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Antibiotics against bovine mastitis pathogens: resistance and novel targets

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Abstract

Bovine mastitis is the most frequent disease of dairy cattle and the major cause of antibiotic usage in dairy production. It causes impaired animal wellbeing, economical losses and food waste all over the world. This thesis contains two subprojects related to antibiotics and antibiotic resistance among mastitis-associated bacteria.

The microbiota of the healthy udder and different pathogens associated with mastitis have been studied previously, but there is still limited knowledge about antibiotic resistant bacteria in this environment. In this work, resistant bacteria of the healthy udder have been investigated by screening milk samples for isolates resistant to penicillin G and amoxycillin-clavulanic acid, two antibiotics commonly used to treat bovine mastitis. To identify these bacteria, MALDI-TOF MS and Illumina 16S rRNA gene sequencing, were used. Resistant strains of common mastitis pathogens, such as *Staphylococcus aureus* and *Corynebacterium bovis*, environmental bacteria, such as *Escherichia coli* and *Enterococcus faecalis,* and bacteria known to be part of the healthy microbiota, such as *Staphylococcus thermophilus* were identified. The minimal inhibitory concentrations of the antibiotics of some of these strains were also found, resulting in surprising findings with some strains displaying very high MIC_{50}-values.

Another aspect of bovine mastitis that were studied was how genes of the major mastitis pathogen *S. aureus* are involved in susceptibility towards the commonly used antibiotics penicillin G and trimethoprim-sulfamethoxazole (TMP-SMX). For this purpose, a genome-wide CRISPRi-library was used to downregulate each gene and investigate the fitness effect of every gene in *S. aureus* during exposure to antibiotics. The method was found to be a useful tool for screening all the genes of *S. aureus*. However, this work also illustrated the importance of doing follow-up experiments to verify these findings. Several genes already known to be involved in tolerance to antibiotics were found, such as *glmS*, *feoB* and *pbp4*, which are involved in penicillin G resistance. Additionally, new genes were found to make the cell more sensitive to the antibiotics when downregulated. For instance, genes putatively involved in DNA metabolism, either by uptake of nucleoside (*nupC* and *nupG*), DNA repair (*ung* and *polA*) or DNA segregation (*noc*), were found to be essential upon TMP-SMX treatment. Moreover, a novel gene, not yet characterized, SAOUHSC_02121 was found to make the cell more sensitive to TMP-SMX when depleted. This gene´s functionality should be investigated further.

Sammendrag

Mastitt er den mest utbredte sykdommen blant melkekyr og den vanligste årsaken til antibiotikabruk i melkeproduksjon. Sykdommen forårsaker nedsatt dyrevelferd, økonomiske tap og matsvinn over hele verden. I denne oppgaven har to delprosjekter relatert til antibiotika og antibiotikaresistens blant mastitt-assosierte bakterier blitt gjennomført.

Mikrobiotaen i friske jur og patogener knyttet til mastitt har blitt studert tidligere, men det er fremdeles lite kunnskap om antibiotikaresistente bakterier i dette miljøet. I dette arbeidet har resistente stammer fra friske jur blitt undersøkt ved å screene melkeprøver for bakterier resistente for penicillin G og amoxicillin-klavulansyre, to antibiotika som er vanlige å bruke i behandling av mastitt. For å identifisere disse bacteriene ble , MALDI-TOF MS og Illumina 16S rRNA gensekvensering, benyttet. Vanlige mastitt-patogener, som *Staphylococcus aureus* og *Corynebacterium bovis,* miljøbakterier, som *Escherichia coli* og *Enterococcus faecalis,* og bakterier kjent for å være en del av frisk mikrobiota, som *Staphylococcus thermophilus* ble funnet å være resistente bakterier i melka fra friske kyr. For antibiotikaene brukt ble minste inhiberende konsentrasjoner (MIC) også funnet, noe som resulterte i overaskende funn der noen stammer hadde svært høye MIC_{50}-verdier.

Videre ble også *S. aureus,* et viktig mastitt-patogen studert ved å undersøke hvordan genene påvirker følsomheten for de ofte brukte antibiotikaene penicillin G og trimetoprim-sulfametoksazol (TMP-SMX). Til dette formålet ble et helgenom CRISPRi-bibliotek benyttet, for å nedregulere hvert gen, slik at genets effekt på cellens fitness ved påvirkning av antibiotika kan undersøkes. Denne metoden viste seg å være en nyttig metode for å screene alle genene til *S. aureus*. Samtidig viste dette arbeidet viktigheten av å gjøre oppfølgingsforsøk for å verifisere funnene fra screeningen. Flere gener som allerede er kjent for å være involvert i antibiotikaresistens ble funnet, slik som *glm, feoB* og *pbp4*, som alle er involvert i penicillin G resistens. I tillegg ble det funnet nye gener som ved nedregulering gjør cellen mer sensitiv for antibiotika. For eksempel ble gener som er antatt å være involvert i DNA metabolismen funnet å være essensielle ved TMP-SMX-behandling, enten ved opptak av nukleosider (*nupC* og *nupG*), DNA reparasjon (*ung* og *polA*) eller DNA segresjon (*noc*). Dessuten ble et nytt gen, SAOUHSC_02121, som enda ikke har blitt karakterisert, funnet å gjøre cellen mer sensitiv for TMP-SMX ved deplesjon. Spesielt dette genets funksjonalitet burde undersøkes videre.

Table of content

Abbreviations

AMC	Amoxycillin-clavulanic acid
aTc	Anhydrotetracycline
BHI	Brain Heart Infusion
Cam	Chloramphenicol
Cas9	CRISPR associated protein 9
CFU	Colony forming units
CNS	Coagulase negative staphylococci
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
dCas9	Catalytically inactive Cas9 protein
ddNTP	Di-deoxynucleotide triphosphate
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
dTTP	Thymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EM	Erlen Meyer
LA	Lysogeny agar
LB	Lysogeny broth
Log2FC	Log_2 fold change
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass
MIC	minimal inhibitory concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
Nup	Nucleoside permease
OD	Optical density
PAM	Protospacer adjacent motif
PBP	penicillin-binding-protein
PCR	Polymerase chain reaction
PenG	Penicillin G
PMA	Propidium Monoazide
qPCR	Quantitative PCR
RNA	ribonucleic acid
S.O.C.	Super Optimal broth with Catabolite repression
SCC	Somatic cell count
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDS	Sodium dodecyl sulfate
sgRNA	Single guide RNA
SMX	Sulfamethoxazole
ssDNA	Single stranded DNA
TK	Thymidine kinase
TMP	Trimethoprim
tracrRNA	Trans-activating CRISPR RNA
TS	Thymidylate synthase
TSASB	Tryptic soy agar with sheep blood
TSB	Tryptic soy broth
UDG	Uracil-DNA glycosylase
UHT	Ultra high temperature

1 Introduction

1.1 Bovine mastitis

Mastitis is the most frequent disease of dairy cattle and has likely been around since humans first started milking cows 3100 B.C. It is an inflammation of the mammary gland, caused by infectious microbes or trauma. This disease affects the animal wellbeing and dairy farm profitability as it causes decreased milk production and quality, veterinarian costs and premature culling (1). Infectious mastitis evolves by organisms invading the udder through the teat canal. There they colonize the secretory cells and produce toxic substances in the milk. This creates inflammation in the udder and the teat with release of leucocytes which affects the milk quality (2).

Bovine mastitis is classified as either clinical or subclinical, based on the degree of inflammation (3). Clinical mastitis is recognized with a sudden onset of visible abnormalities such as a swollen and red udder, pain, fever and alterations in milk appearance and composition, such as flaky and clotty milk or milk with a watery consistency. The milk production is also lowered during such an infection. Unlike clinical mastitis, subclinical mastitis shows no abnormalities on the udder or the milk but can be detected by an increase in somatic cell count (SCC) in the milk. The increased number of SCCs in the milk is due to the inflammation of the udder, where immune cells are recruited to the site of infection and epithelial cells are shed from the inner surface of the mammary gland to remove intracellular pathogens (4). Even though subclinical mastitis does not show severe symptoms, it causes a decrease in milk production (1). Infected mammary glands do not only affect the milk production and the udder health, but also the overall cow health. It has been established that even modest inflammation affects the fertility of the cow (5).

1.1.1 Udder microbiome

The microbial content of the udder has been discussed among researchers, and for long the milk was considered to be sterile inside the mammary gland. This has been disclaimed, and research has shown that the internal part of the mammary gland has its own microbiome that affects the udder health (6, 7). Mastitis was considered an infection driven only by the interaction between host and pathogens, but lately the involvement of the udder microbiota to the host-pathogen interaction has been taken into account by researchers (6–8). Investigating the bovine udder microbiome is challenging, as external contamination during sampling is a considerable

problem (9). Different sampling strategies can be found in the literature, resulting in different microbes being discovered and considered as part of the bovine udder microbiome. To overcome the contamination problem, Porcellato et al. (2020) did the sampling after the regular milking of the cow to remove contaminants from the outer part (teat cistern) of the udder before sampling the milk from deeper within the udder. This led to low amounts of environmental associated taxas such as *Bacillaceae* and *Pseudomonadaceae*. In their study they found a microbiota dominated by two families - *Corynebacteriaceae* and *Staphylococcaceae* (7). *Corynebacterium*, a part of the human skin microbiome and also found in bovine teat canal and uterus, was found in all the samples in the study of Porcellato et al. (2020) and can therefore be suggested to be part of the udder core microbiome. Also, the *Staphylococcus* genus was abundant in the samples, especially the non-aureus staphylococci. These species are commonly found in bovine milk and in the teat canal and skin (10). The well-known mastitis pathogen *S. aureus* is further discussed in section 1.1.2.1. They also found that the abundance of *Staphylococcus* and *Corynebacterium* was negatively correlated, meaning there must be some interaction between the two. These two genera were found in 98% of the quarter milk samples analyzed by Porcellato et al. (2020) and accounted for almost 50% of the udder microbiota. Other typical genera found in bovine milk are *Bacteroides, Comamonas, Enterococcus, Fusobacterium, Lachnospiraceae, Lactobacillus, Propionibacterium, Pseudomonas, Ruminococcaceae Stenotrophomonas and Streptococcus* (11).

1.1.2 Bovine mastitis pathogens

The organisms causing mastitis are broadly classified as either environmental or contagious. As the name implies, environmental bacteria come from the cow´s environment, e.g., soil, manure and bedding. *Streptococcus uberis*, coliform species as *Escherichia coli* and *Klebsiella* spp., *Enterococcus* spp., *Corynebacterium* and coagulase negative Staphylococci (CNS) are examples of pathogens considered as environmental pathogens (3, 12). To cause mastitis, large amounts of these microbes are necessary (12). The impact of environmental bacteria is controlled by sufficient hygiene of the cows and its surroundings. The other group, contagious bacteria, are transferred from a cow with mastitis to a healthy cow via for example milking equipment, humans, spilled milk and cleaning cloths (12–14). Important contagious pathogens are *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Staphylococcus aureus*. Contagious mastitis is controlled by national mastitis prevention programs and good herd management, including proper maintenance of milking equipment and post-milking teat disinfection (12, 15, 16).

1.1.2.1 *Staphylococcus aureus*

Approximately 20-30% of all humans carry *S. aureus* asymptomatically in their nasopharynx and/or other body sites (17). In addition of being a harmless colonizer of healthy humans, *S. aureus* also causes a variety of infectious diseases in humans and animals, such as skin and soft tissue infections, mastitis, bone, joint and implant infections, pneumonia, and septicemia (18). *S. aureus* is recognized as one of the major agents of contagious bovine mastitis and is the main reason for therapeutic and prophylactic use of antibiotics on dairy farms. *S. aureus* has a great advantage of being able to form biofilms and invade mammary cells, and in this way protect itself from the cow´s immune system and antibiotic treatment (19–21). *S. aureus* does not initiate an immune response as strong as, for example *E. coli* and endotoxin producing organisms. Therefore, the symptoms of staphylococcal mastitis are milder, and the disease often turn chronic. The toxins and enzymes produced by *S. aureus* such as enterotoxins, hemolysins, and leucocidins help the bacteria to colonize and cause irreversible damage to the milking tissue, which in the end will decrease milk production (3, 22). Milk is a good medium for growth of *S. aureus*, that can hydrolyze casein and ferment lactose (23). The use of antibiotics, especially β-lactams, have been the go-to option for treating *S. aureus* mastitis for decades, which has contributed to the global challenge of antibiotic resistance.

1.1.3 Treatment of bovine mastitis in Norway

Development of antibiotic resistance challenge our ability to cure infectious diseases, both in humans and in animals. Mastitis is no exception to this. Choice of antibiotics for treatment of bovine mastitis is based on microbial examination, resistance determination, evaluation of the effectivity of previous treatments as well as the health and health history of the cow and the herd (24). Often, knowledge about sensitivity of the predominant strains, at herd-level, can be used when deciding for treatment of clinical mastitis. For subclinical mastitis, the treatment can be postponed until results on the cow-level sensitivity testing is available (25). In Norway, β-lactamase sensitive penicillins are by far the most frequently used antibiotic for cattle (Figure *1.1*) as a majority of the Gram-positive coccus are sensitive to penicillin (26). For infections caused by penicillin sensitive bacteria, intramuscular penicillin is the initial choice. Then, from day 1 to day 5, penicillin is given intramammary. If the infection is caused by a penicillin resistant *Staphylococcus,* the combinations trimethoprim-sulfamethoxazole (TMP-SMX) and amoxycillin-clavulanic acid (AMC) are used. TMP-SMX is given systemically, while AMC is

given intramammary from day 1 to 5. For other causes of mastitis, other treatment regimens are chosen, and in some cases, antibiotics are not the preferred treatment, for example for mild to moderate infections caused by most environmental microbes. In worst case, the only solution is culling of the infected cows (24).

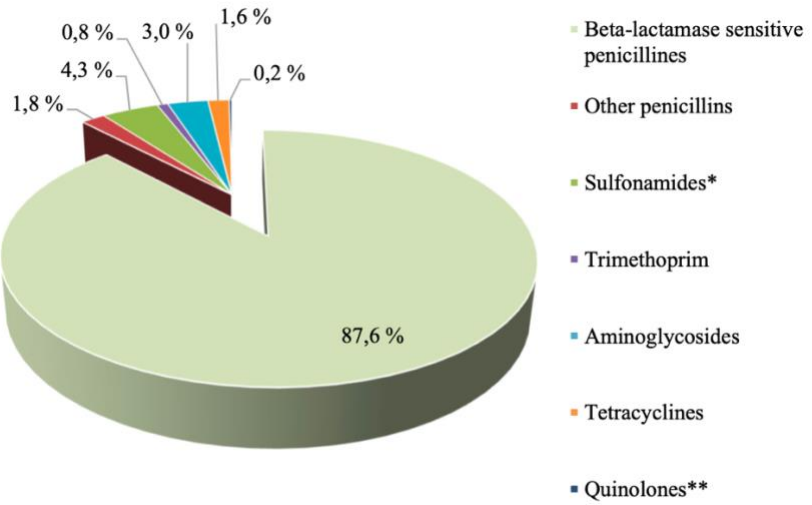

Figure 1.1: Prescribing patters for antibacterial medicines for cattle in Norway in 2020. Data from the Veterinary Prescription Register (not including intramammaries). * Only in combination with trimethoprim. **Only fluoroquinolones. Figure from NORM-VET 2020 (26).

Whether the cow is cured from mastitis or not, depends on the individual animal, the pathogen, and the treatment factors. The likelihood of being cured decrease with increasing age, duration of infection, increasing somatic cells in the milk, and number of quarters of the udder that is infected (25). An important factor regarding the pathogen is whether the infecting agent is resistant to antibiotics or not. An infection with penicillin-resistant *S. aureus* decreases cure rate when treating with either β-lactam or non-β-lactam antibiotics. Treatment duration is the most important treatment factor. A higher treatment duration results in increased chance to cure. These factors must be considered when choosing mastitis therapy and the duration of the treatment. For example, treatment of older cows, with chronic infection of penicillin-resistant strains should be discouraged (25).

1.1.4 Antibacterial resistance in bovine mastitis

The NORM-VET (Norsk overvåkingsprogram for antibiotikaresistens i mikrober fra fôr, dyr og næringsmidler) program was established by the Norwegian Ministry of Health and Social Affairs in 2000 to monitor the usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway as part of the action plan to reduce antimicrobial resistance. The target set

in 2015 was a 10% reduction in antibiotic use for food-producing animals (except fish) by 2020 compared to 2013. The sales (in kg) of antibacterial veterinary medicines declined by 23% from 2013 to 2020, and when measured in mg per population correction unit (mg/PCU) the decline was 18% in the same period.

In the NORM-VET study of 2015, 170 cattle herds were screened for MRSA. The suspected colonies from the sample incubation and preparation were identified by PCR for the detection of the *mecA/nuc* genes (*mecA* is discussed in section 1.2.3.1 and *nuc* is a *S. aureus* specific marker gene (27)). Only one positive sample was detected (28). This result is quite a contrast to other countries, such as Belgium, where one study found 9.3% of *S. aureus* isolates from bovine mastitis to be MRSA (29, 30). The presence of other *S. aureus* resistance mechanisms to β-lactams are also very low in Norway. According to Helsekortordningen for 2020, only 0.71% of the strains identified from teat samples of clinical mastitis cattle, were penicillin resistant *S. aureus*. In 2010 the percentage was 2.8% (31). The situation is also totally contrary in other parts of the world; in a Chinese study by Cheng et al. (2018), 541 Chinese mastitis isolates of the five most common mastitis species (*S. aureus,* non-*aureus* staphylococci, *Streptococcus* spp., *Klebsiella* spp. and *E. coli*), revealed a multidrug resistance of 27% (32). Even though the antibacterial resistance remains low in Norway, the emergence of resistance worldwide and among animal pathogens are of large concern as it can compromise the antimicrobial efficacy of our defense against potential lethal infections both in humans and animals and thereby public health.

1.2 Antibiotics and resistance

The discovery and introduction of antibiotics were one of the greatest medical breakthroughs of the 1900´s. In addition to treating infectious diseases, it made modern medical procedures as surgery, cancer treatment and organ transplant possible (33). Shortly after antibiotics got into use for treating bacterial diseases in human, veterinary medicine followed. Already in the beginning of the 1940´s, infusions of penicillin were used for treating mastitis in lactating animals (34). Since then, antibiotics with different properties and mechanisms of actions have been identified and developed. One such mechanism is the inhibition of protein synthesis, examples of antibiotics being aminoglycoside, chloramphenicol, macrolides, streptozotocin, and tetracycline. Another mechanism is inhibition of DNA or RNA synthesis as quinolone,

rifampin, sulfonamides and trimethoprim. β-lactams and glycopeptide are examples of antibiotics that damage or inhibit the synthesis of the bacterial cell wall (35).

1.2.1 β-lactam antibiotics

Both penicillin and amoxycillin, two antibiotics used in this work, are β-lactam antibiotics in the class penicillins. The key structural feature of the β-lactams is the β-lactam ring (indicated in Figure *1.2*) that is critical for the mechanism of these compounds which inhibit normal cell wall synthesis. An important structure of the cell wall is the peptidoglycan layer that surrounds the plasma membrane and is essential for maintaining the structural integrity of the bacterial cell and prevention of osmotic lysis (36). Gram-negative bacteria have a cytoplastic membrane, covered by a peptidoglycan layer and an outer membrane consisting of lipopolysaccharides (LPS). Gram-positive bacteria, such as *S. aureus*, lack this protective LPS membrane but have a much thicker peptidoglycan layer compared to Gram-negative bacteria (37). Peptidoglycan synthesis is essential for viability and is highly conserved in both Gram-positive and -negative bacteria and is therefore the target of many clinically used antibiotics (38). The peptidoglycan layer consists of chains of altering N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) coupled by a β-1-4-linkage (36). The chains are cross-linked by short peptides (38). The formation of these cross-links is catalyzed by transpeptidases called penicillin-binding-proteins (PBPs), which are the targets of the β-lactams (37, 39). The structure of β-lactams is an analogue to the peptide side chain of the peptidoglycan. The β-lactam bond (CO-N) in the β-lactam ring lies on the same position as the CO-N in the side chain, and the bacterial PBP will therefor hydrolyze this bond of the β-lactam and bind covalently to the β-lactam (Figure *1.2*). This results in inhibition of the PBP (40, 41). Susceptibility to β-lactams is determined by the β-lactam´s affinity for the PBPs and its ability to diffuse through the cell wall. Due to its thick layer of lipopolysaccharides, Gram-negative bacteria has a decreased level of penetration by β-lactams to the cell wall, and are in general less susceptible to β-lactams compared to Gram-positive bacteria, although there also exist β-lactams targeting primarily Gram-negative bacteria. (42).

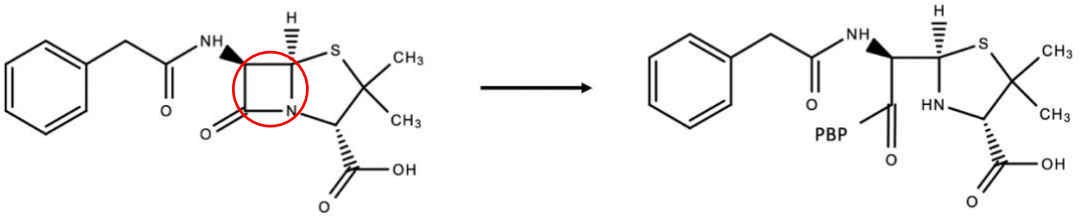

Figure 1.2: Chemical structure of penicillin and the hydrolyzation and binding of transpeptidase to penicillin, resulting in inhibition of transpeptidase. The β-lactam ring is indicated by the red circle.

1.2.1.1 Penicillin

Penicillin was first discovered by Alexander Flemming in 1928 by coincidence. A substrate produced by the fungus *Penicillium* inhibited the growth of *S. aureus*. A couple of decades later, the substrate´s potential for medical use was brought to realization (43). The first discovered penicillin, benzylpenicillin, also called penicillin G, is categorized as a natural penicillin, and is still the initial drug of choice when treating many bacterial infections (42, 44).

1.2.1.2 Amoxycillin-clavulanic acid

Amoxycillin-clavulanic acid (AMC), acid is a well-established combination of the antimicrobial agent amoxycillin and the β-lactamase inhibitor clavulanic acid. It is a moderate-spectrum antibiotic with activity against a wide range of Gram-positive and -negative bacteria, including infections such as middle ear infections, urinary tract infections and pneumonia in humans, and penicillin-resistant bovine mastitis (24, 45, 46). Amoxycillin (α-amino-*p*-hydroxybenzyl-penicillin) is classified as an aminopenicillin, a semi synthetic penicillin containing an amino group (marked on Figure *1.3*A). The amino group causes a polar charge and thereby activity against Gram-negative microbes (44, 47). Amoxycillin differs from the other aminopenicillin, ampicillin, by having a hydroxyl group on the benzene side chain (47).

Clavulanic acid is an irreversible inhibitor of β-lactamases, including those of staphylococci. β-lactamases are further explained in section 1.2.3.1. Clavulanic acid protects the amoxycillin against inactivation by having structural similarities to β-lactams (Figure *1.3*B) and therefor binds to β-lactamases instead of amoxycillin. It has some antibacterial effect alone, but in combination with amoxycillin, even β-lactamase producing amoxycillin-resistant bacteria experience increased susceptibility towards amoxycillin, including *S. aureus* (not MRSA). The addition of clavulanic acid does not affect the susceptibility of amoxycillin-sensitive strains (48).

