gram-positive bacteria being capable of developing compelling evidence of ethanol tolerance. The evidence of Gram-positive species developing ethanol tolerance in a short period of time reinforces the need for this research to be expanded. To keep track of ethanol tolerance in dangerous pathogens, isolates in healthcare settings should be regularly monitored to ensure that methods of disinfecting remain effective. It is imperative that healthcare workers and the public are aware of the effects of repeated ethanol exposure to prolong further ethanol tolerance from occurring.

List of Figures

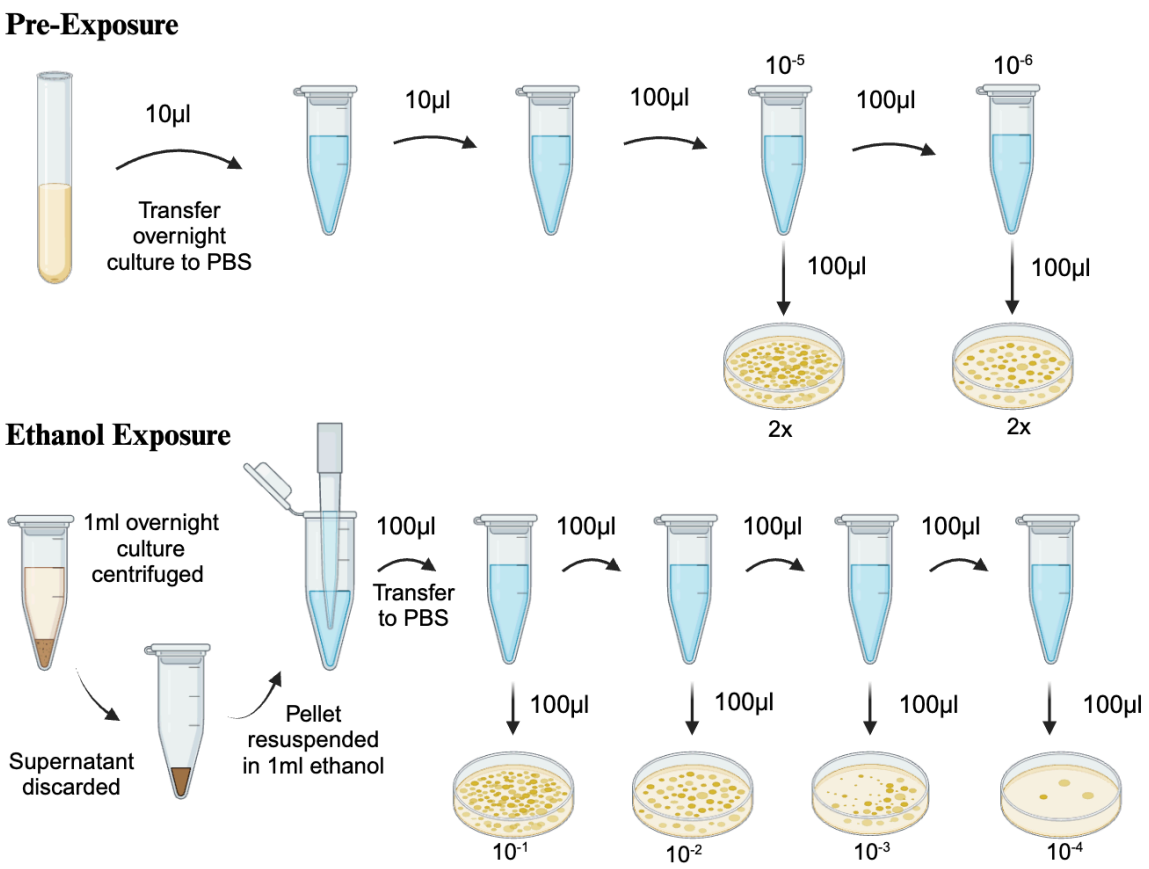

Figure 1: Protocol for determining ethanol susceptibility of ancestral strains. The assay was performed on each of the six ESKAPE pathogens, at ethanol concentrations ranging from 30%-60%. Colony counts of the ethanol-exposed bacteria was compared to the pre-exposure colony counts to determine the survival for each ethanol concentration within each species. Created with BioRender.com.

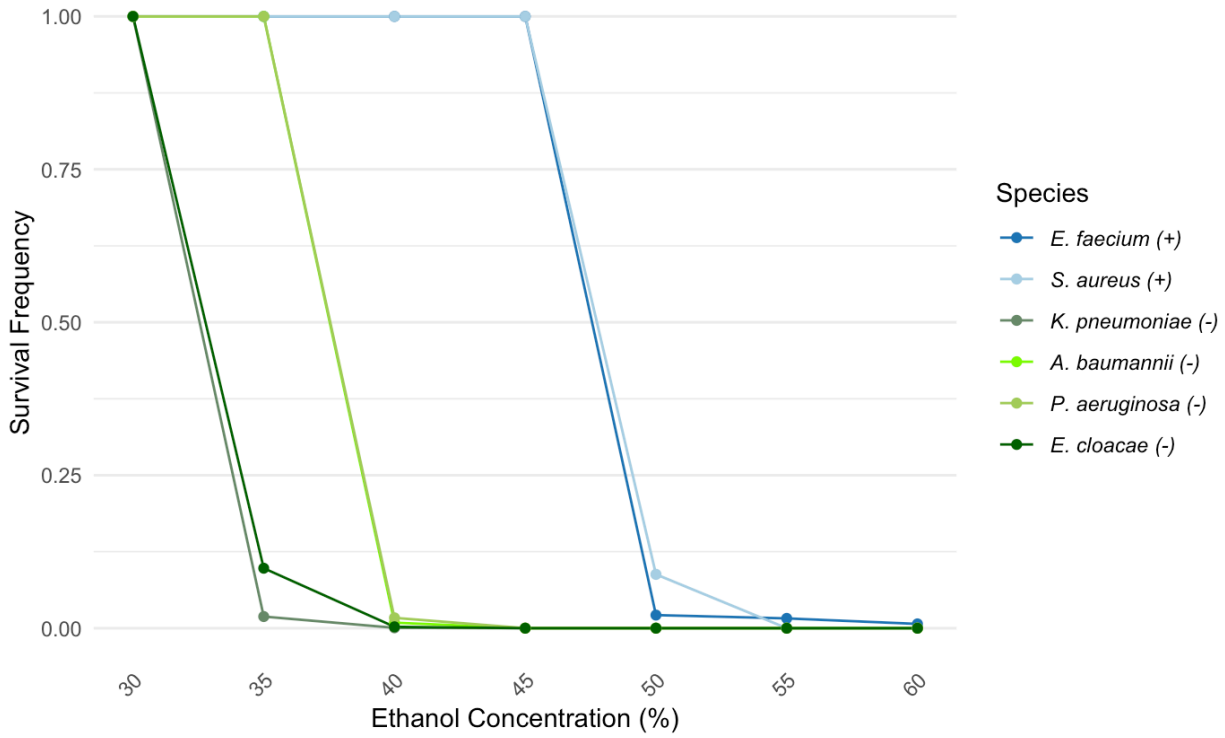

Figure 2: Survival frequency of the ESKAPE pathogen ancestral strains after ethanol exposure at concentrations ranging from 30%-60%. Cell counts were collected via quantitative plate counts. Survival frequency was determined by dividing the number of bacteria (CFU/ml) following ethanol exposure by the number of bacteria prior to exposure. Gram-positive species (*S. aureus and E. faecium*) exhibited sensitivity to ethanol in the 45-50% range. Gram-negative species (*K. pneumoniae, A. baumannii, P. aeruginosa, and E. cloacae*) exhibited sensitivity to ethanol in the 30-40% range.

