Norges miljø- og biovitenskapelige universitet

Master's Thesis 2019 60 ECTS

Faculty of Chemistry, Biotechnology and Food science

Purification and characterization of streptovoracin, a novel antibiotic produced by a *Pseudomonas* species isolated from the Oslo fjord.

Malene Tidemann Nesdal

Biotechnology

Acknowledgment

The work presented in this master thesis was carried out in collaboration with the research group molecular microbiology lead by Dr. Leiv Sigve Håvarstein, at Faculty of Chemistry, Biotechnology and Food science at Norwegian university for Life Science (NMBU). The thesis was written in the period August 2018 to May 2019 and was supervised by both Dr. Leiv Sigve Håvarstein and Dr. Daniel Straume.

I am very grateful for my supervisor Dr. Leiv Sigve Håvarstein for helping me write this thesis and for the opportunity to be working on such an interesting topic. I will also wish to thank my other supervisor Dr. Daniel Straume for his patient helping me accustom to the laboratory's intricate instruments and for guidance during laboratory work. The best of wishes to the both of you continuing the research on this fascinating antibiotic.

In addition, must department engineer Zhian Salehian be mentioned for his immense positivity and eager in helping me preform the many experiment included in this thesis. His early hours at the laboratory growing cultures greatly contributed to efficiency of laboratory work. Also thank you to Dr. Morten Kjos for help with the electron microscope.

Over the course of this master thesis there have been many tears, much laughter and unpleasant smells soaring through the halls of the Molecular Microbiology lab. I wish to thank my friends and family for mental and moral support throughout my thesis. Lastly, I will thank my wonderful boyfriend Sondre Bredesen for always keeping my spirit up and for surviving the ups and downs expected when living with a master student.

May 2019

Malene Tidemann Nesdal

Sammendrag

Antibiotikaresistent *Streptococcus pneumoniae* er en av de 18 største bakterielle truslene verden står ovenfor. Bakterien er alene ansvarlig for oppunder 2 millioner dødsfall hvert år på verdensbasis. På grunn av stadig økende antibiotikaresistens hos *S. pneumoniae* og andre viktige patogener er det nødvendig å oppdage nye antibiotika for å ligge i forkant av resistensutviklingen. Forskningsgruppen Molekylær Mikrobiologi ved NMBU isolerte derfor bakterier fra Oslofjorden i håp om å finne nye antibiotika som er aktive mot *S. pneumoniae.* De fant en bakterie (*Pseudomonas* isolat nr. 33) som hemmet vekst av *S. pneumoniae* ved å produsere det som så ut til å være et nytt antibiotikum. Antibiotikumet fikk navnet streptovoracin og ble identifisert som et «cyclic lipopeptide» (CLP).

I de innledende studiene av streptovoracin så det ut til at antibiotikumet er svært smalspektret og kun aktivt mot enkelte medlemmer av slekten *Streptococcus*. Ett av hovedmålene med denne oppgaven var å undersøke sensitiviteten til et vesentlig større antall bakterier for å kartlegge aktivitetsspekteret til streptovoracin. Resultatene viste at de fleste streptokokker hemmes av streptovoracin. Et klart unntak er *Streptococcus mutans*, bakterien som forårsaker tannråte. Utenom slekten *Streptococcus,* var det bare *Enterococcus faecalis* som viste seg å være sensitiv blant de Gram-positive bakteriene som ble testet. De Gram-negative bakteriene blir trolig ikke hemmet av streptovoracin, men siden jeg kun testet *Escherichia coli* bør flere arter undersøkes før en kan si dette med sikkerhet. På bakgrunn av disse resultatene var det svært overraskende at to arter innen fylum Actinobacteria ble hemmet av streptovoracin, nemlig en art innen slekten *Dietzia* og *Mycobacterium smegmatis.* Sistnevnte er i nært slekt med *Mycobacterium tuberculosis*, noe som tyder på at streptovoracin er aktivt også mot dette viktige humanpatogenet. Siden *S. pneumoniae* og M. *smegmatis* står veldig langt fra hverandre fylogentisk, er det mulig at de hemmes ved ulike mekanismer

Et annet hovedmål ved oppgaven var å bestemme MIC-verdien til streptovoracin mot *S. pneumoniae*. Følgelig måtte det produseres så store mengder av rent streptovoracin at stoffet kunne veies på en analysevekt. For å klare dette måtte produksjonen av streptovoracin optimaliseres og renseprosedyren forbedres. *Pseudomonas* isolat nr. 33 ble dyrket under forskjellige betingelser for

å teste hva som gav best produksjon av antibiotikumet. Det viste seg at de viktigste faktorene for god streptovoracinproduksjon er tilgang på oksygen og et rikt medium.

Etter optimalisering av produksjonen måtte streptovoracin renses. I utgangspunktet ble ammoniumsulfatfelling brukt som første trinn i renseprosedyren, noe som førte til et tap på ~40%. Jeg benyttet i stedet amberlite XAD 16N. Amberlite som første rensetrinn gav maksimum 10% tap av streptovoracin, Når amberlite-trinnet ble kombinert med revers fase HPLC, ble det mulig å fremstille milligrammengder av rent streptovoracin. Som resultat av dette ble det funnet MIC verdier for *S. pneumoniae* og *M. smegmatis* på henholdsvis 10-20 µg/ml og 20-40 µg/ml.

Et delmål ved oppgaven var å identifisere *Pseudomonas* isolat nr. 33, helst til artsnivå. Før jeg startet på oppgaven var isolat nr. 33 allerede plassert i *Pseudomonas fluorescens* gruppen ved hjelp av 16S rRNA sekvensering. 16S rRNA sekvenseringen gav best treff på arten *Pseudomonas brenneri*, men når jeg senere testet isolatet ved MLST, var ikke *P. brenneri* eller noen andre arter gjennomgående beste treff for kombinasjonen av genene. Det er dermed trolig at isolat nr. 33 er en ny art innen slekten *Pseudomonas*.

Abstract

Antibiotic resistant *Streptococcus pneumoniae* is among the 18 most life-threatening human pathogens in the world. This bacterium alone is responsible for almost 2 million deaths every year worldwide. Due to the ever-increasing antibiotic resistance observed for *S. pneumoniae* and other important pathogens, it is crucial to discover new antibiotics in order to be one step ahead of the resistance development. For this reason, the Molecular Microbiology group at NMBU isolated bacteria from the Oslo fjord with the aim to find new antibiotics that are active against *S. pneumoniae*. A *Pseudomonas* strain termed isolate no. 33 was identified and shown to produce a substance that inhibited the growth of pneumococci. The potentially novel antibiotic was named streptovoracin and was identified as a cyclic lipopeptide.

In the initial studies performed on streptovoracin, it looked like the antibiotic is very narrow-spectered and that it is only active against members of the genus *Streptococcus*. One of the main goals of my thesis was to perform a more comprehensive screening of target bacteria to map the inhibition spectrum of streptovoracin. The results showed that the growth of most streptococci are inhibited by streptovoracin. A notable exception is *Streptococcus mutans*, the bacterium causing tooth decay. Apart from the genus *Streptococcus*, only *Enterococcus faecalis* turned out to be sensitive among the Gram-positive bacteria tested. Gram-negative bacteria are probably insensitive to streptovoracin, but since I only tested *Escherichia coli* additional species must be examined before reliable conclusions can be drawn. On the basis of these findings, it came as a big surprise that two species belonging to the phylum Actinobacteria were sensitive to streptovoracin, namely a member of the genus *Dietzia* and *Mycobacterium smegmatis*. The latter is closely related to *Mycobacterium tuberculosis*, indicating that streptovoracin is active against this important human pathogen as well. Since *S. pneumoniae* and *M. smegmatis* belongs to different phyla and consequently are distantly related, their growth is probably inhibited by different mechanisms.

A second major goal of my thesis was to determine the minimal inhibitory concentration (MIC) of streptovoracin against *S. pneumoniae*. Consequently, quantities of streptovoracin large enough to be weighed on an analytical scale had to be produced. To manage this the production of streptovoracin had to be optimized, and the purification protocol had to be improved. *Pseudomonas* isolate no. 33 was cultivated under different conditions to determine which method gave the best

result with respect to yield. My results identified two factors that were essential for obtaining a high production of streptovoracin, namely aerobic growth conditions and a rich medium.

After optimizing the production of streptovoracin, the next step was to develop a protocol for intermediate scale purification of the antibiotic. During the initial attempts to purify streptovoracin, ammonium sulfate precipitation was used as a first step. However, this procedure gave rise to a 40% loss of activity. I therefore tested whether streptovoracin would bind to amberlite resin XAD 16N. It turned out that the antibiotic binds avidly to this resin, and that the loss in yield was only 10%. By combining the amberlite step with reverse phase HPLC it became possible to produce milligram amounts of pure streptovoracin. As a result, the MIC against *S. pneumoniae* and *M. smegmatis* was determined to be 10-20 µg/ml and 20-40 µg/ml, respectively.

Another goal of my thesis was to classify *Pseudomonas* isolate no. 33 to the species level. Before I started my work, 16S rRNA sequencing had revealed that isolate no. 33 belongs to the genus *Pseudomonas,* and that it is a member of the *Pseudomonas fluorescence* subgroup within this genus. Based on the 16S rRNA sequence alone, the closest relative of isolate no. 33 appeared to be a species named *Pseudomonas brenneri*. However, after I used MLST to analyze the phylogenetic relatedness between isolate no. 33 and other members of the *P. fluorescence* group it became clear that the streptovoracin-producing isolate is not very similar to any of them. Hence, it is likely that isolate no. 33 represents a new species within the genus *Pseudomonas*.

Table of content

1. Introduction

1.1 *Streptococcus pneumoniae* and the antibiotic crisis

1.1.1 *Streptococcus pneumoniae*, the great killer

Lower respiratory infections (LRI) are the fifth leading cause of death annually, and the major cause of bacterial deaths. Numbers from 2015 indicated that LRI caused up to 2,7 million deaths worldwide, and the Global Burden of Disease study (GBD) further estimated that 55% were caused by *Streptococcus pneumoniae* (GBD 2015 LRI Collaborators, 2017). As the leading cause of community-acquired pneumonia (CAP), *S. pneumoniae* is the largest cause of death in children under the age of 5 (Martens & Demain, 2017) These numbers were collected in 75 different countries all around the world in a survey called 'Countdown to 2015'. The survey tracked the numbers of CAP deaths in children under the age of 5 during a period of 15 years. Although they saw a decline in the numbers of deaths from 2000 to 2015, CAP still caused over 15% of the deaths in children under 5 in 2015. These numbers were highest in developing countries in sub-Saharan Africa and the south-Asia region (Victora et al., 2016). The numbers are most likely underestimated due to low sensitivity of diagnostic tools and sparse access to care in some part of the world (O'Brien et al., 2009). Nonetheless, *S. pneumoniae* greatly affects communities and societies all around the world despite introduction of childhood vaccination programs in seven European countries using the pneumococcal conjugated vaccine (PCV). The vaccination program did prove effective against the serotypes of *S. pneumoniae* included in the vaccine, but also resulted in an increase in infections caused by other less common serotypes. This raised the question of whether PVC does more harm than good by increasing infections of less common strains that the community have less immunity against (Savulescu et al., 2017).

S. pneumoniae is a Gram-positive ovoid bacterium that grows in pairs or short chains. The bacterium is a common inhabitant of the human oral cavity and upper respiratory tract. The carrier rate of adults is <10%, while it is higher in infants at 27-65% (Ramos-Sevillano et al., 2019). These healthy carriers contribute to the spread of *S. pneumoniae,* causing infections in mostly younger children, elderly and the immune suppressed. *S. pneumoniae* behaves like an opportunistic pathogen that can cause disease in both carriers and non-carriers under conditions that is not well

understood. In carriers, *S. pneumoniae* may cause disease if the immune system is supressed in some ways, for instance during cancer treatment, pregnancy or other infections. While pneumonia is the pneumococcal disease that kills most humans, the bacterium can also infect other parts of the body causing sepsis, meningitis, otitis media and sinusitis (van der Poll & Opal, 2009).

Before antibiotics, *S. pneumoniae* was known as the great killer, and it was therefore a landmark event in medicine when Alexander Fleming discovered penicillin in 1928. Penicillin inhibits the synthesis of the bacterial cell wall, and do not affect the host's own cells. Penicillin is widely used since the second world war, and since then pneumonia has no longer been the death sentence it was previously. Penicillin resistant *S. pneumoniae* was first documented in laboratory research in 1943, and again in 1967 when penicillin resistant strains of *S. pneumoniae* were found in Australia (Watson et al., 1993). In addition to being resistant to beta-lactams, these strains were also tolerant to tetracycline. More recent research has shown that antibiotic resistance can develop through different mechanisms. Common mechanisms are: i) active secretion of the antibiotic, ii) prevention of antibiotic internalization, iii) remodelling of the antibiotic target through mutation, and iv) enzymatic degradation of the antibiotic. In the treatment of pneumococcal infections, beta-lactams are the first choice for medical doctors. Unfortuntely, the resistance of clinical isolates of *S. pneumoniae* to beta-lactams and other antibiotics have been steadily increasing during the last fifty years. In most bacteria, resistance against beta-lactams such as penicillin is due to the expression of beta-lactamases, enzymes that cleave the beta-lactam ring and inactivate members of this family of antibiotics. In the case of *S. pneumoniae*, however, beta-lactam resistance is not due to beta-lactamases, but to mutated penicillin-binding proteins (PBPs) with reduced affinity for beta-lactams. The function of PBPs, which are the target proteins of beta lactams, is to synthesize the peptidoglycan layer that makes up most of the bacterial cell wall. Currently, in the USA, beta-lactam resistance is found in 20-30% of *S. pneumoniae* infections, while second line drugs such as macrolides, trimethoprims, lincosamides and tetracyclines have resistant levels at 20-40%, 25-45%, 20-25% and 20-25%, respectively (Cherazard et al., 2017).

1.1.2 The antibiotic resistance crisis

The trend of increasing antibiotic resistance is to a large extent due to the overuse and misuse of antibiotics. Patients are often given antibiotics based on an uncertain diagnosis, and it has been

estimated that 30-50 % of antibiotic treatments are inappropriately prescribed globally (Ventola, 2015). Another significant contributing factor is the extensive use of antibiotics in agriculture and veterinary medicine, e. g. to improve crop yields and to promote growth in livestock's (Martens & Demain, 2017). In 2013 the Centers of Disease Control and Prevention (CDC) classified the 18 most threatening bacterial pathogens in the U.S by the level of concern; urgent, serious or concerning. *S. pneumoniae* was one of twelve bacteria classified as a serious threat, with 1.2 million cases of drug resistant *S. pneumoniae* infections annually (Eurosurveillance-Editorial-Team, 2013). These numbers are limited to the U.S.A. only, and do not tell the full story of drug resistant *S. pneumoniae*. When faced with infections of multi-drug resistant bacteria, clinicians may use a mixture of different antibiotics or a broad-spectrum last-line antibiotic. Extensive use of broad spectrum antibiotics drives the development of resistance in the patients normal flora, and thereby contributes to increase general drug resistance among bacteria living in the human body (Perry et al., 2018). To stem the current development, improved antibiotic stewardship and surveillance, cheaper and more rapid methods to identify specific bacterial infections and detecting antibiotic resistance and new antibiotics are required. If not, the world is left with a rising numbers of "untouchable" pathogenic bacteria, and a declining number of effective drugs (Ventola, 2015).

1.1.3 The need for precision drugs

As mentioned above, broad spectrum antimicrobial drugs have negative side effects. They contribute to increased antibiotic resistance among members of the human microbiota and can even damage human health through dysbiosis (Spaulding et al., 2018). Dysbiosis (also called dysbacteriosis) is a term for a microbial imbalance or maladaptation on or inside the body. When undergoing treatment with a broad-spectrum drug, useful and important bacterial commensals in the intestine might be killed and replaced by more resistant bacteria. There is growing evidence that this is associated with both intestinal and extra-intestinal disorders. It often elicits a prolonged inflammatory reaction and can be damaging for patients of all ages. In young children dysbiosis affecting their microbiota can influence and prevent proper growth and development. Without a normal host-microbiota interaction during childhood the immune system can become underdeveloped and muscle and bone growth inhibited. Thus, although rare, unwanted side effects of broad-spectrum antibiotics might affect human health and should if possible be prevented. To prevent the antibiotic resistance crisis to get out of hand, and to reduce the negative effect of broad-

spectrum antibiotics they should only be used when absolute needed. Moreover, future research should focus more on identifying and developing narrow-spectrum antibiotics, also called precision antibiotics. Precision antibiotics targets a narrow range of bacteria. Hence, they do not affect most of the body's normal flora, which means they are unlikely to cause super-infections or contribute to increased resistance development among other species present in the host's microbiota (Spaulding et al., 2018).

Most antibiotics prescribed by medical doctors are old broad spectrum. Such drugs are often preferred due to their low cost per treatment. In contrast to the 1950s and 1960s, when a lot of pharmaceutical companies were screening for new antibiotics, the industry is not so interested anymore drugs. There are several reasons for this: i) it takes a long time from a drug is discovered until it is available on the medical market, ii) there is very high costs associated with the development of a new drug, iii) the patient only takes the antibiotic for a short period of time, iv) a patent is only granted for 20 years, and v) it is more profitable to develop other types of medicines. These concerns might be the reasons why 15 out of the world's 18 largest pharmaceutical companies have left the antibiotic field (Ventola, 2015). However, small biotechnology companies and academics are still searching for new drugs to fight the antibiotic crisis (Martens & Demain, 2017). At the Norwegian University of Life Science, the Molecular Microbiology group has been searching for new antibiotics to be used against *S. pneumoniae*. The team found a promising candidate that is highly active against the pneumococcus. Preliminary results, obtained before I started my master thesis, indicated that this antibiotic has a narrow target range and therefore could be classified as a precision drug. The new compound, which was named streptovoracin, is the subject of the present thesis.

1.2 new antibiotic Streptovoracin

Streptovoracin is a novel antibiotic compound produced by a bacterium identified as a *Pseudomonas* species based on the sequence of its 16 S rRNA gene. The closest species identified in BLAST searches was *Pseudomonas brenneri*. The *Pseudomonas* strain (Fig. 1) was identified

in a screening of bacteria collected from the Oslo fjord. The aim was to find bacteria that produce antimicrobial compounds inhibiting the growth of *S. pneumoniae*. Based on tests performed prior to this thesis, streptovoracin is believed to belong to a family of antibiotics called cyclic lipopeptides (CLPs). CLPs generally consist of a short chain of amino acids, some of which form

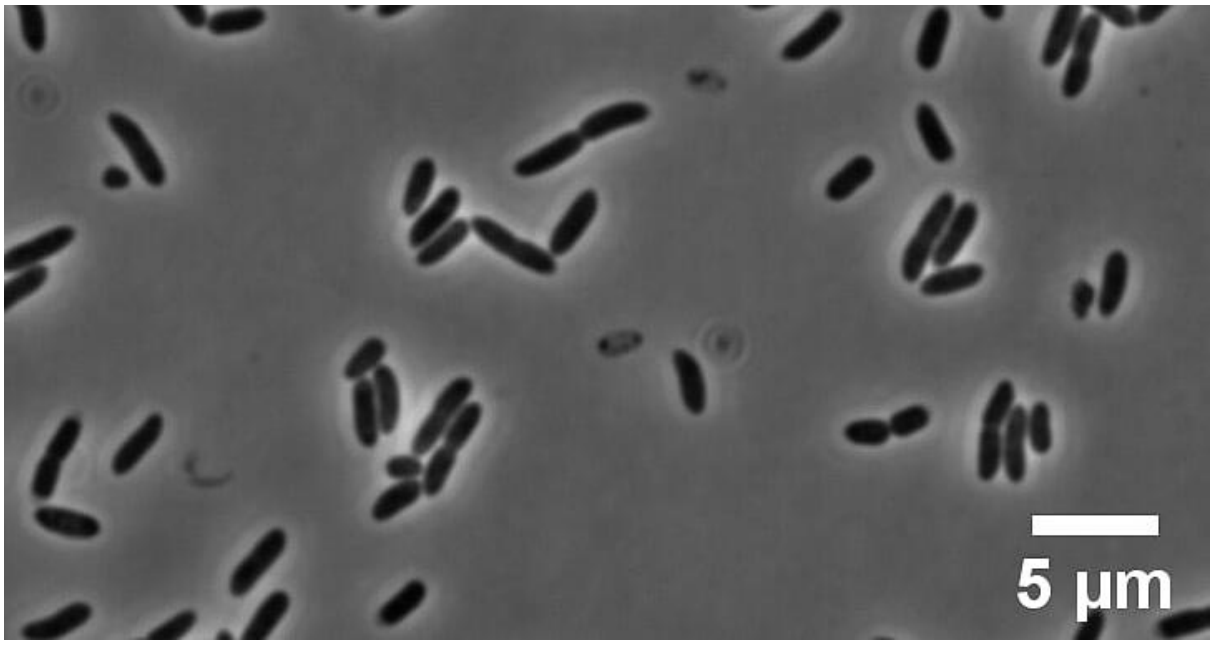


Figure 1. Light microscope picture of Pseudomonas isolate no. 33, the strain producing Streptovoracin. The picture was taken with a phase-contrast objective at 1000 x magnification.

a circle, with an N-terminally attached lipid-chain. Daptomycin, for instance, consists of 13 amino acids, 10 of which are arranged in a cyclic fashion, and three in an exocyclic tail (Fig. 2). Previous small-scale purification of streptovoracin had shown that it elutes as two peaks from the reverse phase column during High Performance Liquid Chromatography (HPLC). Subsequent analysis by mass spectrometry revealed that the masses of the compounds eluting in the two peaks are 1148,8 Da and 1176,8 Da. The only difference between these peaks is believed to be an extra pair of hydrocarbons ($-CH_2-CH_2-$) extending the fatty acid acyl chain. None of the masses matched any cyclic lipopeptide compound registered in the Norine database, and it is therefore reasonable to assume that streptovoracin is a new compound in this family. Before I started working on my thesis, a number of experiments had already been conducted in an initial characterization of streptovoracin. Despite its small mass, dialysis performed with a membrane with a cut-off of 10

kDa failed to let the compound though, suggesting that it forms micelles in aqueous solution. As CLPs are known to form micelles this finding supported the notion that streptovoracin might be an amphiphilic lipopeptide. Furthermore, it was attempted to inactivate the compound with proteolytic enzymes. In accordance with what has been found for other CLPs, trypsin and proteinase K failed to inactivate streptovoracin, while some inactivation was detected after prolonged incubation with pronase, a mixture of different proteases. Interestingly, digestion with polymyxine acylase, an enzyme that deacylates lipopeptides and inactivates CLPs, also inactivated streptovoracin. Together these results, which was obtained before I started working on my thesis, strongly suggested that streptovoracin is a cyclic lipopeptide (Håvarstein, 2018).

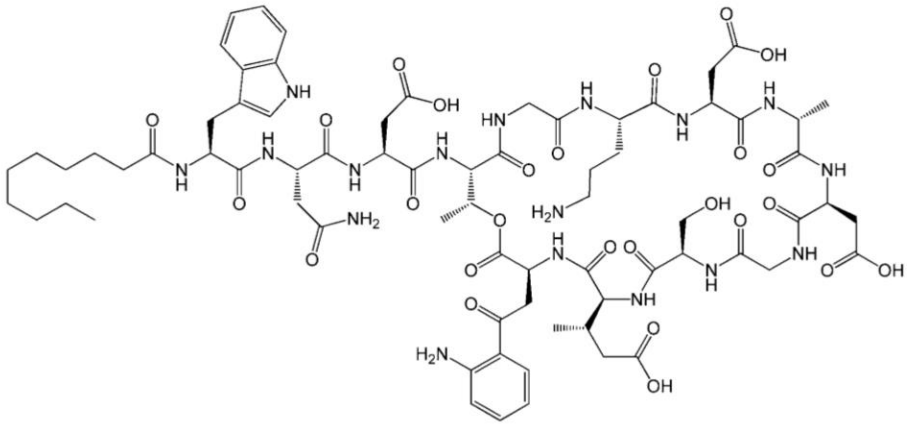


Figure 2. Structure of daptomycin (Hamley, 2015).