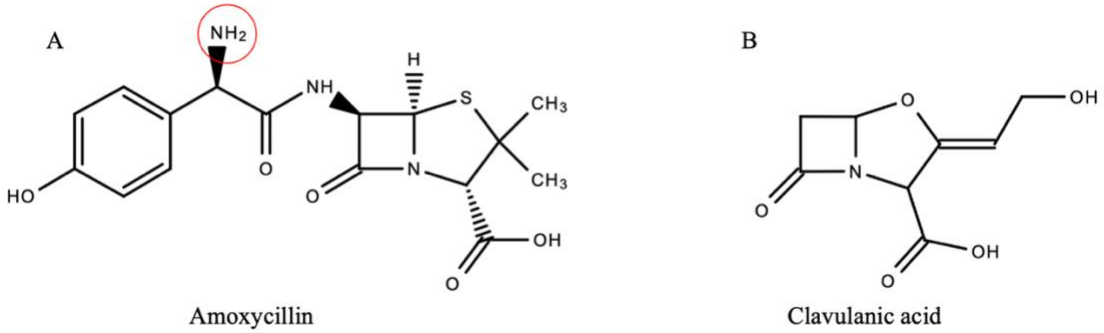

Figure 1.3: Chemical structures of A) amoxycillin and B) clavulanic acid. The red circle indicates the amino group, giving the chemical a polar charge.

1.2.2 Trimethoprim-sulfamethoxazole

The other antibiotic combination used in this work, trimethoprim-sulfamethoxazole (TMP-SMX), are two compounds both targeting the synthesis of the active form of folic acid, tetrahydrofolic acid, and are examples of bacteriostatic antibiotics (49). Contrary to mammalian cells, prokaryotes depends on endogenous synthesis of folic acid (50). TMP-SMX, has mechanisms that targets the synthesis of this essential folic acid.

Sulfamethoxazole is a sulfonamide which is an antimicrobial agent that target the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway (Figure *1.4*). Sulfamethoxazole is a structural analog to p-aminobenzoate (PABA) and will competitively inhibit the synthesis of dihydropteroic acid, and thereby dihydrofolic acid, from PABA.

Trimethoprim is often used in combination with sulfamethoxazole and also targets the folic acid pathway. Trimethoprim, as a partly structural analogue of dihydrofolic acid, inhibits dihydrofolate reductase (DHFR), and the synthesis of tetrahydrofolic acid (Figure *1.4*) (51, 52). Tetrahydrofolic acid is an important co-factor for thymidylate synthase (TS), encoded by *thyA* which in turn is an important precursor of purines, thymidine and DNA. The disrupted biosynthesis of nucleic acid leads to a cessation of cell growth and division. The combination of trimethoprim and sulfamethoxazole is synergic and bactericidal (52), and can be used to treat skin and soft tissue infections, pneumonia, urinary tract infections, pyelonephritis and upper respiratory tract infections in addition to penicillin resistant mastitis (51).

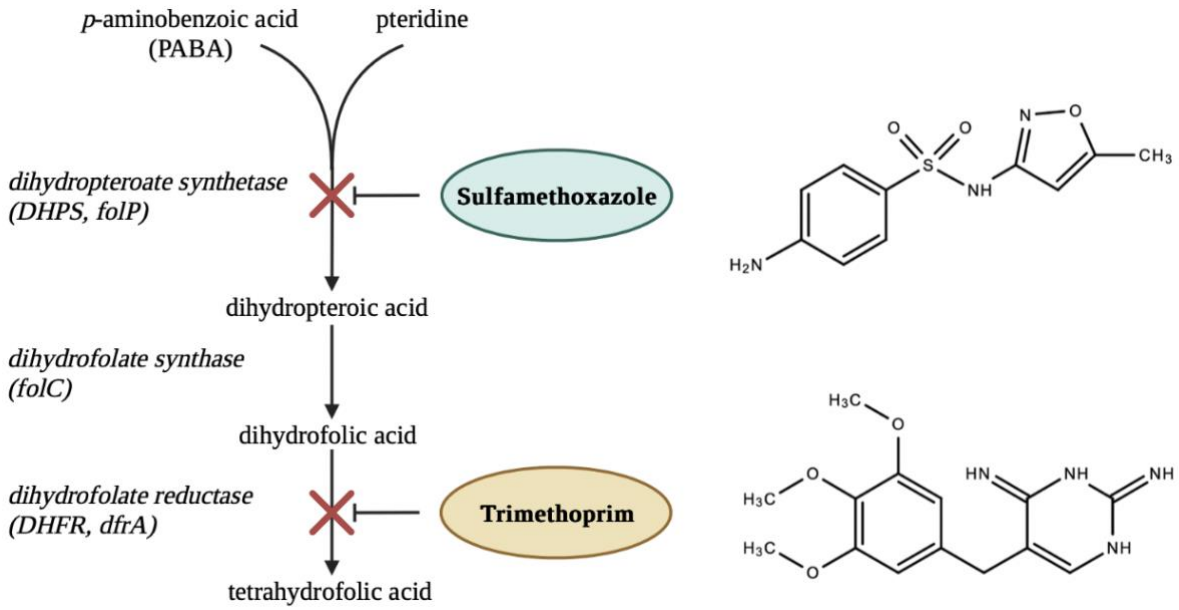

Figure 1.4: Mechanism of action and structures of trimethoprim and sulfamethoxazole.

1.2.3 Resistance mechanisms

The use and overuse of antibiotics have led to a rapid rise of antibiotic resistance. Bacteria do either have an intrinsically resistance towards the action of the antibiotics, for example due to the absence of a susceptible target of the antibiotic, or acquire resistance by genomic mutations or horizontal gene transfer. There are different mechanisms that bacteria use in order to be resistant. These mechanisms are usually grouped into those that minimizes the intracellular concentration of the antibiotic, those that modify the target gene by mutations or post-translational modifications, and those that inactivate the antibiotic (53).

1.2.3.1 Resistance to β-lactams

There are three main mechanisms of resistance to β-lactams: alteration of target site (PBPs), destruction of β-lactam and decreasing the concentration of β-lactams inside the cell. Resistance to penicillin is the most well-known antibiotic resistance of *S. aureus* (25), and an increase of infections caused by penicillin-resistant strains was observed in hospitals in the middle of the 1940s. These strains produced penicillinase, an enzyme that modify the antibiotic by hydrolyzing the β-lactam ring, an essential part of the structure of penicillin (54). β-lactamases, like penicillinase, have likely coevolved with bacteria as a response to naturally occurring antibiotics over time, but the selective pressure exerted by the use of antibiotics in modern medicine has accelerated their development and spread (55). β-lactamases are plasmid

encoded by e.g., the gene *blaZ,* and inhibit the antimicrobial effect of β-lactam antibiotics by hydrolyzing the cyclic amide bond of the β-lactam ring (indicated in Figure *1.5*) (42, 53, 56).

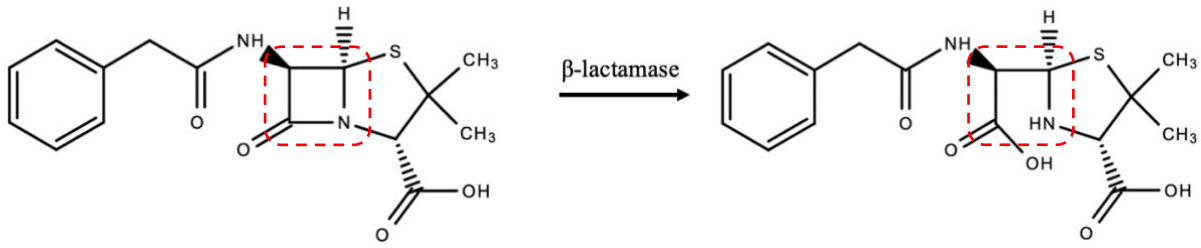

Figure 1.5: Structural illustration of the hydrolyzation of penicillin by β-lactamase.

After the discovery of penicillinase producing bacteria, other β-lactam antibiotics were developed, such as methicillin, cephalosporins and oxacillin, which seemed to tolerate the action of penicillinase (54). Shortly after the introduction of these antibiotics, however, the first cases of methicillin resistant *S. aureus* (MRSA) were detected (18). MRSA is a well-known example of how modification of the target results in resistance. It is the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) with the gene *mecA* that gives rise to MRSA. The *mecA* gene encodes a β-lactam-insensitive protein PBP2a. This protein enables synthesis of cell wall even when the native PBP proteins are inhibited by penicillin (53). Unlike penicillinase-mediated resistance, which is narrow spectrum, methicillin resistance is a broad-spectrum resistance to penicillins, cephalosporins and carbapenems (54). Another way that resistant bacteria alter the target site, is by modification of the existing PBPs, e.g. by creating a mosaic PBP by inserting nucleotides obtained from neighboring bacteria, as done for example by resistant *Streptococcus pneumonia* (55). Also overexpression of the *pbp* gene can result in resistance to β-lactams (57). In addition to alteration of target site and β-lactamase production, bacteria can decrease the concentration of the antibiotic. This can either be done by decreasing the permeability of the cell envelope by reduction of porins, or by the use of efflux pumps (55).

1.2.3.2 Resistance to TMP-SMX

Since both trimethoprim and sulfamethoxazole are synthetic antimicrobial agents, they are not very prone to naturally occurring degradation or alternation (58, 59). Instead, resistance is caused by nonallelic and drug-resistant variants of the drug targets DHPS and DHFR, transferred by plasmids (59). Many different resistance mechanisms to trimethoprim are known. A major mechanism of trimethoprim resistance is the production of an altered DHFR. This

feature is encoded by the *dfrA* and *dfrD* genes located on plasmids and makes trimethoprim unable to bind DHFR (40, 56, 60). Another mechanism is overproduction of the normal, chromosome-encoded DHFR, caused by the *dfrB* gene (40, 56, 61).

Most resistance to sulfonamides is caused by an additional plasmid-encoded DHPS with lowered affinity for sulfonamides, e.g. encoded by the *sulB* gene (40, 56). Resistance can also be caused by increased production of p-aminobenzoic acid (encoded by *pabA*), pteridine or mutations causing sulfonamides resistant DHPS (encoded by *folP*) (40, 56, 62). The mutated variant of DHPS has been found to exhibit a decreased V_{max} and/or a lowered affinity to the substrate, which also cause the overproduction of p-aminobenzoc acid (58).

1.3 CRISPR interference for gene expression studies

Different techniques have been developed for studying genes of various species. The Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) technology used by scientists for gene knockout originates from some bacteria's and archaea's adaptive immune response to foreign DNA elements (63). After being infected by a virus or a phage, spacers derived from phage genomic sequences are integrated into the CRISPR loci of bacteria. The CRISPR loci consist of several non-continuous direct repeats, separated by these variable spacers, homologues to the phage or plasmid sequences. The CRISPR loci are often found adjacent to *cas* genes. One of these encodes Cas9, an endonuclease that cleaves doble stranded DNA (64). The bacterial system also consists of two RNAs which recognized and directs the Cas9 endonuclease to the foreign DNA; a mature CRISPR RNA (crRNA) and partially complementary trans-activating CRISPR RNA (tracrRNA) (65). When binding to the DNA, the Cas9 will make a double stranded break and deactivate the phage-DNA. When applying the CRISPR/Cas9-technology *in vitro*, a small engineered single-guide RNA (sgRNA) replaces the crRNA-tracrRNA-complex. In addition to the sgRNA, a conserved region juxtaposed to the target sequence, the protospacer adjacent motif (PAM) sequence, determines the specificity of the DNA binding (66). What makes the CRISPR technology so practical is the precision and simplicity of the system (small adjustments necessary to target different genes), which makes it less time-consuming and more cost-effective compared to other alternatives.

1.3.1 CRISPR interference

A constraint with the CRISPR technology and other gene modification approaches, is that it cannot be used to investigate essential genes, as the knockout of the gene would be fatal for the cell. A solution to this problem is the CRISPR interference (CRISPRi) technique, that is a powerful silencing method already used in many species and in high throughput screens (67). In CRISPRi, the Cas9 is deactivated, such that it does not cut the double-stranded DNA. Instead, the CRISPR/dCas9 complex binds and prevent transcription by blocking elongation of RNA polymerase at the site where it is designed to bind, usually close to the start codon of a gene of interest (right side of Figure *1.6*). A sgRNA leads the dCas9 to sequence complementary to the sgRNA, next to a PAM. The sgRNAs can be designed to target any desired open reading frame with an appropriate PAM, by changing the 20 nt base-pairing region. The advantage with the CRISPRi system, is that it can down-regulate genes, allowing studies of both essential and non-essential genes by controlling the inducible dCas9 promotors (68).

1.3.2 The CRISPRi-system and genome-wide CRISPRi-library in *S. aureus*

For the CRISPRi-system to work in *S. aureus* strain NCTC8325-4, which does not have a native CRISRP/Cas9-system, both dCas9 and sgRNAs must be expressed (Figure *1.6*). In the designed inducible CRISPRi-system used in the current work (constructed by Marita Torrisen Mårli) the *dCas9* gene is incorporated into the bacterial chromosome downstream of the promotor P_{tet}. In addition, the repressor gene *tetR* and the tetracyclin resistance gene, *tetM* are also integrated on the chromosome. This design allows control of the expression of *dCas9* by using the inducer anhydrotetracycline, aTc. TetR binds to the P_{tet} promotor and inhibits expression, but when binding to TetR, aTc will cause release of TetR from the promotor and the transcription of *dCas9* is thus activated. The sgRNAs in turn, are cloned on the plasmid pVL2336, and expressed from the constitutive promotor P_3. The plasmid also contains a chloramphenicol resistant gene, cam^R, which makes it possible to select strains with adapted chloramphenicol resistance, and thereby the sgRNA.

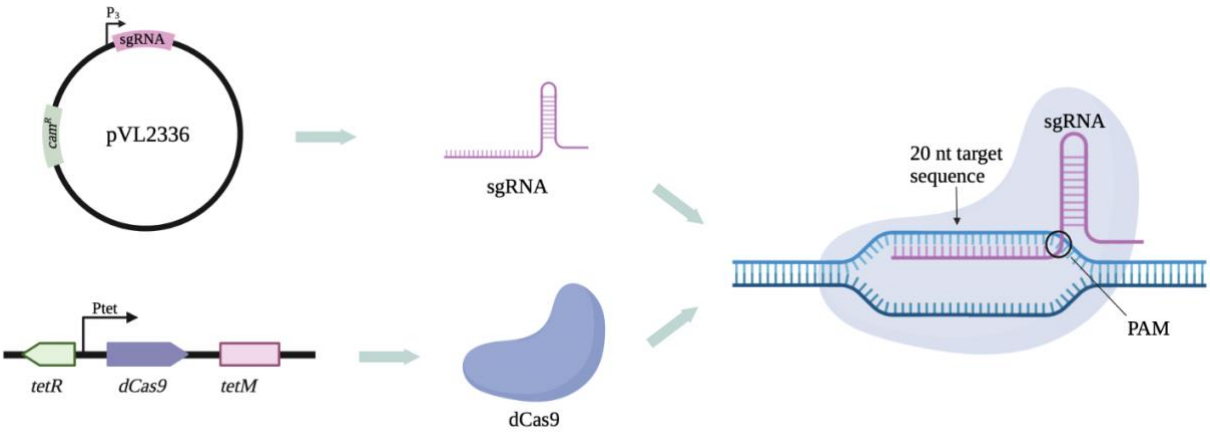

Figure 1.6: Illustration of the CRISPRi-system and the construction of the CRISPRi-library with the plasmid containing the sgRNA gene and the chromosomal dCas9 gene. The dCas9 and sgRNA will form a complex, that will bind the target sequence and by steric hindrance, obstruct replication of this gene.

Based on this CRISPRi-system, a pooled CRISPRi-library of 1928 different strains were prepared by Marita Torrisen Mårli (unpublished). In total 1928 different sgRNAs, together covering almost all of the transcripts of *S. aureus* NTCT8325-4 were cloned into plasmids, that then have been electroporated into the *S. aureus* strain NCTC-8325-4 (Figure *1.7*). This pooled library enables downregulation and investigation of gene fitness for all the genes in this strain simultaneously, since sgRNA counts can reflect the fitness of a gene. I.e., gene essentiality is observed by underrepresentation of the sgRNA encoding this gene in the population of sgRNAs, as illustrated in the bar plot in Figure *1.7*. In this work, the differences in the essentiality of the gene when grown with or without antibiotic treatment, was used to identify genes affecting antibiotic susceptibility.

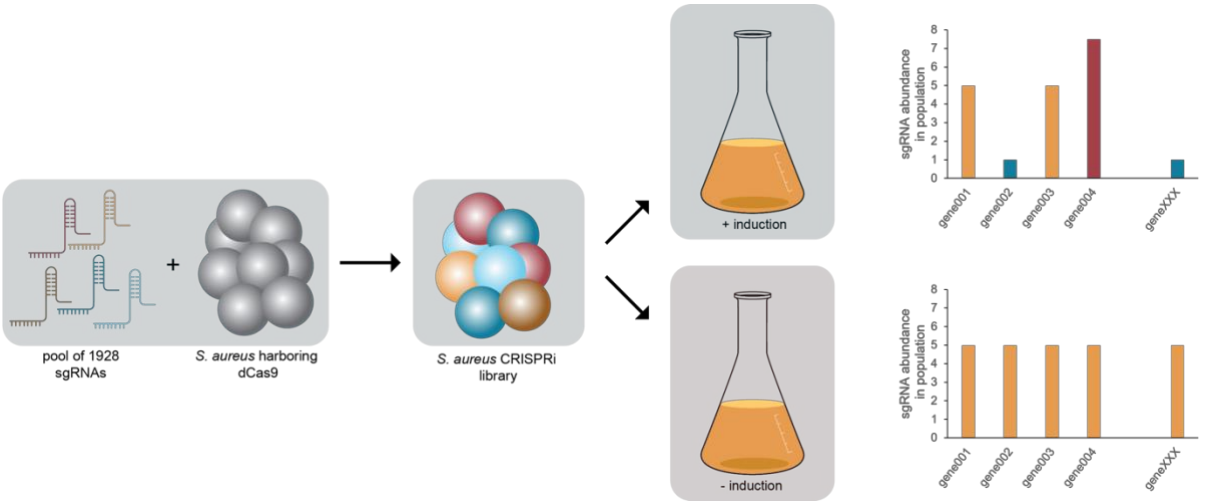

Figure 1.7: Illustration of the CRISPRi-library. 1928 sgRNAs are incorporated in *S. aureus* NCTC8325-4 harboring inducible dCas9. Induction and antibiotic exposure results in different abundances of the sgRNAs related to the essentiality of the genes. Created by Marita Torrisen Mårli.

1.4 Aim of thesis

Bovine mastitis is a prevalent disease in dairy production, causing economical losses, reduced animal welfare and food waste all over the world. Despite this, there is a lack of knowledge about how the disease develop and about the mammary microbiome, especially after antibiotic treatment. In an attempt to close parts of this knowledge gap two subprojects regarding bovine milk microbiota and antibiotic resistance in bovine mastitis were performed.

- Previous studies of the bovine milk microbiome have provided answers regarding the composition of the udder microbial community, but there is a lack of knowledge about the antibiotic resistant strains present. The aim of this project was to get more insight into the occurrence of antibiotic resistant strains of healthy Norwegian cows by screening the microbiota of bovine milk from healthy udders for antibiotic resistant strains. The antibiotics used in this project, penicillin G and the β-lactamase-inhibitor combination AMC, which are commonly used antibiotics to treat bovine mastitis in Norway.
- In the second part, *S. aureus* was specifically studied, and the aim was to get insight into genetic factors that affect the susceptibility towards two of the main antibiotics used to treat bovine mastitis; penicillin G and TMP-SMX. An already-designed genome-wide CRISPRi-library in *S. aureus* was used for this purpose, which allows determination of gene fitness of all genes during antibiotic treatment. The approach will identify genes that modifies the susceptibility towards the antibiotics, and can potentially be used to suggest improved treatment strategies.

2 Materials

2.1 Strains

Table 2.1: List of strains used in this work, with a short description of their relevant genotype and characteristics.

Strain	Genotype and characteristics	Reference
S. aureus		
MM267	NCTC8325-4, tetR_Ptet_dCas9, tetM, pCG248-sgRNA, cam^R	Lab collection
MM268	MM267, but pVL2336(notarget)	Lab collection
AN11	MM267, but pVL2336(polA), cam^R	This work
AN12	MM267, but pVL2336(nupC), cam^R	This work
AN13	MM267, but pVL2336(nupG), cam^R	This work
AN14	MM267, but pVL2336(murB), cam^R	This work
AN15	MM267, but pVL2336(ung), cam^R	This work
AN16	MM267, but pVL2336(02121), cam^R	This work
E. coli		
MK1518	pVL2336, amp^R	Lab collection
IM08B	DH10B, *Δdcm*, P_{help}-*hsdMS*, P_{N25}-*hsdS* (expressing the *S. aureus* CC8 specific methylation genes)	Monk et al., (2015) (69)
AN5	IM08B, but pVL2336(polA), amp^R	This work
AN6	IM08B, but pVL2336(nupC), amp^R	This work
AN7	IM08B, but pVL2336(nupG), amp^R	This work
AN8	IM08B, but pVL2336(murB), amp^R	This work
AN9	IM08B, but pVL2336(ung), amp^R	This work
AN10	IM08B, but pVL2336(02121), amp^R	This work

2.2 Primers and oligoes

Table 2.2: List of primers used in this work.

Name	Sequence 5´-3´
Primers for 16S amplification	
Uni340F	CCTACGGGRBGCASCAG
Bac806R	GGACTACYVGGGTATCTAAT
Primers for validation of sgRNA plasmids	
mk25_pCG248_r_check	AAATCTCGAAAATAATAGAGGGA
mk26_pCG248_f_up_check	GGATAACCGTATTACCGCCT
Oligoes for Golden gate cloning	
polA Forward	TATATGCAAAACCATATACTGCAT
polA Reverse	AAACATGCAGTATATGGTTTTGCA
nupC Forward	TATAAAGAAGAATGGTGGTTGCTT
nupC Reverse	AAACAAGCAACCACCATTCTTCTT
nupG Forward	TATATAAAGAACCATGCTAAAAAC
nupG Reverse	AAACGTTTTTAGCATGGTTCTTTA
murB Forward	TATAGTATAAGTGTATCGTTTTAA
murB Reverse	AAACTTAAAACGATACACTTATAC
ung Forward	TATAGATATATATTTTCCCTATCA
ung Reverse	AAACTGATAGGGAAAATATATATC
SAOUHSC_02121 Forward	TATATTCCAGCCTGGTCATCCTTA
SAOUHSC_02121 Reverse	AAACTAAGGATGACCAGGCTGGAA

2.3 Antibiotics

Table 2.3: List of antibiotics used in this work.