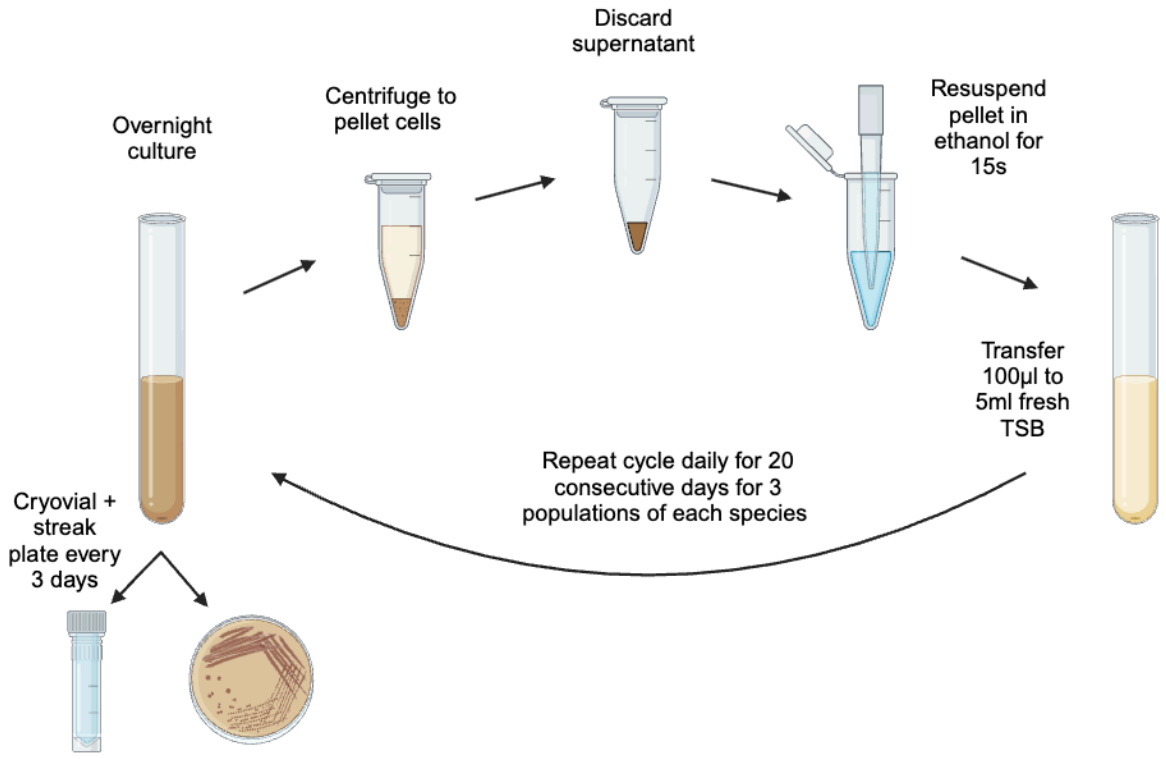

Figure 3: Protocol for 20-day evolution experiment. Three replicate populations for each ESKAPE pathogen were generated at the start of the evolution experiment, totaling 18 populations. Each species was exposed to an ethanol concentration that causes a 2- to 4-log reduction in cells for a total of 20 days. Created with BioRender.com.

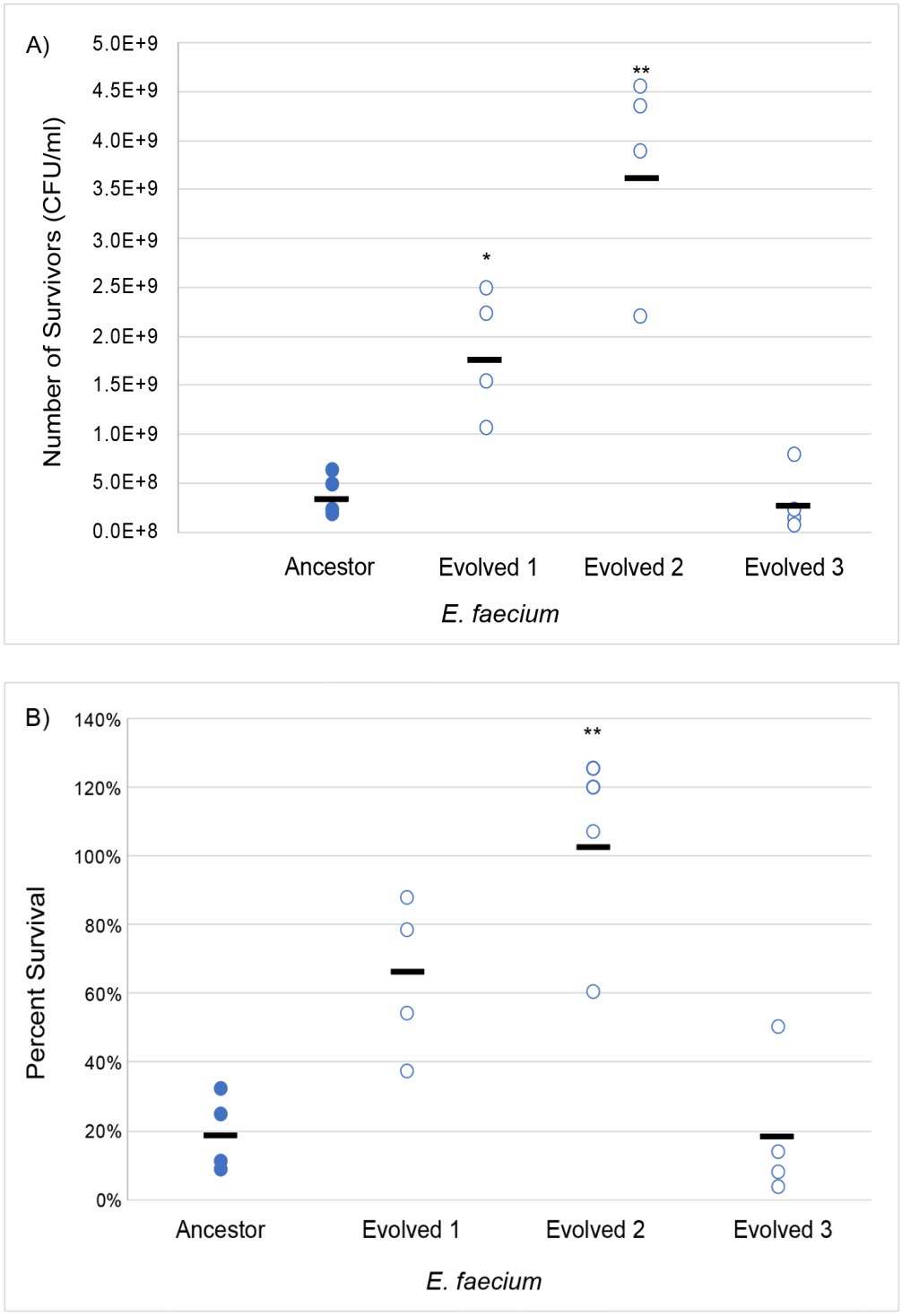

Figure 4: Survival of *E. faecium* ancestral and evolved populations after exposure to 50% ethanol. A) Total number of survivors. B) Percent survival. Open circles correspond to evolved populations while closed circles correspond to the ancestor. Each population consists of four replicates. Asterisks indicate level of statistical significance in comparison to ancestor: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

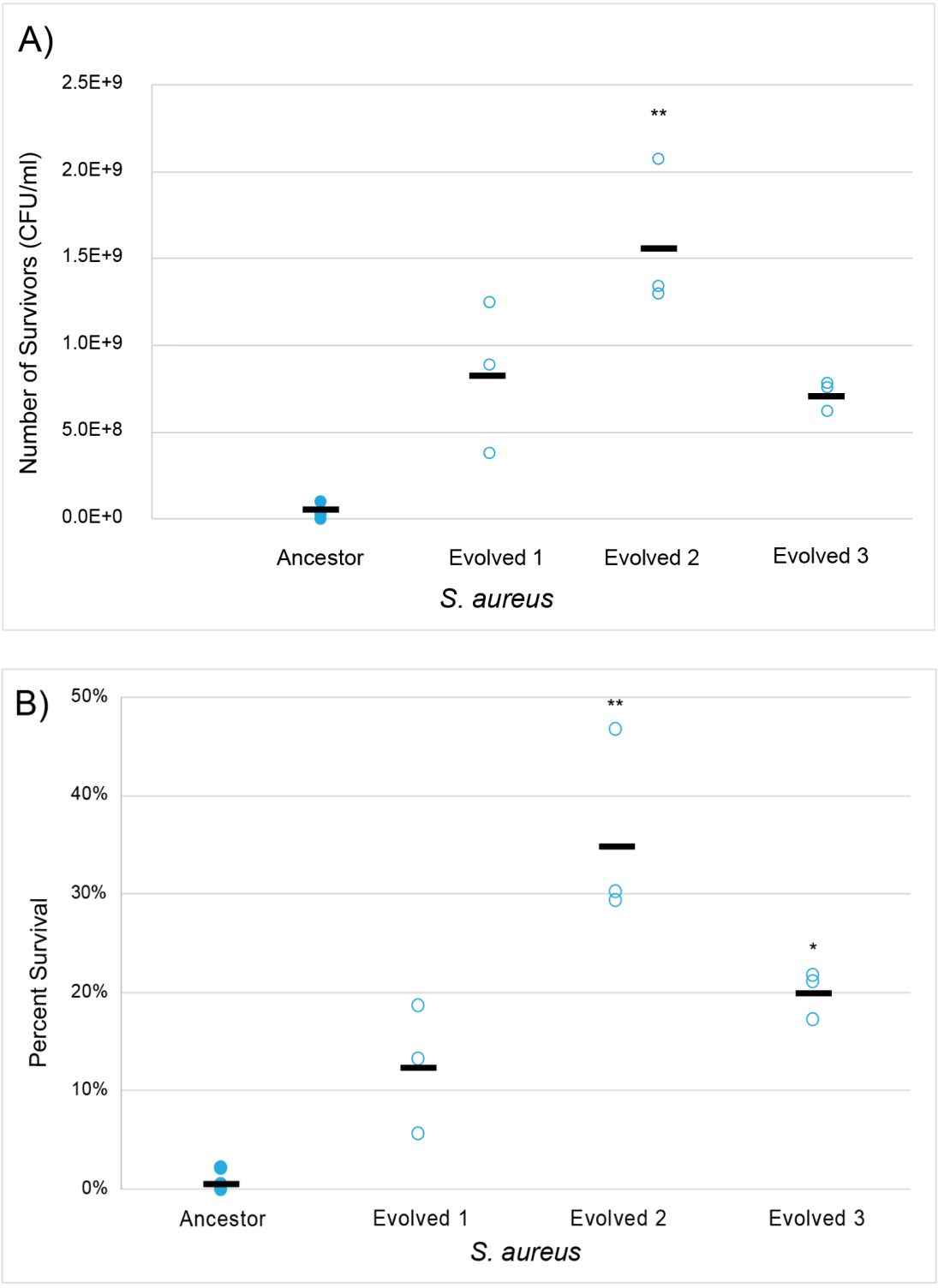

Figure 5: Survival of *S. aureus* ancestral and evolved populations after exposure to 50% ethanol. A) Total number of survivors. B) Percent survival. Open circles correspond to evolved populations while closed circles correspond to the ancestor. Each population consists of three replicates. Asterisks indicate level of statistical significance in comparison to ancestor: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