1.3 Cyclic lipopeptide antibiotics

1.3.1 General structure, production and properties of cyclic lipopeptides

Cyclic lipopeptides are amphiphilic compounds that are produced as secondary metabolites by a variety of aerobic microorganisms such as fungi, yeast and bacteria. They have a very low critical micelle concentration, meaning that the monomers self-assemble into micelles at a low concentration (Ines & Dhouha, 2015). CLPs are composed of a lipid tail linked to a short cyclic oligopeptide. The lipid part varies by the length of the alkyl chain, the content of cis and trans

isomers, and the extent of chain branching. The peptide part of CLPs is synthesized by very large enzymes called nonribosomal peptide synthetases (NRPSs) (Schneider et al., 2014). These enzymes have a modular structure where each module function as a mould for the stepwise incorporation of amino acids during CLP synthesis. The order and number of NRPS modules are colinear with the amino acid sequence of the CLP. NRPSs also contain thioesterase domains that cleave the lipopeptides at the end of the assembly line and catalyse the cyclization of the mature peptide products. Epimerization domains that determine the configuration (D or L) of the incorporated amino acids may also be present (Raaijmakers et al., 2010). CLPs vary by the nature, number, L- and D- configuration of their amino acid constituents and the size of the oligopeptide ring. In addition, most CLPs contain non-natural amino acids, as for example Dba (2,4-diamino butyric acid) in polymyxin B and Kyn (3-aminobenzoyl-alanine) in daptomycin (Schneider et al., 2014). By combining the components described above in different ways, microorganisms produce a large array of different CLPs with different properties, indicating that they serve different and possibly multiple purposes in nature. However, as CLPs have antimicrobial and antifungal properties, it is very likely that their main biological function is to act as weapons against competing bacteria and/or fungi. In addition, due to their amphiphilicity most CLPs have surfactant properties that might help the producer strain to utilize hydrophobic nutrients that would have been less available without the emulsifying effect of the biosurfactant. It is also possible that some CLPs are used for communication between bacteria and plants (Cochrane & Vederas, 2016). The interesting and largely unexplored properties of CLPs should make them attractive for number of applications relevant for the agricultural, food, chemical and pharmaceutical industries (Ines & Dhouha, 2015).

1.3.2. General antimicrobial properties of CLPs

The mechanism by which CLPs kill susceptible bacteria is known in a few cases (see section 1.5 for more details), but generally their mode of action is not well understood (Ines & Dhouha, 2015). The fatty acid tail of CLPs, which is essential for activity, inserts itself into the lipid bilayer of bacterial membranes. Apparently, the specific molecular structure of the tail is less important than its overall hydrophobicity. However, it has been shown that the fatty acyl chain length of CLPs can influence the MIC in some cases (Magetdana et al., 1988; Morikawa et al., 2000). A general property of CLPs is that they disturb the organization and integrity of bacterial membranes, and

several are known to create pores or ion channels (Epand et al., 2016). However, experiments have shown that CLPs inhibit susceptible bacteria at much lower concentrations than those required to permeabilize or disrupt membranes in an unspecific manner. Furthermore, CLPs varies with respect to target range, some are broad-spectrum antibiotics while others are more specific. Hence, their antibacterial activity cannot be explained by an unspecific detergent-like mechanism (Reder-Christ et al., 2012). It is likely that the large structural variation found in CLPs discovered and characterised in recent decades reflects that they kill target bacteria through many different mechanisms. If so, CLPs represent a treasure trove for future drug development.

1.3.3. CLPs in medicine

CLPs are very stable within the body, possibly due to their cyclic structure, their content of non-natural amino acids or their resemblance to human antimicrobial peptides (Falanga et al., 2017). Unfortunately, although CLPs remain stable during passage through the digestive system, they are poorly absorbed in the intestine. This means that treatments must be given intravenously to reach different parts of the body (Kleijn & Martin, 2018). Although CLPs have been known for decades, their clinical applications have mostly been limited to topical use. Their clinical use has been hampered by studies raising concerns about their neuro- and nephrotoxicity. However, recent case studies suggest that they are considerably less toxic than previously reported (Sauermann et al., 2008).

Despite of CLPs great potential as last resort antibiotics that can be used for treating difficult infections caused for instance by antibiotic resistant bacteria, only a few have reached the medical market. Schneider et al. wrote in 2014 that only two of the CLPs they reviewed were approved, but that several promising CLPs were in the preclinical phase and looked promising (Schneider et al., 2014). Since Schneider et al. published this paper none of these promising drugs have to my knowledge reached the market. The only CLPs Schneider and co-workers described that is currently approved are daptomycin and polymyxin, which were already in use at the time the paper was published. However, ramoplanin, which in 2014 was in phase III clinical trials, is somewhat in use, but under a very strict regime. Ramoplanin is structurally identical to CLPs, except that it has a mannosyl-(1,2)-α-D-mannose disaccharide attached to the oligopeptide ring. In addition, MX-2401, a synthetic modification of amphomycin, is still in the preclinical phase. In addition to their antimicrobial activity, it has been discovered that some CLPs have anti-inflammatory,

antitumor or pro-biotic properties (Patel et al., 2015). Hence, they may have a broader medical potential than initially believed.

In recent years, many CLPs have been identified and characterised. They are placed in groups based on structure and mode of action. Without knowing the structure of streptovoracin, it is not possible to know which group it belongs to. But based on its molecular mass and the fact that it is produced by a *Pseudomonas* species, we speculate that Streptovoracin might belong to the viscosin group of CLPs. However, this is largely based on guesswork, and very little is known about the medical potential of members of the viscosin group. In the more detailed description given in the next section (1.4), I will therefore focus on the best studied and medically most relevant CLPs (Table 1).

Table 1. Streptovoracin compared to the best characterized and medically relevant CLPs.

Cyclic lipopeptides	**Molecular weight (Da)**	**Producer**	**Target**
Streptovoracin	1149/1177	*Pseudomonas sp.*	unknown
Daptomycin	1620	*Streptomyces roseosporus*	Binds to phosphatidylglycerol in Gram-positive bacteria's cytoplasmic membrane, depolarizes cells and inhibit macromolecular biosynthesis.
Amphomycin/ MX-2401	1290/1468	*Streptomyces canis* ATCC 12237	C_{55}-P in Gram-positive bacteria. Inhibits peptidoglycan biosynthesis
Polymyxin (B_1)	1188	*Bacillus polymyxa*	Outer membrane on Gram-negative bacteria.
Ramoplanin	2254	*Actinoplanes sp.*	Lipid II on Gram-positive bacteria's cytoplasmic membrane, inhibits peptidoglycan biosynthesis

1.4 Well characterized medically important or promising CLPs

1.4.1 Daptomycin – history and clinical use

Daptomycin is the best studied CLP, and most research on other CLPs is based on and inspired by the early daptomycin studies. Daptomycin was originally discovered and developed by the Eli Lilly and Company in the early 1980s. It showed promising results against Gram-positive pathogens and was further investigated by clinical trials in the early 1990s. The trials involved several hundred subjects and a large variety of diseases. Positive effects were obtained for blood stream infections, endocarditis and complicated skin and soft tissue infections. However, some side effects were detected which indicated that daptomycin is too toxic for use in humans. It was for instance found that it might cause forearm weakness, myalgia and elevated creatine kinase levels. These problems were trace to the dose intervals used, i.e. 4 mg/kg every 12 hours. By increasing the dose to 6-8 mg/kg and prolonging the administration interval to 24 hours, many of these side effects disappeared. However, despite of this improvement, Eli Lilly put the clinical trials on hold. No more research on daptomycin was performed until Cubist Pharmaceuticals licensed the drug from Eli Lilly and Company in 1997. Then, from 1999 and onwards clinical trials were conducted both in the US and Europe. In September 2003 daptomycin was approved by the U.S. Food and Drug Administration (FDA) for the treatment of complicated skin infections caused by the following Gram-positive bacteria: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae* subsp. *equisimilis* and *Enterococcus faecalis*. Later on, in 2006, it was approved for treatment of *Staphylococcus aureus* bloodstream infections in adult patients, including those with right-sided infective endocarditis, caused by methicillin-susceptible and methicillin-resistant isolates (Vilhena & Bettencourt, 2012). As the first approved CLP it is now used as a new last resort weapon against multidrug resistant bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA) and vancomycin resistant *enterococci* (VRE) (Straus & Hancock, 2006).

1.4.2 Daptomycin – structure and mode of action

Daptomycin is produced during fermentation by the soil actinomycete bacteria *Streptomyces roseosporus*. It consists of 13 amino acids, six of which are non-proteogenic. A fatty acid is

attached to the N-terminal end, and the ten C-terminal amino acids form a ring which is closed by an ester bond between the side chain of Thr-4 and the α-COOH group of the C-terminal amino acid kynurenine (Kyr-13). Daptomycin has an overall negative nett charge of -3 and is highly water soluble because of the five amino acids that are charged at neutral pH. It aggregates in oligomers and micelles and is highly dependent on calcium ions to be activated (Schneider et al., 2014).

Many studies have been performed on daptomycin to elucidate its mode of action. The various hypothesis proposed have shifted through the years, but daptomycin's action mechanism is still not fully understood. Daptomycin specifically targets the cytoplasmic membrane of Gram-positive bacteria. Gram-negative bacteria are not sensitive, probably because their outer membrane lack the molecule(s) recognized and targeted by daptomycin. Daptomycin is first activated by binding 1:1 to Ca^{2+} where it undergoes conformation changes that locks it in an active position (Ho et al., 2008). Evidence indicate that daptomycin then interacts, probably stoichiometric, with phosphatidylglycerol (PG) in the cytoplasmic membrane (Muraih et al., 2011). Moreover, Straus and Hancock suggested that this interaction drives a second configuration change (Straus & Hancock, 2006). The interaction with PG appears to be essential for activity, and the lack of this phospholipid in the outer membrane of Gram-negative bacteria is probably the reason why daptomycin is not active against them (Miller et al., 2016). After inserting into the cytoplasmic membrane, it seems to oligomerize to form pores selective for cations, with permeabilities being highest for Na^+ and K^+. This depolarizes the cell, and thereby interferes with and inhibits active transport and eventually causes cell death. However, the antibiotic does not induce lysis, unlike many other antibiotics targeting the cell membrane.

Sublethal concentrations of daptomycin changes the morphology of affected bacteria. This has led many to believe that the antibiotic is not only making pores in the membrane, but also effects peptidoglycan synthesis. However, conclusive evidence for this is lacking (Taylor & Palmer, 2016). Moreover, previously it was believed that daptomycin inhibits lipoteichoic acid biosynthesis in *Staphylococcus* and *Enterococcus*. However, this theory was weakened when daptomycin showed activity against bacteria without lipoteichoic acid biosynthesis (Tedesco & Rybak, 2004).

As mentioned above, daptomycin is a broad spectre antibiotic that kills most Gram-positive pathogens, including *S. pneumoniae*. However, it turned out to be ineffective against pneumococcal

pneumonia. Daptomycin was thereby the first antibiotic proven to be tissue specific. In lung tissue, pulmonary surfactants primarily consist of dipalmitoylphosphatidyl chloline (PC), but they also contain approximately 10 % PG (Silverman et al., 2005). It is believed that daptomycin binds to PG in lung surfactants, thereby getting sequestered and prevented from interacting with target bacteria. This is believed to be the reason that daptomycin is ineffective against lung infections.

1.4.3 Amphomycin MX-2401

Amphomycin is a calcium dependent CLP first discovered as a secondary metabolite produced by the bacterium *Streptomyces canus* ATCC 12237. It was discovered in the early 1950s and after a few years several other closely related peptides were also isolated. The amphomycin's were split in three groups; friulimicins, amphomycins and laspartomycins, all of which are active against Gram-positive bacteria. The three groups are characterised by their common cyclic decapeptide core, and that they have only one exocyclic amino acid linked to the N-terminal lipid chain. The lipid-chain varies between C_{12}- C_{15} in length, with several different branching patterns. Experimental evidence indicates that amphomycins, friulimicins and laspartomycins kill susceptible bacteria by interfering with peptidoglycan and teichoic acid synthesis. They bind to and sequester the lipid carrier undecaprenyl-phosphate (C_{55}-P), which is essential for both of the above-mentioned processes. (Schneider et al., 2014).

Amphomycin was thought to be too toxic for human use because of its haemolytic effect on blood cells. In an attempt to improve its properties, the semi-synthetic amphomycin MX-2401 was synthesized. Amphomycin was first deacylated after which the fatty acyl tail was replaced with an aromatic linker and a C_{12} lipid side chain. MX-2401 turned out to be non-haemolytic, and to have twice the half-life in the human body compared to amphomycin (Butler et al., 2016). Another important characteristic of amphomycin MX-2401 is that, in contrast to daptomycin, it is able to retain its activity in the presence of lung surfactant (Craig et al., 2010). Although MX-2401 was recently brought to clinical trials by BioWest Therapeutics Inc., it has so far not been tested on human patients in a clinical setting.

1.4.4 Polymyxins - history and clinical use

Polymyxins are a group of CLPs produced by different strains of *Bacillus polymyxa*. There are 11 different types of polymyxin, called A, B, C, D, E, F, K, M, P, S and T. Polymyxins inhibit a broad spectrum of infections caused by Gram-negative bacteria. However, out of the 11 types only two are approved for clinical use, namely polymyxin B and polymyxin E (better known as Colistin). Polymyxins was first discovered in 1947. However, they were withdrawn from clinical use in the 1970s because 40-60% of all patients who received treatment with the drug experienced acute kidney injury. Unfortunately, a method to avoid such side effects, as was established for daptomycin, has not been found for polymyxins. Nevertheless, from the mid-1990s there was a renewed interest in these drugs and new clinical trials were initiated. This interest was to a large extent due to the emergence of multidrug resistant Gram-negative bacteria. Polymyxin B and colistin are currently used as two last-resort broad-spectrum drugs against severe infections with multidrug resistant *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Actinobacteria spp*. and *Pseudomonas. aeruginosa*, along with many others (Zavascki et. al., 2007). Several of which joins *S. pneumoniae* on CDC's list of the 18 most concerning drug resistant bacteria in the U.S. (Eurosurveillance-Editorial-Team, 2013).

1.4.5 Polymyxins-structure and mode of action

Polymyxins consist of ten amino acids with a characteristic polycationic heptapeptide ring and an N-terminal fatty acid modification. They consist of many different chemical forms, but only polymyxins B and Colistin are used clinically. There are four variants of polymyxin B (B_1-B_4), with B_1 and B_2 as the two main components. B_1 and B_2 differ only by an extra carbon atom in the lipid chain. Colistin consists mainly of an A and B variant, which differs in the same way as polymyxin B_1 and B_2. Polymyxin B and Colistin differ only in one amino acid situated in the heptapeptide ring. In Colistin, a D-phenylalanine has been replaced by a D-leucine.

Polymyxin B and Colistin have a similar spectrum of activity and mode of action (Schneider et al., 2014). They bind to lipid A and thereby competitively displaces divalent cations from membrane lipids in the outer membrane of Gram-negative bacteria. This interaction between the CLPs and the negatively charged lipopolysaccharides (LPS) leads to disruption of the outer cell membrane, leakage of periplasmic contents and cell death (Olaitan et al., 2014). The amphipathicity of polymyxins, and possibly their ability to form pore-like oligomers, may be

responsible for their ability to permeabilize the outer membrane. The specific interaction between lipid A and polymyxins explains why these drugs are not active against Gram-positive bacteria.

1.4.6 Ramoplanin

Ramoplanin A1, A2, and A3, which is structurally very closely related to CLPs, belong to a group of antibiotics called lipoglycodepsipeptides. They were discovered in 1984 and are produced by *Actinoplanes sp.* ATCC 33076. The A1, A2, and A3 variants differ only in the length of their N-terminal acyl chains (Schneider et al., 2014). They consist of 16 amino acids, one exocyclic and 15 that form the oligopeptide ring. Ramoplanins are different from CLPs in that they have the disaccharide mannosyl-(1,2)-α-D-mannose bound to the oligopeptide ring at position 11. The ramoplanins are further characterized by containing several unusual amino acids. They kill Gram-positive bacteria by inhibiting the N-acetylglucosaminyl transferase-catalysed conversion of lipid I to lipid II, a step that occurs before transglycosylation and transpeptidation reactions. By inhibiting this reaction, the bacterial cell becomes unable to build the cell wall. At three times the MIC, ramoplanins will insert and aggregate in the cell membrane causing the cell to depolarize. Ramoplanin A_2 is a promising drug for the treatment of MRSA and VRE infections. It is more potent than for example vancomycin and has showed excellent activity *in vitro* and *in vivo* against *Enterococci*, *Staphylococci*, *Bacilli*, *Streptococci*, *Listeria monocytogenes*, and anaerobes such as *Clostridrium difficile*. However, it shows no activity against Gram-negative pathogens. Currently, ramoplanin is in phase III clinical trials. (Kleijn & Martin, 2018)

1.5 Resistance against CLPs

Similar to vancomycin, ramoplanin inhibits peptidoglycan synthesis in susceptible bacteria. However, in contrast to vancomycin no resistance has been reported for ramoplanin. This is also the case for several other CLPs, implying that resistance against CLPs is very hard to obtain (Kleijn & Martin, 2018). This is illustrated by Hover's attempt to induce resistance against malacidin A by exposing sensitive bacteria to a sub-lethal dose continuously for 20 days (Hover et al., 2018). At the end of the experiment no resistant colonies were observed. However, for both daptomycin and polymyxin, which are the only CLPs in commercial use, resistance has been reported. Resistance against polymyxin always involves changes in the activity of proteins that directly or

indirectly alter the structure of lipid A or LPS, resulting in loss of binding between polymyxin and its target molecule lipid A (Olaitan et al., 2014). This resistance threatens Polymyxin's status as a last-resort drug against multidrug-resistant Gram-negative bacteria, especially since the resistance genes are plasmid borne (Liu et al., 2016).

Resistance against daptomycin have appeared sporadically during treatments. Such incidents are rare, and usually happens when patients have been treated with other antibiotics prior to daptomycin, for instance vancomycin. The exact mechanisms giving rise to resistance against daptomycin vary among bacterial species, but generally includes changes in the composition of the cytoplasmic membrane. A common theme that mechanistically links various pathways leading to resistance is a change in the net charge of the bacterial surface leading to electrostatic repulsion of daptomycin. An example of this is the conversion of phosphatidylglycerol, the phospholipid targeted by daptomycin, into its positively charged derivative lysyl-phosphatidylglycerol. Other, mechanisms involve the activation of general cell envelope defence systems such as the two-component regulatory system LiaSR and/or alterations in the activity of enzymes involved in the metabolism of phospholipids. (Kleijn & Martin, 2018)

1.6 CLPs produced by *Pseudomonas* spp.

CLP-producers are found in soils, freshwater and marine environments. Most are members of the following genera*: Bacillus, Paenibacillus, Pseudomonas* and *Streptomyces*. The bacilli and *Streptomyces* are Gram-positive bacteria that belong to the phyla Firmicutes and Actinobacteria, respectively, while the pseudomonades are Gram-negative and belong to the phylum Proteobacteria. It is not always clear how bacteria producing CLPs avoid being killed by their own antibiotic. In the case of polymyxin, which is synthesized by the Gram-positive bacterium *Bacillus polymyxa*, the producer bacterium is presumably resistant because it does not possess an outer membrane containing LPS and lipid A. Similarly, *Pseudomonas* spp. producing CLPs attacking the cytoplasmic membrane of Gram-positive bacteria, are probably protected by their own outer membrane. However, it is not uncommon that Gram-positive bacteria, e.g. *Streptomyces* spp., produce CLPs active against other Gram-positive bacteria. How the producer bacteria protect themselves in these cases are not known. (Schneider et al., 2014)

Pseudomonas species are known to produce a large diversity of CLPs. After the year 2000, 40 new CLPs produced by *Pseudomonas* strains have been described. They are classified into six families: viscosins, syringomycins, amphisins, xantholysin, tolaasins, and syringopeptins (Janek et al., 2010Geudens et al., 2017; Ines & Dhouha, 2015). Most CLPs produced by the pseudomonades are only active against Gram-positive bacteria. However, it has recently been reported that CLPs of the tolaasin and xantolysin groups are able to inhibit Gram-negative bacteria (Geudens & Martins, 2018). Thus, in conclusion, the antimicrobial activity of CLPs produced by *Pseudomonas* spp. is not limited to Gram-positive bacteria. Nor it is limited to bacteria. It has been reported that several *Pseudomonas*-CLPs show activity against fungi and protozoa (Geudens & Martins, 2018). Another exciting but largely unexplored field is antiviral therapy. It has for example been shown that some *Pseudomonas*-CLPs have modest activity against enveloped viruses, e.g. HIV (Xue et al., 2018).

The *Pseudomonas* species producing Streptovoracin was isolated from the Oslo fjord. Before I started on the current study, sequencing of its 16S rRNA gene followed by BLAST searches suggested that the new isolate is closely related to *Pseudomonas brenneri*, a species originally isolated from French natural mineral waters (Baïda et al., 2001).Phylogenetic analysis shows that *P. brenneri* belongs to the *Pseudomonas fluorescence* lineage, also called the *P. fluorescence* cluster (Baïda et al., 2001). Species belonging to this cluster are aerobic bacteria that can be found all around the world from Svalbard (Janek et al., 2010) to Antarctica (Vasquez-Ponce et. al., 2018). The bacteria are rod-shaped and usually move by polar flagella. Many fluoresce under UV light. Members of the *P. fluorescence* cluster, and pseudomonas in general, have fairly large genomes (5.2-7 Mb). They are able to use a wide variety of organic compounds as carbon and energy sources. Moreover, *Pseudomonas* as well as members of the *P. fluorescence* cluster are associated with the production of CLPs (Raaijmakers et. al., 2006). However, there are no studies in the literature reporting on antimicrobial CLPs produced by *P. brenneri*.

1.7 Main goals for this thesis.

Some initial studies had been performed before I started on my master thesis. By screening a collection of bacteria isolated from the Oslo fjord, an isolate producing a compound inhibiting the growth of *S. pneumoniae* was discovered. The active compound, which was named streptovoracin, was purified in tiny amounts in order to determine its mass by mass spectrometry. Furthermore, streptovoracin was tested for its sensitivity to various proteases and polymyxin acylase, an enzyme that deacylates lipopeptides. The results of this preliminary characterisation indicated that streptovoracin is a cyclic lipopeptide. In addition, a limited screening of bacterial species had been carried out to get an impression of the target range of the active compound.

The major goal of my thesis work was to develop better protocols for production and purification of streptovoracin. Purification of milligram amounts would be necessary in order to determine an approximate minimal inhibitory concentration (MIC) of streptovoracin against *S. pneumoniae*. Another important goal was to perform a comprehensive screening of streptococci and other bacteria of medical interest to identify those that are sensitive to streptovoracin. In addition, it was of interest to learn more about the properties and mode of action of streptovoracin, and to further study *Pseudomonas* isolate no. 33 in order to determine its phylogenetic position.

2. Materials and methods.

2.1 Glycerol stocks and cultures

2.1.1 Frozen stocks

New glycerol stocks of the strains used in this thesis (appendix C) were made by taking a small sample from the top of an old frozen glycerol stock with an inoculation loop and transfer it to a 15 ml Falcon tube with 2-3 ml sterilised C-medium. The inoculation loop was sterilized with a Bunsen burner between each new sample. The falcon tubes were then placed in a 37°C water bath until the cultures had grown to OD_{550} ~ 0,3. Next, 1 ml samples from each culture were transferred to a 2 ml cryo-tube, and 500 µl 50% glycerol (v/v) was added. The mixtures then contained 15% glycerol and was ready to be frozen at -80°C. *Streptococcus* species were grown in C-medium at 37°C. Media and growth conditions for non-*Streptococcus* species are listed in table 2. Frozen glycerol stocks were made for every strain used in this thesis.

Table 2. Growth condition and media used for cultivation of non-*Streptococcu*s species. Media were mixed according to appendix A

Growth medium	**Bactria**	**Growth temperature**
MRS	2312 *Lactobacillus sake* NCDO 2714	30 °C
M17	1403 *Lactococcus lactis*	
	1363 *Lactococcus lactis subsp. Cremoris*	
	2708 *Enterococcus faecalis* EF BRIDGE (B)	
BHI	2787 *Enterococcus faecium*	
	Bacillus cereus	30 °C shaken
	Bacillus subtilis	
	Listeria monocytogenese	37 °C shaken
	D5Hα *Escherichia coli*	
	Mycobacterium smegmatis	
	Dietzia sph 492	

2.1.2 Starter cultures

Starter cultures that were used in this thesis was made by taking a small sample from the top of a frozen glycerol stock with an inoculation loop and transferring it to a 15 ml Falcon tube with 3 ml sterile medium. The inoculation loop was sterilized with a Bunsen burner between each new sample. After inoculation was the Falcon tube placed on a 37°C water bath until the stocks had grown to OD_{550} ~0,2. Next, 1.3 ml 50% glycerol were added to each stock and the cultures was frozen at -80°C. *Streptococcus* species were grown in C-medium at 37°C, media and incubation conditions for non-*Streptococcus* species are listed in table 2. Starter cultures were thawed and diluted in fresh media when needed

2.1.3 Pure cultures.

Pure cultures were made by taking a small sample from the top of a frozen glycerol stock with a sterile inoculation loop. The inoculation loop was sterilised with a Bunsen burner for each of the three rotation of the streaking pattern illustrated in Fig 3. Next, the plates were stored in an unsealed box at 4°C. Due to overgrowth on the plate and reduced numbers of single pure cultures were clean plates re-stroked every few months.