Antibiotics	Product number	Supplier
Amoxicillin trihydrate: potassium clavulanate (4:1), $C_{24}H_{27}KN4O_{10}S$	SMB00607	Sigma-Aldrich
Ampicillin, $C_{16}H_{19}N_3O_4S$	A-9518	Sigma-Aldrich
Chloramphenicol, $C_{11}H_{12}Cl_2N_2O_5$	C-0378	Sigma-Aldrich
Penicillin G, $C_{16}H_{18}N_2O_4S$	13752	Sigma-Aldrich
Spectinomycin, $C_{14}H_{24}N_2O_7$	S6501	Sigma-Aldrich
Sulfamethoxazole, $C_{10}H_{11}N_3O_3S$	S7507	Sigma-Aldrich
Trimethoprim, $C_{14}H_{18}N_4O_3$	T-7883	Sigma

2.4 Kits

Table 2.4: List of kits used in this work.

Kit	Product number/Model	Supplier
DNeasy® UltraClean® Microbial Kit (250)	12224-250	QIAGEN
E.Z.N.A.® Plasmid DNA Mini Kit	D6943-02	Omega Bio-tek
NucleoSpin® Gel and PCR Clean-up	740609.250	Macherey-Nagel
Qubit dsDNA HS Assay Kit	Q32854	Invitrogen
SequalPrep™ Normalization Plate (96) Kit	A1051001 (lot 2319208)	Invitrogen

2.5 Equipment and instruments

Table 2.5: List of laboratory equipment and instruments used in this work.

Equipment/instrument	Product number/Model	Supplier
96-well polysterine microtiter plates	82.1581.001	Starstedt
Electrophoresis cell	Mini-Sub Cell® GT	BioRad
Electroporation device	Gene Pulser™	BioRad
Gel Imager	GelDoc-1000	BioRad
Incubator	INFORS HT minitron	ACD Pharmaceuticals
Magnetic stand - 96	AM10027	Ambion
VITEK® MS-DS slide	410893	BioMérieux
VITEK® MS		BioMérieux
Microtiter plate reader	Synergy H1 hybrid reader Hidex Sense	BioTek® Hidex
Nanodrop	NanoDrop 2000	Thermo Fisher Scientific
PCR machine	ProFlex PCR systems	Applied Biosystems
Plate spinner	PCR Plate Spinner	VWR
qPCR machine	LightCycler® 480 II	Roche
Qubit	Qubit® 2.0 Fluorometer	Invitrogen

2.7 Chemicals and solutions

Table 2.6: List of chemicals and solutions used in this work.

Compound	Start concentration	Product number	Supplier
1 kb DNA ladder	500 µg/mL	N3232s	New England BioLabs
10x NEBbuffer™ 3.1		B7203S	New England BioLabs
Acetic acid, CH_3COOH		1.00063.2500	Merck
Agarose		15510-027	Invitrogen
Agar powder		20767.298	VWR
anhydrotetracycline		37919	Sigma-Aldrich
Bacto™ Tryptic Soy Broth		211825	BD Diagnostics
Bacto™ Yeast Extract		212750	BD Diagnostics
Brain Heart Infusion		CM1135	Oxoid
Bromophenol blue		B-5525	Sigma-Aldich
BsmBI		R0580	New England BioLabs
dNTPs	10 mM	N0447L	NEB
Ethylenediaminetetraacetic acid (EDTA)		20 296.360	VWR
Elution buffer		19086	Qiagen
Evagreen®	20x	31000	Biotium
Glucose, $C_6H_{12}O_6$		10117gK	VWR
Glycerol, $C_3H_8O_3$	85%	1.04094.1000	Merck
HotFirePol ® DNA polymerase	5 U/µL	01-02-01000	Solis BioDyne
HotFirePol ® buffer B2	10x	01-02-01000	Solis BioDyne
Lysostaphin	10 mg/ml	L9043	Sigma-Aldrich
Lysozyme	100 mg/ml	L2979	Sigma-Aldrich
Mag-Bind® TotalPure NGS			Omega Bio-tek
Magnesium chloride, $MgCl_2$	25 mM	M3634 (	Sigma
peqGreen		PEQL37-501	Saveen Werner
PMAxx (Propidium Monoazide)	20mM		Biotium
Potassium chloride, KCl		1.04936.1000	Merck
Q5® Hot Start, High-Fidelity 2X Master Mix	2x	M0494X	BioLabs
Red Taq 2X Mastermix, 1.5 mM $MgCl_2$	2x	5200300-1250	VWR Life Science
Sodium dodecyl sulfat (SDS)		05030	Fluka
Sodium chloride	>=99%	S9625	Sigma-Aldrich
Sucrose, $C_{12}H_{22}O_{11}$		J65148	Alfa Aesar
T4 ligase reaction buffer	10x	B0202S	New England BioLabs
T4 DNA ligase		M0202L	New England BioLabs
Tris, $C_4H_{11}NO_3$		T1503	Sigma-Aldrich
Tris-HCl		T3253	Sigma-Aldrich
Trisodium citrate dihydrate, $Na_3C_6H_5O_7 \cdot 2H_2O$		1.06448.1000	Merck
Tryptone		LP0042	Oxoid
Tryptic soya agar with sheep blood (TSASB)		PB5012A	Oxoid
UHT milk			TINE
VITEK® MS-Formic acid		411072	BioMérieux
VITEK® MS CHCA matrix		411971	BioMérieux

2.8 Growth mediums, buffers and solutions

1% agarose gel

0.5 g agarose was dissolved 50 mL 50x Tris-Acetate-EDTA (TAE) buffer by boiling. 1 µl peqGreen (1 µl/50 mL) was then added. The agarose solution was then poured into a cast, and combs were placed in the gel to create wells. The gel had to set before using it for gel electrophoresis.

1 kb DNA ladder

50 µL DNA ladder, 200 µL 10x loading buffer, 750 µL dH_2O.

6x loading buffer

10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 40% sucrose, 0.01% Bromophenol blue

10xTEN buffer

100mM Tris-HCl, 10 mM EDTA, 1 M NaCl.

50x TAE

424 g Tris base, 57.1 ml Acetic acid, 100 ml 0.5 M EDTA, pH 8.0.
Adjusted to 1 L with dH2O

Brain Heart Infusion (BHI) medium

Autoclaved: 37g/L BHI-powder, 1 L dH_2O.

BHI agar

Autoclaved: 37g/L BHI-powder, 1.2% (w/v) agar powder, 1 L dH_2O.

Citrate water

20g Trisodium citrate dihydrate, 1000 mL dH_2O. The salt was dissolved in water by heating the solution to 45-50°C. The pH was adjusted to 7,5 at 25°C before autoclaving the solution at 121°C for 15 minutes.

Lysogeny agar (LA)

Autoclaved: 10 g/L NaCl, 10 g/L Tryptone, 5 g/L yeast extract and 1.2% (w/v) agar powder.

Lysogeny broth (LB)

Autoclaved: 10 g/L NaCl, 10 g/L Tryptone, 5 g/L yeast extract.

Staphylococcus lysis buffer

40 mM NaOH, 0.2% SDS

Super Optimal broth with Catabolite repression (SOC) medium

Autoclaved: 2 g Tryptone, 0.5 g yeast extract, 333.3 µL 3 M NaCl, 83.3 3 M KCl, 96 mL dH_2O. After autoclaving, 2 mL sterile filtrated 1 M $MgCl_2$ and 1 sterile filtrated mL 2M glucose were added.

Tryptic soy broth (TSB) medium with 0.5 M sucrose

30 g TSB powder dissolved in 1 L dH_2O.

3 Methods

3.1 Growth and storage of bacteria

3.1.1 Growth of bovine milk cultures and isolates picked from tryptic soy agar with sheep blood

The bovine milk samples were grown in ultra-high temperature (UHT) milk with the preferred antibiotics at 37°C. The samples and the cultures were also grown aerobically at 37°C on tryptic soy agar with sheep blood (TSASB) plates. Colonies picked from these agar plates were grown in liquid BHI medium at 37°C. The samples used for MALDI-TOF were also grown on TSASB plates prior to the analysis.

3.1.2 Growth of *S. aureus*

In this work, most experiments were performed in the *S. aureus* strain NTCT8325-4. For the CRISPRi-seq experiment and for the antibiotic susceptibility testing of single CRISPRi strains, the strains were grown in UHT milk and on BHI agar, both supplemented with 5 μg/mL or 10 μg/mL chloramphenicol for selection. The milk cultures were grown in 37°C without shaking, unless otherwise stated, and the plates were incubated anaerobically at 37°C. In other parts of this work, where the NTCT8325-4 was used, liquid BHI medium with 10 μg/mL chloramphenicol was used as growth medium, and the cultures were incubated with shaking.

3.1.3 Growth of *E. coli*

Cloning experiments were performed in the IM08B strain. This strain was grown in lysogeny broth (LB) or on lysogeny agar (LA), both supplemented with 100 μg/mL ampicillin for selection. LB was incubated at 37°C with shaking and LA was incubated aerobically at 37°C.

3.1.4 Long term storage

For all the isolates in this work, frozen stocks for long term storage were prepared by adding glycerol to the overnight culture to the final concentration of 17% before storing in Nunc® CryoTubes® at -80°C.

3.2 Screening for antibiotic resistant bacteria in bovine milk

Milk samples from the Jurfrisk project and from a project at the Faculty of Veterinarian Medicine at NMBU were used in this work (70). The samples from the Jurfrisk project were collected post-milking from 10 cows, at quarter level, where different parameters such as SCC had been measured. Before sampling, after removal of the milking apparatus, the teats were cleaned with iodine, then alcohol. Samples from 3 cows were taken by the Faculty of Veterinarian Medicine without the sampling preparations. Out of these samples, 18 samples were selected for this work. In this experiment (Figure *3.1*) the milk samples (100 µL) were first plated on TSASB plates and incubated aerobically at 37°C over night. Cultures were prepared from the same sample with antibiotics in the following way. The samples (1 mL) were cultivated in UHT milk (9 mL), with penicillin (0.125 µg/mL), AMC (0.250 µg/mL) and without antibiotics, separately. A few samples were also treated with chloramphenicol (10 µg/mL). The three following days, cultures (100 µL) were plated on TSASB and cells were isolated (section 3.2.1). The number of colonies on the TSASB plates was recorded, and isolates with different colony morphologies were picked and grown in liquid BHI medium before glycerol stocks were made.

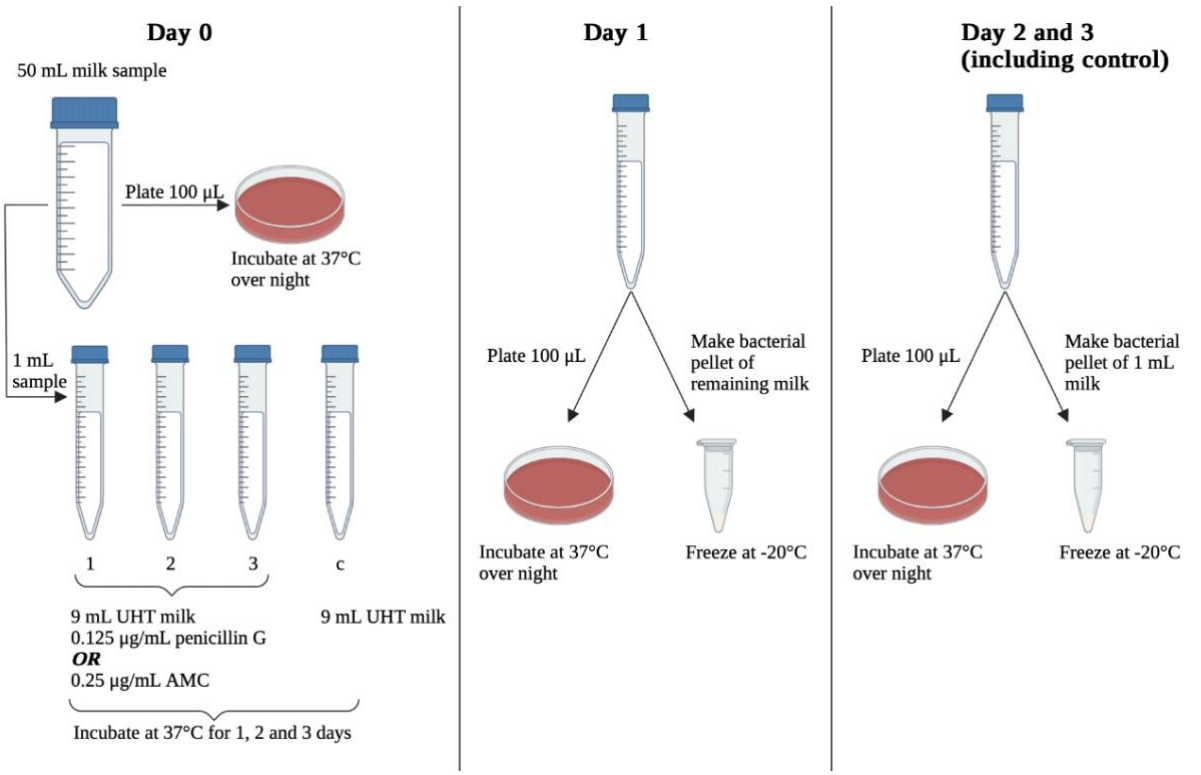

Figure 3.1: Workflow of cultivation of bovine milk samples with antibiotics. The 50 mL milk sample represents the quarter sample taken directly from the cow.

3.2.1 Isolating bacteria from milk with Propidium Monoazide (PMA) treatment

From the cultures incubated for one day, 10 mL was used to make a bacterial pellet, while for the rest of the cultures that were incubated for two or three days, 1 mL was used. To make a bacterial pellet from milk, the culture was centrifuged (8000 *x* g, 10 min, 4°C). The fat layer on top of the supernatant and the supernatant were discarded, and the pellet was resuspended in citrate water (1 mL) before centrifugation (10,000 *x* g, 5 min) to increase the cell yield. Milk contains a large amount of the protein casein, which gives the white color and the opaque appearance of milk. In milk, casein forms micelles when combined with calcium and phosphorus (71). These micelles decrease the extracted cell and DNA yield and must therefore be dissociated before isolating the plasmids (72). For this purpose, citrate water was used, which works as a chelating agent that binds the calcium ions. This washing step was repeated once or twice, depending on the amount of casein aggregation left with the pellet. The pellet was then resuspended in water (500 µL) and 1.25 µL of 10 mM Propidium Monoazide (PMAxx) was added to the cell suspension in a dark room. Propidium Monoazide is a photo-reactive compound with high affinity towards DNA, that penetrates damaged cell membranes. When exposed to visible light, PMA forms covalent bonds to the DNA, which results in irreversible damage to the DNA, and prevents DNA from dead cells to undergo amplification by PCR. This way, only intact DNA from living cells will be amplified (73). The samples were wrapped in foil and incubated for 10 min with agitation, before being treated with light of wavelength 465-475 nm for 20 minutes. Then the samples were centrifuged (10,000 *x* g, 5 min), the supernatant was removed, and the pellets were stored at -20°C.

3.2.2 Isolating DNA with DNeasy® UltraClean® Microbial Kit (250)

Before 16S rRNA gene amplification, DNA from the bacterial pellet of the bovine milk cultures (section 3.2.1) had to be isolated. DNeasy® UltraClean® Microbial Kit (QIAGEN) was used for this purpose. The pellet was resuspended in PowerBead Solution (300 µL) and transferred to a PowerBead Tube. Solution SL (50 µL) was then added, and the tube was vortexed at max speed for 10 minutes. The tube was then centrifuged (10,000 *x* g, 30 s). The supernatant was transferred to a 2 mL collection tube and Solution IRS (100 µL) was added before vortexing and incubation (5 min, 4°C). After incubation, the tube was centrifuged (10,000 *x* g, 1 minute). The supernatant was transferred to a clean tube and Solution SB (900 µL) was added. The solution was loaded into a MB Spin Column and centrifuged (10,000 *x* g, 30 s). Solution CB (300 µL) was added to the column and centrifuged (10,000 *x* g, 30 s) before the empty column

was centrifuge once more before elution with Solution EB (50 µL). After centrifugation, the DNA in the tube was stored at -20°C. The DNA was now ready for 16S rRNA gene qPCR (section 3.3.1.1)

3.3 PCR, qPCR, clean-up and normalization

Polymerase chain reaction (PCR) is a much-used molecular method for amplification of specific DNA sequences *in vitro* and is also used in this work for several purposes. The standard PCR method consists of three temperature-specific steps that proceed in cycles: denaturation of the target DNA, annealing of synthetic oligonucleotide primers, and elongation by DNA polymerase. Each cycle will double the amount of PCR product. For the amplification to occur, deoxyribose nucleotide triphosphates (dNTPs), primers, template DNA, thermostable DNA polymerase, salts and buffers must be included. In the denaturing step a temperature of 95°C is used to denature the double stranded DNA. An initial denaturing step is often used, for adequate DNA denaturing. In the primer annealing step, the temperature is lowered. The temperature depends on the melting point of the primers used. The final step is the elongation step at a polymerase dependent temperature, typically 68°C or 72°C, which is the optimum temperature for the DNA polymerase. For the next cycle, the newly synthesized double-stranded PCR products serve as templates for the reaction (74). In this work, both standard PCR and quantitative PCR have been used.

3.3.1 qPCR

In this work, quantitative PCR (qPCR) was used for amplification of 16S rRNA gene sequences for identification of bacteria strains in bovine milk (section 3.2), and for indexing sgRNAs and 16S rRNA gene sequences before Illumina sequencing. In this method, a fluorescent dye is added to the standard PCR reaction and the fluorescence is monitored after each cycle, meaning the amplification can be followed in real time. In this work, Evagreen, that binds to double-stranded DNA, was used as fluorescent dye. This dye can be easily added to an already established PCR assay but can result in both specific and non-specific detection of PCR-products (75). To rule out non-specific PCRs, a melting curve analysis was performed after the amplification cycles are done. The temperature is increased slowly after amplification. The DNA strands denatures at different temperatures according to size, and the fluorescence reduces sharply at the melting temperature (T_m) of the PCR product. This creates a peak in the negative derivative of the melting curve ($-dF/dT$). This way, fragments of different sizes and different

melting temperatures are represented with different peaks, and specific products can be distinguished from non-specific products (76). Even though dyes like Evagreen result in non-specific binding, optimalization of the conditions and use of melting curve analysis lead to adequate results.

3.3.1.1 16S rRNA gene qPCR

16S rRNA gene qPCR was used in this thesis to amplify 16S rRNA genes of the surviving bacteria from the bovine milk cultures, prior to sequencing. After isolating DNA from the bovine milk cultures (section 3.2), the V3 and V4 region of the 16S rRNA was amplified using the primers Uni340F and Bac806R in the same matter as Porcellato et la. (2020) (7). A high-fidelity, hot start master mix (Q5 master mix) containing dNTPs, Mg^{2+} and buffer was used. First a master mix of Q5, primers, Evagreen and water was made according to Table *3.1* and added to the 96-well PCR plate (18 µL per well). 2 µL of template DNA and control (water) were added before running the qPCR with the conditions listed in Table *3.2*.

Table 3.1: Components of 16S rRNA PCR. For samples giving negative qPCR results, the qPCR was repeated with lower concentrations of primer and higher concentrations of template.

Reagent	Final concentration	Volume pr. reaction [µL]
Q5 master mix	1x	10
Primer 1 (Uni340F)	0.2 or 0.1 µM	1 or 0.5
Primer 2 (Bac806R)	0.2 or 0.1 µM	1 or 0.5
Evagreen	1x	1
Template DNA	7.5 – 150 ng	2 or 4
PCR grade H_2O		5 or 4
In total		20

Table 3.2: Thermocycling conditions of 16S rRNA PCR.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	00:30	
Denaturation	98°C	00:10	
Annealing	53°C	00:30	x35
Elongation	72°C	00:20	
Final extension	72°C	05:00	
Melting curve	-	∞	

3.3.1.2 Illumina indexing qPCR

Before Illumina sequencing, indexes must be added to the sgRNAs from the CRISPRi-seq experiment and the 16S rRNA gene sequences from the bovine milk experiment. Indexes are used in order to tell what sequences originates from what sample as sequencing is done from a

pooled library of all the samples of one experiment. For this purpose, qPCR with the reagents listed in Table *3.3* were used, in the same matter at Porecellato et al. (2020) (7). A master mix of polymerase, buffer, $MgCl_2$, dNTPs, Evagreen and water was made and kept on ice. In each well of the 96-well PCR plate, 19µL master mix, 2µL of two different indexing primers and 2 µL DNA template were added. Two controls were also used, one PCR control and water. The thermocycling conditions used to amplify the DNA in this work is listed in Table *3.4*

Table 3.3: Components of Illumina indexing qPCR.

Reagent	Final concentration	Volume pr. reaction [µL]
HotFirePol® DNA polymerase	1.25 U	0.25
HotFirePol® buffer B2	1x	2.5
$MgCl_2$	2.5 mM	2.5
dNTPs	200 µM	0.5
Evagreen	1x	1
Index forward primer	0.25 µM	2
Index reverse primer	0.25 µM	2
Template DNA	7.5 – 150 ng	4
PCR grade H_2O		10.25
In total		25

Table 3.4: Thermocycling conditions of Illumina indexing qPCR.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	15:00	
Denaturation	95°C	00:30	
Annealing	55°C	01:00	x30/12*
Elongation	72°C	00:45	
Final extension	72°C	07:00	

* 12 cycles were used to index the 16S rRNA gene library.

3.3.2 PCR clean-up using Mag-Bind® TotalPure NGS beads

To remove contaminants, such as primer dimers, dNTPs, salts, and to select the wanted fragment sizes for the sequences after 16S rRNA gene qPCR (section 3.3.1.1) and before Illumina indexing PCR (section 3.3.1.2), Mag-Bind® TotalPure NGS beads were used. The beads have reversible nucleic acid-binding properties and are used to clean up the PCR product before the indexing PCR. To each well in the 96-well PCR plate (containing PCR products), 14 µL of the beads were added and mixed before transferring the solutions to a new plate. After incubation (5 min), the plate was placed on a magnetic stand for another incubation (2 min). The supernatant was discarded before two washing steps with freshly made 80% ethanol (100

μL) were performed while the plate was standing on the magnetic stand. As much ethanol as possible was removed before the samples were air dried for 10 minutes. The plate was removed from the magnetic stand and Elution buffer (20 μL) was added to each well. The samples were incubated (2 min) before being placed back onto the magnetic stand and incubated (5 min). 16 μL of the eluate of each well was transferred into a new plate before proceeding with Illumina indexing PCR (section 3.3.1.2).

3.3.3 Normalization of qPCR-product

After Illumina indexing PCR the libraries of the CRISPRi sgRNAs and the 16S rRNA gene amplification were pooled, separately, before sequencing. Therefore, the amplicons had to be of the same concentrations and a clean-up and normalization step were performed using SequalPrep™ Normalization Plate Kit (ThermoFisher Scientific) after the indexing qPCR. This kit includes a SequalPrep™ Normalization Plate that can bind and elute ~25ng of PCR product via a limited binding capacity solid phase (77). The PCR-products (20μL) and SequalPrep ™ Normalization Binding Buffer (20 μL) were transferred to the SequalPrep ™ plate 96-wells. The content of the wells was mixed completely by pipetting up and down, and foiled before incubation (1 hour, room temperature). After incubation, the buffer was removed and discarded. SequalPrep ™ Normalization Wash Buffer (50 μL) was added to the wells and mixed by pipetting. The buffer was completely aspirated from the wells and discarded. To obtain a high amplicon concentration the same 20 μL elution buffer was used to sequentially elute multiple columns of the plate. This was carried out by adding SequalPrep ™ Normalization Elution Buffer (20 μL) to columns 1 and 4 before a 5-minute incubation. After incubation, the elution buffer in column 1 were transferred to column 2, and the content in column 4 were collected in an Eppendorf tube. Another 5-minute incubation was conducted before the buffer in column 2 were transferred to column 3. Another 5 minutes incubation followed. The solutions in column 3 were in the end collected in the same Eppendorf tube (= pooled library). After normalization the pooled library was quantified by using Quibit (section 3.4.13) to ensure adequate concentration before sequencing.