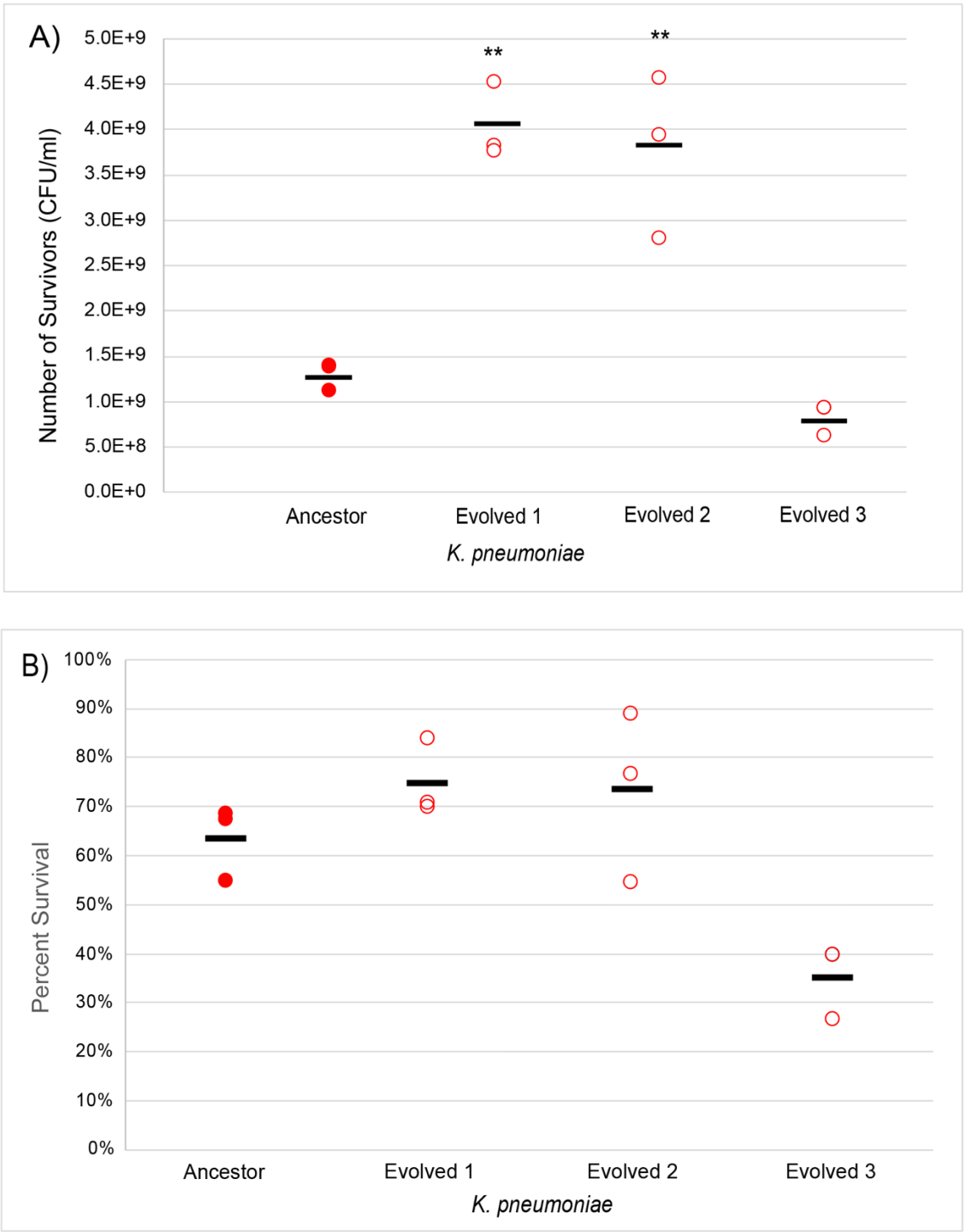

Figure 6: Survival of *K. pneumoniae* ancestral and evolved populations after exposure to 35% ethanol. A) Total number of survivors. B) Percent survival. Open circles correspond to evolved populations while closed circles correspond to the ancestor. Each population consists of three replicates. Asterisks indicate level of statistical significance in comparison to ancestor: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

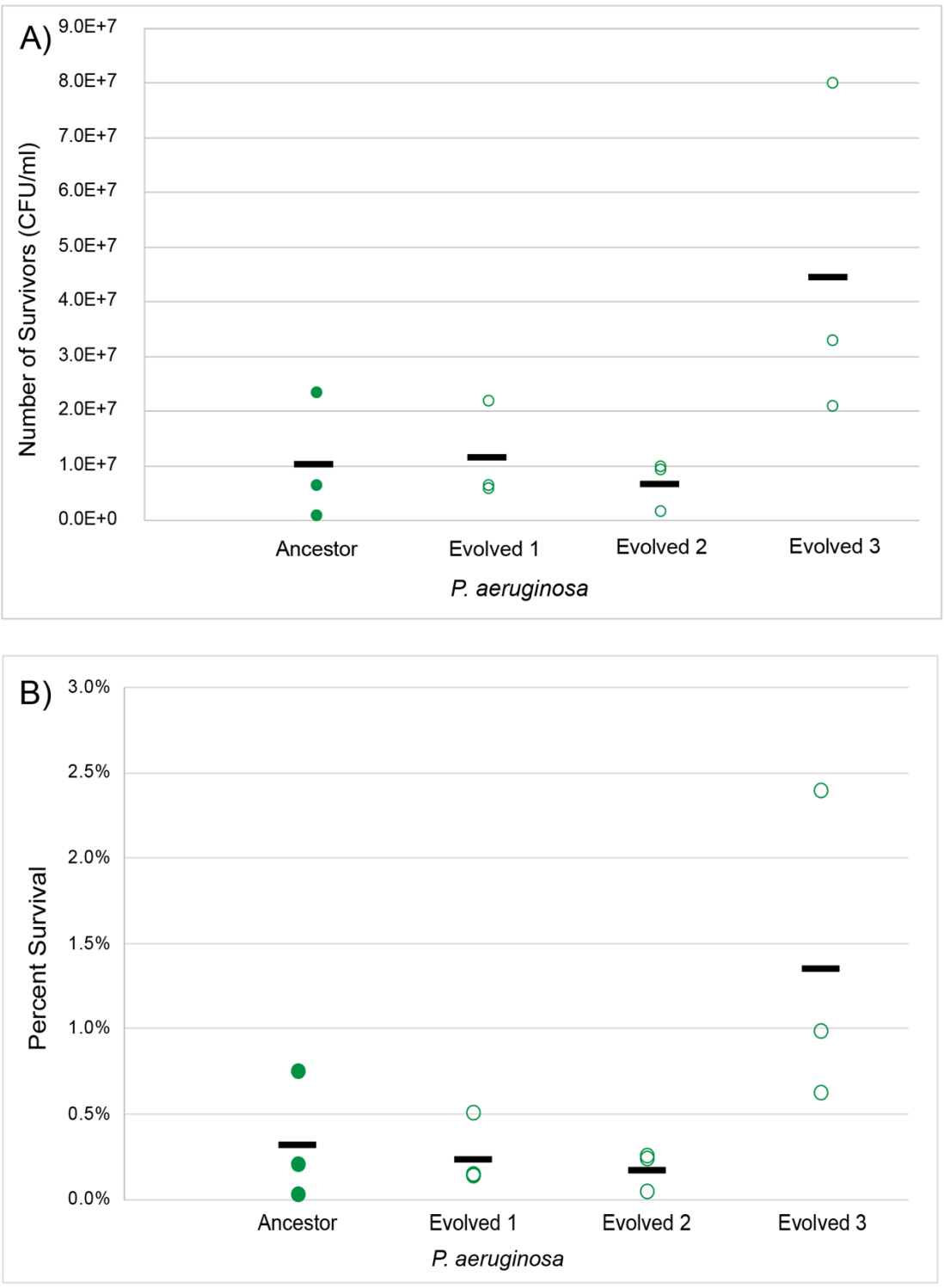

Figure 7: Survival of *P. aeruginosa* ancestral and evolved populations after exposure to 35% ethanol. A) Total number of survivors. B) Percent survival. Open circles correspond to evolved populations while closed circles correspond to the ancestor. Each population consists of three replicates. Asterisks indicate level of statistical significance in comparison to ancestor: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