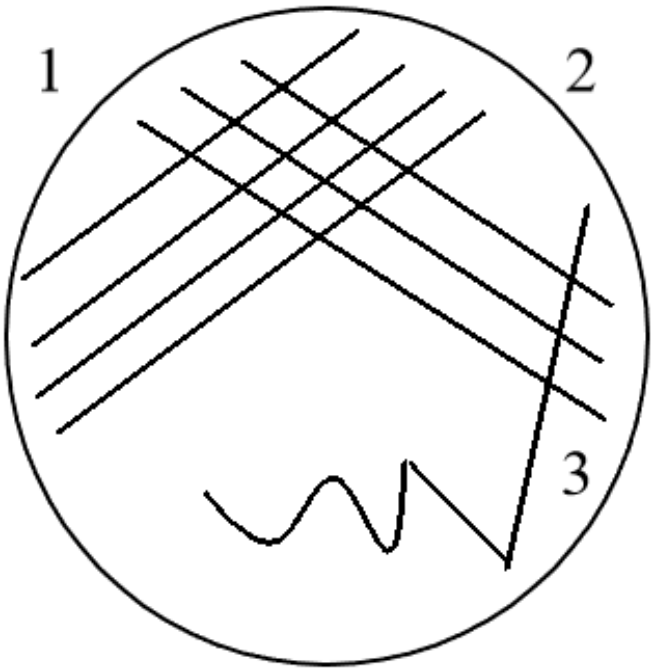


Figure 3. Streaking pattern used to obtaining single pure cultures. Number indicate rotations done to streak the lines onto the plate.

2.2 Screening bacteria for sensitivity to streptovoracin

2.2.1 Overlay assay

Agar plates were made by mixing media according to Appendix A. The agar was autoclaved and distributed onto clean petri dishes. Once the agar had solidified, the plates were stacked, placed in plastic bags and stored at 4℃.

To inoculate a single culture of *Pseudomonas* isolate no. 33 on agar plates were the plates first placed in room temperature for at least half an hour to avoid condensation. Then a single colony was picked from the pure culture plate and prick onto the middle of a clean BHI-agar plate. One toothpick was used to inoculate three clean agar plates with the same picked colony. The plates were marked with date and colony before it was incubated aerobic at room temperature for four days.

Soft ager was prepared for overlay by mixing the media according to appendix A. The autoclaved soft agar was distributed as 5 ml fraction into autoclaved test tubes. A Bunsen burner was used to maintain a sterile environment. The test tubes containing ready soft agar were stored in a 50℃ water-bath until needed.

The strains used for overlay was prepared by thawing a frozen glycerol stock of the strain. The stock was spun at 5000 rpm for 5 minutes before the supernatant was removed. The cell pellet was then dissolved in 1-3 ml clean C-medium and place on a 37 ℃ water bath. The strain grew to OD_{550} ~0.3. If several strains were prepared at the same time, they would grow irregularly. The ready strain was then placed on ice once the colony reached OD_{550} ~0.3, growth would then halter until the stocks were placed as overlay.

On the fourth day after incubation of the *Pseudomonas* strain was soft-agar, and stocks prepared. After the *Pseudomonas* cultures on agar plates had grown for four days, they were ready for overlay. In order to place overlay, the test tube was first removed from the water-bath and water dried off with a paper towel. Next, were 40 µl stock solution with OD_{550} ~0.3 added to the soft agar. The mixture was vortexed for a couple before the colony was gently poured over the existing ager. Once the soft agar had solidified the plates were marked with the additional species. If overlay was laid correctly should the horizontal cross section of the plate look according to Fig. 4, with the soft agar containing the strain tested for streptovoracin sensitivity. During the process of placing

overlay, sterile conditions were maintained to avoid contamination. The process was also performed over a duration of 15-20 seconds. This because soft ager cools quickly and must not solidify before properly placed over the existing agar. All the species in appendix C were placed in overlays, with the exception of *Pseudomonas* isolate no. 33.

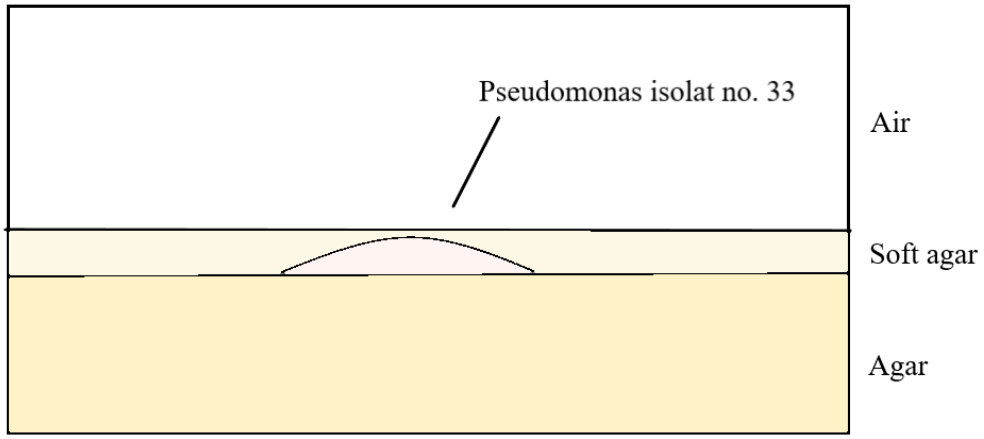


Figure 4. Horizontal cross section of an agar plate with finished overlay. Inhibition zone is not included in illustration. The *Pseudomonas* isolate no. 33 culture is in the middle, laying on top of the agar and covered by the soft agar. The *Pseudomonas* isolate had grown for four days before the overlay was placed on top.

All overlay assay involving *Streptococcus* species were incubated anaerobically. The anaerobic environment was obtained by stacking the plates in an airtight box with one or two AnaeroGen bags to remove all remaining O_2. The number of bags depended on the size of the box, where a larger box would need two bags to remove all O_2. The sealed box was placed in a 37 ℃ incubation cabinet overnight. The plates were removed from the cabinet the next morning and visible inhibition zones measured with a ruler.

2.2.2 Protocol adjustment to fit non-streptococcus species.

The above-mentioned protocols for overlay was written in the perspective of streptococci species. Protocol for non-streptococcus species was adjusted to fit the given species. Adjustments to protocol are listed in table 3. In addition to the changes in table 3 was the *Mycobacterium smegmatis* stock spun briefly at 200 rpm right before overlay to avoid large clumps of cells to be transferred to the soft agar.

Table 3. Alterations done to the protocol in section 2.2.1 made in order to fit the non-streptococcus species used in this thesis. With the exceptions of media, volumes and incubation conditions were the protocol in section 2.2.1 followed for these species as well.

Broth used for growth and soft agar	**Bactria**	**Volumes added to overlay (µL)**	**Incubation before overlay**	**Incubation overlay**
MRS	2312 *L. sake* NCDO 2714	20	30 °C	30 °C
M17	1403 *L. lactis*	20		
	1363 *L. lactis subsp. Cremoris*	20		
	2708 *E. faecalis* EF BRIDGE (B)	20		
BHI	2787 *E. faecium*	50		
	B. cereus	20	30 °C shaken	
	B. subtilis	20		
	L. monocytogenese	20	37 °C shaken	37 °C
	D5Hα *E. coli*	20		
	M. smegmatis	100		
	Dietzia sph 492	40		

2.3 Culturing condition for production of streptovoracin

2.3.1 Culturing condition assay

Media were mixed according to appendix A, autoclaved and cooled to room temperature. The medium was inoculated by taking a small sample from a frozen glycerol stock using a sterilized inoculation loop without thawing the stock and transferring the sample to the medium. All cultures were grown in 500 ml BHI-medium at room temperature for four days unless otherwise is stated. The following conditions were tested:

1. **Anaerobic incubation:** The inoculated medium was placed in a flask with the lid loosely screwed on. The flask was placed in an airtight box with an AnaeroBag. The bag would remove the O_2 from around and within the flask.
2. **Aerobic incubation:** The inoculated medium was grown in a 3-litre Erlenmeyer flask with a sterile filter lid. Allowing the culture free air exchange.
3. **Surface enhancement**: Glass wool was added to the medium before autoclavation, 12.5 g per 500 ml medium.
4. **Growth during shaking:** Culture was grown on a shaker at 100 rpm.
5. **Semi-synthesised media**: *Pseudomonas* broth and M9 minimal medium were prepared according to appendix A.
6. **Mapping production over eight days**: Production of streptovoracin was tracked over the duration of eight days. One millilitre samples were removed for all 8 days, spun at 5000 rpm for 5 minute and transferred to a clean Eppendorf tube. The samples were stored at -20 ℃ until all samples were collected.

Production of streptovoracin was tested by removing 1 ml from the growing culture by a long pipet. The samples were transferred to clean Eppendorf tubes and spun for 5 minutes at 5000 rpm. Once spun, the cell free media containing the antibiotic were transferred to clean Eppendorf tubes and diluted in two-fold dilution series. Activity was tested against an indication strain on a microtiter plate.

2.3.2 Testing activity of samples by kinetic absorbance

Activity was determined by the bacteria culture's inability to grow in the presence of the streptovoracin. Each microtiter plate contained 96 wells and could be read by a microtiter plate reader. In the lab were both a Synergy H1 and a Hidex microplate reader, but both instruments had the same function and were run on the same program. The instrument was set to preheating at 37℃, a temperature that was maintained throughout the run and readings were set to measure absorbance at λ 550 nm every five minutes. One second of linear shaking was added before every reading to homogenize the sample and reduce inaccuracies.

First a started culture was prepared by thawing and diluting the culture to 10-30 ml using fresh C-medium. Dilution volume would depend on the number of wells intended to use. The culture was distributed on the microtiter plate as 270 µl fraction in each well. Every plate had a well with clean C- medium as blank. The microtiter plate was then placed in one of the microtiter plate reader. Once the culture reached OD_{550} ~0.2 the wells were added 30 µl from each of the sample dilutions making the final well volume 300 µl. One of the wells containing a growing culture was left untouched as a control sample. Readings continued until the bacteria reaches stationary phase or growth curve levelled out.

2.4 Developing a purification method for streptovoracin

2.4.1 Extraction of streptovoracin by ammonium sulphate precipitation

Preliminary streptovoracin was extracted by ammonium sulphate precipitation. Throughout this thesis work was a few modifications done to the original method achieve higher yield. The following protocol contains these modifications.

To begin, was a four-day old *Pseudomonas* culture spun at 10 000 rpm for 10 minutes. The cell free supernatant[1] was transferred to a clean container and added 40 g ammonium sulphate for every 100 ml cell free supernatant[1]. The cell-free medium and the ammonium sulphate was mixed until all had dissolved. The mixture was left to rest at 4 °C for 30 minutes to 2 hours. After incubation was the mixture spun at 12 000 rpm for 30 minutes and the precipitation supernatant[2] was discharged. The precipitate was dissolved in 10 ml dH_2O and transferred to a 15 ml Falcon tube. The Falcon tube was spun at 4000 rpm for 10 minutes. The pellet and the supernatant would at this point both contain streptovoracin. Further was the water phase supernatant[3] transferred it to a clean falcon tube. The remaining precipitate pellet was added 4 ml 100% MeOH. The methanol mixture was mixed well by pipetting up and down several times before the mixture would rest for 10 minutes. The methanol mixture was spun at 4 000 rpm for 10 minutes, the methanol supernatant[4]

[1] Cell-free supernatant from spun bacteria culture
[2] Supernatant from ammonium sulphate precipitation
[3] Water phase from the precipitation pellet
[4] Methanol phase from the precipitation pellet

was kept. The methanol was evaporated from the sample *in vacuo* and the dried pellet dissolved in 100 µl dH_2O.

2.4.2 Extraction of streptovoracin by Amberlite® XAD16N.

The first time the amberlite XAD 16N resin were used the following protocol was followed.

A four-day old *Pseudomonas* culture was first spun 10 000 rpm for 10 minutes to obtain cell-free medium. The cell-free supernatant was transferred to a clean Erlenmeyer flask and added 1 g amberlite resin for every 10 ml medium. The flask was placed on a shaker at 100 rpm for two hours. After two hours were the mixture poured onto a column, the specific column that were used would depend on the sample size. Smaller samples would be loaded little by little onto a 20 ml disposable column. For the most part were the samples pulled through by gravity, but occasionally were additional pressure added using a pipette. The column was washed three times with column volume of 30% MeOH. Lastly was streptovoracin eluted from the resin by two times column volumes of 100% MeOH. The methanol was evaporated *in vacuo* and the dried pellets were dissolve in 100 µl dH_2O.

A few modifications were done to the protocol. These modifications were:

1. The cell-free supernatant was added methanol to a final concentration of 30% MeOH. This to avoid impurities from binding to the resin.
2. The dried pellet was dissolved in 100 µl 50% ACN instead of 100 µl dH_2O. This simplified dissolving the dry pellet (This step was also applied to pellets from ammonium sulphate precipitation).

2.4.3 Purification streptovoracin by High Performance Liquid Chromatography (HPLC)

The dissolved pellets from the extraction steps were spun at 13 200 rpm (max speed) for 5 minutes before 65 µl from the sample was transfer to snap ring micro-viral tubes. The C18 (vydac 218TP C_{18} 5 µm) column were inserted in the HPLC machine and saturated with either 0% ACN 0.1% TFA or 50% ACN 0.1% TFA for 10 minutes. Concentration of ACN used for saturation depended on the samples' ACN concentration. The samples were then placed in the instrument and 50 µl of each sample was run through the column with a gradient of either 0-100% ACN or 50-100% ACN.

Streptovoracin was collected by a sample collector. In order to obtain a known concentration of highly pure streptovoracin, the two peaks from the HPLC were collected and the ACN evaporate *in vacuo*. The dried pellets of purified streptovoracin was weigh before they were subsequently attempted dissolved in 200 µl of all the solutions shown in table 4.

Table 4. Solutions attempted to dissolve streptovoracin

100% ACN
100% MeOH
50% MeOH
50% Glycerol
Phosphate-buffer saline (PBS)
Acetate buffer pH 5
H_2O
DMSO
H_2O with added $NaCO_3$ to PH 11
PH 11, downregulated to pH 8 with Tris-HCL pH 7,4
PH 11, downregulated to pH 8 with 0,1 M HCL pH 1

2.4.6 Extraction efficacy of the two methods

Streptovoracin was originally purified by ammonium sulphate precipitation, but amberlite resin was tested in order to test if it would achieve a higher purification yield. However, the strength of the bond between the amberlite resin and streptovoracin was unknown. Therefore, different concentrations of MeOH were used as washes and tested according to section 2.3.2 in order to find the highest concentration that could wash the resin without coeluting streptovoracin.

In addition, an experiment was set up to test if the use of amberlite resin had improved extraction yield. The was done according to the illustration in Fig 5. A 1 ml sample was taken from the cell-free medium before the medium was separated into three equal parts of 50 ml. Subsequently, two parts were purified by ammonium sulphate precipitations, one with 40 g per 100 ml cell-free supernatant and one with 60 g. The last part was treated with amberlite resin. The parallels then followed the protocols accordingly to sections 2.4.1 and 2.4.2. One millilitre samples were collected for every purification step to track antibiotic loss. All samples were evaporated *in vacuo* before dissolved in 100 µl 50% ACN. The samples were then run in the HPLC machine according to section 2.4.4, and efficacy was calculated up against the original cell-free medium of multiplied to represent the starting solution of 50 ml.

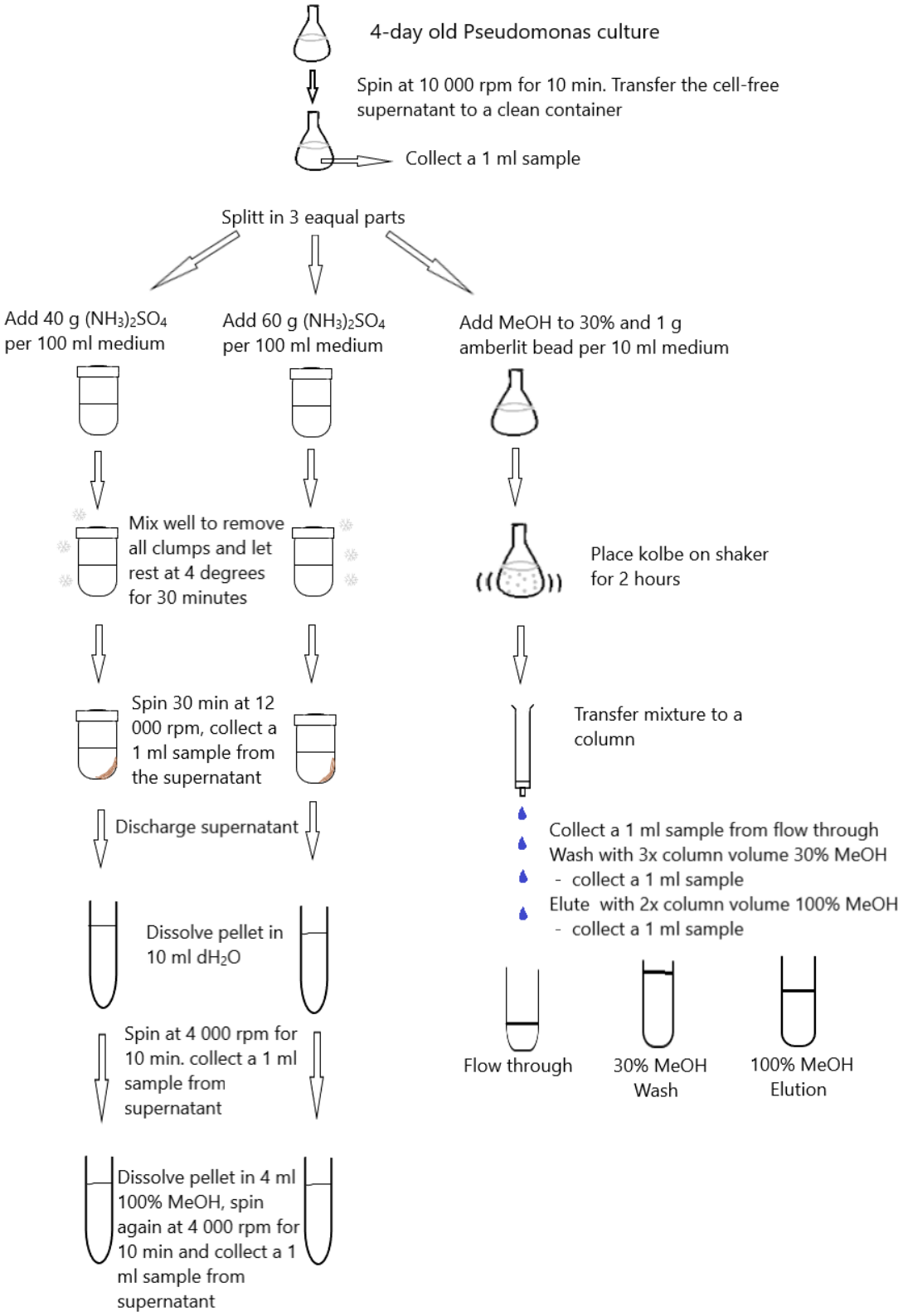


Figure 5. Extraction efficacy experiment setup. Each of the three equal parts contained 50 ml.

2.5 Activity of purified streptovoracin.

Known concentration of highly purified streptovoracin was used to find MIC for *S. pneumoniae* and *M. smegmatis.* This experiment was conducted according to protocol for kinetic absorbance, described in section 2.3.4. The protocols for *S. pneumoniae* and *M. smegmatis* were the same with the exception that *S. pneumoniae* was grown in C-medium and *M. smegmatis* grown in BHI-medium.

The two homologs were also tested for activity separately. The homologs were then collected and separated by HPLC and evaporated *in vacuo*. The dried pellets were dissolved in dH_2O pH 11 and the peak with higher peak volume (mAU*min) on the HPLC spectra would be diluted to match the concentration of the other. The peaks were then tested for activity both separately and in a 1:1 mixture according to section 2.3.2.

2.6 Transformation assay

2.6.1 Extracting genomic DNA.

In order to perform the transformation assay, genomic DNA was needed. The genomic DNA was transformed into the RH425 strain. But first, was the DNA from already resistant or low sensitive strains extracted. The exact strains are marked with a raised (t) in Appendix A. The DNA extraction was done by using the kit "NucleoBond® AXG and NucleoBond® Buffer Set" (table 5).

Table 5. NucleoBond® AXG and NucleoBond® Buffer Set. Table over the component included in the genomic DNA extraction and isolation kit. The kit specify that user need to supply isopropanol, ethanol 70% & 96%, buffer for reconstitution of DNA, in this case dH_2O and lysozyme. The cells in this thesis had a hard time lysing and both mutanolyzym and fast prep was used in addition to the kit in order to lyse the cells.

Kit content
Buffer G3
Buffer G4
Buffer N2
Buffer N3
Buffer N5

RNase A
Proteinase K
Proteinase Buffer PB
NucleoBond® AXG 100 columns

The kit was used to extract and isolate genomic DNA from bacteria cells. The kits protocol was followed, although a few alterations were needed. These alterations are described in detail in the following protocol.

To start the experiment were a strain grown to very high OD in 20 ml C-medium. The culture was transferred to the medium in the same manner as described according to section 2.2.4. The exact OD were not measured, but the media would be clearly fogged by the number of cells in the sample. A large quantity of cells before extraction was wanted, since DNA loss during the process would be expected. When cell density was high enough, the cells were pellet by centrifugation at 4000 rpm for 10 min. The cell-free supernatant was discharged.

The pellet was resuspended in 5 ml G3 buffer. This buffer contains RNase, which breaks down RNA in the cells after the they have been lysed. Further was 50 µl lysozyme and 100 µl proteinase K and 50 µl mutanolysozym added to induce enzymatic lysis. The volumes of enzymes were in this thesis adjusted from the kits protocol, and mutanolysozym was added in addition because *Streptococcus* species as Gram-positive bacteria can be harder to lyse enzymatic. The mixture was placed in a 37°C water bath for 40 minutes. Samples that had not thicken in consistency after 40 minutes would have to sit for a while longer. If samples had not thickened after 2 hours on the water bath, was transferred to fast prep tubes containing glass beads. The tubes would be run on the fast prep two times 20 seconds. The tubes were then spun briefly to pellet the beads before the supernatant containing DNA was collected. The supernatant would be transferred to clean container.

The lysed samples were then added 1,2 ml G4 buffer. G4 buffer would dissolves remaining proteins in the sample. The samples were then vortexed and placed in a 50°C water bath for 30 minutes. While the mixture incubated on water bath could the kits columns be equilibrated with 2 ml N2 buffer. After incubation was 5 ml N2 buffer added to the samples. The mixtures were vortexed for 15 seconds and loaded onto the columns. The columns were washed three timed with 4 ml N3 buffer. The columns were then transferred to clean centrifugation tubes. Once placed in

the centrifugation tubes were the genomic DNA eluted with 4 ml N5 buffer. For every wash and elution, gravity pulled liquid through the columns.

Next, the eluted DNA was precipitate by adding 3,5 ml room tempered 100% isopropanol. The mixtures were incubated at room temperature for 30-60 minutes. After incubation, the mixtures were centrifugated at 4 °C at 15 000 rpm for 25 min then the supernatants were carefully discharge and the pellets air dried. The pellets were then carefully washed with 1 ml cold 70- and 96 % ethanol. The precipitate should not loosen from the centrifugation tube wall. The pellet was air dried once more before it was resuspending in 50-100 µl dH_2O. Concentration of genomic DNA was checked by nanodrop. (Philippe Desjardins, 2010)

2.6.2 Transformation

Transformation experiments were performed with DNA isolated from already resistant or low sensitive *Streptococcus* strains. The genomic DNA from section 2.6.1 was used to transform the *S. pneumoniae* RH425 strain.