3.4 Quantification of DNA

In this work, quantification of DNA was necessary after different processes, such as isolation and amplification of DNA. This was to make sure the samples contained enough DNA for further analysis and experiments, such as amplicon sequencing and transformation.

3.4.1 Qubit

After Illumina indexing qPCR and normalization, Qubit 2 fluorometer with the dsDNA HS kit (ThermoFischer Scientific) was used to make sure the concentration of the library was sufficient for sequencing. This mode allows precise measurement of low concentrations of double stranded DNA. Illumina sequencing at Novogene requires concentrations >0.5 ng/mL in volumes >30 µL. The Qubit fluorometer utilizes a fluorescent dye to determine the concentration of nucleic acids. The dye is added to the PCR product and binds the DNA molecules within seconds. The concentration is proportional to the fluorescens measured and is calculated by using specific standards (78).

3.4.2 Nanodrop

Nanodrop (ThermoFisher Scientific) was also used for quantification of nucleic acids. After isolation of plasmid DNA (section 3.9 and 3.9.2), prior to the sequencing preparation and golden gate cloning, Nanodrop was used. The Nanodrop spectrophotometer measures any molecules that absorbs a specific wavelength, in this work 260nm. These molecules include nucleotides, RNA, ssDNA and dsDNA (78).

3.5 Sequencing

In this work, Sanger sequencing was used for sequencing and thereby verifying correct construction of sgRNA plasmids. This was performed by Eurofins genomics. Illumina sequencing was used for sequencing all the sgRNA in the CRISPRi-seq experiment and for identification of microbes in the antibiotic treated bovine milk. This was performed by Novogene.

3.5.1 Sanger sequencing

Sanger sequencing, also referred to as First-Generation Sequencing, is a sequencing by synthesis method developed by Frederick Sanger and colleagues in the 1970´s (79). A DNA dependent polymerase generates a complimentary copy of the single stranded template DNA by adding deoxynucleotide triphosphate (dNTPs) one by one, starting at the 3´end. During this *in vitro* DNA replication, chain terminating di-deoxynucleotide triphosphate (ddNTPs) is incorporated. The ddNTPs lack the hydroxyl group required for creation of the phosphodiester bond between two nucleotides and will thereby terminate the addition of nucleotides to the

strand. Four reactions, containing template DNA, dNTPs, DNA polymerase and a primer are prepared. To each reaction one of the four ddNTPs (ddATP, ddGTP, ddCTP, ddTTP), whom are radioactively or fluorescently labeled, are added. This will terminate elongation of the DNA strand copy and makes it possible to determine the DNA sequence by polyacrylamide gel electrophoresis (80).

3.5.2 Illumina sequencing

For sequencing of the bacterial 16S rRNA genes from the bovine milk (section 3.2) and the sgRNAs from the CRISPRi-seq experiment (section 3.8) sequencing was performed by Novogene using a NovaSeq system with paired end reading of 250 base pairs (www.novogene.com/). Analysis of the data from the CRISPRi-seq experiment was performed by Marita Torrisen Mårli (NMBU) and of the bovine milk experiment in collaboration with Davide Porcellato.

Illumina sequencing is a part of Next-Generation or Second-Generation Sequencing, that is characterized by massively parallel sequencing of numbers of templates from the same sample in a single run, creating enormous volumes of data (79). In short, Illumina is also a sequencing by synthesis method, where successive dNTPs are added and detected by fluorescence signals. The workflow includes library generation, cluster generation, sequencing, and data analysis. When doing whole genome sequencing, the library is prepared by randomly fragmenting the DNA sample before 5´and 3´end adaptor ligation. In this work, amplicon sequencing was performed and the library was prepared by qPCR (section 3.3.1). The cluster generation is done by applying the library to a glass slide containing a surface-bound lawn of oligonucleotides, that are complimentary and bind to the terminus of the adaptor. Bridge amplification generates a monoclonal cluster of replicated fragments around each original library fragment (81, 82). The clusters are exposed to a mixture of reversable terminator-bound dNTPs. These nucleotides are added by the polymerase and a blocking group to make sure only one nucleotide is added at a time. Each of the four nucleotides are labeled with different fluorescent tag. Each cluster has a fixed position, so by imaging, the distribution of colors identifies which base was added to each cluster. After being excited, the blocking group is removed, and the cycle is repeated. The end result is a kaleidoscopic movie of shifting colors, one frame per position, one color per cluster, generating the separate sequences of the library fragments (82). Data analysis and alignment consist of aligning the newly synthesized reads to a reference genome. Following this, many different analyses are possible (83).

3.6 MALDI-TOF MS

In addition to using Illumina sequencing for identification of all bacterial strains that grew in milk, Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was used in order to identify the isolates that grew on the TSASB plates. This is a rapid method for identification, differentiating bacteria based on chemotaxonomy in a few minutes. It can be used for detection of microbial proteins, DNA, RNA, and metabolites (84). The introduction of MALDI-TOF MS devices revolutionized the routine identification of microorganisms in clinical microbiology laboratories, that relied on conventional phenotypic and gene sequencing methods. This technique is cost efficient, easy, rapid, high-throughput, and can identify multiple components in the same sample (85). The day prior to the MALDI-TOF MS analysis, the samples were plated on TSASB plates, as they must be freshly grown. After incubation over night colonies were picked with a 1 µL loop and applied on a VITEK® MS-DS slide (BioMérieux), one colony per sample spot. VITEK® MS CHCA matrix (1 µL) was applied on the cells and air dried. When the MS-CHCA crystalizes, it appears as a yellow film, and the samples are ready for identification. In the spectrometer, a laser pulse hits the matrix that absorbs the energy and vaporizes and ionizes the proteins in the sample. This matrix moderates the delivery of energy, and therefore MALDI-TOF MS is called a "soft-ionization" method, as the proteins get ionized without being damaged (82, 85). The ions are accelerated through an electromagnetic field before being sent through a vacuumized metal flight tube. According to size, smallest to largest, the ions reach a detector, leading to the generation of a mass spectrum composed of mass to charge ratio peaks. These mass spectral fingerprints are unique for each microorganism, making it possible to determine the identity on the genus and species level, potentially also typing and identify strains (85). The fingerprints are compared to databases of fingerprints generated from known microorganisms to make identifications.

3.7 Microtiter assay for minimal inhibitory concentration determination of antibiotics

To determine the lethal concentration of penicillin G and AMC for the identified strains from the bovine milk, and for determination of the concentrations of antibiotics to use in CRISPRi-seq experiments, a minimal inhibitor concentration (MIC) assay was performed. For the CRISPRi-seq experiment the CRISPRi control strain MM268 (carrying a nontarget sgRNA) was used. To each well in the microtiter plate, except well 1, 100 µL BHI medium was added.

A stock of BHI medium and twice the wanted start concentration of the antibiotic were prepared. 200 µL of the stock was added to each well 1. To obtain a two-fold dilution series, 100 µL was transferred from well 1 to well 2. After mixing by pipetting up and down, 100 µL was transferred to well 3. This procedure was repeated until well 11, and the remaining 100 µL was discarded. Well 12 served as control, containing no antibiotics. The culture was prepared by diluting 1/100 in BHI, before adding 100 µL to each well. For MIC determination for the CRISPRi-seq experiment, 10 µg/mL chloramphenicol was added for selection. The microtiter plate was placed in a plate reader and the optical density (OD) was measured spectrophotometrically at 600 nm every 10 minutes for 20 hours, creating a growth curve for the bacteria growing in each well in the plate. From these growth curves, the MIC was determined. The MIC was defined as the concentration of antibiotics that gave 50% growth inhibition (MIC_{50}) compared to the control strain growing without exposure to antibiotics (further explained for the bovine milk strains in section 4.1.2).

3.8 CRISPRi-seq experiment in milk with antibiotics

One of the aims of this work was to identify essential genes of *S. aureus* exposed to antibiotics used for treating bovine mastitis. To meet this, a CRISPRi-library of the NCTC8325-4 strain was grown in UHT milk (Figure *3.2*). First the CRISPRi-library was diluted 1/1000 in 500 mL milk and added antibiotics (for selection and testing) and aTc (for induction) were added according to Table *3.5*. The cultures (four parallels of 100 mL) were transferred to 250 mL Erlenmeyer (EM) flasks.

Table 3.5: Concentrations of antibiotics and aTc used in CRISPRi-seq experiment. Six combinations of antibiotics and aTc were examined in this experiment, A-F. A and B serves as controls, without and with induction and without antibiotics besides of chloramphenicol used for selection.

	A	**B**	**C**	**D**	**E**	**F**
Cam	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
aTc	-	30 ng/mL	-	30 ng/mL	-	30 ng/mL
PenG	-	-	-	-	0.008 µg/mL	0.008 µg/mL
TMP	-	-	0.1 µg/mL	0.1 µg/mL	-	-
SMX	-	-	0.5 µg/mL	0.5 µg/mL	-	-

Cam: chloramphenicol, PenG: penicillin G, TMP: trimethoprim, SMX: sulfamethoxazole.

The milk cultures were incubated at 37°C for 7 hours before being rediluted 1/1000 in the same medium. After another 6 hours of incubation at 37°C, 5 mL of the cultures were transferred to a large Eppendorf tube (for plasmid isolation), 50 mL to a Nunc tube (for storage at -80°C) and

the remaining 45 mL was discarded. To determine the colony forming units per milliliter (CFU/mL) in the final cultures, a 10-fold dilution series of each parallel was made, and the dilutions were spotted on BHI agar with 10 µg/mL chloramphenicol and incubated anaerobically over night at 37°C. The next day, the colonies were counted to determine CFU/mL. The resulting plasmids in the cultures in the 5 mL tubes were isolated (section 3.9.2) and quantified (section 3.4.2) before the samples were prepared by qPCR (section 3.3.1.2) and sent to Novogene for sequencing (section 3.5.2). The analysis of the resulting data was done by Marita Torrisen Mårli before a follow-up experiment was conducted (section 3.10 and section 3.11).

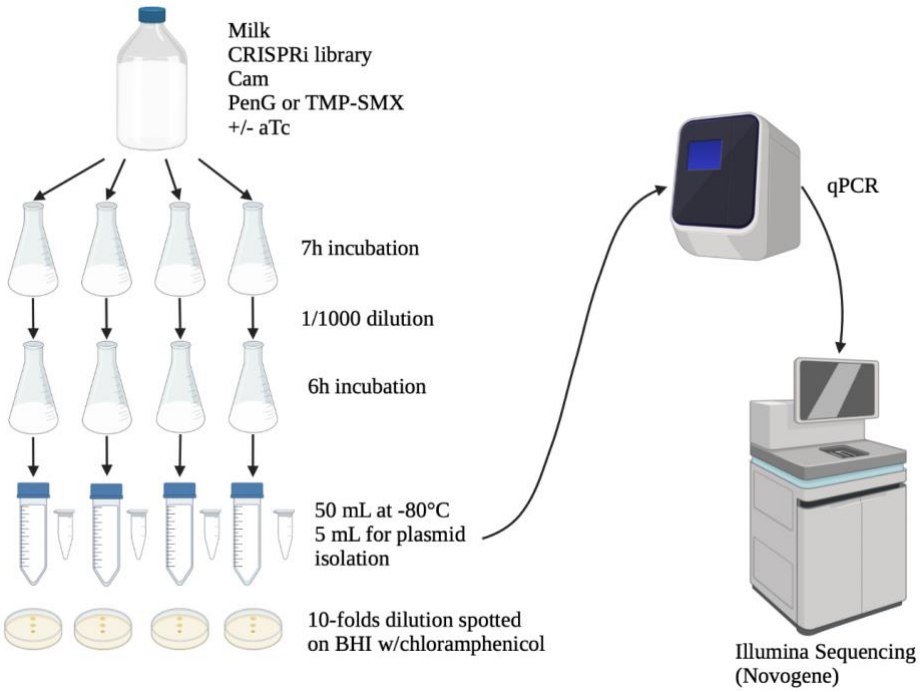

Figure 3.2: Schematic overview of CRISPRi-seq experiment in milk.

3.9 Plasmid isolation

3.9.1 Isolation of plasmids from *E. coli*

The *E. coli* plasmid pVL2336 was used in construction of single CRISPRi strains (section 3.10). To isolate these plasmids, E.Z.N.A.® Plasmid DNA Mini Kit I (Omega Bio-tek) was used, following the supplier´s protocol. Culture (5 mL) was grown over night and the cells were harvested by centrifuging (10.000 *x* g, 1 min) 1-1.5 mL of culture in an 1.5 mL Eppendorf tube. The cell pellet was resuspended in Solution I (250µL), containing RNase, that degrades the RNA in the sample. Solution II (250 µL) was added, for further lysis. When a clear lysate was obtained, Solution III (350µL) was added before inverting the tube several times and a

flocculent white precipitate was formed. This consists of chromosomal DNA and debris, while the plasmid DNA remains in the solution. The debris was removed by first centrifuging (10.000 *x* g, 1 min) the solution, then transferring the supernatant, that contained the plasmids, to the HiBind® DNA mini column before another centrifugation. HBC Buffer (500 μL) was added to the column, binding the DNA to the column. The column was centrifuged and added DNA Wash Buffer (700 μL). To remove remaining ethanol from the column, the empty column was centrifuged. To elute the DNA, the column was placed in a new Eppendorf tube and Elution buffer (30 μL) was added before centrifuging. This elutes the plasmids from the column, leaving the isolated plasmids and elution buffer in the tube, which can be stored at -20°C.

3.9.2 Isolation of plasmids from *S. aureus*

For isolating plasmids from the *S. aureus* used in the CRISPRi-seq experiment (section 3.8), E.Z.N.A.® Plasmid DNA Mini Kit I and its protocol for isolating *E. coli* was used, with some adjustments to increase the DNA yield. To harvest the cells, the 5 mL Eppendorf tube with milk culture was centrifuged (8000 rpm, 4°C, 10 min) and the fat layer on the side of the tube was removed with a swab before the pellet was resuspended and washed with 5 mL of sodium citrate water, for reasons described in section 3.2.1. The suspension was centrifuged (8000 rpm, 4°C, 5 min), the supernatant and the fat were removed, and the pellet was washed with sodium citrate water (1 mL) and transferred to a 1.5 mL Eppendorf tube. After another centrifugation (17,000 *x* g, 3 min), the cell pellet was resuspended in Solution I (250 μL), lysozyme (2 μL) and lysostaphin (1μL), before being incubated in water bath (37°C, 30 min). *S. aureus* is rather insensitive to lysozyme, which often works as the cell degrading agent in isolation techniques, due to its peptidoglycan degrading activity. Therefore, lysostaphin, is included as this lytic enzyme cleaves glycylglycine bonds that serves as cross-links between glycopeptide chains in *S. aureus* (86). After incubation the manufacture's protocol for plasmid isolation was followed, as described in section 3.9.1. Water was used for elution.

3.10 Construction of single CRISPRi depletion strains in *S. aureus*

Sequencing of the resulting sgRNAs from the CRISPRi-seq experiment (section 3.8) revealed genes being essential when treated with TMP-SMX. To investigate this further single CRISPRi depletions strains were constructed. Golden gate cloning was used to construct sgRNA plasmids that later were transformed into the *S. aureus* strain MM267. Golden gate cloning is a seamless cloning technique that utilizes type II restriction enzymes, that digest nucleotides close to the

recognition site (87). This leaves a final product without recombination site sequences. Type IIs restriction enzymes can cleave DNA outside of their restriction sites, leaving 5´or 3´overhangs. The same restriction enzyme can cut different DNA fragments, that later can be ligated. This leaves a final product without recombination site sequences (88).

3.10.1 Oligo annealing

New 20 bp sequences in the sgRNAs were introduced using oligos. To anneal the forward and reverse oligo of each sgRNA, to produce an annealed product with overhangs, reactions were sat up according to Table *3.6*. The solutions were incubated at 95°C for 5 minutes before slowly cooling down to room temperature.

Table 3.6: Components of sgRNA oligo annealing.

Component	Volume
Forward Oligo (100 μM)	2.5 μL
Reverse Oligo (100 μM)	2.5 μL
10XTEN buffer	4 μL
ddH_2O	40 μL
In total	50 μL

3.10.2 Preparation of vector

The vector backbone was the plasmid pVL2336. This plasmid contained a fragment encoding mCherry in the site where the sgRNA will be inserted. This can be used to select correct transformants, as unsuccessful removal of mCherry from the cloning site will result in pink colonies. The plasmid was first isolated using the protocol described in section 3.9.1. This plasmid was digested with the type II restriction enzyme BsmBI, by mixing the components listed in Table *3.7*, and incubated at 55°C for two hours. The digestion leaves two products, the 5935 bp vector backbone and the 739 bp fragment encoding mCherry. The backbone was isolated from gel (section 3.10.2.2).

Table 3.7: Components of digestion using BsamBI restriction enzyme.

Component	Volume
Plasmid (pVL2336)	10 μL
BsmBI	1 μL
10xNEB 3.1	5 μL
ddH_2O	34 μL
In total	50 μL

3.10.2.1 Agarose gel electrophoresis

In this work, agarose gel electrophoresis was used for separating DNA fragments. Agarose is a linear polymer extracted from seaweed. Agarose powder was heated in TAE buffer and a gel matrix was formed by hydrogen-bonds whose pore sizes determine the molecules migration through the gel (89). Before the gel cooled down and sat, a fluorescent dye was added, enabling visualization of the DNA fragments using UV-light (90).

The sample was added a loading buffer before application. This buffer contains glycerol and bromophenol blue, making loading of the sample easier. The sample and a ladder with fragments of known sizes were applied to the gel, before 90V current was applied for approximately 30 minutes. Since DNA and RNA consist of a phosphate backbone, they are negatively charged and will move to the positively charged anode when exposed to current. The gel network will slow down molecules with higher molecular weight while smaller molecules will move faster towards the anode since the mass/charge ratio of DNA/RNA is uniform. GelDoc-1000 was used to visualize the fragments, and they were cut from the gel and extracted using the protocol described in section 3.10.2.2.

3.10.2.2 Extraction of DNA from agarose gel

The backbone DNA fragment was extracted from agarose gel using NucleoSpin® Gel and PCR Clean-up (Machnery-Nagel). The agarose piece containing the DNA fragment was dissolved in NTI (200 μL per 100 mg gel) at 55°C. A column was placed in a collection tube and the dissolved gel was transferred into the column and centrifuged (11,000 *x* g, 30 s). The silica membrane of the column was then washed by adding NT3 washing solution (700μL) and centrifuged (11,000 *x* g, 30 s). To dry the silica membrane, the empty column was centrifuged another round (11,000 *x* g, 1 min). The elution was done by adding NE elution buffer (30 μL), followed by a short incubation (1 min, room temperature) and centrifugation (11,000 *x* g, 1 min) to elute the DNA into an Eppendorf tube.

3.10.3 Ligation

The annealed oligoes from section 3.10.1 were ligated to the digested vector by mixing the components listed in Table *3.8*. The mixes were then incubated in water bath at 16 °C over night.

Table 3.8: Components of ligation reaction of sgRNA into plasmids.

Component	Volume
Vector backbone	1 μL
Annealings	15 μL
T4 ligase	1 μL
T4 ligase reaction buffer	2 μL
In total	19 μL

3.10.4 Transformation in *E. coli* by heat shock

S. aureus has restriction modification barriers that prevent introduction of DNA without host-specific methylation profiles. Therefore, the cloned vector cannot be transformed directly into a *S. aureus* strain. To by-pass this complicating factor, the plasmid is introduced into an *E. coli* strain heterogeneously expressing methyltransferases of the target host, thus resulting in methylation of the DNA (69). The ligation products were transformed into modified *E. coli* by heat shock. This was done by mixing 14 μL ligated product with 40 μL of the chemically competent *E. coli* strain IM08B (prepared by Zhian Salehian) and incubating it on ice for 30 minutes. Then the cells were heat shocked at 42°C for 30 seconds and cooled on ice for 1 minute. 250 μL S.O.C medium was added, and the cells were incubated at 37°C with shaking for one hour. The cultures were plated (50 μL and 200 μL) on LB agar plates with 10 μg/mL ampicillin for selection and incubated over night. Transformants were picked and grown in LB medium with 10 μg/mL ampicillin until the day after. Plasmids were isolated using E.Z.N.A.® Plasmid DNA Kit, as described in section 3.9.1, and verified by Sanger sequencing.

3.10.5 Transformation in *S. aureus* by electroporation

In this work, electroporation was used for introducing plasmids to *S. aureus*. *S. aureus* was made electrocompetent prior to electroporation by Zhian Salehian, using the protocol of Löfbom et al. (2007) (91). First, over night culture was diluted in 100 mL BHI to a final OD_{600} of 0.5-0.6, and then incubated for 30 minutes at 37°C with shaking. Before harvesting the cells, the culture was kept on ice for 10 minutes. The culture was transferred to two 50 mL Nunc-tubes before centrifuging (4000 rfc, 10 minutes, 4°C). The supernatant was discarded, and the pellet was washed with 35 mL ice cold H_2O. This was done twice. Then three washing steps with 25 mL 10% glycerol were done. The final pellet was resuspended in 2-3 mL ice cold 10% glycerol with 0.5M sucrose. The cells were stored at -80°C.

For electroporation, 50 µL of competent cells were mixed with 5 µL plasmid DNA. The mix was transferred to a 1 mm cuvette and using setting 2.1V, 100Ω and 25 µF. Quickly, 950 µL of cold TSB with 0.5 sucrose was added. The cells were then incubated for 2 hours at 37°C with shaking before being plated on BHI agar with 10 µg/mL chloramphenicol.

3.10.5.1 PCR screening of *S. aureus*

To verify the electroporation and the prior steps of making transformants, a PCR-screening with RedTaq® of the colonies was performed. Due to *S. aureus* being difficult to lyse, a pre-PCR lysis step was included before the screening using the protocol of Lee et al. (2018) (92). The colony was first resuspended in 30 µL lysis buffer (40 mM NaOH, 0.2% SDS) and incubated at 98°C for 5 minutes. Cells was also harvested and grown in liquid BHI with 10 µg/mL chloramphenicol for selection, at 37°C overnight. The lysate was cooled on ice before 200 µL dH_2O was added. Then, the regular PCR protocol was proceeded. RedTaq® consists of buffer, polymerase, nucleotides and loading buffer. For this screening, primers MK25 and MK26 were used, which hybridizes specific DNA-sequences in the transformed pVL2336-plasmids. The reactions were mixed according to Table *3.9* and the thermocycling conditions listed in Table *3.10* was used for the PCR. The PCR products were verified using agarose gel electrophoresis (section 3.10.2.1).

Table 3.9: Components of RedTaq® PCR.