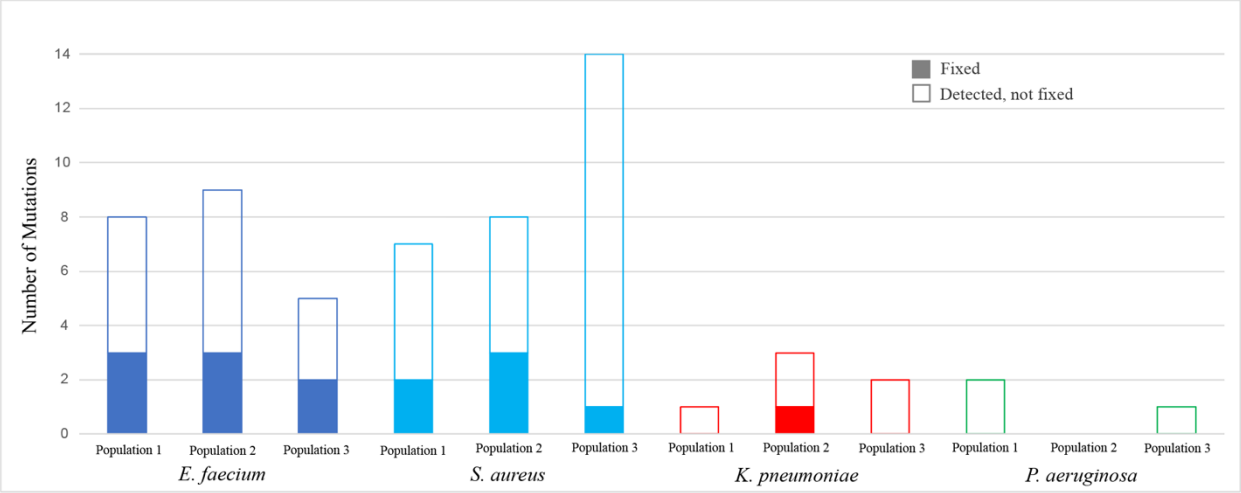

Figure 8: Number of fixed and detected mutations in all populations. Fixed mutations are those that are present at 100% frequency in an evolved population. Mutations that were detected but not fixed were present in only a portion of the population. Only mutations detected above a 5% threshold were considered.

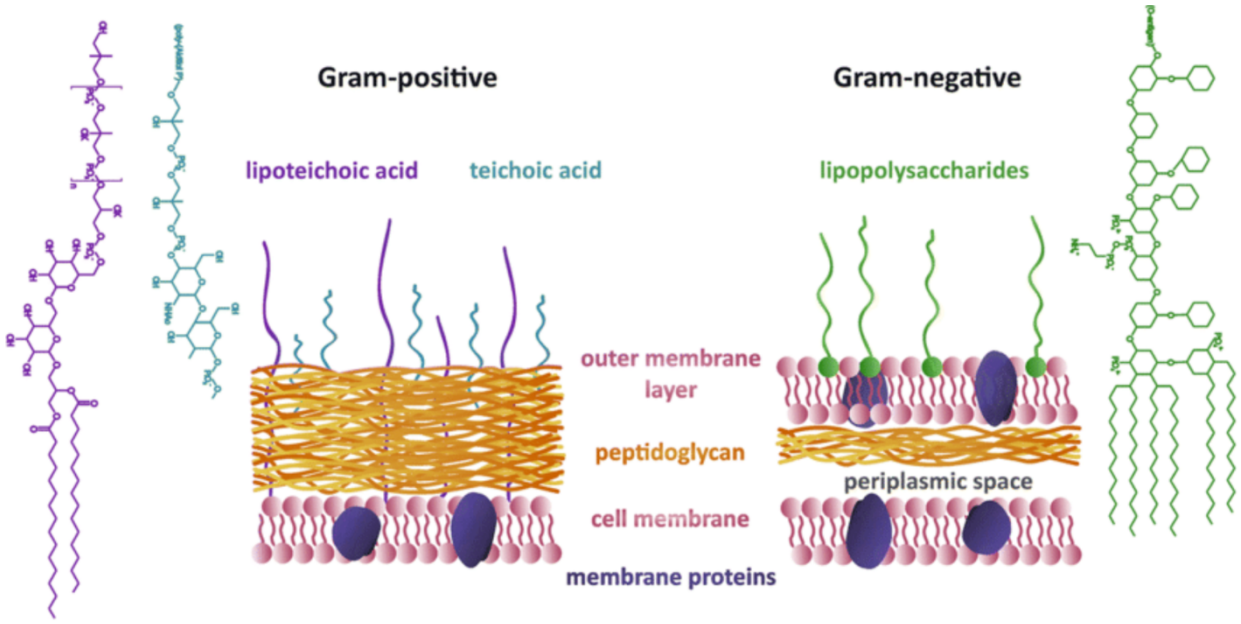

Figure 9: Gram-positive and Gram-negative cell wall. Differences between Gram-positive and Gram-negative bacterial cell walls. Source: Research Gate.

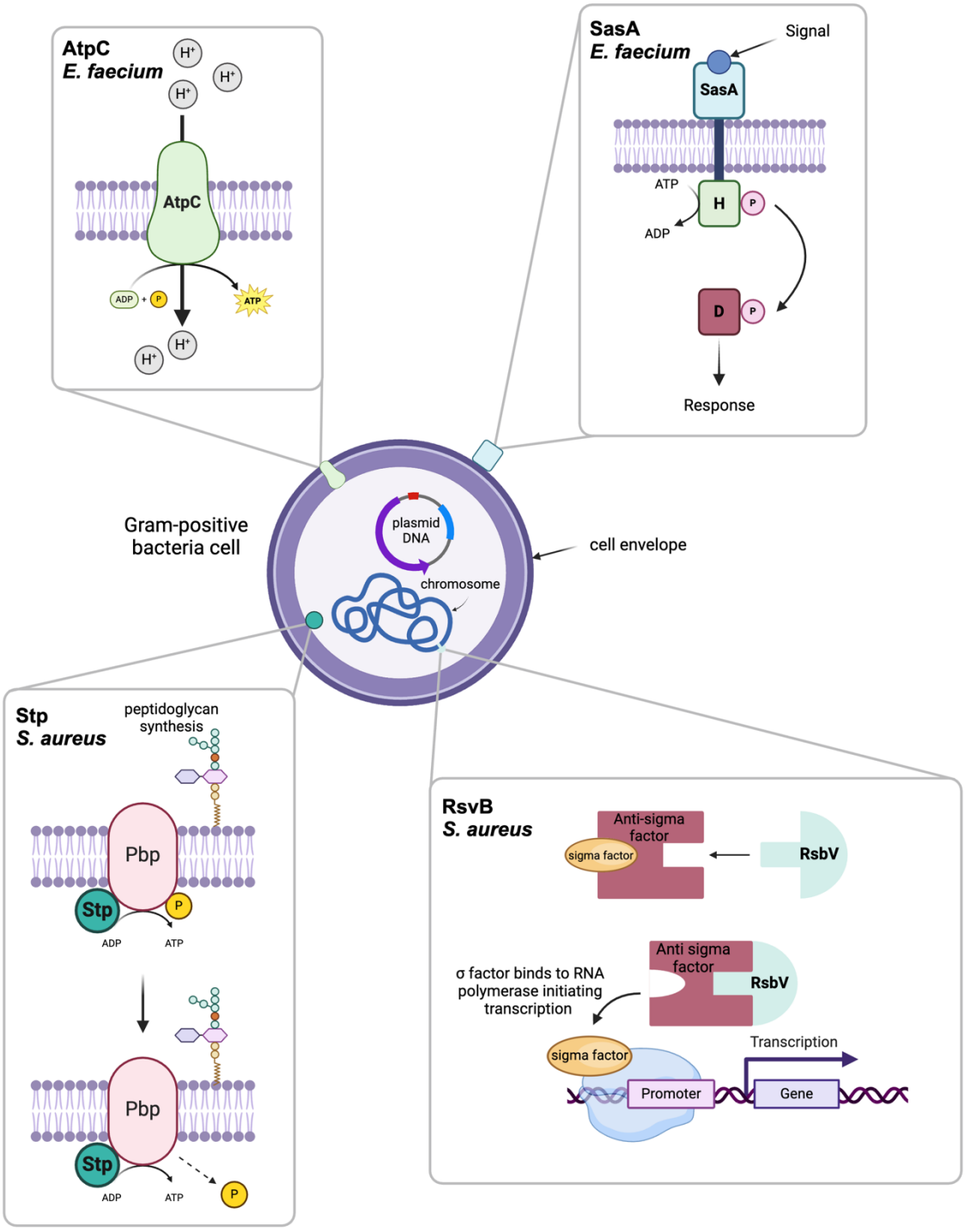

Figure 10: Putative tolerance mechanisms in Gram-positive species. Proposed function of proteins encoded by mutated genes *atpC* and *sasA* in *E. faecium* and *stp* and *rsvB* in *S. aureus*. Created with BioRender.com.