Transformation was performed by taking a RH425 starter culture, thawing it and increasing the volume to 10 ml with fresh C-medium. This lowered the OD_{550} to 0,05. The diluted culture was then split into 1 ml fraction in sterile Eppendorf tubes and placed in a 37°C water bath for 15 minutes. After 15 minutes the cultures had woken up and would have started early exponential growth. Next, 1 ug genomic DNA from the resistant strains were added each sample along with 250 ng CSP. The mixtures were placed back on the 37°C water bath for 2 hours. After 2 hours the strain was done transforming and genomic DNA should be incorporated. The transformed RH425 strain were then laid as overlay and incubated according to section 2.2.1.

2.6.3 Transforming *S. mitis* SK564

The strain *S. mitis* SK564 was attempted to induce transformation. A positive and negative control was tested to see if the strains CSP would induce transformation. The strain was transformed according to section 2.6.2 by CSP specific for the SK564 strain with genomic DNA from a *S. pneumoniae* strain contained the gene for novobiocin resistance. Instead of laying the transformed strain as overlay over the *Pseudomonas* strain was the transformed SK564 streaked onto TH-plates

containing 2.5 µg/ml novobiocin. Two plates were spread for both positive and negative control, one with 20 µl culture spread and one with 50 µl. The plates were then incubated anaerobic at 37℃.

2.7 Investigating how streptovoracin affects inhibited cells.

2.7.1 Sytox staining done to test membrane integrity of inhibited *S. pneumoniae*

Sytox staining was done to see if the membrane of inhibited *S. pneumoniae* cells would stay intact when streptovoracin was added to the culture. In order to perform sytox staining was a starter culture of *S. pneumoniae* thawed and diluted to 10 ml with fresh C-medium. The diluted culture was distributed as 250 µl fraction into the wells on the microtiter plate. One well was added 250 µl clean C-medium as a blank. Next, to prepare the colour was 3 µl sytox dye added to 497 µl clean C-medium and mixed well. Lastly was 20 µl of the coloured C-medium added to every well containing sample on the microtiter plate. The final volume in each well would add up to 300 µl after dilution of streptovoracin was added, except for the blank which would have final volume of 270 µl. Once the colour was added were the same procedure followed as described in section 2.3.2 with the exception that the instrument would read fluorescence signal excited 485 nm in addition to measuring OD at 550 nm.

2.7.2 Examination of inhibited *S. pneumonia* and *M. smegmatis* in microscope

In order to take good pictures of the colonies in the microscope were the cells transferred to a glass slide coated with a thin layer of 0,5 % agarose agar. The agar coating on the glass would prevent the cells from moving too much which could blur photos. The agar was a mixture of 2,5 agarose agar and 50 ml TEA buffer. This was mixed and heated in a microwave until all was dissolved. Once the agar was completely dissolved it was placed in a 50°C water bath until needed. To coat the glass slide with agar was the slide first covered in 1 ml agar before another slide was pressed against it, this would spread the agar as thin as possible. Excess agar spilling out from the slide were removed. Further was 0.5-1 µl of the culture transferred onto the agar. Pictures were taken in phase contrast by a x1000 magnification objective.

2.8 Identification assay: MLST of *Pseudomonas* isolate no. 33

2.8.1 Polymerase Chain Reaction (PCR)

In order to sequence genes from a species, must genomic DNA from the species be available. This was obtained by following the protocol described in section 2.6.1. PCR was then used to amplify the specific genes that would be sequenced. PCR uses two primers which matches the DNA on either end of a target sequence, a forward and a reverse primer. In theory would only the target sequence be amplified in large amounts when the polymerase extends the sequence starting from the primers. The following program was used for PCR during this thesis (Fig. 6).

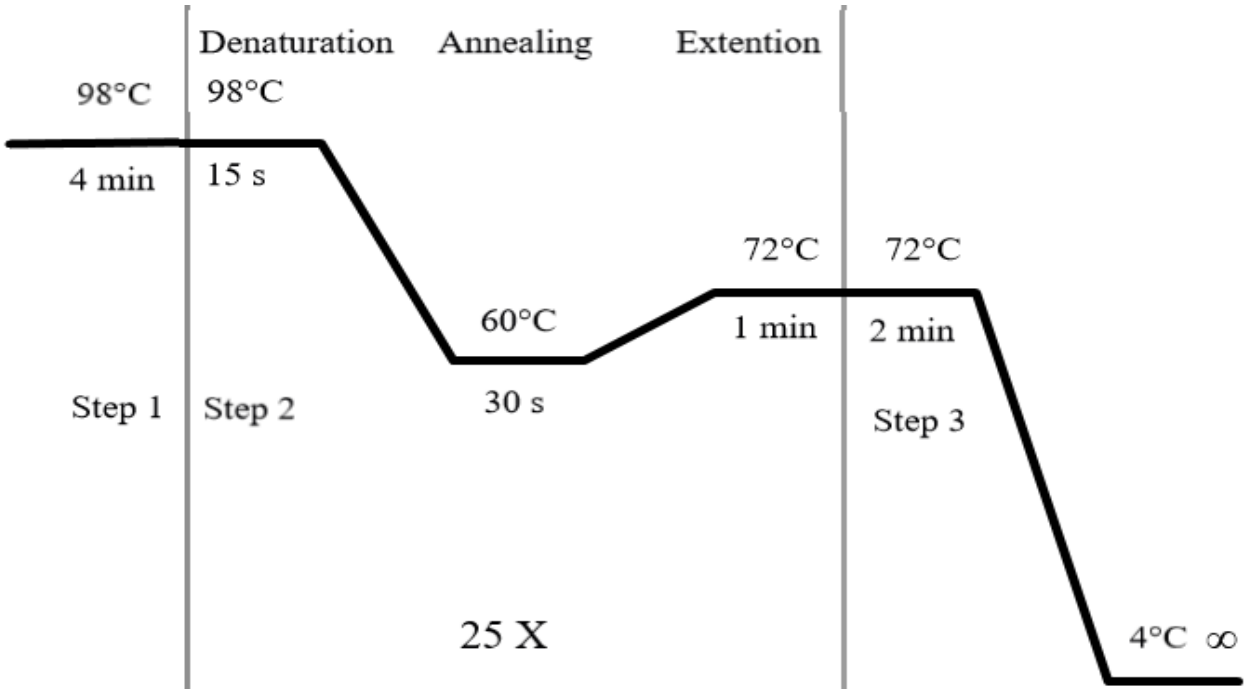


Figure 6. Base for all PCR cycles used in this thesis. Denaturation annealing and extension (step 2) were repeated 25 times.

Annealing temperature would be adjusted to fit the given gene. Annealing temperatures used to amplify the genes can be seen in table 6. Additionally, the table contains primer sequences and the full name of the genes.

Table 6. Primers listed below was specifically ordered for this thesis and gives the sequence for the seven housekeeping gene used to identification species within the *Pseudomonas fluorescence* complex.

Primer	**Sequence (5'-3')**	**Gene product**	**Annealing temperature**
GlnS_F	ACCAACCCGGCCAAGAAGACCAGG	Glutaminyl-tRNA synthetase	65°C
GlnS_R	TGCTTGAGCTTGCGCTTG		
GyrB_F	GGTGGTCGATAACTCCATCG	DNA gyrase subunit B	60°C
GyrB_R	CGCTGAGGAATGTTGTTGGT		
IleS_F	TTCCCAATGAARGCCGGCCTGCC	Isoleucyl-tRNA synthetase	65°C
IleS_R	GGGGTGGTGGTCCAGATCACG		
NuoD_F	GAAGTCCTGACCTTCCTGC	NADH dehydrogenase subunit D	60°C
NuoD_R	GAAGAACTCGGCCATCATG		
RecA_F	TGGCTGCGGCCCTGGGTCAGATC	Recombinase A	60°C
RecA_R	ACCAGGCAGTTGGCGTTCTTGAT		
RpoB_F	TGGCCGGTCGTCACGGTAACA	DNA-directed RNA polymerase subunit beta	60°C
RpoB_R	CCGAAACGCTGACCACCGAAC		
RpoD_F	CTGATCCAGGAAGGCAACATCGG	RNA polymerase sigma factor	60°C
RpoD_R	ACTCGTCGAGGAAGGAGCG		

The reagents used for each PCR run are listed in table 7. The reagents were mixed in PCR-tubes with no preferred order, with the exception that Phusion polymerase would be the last reagent added. The reagents were kept on ice during preparation. Once the mixture was done, the PCR tubes were placed in the PCR machine. During this thesis was a ProFlex PCR machine used. For the specific primers used in this thesis would annealing temperature be adjusted according to table 6, otherwise was the program followed according to Fig. 6.

Table 7. Reagent needed to perform PCR. DMSO was added to easier separate the strands during denaturation.

Reagents	Quantities
dNTP 10 mM	1 µl
5X Phusion reaction buffer	10 µl
Primer 1 forward 10 mM	1 µl
Primer 2 reverse 10 mM	1 µl
Genomic DNA	1 µl
Phusion DNA polymerase	0,5 µl
dH_2O	34 µl
DMSO	1 µl
Total	**50 µl**

2.8.2 Gel electrophorese

A 1% agarose gel was prepared by mixing 5 g agarose agar and 50ml TEA. The mixture was boiled in a microwave until all the agarose agar had dissolved. The gel was then cooled to approximately 50°C before it was added 2 µl peqGREEN dye. The mixture was then poured onto a gel casting trays and combs were placed in the gel in order to make chambers for the samples. Once the gel had cooled down the combs were removed and the gel, still in the casting tray, was placed in an electrolysis bath filled with TEA buffer so that the gel was completely covered. The PCR product was prepared by mixing in 5X loading dye right before loading, 2 µl dye with 8 µl PCR product. The gel wells were then loaded with 10 µl PCR product mixed with loading dye. For every run was one well added 5µl of a 1 kb DNA ladder, in order to have a reference point for band length. The gel electrophoresis was then run at 90V for 30 minutes to an hour.

2.8.3 Purifying PCR product from gel

After the PCR-products was approved by length and intensity were the appropriate bands cut from the gel. The PCR bands were visualised under UV-light at 490 nm and cut from the gel with a clean

scalpel. The reference length of the gene made it easier to identify the correct band when comparing it to the 1 kb DNA ladder.

The PCR product were purified from the agarose gel by the kit "NucleoSpin® Gel and PCR Clean-up", components are listed in table 8. The cut-out gel was placed in clean Eppendorf tubes and added 500 µl NTI buffer. The NTI buffer was added to dissolve the gel. The samples were incubated in a 50°C water bath until the gel had completely dissolved. While the sample incubated were the kits columns placed in collection tubes. Once the samples had dissolved were the sample loaded onto the column 700 µl at a time and spun down at 11,000 x g for 30 seconds. If the sample contained more than 700 µl was the process repeated. The supernatant was discharged. The column was then washed twice with 700 µl NT3 buffer, both times were the column spun at 11,000 x g for 30 seconds and supernatant discharged. The column was dried by spinning it for 1 minute at 11,000 x g. The PCR product was then eluted by adding 15-30 µl NE buffer. The elution buffer would sit in the column for one minute. In the meantime, was the column transferred to a clean Eppendorf tube. The purified PCR product were collected by spinning the column at 11 000 x g for 1 minute. Final concentration was measured by nanodrop using NE buffer as blank. The kits protocol was followed.

Table 8. NucleoSpin® Gel and PCR Clean-up. User of the kit needed to supply with 96-100% ethanol, pipette tips, and Eppendorf tubes.

Content
Binding buffer NTI
Wash Buffer NT3 (concentrate)
Elution buffer NE (5 mM Tris/HCl, pH8,5)
NucleoSpin® Gel and PCR Clean-up Columns
Collection Tubes (2 ml)

2.8.4 Sequencing

The sequences were all sent to be sequenced by the company Eurofins. The samples therefore had to be strictly prepared for the process to go as smoothly as possible. Each PCR product was measured with Nanodrop in order to find the concentrations. This was important because the company required between 50-100 ng of DNA in each sample. Each sample were split in two, so that every primer was sent with its belonging PCR-product. The mixtures would contain 50-100 ng PCR-product and 2 µl primer (either forward or reverse), with a total volume of 10 µl. If the sample had very high concentration of PCR product was some of the product replaced with sterile dH_2O to stay within the company's demand. Each primer was then sent in separate Eppendorf tubes with its belonging PCR-product.

3. Results

3.1 Determination of the target range of streptovoracin

3.1.1 Streptococcus species

A screening was performed to establish the target range of streptovoracin. The diameter of the inhibition zones of all bacteria tested can be found in appendix D. Most of the species tested belong to the *Streptococcus* genus. Species belonging to the mitis- and anginosus phylogenetic groups within the genus *Streptococcus* were streptovoracin sensitive (appendix D). The only exceptions were *S. anginosu*s NCTC 10713 and *S. constellatus* NCDO 2226, whose inhibition zones were too narrow to be properly measured, and *S. criceti* ATCC 19642, which was resistant to streptovoracin. Inhibition zones tended to become smaller in the species that are further away from *S. pneumoniae* on the phylogenetic tree. However, in cases where several strains of the same species were tested, large variations were found within each species. The largest diversity within a species was observed for *S. pneumoniae's* closest relative, *S. mitis*. The *S. mitis* strains tested had inhibition zones ranging from 1.1 cm to 4.5 cm. This latter must be considered to be a hypersensitive strain. Outside of the mitis- and anginosus groups was resistance more common and all the *S. mutans* strains tested were completely resistant to streptovoracin.

All *S. pneumoniae* strains tested were sensitive, including highly pathogenic encapsulated strains such as A66 and TIGR4. Moreover, the polysaccharide capsule of pathogenic *S. pneumoniae* strains did not seem to significantly affect inhibition. This was demonstrated by comparing the sensitivity of a capsule-less mutant of the A66 strain with the parental strain. The diameter of the inhibition zone obtained when the A66 wild-type strain was used as indicator for susceptibility, was approximately the same as the inhibition zone obtained with the capsule-less mutant (see appendix D). However, when grown on blood agar, which stimulates capsule production, the TIGR4 and A66 strain displayed somewhat smaller inhibition zones. Interestingly, the results of the screening showed that streptovoracin inhibits the growth of all the important streptococcal pathogenic species, except *S. mutans* (Fig. 7). These included *S. pneumoniae*, exemplified by the highly pathogenic TIGR4 strain, and *S. pyogenes* and *S. agalactiae*. All tested pathogenic strain of *S. pneumoniae* were found sensitive, including *S. pneumoniae* Type 4, *S. pneumoniae* B14, *S. pneumoniae* D36 and *S. pneumoniae* A66

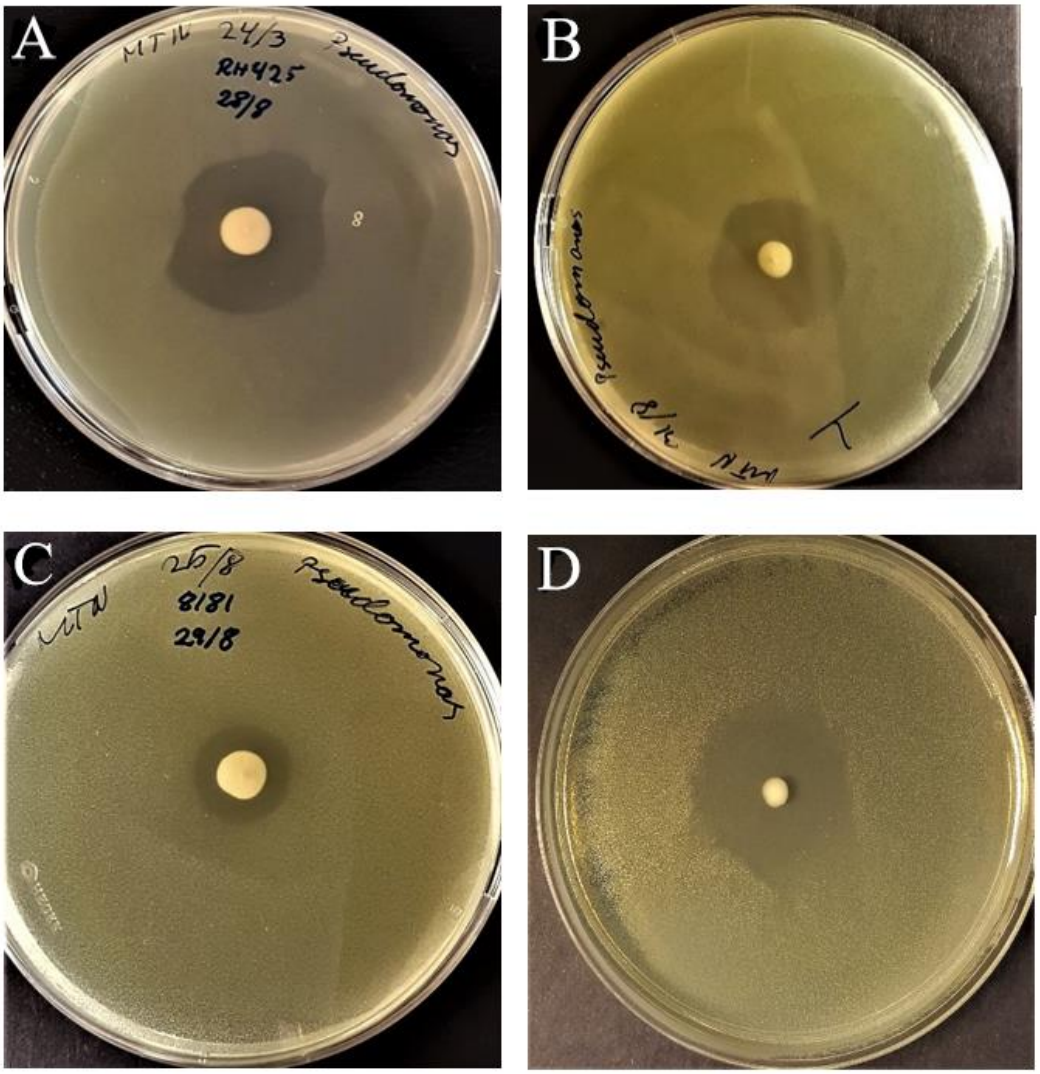


Figure 7. Pathogenous *Streptococcus* species in overlay over a colony of *Pseudomonas* isolate 33. A) *S. pneumoniae* RH425, control strain. B) *S. pneumonia* TIGR4. C) *Streptococcus agalactica* NCTC 8181. F) *S. pyogenes* NCTC 8198.

3.1.2 Non-streptococcus species

A number of non-streptococcus species were also screened to determine if streptovoracin really is a narrow-spectrum antibiotic as initially believed. In other words, these species were tested to see if sensitivity is limited to the genus *Streptococcus*, or if sensitivity to streptovoracin is more widespread than previous tests showed. The results of the overlay assay for the non-streptococcus species are listed at the end of the table in appendix D. *Lactococcus lactis*, a close relative of

streptococci (formerly *Streptococcus lactis*), was not susceptible to the antibiotic. The same was true for the following Gram-positive bacteria: *Lactobacillus sake, Enterococcus faecium, Listeria monocytogenes, Bacillus subtilis, and Bacillus cereus*. Interestingly, *E. faecalis* EF 2708 was found to be sensitive to streptovoracin. It was the only susceptible Gram-positive bacterium tested that did not belong to the genus *Streptococcus* (Fig. 8A). This particular bacterium was an exception. The only Gram-negative bacterium tested was *E. coli*, which was found to be resistant to the antibiotic.

At the time the overlay assay was performed, my supervisor was growing a culture of a Dietzia species in the laboratory. Out of curiosity, this strain was included in my screening, and to our great surprise it was sensitive (Fig. 8B). Similar to *Mycobacteria*, does members of the genus *Dietzia* contain lipoglycans such as mycolic acid in their outer cell envelopes (Sutcliffe, 2000). Hence, this finding opens up the possibility that *Mycobacterium tuberculosis* and *Mycobacterium bovis*, the bacteria causing tuberculosis in humans, are sensitive as well. Since propagation and manipulation of cultures of *M. tuberculosis* and *M. bovis* requires biosafety level 3 practise, I was not able to test streptovoracin against these species. Instead, was *Mycobacterium smegmatis* used. As it is not pathogenic to healthy individuals and is much easier to cultivate than *M. tuberculosis* and *M. bovis*, this species is commonly used as a substitute for its highly pathogenic relatives. As shown in Figure 8C, the growth of *M. smegmatis* is inhibited by streptovoracin, making it highly likely that *M. tuberculosis* and *M. bovis* is sensitive as well. Interestingly, similar to what was observed for *S. pneumoniae*, no spontaneous resistant colonies were observed inside the inhibition zone of *M. smegmatis*. This suggest that resistant against this antibiotic will be slow to develop. In sum, the screening results indicate that streptovoracin has a relatively narrow inhibition spectrum and is mainly active against members of the genus *Streptococcus* in the phylum Firmicutes. In addition, it is active against some members of the phylum Actinobacteria, possibly those that have lipoglycans in their outer envelope. The fact that streptovoracin is active against *S. pneumoniae*, and probably also *M. tuberculosis/M. bovis*, two of the most important human pathogens, makes it an exciting and promising candidate for future drug development.

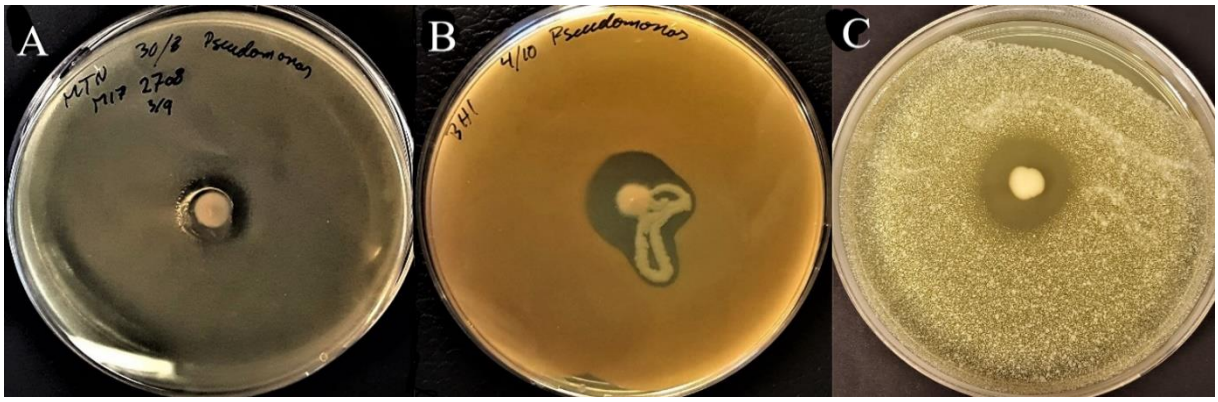


Figure 8. Overlay assay showing streptovoracin-mediated growth inhibition of non-*Streptococcus* strains incorporated in the top-agar layer. The *Pseudomonas* producer strain was spotted in the middle of each plate and was left to grow for four days in room temperature before it was overlaid with top-agar. Then depending on the growth rate of the indicator strain, the plated were further incubated for 1-2 days before a picture was taken. A) *Enterococcus faecalis* EF BRIDGE EF 2708. B) *Dietzia* SPH 492 C) *Mycobacterium smegmatis* NCTC 8159.

3.2 Optimization of culture conditions for streptovoracin production.

3.2.1 Cultures grown with and without oxygen

Prior to this study, the amount of streptovoracin presented in the medium of *Pseudomonas* cultures varied a lot from batch to batch, and in general the streptovoracin concentration was too low to be detectable when medium was added to pneumococcal cultures grown in 96-well microtiter plate. It was therefore important to optimize the culture condition in order to get higher and more reproducible yields. In the beginning of my work the *Pseudomonas* isolate was grown in BHI medium in 500 ml flasks. Unfortunately, the streptovoracin production varied a lot from flask to flask. A possible explanation for this variation was that the lid on the flasks had sometimes been screwed on loosely while it was strongly tightened at other occasions. If so, this would lead to different levels of oxygen in the cultures, a factor that could influence streptovoracin production. Therefore, experiments comparing streptovoracin production in aerobic and anaerobic cultures were conducted. In the aerobic culture, where the growing *Pseudomonas* producer had free access to oxygen through a sterile filter lid, lysis or growth inhibition was detected when 4-day-old medium was added to pneumococcal cultured (Fig. 9A). The growth medium was diluted in a two-

fold series, and 30 µl of each diluted sample was added to 270 µl test culture grown in a microtiter plate at 37℃ in a microplate reader. A clear growth inhibitory effect was seen down to approximately 1:8 dilution of the medium. In contrast, the anaerobic culture had no measurable production of streptovoracin (Fig 9B). The reason for pneumococcal culture lyse when undiluted and 1:2 diluted growth medium is added is because streptovoracin triggers the action of the autolysins LytA. In a *ΔlytA* mutant however, they just stop growing. This observation was done before I started my thesis work. In sum, my results clearly show that the presence of oxygen is absolutely necessary for the production of streptovoracin.