Reagent	Final concentration	Volume pr. reaction [µL]
RedTaq 2x master mix	1x	5
Forward Primer	0.2 µM	0.2
Reverse Primer	0.2 µM	0.2
Template DNA		2
dH_2O		7.6
In total		10 µL

Table 3.10: Standard thermocycling conditions for RedTaq® PCR.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	10:00	
Denaturation	95°C	00:30	
Annealing	55°C	00:30	x30
Elongation	72°C	01:00	
Final extension	72°C	10:00	
	16°C	∞	

3.11 Antibiotic susceptibility testing of single CRISPRi strains in milk

To verify the results from the TMP-SMX CRISPRi-seq experiment in milk (section 3.8), the sensitivity of the strains was tested. Over night cultures of each single CRISPRi strain (section 3.10), including a CRISPRi control strain (carrying a non-target sgRNA), was diluted 1/1000 in UHT milk containing 10 μg/mL chloramphenicol and 30 ng/mL aTc. In one of the parallel of each strain, 0.1 μg/mL trimethoprim and 0.5 μg/mL sulfamethoxazole were added. The cultures were incubated for 7 hours at 37°C before being rediluted 1/1000 in milk with the same conditions. The diluted cultures were incubated for another 6 hours at 37°C. After incubation, a 10-fold dilution series of the cultures were plated on BHI agar with 10 μg/mL chloramphenicol and incubated aerobically at 37°C over night. The next day, the colonies were counted to determine the CFU/mL.

4 Results

4.1 Screening for antibiotic resistant bacteria in bovine milk

Bovine mastitis is the main cause of antibiotic use in dairy production, and two of the most commonly used treatments are penicillin G and AMC. Both drugs target the cell wall synthesis, whereas amoxycillin always is combined with the β-lactamase inhibitor clavulanic acid for broader spectrum. To get some deeper insight into the fate of the bovine milk microbiota upon antibiotic treatment 18 milk samples from healthy cows were screened for antibiotic resistant bacteria (four of them by A. R. Winther) (Figure *3.1*). This resulted in the isolation of over 200 bacterial isolates that were able to grow in milk in the presence of one of the two antibiotics. To identify the bacteria that grew on agar-plates after incubation with penicillin G or AMC, MALDI-TOF MS was used. Illumina sequencing was used to identify the resistant bacteria directly from liquid cultures of milk with antibiotics (hereby referred to as cultures). Isolates and cultures for identification were selected based on colony counts and morphology. The isolates having increasing numbers of colonies over the tree days of incubation and different morphologies were selected for MALDI-TOF MS identification, which included 34 isolates. The milk cultures selected for 16S rRNA gene sequencing displayed increasing number of colonies over the three incubation days, leading to 56 milk cultures being sequenced after DNA isolation and 16S rRNA gene amplification.

4.1.1 Identification of isolates with MALDI-TOF MS

Samples that showed a clear increase in number of colonies between one and three days of cultivation with penicillin G or AMC were selected for MALDI-TOF MS identification. Samples were plated from glycerol stocks on TSASB plates and incubated overnight. A total of 38 colonies, originating from 34 different glycerol stocks, were analyzed by MALDI-TOF MS for identification. Freshly grown colonies were picked from TSASB plates, smeared onto the MALDI-TOF MS slide and fixed by addition of the matrix solution. Samples that were not identified and those identified as the same species from the same quarter sample, were excluded (data not shown). The identified strains are presented in Table *4.1* and Table *4.2* for AMC and penicillin G, respectively. Out of the 38 colonies analyzed by MALDI-TOF MS, 27 isolates were chosen for analysis by determination of MIC.

4.1.2 Minimal inhibitory concentration (MIC) determination of antibiotic resistant isolates

To determine the minimal inhibitory concentration of the antibiotics (penicillin G and AMC) for the resistant isolates, a microtiter assay was performed (section 3.7). A two-fold dilution series with start concentrations of 32 or 64 µg/mL were prepared on the microtiter plate. The 12th well served as control and did therefore not contain any antibiotic. The 27 strains selected after MALDI-TOF identification were grown over night and diluted 1/100 before added to the plate. The isolates were exposed to the same type of antibiotic as the original sample were exposed to when being cultivated in milk. A plate reader was used for OD_{600} measurements every 10 minutes for 20 hours. Due to the large variation in growth between strains, the MIC_{50} was determined as the concentration giving a maximum OD that equals half of the maximum OD of the control (Figure *4.1*). The identification by MALDI-TOF MS and the MIC results are presented in Table *4.1* and Table *4.2* for AMC and penicillin G, respectively. The susceptibility breakpoints of some of the species are specified beneath the tables, retrieved from the The European Committee on Antimicrobial Susceptibility Testing 2022 (93)

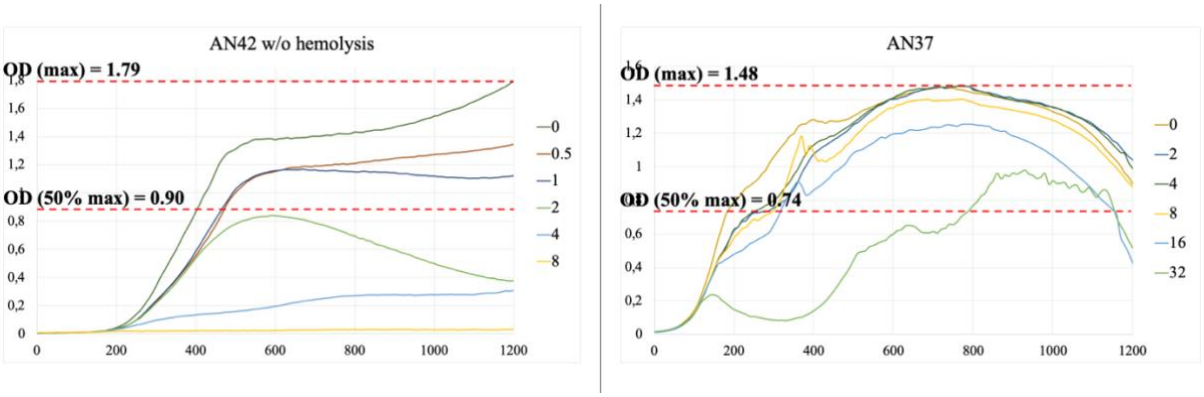

Figure 4.1: Determination of MIC_{50} exemplified by the growth curves of two of the isolates, AN42 and AN37. Concentrations of antibiotics (in µg/mL) are shown on right side of the charts. MIC_{50} was defined as the concentration reducing the maximum OD to half the maximum OD of the control during the course of the experiment (20 hours). For AN42 the maximum OD of the control was 1.79. The concentration giving half of this OD (=0.90) was 1.75 µg/mL. For AN37 all the concentrations gave a higher OD than half of the maximum OD of the control, and the MIC_{50} was there for determined as >32µg/mL.

Table 4.1: Results of MALDI-TOF MS identification and MIC analysis of 14 isolates treated with AMC.

Isolate ID	MALDI-TOF MS result (confidence)	MIC_{50} (µg/mL)
AN32 large	*Escherichia coli* (100%)	>64[1]
AN32 small	*Stenotrophomonas maltophilia* (100%)	16
AN34 w/hemolysis	*Staphylococcus aureus* (100%)	8[2]
AN34 w/o hemolysis	*Enterococcus faecalis* (100%)	52[3]
AN42 w/hemolysis	*Staphylococcus aureus* (100%)	11[2]
AN42 w/o hemolysis	*Streptococcus dysgalactiae ssp. equisimilis* (50%)/*dysgalactiae* (50%)	1.75
AN59	*Staphylococcus epidermidis* (100%)	11[2]
AN60	*Corynebacterium amycolatum* (100%)	3
AN86	*Staphylococcus simulans* (100%)	3[2]
AN129	*Micrococcus luteus* (100%)	6
AN169	*Staphylococcus chromogenes* (100%)	1.75[2]
AN170	*Staphylococcus chromogenes* (100%)	4[2]
AN171	*Staphylococcus saprophyticus* (100%)	44[2]

[1] Susceptibility breakpoint: ≤ 8 µg/mL
[2] Susceptibility breakpoint: ≤ 0.25 µg/mL
[3] Susceptibility breakpoint: ≤ 4 µg/mL

Table 4.2: Results of MALDI-TOF MS identification and MIC analysis of 14 isolates treated with penicillin G.

Isolate ID	MALDI-TOF MS result (confidence)	MIC_{50} (µg/mL)
AN31	*Enterococcus faecalis* (100%)	6
AN33	*Enterococcus faecalis* (100%)	9
AN35	*Enterococcus durans* (100%)	>32
AN37	*Escherichia coli* (100%)	>32
AN43	*Staphylococcus aureus* (100%)	0[1]
AN45	*Staphylococcus epidermidis* (100%)	44[1]
AN61	*Enhydrobacter aerosaccus* (50%)/*Moraxella osloensis* (50%)	0
AN62	*Micrococcus luteus* (100%)	0,125
AN158	*Micrococcus luteus* (100%)	0,03
AN160	*Enhydrobacter aerosaccus* (50%)/*Moraxella osloensis* (50%)	*
AN190	*Micrococcus luteus* (100%)	0,03
AW24 w/hemolysis	*Bacillus cereus group* (100%)	>64
AW24 w/o hemolysis	*Staphylococcus epidermidis* (100%)	>64[1]
AW41	*Escherichia coli* (100%)	>64

* No growth

[1] Susceptibility breakpoint: ≤ 0.125 µg/mL

4.1.3 Identification of species by 16S Illumina sequencing

To identify and get an overview of all resistant bacteria, including those that were not able to grow with normal cultivation methods in the laboratory, such as agar plates, sequencing of 16S rRNA genes was performed directly on the bacteria in the milk samples. The relative abundances of the 30 most abundant species in the samples are presented in Figure *4.2*. In the figure the samples exposed to the same antibiotic were grouped together; 28 samples treated with AMC, two samples treated with chloramphenicol and 23 samples treated with penicillin

G. The sequences that were the most abundant belonged to *Staphylococcus caprae/epidermidis/capitis*, followed by the *Streptococcus mitis* group and *Corynebacterium bovis* when doing a BLAST-search of the Illumina sequences. Many of the samples were dominated by a few species. Some of the samples showed similar abundance patterns, such as samples 15 and 16 (day 1 and 3, AMC), and 17 and 18 (day 1 and 3, penicillin G), which originates from the same milk sample (JF-534).

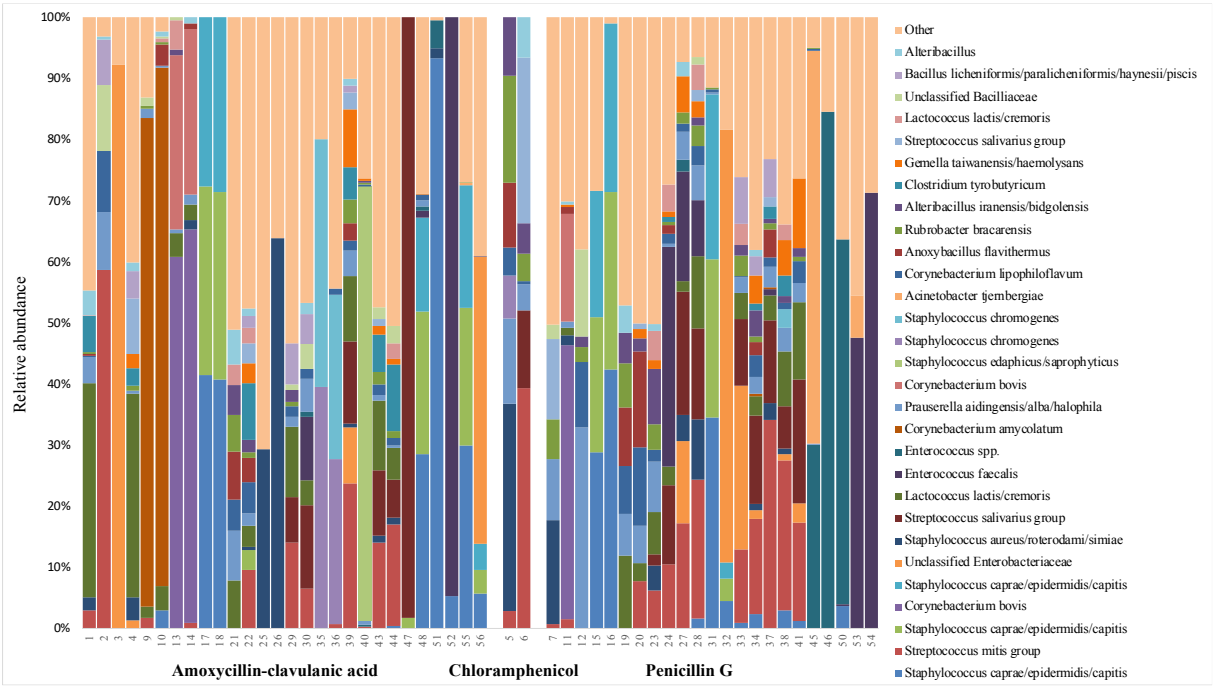

Figure 4.2: The relative abundance of the 30 most abundant genera/species plotted in groups based on type of antibiotic treatment. A total of 53 samples are plotted, whose 28 of the samples were exposed to AMC, 2 to chloramphenicol and 23 to penicillin G.

4.2 CRISPRi-seq for identification of genes involved in susceptibility to antibiotics

In addition to penicillin G and AMC, TMP-SMX is also a commonly used antibiotic combination for treating bovine mastitis. While penicillin G and AMX target the cell wall biosynthesis, TMP-SMX target folate synthesis (41, 59). To get some further insight into the genetic factors of *S. aureus* that are affecting the susceptibility to these antibiotics during exposure in milk, a pooled CRISPRi-library, together targeting all genes in the *S. aureus* genome was utilized (designed by Marita Torrisen Mårli). When using the CRISPRi-library, the fitness effect of depletion of all genes can be studied simultaneously in one experiment. Using Illumina sequencing, the abundance of each sgRNA in the population can be determined and this abundance reflects the essentiality of the gene represented by the sgRNA. Hence,

sgRNAs targeting essential genes will have reduced abundance compared to sgRNAs targeting non-essential genes. Thus, by comparing sgRNA abundances in CRISPRi-library cultures treated or not treated with the sublethal concentrations of antibiotics (penicillin G and TMP-SMX), it is therefore possible to identify genes whose fitness is altered upon antibiotic exposure. Such altered fitness suggest that the gene is involved in susceptibility to antibiotic, by either becoming more or less essential in the presence of the antibiotic. A schematic overview of the experiment is presented in Figure *3.2*.

4.2.1 Determination of MIC for penicillin G, trimethoprim and sulfamethoxazole

To determine the concentration of antibiotics to be used in the CRISPRi-library exposure experiments, the MIC_{50} of the antibiotics (penicillin G and TMP-SMX) was determined for the CRISPRi control strain *S. aureus* MM268. The MM268 CRISPRi control strain carries a plasmid with a non-targeting sgRNA, pVL2336 (non-target). Microtiter assays were performed in BHI medium and the MIC_{50} was found to be 0.008 µg/mL for penicillin G (Figure *4.3*). For the TMP-SMX combination, the MIC_{50} was found to be 0.1 µg/mL trimethoprim and 0.5 sulfamethoxazole in combination (Figure *4.4*). These concentrations of penicillin G and TMP-SMX were used in the CRISPRi-seq experiment (section 3.8).

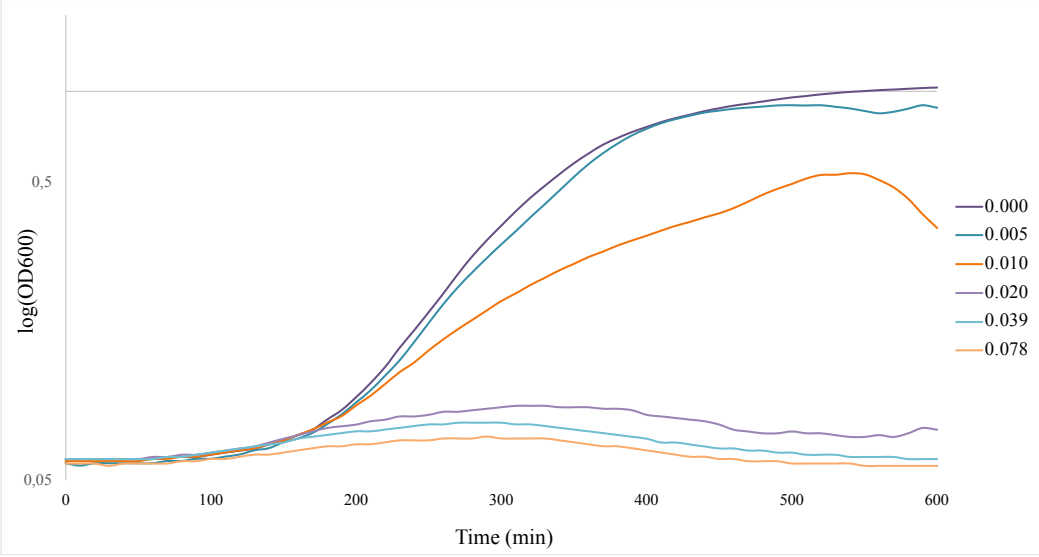

Figure 4.3: Growth curves of *S. aureus* MM268 grown with different concentrations of penicillin G. Concentrations in µg/mL are shown on the right side. MIC_{50} was 0.008 µg/mL

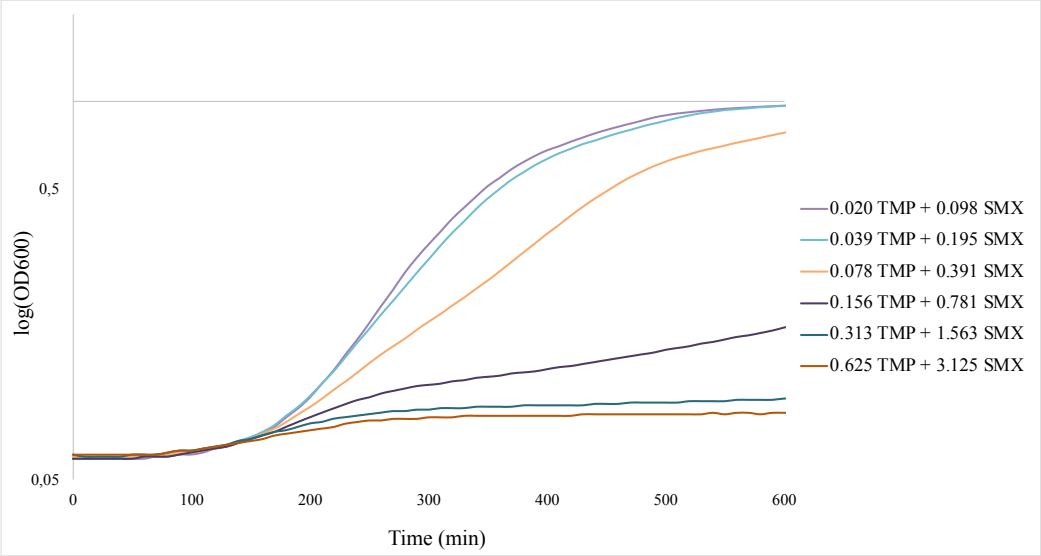

Figure 4.4: Growth curves of *S. aureus* MM268 grown with different concentrations of trimethoprim and sulfamethoxazole. Concentrations in µg/mL are shown on the right side. MIC_{50} was 0.1 µg/mL trimethoprim and 0.5 µg/mL sulfamethoxazole.

4.2.2 Viable counts after treatment with sublethal concentrations of penicillin G and TMP-SMX

For the CRISPRi-seq experiment, the CRISPRi-library was cultivated in milk with and without exposure to antibiotics (penicillin G and TMP-SMX) and with and without induction, following the experimental setup with four parallels for each condition described in section 3.8 and illustrated in Figure *3.2*. To determine the number of viable cells after antibiotic exposure, 10-folds dilution series of the cultures were prepared and spotted on BHI agar with 10 µg/mL chloramphenicol for selection. After incubation of the spotted library cultures, the colonies were counted. The colony forming unit per milliliter (CFU/mL) was calculated (results in Table *4.3*). As expected, reduced growth is seen for the antibiotic treated libraries, than the control. The growth of the library with penicillin varies more between the parallels than with TMP-SMX.

Table 4.3: CFU/mL of the parallels (1-4) of the CRISPRi-seq experiment with TMP-SMX and penicillin G.

		Control	**Penicillin G**	**TMP-SMX**
CRISPRi library - aTC	1	5×10^7	1.5×10^5	2.5×10^5
	2	5.5×10^7	8.5×10^4	2×10^5
	3	5×10^7	8×10^4	2.5×10^5
	4	4×10^7	2×10^5	5×10^5
CRISPRi library + aTc	1	7×10^7	1.5×10^5	2.5×10^5
	2	6×10^7	4×10^4	3.5×10^5
	3	6.5×10^7	3.5×10^4	3.5×10^5
	4	6.5×10^7	7.5×10^4	3×10^5

4.2.3 CRISPRi-seq analysis to determine difference in gene fitness upon treatment with penicillin and TMP-SMX

After incubation of the CRISPRi cultures, the plasmids were isolated and the resulting sgRNAs were amplified and indexed for Illumina sequencing. Illumina sequencing of the pooled amplified sgRNAs were performed by Novogene. The resulting data was analyzed by Marita Torrisen Mårli (NMBU), using the DESeq2 package in R (94) and the CRISPRi-seq analysis pipeline developed by the Veening lab (68).

4.2.3.1 Principal component analysis of CRISPRi-seq results after treatment with TMP-SMX

A Principal Component Analysis (PCA) was performed first to assess the data quality. The PCA plot illustrates the difference in sgRNA count between the parallels, treatments (with or without aTc) and conditions (with or without TMP-SMX) (Figure 4.5). Illustrating all sgRNAs in all samples would be overwhelming, and the PCA plot reduces all these dimensions by constructing principal components (PCs). This leaves a plot with clusters, where differences and similarities are represented by the distance in the plot.

The PCA-plot for the CRISPRi-seq with TMP-SMX is shown in Figure 4.5. Ideally, the four parallels of each treatment-condition combination (with/without aTc and TMP-SMX) should be similar and lay on top of each other in the plot. In this case, this applies especially for the non-induced samples, shown as minus signs (-). The samples with induction (+) also clusters well, with only small distances between the parallels. The main source of variation is the CRISPRi induction, with PC1 accounting for 87% of the variance. This is the variance between induced and non-induced samples and is caused by the reduced counts of sgRNA targeting essential genes that are common in all samples. It can also be observed that there is larger distance between the treated and non-treated samples after induction. This strongly suggests that there are some differences in fitness due to different genes/operons being knocked down between the two treatments (with/without TMP-SMX). Taken together, the PCA analysis suggests that the experiment was successful with parallels clustering together and that the induction with aTc and treatment with TMP-SMX had effects on the sgRNA distributions.