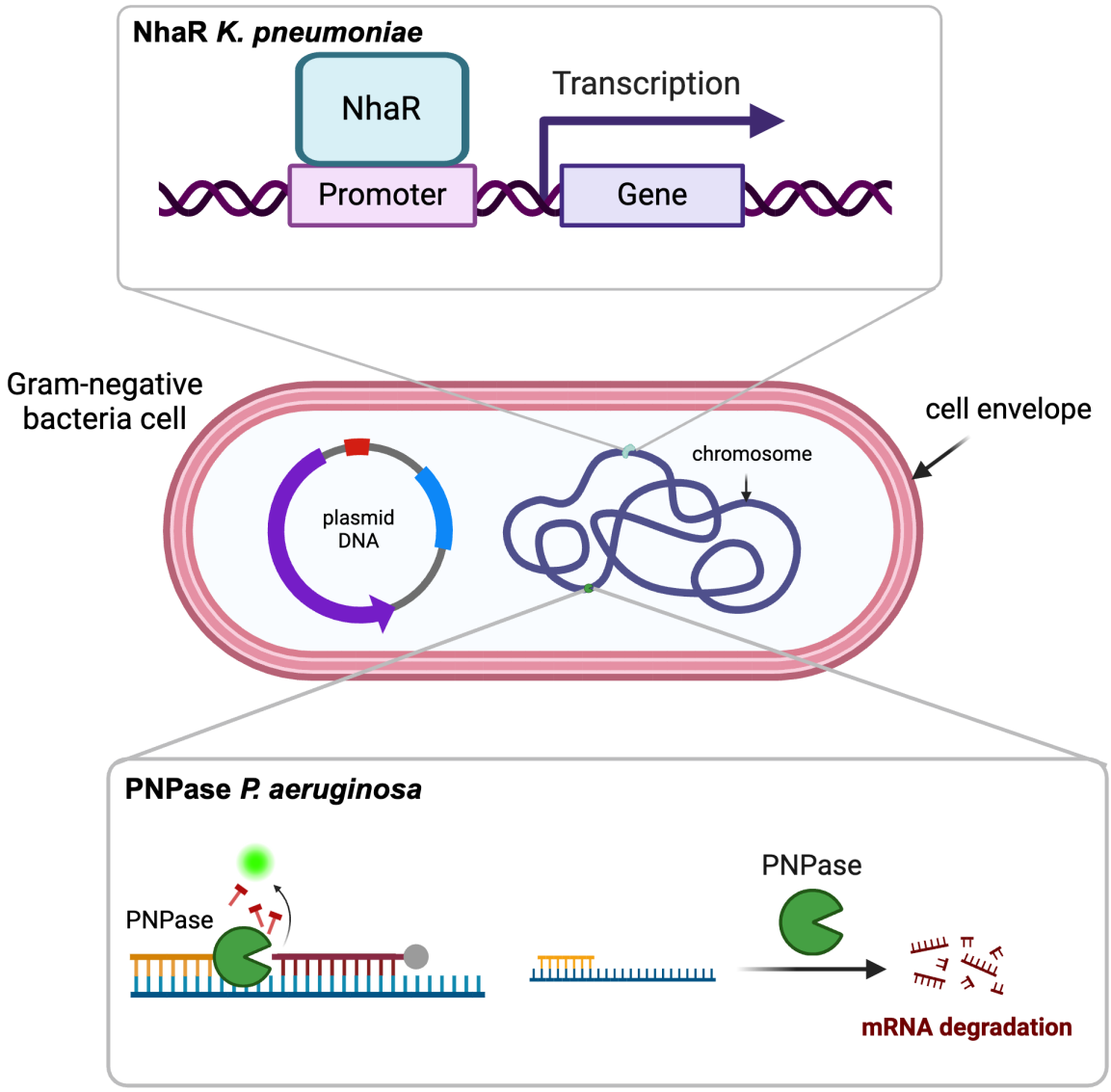

Figure 11: Putative tolerance mechanisms in Gram-negative species. Proposed function of proteins encoded by mutated genes *nhaR* in *K. pneumoniae* and *pnp* in *P. aeruginosa*. Created with BioRender.com.

Figure 12: Opentrons OT-2 liquid handling robot. Picture of the liquid handling robot used to perform the automated ethanol sensitivity assays. Source: Opentrons (2023).

List of Tables

Table 1: ESKAPE pathogen strain classification.

Bacteria Name	ATCC ID	Common Strain Name	Gram Classification
Enterococcus faecium	19434	NCTC 7171	positive
Staphylococcus aureus	12600	NCTC 8532	positive
Klebsiella pneumoniae	13883	NCTC 9633	negative
Acinetobacter baumannii	19606	2208	negative
Pseudomonas aeruginosa	10145	NCTC 10332	negative
Enterobacter cloacae	13047	CDC 442-68	negative

Table 2: Percent of reads mapped in the genomes of each species.

Percent Mapped Reads				
	Ancestor	Population 1	Population 2	Population 3
E. faecium	99.4%	99.5%	99.4%	99.4%
S. aureus	80.4%	99.5%	99.5%	99.4%
K. pneumoniae	95.2%	97.5%	95.8%	97.7%
P. aeruginosa	95.2%	95.2%	99.3%	99.3%

Table 3: Average sequence coverage across each genome.

Average Coverage Across Genome				
	Ancestor	Population 1	Population 2	Population 3
E. faecium	657.6x	576.1x	317.1x	280x
S. aureus	518.3x	637.1x	607.5x	676.1x
K. pneumoniae	90.6x	91.8x	111.7x	108.5x
P. aeruginosa	113.9x	118x	93.7x	136.4x

Table 4: DNA concentrations and purity of evolved and ancestral populations.

Species	Population	Concentration (ng/µl)	260/280	260/230
E. faecium	Ancestor	147.3	1.95	2.03
	1	32.5	1.94	2.50
	2	82.4	1.97	1.96
	3	166.7	1.91	2.10
S. aureus	Ancestor	25.1	1.68	0.69
	1	22.1	1.68	0.81
	2	22.7	1.73	0.82
	3	31.7	1.89	1.01
K. pneumoniae	Ancestor	46.5	1.72	0.71
	1	28.3	1.65	0.52
	2	26.5	1.56	0.44
	3	30.1	1.68	0.50
A. baumannii	Ancestor	113.1	1.86	1.37
	1	21.3	1.99	0.59
	2	19.5	1.74	0.51
	3	13.3	1.99	0.40
P. aeruginosa	Ancestor	106.1	1.88	1.32
	1	170.5	1.88	1.61
	2	68.5	1.87	1.31
	3	99.4	1.85	1.48
E. cloacae	Ancestor	2679.7	1.69	0.78
	1	34.5	1.92	0.51
	2	25.7	1.95	0.48
	3	22.5	1.69	1.31

Table 5: Percent survival of ancestral and evolved populations after exposure to ethanol.

		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean
E. faecium	Ancestor	32.5	24.9	11.1	8.70	19.3
	Evolved 1	87.8	54.1	78.7	37.3	64.5
	Evolved 2	120	126	60.6	107	103
	Evolved 3	8.10	3.60	50.3	14.1	19.0
S. aureus	Ancestor	2.25	0.04	0.55	n/a	0.95
	Evolved 1	18.7	5.68	13.3	n/a	12.6
	Evolved 2	29.4	46.7	30.3	n/a	35.4
	Evolved 3	21.2	21.7	17.3	n/a	20.1
K. pneumoniae	Ancestor	55.0	67.6	68.6	n/a	63.8
	Evolved 1	71.0	70.1	84.0	n/a	75.0
	Evolved 2	76.8	54.6	89.0	n/a	73.5
	Evolved 3	26.6	39.8	39.8	n/a	35.4
P. aeruginosa	Ancestor	0.03	0.75	0.21	n/a	0.33
	Evolved 1	0.14	0.15	0.51	n/a	0.27
	Evolved 2	0.05	0.26	0.24	n/a	0.18
	Evolved 3	0.63	0.99	2.40	n/a	1.34

Table 6: Genes mutated in multiple evolved populations.

Species	Gene	Gene Description	Population 1	Population 2	Population 3
E. faecium	*atpC*	ATP synthase epsilon chain	Δ33 bp (100%)	33 bp duplication 1 → 2 (80.7%) *	33 bp duplication 1 → 2 (36.8%) *
E. faecium	*sasA_3*	Adaptive-response sensory-kinase SasA	D232G (100%)	F23V (100%)	-
E. faecium	intergenic (ACDLBFJA_02240 ← / → ACDLBIA_02241)	Hypothetical Proteins	substitution (100%) *	substitution (100%) *	-
S. aureus	*stp*	Serine/threonine phosphatase stp	V52L (100%)	Δ2 bp (100%)	-
S. aureus	*rsbV*	Anti-sigma-B factor antagonist	Q10 (100%)	E29 (100%)	E106 (13%)
S. aureus	*HHPGJKJE_01811*	Hypothetical Protein	-	A55D (10%)	+A (100%)
K. pneumoniae	*nhaR*	Transcriptional activator protein NhaR	-	Δ11 bp (100%)	D96G (42.4%)
P. aeruginosa	*intergenic (pnp ← / ← rpsO*	Polyribonucleotide nucleotidyltransferase / 30S ribosomal protein S15	Δ71 bp (83.5%) *	-	Δ71 bp (72.6%) *

* indicates identical mutation appeared in multiple populations
Δ bp indicates a deletion of base pairs
% refers to the percentage of cells that possess the mutation within the corresponding populations