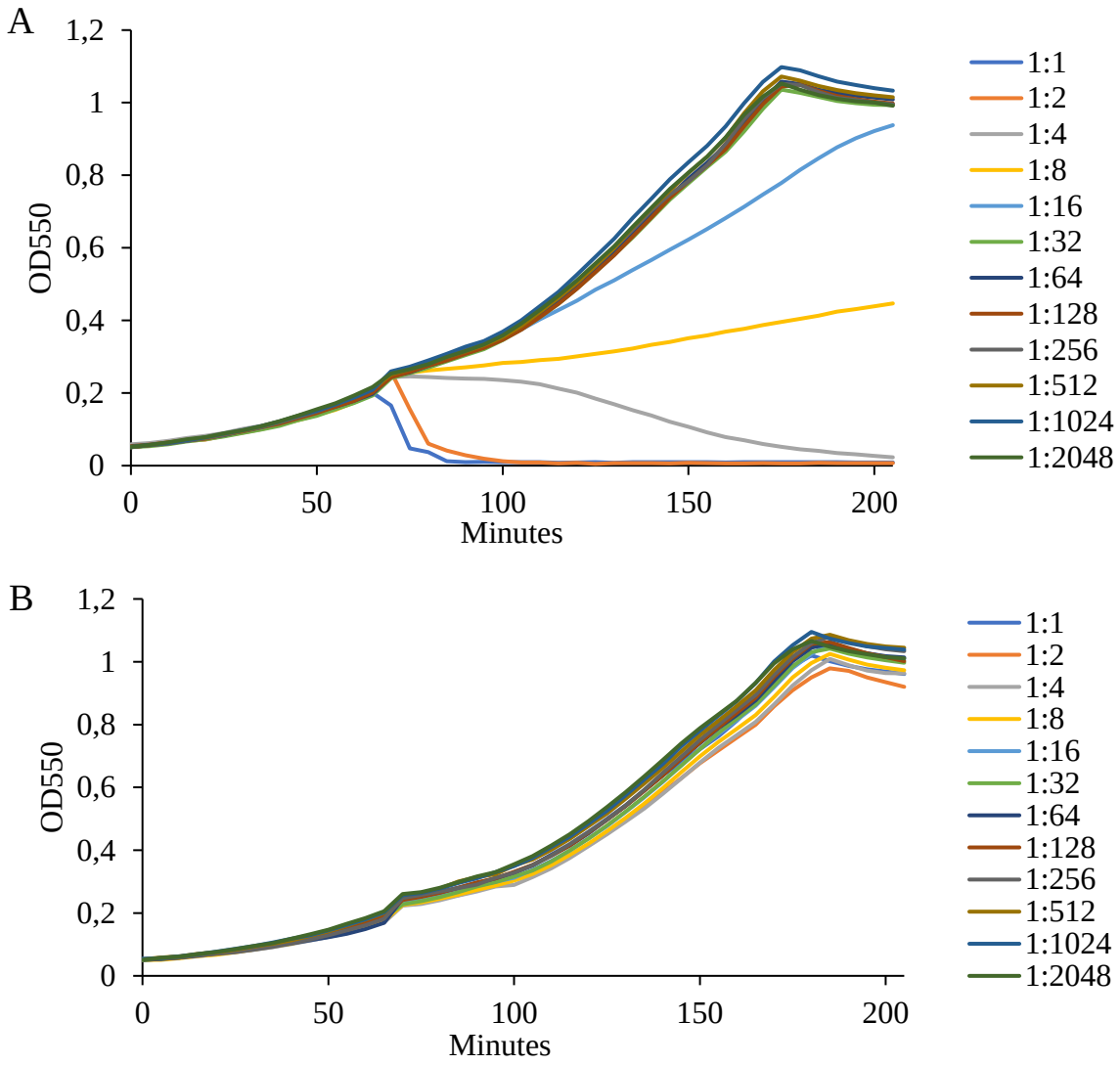
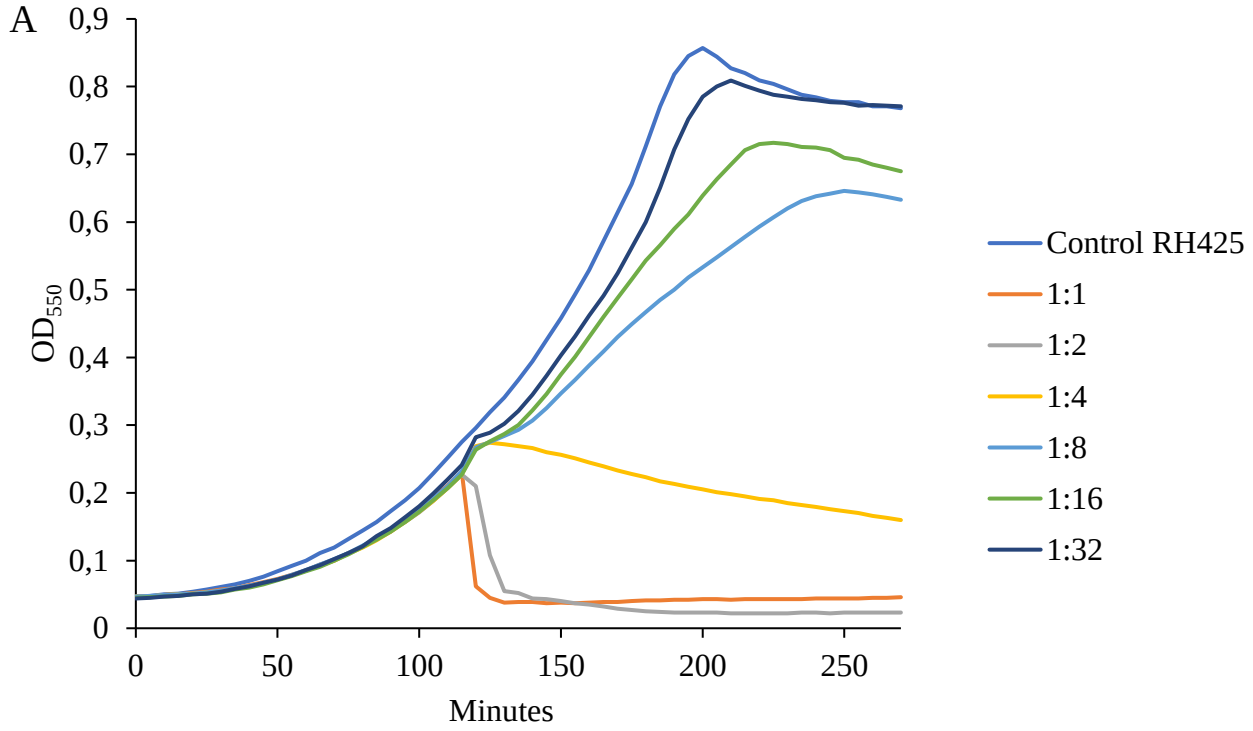


Figure 9. Comparison of the amount of streptovoracin produced by aerobic (A) and anaerobic (B) cultures of the *Pseudomonas* producer strain grown in BHI medium at room temperature. Streptovoracin was added after 70 minutes when the cultured had grown toOD_{550} ~0,24.

After it was established that oxygen is a necessary for streptovoracin production all subsequent cultured were grown aerobically. This was done by inoculating 500 ml medium in 3 litre Erlenmeyer flasks with a wide bottom and a filter lid. The filter lid allows air to pass through while the wide bottom created a large surface area that gives better aeration of the culture.

3.2.2 Cultures grown with shaking

The *Pseudomonas* producer strain was isolated from the littoral zone of the Oslo fjord where the waves and constant stirring of the water increase its oxygen content and sometimes expose bacteria directly to air. To simulate these conditions, the inoculated Erlenmeyer flask was placed in a shaker. Shaking will stir the culture and bring more bacteria close to the surface than under stagnant culture conditions. In theory, this should increase the access of oxygen to the bacteria in the *Pseudomonas* culture. However, as seen in Figure 10, shaking did not further improve streptovoracin production compared with a stagnant culture. In both cases, complete inhibition were obtained at 1:4 dilution of the growth medium. Therefore, shaking was considered superfluous and omitted in later experiments.



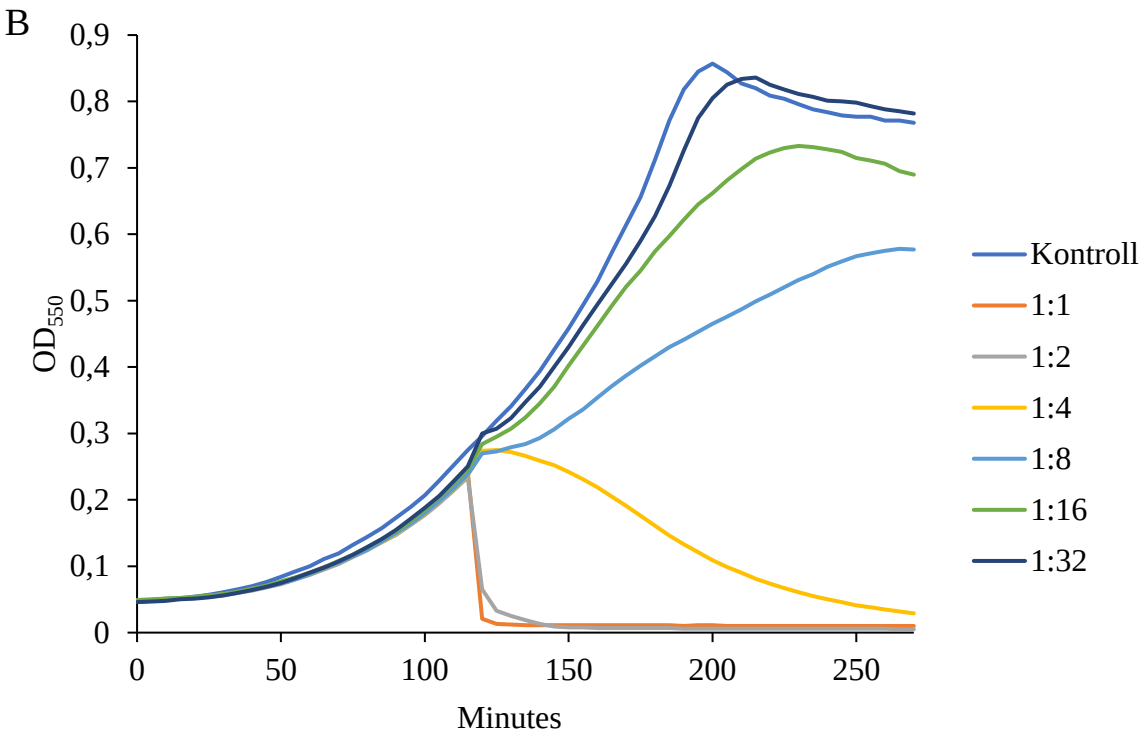


Figure 10. Comparison of the amount of streptovoracin produced in (A) a shaken culture (100 rpm) and (B) a still culture of the *Pseudomonas* producer strain grown in BHI medium at room temperature. The experiments were performed as explained in section 3.2.1. Streptovoracin was added when the culture had grown toOD_{550} ~ 0,27.

3.2.3 Cultures grown in the presence of glass wool

As mentioned above, the streptovoracin producer strain was isolated from the littoral zone, and we therefore speculated that its natural habitat is biofilms on rocks and vegetation in shallow water along the waterfront of the Oslo fjord. It was therefore possible that growth on solid surfaces would stimulate streptovoracin production. Thus, in another attempt to simulate the original environment of *Pseudomonas* isolate no. 33, glass wool was mixed into the growth medium to enhance the available surface area. It is known that biofilm formation and CLP production goes hand in hand for many CLP producers (Geudens & Martins, 2018). By visual inspection, it appears that the *Pseudomonas* strain attached to the glass wool as it became less lustre and more opaque, but the degree of biofilm formation was not studied any further. However, when glass wool was added to the growth medium it approximately doubled the production of streptovoracin (Fig. 11). In the culture without glass wool, 1:4 dilution of the medium completely inhibited growth of the

pneumococcus indicator strain. In contrast, 1:8 dilution of the medium was sufficient to fully inhibit growth of the indicator strain in the culture containing glass wool (Fig. 11).

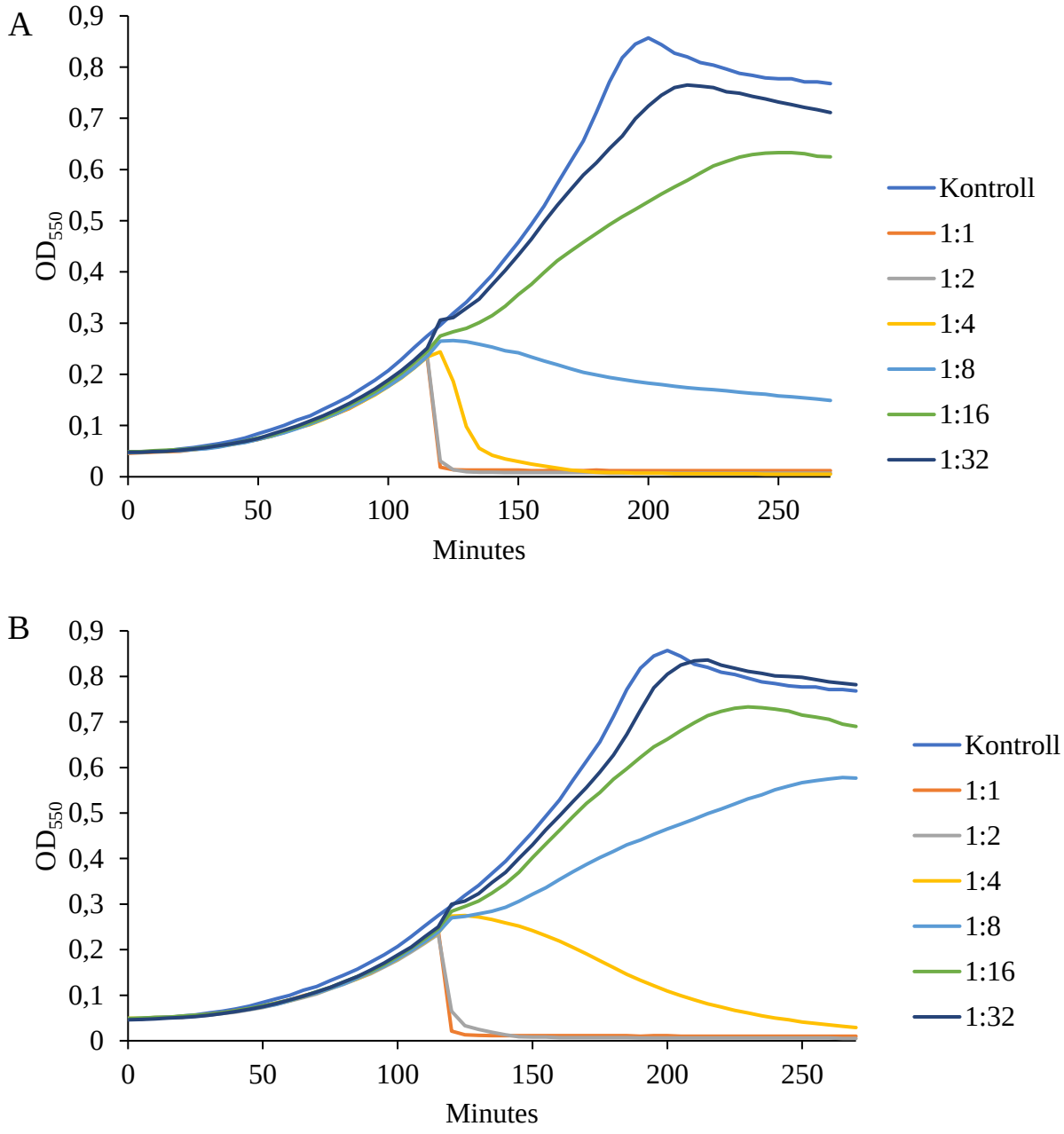
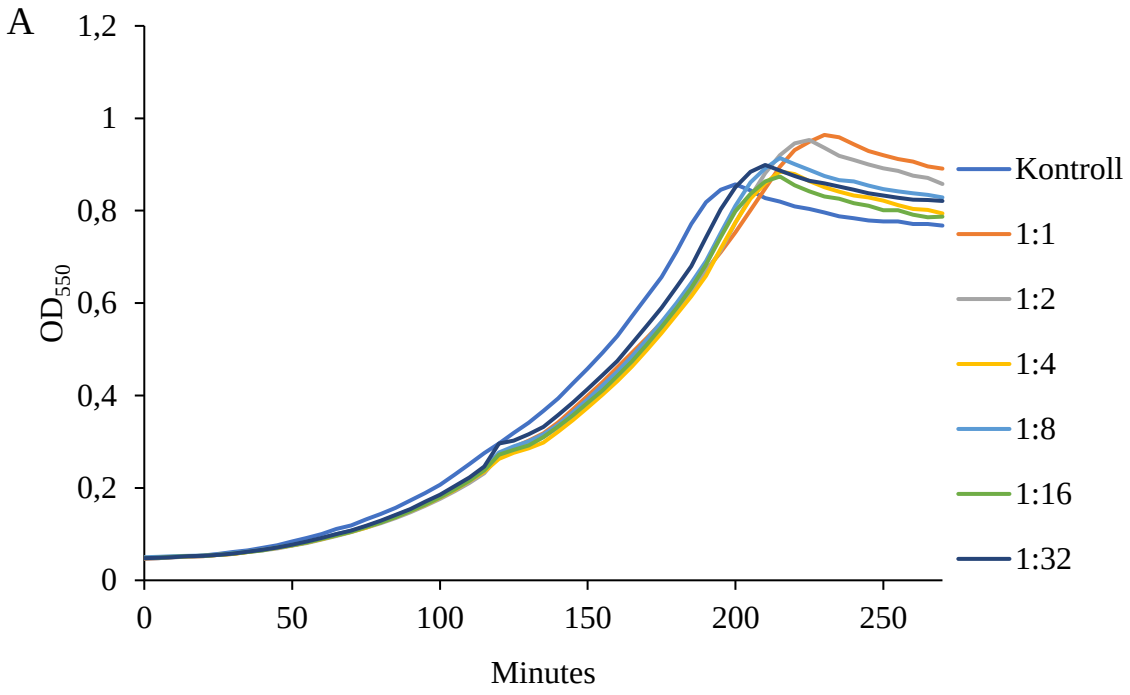


Figure 11. Comparison of the amount of streptovoracin produced in (A) a culture containing glass wool and (B) a still culture of the *Pseudomonas* producer strain grown in BHI medium at room temperature. The experiments were performed as explained in section 3.2.1. Streptovoracin was added when the cultures had grown to OD_{550} ~0,27.

3.2.4 Cultures grown on different media

In the experiments described above the cultures were grown in BHI medium, which is a very rich medium. This medium was used in the initial studied preformed before I started my thesis work, because the bacteria grew well in it. However, since BHI is made from cow's brain and heart, it contains a lot of lipids and hydrophobic constituents which carried along the streptovoracin during purification. With the aim to reduce these impurities, attempts were made to grow *Pseudomonas* isolate no. 33 on other less complex media with less lipid content. Two different media were tested, namely *Pseudomonas* broth and M9 minimal medium (see appendix A). The two media were tested along with the glass wool and control cultures described under 3.2.3. Hence the same control culture was used for all experiments. The experiments involving *Pseudomonas* broth and M9 medium were performed in the same manner as previously described for BHI medium using the same growth period for the *Pseudomonas* producer strain. The M9 minimal medium showed no measurable activity after four days (Fig. 12A). The culture was therefore left for four additional days, but no streptovoracin production was detected with the method used. Similarly, less streptovoracin was produced in *Pseudomonas* broth. Compared to BHI medium, less than half the amount of streptovoracin was produced in *Pseudomonas* broth (Fig. 12B)



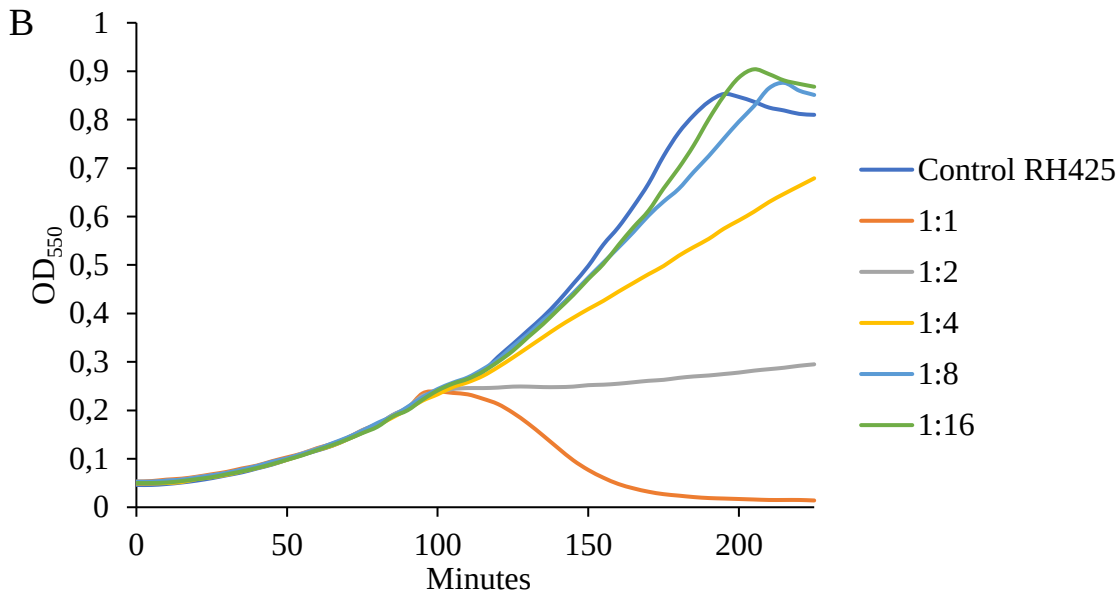


Figure 12. Production of streptovoracin by *Pseudomonas* cultures grown in M9 minimal medium (A) and *Pseudomonas* broth (B). The experiments were performed as explained in section 3.2.1. Streptovoracin was added when the cultures had grown to OD_{550}~0,22.

3.2.5 Production of streptovoracin tracked over an eight-day period.

Of the media tested, the BHI medium came out on top. Furthermore, the use of the still cultures cultivated in Erlenmeyer flask with a filter lid for good aeration was as good as corresponding cultures grown in a rotary shaker. Since the use of glass wool was cumbersome to use, and only resulted in approximately a doubling of streptovoracin production, I decided not to use glass wool in later experiments. In all the optimization experiments described above the cultures were harvested after four days. I therefore decided to follow streptovoracin production over an eight-day period to determine when the production peaks. The culture was grown in 500 ml BHI medium in a 3 litre Erlenmeyer flask with a filter lid at room temperature. One millilitre samples were collected every day and kept frozen until use. When tested they were diluted in a 2-fold dilution series, and 30 µl of the dilutions were added to 270 µl of the pneumococcus indicator stain as described above. The dilution 1:2 gave a good representation of the entirety of the samples. The result from dilution 1:2 presented in figure 13 show that streptovoracin production continue to rise until day six, after which it levels off. The slight reduction in activity see for day seven and eight compared to day six might be accidental or could indicate that there is some loss in activity after the production slows down.