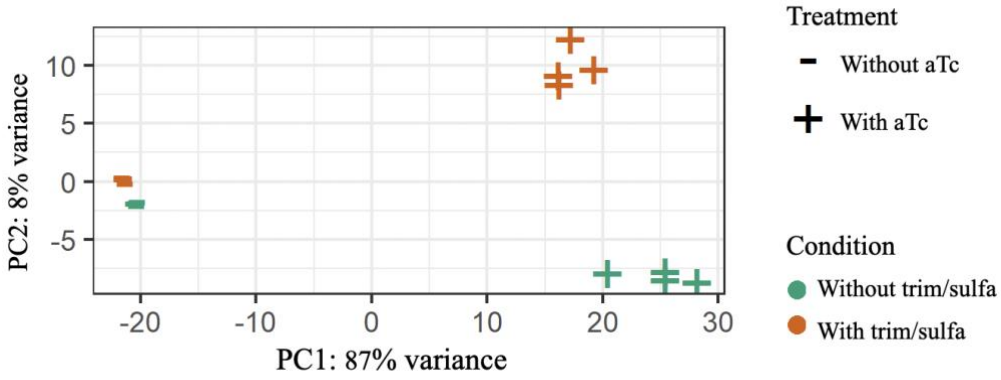

Figure 4.5: PCA-plot for CRISPRi-seq with TMP-SMX.

4.2.3.2 97 genes alter the susceptibility of *S. aureus* to TMP-SMX

To determine the fitness of all genes under the two conditions (treated and non-treated with antibiotics) individually, the change in sgRNA counts between the non-induced and induced samples are compared. This is calculated as $\log_2$ of the fold-change (log2FC) in sgRNA counts between the induced and non-induced condition (data not shown). Then, to quantify the difference in fitness for the genes between the two conditions, a so-called interaction log2FC between the non-treated control and treated samples was determined for each sgRNA. A negative interaction log2FC means that the genes become more essential when treated with the antibiotic. A positive log2FC on the other hand, is a result of genes that become less essential when treated with the antibiotic compared to the non-treated control.

Figure *4.6* shows a volcano plot of all the 1928 genes in the *S. aureus* CRISPRi-library with the interaction log2FC between treated and non-treated samples on the x-axis and the negative adjusted p-values on the y-axis. This threshold for significant differential fitness was set to log2FC <-2 or >2, and an adjusted p-value <0.05. The majority of the sgRNAs (n=1831) were, as expected, not significantly different between the treatments (represented by green dots) and are therefore considered neutral when treated with TMP-SMX. These are genes that have similar fitness effect in the two conditions. In total 97 sgRNAs were determined to be significantly different between conditions. The orange dots on the right side of the threshold represent the 89 genes being less essential when treated with TMP-SMX (listed in Appendix B). On the other hand, the 8 orange dots on the left side of the threshold (representing genes shown in Figure *4.6* and listed in Table *4.4*, represent genes being more essential when grown with TMP-SMX. Knockdown of these genes are thus expected to increase the susceptibility to the antibiotic (se section 4.2.4).

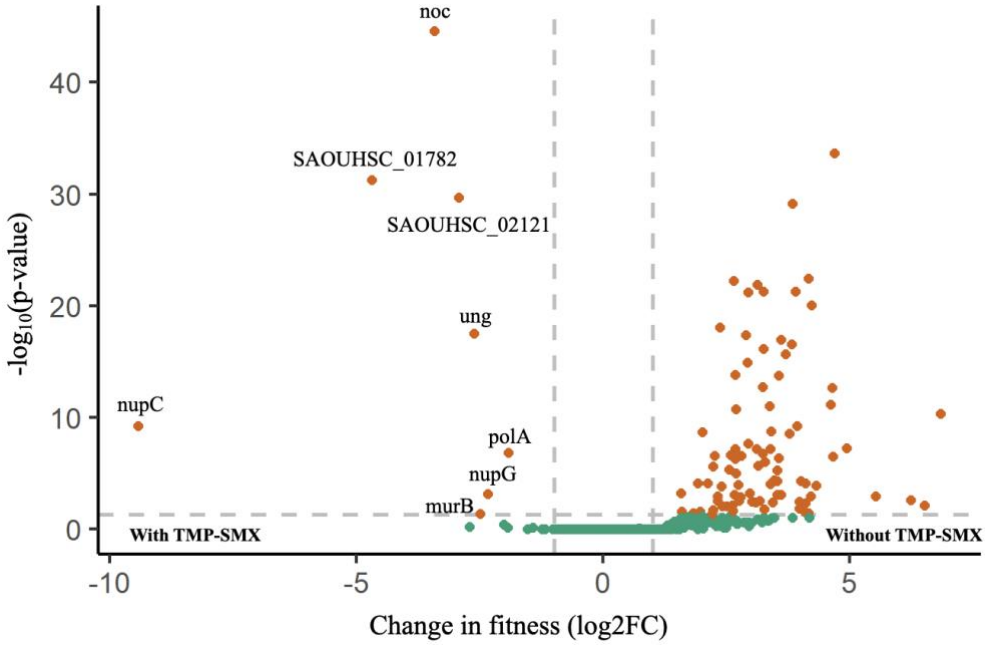

Figure 4.6: Volcano plot of genes that are more and less essential in the presence of TMP-SMX. One dot represents one sgRNA variant.

4.2.3.3 A large number of genes modulates the susceptibility to penicillin G

In a similar fashion as for TMP-SMX, the CRISPRi-seq experiment were performed when treated with sublethal concentrations of penicillin G. The PCA-plot for the CRISPRi-seq with penicillin G is shown in Figure *4.7*. As in the TMP-SMX experiment, the main source of variation is the CRISPRi induction, with PC1 accounting for 79% of the variance. There is also larger distance between the penicillin G treated and non-treated samples after induction, indicating that there are differences in fitness due to knocked down of different genes between the two conditions (with/without penicillin G). As for the TMP-SMX experiment, the PCA analysis suggests that the experiment was successful.

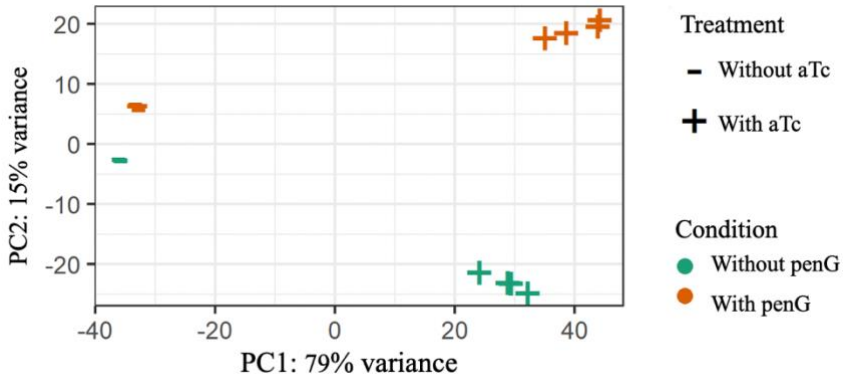

Figure 4.7: PCA-plot for CRISPRi-seq with penicillin G.

CRISPRi-seq analysis of penicillin G treated cultures resulted in a long list of genes with significantly altered fitness between the treatment, both genes being significantly more essential (n=99) and less essential (n=51) when treated with penicillin G (listed in Appendix C and Appendix D). Figure *4.8* shows a volcano plot with the interaction log2FC on the x-axis and the negative adjusted p-values on the y-axis. These genes represent interesting candidates for follow up work but were not investigated further in this work.

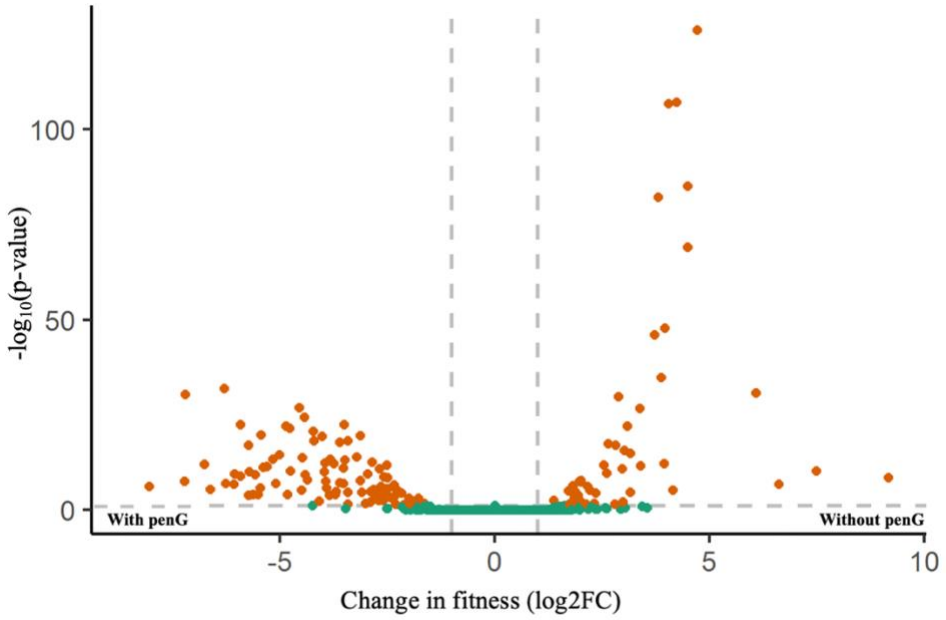

Figure 4.8: Volcano plot of genes that are more and less essential in the presence of penicillin G. One dot represents one type of sgRNA.

4.2.4 Downregulation of genes involved in nucleoside uptake and DNA repair causes increased susceptibility to TMP-SMX

The eight genes that were significantly more essential when treated with TMP-SMX (Figure *4.6*), are listed in Table *4.4*. This group of genes is particularity interesting, as targeting of these genes result in increased susceptibility to TMP-SMX. As shown in Table *4.4*, seven of these genes were non-essential for growth in milk but became essential upon treatment with TMP-SMX. *murB* is essential when the strain was grown in milk alone. The predicted function of the genes based on annotation in Uniprot (www.uniprot.com) are shown in Table *4.5*. Two of the genes (SAOUHSC_02121 and SAOUHSC_01782) are fully uncharacterized. Five out of the remaining six genes are putatively involved in DNA metabolism, either by uptake of nucleoside (*nupC* and *nupG*), DNA repair (*ung* and *polA*) or DNA segregation (*noc*).

Table 4.4: Genes found to have a log2FC <-2 and therefore must be essential when *S. aureus* is treated with TMP-SMX

Locus tag	Gene name	Essentiality, milk	Essentiality, with TMP-SMX	Log2FC interaction
SAOUHSC_01797	*polA*	neutral	essential	-1,91456
SAOUHSC_00501	*nupC*	neutral	essential	-9,41777
SAOUHSC_00648	*nupG*	neutral	essential	-2,34418
SAOUHSC_00752	*murB*	essential	essential	-2,50179
SAOUHSC_00564	*ung*	neutral	essential	-2,61561
SAOUHSC_02121	Hypothetical	neutral	essential	-2,93375
SAOUHSC_03049	*noc*	neutral	essential	-3,42614
SAOUHSC_01782	Hypothetical	neutral	essential	-4,68662

Table 4.5: List of genes in *S. aureus* found to be essential when being exposed to TMP-SMX. Protein and function information retrieved from www.uniprot.com

Gene name/locus tag	Protein	Function
polA	DNA polymerase I	DNA replication and repair
nupC	Uncharacterized	Nucleoside transmembrane transport
nupG	Uncharacterized	Nucleoside transmembrane transport
murB	UDP-N-acetylenolpyruvoylglucosamine reductase	Peptidoglycan synthesis
ung	Uracil-DNA glycosylase	DNA repair
SAOUHSC_02121	Uncharacterized	Unknown
noc	ParB domain-containing protein	DNA segregation
SAOUHSC_01782	Nudix hydrolase domain-containing protein	Unknown

To verify the results above (Table *4.4*), single CRISPRi-depletion strains were constructed of six out of the eight genes. sgRNA plasmids were constructed by Golden Gate cloning (section

3.10) and the resulting CRISPRi-strains were induced with aTc and exposed to TMP-SMX in a similar fashion as was done for the library (se section 3.8 and section 3.11 for methodology). The CRISPRi(non-target) strain MM268 was included as control. The milk cultures were spotted on BHI after treatment and the CFU/mL was determined. Images of the spotting assay and estimated CFU/mL are shown in Figure *4.9* and Table *4.6*, respectively.

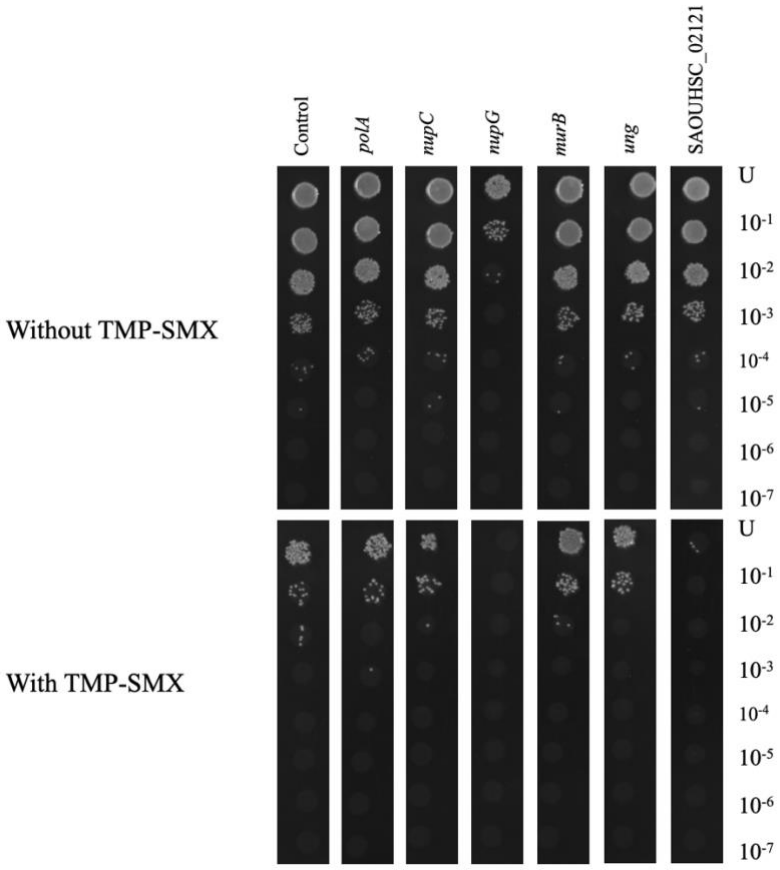

Figure 4.9: Pictures of spotting of single CRISPRi-depletion strains cultivated in milk, taken with GelDoc-1000. The upper pictures are of the strains cultivated with TMP-SMX, while lower pictures are of the strains cultivated without TMP-SMX.

Table 4.6: CFU/mL of single CRISPRi-strains cultivated in milk with TMP-SMX, spotted on BHI agar with 10 µg/mL chloramphenicol.

Depleted gene	**With TMP-SMX**	**Without TMP-SMX**	**Fold reduction in CFU/mL upon treatment**
Control	2.5×10^5	5×10^7	200
polA	6×10^4	4.5×10^7	750
nupC	6.5×10^4	1×10^8	2000
nupG	0	1.5×10^5	*
murB	1.5×10^5	5×10^7	333
ung	8.5×10^4	1.5×10^7	176
SAOUHSC_02121	1.5×10^3	5×10^7	33333

As expected, the growth and the CFU/mL are reduced upon treatment with TMP-SMX, compared to the samples not being exposed to the antibiotics for all strains including the control. For the control strain, this reduction was 200-fold. *murB* and *ung* do not deviate notably from the control, having fold reductions of 333 and 176, respectively, suggesting that knockdown of these genes did not clearly cause increased susceptibility to TMP-SMX. For the remaining strains, on the other hand, the knockdown resulted in strains with increased susceptibility to TMP-SMX, as would be expected from the CRISPRi-seq results. Two genes stand especially out – *nupG* and SAOUHSC_02121. The strains with these depleted genes show a significant poorer growth than the control strain, with a much lower CFU/mL. When looking at the growth of these strains without TMP-SMX, it appears that *nupG* grows quite poorly even without the antibiotic compared to the control with only 1.5×10^5 CFU/mL versus 3.5×10^7 CFU/mL, suggesting that *nupG* is necessary for normal growth in milk. This is not the case for SAOUHSC_02121, which has almost the same CFU/mL as the control, when not being exposed to TMP-SMX. Thus, by this approach staphylococcal genes involved in susceptibility to TMP-SMX when grown in milk were identified.

5 Discussion

5.1 Evaluation of the methods

5.1.1 Milk as medium

The medium used in the screen for antibiotic resistant strains and in the CRISPRi-seq experiment was UHT milk. The conditions for bacterial growth in milk are different than in broths such as BHI medium, creating an environment closer to that of the udder. On the other hand, UHT milk is not as complex as raw milk. UHT milk goes through high heat treatment, meaning it is heated to 130-145°C under pressure before being cooled and packed in aseptic containers (95). The whey proteins of the milk get denatured during this process, but the casein remains intact, so the difference between the protein composition of UHT milk and raw milk is insignificant. This process also kills all microorganisms in the raw milk. The absence of other microorganisms in the milk is necessary for our experimental design, in order to study only the microbes from the bovine samples and only the *S. aureus* genes in the CRISPRI-library. On the other hand, this sterile environment makes the conditions not fully comparable to the conditions in the udder. *S. aureus* genes might show different expression patterns when affected by other bacterial strains. Another important disadvantage of using milk as medium is that it is not possible to monitor the growth of the bacteria by OD measurements, making it difficult to determine the growth phase of the bacteria. Prior to the CRISPRi-seq experiment a similar experiment with BHI instead of milk was conducted (data not shown) and the incubation periods used in this experiment were transferred to the experiment using milk. The aim was to reach a cell density corresponding to an OD of 0.4-0.6 before ending the incubations. BHI is a rich medium and the strain might grow faster in BHI than milk, resulting in lower bacterial counts and another growth phase when grown using milk, different from what intended. However, comparisons of viable count during growth in milk and BHI suggest that these are indeed similar (personal communication, M. Kjos). Despite these disadvantages, UHT milk was selected best option regarding growth medium similar to what bacteria encounters in the bovine udder, as it contains components found in raw milk, such as calcium, proteins, phosphorus and vitamins.

Working with milk in the laboratory is challenging because of the casein and fat aggregations. These aggregates can capsulate bacterial cells of interest. In the protocols for cell and plasmid isolation from milk culture (section 3.2.1 and section 3.9.2) a washing step with citrate water

was included. This deals with most of the problem by dissolving most of the casein micelles but cannot prevent all cells from being trapped in these aggregates. These cells got swabbed away together with the casein and fat. This should not be an issue when the samples are incubated for some time, as the milk would contain enough bacterial cells for isolation.

5.1.2 Antibiotic concentrations

The antibiotic concentrations for the CRISPRi-seq experiment were determined by a microtiter assay, where a control strain not targeting any sgRNA was grown in BHI together with chloramphenicol for selection, aTc and the chosen antibiotic. The concentrations to use in the CRISPRi-seq experiment were determined as the concentration giving 50% growth inhibition. For penicillin G, MIC_{50} was 0.008 μg/mL. Trimethoprim and sulfamethoxazole were used both separately and in combination in the microtiter assay. They showed a clearly synergetic effect as previously reported. Trimethoprim alone gave a MIC_{50}-value of 0.5 μg/mL, whereas sulfamethoxazole did not inhibit the growth even at 25 μg/mL. Together they gave a MIC_{50} of 0.1 μg/mL trimethoprim and 0.5 μg/mL sulfamethoxazole. To see a clearer effect of the antibiotic, the chosen concentrations could have been higher. On the other hand, based on the antibiotic susceptibility testing of single CRISPRi-strains, the concentrations seemed to be appropriate as we wanted to see some growth.

For the screen of bovine milk, concentrations of antibiotics (penicillin G and AMC) were selected based on previous literature and the values from the European Committee on Antimicrobial Susceptibility Testing (93, 96). It is difficult to conclude whether appropriate concentrations were used, as the freshest samples (sampled by the Faculty of Veterinary Medicine) resulted in overgrown agar plates and some of the oldest samples (from Jurfrisk) did not grow at all. Ideally all the samples should have been sampled in the same way and stored for the same period of time prior to the screen.

An important factor that affected the MIC results of the resistant isolates was the number of bacteria in the overnight culture. OD measurements of the culture were not conducted prior to the microtiter assays but the differences in OD between the cultures were visible, some cultures being denser than others. This was especially prominent for the isolates from the bovine milk. Normalizing the OD before making the two-fold dilution series with antibiotics would have been advantageous to make the MIC determinations more standardized and comparable.

5.1.3 Strain identification methods

Two methods for identification of surviving bacteria from bovine milk upon antibiotic treatment were used, a culture dependent (MALDI-TOF MS) and a culture independent method (16S rRNA gene sequencing). MALDI-TOF MS, a rapid method using protein fingerprints, was used for isolated bacteria grown on blood agar. The other method identified species by 16S rRNA gene sequencing of bacteria in the liquid milk cultures. The latter method is more time-consuming method as sequencing was performed by an external company, but this allowed identification of the entire population, including bacteria not able to grow on agar plates. Some of the identified isolates from MALDI-TOF MS were not identified by 16S rRNA gene sequencing (see more discussion below), and some of these originated from samples that caused overgrowth on the agar plates. This indicate high numbers of these bacteria in the milk and should have been identified by sequencing. Likely reasons for this discrepancy between the methods could be the database used by MALDI-TOF MS. As the database does not include all bacterial species it can cause some misidentifications. In this assay a database of clinical microbes was used. Another reason could be the depth of the sequencing, meaning that too few of a bacteria were present in the sequencing sample it would not be detected, but this should not be an issue for the overgrown cultures.

Amplicon sequencing of the 16S rRNA gene results in counts of different nucleotide sequences. More than one species can match the sequences found by Illumina sequencing when doing BLAST-searches. To determine the exact species the MALDI-TOF MS results can be used. For example, 16S rRNA gene sequencing of the cow sample JF-562 detect *Staphylococcus caprae/epidermidis/capitis* which can be confirmed by the MALDI-TOF MS identification of *S. epidermidis*. By only using 16S rRNA gene sequencing the exact species are not necessary identified. The primers used for sequencing amplify over 99% of all the 16S rRNA gene equences, but as different species have different numbers of copies of the 16S rRNA gene in the genome, those with more copies will get overestimated, being a cause of bias for this method. Another possible bias results from the interactions between the different bacterial strains in the sample. By having some bacteria inhibiting other resistant ones the diversity of the resistant bacteria might be underestimated.

Many of the bovine milk samples used in this work (from the Jurfrisk project) had been stored in -80°C freezer for a long period of time, resulting in many dead bacteria. This issue was faced

when amplifying DNA from these samples using qPCR, where the normal protocol resulted in many negative results. To solve this, lower concentrations of primers and higher concentrations of template DNA were used (Table *3.1*).

To sum up, this experiment illustrated how MALDI-TOF MS and 16S rRNA gene sequencing complement each other in strain identification. MALDI-TOF MS allows quick identification of bacterial isolates on species level. However, growth of colonies is needed for this approach, and abundant strains in the culture not necessary grow on agar plates, while bacteria with low abundance may grow. This is because different species require different conditions to grow, and it is difficult to meet all these conditions by using agar plates. Thus, molecular approaches to microbial population studies should be conducted to get a better overview of the diversity and abundance of bacteria in the samples.