Table 7: Confirmed mutations in one or more evolved populations *E. faecium.*

Strain	Evidence	Position	Mutation	Frequency	Annotation	Gene	Description
pop1	MC JC	744,155	Δ33 bp	100%	coding (266 -298/423 nt)	atpC →	ATP synthase epsilon chain
pop1	RA	778,552	A→G	60.20%	M19V (ATG →GTG)	ccpA_2 →	Catabolite control protein A
pop1	RA	1,702,504	T→C	100%	D232G (GAT →GGT)	sasA_3 ←	Adaptive-response sensory-kinase SasA
pop1	RA	2,307,195	G→A	100%	intergenic (-43/-125)	ACDLBFJA_02240 ← / → ACDLBFJA_02241	hypothetical protein/hypothetical protein
pop1	RA	2,359,981	C→T	7.70%	A452T (GCT→ACT)	mfd ←	Transcription-repair-coupling factor
pop2	JC	744,175	(GTGCGGAACGTGA ACGTGATATCG ATGTATCTC) 1→2	80.70%	coding (286/423 nt)	atpC →	ATP synthase epsilon chain
pop2	JC	1,018,020	+GACA	43.60%	coding (629/1044 nt)	hrcA →	Heat-inducible transcription repressor HrcA
pop2	RA	1,169,075	G→A	100%	D2N (GAT→AAT)	uviB →	Bacteriocin UviB
pop2	RA	1,703,132	A→C	100%	F23V (TTT→GTT)	sasA_3 ←	Adaptive-response sensory-kinase SasA
pop2	RA	2,305,825	A→T	47.80%	H331Q (CAT→CAA)	licR_2 ←	putative licABCH operon regulator
pop2	RA	2,307,195	G→A	100%	intergenic (-43/-125)	ACDLBFJA_02240 ← / → ACDLBFJA_02241	hypothetical protein/hypothetical protein
pop3	JC	744,175	(GTGCGGAACGTG AACGTGATATC GATGTATCTC)1→2	36.80%	coding (286/423 nt)	atpC →	ATP synthase epsilon chain
pop3	RA	949,564	C→A	100%	Y129* (TAC→TAA)	ACDLBFJA_00910 →	hypothetical protein
pop3	RA	1,920,467	C→T	100%	R28C (CGT→TGT)	clpP_2 →	ATP-dependent Clp protease proteolytic subunit

Table 8: Confirmed mutations in one or more evolved populations of *S. aureus.*

Strain	Evidence	Position	Mutation	Frequency	Annotation	Gene	Description
pop1	RA	1,199,251	G→C	100%	V52L (GTT→CTT)	stp →	Serine/threonine phosphatase stp
pop1	RA	2,065,425	G→A	100%	Q10* (CAA→TAA)	rsbV ←	Anti-sigma-B factor antagonist
pop1	JC	2,382,007	Δ101 bp	63%	intergenic (+1100/-288)	HHPGJKJE_02251 → / → HHPGJKJE _02252	hypothetical protein/hypothetical protein
pop1	RA	2,382,142	C→A	8%	intergenic (+1235/-253)	HHPGJKJE_02251 → / → HHPGJKJE_02252	hypothetical protein/hypothetical protein
pop1	RA	2,382,174	T→C	11%	intergenic (+1267/-221)	HHPGJKJE_02251 → / → HHPGJKJE_02252	hypothetical protein/hypothetical protein
pop2	RA	928,552	G→A	100%	G151S (GGT→AGT)	ydjZ →	TVP38/TMEM64 family inner membrane protein YdjZ
pop2	RA	1,199,165	Δ2 bp	100%	coding (68-69/744 nt)	stp →	Serine/threonine phosphatase stp
pop2	RA	1,929,203	C→A	10%	A55D (GCT→GAT)	HHPGJKJE_01811 →	hypothetical protein
pop2	RA	1,934,836	A→C	6%	D134A (GAC→GCC)	yfhP →	putative protein YfhP
pop2	RA	2,065,368	C→A	100%	E29* (GAA→TAA)	rsbV ←	Anti-sigma-B factor antagonist
pop3	RA	199,780	G→A	14%	R272Q (CGA→CAA)	uhpT →	Hexose-6-phosphate:phosphate antiporter
pop3	RA	402,735	T→A	14%	Y66N (TAT→AAT)	hsdM →	Type I restriction enzyme EcoKI M protein
pop3	RA	670,584	T→C	5%	L50S (TTG→TCG)	graR_1 →	Response regulator protein GraR
pop3	RA	692,932	C→T	5%	L18L (TTG→TTA)	HHPGJKJE_00629 ←	hypothetical protein
pop3	RA	1,340,424	T→C	13%	F6S (TTC→TCC)	HHPGJKJE_01269 →	hypothetical protein

pop3	RA	1,576,905	G→A	10%	S43L (TCA→TTA)	comGC ←	ComG operon protein 3
pop3	RA	1,825,086	C→T	76%	G267E (GGA→GAA)	atl_2 ←	Bifunctional autolysin
pop3	RA	1,929,716:1	+A	100%	coding (677/1146 nt)	HHPGJKJE_01811 →	hypothetical protein
pop3	RA	1,975,762	C→T	10%	M137I (ATG→ATA)	purB ←	Adenylosuccinate lyase
pop3	RA	2,065,137	C→A	13%	E106* (GAG→TAG)	rsbV ←	Anti-sigma-B factor antagonist
pop3	RA	2,165,960	A→T	12%	D205E (GAT→GAA)	HHPGJKJE_02027 ←	hypothetical protein
pop3	RA	2,169,033	G→A	8%	Q45* (CAA→TAA)	cdaR ←	CdaA regulatory protein CdaR
pop3	JC	28,116	54141	76%	26025 bp deletion	23 gene deletion	starts within yycH gene and includes yycI

Table 9: Confirmed mutations in one or more evolved populations of *K. pneumoniae.*

Strain	Evidence	Position	Mutation	Frequency	Annotation	Gene	Description
pop1	JC	2062899-2077246	14347 bp deletion	78.20%		22 gene deletion	include genes involved in acid stress response and capsule biosynthesis and genes involved in glycerol metabolism
pop2	JC	3,548,794	Δ11 bp	100%	coding (273-283/ 897 nt)	nhaR →	Transcriptional activator protein NhaR
pop2	RA	5,095,022	G→C	15.80%	Q31H (CAG→CAC)	JLEILMAA_04781 →	hypothetical protein
pop3	RA	3,548,808	A→G	42.40%	D96G (GAC→GGC)	nhaR →	Transcriptional activator protein NhaR
pop3	JC	1,899,969	Δ9 bp	17.90%	coding (135-143/714 nt)	nlpD_2 ←	Murein hydrolase activator NlpD

Table 10: Confirmed mutations in one or more evolved populations of *P. aeruginosa.*

Strain	Evidence	Position	Mutation	Frequency	annotation	gene	description
pop1	JC	5,363,882	Δ71 bp	83.50%	intergenic (-89/+15)	pnp ← / ← rpsO	Polyribonucleotide nucleotidyltransferase/30S ribosomal protein S15
pop1	RA	5,167,696	G→A	22.90%	P373L (CCG→CTG)	norR_3 ←	Anaerobic nitric oxide reductase transcription regulator NorR
pop3	JC	5,363,882	Δ71 bp	72.60%	intergenic (-89/+15)	pnp ← / ← rpsO	Polyribonucleotide nucleotidyltransferase/30S ribosomal protein S15

References

1. Adobe Inc. (2019). *Adobe Illustrator*. Retrieved from https://adobe.com/products/illustrator

2. Agudelo Higuita, N. I., & Huycke, M. M. (2014). Enterococcal Disease, Epidemiology, and Implications for Treatment. In M. S. Gilmore, D. B. Clewell, Y. Ike, & N. Shankar (Eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary. http://www.ncbi.nlm.nih.gov/books/NBK190429/

3. Ashurst, J. V., & Dawson, A. (2023). Klebsiella Pneumonia. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK519004/

4. Barrick, J. E., & Lenski, R. E. (2013). Genome dynamics during experimental evolution. *Nature Reviews. Genetics*, *14*(12), 827–839. https://doi.org/10.1038/nrg3564

5. Breijyeh, Z., Jubeh, B., & Karaman, R. (2020). Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. *Molecules*, *25*(6), 1340. https://doi.org/10.3390/molecules25061340

6. Chen, J., Tian, S., Nian, H., Wang, R., Li, F., Jiang, N., & Chu, Y. (2021). Carbapenem-resistant Enterobacter cloacae complex in a tertiary Hospital in Northeast China, 2010-2019. *BMC infectious diseases*, *21*(1), 611. https://doi.org/10.1186/s12879-021-06250-0

7. De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., Paterson, D. L., & Walker, M. J. (2020). Antimicrobial Resistance in ESKAPE Pathogens. *Clinical microbiology reviews*, *33*(3), e00181-19. https://doi.org/10.1128/CMR.00181-19

8. Deatherage, D. E., & Barrick, J. E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods in Molecular Biology, 1151,* 165–188.