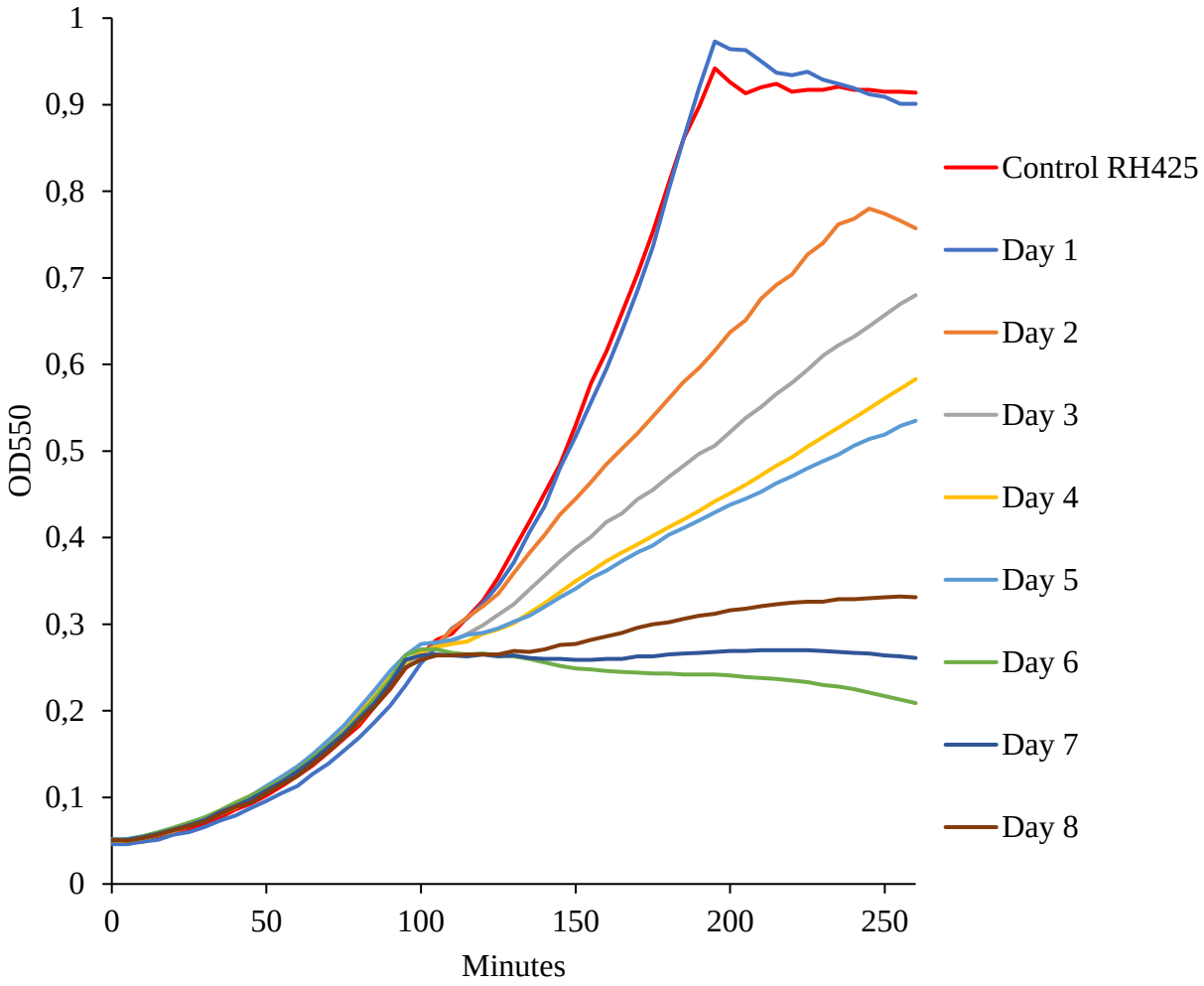


Figure 13. Tracking streptovoracin production over 8 days. Samples were collected once a day, diluted 1:2 in BHI medium, and tested as explained in section 3.2.1. Streptovoracin was added when the cultures had grown to OD_{550}~ 0,27.

In this particular experiment all samples displayed a somewhat lower activity than observed for previous experiments conducted in the same way. In previous experiments 1:2 dilutions of the growth medium caused all the indicator bacteria to lyse (see figure 10 and 11), while in the present experiment they did not. The reason for this is not known. However, as the experiment gave a clear-cut result, it was not repeated.

3.3 Developing a purification method for streptovoracin

3.3.1 Amberlite resin

In the studies done before my thesis work, streptovoracin in the growth medium was precipitated by 40% (w/v) ammonium sulphate. The precipitation was dissolved in distilled water and subjected to reverse phase HPLC chromatography on a C18 column. Material that bound to the column was eluted by a 0-100% acetonitrile/0.1% trifluoracetic acid gradient. Ammonium sulphate precipitated protein by making the protein less soluble in the aqueous solution. During centrifugation the precipitate will stick to the wall of the centrifugation bottle. However, since a high concentration of ammonium sulphate is needed to precipitate streptovoracin, additional steps such as dialysis is required to remove the salt in the precipitate. In the long run this procedure would be expensive and time consuming if production were to be scaled up. Therefore, it was decided to test whether streptovoracin binds to the amberlite resin XAD 16N. Amberlite XAD 16N is made from styrene-divinylbenzene and is hydrophobic polyaromatic resin that absorbs and releases molecule through hydrophobic and polar interactions. Hence, it should bind streptovoracin, which was known from initial studied to be a very hydrophobic compound. The resin was mixed directly with the growth medium in an Erlenmeyer flask after the producer strain had been removed by centrifugation. Then, the mixture was put on a rotary platform for approximately two hours. Next, the medium was removed, and the resin was washed with 30%, 60% and 100% methanol. The different wash solutions were tested for antimicrobial activity by preforming dilution series on microtiter plate as described above. 30 microliters of each washing solution were added to 270 µl S. pneumoniae culture. As a control, a two-fold dilution series of 100% methanol was made and run in parallel to ensure that any observed growth inhibition was caused by streptovoracin and not by methanol. The control showed that a 10% final solution of methanol inhibited growth pneumococci, whereas they tolerated 5%. It was also observed that 10% methanol did not induce LytA-mediated autolysin. The result of the experiment showed that streptovoracin was not released by 30% methanol, but that some antimicrobial activity was released by washing with 60% methanol. However, the majority of streptovoracin was released with 100% methanol. Next, the concentrations between 30- and 60% methanol was tested with a 5 % interval to find the maximum methanol concentration that could be used for washing. The results in figure 14 shows that traced of antibiotic could be found already in the 40% solution, and the amberlite resin could therefore not be washed with concentrations higher than 35% to prevent loss during this purification step.

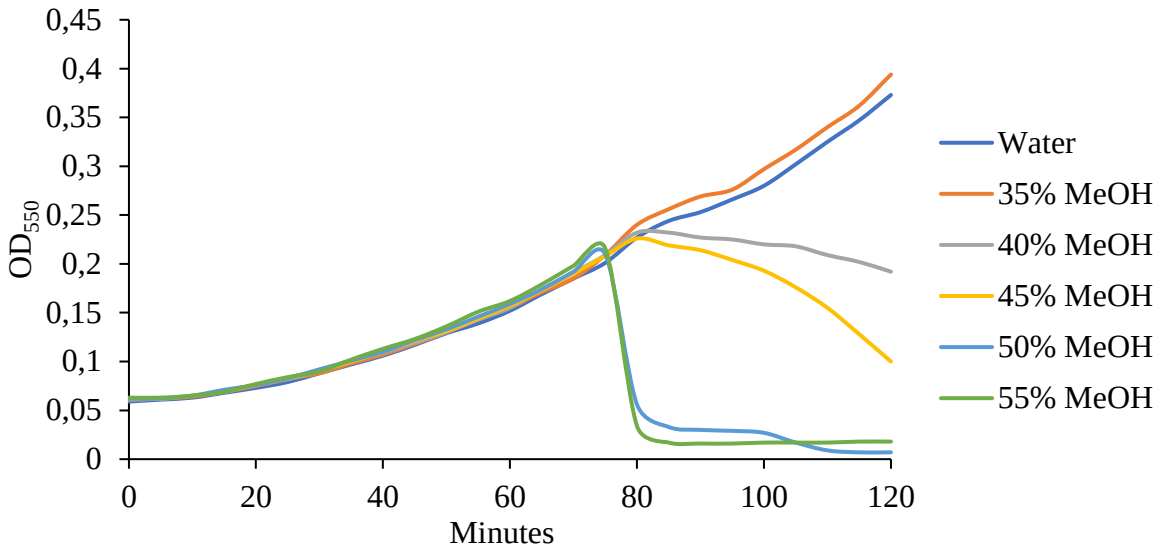


Figure 14. Determination of streptovoracin activity in washing solutions with different concentrations of methanol. Streptovoracin in the medium was bound to amberlite XAD 16N resin. To get rid of as many contaminants as possible, methanol was included in the washing solution. The purpose of the experiment was to determine the maximum methanol concentration that could be used without co-eluting the antibiotic. Serially diluted washing solutions (30 µl) was added to 270 µl cultures when the cultures had grown to OD_{550} ~ 0,22. Control experiments showed that a final concentration of 5% methanol does not significantly inhibit the growth of pneumococci or cause LytA-mediated lysis of the culture. The finial concentrations of the washing solutions listed above were as follows: 35% (3.5% final); 40% (4% final); 45% (4.5% final); 50% (5% final) and 55% (5.5% final).

To be in the safe side it was decided to use washing with 30% methanol and elution of streptovoracin with 100% methanol as the standard protocol. The amberlite resin was mixed with growth medium in an Erlenmeyer flask, but to make the washing steps and elution easier the resin was transferred to a column after incubation on a rotary shaker. To get rid of as many contaminants as possible the amberlite resin was washed with 3 column columns of 30% methanol during each run. The elution step was always performed with two column volumes. The highest concentrations of streptovoracin was found in elution number two. A third elution step was not necessary, as little to no streptovoracin found in the third eluate (results not shown).

Moreover, as a 30% methanol solution did not prevent the binding of streptovoracin, the cell free medium containing streptovoracin was mixed with methanol to a concentration of 30% before the medium was mixed with the amberlite resin. Again, this was to minimize the binding of

contaminants that otherwise would compete with streptovoracin for binding site on the amberlite resin. The strategy proved highly effective as far less impurities were observed in the successive HPLC run.

3.3.2 HPLC

Before eluates from the amberlite step were ready for reverse phase HPLC the methanol was removed by vacuum evaporation. Then, the fully dried pellets were dissolved in 100 µl of 50% acetonitrile in water with 0.1% trifluoracetic acid added. HPLC was performed by injecting 50 µl samples into a C18 reverse phase column (vydac 218TP C_{18} 5 µm) followed by elution of streptovoracin with a 50-100% gradient of acetonitrile/0.1% trifluoracetic acid. Streptovoracin was released at very high concentration of acetonitrile at two separate peaks (Fig. 15). The indicated masses were known before I started on my thesis work, and were determined by mass spectroscopy (Fig. 15)

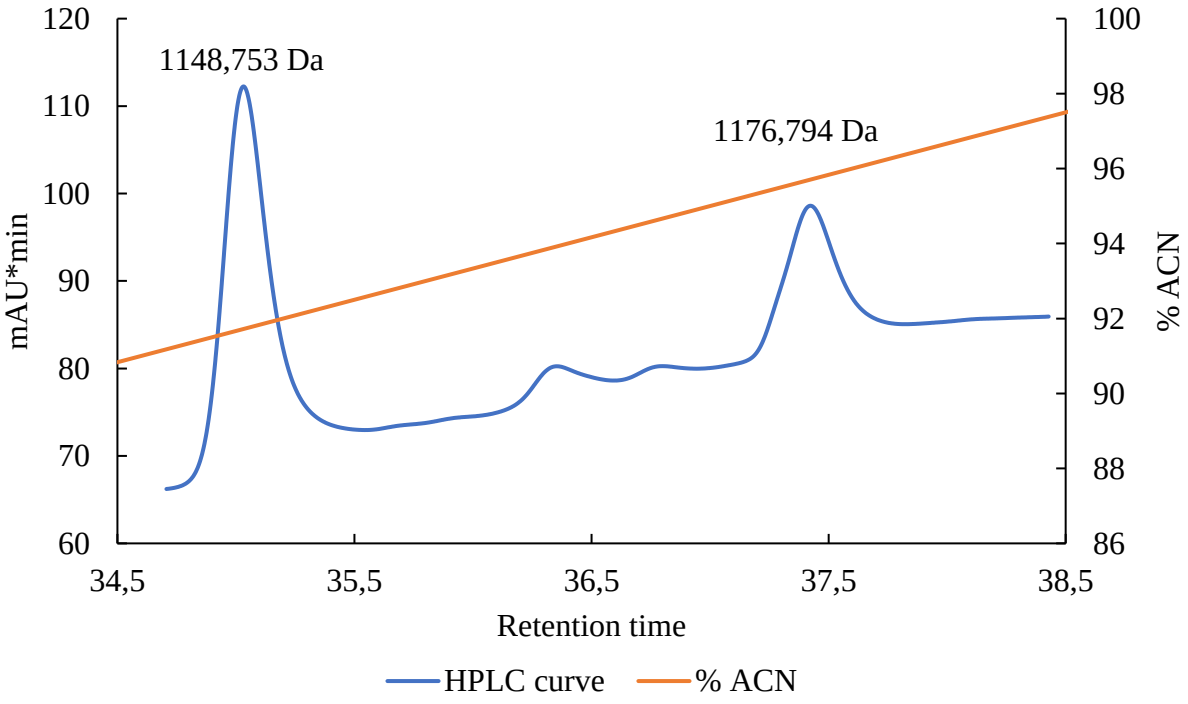


Figure 15. Purification of streptovoracin by reverse phase HPLC. The figure shows only the part of the chromatogram where streptovoracin elutes. The orange line indicates the concentration of acetonitrile (ACN), while the blue line indicates absorption at 220 nm (A_{206}).

Although streptovoracin is thought to be amphiphilic, the dried pellets were insoluble in water. They were therefore dissolved in 50% ACN instead of pure dH_2O. Because of this the acetonitrile gradient used to elute streptovoracin from the C18 column was adjusted to run from 50-100% instead of 0-100%.

3.3.3 Comparing the developed purification method to the original

To ensure that the new method for purifying streptovoracin was equal to or better than the method used before, the two methods were compared. This experiment also included a modified version of the ammonium sulphate precipitation method in which a higher concentration of ammonium sulphate was used to see if it would give a better yield. A culture was split in three equal parts of 50 ml after removal of the bacteria by centrifugation (see Fig. 5 for details). Two of the samples were subjected to ammonium sulphate precipitation (40% and 60% w/v), while the third was mixed with amberlite resin. All samples were treated and purified as described in Fig. 5 and section 2.4.6. For each purification step 1 ml fractions were collected, dried *in vacuo* and dissolved in 50 % acetonitrile before being subjected to HPLC chromatography as described above. Comparisons of yields between the three methods were calculated based on the area under the two peaks representing streptovoracin (see Fig. 15). Because of manual adjustment it is not possible to get the peak-area measurement 100% accurate. Probably because of this none of the purification protocols gave values of exact 100% when added together.

The results in table 9 show that at least 40 % of the streptovoracin initially present in the growth medium was lost in the supernatant after ammonium sulphate precipitation. In contrast, due to minimal loss of streptovoracin in the flow-through and washes, less than 10% was lost when the amberlite-base purification method was used. To get more reliable results this experiment should have been repeated several times. However, although the number may not be very accurate, I think that it is safe to conclude that the yield of streptovoracin is higher when using the amberlite method compared to ammonium sulphate precipitation.

Table 9. Comparison of yields of streptovoracin after absorption to amberlite resin or ammonium sulphate precipitation.

Purification stage[a]	Sum of the total areas of peaks 1[b] and 2[b] after HPLC analysis[c]	Yield (%)
Sample (1 ml) withdraw from cell-free growth medium in a 4 days old culture of the streptovoracin producer strain.	2661	100%
Amberlite purification		
Flow through	142	5.3%
Wash solution (30% methanol)	64	2.4%
Eluate (100 % methanol)	2601	**97.7%**
Ammonium sulfate precipitation (40% w/v)		
Supernatant collected after precipitation and centrifugation	2140	80.4%
Precipitate dissolved in dH_2O	634	23.8%
Water-insoluble precipitate dissolved in 100% methanol	1129	42.4%
Yield of streptovoracin extracted from precipitates		**66.2%**
Ammonium sulfate precipitation (60% w/v)		
Supernatant collected after precipitation and centrifugation	1111	41.7%
Precipitate dissolved in dH_2O	190	7.1%
Water-insoluble precipitate dissolved in 100% methanol	1477	55.5%
Yield of streptovoracin extracted from precipitates		**62.6%**

[a]See figure 5 and section 2.4.6 for details.

[b]Streptovoracin elutes as two different peaks in HPLC, designated peak 1 and peak 2.

[c]1 ml samples were withdrawn, dried *in vacuo*, dissolved in 50% acetonitrile and subjected to HPLC analysis. However, the calculations are based on the total volume of liquid from each purification stage, and not only on the 1 ml that was analyzed by HPLC.

3.3.4 The solubility of streptovoracin in water

The HPLC peaks corresponding to streptovoracin were collected and dried *in vacuo* to get rid of the toxic acetonitrile. The dried pellets were then weighed on an analytical scale. In a standard purification experiment between 1.5 to 3.2 mg of pure streptovoracin was obtained. Unfortunately, it turned out that solubility of streptovoracin in pure water is low after it has been dissolved in acetonitrile and dried. Therefore, several other possible solvents were tested. DMSO, acetonitrile and alcohols were highly effective in dissolving streptovoracin. However, if streptovoracin is to be used as an antibiotic for treatment of human infection it ought to be soluble in water. I therefore tested whether dried streptovoracin pellet would be soluble in aqueous solutions at pH 5 or pH 11. It turned out that streptovoracin was readily soluble in dH_2O adjusted to pH 11 with NCO_3. The solution was subsequently adjusted to pH 8 by the addition of small volumes of 1 M HCl. Streptovoracin dissolved in aqueous solution at pH8 was used to determine the MIC of the antibiotic against *S. pneumoniae* and *M. smegmatis.*

3.4 Activity of purified streptovoracin

3.4.1 Minimun inhibitory concentration (MIC)

After the pelleted were weight and dissolved in aqueous solutions at pH 8 the concentration could be calculated. This was used to find the MIC value of streptovoracin against *S. pneumoniae* RH425, one of the main goals for this thesis. The MIC was determined by subjecting cultures of the pneumococcus indicator strain to various concentrations of streptovoracin. The experiment was carried out in a microplate as described above. The results showed that the MIC value of streptovoracin against *S. pneumoniae* RH425 is in the range of 12 and 24 ug/ml (Fig. 16).

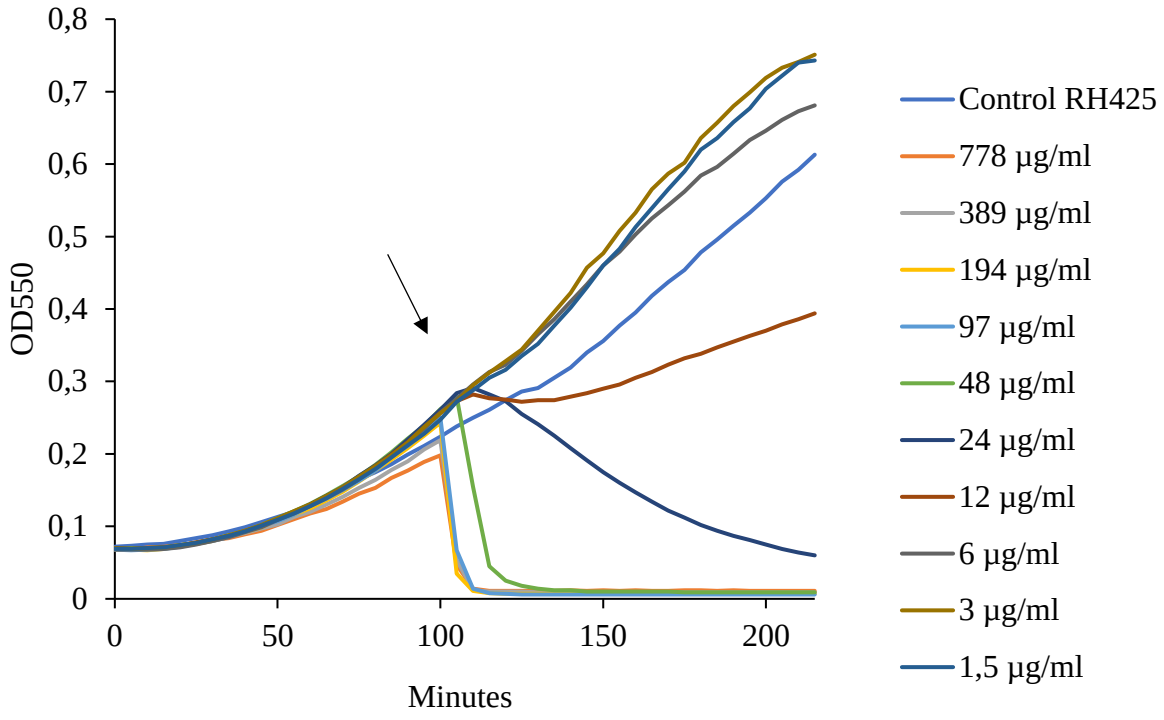


Figure 16. Determination of the MIC value of streptovoracin against *S. pneumoniae* RH425. Streptovoracin was added at the listed concentrations as indicated by the arrow.

Due to the fact that *M. smegmatis* is a close relative of *M. tuberculosis* and is sensitive to streptovoracin, it was of interest to determine the MIC value of streptovoracin against this species. Based on the results depicted in figure 17, the MIC value of streptovoracin against *M. smegmatis* is somewhere between 20-40 µg/ml, which is about the double of its MIC against *S. pneumoniae* RH425. However, determination of the MIC against *M. smegmatis* was more technically challenging than against *S. pneumoniae*. The bacteria grow much slower than *S. pneumoniae*, and similar to other *Mycobacteria* they grow in large clumps. The microplate reader in which the bacterial cultures grow, shakes the plates before each reading to avoid biased readings due to sedimentation of the bacteria. The result is smooth growth curves. However, in the case of *M. smegmatis* the growth curves became highly irregular due to extensive clumping that disturbs the absorbance reading in the Synergy H1. Nevertheless, in this particular experiment a concentration of streptovoracin of 41 µg/ml is clearly inhibitory, while a concentration of 20 µg/ml is not.