5.1.4 CRISPRi-seq was a successful tool for identification of genes involved in antibiotic susceptibility

In general, the CRISPRi-library and the CRISPRi-seq was successful as it produced a list of genes putatively involved in penicillin G and TMP-SMX susceptibility. In the CRISPRi-seq experiment with penicillin G (which was not discussed further here), several of the genes in the list of hits are already known to be involved in susceptibility to β-lactams, including *glmS* (peptidoglycan synthesis), *feoB* (iron permease) and *pbp4* (peptidoglycan synthesis) (97–99). Though these genes were not examined in this work, the findings of these genes shows that the method was successful. Nevertheless, the importance of investigating these hits further with additional experiments to make conclusions about the gene essentialities must be emphasized (see section 5.3.1). This method is only a screening approach and cannot be used as a tool alone for identifying genes involved in susceptibility to antibiotics. Nevertheless, this CRISPRi-seq method is a simple and useful tool for screening of the entire genome of the *S. aureus* NCTC8325-4 strain.

5.2 Resistant strains in the healthy udder microbiota

More knowledge about the udder microbiota is necessary to understand the pathogenesis of bovine mastitis. With a better understanding of this topic, the incidence of mastitis and the use of antibiotics can perhaps be reduced by the development of novel prophylactic and therapeutic strategies as alternatives to the treatments we use today (11). The composition of the udder

microbiome and mastitis pathogens have been investigated earlier (7, 10, 11, 70), but in this work the aim was to get more insight into the antibiotic resistant strains of healthy udders. We wanted to see how commonly used antibiotics affects the microbiota in the milk and if there are strains found commonly in healthy udders that are resistant to these antibiotics. Even though the antimicrobial use in dairy industry in Norway is low, antimicrobial resistance in food production animals is of large concern, as resistant strains and genes can be transferred to humans and human pathogens and thereby compromise the effectiveness of the antibiotics used to treat infections today.

Milk samples from 13 different cows, collected from different quarters (in total 18 samples) were exposed to antibiotics (penicillin G and AMC) at concentrations similar to those expected to be found in milk during treatment (Figure *3.1*). Strains that were able to grow on TSASB plates were identified by MALDI-TOF MS, resulting in the identification of 13 AMC resistant strains (Table *4.1*), and 14 penicillin G resistant strains (Table *4.2*). The MIC values of the antibiotic used on these strains was also determined. In addition, the total composition of the samples treated with antibiotics (including strains that were not able to grow on agar plates) was determined by amplicon sequencing of the 16S rRNA genes (Figure *4.2*).

5.2.1 Mastitis and environmental associated strains were identified

Most of the isolates and samples identified originated from high SCC quarters. Several species known to cause mastitis were identified by MALDI-TOF MS, such as *E. coli*, *Enterococcus faecalis* and *S. aureus*, found in both penicillin G and AMC treated isolates. Some of these findings were confirmed by the results from the Illumina sequencing, where sequences belonging to unclassified *Enterobacteriaceae, Enterococcus* spp. and *S. aureus* were of the 30 most abundant species identified. In addition, CNSs (*Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus saprophyticus* and *Staphylococcus simulans*) were found to survive in the milk with antibiotic, *S. chromogenes* and *S. simulans* only in the AMC treated samples, by MALDI-TOF MS. These species, especially *S. chromogenes*, *S. epidermidis* and *S. simulans* are highly associated with mild to moderate bovine mastitis (100). However, some of them are also known to serve protective roles against e.g. *S. aureus* infections (101). CNSs were also the overall most abundant group of species found by Illumina sequencing (Figure *4.2*), especially *S. epidermidis, S. caprae* and *S. capitis*. Even though *S. epidermidis* is considered a minor mastitis pathogen, this is also a normal species to find in healthy udders. The results from MALDI-TOF MS can confirm the finding of *S. epidermidis*

by sequencing for some of the samples. *Corynebacterium bovis,* another minor contagious mastitis pathogens, were identified by sequencing in three samples, both treated with penicillin G and AMC. Other *Corynebacterium* spp. (*Corynebacterium amycolatum* and *Corynebacterium lipophiloflavum)* were also detected, though these are not commonly mastitis causing pathogens. Another typical mastitis pathogen, *S. dysgalactiae*, was found in a couple of samples treated with AMC. Bacteria assumed to be harmless were also found to be abundant by sequencing, including *Lactobacillus* spp*., Streptococcus salivarius* and *Streptococcus thermophilius*. These do not cause infections and are even viewed as probiotic, preventing colonization and infection by pathogenic bacteria, however, their resistance is a treat to human and animal health.

E. coli is a typical bacterium to find in the cow´s environment and was identified by both MALDI-TOF MS and 16S rRNA gene sequencing. Especially in the samples taken by the Faculty of Veterinary Medicine without the milking and disinfecting washing of the teats prior to the sampling, the presence of bacteria associated to the environment is not surprising. From these samples, environmental bacteria, such as the family *Enterobacteriaceae* and the genera *Enterococcus* and *Acinetobacter* (that all are associated with mastitis) were the most abundant according to sequencing. Other bacteria that were found in the majority of the samples were *Prauserella aidingensis/alba/halophila.* Their association to mastitis is not found in current literature, but this group of microbes have earlier been isolated from soil and water samples (102, 103). This shows the importance of sampling method when examining the population of microbiota.

The *E. coli* isolates found in this experiment exhibited high MIC values, being able to grow even with the highest concentrations used in the assay. Even though *E. coli* is an environmental bacterium and most likely a contamination in our samples, the results show that *E. coli* with high MIC values are present in the cow's environment. This is a potential problem, either when the *E. coli* (or the other resistant environmental bacteria) is able to breach the cow's defenses and cause an infection in the udder, or if the resistance genes it carries are transferred to other bacteria that are able to cause an infection in the udder.

Micrococcus luteus strains were also found by MALDI-TOF MS, in both penicillin and AMC treated samples. This species is often found in soil and water and do normally not cause infections but can be an opportunistic pathogen (104). Other *Micrococcus* species are more

often found in mastitis cases, such as *Micrococcus pyogenes* (105). *M. luteus* was identified by sequencing also but in a too low number to be included in Figure *4.2*. This specie shows an ability to grow and outcompete other species on blood agar. Another bacterium not commonly associated with mastitis is *Stenotrophomonas maltophilia*, identified by MALDI-TOF MS and of low numbers by 16S rRNA gene sequencing. *S. maltophilia* has rarely been isolated from bovine mastitis and has been considered a nonpathogenic environmental microbe. Its involvement in bovine mastitis has been unclear but Ohnishi et al. (2012) found isolates being somewhat involved in an outbreak of mild bovine mastitis (106). Even though these species are not especially known for being the cause of mastitis, their resistance genes can be transferred to pathogenic bacteria that can cause harm.

Overall, we expected to see more inhibition of growth for the cultures exposed to AMC than penicillin G, due to the β-lactamase inhibitor making the AMC combination able to be effective on β-lactamse producing bacteria. To compare the difference in strain composition of the samples treated with penicillin G versus the samples treated with AMC, the resulting taxas from the same quarter sample with different treatments can be compared (results from the 16S rRNA sequencing). One example is when comparing the treatments of JF-523 (number 7-10 in Figure *4.2*). The cultures treated with AMC has *C. amycolatum* as the most abundant specie, known to display multidrug-resistant phenotypes e.g., to β-lactams (107), while penicillin G treated cultures has a more diverse composition of species, *Staphylococcus aureus/roterodami/simiae* being the most abundant. For JF-540 (number 23-26 in Figure *4.2*) *Staphylococcus aureus/roterodami/simiae* are the most abundant species resistant to AMC, while a larger variety of species are resistant to penicillin G, e.g., species from the *Streptococcus mitis* group. In JF-566, *Staphylococcus chromogenes* were the most abundant specie resistant to AMC, while *Streptococcus mitis* group and species belonging to the *Enterobacteriaceae* family were the most abundant penicillin G resistant strains. *Enterococcus* spp. seem to be more tolerant to penicillin G than AMC, as these species are abundant in the penicillin G treated cultures of 6583 (45 and 46 in Figure *4.2*) and barely present in the AMC treated cultures (47 and 48 in Figure *4.2*). *S. chromogenes* and *S. edaphicus/saprophyticus* are only identified in the AMC treated samples, which do not correspond the expectations of AMC being more effective than penicillin G. This expectation was overall not met, as a clear trend of more inhibition of growth among the AMC treated cultures was not observed. However, these results are not sufficient to

make final conclusions, and screening of more and fresher samples in addition to more data analysis of the differential abundances is necessary.

The strains found in this work might be apathogenic as none of the cows had an ongoing infection at the sampling time, or they were present in too few numbers to be able to cause an infection. It is also conceivable that some of these cows had an ongoing mild infection, as subclinical mastitis can be difficult to detect and diagnose. However, many of the species found that are associated with mastitis are also found in the microbiota of healthy udders. Though this experiment shows that these species are able to be resistant to antibiotics and therefore, if harboring the right virulence genes, could cause mastitis after an antibiotic treatment where host symbiotic and competitive microbes are compromised. The MICs of the antibiotics varied between the species but several survived surprisingly high concentrations of antibiotics. Several species found are not commonly treated with penicillin G and/or AMC. Examples are mastitis caused by *E. coli* or other Gram-negative microbes which are rarely treated with antimicrobial drugs at all. *S. aureus* and other *Staphylococcus* spp. were also identified among the resistant isolates. *Staphylococcus* spp. are determined sensitive if inhibited by ≤ 0.125 μg/mL penicillin G and ≤ 0.25 μg/mL AMC. The concentrations found in the MIC_{50} assays (section 4.1.2) were higher for all *Staphylococcus* spp. tested, except one that were totally inhibited (isolate AN43). The presence of bacteria being highly resistant, like some of these strains, is worrying, even though they are not causing infections. The strains can harbor resistance genes that can be easily transferred to more pathogenic strains with virulence factors giving them the ability to invade the udder. Additionally, some of them might be opportunistic, meaning they might be able to cause an infection if the host is somehow compromised.

5.3 CRISPRi-seq to investigate *S. aureus* genes involved in antibiotic susceptibility

In the second part of this project, we wanted to investigate how different genes in *S. aureus*, an important mastitis pathogen, impact the susceptibility to antibiotics during treatment. The fitness of all the genes in the *S. aureus* upon antibiotic treatment with penicillin G and TMP-SMX was analyzed using a CRISPRi-sequencing approach (Figure *1.7* and Figure *3.2*). A CRISPRi-library targeting all genes of *S. aureus* was cultivated in UHT milk with and without antibiotics (penicillin G and TMP-SMX). If downregulation of a gene (sgRNA) causes increased susceptibility to the antibiotic, strains having this sgRNA will have decreased fitness

and become underrepresented in the total composition of sgRNAs upon antibiotic treatment. In this thesis, only the underrepresented genes from the TMP-SMX exposure were studied further. However, tables of all genes putatively affecting susceptibility to both TMP-SMX and penicillin G are shown in Table *4.4* and Appendix B-Appendix D.

5.3.1 Genes involved in susceptibility to TMP-SMX identified by CRISPRi-seq

Eight genes were found to be more essential and 89 genes less essential for *S. aureus* when grown in milk with TMP-SMX. The less essential genes were not investigated further in this work. Out of the eight more essential genes, six genes were further examined to verify their essentiality. Downregulation of these genes was expected to increase the susceptibility towards TMP-SMX. To verify this, single CRISPRi-strains (including a non-targeting sgRNA strain) were constructed and grown with and without TMP-SMX. We expected to see more inhibition of growth (increased sensitivity) for the new CRISPRi-constructs than of the control strain. Most of the depleted strains showed this trend – especially *nupC, nupG, polA* and SAOUHSC_02121 (Table *4.6*Table *4.4* and Figure *4.9*). These four strains had a notably poorer growth when grown with TMP-SMX compared to the control. It must be emphasized that this experiment is only conducted once, and repetition of the experiment is necessary to make conclusions.

The same was, however, not the case for *murB* and *ung*. Neither of these deviated clearly from the control strain (Table *4.6* and Figure *4.9*) upon antibiotic exposure, although the CRISPRi-seq results suggested that knockdown of these genes should confer increased sensitivity to TMP-SMX. These genes are possibly false positives from the CRISPRi-seq. To make a final conclusion to this statement, a repetition of the experiment should be conducted. In future experiments a parallel of non-induced strains should also be included, to compare induced and non-induced depletion strains in presence of TMP-SMX. It should be noted that the results for the *murB* depletion are unexpected also for other reasons. *murB* is an essential gene and one would expect the *murB* depleted strain to show poorer growth than the control strain also when not being treated with TMP-SMX as this was the only gene showing essentiality when grown in milk alone. The growth did, however, not deviate notably from the control strain, neither with nor without antibiotic exposure.

5.3.1.1 Nucleoside transport genes, *nupC* and *nupG,* are involved in extracellular uptake of thymidine

Knockdown of *nupC* and *nupG* were found to result in increased sensitivity to TMP-SMX by CRISPRi-seq, and this was later verified in the single construct assay. Both genes are nucleoside transport proteins (108, 109).

In CRISPRI-seq *nupC* was the gene showing the most dramatic effect on interaction log2FC when treated with TMP-SMX (Table *4.4*), and although the strain showed some decreased growth compared to the control strain in the verification assay (Table *4.6*), the inhibition of growth was less notable than expected.

As mentioned in section 1.2.2, *S. aureus* has an endogenous synthesis of tetrahydrofolic acid. Tetrahydrofolic acid serves as a cofactor for TS which catalyzes the last step of the thymidine synthesis, the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (Figure *5.1*). dTMP is the precursor of thymidine triphosphate (dTTP) and is essential for DNA synthesis and replication. Therefore *S. aureus* is normally susceptible to TMP-SMX as this compound combination depletes the cells for tetrahydrofolic acid (110). When there are no available TS in the cell, due to TMP-SMX treatment, the cell needs to take up thymidine from the surroundings to maintain DNA synthesis. For patients being treated with TMP-SMX for an extended period, thymidine-dependent (TD) small-colony variants (SCVs) have been reported (111). Different mechanisms can cause this feature, one of them being an increased expression of the nucleoside transporter NupC, encoded by *nupC* (112). The function of NupC in *S. aureus* was proven by Kriegeskorte et al. (2014) to be transportation of pyrimidine, similar to what was also shown for its homologue NupC in *Bacillis subtilis* (113, 114). They confirmed this by growing a *S. aureus nupC* mutant and wildtype under TMP-SMX conditions in presence of extracellular thymidine. The wildtype strain revealed SCV-variants that were able to utilize the thymidine, while the mutant was not able to grow. This strongly suggested that NupC is the major transporter of extracellular thymidine, and is essential for bypassing the TMP-SMX effect on *de novo* thymidine synthesis in *S. aureus* (114). This is in line with the results obtained in this thesis. NupC could therefore be a potential target for a combination therapy with TMP-SMX, in order to improve its antibiotic effect and avoid development of TD-SCV, especially if thymidine is present.

The depletion of *nupG* did also result in reduction of growth compared to the control strain and is also suggested to be essential for cell metabolism. This depleted strain grew poorly even without exposure to TMP-SMX, making it difficult to determine whether the depletion actually affects the susceptibility to TMP-SMX or if it is the silencing of the gene itself that makes the cell susceptible. The exact function of *nupG* has not yet been examined in *S. aureus* but has been more studied for *E. coli.* NupG in *E. coli* transports a wider range of nucleosides than NupC, including both guanosine and inosine, that NupC transports poorly. Unlike NupC, NupG is not able to transport the nucleoside analogue showdomycin which is an antitumor and antibacterial compound (115, 116). The two proteins have a sequence similarity of 33.09% when aligned with the Clustal Omega program (115). It is possible the NupG also transports thymidine in *S. aureus,* but this must be further studied.

If NupC and NupG should be developed as potential antimicrobial targets in the future, it is important to know whether these proteins are also found in human. Regarding human homologs, NupC (of *E. coli*) shows sequence similarities to mammalian nucleoside transporters, while NupG only possesses distantly related homologues in eukaryotes, including humans (115, 117, 118). Doing a BLAST-search of the protein sequences of NupC and NupG to human proteins, 31% and 23% identities to a human concentrative nucleoside transporter (hCNT3) were found, respectively. It should therefore be carefully tested whether potential nucleoside transport inhibitors targeting *S. aureus* (or other bacteria) have cross-activity against human homologs before considering this an antimicrobial therapy.

In addition to transporting nucleosides for cell metabolism, nucleoside transporters may serve as a route of entry of cytotoxic compounds, analogues to nucleotides. These compounds have been of interest as antimicrobial, antiviral and anticancer agents because of their ability to interfere with DNA and RNA synthesis and thereby disturb cell growth (118). Also inhibition of nucleoside transporters of humans is commonly used in treatment regiments of solid tumors and leukemias, and in AIDS-therapy (119, 120).

Thymidin kinase (TK), encoded by *tdk*, is also an important enzyme during uptake of thymidine from the surroundings. TK catalyzes the phosphorylation of thymidine to dTMP (Figure *5.1*). *tdk* is not found on the list of hits from the CRISPRi-seq in this work, although this would be expected based on the *nupC* and *nupG* results. A likely reason for this is probably found in the

makeup of the CRISPRi-library. *tdk* is a part of a 5-gene operon. Thus, there is no sgRNA in the library that targets only the *tdk* gene.

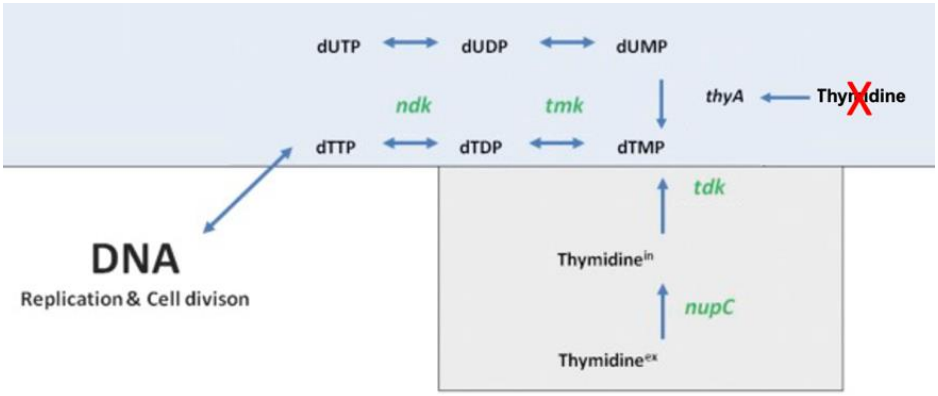

Figure 5.1: Parts of *S. aureus* pyrimidine pathway. When the *in novo* synthesis of thymidine is deprived due to TMP-SMX treatment, the cell rely on extracellular uptake of thymidine. Figure adapted from Kriegeskorte et al. (2014) (114).

The genes *folP* and *dfr* are genes encoding DHPS and DHFR (Figure *1.4*), the two targets of TMP-SMX. These genes were not hits from the CRISPRi-seq, i.e., their downregulation did not result in increased sensitivity to TMP-SMX. The reason for this might be that the enzymes are already inactivated by the antibiotic and absence of these genes will therefore not play an additional role for the cell. Another enzyme involved in the folate biosynthesis is FolC, encoded by *folC*, is not targeted by TMP-SMX (Figure *1.4*). It was expected that depletion of this gene would show synergy with the TMP-SMX treatment (i.e., that cells depleted of *folC* would be more sensitive to TMP-SMX compared to the wild-type). Instead, however, the CRISPRi-seq suggest that this is not the case. While *folC* is essential for growth in both conditions (with and without TMP-SMX), the *folC* depletion surprisingly appears to make the strain somewhat less sensitive TMP-SMX (Appendix B). The reason for this is unknown. One could speculate that since the folate biosynthesis is already fully inhibited by TMP-SMX, the expression of *folC* is costly to the cells as this enzyme is not needed. However, this needs to be verified and tested further. *folK* is also involved in the folate pathway but as part of a larger operon and has therefore not a sgRNA targeting only this gene, as explained for *tdk*.

5.3.1.2 *polA* and *ung* are genes involved in DNA repair

Other genes found to be essential by CRISPRi-seq when treated with TMP-SMX were *polA* and *ung*. The single depletion strain of *polA* had clear inhibited growth upon antibiotic treatment, with a 750-fold reduction in CFU/mL compared to the 200-fold reduction of the control strain. The growth of the *ung* depleted strain did not deviate that much from the control, with only a 176-fold reduction in CFU/mL and is potentially a false positive hit (see section

5.3.1). *polA* encodes a repair polymerase (Pol I) that is involved in DNA repair. When one of the DNA strands are damaged, an excision repair mechanism called base excision repair (BER) removes and replace the non-bulky single-base lesion or the single strand break in the DNA. Non-bulky lesions are caused by different chemical alterations, such as alkylation, oxidation, depurination/depyrimidination, deamination and deoxyuridine triphosphate (dUTP) incorporation during DNA replication (121, 122). The initial step is the recognition of the damaged base by a damage specific DNA *N*-glycosylase that removes the damaged base by hydrolyzing the N-glycosidic bond between the 2´-deoxyribose and the base (122). This leaves an apurinic/apyrimidinic (AP) site. AP endonucleases and AP lyases cuts the 5´and the 3´ ends of the AP site, respectively, allowing further processing by exonucleases or deoxyribophosphodiesterase (dRpase). It is this small gap that is repaired by the repair polymerase, Pol I, and ligated by DNA ligase (121).

The *ung* gene is also involved in the BER pathway of DNA repair is *ung* and encodes uracil-DNA glycosylase (UDG). This protein takes part in the repair of DNA damage where uracil residues are incorrectly incorporated, by specifically removing the uracil from the DNA. The incorporation of uracil arises because of misincorporation of dUMP residues by DNA polymerase or due to deamination of cytosine (121, 123). The TMP-SMX treatment is likely to cause more mistakes during DNA replication due to lack or imbalance of nucleotides. A plausible interpretation of the results is therefore that DNA repair is more essential for the cells during TMP-SMX exposure, and therefore these genes (*ung* and *polA*) are more essential for survival in these conditions. Having inhibitors of these proteins in combination of TMP-SMX could therefore increase the cells susceptibility towards TMP-SMX by obstructing important DNA repair mechanisms in the cell.

5.3.1.3 Depletion of SAOUHSC_02121 results in increased susceptibility to TMP-SMX in *S. aureus*

SAOUHSC_02121 was also found to be more essential upon TMP-SMX treatment. This was also verified by cultivation of the single CRISPRi-construct with and without TMP-SMX, being the strain having the largest fold reduction in CFU/mL upon treatment. SAOUHSC_02121 is an uncharacterized gene, and the protein function is unknown (124). By searching the pfam-database a significant match to the “CamS protein family” was found. This family includes CamS, a precursor of the *S. aureus* sex pheromone staph-cAM373, a signaling molecule that induces conjugal transfer of plasmids between cells (125). Other predictions can also be made

bioinformatically. LocateP is a location predictor of bacterial proteins that predicts the location of SAOUHSC_02121 to be the outside of the cell, lipid anchored to the membrane. A BLAST search of the protein sequence results in alignments of high identity with CamS family proteins from other strains, such as *E. coli*, *Pseudomonas aeruginosa* and *Sulfitobacter donghicola.* To get a deeper understanding of the function of this hypothetical gene, including why it influences the TMP-SMX susceptibility, functional studies need to be performed, including phenotypic studies of mutants and protein-protein interaction studies.