9. Denissen, J., Reyneke, B., Waso-Reyneke, M., Havenga, B., Barnard, T., Khan, S., & Khan, W. (2022). Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. *International Journal of Hygiene and Environmental Health*, *244*, 114006. https://doi.org/10.1016/j.ijheh.2022.114006

10. Durr-e-Shahwar, S., Atia-tul-Wahab, Choudhary, M. I., & Jabeen, A. (2019). Cloning, purification, structural, and functional characterization of methicillin-resistant *Staphylococcus aureus* (MRSA252) RsbV protein. *International Journal of Biological Macromolecules*, *134*, 962–966. https://doi.org/10.1016/j.ijbiomac.2019.05.034

11. Dvornyk, V., Deng, H.-W., & Nevo, E. (2004). Structure and Molecular Phylogeny of sasA Genes in Cyanobacteria: Insights into Evolution of the Prokaryotic Circadian

System. *Molecular Biology and Evolution*, *21*(8), 1468–1476. https://doi.org/10.1093/molbev/msh106

12. Eberlein, C., Baumgarten, T., Starke, S., & Heipieper, H. J. (2018). Immediate response mechanisms of Gram-negative solvent-tolerant bacteria to cope with environmental stress: Cis-trans isomerization of unsaturated fatty acids and outer membrane vesicle secretion. *Applied Microbiology & Biotechnology*, *102*(6), 2583–2593. https://doi.org/10.1007/s00253-018-8832-9

13. Fan, Z., Chen, H., Li, M., Pan, X., Fu, W., Ren, H., Chen, R., Bai, F., Jin, Y., Cheng, Z., Jin, S., & Wu, W. (2019). Pseudomonas aeruginosa Polynucleotide Phosphorylase Contributes to Ciprofloxacin Resistance by Regulating PrtR. *Frontiers in Microbiology*, *10*, 1762. https://doi.org/10.3389/fmicb.2019.01762

14. Feng, L., Xu, M., Zeng, W., Zhang, X., Wang, S., Yao, Z., Zhou, T., Shi, S., Cao, J., & Chen, L. (2022). Evaluation of the antibacterial, antibiofilm, and anti-virulence effects of acetic acid and the related mechanisms on colistin-resistant Pseudomonas aeruginosa. *BMC Microbiology*, *22*(1), 306. https://doi.org/10.1186/s12866-022-02716-6

15. Forman, M. E., Fletcher, M. H., Jennings, M. C., Duggan, S. M., Minbiole, K. P. C., & Wuest, W. M. (2016). Structure–Resistance Relationships: Interrogating Antiseptic Resistance in Bacteria with Multicationic Quaternary Ammonium Dyes. *ChemMedChem*, *11*(9), 958–962. https://doi.org/10.1002/cmdc.201600095

16. He, Shoukui, Yan Cui, Rui Dong, Jiang Chang, Hua Cai, Hong Liu, and Xianming Shi. "Global Transcriptomic Analysis of Ethanol Tolerance Response in Salmonella Enteritidis." *Current Research in Food Science* 5 (May 6, 2022): 798–806. https://doi.org/10.1016/j.crfs.2022.04.011.

17. Heesterbeek, D. A. C., Martin, N. I., Velthuizen, A., Duijst, M., Ruyken, M., Wubbolts, R., Rooijakkers, S. H. M., & Bardoel, B. W. (2019). Complement-dependent outer membrane perturbation sensitizes Gram-negative bacteria to Gram-positive specific antibiotics. *Scientific Reports*, *9*, 3074. https://doi.org/10.1038/s41598-019-38577-9

18. Horinouchi, T., Maeda, T., & Furusawa, C. (2018). Understanding and engineering alcohol-tolerant bacteria using OMICS technology. *World Journal of Microbiology & Biotechnology*, *34*(11), 157. https://doi.org/10.1007/s11274-018-2542-4

19. Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). Acinetobacter baumannii. *Virulence*, *3*(3), 243–250. https://doi.org/10.4161/viru.19700 Jadimurthy, R., Mayegowda, S. B., Nayak, S. C., Mohan, C. D., & Rangappa, K. S. (2022). Escaping mechanisms of ESKAPE pathogens from antibiotics and their targeting by natural compounds. *Biotechnology Reports*, *34*, e00728. https://doi.org/10.1016/j.btre.2022.e00728

20. Iglewski, B. H. (1996). Pseudomonas. In S. Baron (Ed.), *Medical Microbiology* (4th ed.). University of Texas Medical Branch at Galveston. http://www.ncbi.nlm.nih.gov/books/NBK8326/

21. Jadimurthy, R., Mayegowda, S. B., Nayak, S. C., Mohan, C. D., & Rangappa, K. S. (2022). Escaping mechanisms of ESKAPE pathogens from antibiotics and their targeting by natural compounds. *Biotechnology reports (Amsterdam, Netherlands)*, *34*, e00728. https://doi.org/10.1016/j.btre.2022.e00728

22. Jagdish, T., & Nguyen Ba, A. N. (2022). Microbial experimental evolution in a massively multiplexed and high-throughput era. *Current Opinion in Genetics & Development*, *75*, 101943. https://doi.org/10.1016/j.gde.2022.101943

23. Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I., & Whitlock, M. C. (2012). Experimental evolution. *Trends in Ecology & Evolution*, *27*(10), 547–560. https://doi.org/10.1016/j.tree.2012.06.001

24. Khoshnood, S., Sadeghifard, N., Mahdian, N., Heidary, M., Mahdian, S., Mohammadi, M., Maleki, A., & Haddadi, M. H. (2023). Antimicrobial resistance and biofilm formation capacity among Acinetobacter baumannii strains isolated from patients with burns and ventilator-associated pneumonia. *Journal of clinical laboratory analysis*, *37*(1), e24814. https://doi.org/10.1002/jcla.24814

25. Kim, E. B., & Marco, M. L. (2014). Nonclinical and clinical Enterococcus faecium strains, but not Enterococcus faecalis strains, have distinct structural and functional genomic features. *Applied and environmental microbiology*, *80*(1), 154–165. https://doi.org/10.1128/AEM.03108-13

26. Kojic, M., & Venturi, V. (2001). Regulation of rpoS Gene Expression in Pseudomonas: Involvement of a TetR Family Regulator. *Journal of Bacteriology*, *183*(12), 3712–3720. https://doi.org/10.1128/JB.183.12.3712-3720.2001

27. Lander, E. S., & Waterman, M. S. (1988). Genomic mapping by fingerprinting random clones: A mathematical analysis. *Genomics, 2*(3), 231-239. https://doi.org/10.1016/0888-7543(88)90007-9

28. Liang, C., Rios-Miguel, A. B., Jarick, M., Neurgaonkar, P., Girard, M., François, P., Schrenzel, J., Ibrahim, E. S., Ohlsen, K., & Dandekar, T. (2021). Staphylococcus aureus Transcriptome Data and Metabolic Modelling Investigate the Interplay of Ser/Thr Kinase PknB, Its Phosphatase Stp, the glmR/yvcK Regulon and the cdaA Operon for Metabolic Adaptation. *Microorganisms*, *9*(10), 2148. https://doi.org/10.3390/microorganisms9102148

29. Lenski, R. E., Rose, M. R., Simpson, S. C., & Tadler, S. C. (1991). Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000

Generations. *The American Naturalist*, *138*(6), 1315–1341. https://doi.org/10.1086/285289

30. Liu, S., Fang, R., Zhang, Y., Chen, L., Huang, N., Yu, K., Zhou, C., Cao, J., & Zhou, T. (2021). Characterization of resistance mechanisms of Enterobacter cloacae Complex co-resistant to carbapenem and colistin. *BMC microbiology*, *21*(1), 208. https://doi.org/10.1186/s12866-021-02250-x

31. Liu, S., Skory, C., Liang, X., Mills, D., & Qureshi, N. (2019). Increased ethanol tolerance associated with the pntAB locus of Oenococcus oeni and Lactobacillus buchneri. *Journal of Industrial Microbiology & Biotechnology*, *46*(11), 1547–1556. https://doi.org/10.1007/s10295-019-02209-y

32. Liu, W. Y., Wong, C. F., Chung, K. M., Jiang, J. W., & Leung, F. C. (2013). Comparative genome analysis of Enterobacter cloacae. *PloS one*, *8*(9), e74487. https://doi.org/10.1371/journal.pone.0074487

33. Lovering et al. 2010; Clifton et al. 2013. Attachment efficiency of gold nanoparticles by Gram-positive and Gram-negative bacterial strains governed by surface charges: *Differences between Gram-positive and Gram-negative bacterial cell walls.* Research Gate. https://www.researchgate.net/figure/Differences-between-Gram-positive-and-Gram-negative-bacterial-cell-walls-Lovering-et-al_fig4_335226853

34. Maddamsetti, R. (2021). Selection Maintains Protein Interactome Resilience in the Long-Term Evolution Experiment with Escherichia coli. *Genome Biology and Evolution*, *13*(6), evab074. https://doi.org/10.1093/gbe/evab074

35. Mai-Prochnow, A., Clauson, M., Hong, J., & Murphy, A. B. (2016). Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Scientific Reports*, *6*(1), 38610. https://doi.org/10.1038/srep38610

36. Mancuso, G., Midiri, A., Gerace, E., & Biondo, C. (2021). Bacterial Antibiotic Resistance: The Most Critical Pathogens. *Pathogens (Basel, Switzerland)*, *10*(10), 1310. https://doi.org/10.3390/pathogens10101310

37. Masoud, S. S., Kovacevich, A., Gangji, R., Nyawale, H., Nyange, M., & Ntukula, A. (2022). Extent and Resistance Patterns of ESKAPE Pathogens Isolated in Pus Swabs from Hospitalized Patients. *Canadian Journal of Infectious Diseases & Medical Microbiology*, 1–7. https://doi.org/10.1155/2022/3511306

38. Maynard, J., & Haigh, J. (2007). The hitch-hiking effect of a favourable gene. *Genetics Research*, *89*(5–6), 391–403. https://doi.org/10.1017/S0016672308009579

39. Microsoft Corporation. (2018). *Microsoft Excel*. Retrieved from https://office.microsoft.com/excel

40. Motiwala T, Mthethwa Q, Achilonu I, Khoza T. ESKAPE Pathogens: Looking at Clp ATPases as Potential Drug Targets. *Antibiotics*. 2022; 11(9):1218. https://doi.org/10.3390/antibiotics11091218

41. Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Frontiers in Microbiology*, *10*, 539. https://doi.org/10.3389/fmicb.2019.00539