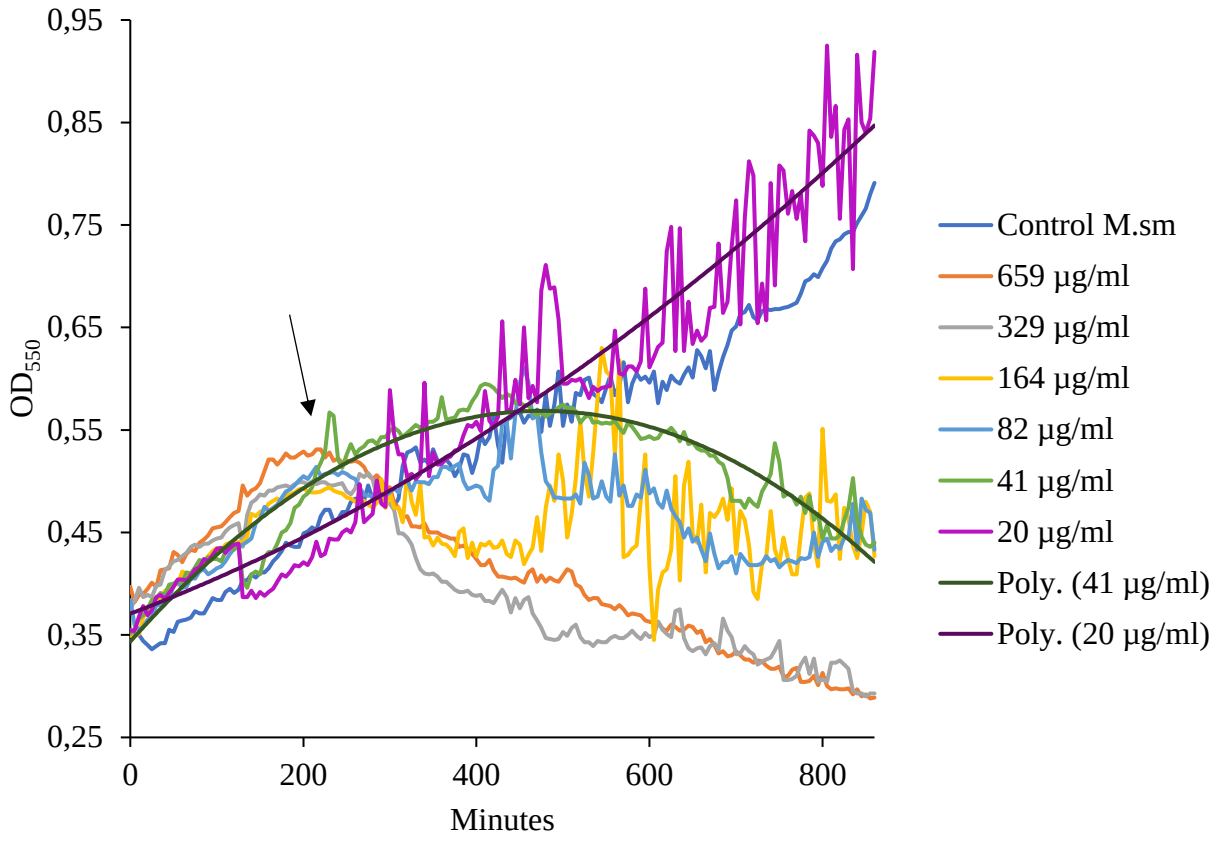


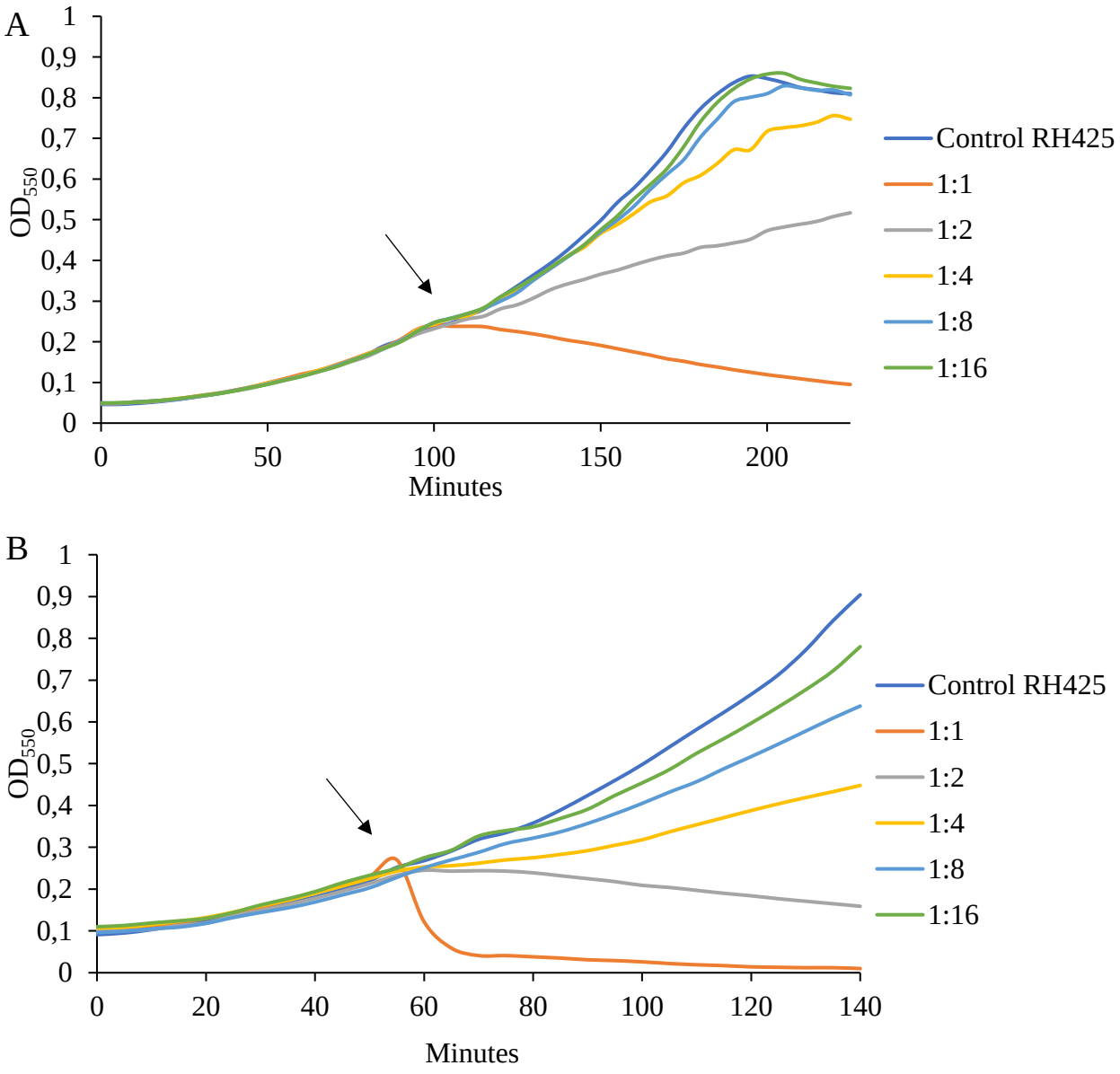
Figure 17. Determination of the MIC value of streptovoracin against *M. smegmatis*. Streptovoracin was added at the listed concentrations as indicated by the arrow. To highlight the difference of the cultures receiving 20 and 41µg/ml of streptovoracin, smooth artificial lines were drawn on top of the irregular growth curves.

3.4.2 Antimicrobial potency and production of peak 1 and 2.

As shown in Fig. 15, streptovoracin elutes as two peaks from the C18 reverse phase column. Peak 1 (molecular mass = 1148.753 Da) elutes first, followed by peak 2 (molecular mass = 1176.794 Da). It was obviously of great interest to determine if these two versions of streptovoracin is equally active against *S. pneumoniae*, or if they differ in potency. Hence, the two peaks were separated by HPLC, dried and dissolved dH_2O (pH 11) to obtain the same concentration. Estimations of the amounts of streptovoracin eluting in peak 1 and 2 were based on the calculated area of each peak in the chromatogram. Provided that streptovoracin 1 and streptovoracin 2 have the same mass extinction coefficient this method is fairly accurate. The results shown in Fig. 18 strongly indicate that streptovoracin 2 is the most active compound with about twice the activity of streptovoracin 1

per unit mass. The 1:2 dilution of streptovoracin 2 was able to completely inhibit the growth of *S. pneumoniae* RH425 (Fig. 18 B), whereas streptovoracin 1 was only able to inhibit growth at the 1:1 dilution (Fig. 18 A).

In addition, streptovoracin 1 and 2 were tested as a 1:1 mixture in case they are more active together than on their own. The MIC and growth inhibition pattern observed with the mixture were identical to that obtained with streptovoracin 1, demonstrating that the two versions of streptovoracin do not act synergistically (Fig. 18 C).



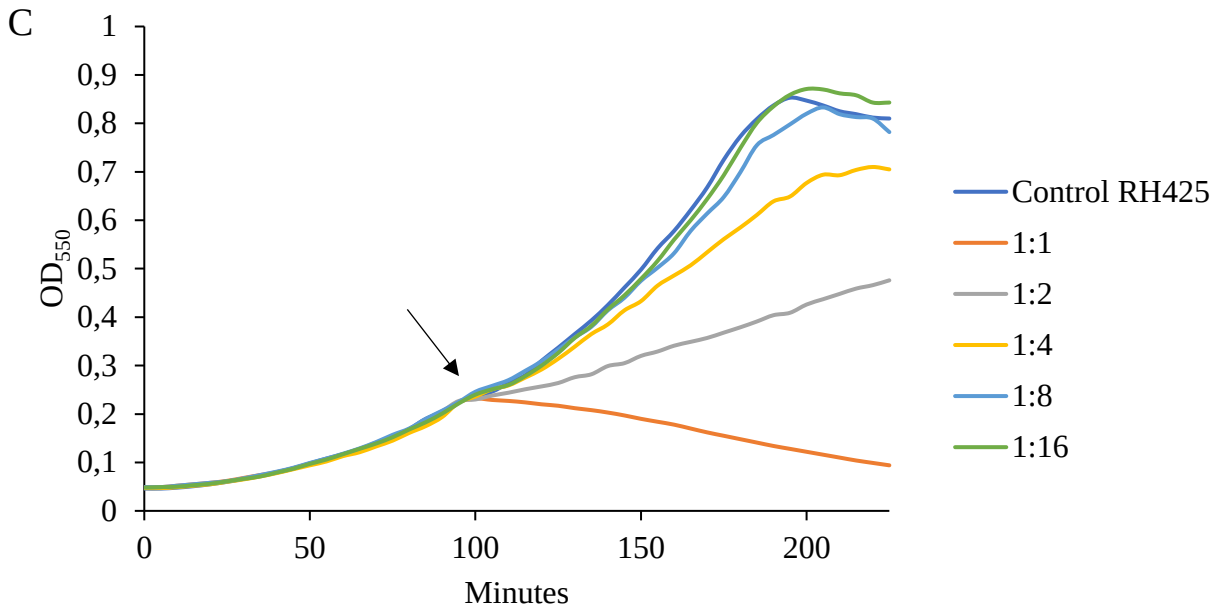


Figure 18. Comparison of the potency of streptovoracin 1 (panel A), streptovoracin 2 (panel B) and a 1:1 mixture of the two (panel C). Equal amounts of streptovoracin 1 (peak 1), streptovoracin 2 (peak 2) and a mixture of the two was tested in two-fold dilution steps for antimicrobial activity agains *S. pneumoniae* RH425 using the same experimental setup as described above.

Since streptovoracin 2 appears to be more potent than streptovoracin 1 it was of interest to follow the production of the two over time. Perhaps the ratio of the two peaks would vary depending on the age of the culture. The results depicted in figure 19 show that streptovoracin 2 is starting to be produced already at day one, a day earlier than streptovoracin 1. However, at day two, streptovoracin 1 production has surpassed production of streptovoracin 2, and is the dominant product for the next six days. This means that it is not possible to improve the yield of streptovoracin 2 compared to streptovoracin 1 by harvesting the culture at a time that maximizes streptovoracin 2 production.

Interestingly, in all of the HPLC chromatograms, a small peak (peak 3) eluted in the middle of peak 1 and peak 2 (Fig. 19). This could be a contaminant or a third version of streptovoracin produced in very small quantities. The fact that peak 3 increases in step with the increases in production of streptovoracin 1 and 2 during the eight-day period argues against that it is a

contaminant. In future experiments peak 3 should be collected and analysed by mass spectrometry. Moreover, by scaling up the production, it should be possible to determine whether the material eluting in peak 3 has antimicrobial activity.

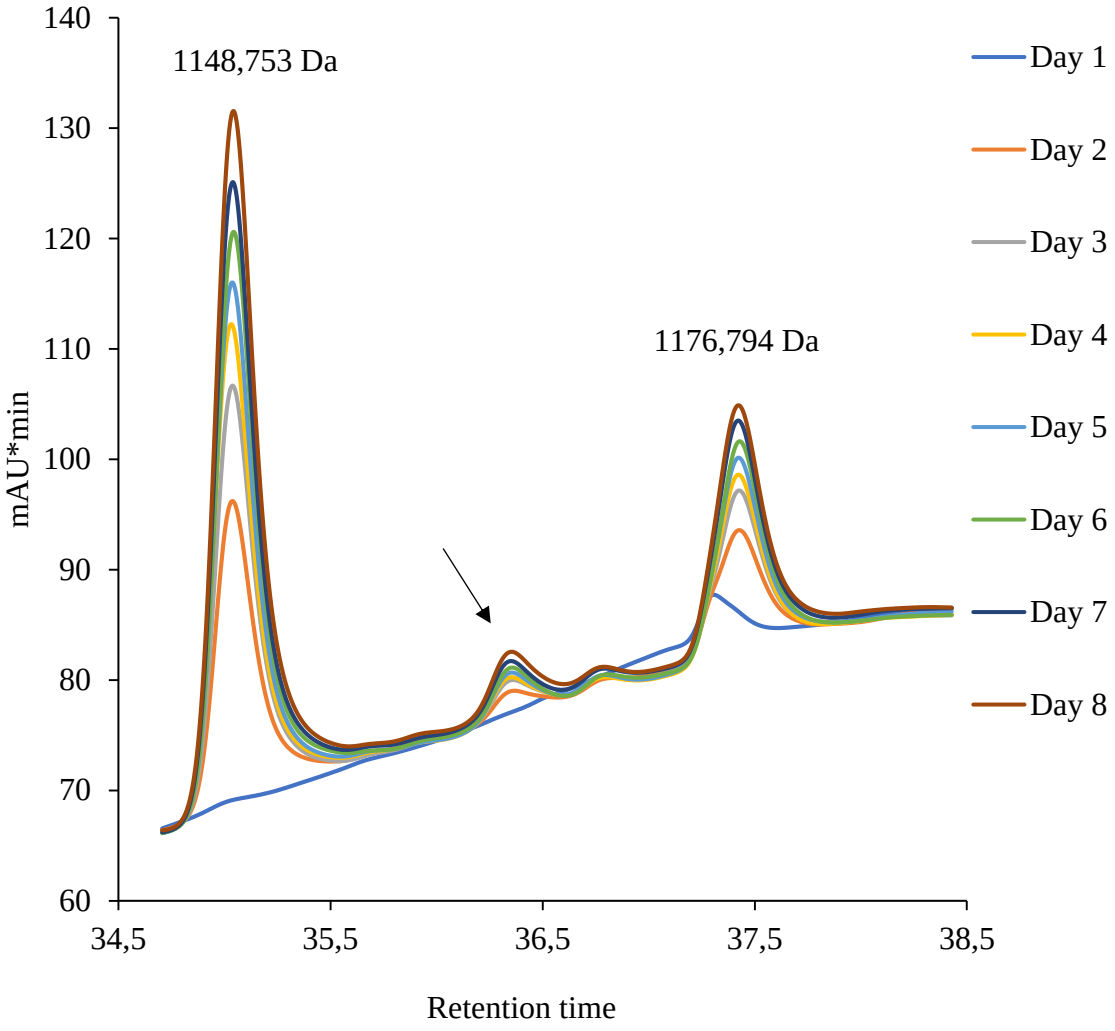


Figure 19. HPLC chromatograms showing the separation of peak 1 (1148. 753 Da) and peak 2 (1176.794 Da). The analysed samples were taken directly from cell-free medium one to eight days post inoculation with *Pseudomonas* strain nr. 33. A third peak (peak 3) is indicated by the arrow and might represent a third version of streptovoracin.

3.5 Investigations of the mechanism of action of streptovoracin.

3.5.1 Resistance against streptovoracin

To understand the mechanism of action of streptovoracin is a highly important but probably also a very difficult task. Nevertheless, I did some experiments to gain at least some insights into the mode of action of streptovoracin. When performing an overlay assay there will be a gradient of decreasing streptovoracin concentration towards the edge of the inhibition zone. This will generate a selection pressure gradient where some *S. pneumoniae* RH 425 target bacteria might mutate and thereby become more resistant to the antibiotic. Usually, such spontaneously resistant mutant colonies will appear close to the rim of the zone. The plan was to pick resistant mutants, grow them, and test them by a second screening to verify that they really are more resistant than their parental strain. If so, the resistant mutants and the parental strain would be genome sequenced to identify the mutation(s) giving rise to the resistant phenotype. If successful, the identification of mechanisms that give rise to resistance against streptovoracin would probably provide important information that can be used to elucidate the mechanism of action of streptovoracin. Unfortunately, no spontaneous mutants appeared in the inhibition zones of plates overlaid with *S. pneumoniae* RH 425. Overlay assay with other streptococcal species as indicator bacteria were also checked for spontaneously resistant mutants. An example is shown in figure 20, where a few colonies are observed inside the rim. In such cases some colonies were re-screened, but they turned out to be as sensitive as their parental strains.

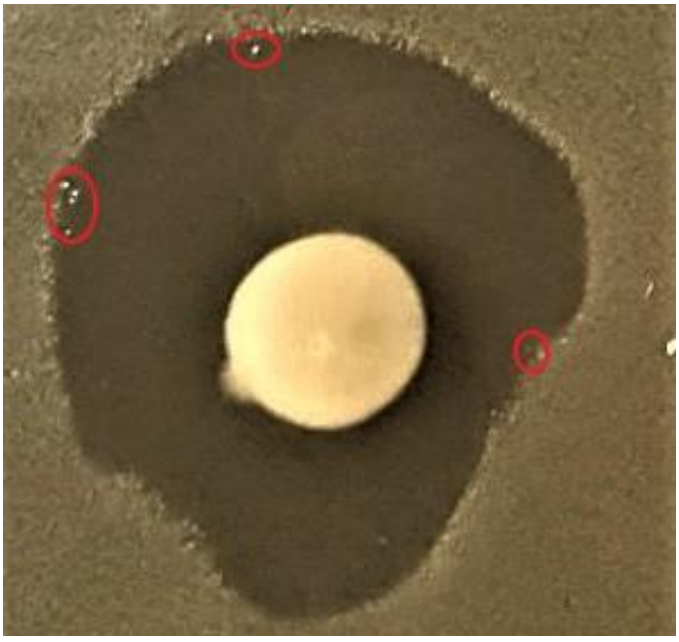


Figure 20. An overlay assay showing a large colony of the streptovoracin producer strain in the middle and an overlay of *Streptococcus equi subsp. zooepidemicus* (NCTC 4676). Colonies growing inside the inhibition zone are marked by circles.

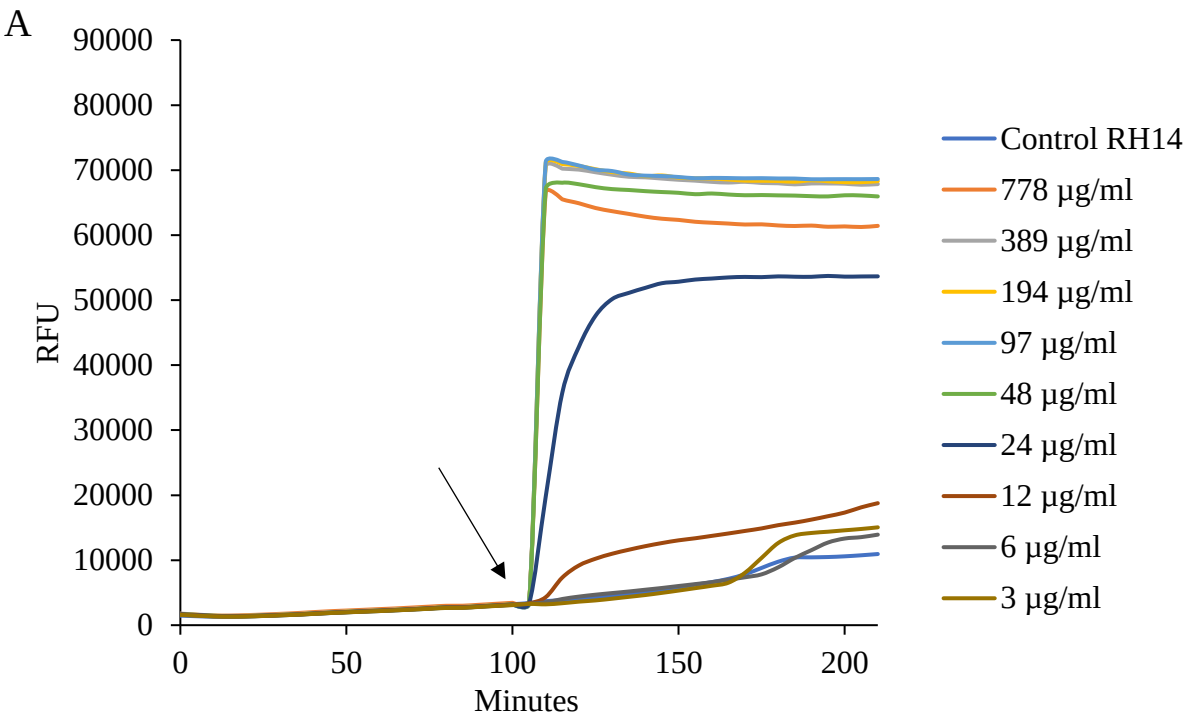
In a different approach to generate resistant mutants, genomic DNA from naturally resistant *Streptococcus* species was used to transform the RH425 strain. Two fully resistant species (*S. mutans* UA130 and *S. anginosus* NCTC 10713) and two low-sensitivity species (*S. sanguinis* SK1, and *S. gordonii* Challis) were chosen. Next, *S. pneumoniae* RH425 was induced to competence by addition of CSP and transformed with genomic DNA from each species. The transformation reactions were mixed with soft agar that was poured on agar plates on top of the streptovoracin producer strain as depicted in figure 20. The hope was that resistance determinants should be transferred from the donor strains to the RH425 strain via homologous recombination, and that mutant colonies should appear within the inhibition zone of the overlay assay. Unfortunately, no resistant mutant colonies were detected.

In a last attempt to produce mutants with increased resistance against streptovoracin, I decided to use the hypersensitive *S. mitis* SK564 strain. A very large inhibition zone (4.5 cm) was observed when this strain was tested in the overlay assay. Although all *S. mitis* strains examined were sensitive to streptovoracin the degree of sensitivity varied significantly (see appendix D). If the *S. mitis* strain could be induced to competence by its cognate CSP it would be possible to transform it with genomic DNA from another low-sensitivity *S. mitis* strain. By using the transformation reaction as indicator in an overlay assay as explained above mutant colonies with higher resistance might appear within the inhibition zone. Regrettably, I did not succeed in inducing the competent state in *S. mitis* SK564, even after addition of CSP. To check for competence, I transformed *S. mitis* SK564 with genomic DNA from *S. pneumoniae* containing a novobiocin marker. Since *S. mitis* and *S. pneumoniae* are very closely related species, it should be easy to introduce this marker into the genome of the *S. mitis* SK564 recipient strain. However, after seeding the transformation reaction on agar plates containing the appropriate concentration of novobiocin, no resistant colonies appeared.

In sum, none of my attempts to generate mutants with increased resistance against streptovoracin were successful, suggesting that it is difficult for pneumococci to develop resistance against this antibiotic.

3.5.2 Sytox green staining of DNA used to study membrane integrity

When testing the activity of streptovoracin against *S. pneumoniae* it was noticed that the culture lysed when subjected to streptovoracin concentrations well above the MIC. It was also discovered that this does not happen in a Δ*lytA* mutant. Hence, the antibiotic somehow triggers the action of the LytA autolysin. However, most of the streptococcal species tested does not produce LytA, so activation of LytA in *S. pneumoniae* must be considered a side effect of the real killing mechanism. Since cyclic lipopeptides in general interfere with the integrity of the membrane or membrane-associated functions, it was of interest to see if streptovoracin makes pneumococcal cells leaky. To avoid autolysis the Δ*lytA* mutant RH14 was used for this purpose. Sytox green is a compound that fluoresces strongly upon binding to DNA when excited at 485 nm. As it is unable to cross the cytoplasmic membrane, it can be used to monitor membrane damage that allows Sytox green to penetrate into the cytoplasm and bind to intracellular DNA. Cultures of the RH14 Δ*lytA* strain were grown in C medium containing 0.33 mM Sytox green at 37 ºC in a Synergy H1 Hybrid Reader. When reaching OD_{550} ~ 0.3, various amounts of streptovoracin were added. The results depicted in figure 21A clearly show that streptovoracin, at concentrations of 12 µg/ml and higher, causes damage to the cytoplasmic membrane that allows Sytox green to diffuse into the cytoplasm and bind to intracellular DNA.



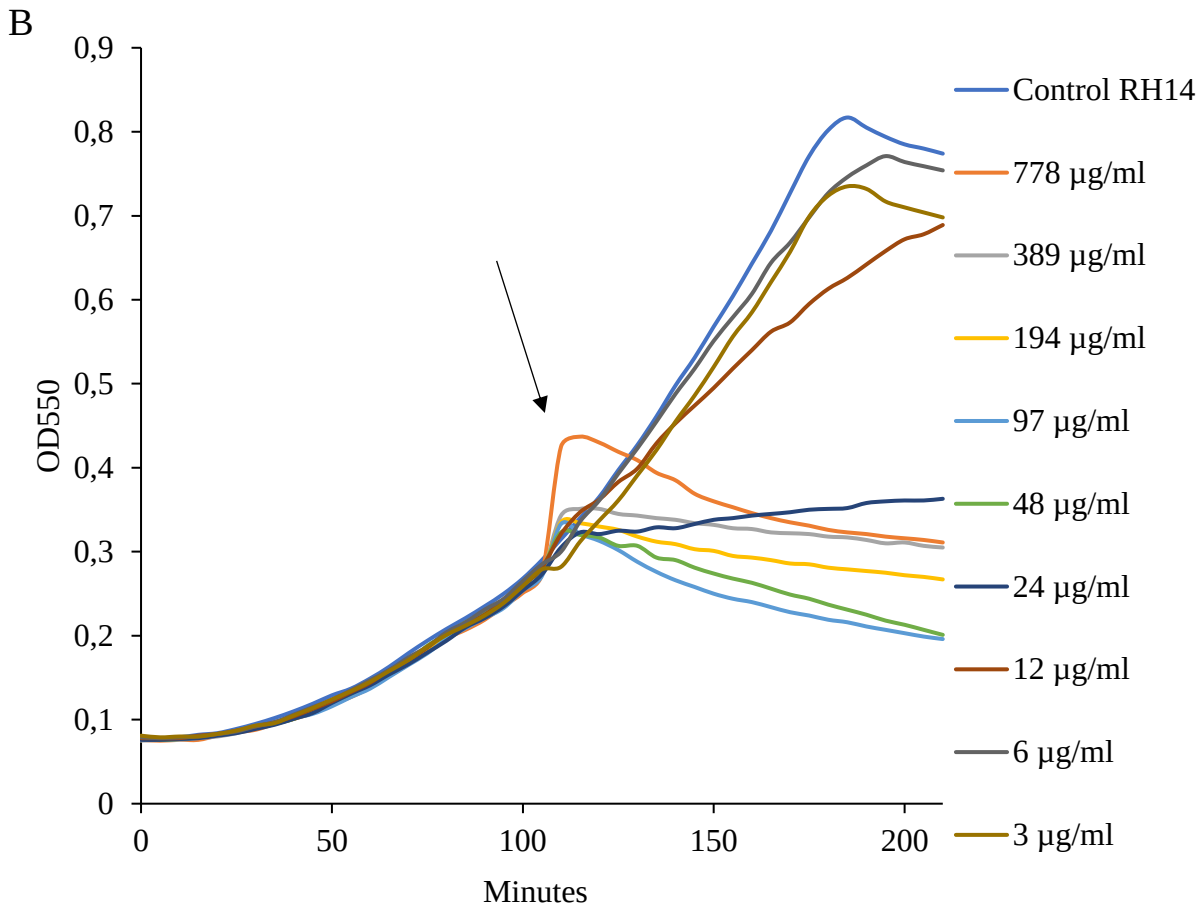


Figure 21. Detection of streptovoracin-induced damage to the cytoplasmic membrane in a Δ*lytA* pneumococcal strain (RH14). Streptovoracin was added to the cultures as indicated by the arrow at the concentrations listed on the right-hand side. Panel A shows fluorescence caused by the binding of Sytox green to intracellular DNA. The cytoplasmic membrane is normally impermeable to Sytox green, but when its integrity is damaged, Sytox green is able to penetrate, bind intracellular DNA and fluoresce. Panel B depicts the growth curves of the cultures whose fluorescence signals are measured in panel A. For every time point, optical density (OD550) as well as fluorescence are measure in each culture.