5.3.2 Genes involved in susceptibility to penicillin G identified by CRISPRi-seq

As for TMP-SMX, a CRISPRi-seq experiment was conducted with penicillin G. From this experiment, 99 and 51 genes came out as more essential or less essential, respectively, upon antibiotic treatment. Many of these hits are already known to be involved in penicillin G susceptibility (as explained in section 5.1.4). With this many hits, it is likely that some of them are false positives. Therefore, these genes must be investigated further in order to make any conclusions about their essentiality in the presence of penicillin G. Findings of genes being more essential upon penicillin G exposure are of particularly interest when it comes to bovine mastitis, as this is the most used antibiotic for treating the disease. The increasing rates of penicillin resistance and MRSA among both humans and animals is a worldwide concern and to find targets that make bacteria more susceptible to penicillin G is a potential approach to the resistance challenge. To uncover these targets, the hits from the CRISPRi-seq with penicillin G can be looked into further (Appendix C and Appendix D).

6 Conclusion and future perspectives

In this thesis two subprojects related to antibiotics and antibiotic resistance in bovine mastitis were performed. In the first part, where milk samples from healthy cows were screened for penicillin G and AMC resistant strains, surprisingly many resistant strains were identified, and some of them displayed high concentrations of antibiotics. Even though none of the strains apparently were causing mastitis in the cows, and though some of the bacteria likely were from the cows´ surroundings, the presence of antibiotic resistance in the herd or in the environment of the herd must be taken seriously. These strains can potentially transfer their resistant genes to more virulent strains that can cause infections to the cows, other animals or humans. Also, the strains can have a more opportunistic character, causing disease under the right conditions. The experiment also emphasized the importance of sampling technique. The samples that were not sampled by the technique described by Porcellato et al. (2020), contained an abundance of environmental associated bacteria contaminating the samples.

In the second part of this thesis, it was shown that the aTc-induced CRISPRi-system could be used in milk for identification of genes involved in susceptibility to TMP-SMX and penicillin G. It also showed the importance of verifying the results from CRISPRi-seq by follow-up experiments with depletion of the genes found to be more or less essential. Using CRISPRi known and novel genes involved in antibiotic susceptibility, including genes with unknown mechanisms, were identified. The two candidates *nupC* and *nupG* are both studied in *B. subtilis* and *E. coli* and are already established targets of anticancer and antivirus treatments. These are also interesting antibacterial targets combined with TMP-SMX, as the lack of thymidine and tetrahydrofolic acid in the cell would be lethal, making the cell more susceptible to TMP-SMX, than when TMP-SMX is used alone. A logical next step is to look further into the results of the CRISPRi-seq experiment with penicillin G, by making single CRISPRi-constructs for cultivation assay in a similar matter as done in this thesis for TMP-SMX essential genes. For future work, it would also be interesting to gain knowledge about the function of the product of the SAOUHSC_02121 gene, another hit from CRISPRi-seq, in order to understand its mechanism behind its essentiality in *S. aureus* when exposed to TMP-SMX. For this, functional studies as phenotypic studies of mutans and protein-protein interactions are necessary. It would also be interesting to do similar CRISPRi-seq experiments in other strains by introducing the CRISPRi-system into other *S. aureus* strains or other mastitis pathogens such as *S. dysgalactiae*,

for then to make depletion constructs for verification. This could be useful in the process of gaining more insight into antibiotic resistance in bovine mastitis.

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Appendix

Appendix A Samples and isolates identified by Illumina sequencing and MALDI-TOF

Sample number	Sample ID	Antibiotic	Days of incubation	Isolate ID
1	JF-503	AMC	1	
2	JF-503	AMC	3	
3	JF-520	AMC	2	
4	JF-520	AMC	3	
5	JF-520	Cam	1	
6	JF-520	Cam	3	AW41*
7	JF-523	PenG	1	
8	JF-523	PenG	3	AN61, AN62
9	JF-523	AMC	1	
10	JF-523	AMC	3	AN60
11	JF-529	PenG	1	
12	JF-529	PenG	3	AN158, AN160
13	JF-529	AMC	1	
14	JF-529	AMC	3	AN169
15	JF-534	PenG	1	
16	JF-534	PenG	3	AN45
17	JF-534	AMC	1	
18	JF-534	AMC	3	AN59
19	JF-537	PenG	1	
20	JF-537	PenG	3	
21	JF-537	AMC	1	
22	JF-537	AMC	3	
23	JF-540	PenG	1	
24	JF-540	PenG	3	AN43*
25	JF-540	AMC	1	AN86
26	JF-540	AMC	3	AN42
27	JF-556	PenG	1	
28	JF-556	PenG	3	AN190
29	JF-556	AMC	1	
30	JF-556	AMC	3	
31	JF-562	PenG	1	
32	JF-562	PenG	3	AW24
33	JF-566	PenG	1	
34	JF-566	PenG	3	
35	JF-566	AMC	1	
36	JF-566	AMC	3	AN170
37	JF-571	PenG	1	

38	JF-571	PenG	3	
39	JF-571	AMC	1	AN129
40	JF-571	AMC	3	AN171
41	JF-577	PenG	1	
42	JF-577	PenG	3	
43	JF-577	AMC	1	
44	JF-577	AMC	3	
45	6583	PenG	1	
46	6583	PenG	3	AN35, AN37*
47	6583	AMC	1	
48	6583	AMC	3	
49	6703	PenG	1	
50	6703	PenG	3	AN33
51	6703	AMC	1	
52	6703	AMC	3	AN34*
53	6708	PenG	1	
54	6708	PenG	3	AN31
55	6708	AMC	1	
56	6708	AMC	3	AN32

* Two isolates

Appendix B Table of genes in *S. aureus* found to be less essential when treated with TMP-SMX

Locus tag	Gene name	Essentiality in milk	Essentiality with TMP-SMX	Log2FC interaction
SAOUHSC_T00033	trnaL	essential	neutral	6,834526158
SAOUHSC_02107	murT	essential	essential	6,509803055
SAOUHSC_00921	fabF	essential	essential	6,236693023
SAOUHSC_00472	prs	essential	essential	5,517156719
SAOUHSC_02860	mvaS	essential	essential	4,9348254
SAOUHSC_00519	rplA	essential	essential	4,679191204
SAOUHSC_02126	purB	essential	essential	4,651516923
SAOUHSC_01350	plsY	essential	essential	4,633603782
SAOUHSC_00934	spxA	essential	neutral	4,598551109
SAOUHSC_01598	rnz	essential	neutral	4,316694408
SAOUHSC_02158	aspB	essential	neutral	4,223390763
SAOUHSC_01373	femA	essential	essential	4,201107644
SAOUHSC_01722	alaS	essential	essential	4,163264663
SAOUHSC_01038	def	essential	neutral	4,16143765
SAOUHSC_01242	rimP	essential	essential	4,095226432
SAOUHSC_00336	thl	essential	essential	4,094357187
SAOUHSC_01807	pfkA	essential	essential	4,093502001
SAOUHSC_01339	SAOUHSC_01339	essential	essential	4,001686703
SAOUHSC_01209	rimM	essential	essential	3,972445161
SAOUHSC_03055	rpmH	essential	essential	3,970048739
SAOUHSC_02859	mvaA	essential	essential	3,926281677
SAOUHSC_00551	SAOUHSC_00551	essential	neutral	3,898052156
SAOUHSC_00412	mpsA	essential	neutral	3,833962567
SAOUHSC_01961	hemH	essential	neutral	3,819931897
SAOUHSC_00922	SAOUHSC_00922	essential	neutral	3,778166302
SAOUHSC_00920	fabH	essential	neutral	3,696682129
SAOUHSC_02102	map	essential	neutral	3,594899687
SAOUHSC_00518	rplK	essential	essential	3,59464818
SAOUHSC_01787	lysP	essential	neutral	3,561619435
SAOUHSC_01772	hemB	essential	essential	3,554025692
SAOUHSC_03054	rnpA	essential	essential	3,528759508
SAOUHSC_01470	dnaD	essential	essential	3,519197812
SAOUHSC_02155	pmtR	essential	essential	3,516796963
SAOUHSC_00525	rpoC	essential	essential	3,465616686
SAOUHSC_01496	cmk	essential	neutral	3,429971778
SAOUHSC_01488	SAOUHSC_01488	essential	neutral	3,402483843
SAOUHSC_02255	groES	essential	essential	3,389365693
SAOUHSC_00414	mpsC	essential	neutral	3,388192304
SAOUHSC_01214	rbgA	essential	essential	3,373208863
SAOUHSC_01777	engB	essential	essential	3,279479874
SAOUHSC_01766	folC	essential	essential	3,266302321
SAOUHSC_01002	qoxA	essential	neutral	3,247752193
SAOUHSC_01962	hemE	essential	neutral	3,240792854

SAOUHSC_00019	purA	essential	neutral	3,230767907
SAOUHSC_01789	SAOUHSC_01789	essential	essential	3,22225174
SAOUHSC_00002	dnaN	essential	essential	3,167362591
SAOUHSC_00573	hemQ	essential	essential	3,140428377
SAOUHSC_01216	sucC	essential	neutral	3,123618078
SAOUHSC_00162	hsdR	essential	essential	3,109883465
SAOUHSC_01909	metK	essential	essential	3,089194089
SAOUHSC_02430	htsA	essential	essential	3,00985962
SAOUHSC_01178	coaBC	essential	essential	2,967535875
SAOUHSC_01064	pycA	essential	neutral	2,93933242
SAOUHSC_01859	SAOUHSC_01859	essential	essential	2,928656253
SAOUHSC_01490	hup	essential	neutral	2,914932016
SAOUHSC_02523	SAOUHSC_02523	essential	neutral	2,885037048
SAOUHSC_00471	glmU	essential	essential	2,79210449
SAOUHSC_01829	rpsD	essential	essential	2,781631657
SAOUHSC_01487	ubiE	essential	neutral	2,750814895
SAOUHSC_01200	SAOUHSC_01200	essential	neutral	2,727936444
SAOUHSC_00574	eutD	essential	neutral	2,694288551
SAOUHSC_01721	SAOUHSC_01721	essential	essential	2,690695234
SAOUHSC_00903	spsB	essential	essential	2,675893385
SAOUHSC_01065	ctaA	essential	neutral	2,67295695
SAOUHSC_01182	def2	essential	neutral	2,665608962
SAOUHSC_00802	est	essential	essential	2,656036758
SAOUHSC_00489	folP	essential	neutral	2,648089123
SAOUHSC_02371	coaA	essential	neutral	2,61928736
SAOUHSC_01100	trxA	essential	essential	2,609062704
SAOUHSC_00223	tagF	essential	essential	2,572869136
SAOUHSC_00652	fhuC	essential	essential	2,546945927
SAOUHSC_00524	rpoB	essential	essential	2,502402459
SAOUHSC_01492	engA	essential	essential	2,411257645
SAOUHSC_02366	fbaA	essential	neutral	2,400569515
SAOUHSC_03053	trmE	essential	neutral	2,359919266
SAOUHSC_01203	rnc	essential	essential	2,317820395
SAOUHSC_01592	fur	essential	neutral	2,317013883
SAOUHSC_03052	gidA	essential	neutral	2,249417579
SAOUHSC_02524	SAOUHSC_02524	essential	neutral	2,230084273
SAOUHSC_01868	SAOUHSC_01868	essential	essential	2,221729755
SAOUHSC_00484	tilS	essential	essential	2,195009856
SAOUHSC_00781	hprK	essential	neutral	2,114125951
SAOUHSC_01008	purE	essential	neutral	2,000098783
SAOUHSC_00269	esaG	essential	neutral	1,959922471
SAOUHSC_01975	SAOUHSC_01975	essential	neutral	1,90917963
SAOUHSC_02125	hisR	essential	neutral	1,889132167
SAOUHSC_01960	hemY	essential	neutral	1,827437911
SAOUHSC_00620	sarA	essential	neutral	1,588578976
SAOUHSC_01809	accD	essential	neutral	1,570966414

Appendix C Table of genes in *S. aureus* found to be more essential when treated with penicillin G

Locus tag	Gene name	Essentiality in milk	Essentiality with penG	Log2FC interaction
SAOUHSC_00724	SAOUHSC_00724	neutral	essential	-1,646745747
SAOUHSC_00723	pabB	neutral	essential	-1,717422765
SAOUHSC_01095	rnhC	neutral	essential	-1,77769661
SAOUHSC_01895	SAOUHSC_01895	neutral	essential	-1,778247611
SAOUHSC_01427	ctpA	neutral	essential	-1,781209177
SAOUHSC_00561	vraX	neutral	essential	-1,7886798
SAOUHSC_01282	bsaA	neutral	essential	-1,794046522
SAOUHSC_00344	SAOUHSC_00344	neutral	essential	-1,963370992
SAOUHSC_A01081	SAOUHSC_A01081	neutral	essential	-1,983110423
SAOUHSC_00427	sle1	neutral	essential	-1,999698126
SAOUHSC_02852	cidR	neutral	essential	-2,03354009
SAOUHSC_00502	ctsR	neutral	essential	-2,116355287
SAOUHSC_02615	SAOUHSC_02615	neutral	essential	-2,176628537
SAOUHSC_00536	ilvE	neutral	essential	-2,293818636
SAOUHSC_02366	fbaA	essential	essential	-2,302295023
SAOUHSC_A01909	SAOUHSC_A01909	neutral	essential	-2,343811077
SAOUHSC_01916	menE	neutral	essential	-2,347840851
SAOUHSC_01403	cspA	neutral	essential	-2,435177505
SAOUHSC_01005	SAOUHSC_01005	neutral	essential	-2,488881813
SAOUHSC_01941	splB	neutral	essential	-2,495440374
SAOUHSC_01269	miaB	neutral	essential	-2,511349685
SAOUHSC_A02801	SAOUHSC_A02801	neutral	essential	-2,522380003
SAOUHSC_00475	pth	essential	essential	-2,535516235
SAOUHSC_02611	lyrA	neutral	essential	-2,590718019
SAOUHSC_01635	aroK	essential	essential	-2,591044807
SAOUHSC_00253	SAOUHSC_00253	neutral	essential	-2,604967402
SAOUHSC_01096	zapA	neutral	essential	-2,650387171
SAOUHSC_02362	rho	neutral	essential	-2,683572191
SAOUHSC_01840	sgtA	neutral	essential	-2,688738682
SAOUHSC_01481	aroA	essential	essential	-2,701143523
SAOUHSC_01223	gid	neutral	essential	-2,707059143
SAOUHSC_01404	SAOUHSC_01404	neutral	essential	-2,787422364
SAOUHSC_00980	menA	essential	essential	-2,819897595
SAOUHSC_00892	SAOUHSC_00892	neutral	essential	-2,853165401
SAOUHSC_01258	SAOUHSC_01258	neutral	essential	-2,864710245
SAOUHSC_00640	tagA	neutral	essential	-2,884816062
SAOUHSC_01028	ptsH	essential	essential	-2,885011254
SAOUHSC_02823	SAOUHSC_02823	neutral	essential	-2,957815054
SAOUHSC_A01041	SAOUHSC_A01041	essential	essential	-3,018436169
SAOUHSC_00918	SAOUHSC_00918	neutral	essential	-3,095044311
SAOUHSC_00718	SAOUHSC_00718	neutral	essential	-3,129701063
SAOUHSC_02589	SAOUHSC_02589	neutral	essential	-3,133625343
SAOUHSC_00836	gcvH	neutral	essential	-3,221828872

SAOUHSC_01216	sucC	essential	essential	-3,418806476
SAOUHSC_A00526	SAOUHSC_A00526	neutral	essential	-3,425207077
SAOUHSC_00878	ndh2	neutral	essential	-3,428424332
SAOUHSC_02407	dacA	neutral	essential	-3,48941593
SAOUHSC_00105	phnD	neutral	essential	-3,500021554
SAOUHSC_01482	aroB	essential	essential	-3,526440178
SAOUHSC_00832	aroD	essential	essential	-3,532996367
SAOUHSC_00251	SAOUHSC_00251	neutral	essential	-3,611528531
SAOUHSC_01798	SAOUHSC_01798	neutral	essential	-3,613654375
SAOUHSC_01724	SAOUHSC_01724	neutral	essential	-3,688518723
SAOUHSC_01659	cshB	essential	essential	-3,720343864
SAOUHSC_02731	SAOUHSC_02731	neutral	essential	-3,746458817
SAOUHSC_01405	SAOUHSC_01405	neutral	essential	-3,831815801
SAOUHSC_01106	murI	neutral	essential	-3,852061897
SAOUHSC_00767	saHPF	neutral	essential	-3,912764742
SAOUHSC_01908	smdA	neutral	essential	-3,942696923
SAOUHSC_01852	aroA2	neutral	essential	-3,952797465
SAOUHSC_00694	mgrA	neutral	essential	-3,971657795
SAOUHSC_01062	SAOUHSC_01062	neutral	essential	-4,014801479
SAOUHSC_02343	atpG	neutral	essential	-4,089521461
SAOUHSC_01109	SAOUHSC_01109	neutral	essential	-4,205865452
SAOUHSC_02801	gtaB	neutral	essential	-4,224596239
SAOUHSC_02533	SAOUHSC_02533	neutral	essential	-4,358242066
SAOUHSC_00466	ipk	neutral	essential	-4,413119739
SAOUHSC_A02013	SAOUHSC_A02013	neutral	essential	-4,430193751
SAOUHSC_02259	SAOUHSC_02259	neutral	essential	-4,490499203
SAOUHSC_01850	ccpA	essential	essential	-4,493476626
SAOUHSC_03049	noc	neutral	essential	-4,553563758
SAOUHSC_01803	aapA	neutral	essential	-4,753974792
SAOUHSC_01050	SAOUHSC_01050	neutral	essential	-4,773586345
SAOUHSC_02337	murA1	neutral	essential	-4,827855716
SAOUHSC_01501	ebpS	neutral	essential	-4,865692913
SAOUHSC_01827	ezrA	neutral	essential	-5,019913729
SAOUHSC_02273	rex	neutral	essential	-5,091706569
SAOUHSC_02650	SAOUHSC_02650	neutral	essential	-5,16812858
SAOUHSC_01285	glnR	neutral	essential	-5,305770268
SAOUHSC_01361	msrR	neutral	essential	-5,386129589
SAOUHSC_01073	SAOUHSC_01073	neutral	essential	-5,449660608
SAOUHSC_00567	SAOUHSC_00567	essential	essential	-5,452351633
SAOUHSC_00867	SAOUHSC_00867	neutral	essential	-5,518017499
SAOUHSC_02316	cshA	neutral	essential	-5,579524372
SAOUHSC_00953	ugtP	neutral	essential	-5,633716196
SAOUHSC_01025	SAOUHSC_01025	neutral	essential	-5,713185194
SAOUHSC_02347	atpF	neutral	essential	-5,734015944
SAOUHSC_00646	pbp4	neutral	essential	-5,738640059
SAOUHSC_00409	SAOUHSC_00409	neutral	essential	-5,91727645

SAOUHSC_00468	yabJ	neutral	essential	-5,917460053
SAOUHSC_00189	SAOUHSC_00189	neutral	essential	-6,061835262
SAOUHSC_A00332	SAOUHSC_A00332	neutral	essential	-6,0835968
SAOUHSC_01154	sepF	neutral	essential	-6,268722075
SAOUHSC_01298	SAOUHSC_01298	neutral	essential	-6,291657763
SAOUHSC_00762	tagO	essential	essential	-6,629610272
SAOUHSC_02864	feoB	neutral	essential	-6,753407248
SAOUHSC_01359	fmtC	neutral	essential	-7,202908777
SAOUHSC_00787	SAOUHSC_00787	essential	essential	-7,226288501
SAOUHSC_02399	glmS	neutral	essential	-8,04109999

Appendix D Table of genes in *S. aureus* found to be less essential when treated with penicillin G

Locus tag	Gene name	Essentiality in milk	Essentiality with penG	Log2FC interaction
SAOUHSC_00659	SAOUHSC_00659	essential	neutral	9,164729658
SAOUHSC_00801	secG	neutral	costly	7,491742555
SAOUHSC_00939	SAOUHSC_00939	neutral	costly	6,615669598
SAOUHSC_00790	clpP	essential	neutral	6,087817458
SAOUHSC_00014	SAOUHSC_00014	neutral	costly	4,717142307
SAOUHSC_01979	SAOUHSC_01979	neutral	costly	4,488265174
SAOUHSC_02012	sgtB	neutral	costly	4,487461727
SAOUHSC_00755	SAOUHSC_00755	neutral	costly	4,236077804
SAOUHSC_01192	SAOUHSC_01192	essential	neutral	4,150961861
SAOUHSC_02899	SAOUHSC_02899	neutral	costly	4,041701444
SAOUHSC_01822	tpx	neutral	costly	3,954342669
SAOUHSC_00162	hsdR	essential	essential	3,934072295
SAOUHSC_02690	SAOUHSC_02690	neutral	costly	3,865225932
SAOUHSC_01821	SAOUHSC_01821	neutral	costly	3,803068335
SAOUHSC_01809	accD	essential	costly	3,725867279
SAOUHSC_02102	map	essential	essential	3,398285875
SAOUHSC_02754	SAOUHSC_02754	neutral	costly	3,38190251
SAOUHSC_01263	rny/cvfA	neutral	neutral	3,15717567
SAOUHSC_02369	rpoE	neutral	costly	3,150899831
SAOUHSC_01624	accB	essential	costly	3,090519656
SAOUHSC_01008	purE	essential	neutral	3,022152266
SAOUHSC_01262	recA	essential	essential	2,98444021
SAOUHSC_00614	SAOUHSC_00614	neutral	costly	2,957704811
SAOUHSC_00342	parB	neutral	costly	2,878668501
SAOUHSC_01761	SAOUHSC_01761	neutral	costly	2,806854954
SAOUHSC_02155	pmtR	essential	essential	2,786911335
SAOUHSC_01746	secF	essential	neutral	2,64315396
SAOUHSC_02618	SAOUHSC_02618	neutral	costly	2,600079833
SAOUHSC_00935	trfA	neutral	costly	2,538671648
SAOUHSC_00637	mntA	essential	neutral	2,353621304
SAOUHSC_01778	clpX	essential	neutral	2,3164847
SAOUHSC_00341	metI	neutral	neutral	2,217045598
SAOUHSC_02909	pyrD	neutral	costly	2,153023448
SAOUHSC_02158	aspB	essential	essential	2,131004918
SAOUHSC_00434	gltC	neutral	costly	2,009193436
SAOUHSC_02319	rodA	neutral	costly	2,001083757
SAOUHSC_01685	hrcA	essential	neutral	1,993990925
SAOUHSC_01810	SAOUHSC_01810	neutral	costly	1,975825956
SAOUHSC_01897	sigS	neutral	costly	1,932394345
SAOUHSC_01657	znuC	neutral	costly	1,912926345
SAOUHSC_00003	SAOUHSC_00003	essential	neutral	1,876377289
SAOUHSC_01718	SAOUHSC_01718	neutral	costly	1,871877898
SAOUHSC_00473	SAOUHSC_00473	neutral	neutral	1,859425576

SAOUHSC_02121	SAOUHSC_02121	neutral	costly	1,812350232
SAOUHSC_02898	gbaB	neutral	neutral	1,793398923
SAOUHSC_01630	SAOUHSC_01630	neutral	costly	1,792073315
SAOUHSC_02990	sasA	neutral	costly	1,745705833
SAOUHSC_00873	nifU	neutral	neutral	1,73004481
SAOUHSC_00291	SAOUHSC_00291	neutral	costly	1,711981697
SAOUHSC_00474	rplY	essential	neutral	1,632140342
SAOUHSC_01971	SAOUHSC_01971	neutral	costly	1,378293677

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