42. Nocera, F. P., Ferrara, G., Scandura, E., Ambrosio, M., Fiorito, F., & De Martino, L. (2021). A Preliminary Study on Antimicrobial Susceptibility of Staphylococcus spp. And Enterococcus spp. Grown on Mannitol Salt Agar in European Wild Boar (Sus scrofa) Hunted in Campania Region-Italy. *Animals: An Open Access Journal from MDPI*, *12*(1), 85. https://doi.org/10.3390/ani12010085

43. Opentrons. (2023). OT-2 Robot [Image]. Opentrons. https://shop.opentrons.com/ot-2-robot/

44. Paauw, A., Caspers, M. P., Schuren, F. H., Leverstein-van Hall, M. A., Delétoile, A., Montijn, R. C., Verhoef, J., & Fluit, A. C. (2008). Genomic diversity within the Enterobacter cloacae complex. *PloS one*, *3*(8), e3018. https://doi.org/10.1371/journal.pone.0003018

45. Paczosa, M. K., & Mecsas, J. (2016). Klebsiella pneumoniae: Going on the Offense with a Strong Defense. *Microbiology and Molecular Biology Reviews*, *80*(3), 629–661. https://doi.org/10.1128/MMBR.00078-15

46. Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). Acinetobacter baumannii: Emergence of a Successful Pathogen. *Clinical Microbiology Reviews*, *21*(3), 538–582. https://doi.org/10.1128/CMR.00058-07

47. Perveiler, Vaughn, "Investigating the development of alcohol tolerance by the opportunistic pathogen Staphylococcus aureus" (2022). *West Chester University Master's Theses*. 257. https://digitalcommons.wcupa.edu/all_theses/257

48. Pidot, S. J., Gao, W., Buultjens, A. H., Monk, I. R., Guerillot, R., Carter, G. P., Lee, J. Y. H., Lam, M. M. C., Grayson, M. L., Ballard, S. A., Mahony, A. A., Grabsch, E. A., Kotsanas, D., Korman, T. M., Coombs, G. W., Robinson, J. O., Gonçalves da Silva, A., Seemann, T. Howden, B. P., … Stinear, T. P. (2018). Increasing tolerance of hospital Enterococcus faecium to handwash alcohols. *Science Translational Medicine*, *10*(452), eaar6115. https://doi.org/10.1126/scitranslmed.aar6115

49. Priyamvada, P., Debroy, R., Anbarasu, A., & Ramaiah, S. (2022). A comprehensive review on genomics, systems biology and structural biology approaches for combating antimicrobial resistance in ESKAPE pathogens: Computational tools and recent

advancements. *World Journal of Microbiology and Biotechnology*, *38*(9), 153. https://doi.org/10.1007/s11274-022-03343-z

50. Qin, S., Xiao, W., Zhou, C., Pu, Q., Deng, X., Lan, L., Liang, H., Song, X., & Wu, M. (2022). Pseudomonas aeruginosa: Pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. *Signal Transduction and Targeted Therapy*, *7*(1), Article 1. https://doi.org/10.1038/s41392-022-01056-1

51. RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

52. Rahav-Manor, O., Carmel, O., Karpel, R., Taglicht, D., Glaser, G., Schuldiner, S., & Padan, E. (1992). NhaR, a protein homologous to a family of bacterial regulatory proteins (LysR), regulates nhaA, the sodium proton antiporter gene in Escherichia coli. *The Journal of Biological Chemistry*, *267*(15), 10433–10438.

53. Ramijan, K., Ultee, E., Willemse, J., Zhang, Z., Wondergem, J. A. J., van der Meij, A., Heinrich, D., Briegel, A., van Wezel, G. P., & Claessen, D. (2018). Stress-induced formation of cell wall-deficient cells in filamentous actinomycetes. *Nature Communications*, *9*, 5164. https://doi.org/10.1038/s41467-018-07560-9

54. Ren, Y., Ren, Y., Zhou, Z., Guo, X., Li, Y., Feng, L., & Wang, L. (2010). Complete genome sequence of Enterobacter cloacae subsp. cloacae type strain ATCC 13047. *Journal of bacteriology*, *192*(9), 2463–2464. https://doi.org/10.1128/JB.00067-10

55. Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011). Integrative genomics viewer. *Nature biotechnology*, *29*(1), 24–26. https://doi.org/10.1038/nbt.1754

56. Roche, F. M., Massey, R., Peacock, S. J., Day, N. P. J., Visai, L., Speziale, P., Lam, A., Pallen, M., & Foster, T. J. (2003). Characterization of novel LPXTG-containing proteins of Staphylococcus aureus identified from genome sequences. *Microbiology*, *149*(3), 643–654. https://doi.org/10.1099/mic.0.25996-0

57. Rohde M. (2019). The Gram-Positive Bacterial Cell Wall. *Microbiology spectrum*, *7*(3), 10.1128/microbiolspec.GPP3-0044-2018. https://doi.org/10.1128/microbiolspec.GPP3-0044-2018

58. Rozman, U., Duh, D., Cimerman, M., & Turk, S. Š. (2022). Hygiene of Medical Devices and Minimum Inhibitory Concentrations for Alcohol-Based and QAC Disinfectants among Isolates from Physical Therapy Departments. *International Journal of Environmental Research and Public Health*, *19*(22), 14690. https://doi.org/10.3390/ijerph192214690

59. Saha, S., Lach, S. R., & Konovalova, A. (2021). Homeostasis of the Gram-negative cell envelope. *Current Opinion in Microbiology*, *61*, 99–106. https://doi.org/10.1016/j.mib.2021.03.008

60. Santajit, S., & Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed research international*, *2016*, 2475067. https://doi.org/10.1155/2016/2475067

61. Schuster, M., Hawkins, A. C., Harwood, C. S., & Greenberg, E. P. (2004). The Pseudomonas aeruginosa RpoS regulon and its relationship to quorum sensing. *Molecular Microbiology*, *51*(4), 973–985. https://doi.org/10.1046/j.1365-2958.2003.03886.x

62. Sielaff, H., Duncan, T. M., & Börsch, M. (2018). The regulatory subunit ε in Escherichia coli FOF1-ATP synthase. *Biochimica et Biophysica Acta. Bioenergetics*, *1859*(9), 775–788. https://doi.org/10.1016/j.bbabio.2018.06.013

63. Silhavy, T. J., Kahne, D., & Walker, S. (2010). The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology*, *2*(5), a000414. https://doi.org/10.1101/cshperspect.a000414

64. Steenackers, H. P., Parijs, I., Foster, K. R., & Vanderleyden, J. (2016). Experimental evolution in biofilm populations. *FEMS Microbiology Reviews*, *40*(3), 373–397. https://doi.org/10.1093/femsre/fuw002

65. Sun, S., & Zhou, J. (2018). Molecular mechanisms underlying stress response and adaptation. *Thoracic cancer*, *9*(2), 218–227. https://doi.org/10.1111/1759-7714.12579

66. Taylor, T. A., & Unakal, C. G. (2023). Staphylococcus Aureus. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK441868/

67. Toesca, I., Perard, C., Bouvier, J., Gutierrez, C., & Conter, A. (2001). The transcriptional activator NhaR is responsible for the osmotic induction of osmCp1, a promoter of the stress-inducible gene osmC in Escherichia coli. *Microbiology*, *147*(10), 2795–2803. https://doi.org/10.1099/00221287-147-10-2795

68. Wickham H (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

69. Wilson, M. G., & Pandey, S. (2023). Pseudomonas Aeruginosa. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK557831/

70. Xavier, B. B., Coppens, J., De Koster, S., Rajakani, S. G., Van Goethem, S., Mzougui, S., Anantharajah, A., Lammens, C., Loens, K., Glupczynski, Y., Goossens, H., & Matheeussen, V. (2021). Novel vancomycin resistance gene cluster in *Enterococcus faecium* ST1486, Belgium, June 2021. *Euro surveillance : bulletin Europeen sur les*

maladies transmissibles = European communicable disease bulletin, *26*(36), 2100767. https://doi.org/10.2807/1560-7917.ES.2021.26.36.2100767

71. Yang, Y., Qian, M., Yi, S., Liu, S., Li, B., Yu, R., Guo, Q., Zhang, X., Yu, C., Li, J., Xu, J., & Chen, W. (2016). Monoclonal Antibody Targeting Staphylococcus aureus Surface Protein A (SasA) Protect Against Staphylococcus aureus Sepsis and Peritonitis in Mice. *PLOS ONE*, *11*(2), e0149460. https://doi.org/10.1371/journal.pone.0149460

72. Yeung, Y. W. S., Ma, Y., Liu, S. Y., Pun, W. H., & Chua, S. L. (2022). Prevalence of alcohol-tolerant and antibiotic-resistant bacterial pathogens on public hand sanitizer dispensers. *The Journal of Hospital Infection*, *127*, 26–33. https://doi.org/10.1016/j.jhin.2022.05.017

Appendix 1: Equations

Equation 1: Number of bacteria (CFU/ml)

$$\frac{\#\ of\ CFUs}{(dilution\ factor)\ x\ \left(volume\ plated(ml)\right)}$$

Equation 2: Percent survival of bacteria following ethanol exposure

$$S = \frac{C_f}{C_i} x\ 100$$

C_i = initial concentration (CFU/ml)

C_f = final concentration (CFU/ml)

S = percent survival