The growth curves depicted in figure 21 B are from the same experiment as the fluorescence curves shown in figure 21 A. As the RH14 Δ*lytA* strain was used in this experiment the pneumococcal cells do not lyse, but just stop growing at streptovoracin levels or above the MIC. Interestingly, the culture receiving 12 µg/ml of streptovoracin continues to grow at a slow rate (Fig. 21 B), even though its cytoplasmic membrane is somewhat leaky (Fig. 21 A).

3.5.3 Morphological changes in *S. pneumoniae* and *M. smegmatis* induced by streptovoracin

To investigate whether treatment with streptovoracin induces any distinguishable morphological changes I used the *S. pneumoniae* RH16 strain. This strain, a Δ*lytA* and Δ*lytB* mutant, was chosen to avoid autolysis when the pneumococcal cells are treated with high concentration of streptovoracin. After treatment with 778 µg/ml of streptovoracin for 4 hours, RH16 cells were spotted on glass slides and examined in a light microscope at 1000 x magnification. Many of the streptovoracin-treated cells lacked the characteristic elongated shape characteristic of pneumococci (Fig. 22 A and B). The cells appeared shorter and more rounded, and grew in somewhat longer chains than untreated cells. Similar to the experiment shown in figure 21, growth curves and DNA staining by Sytox green were carried out with RH16 cultures treated with different concentrations of streptovoracin (see appendix E).

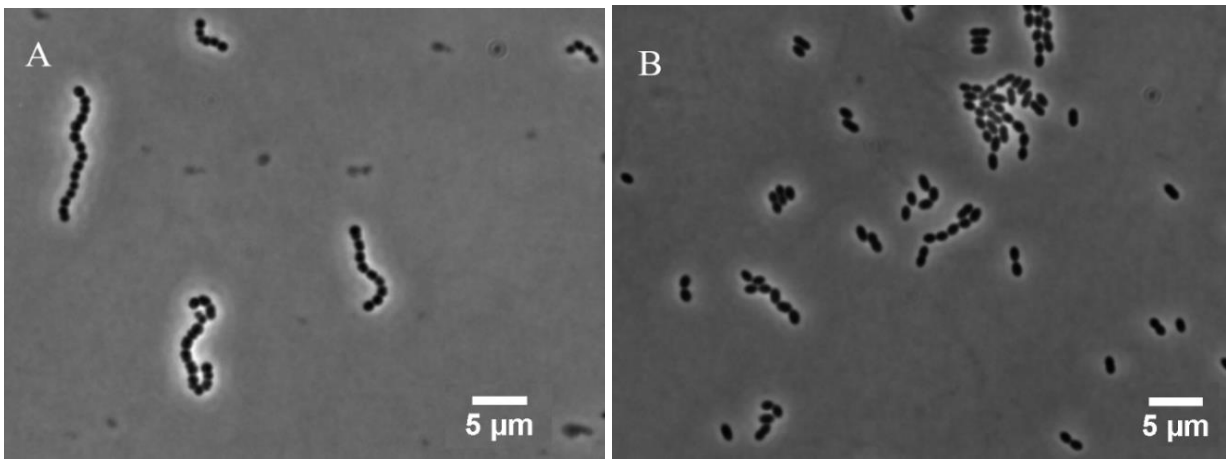


Figure 22. Pictures of *S. pneumoniae* strain RH16 treated with 778 µg/ml streptovoracin (panel A) or untreated (panel B). The pictures were taken with a phase-contrast objective at 1000 x magnification.

A culture of wild-type *M. smegmatis* cells was treated with 778 µg/ml of streptovoracin in the same way as the *S. pneumoniae* RH 16 strain. No cell remains resulting from autolysis was observed in the treated *M. smegmatis* culture. However, clear morphological alteration could be observed. The cells looked misshapen and uneven (Fig. 23 A). Interestingly, while the untreated cells appeared uniformly dark in the phase contrast objective (Fig. 23 B), the treated cells appeared greyish or almost transparent with dark spots (Fig 23 A).

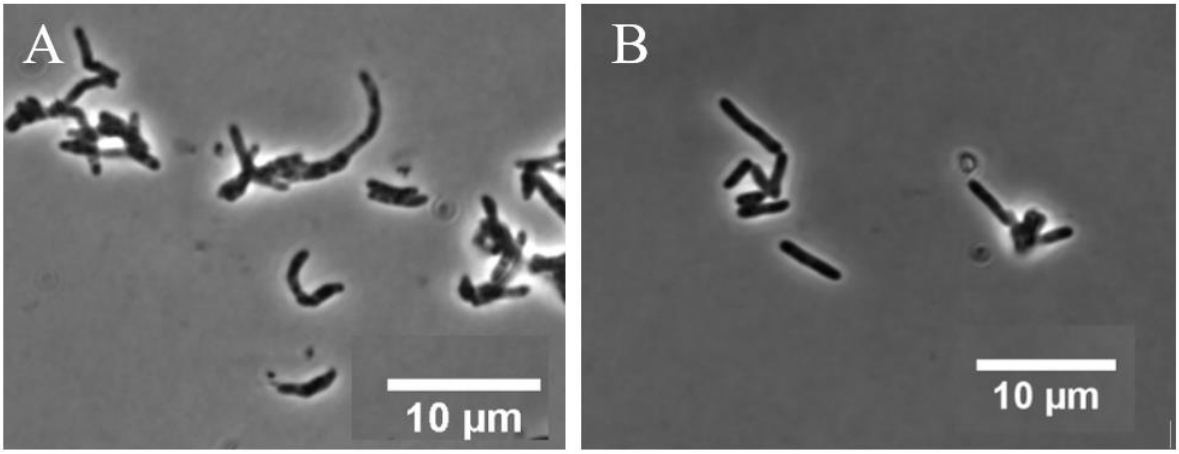


Figure 23. Pictures of *M. smegmatis* treated with 778 µg/ml streptovoracin (panel A) or untreated (panel B). The pictures were taken with a phase-contrast objective at 1000 x magnification

3.6 Pseudomonas isolate 33

Before I started on my thesis work the 16S rRNA gene of *Pseudomonas* isolate no. 33 had been sequenced. However, only the conserved part between variable part three and four was sequenced. This might be insufficient for identifying an unknown isolate to species level. Multilocus sequence typing (MLST) was therefore performed to further characterize the strain. Based on the 16S rRNA sequence data isolate no. 33 belongs to the *Pseudomonas fluorescens* complex and the MLST scheme designed for this group was therefore executed (https://pubmlst.org/pfluorescens/). The following seven housekeeping genes were amplified and sequenced: *glnS, gyrB, ileS, nuoD, recA, rpoB* and *rpoD.*

Sequencing of the *nuoD* was for some reason difficult and gave a very poor sequence that was impossible to read. This gene was also difficult to amplify and gave rise to a weak band. Because of this the *nuoD* gene was not used in the subsequent MLST analysis or BLAST searches.

The remaining gene sequences were used as queries in searches against the MLST database to identify the closest sequence homologs among the *glnS*, *gyrB*, *ileS*, *recA*, *rpoB* and *rpoD* sequences deposited in the database. None of the housekeeping gene sequences from isolate no. 33

were identical to their corresponding gene sequences in the MLST database. The best hit had 4 nucleotide mismatches while the most diverging sequence had 27 nucleotide mismatches to its closest homolog in the database. Furthermore, the best hits of the six genes were distributed randomly among the species registered in the database. Hence, no species in the database stood out as the best match to *Pseudomonas* isolate no. 33.

When the *glnS, gyrB, ileS, recA, rpoB* and *rpoD* gene sequences were blasted against the NCBI database the best hit for each gene was to a different *Pseudomonas* species or isolate. However, all of these species belonged to the *Pseudomonas fluorescens* group. This shows that the 16S rRNA gene sequence analysis correctly placed isolate no. 33 in this group. Interestingly, *P. brenneri*, which was selected as the most closely related species based on 16S rRNA sequence analysis, was not selected as the closest relative based on MLST analysis. None of the housekeeping genes matched particularly well with the corresponding *P. brenneri* genes. All nucleotide sequences and protein sequences can be seen in appendix F, where the sequences are compared to best the hit obtained in BLAST searches against the NCBI database. In sum, the preliminary data obtained in this study indicate that isolate no. 33 represents a new species within the genus *Pseudomonas*.

4. Discussion

4.1 The target range and antimicrobial properties of streptovoracin

Streptovoracin was originally thought to only target *S. pneumoniae* and closely related species in the genus *Streptococcus*, rendering it a precision drug. My results clearly show that streptovoracin is not a broad-spectrum antibiotic. However, the target range is wider than originally believed. The surprising finding that streptovoracin is active against a *Dietzia* sp. and *M. smegmatis*, two members of the phylum Actinobacteria, indicates that streptovoracin is able to kill bacteria by different mechanisms. Streptovoracin has a very limited target range within the phylum Firmicutes, suggesting that it targets a specific molecule embedded in the cytoplasmic membrane. It is unlikely that the same molecular structure is present in the cytoplasmic membranes of *Dietzia* sp. and *M. smegmatis*, especially since these bacteria have a dense outer layer of glycolipids (Sutcliffe, 2000). If it is true that streptovoracin kills streptococci and the two members of the phylum Actinobacteria by different mechanism, it is possible that it could be active also against some species of Gram-negative bacteria. I only tested *E. coli*, which was resistant, but more Gram-negative pathogens should be tested in the future.

In the phylum Firmicutes, only one strain of *E. faecalis* was found to be sensitive outside the genus *Streptococcus*. This suggests that streptovoracin is specific for streptococci. There was a tendency that species that are more distantly related to *S. pneumoniae* were less sensitive to streptovoracin. However, the tendency was not absolute. The important human pathogen *S. pyogenes*, for instance, was quite sensitive. There was also a large variation in sensitivity among strains belonging to the same streptococcal species, particularly in *S. mitis* where the diameters of the inhibition zones ranged from 4.5 cm (*S. mitis* SK564) to 1.1cm (*S. mitis* SK24 and SK102). The SK564 strain had the largest inhibition zone of all species and strains tested, and must be considered to be hypersensitive. Although most members of the genus *Streptococcus* were sensitive to streptovoracin, all *S. mutans* strains tested plus *S. criceti* were resistant. Together, the screening results clearly show that streptovoracin has specificity. In order to kill with high precision streptovoracin must recognize a specific target. Hence, it is likely that it targets a specific docking molecule in the membranes of streptococci, and that this interaction is necessary in order for

streptovoracin to insert its lipid acyl chain into the membrane and make it leaky. The observed variation in sensitivity could be due to variations in the amounts of docking molecule. Alternatively, if the docking molecule is a protein, it could be due to variations in structure and/or amino acid composition. Identification of this hypothetical docking molecule should be the focus of future studies on streptovoracin.

Even though streptovoracin is not active against *S. mutans*, which causes tooth decay, it is active against three very important streptococcal pathogens, namely *S. pneumoniae*, *S. pyogenes* and *S. agalactiae*. These bacteria cause a range of diseases ranging from life threatening diseases such as meningitis, pneumonia to milder diseases such as sinusitis, otitis media and skin infections (van der Poll & Opal, 2009). Hence, if streptovoracin turns out to be too toxic to be used internally, it might be used externally to treat skin infections. If, on the other hand, streptovoracin can be used intravenously to treat blood and lung infections, it was very positive finding that the presence of a polysaccharide capsule in pneumococci did not significantly reduce the effect of streptovoracin (appendix D). This was clearly demonstrated by the *S. pneumoniae* strain A66, which was screened both as the encapsulated wildtype and capsule-less mutant. However, when grown on blood agar, the *S. pneumoniae* A66 (wild type) and the TIGR4 strain both displayed somewhat smaller inhibition zones, indicating that the capsule provides some protection against streptovoracin. Presumably the presence of blood stimulates capsule production, and a thicker polysaccharide layer will probably act as a barrier that reduces the amounts of streptovoracin that reach the cytoplasmic membrane.

It was very interesting that the bacterium *M. smegmatis* was sensitive to streptovoracin. *M. smegmatis* is used as a substitute for *M. tuberculosis* and *M. bovis* when screening for new drugs against tuberculosis (Geudens et al., 2017; Jahanshah et al., 2019; Reder-Christ et al., 2012). *M. smegmatis* is considered safe for healthy humans, and does not require a biosafety level 3 environment like its highly pathogenic relatives. Besides, it grows much more rapidly than *M. tuberculosis* and *M. bovis*, and is therefore much easier to work with. The fact that streptovoracin is active against *M. smegmatis* makes it almost certain that it will also be active against *M. tuberculosis* and *M. bovis.* In 2017, tuberculosis caused about 1.3 million deaths among HIV-negative people and 0.3 million deaths among HIV-positive people, worldwide. Moreover, it has been estimated that in 2017, approximately 10 million people developed tuberculosis globally

(Global Tuberculosis Report, 2018). Standard treatment for tuberculosis disease involves 4 different antibiotics: isoniazid, rifampicin, pyrazinamide, and either ethambutol or streptomycin. The standard six-month course of treatment consists of two phases: the intensive phase (the first two months) and the continuation phase (the last four months). There may be severe side effects such as liver damage, joint pain, peripheral neuropathy (nerve damage), visual impairment and more commonly nausea and abdominal pain. In addition to all this, drug resistant forms of tuberculosis have become a big problem. Each year about 0.6 million people become sick with rifampicin-resistant tuberculosis (Furin et al., 2019 ; Nguyen et al., 2019). Due this this precarious situation, every new drug that is active against *M. tuberculosis* and *M. bovis* is of high interest.

It was a disappointment that daptomycin could not be used to treat pneumococcal pneumonia. Daptomycin is inactivated by the presence of phosphatidylglycerol, which together with dipalmitoylphosphatidyl chloline serve as lung surfactants. Phosphatidylglycerol is also found in the cytoplasmic membranes of daptomycin-sensitive Gram-positive bacteria, where it probably functions as a receptor for daptomycin. However, although daptomycin is inactivated by phosphatidylglycerol in lung tissue, other cyclic lipopeptide antibiotics are not affected. Examples of the latter are the calcium dependent cyclic lipopeptide MX-2401 and the malacidins (Dugourd et al., 2011; Hover et al., 2018). Since the inhibition spectrum of streptovoracin is completely different from that of daptomycin, it is unlikely that it will be inactivated by lung surfactants. However, to answer this question *in vivo* experiments in an animal model will have to be carried out.

4.2 Regulation of streptovoracin synthesis

Bacteria belonging to the genus *Pseudomonas* are in generally obligate aerobes. However, some strains can utilize NO_3 instead of O_2 as a terminal electron acceptor, and at least some strains can grow by fermentation under virtually oxygen free conditions (Schobert & Jahn, 2010). The *Pseudomonas* strain producing streptovoracin was able to grow under anaerobic conditions or at least with very little oxygen. Interestingly, no streptovoracin was produced during anaerobic or microaerobic growth. This indicates that oxygen might be involved in the regulation of streptovoracin production. It has been reported previously that oxygen levels can influence the production of a lipopeptide. Guez and co-workers (2008) showed that production of mycosubtilin,

a lipopeptide synthesized by *Bacillus subtilis* ATCC 6633, increases dramatically under oxygen limitation (Guez et al., 2008).

It is well documented that cyclic lipopeptides is synthesized by large multimodular non-ribosomal peptide synthetases (NRPS), but much less is known about the signal transduction pathways and networks regulating their synthesis (Song et al., 2014). The GacS/GacA two-component regulatory system is a central regulator in *Pseudomonas* spp., which controls the production of secondary metabolites. For some *Pseudomonas* strains it has been reported that mutants disrupted in either one of the two genes are deficient in production of their respective cyclic lipopeptides (Hassan et al., 2010; Raaijmakers et al., 2006). Interestingly, it has been shown that quorum-sensing is involved in the regulation of cyclic lipopeptide synthesis in some species and strains of pseudomonads (Dubern et al., 2006; Hennessy et al., 2017). Since streptovoracin production first became detectable two days post inoculation and increased until day six, i.e. during stationary phase, it might well be that the antibiotic is cell density regulated by a quorum sensing mechanism. Judging from its molecular weight streptovoracin probably belongs to a group of cyclic lipopeptides called viscosins. It has been documented in the literature that the synthesis of viscosins in some cases is quorum-sensing regulated with N-Acyl homoserine lactones as the signalling molecule (Cui et al., 2005; de Bruijn & Raaijmakers, 2009). The possibility that streptovoracin is regulated by an N-Acyl homoserine lactone should be further investigated. If true, the identification and synthesis of the particular homoserine lactone involved would be very useful for controlling and optimizing the production streptovoracin.

Pseudomonas isolate no. 33 grew well in synthetic M9 medium, but it did not produce detectable levels of streptovoracin. This suggests that M9 medium is lacking some specific component(s) that are required to induce production of the antibiotic, or that streptovoracin production just requires a rich medium. Growing isolate no. 33 in the presence of glass wool as a substrate for biofilm formation seemed to increase the production of streptovoracin. Biofilms present on the surfaces of stones and plant material in the littoral zone is presumably the natural habitat of *Pseudomonas* isolate no. 33. Such habitats must be very competitive, and it is therefore likely that isolate no. 33 produce streptovoracin as a weapon to outcompete other bacteria sharing the same niche. Hence, it is not unlikely that growth in biofilms stimulate streptovoracin production. Besides, cell-cell communication by diffusible substances such as N-Acyl homoserine

lactones work more efficiently at lower concentrations in biofilm than in liquid culture. However, further studies are needed to settle these questions.

4.3 Purification of streptovoracin

The early attempts at purifying streptovoracin involved a precipitation step with ammonium sulphate. It was mentioned specifically in a paper by Inès et. al. (2015) that this method has disadvantages and no particular advantages. The precipitation step must be followed by dialysis to remove the high concentration of salt in the precipitate. Dialysis is time-consuming and complicates the purification process if streptovoracin production is to be scaled up. Furthermore, ammonium sulphate will precipitate all proteins in the sample. Considering that a rich medium was used to cultivate isolate no. 33 a lot of contaminants will co-precipitated with streptovoracin and complicate downstream purification steps (Ines & Dhouha, 2015; Rangarajan & Clarke, 2016). In addition, ammonium sulphate precipitation was not particular efficient. My results show that approximately 40% of the streptovoracin present in the growth medium was lost to the supernatant during the centrifugation step succeeding precipitation with ammonium sulphate.

Acid precipitation can be used as an alternative to ammonium precipitation. However, since this would involve working with large quantities of strong acids it was avoided for safety reasons. Instead, the non-ionic amberlite resin XAD 16N was used to adsorb streptovoracin directly from the growth medium. This resin binds hydrophobic and polar molecules through hydrophobic interactions. Hence, it should be suitable for the purification of streptovoracin, since cyclic lipopeptides are amphiphilic compounds. This was confirmed by searches in the literature, which showed that amberlite resin has been used successfully in the purification of other cyclic lipopeptides (Huang et al., 2013). My results showed that the amberlite XAD 16N resin was very effective in binding streptovoracin directly from the cell-free growth medium with a loss of only a few percents. However, since the overall yield from all the amberlite purification steps adds up to >100%, this calculation is not very accurate. Nonetheless, direct extraction with amberlite XAD 16N resin represented a big improvement compared to ammonium sulphate precipitation where only ~60% of the antibiotic was recovered from the precipitate. A disadvantage of the amberlite purification step was that it required the use of a large volume of methanol or other organic solvents for desorption. To proceed to the next purification step methanol had to be removed by evaporation.

This is not a big problem when purifying small amounts of streptovoracin, but special laboratory equipment would be needed for evaporation of large volumes of organic solvents. Moreover, since pneumococci is inhibited by the methanol, desorption with methanol made it more difficult to use a biological assay to directly estimate the amounts of streptovoracin present in the eluate.

Following the amberlite purification step, the methanol used to elute streptovoracin was evaporated in a Speed-Vac centrifuge, and the resulting pellets were dissolved in a small volume of 50% acetonitrile and subjected to reversed-phase HPLC. The final purification protocol in which 30% methanol was added to the growth medium before it was mixed with amberlite, and where 3 washing steps were performed before streptovoracin was eluted with 100% methanol, was superior to earlier protocols. When this method was used, much less contaminating material was present in the sample loaded onto the reversed phase column compared to previous protocols. The HPLC purification step resulted in highly purified streptovoracin 1 and streptovoracin 2. This was demonstrated by mass spectrometry before I started my thesis work. However, the HPLC step has one large drawback, namely low capacity. HPLC is primarily an analytical tool and is not intended for large-scale purification. It should therefore be tested whether ion exchange chromatography might replace reversed-phase HPLC in the future, as a larger volume and much larger amounts of material can be loaded onto an ion-exchange column.

After eluting from the reversed-phase C18 column, streptovoracin 1 and 2 were dissolved in around 90% acetonitrile. The solvent was later removed by evaporation *in vacuo*. For some reason, the resulting pellet was not soluble in dH_2O. This might be due to streptovoracin's amphiphilic nature. Streptovoracin was soluble in the growth medium, where it probably formed micelles with a hydrophilic surface and a hydrophobic core. However, after it had been dissolved in an organic solvent it was virtually insoluble in dH_2O. A possible explanation is that the micelles invert when the antibiotic is dissolved in an organic solvent and form spheres with a hydrophobic surface and a hydrophilic core. Due to their hydrophobic exterior the micelles would have been difficult to dissolve in water. In an attempt to ionize streptovoracin we lowered and raised the pH of the aqueous solution. It turned out that streptovoracin pellets obtained after evaporation of acetonitrile could be dissolved in dH_2O adjusted to pH 11 with $NaCO_3$. Presumably, this is due to the ionization of one or more carboxyl groups, and indicates that streptovoracin contains glutamate and/or aspartate. After it had been dissolved at pH 11, streptovoracin stayed in solution even when

the pH was reduced to pH 8. Later on, I learned that Chen and Juang (2008) had used the same "trick" to get surfactin into aqueous solution (Chen & Juang, 2008).

4.4 Activity of purified streptovoracin

Purified streptovoracin was used to estimate the MICs for both *S. pneumoniae* and *M. smegmatis*. Their MICs were estimated to be in the range of 10-20 µg/ml for *S. pneumoniae* and 20-40 µg/ml for *M. smegmatis*. I suspect that my MIC-estimates for streptovoracin was too high since the pellets looked glossy and did not appear fully dried after the acetonitrile had been evaporated *in vacuo*. Moreover, it was difficult to avoid that some residue from the pellet stuck to the pipette tip when the pellet was resuspended in dH_2O at pH 11. To obtain the exact MIC, larger amounts of streptovoracin must be produced to get a more accurate weight measurement. In addition, smaller concentration intervals should be used. After it was discovered that streptovoracin is active against *M. smegmatis*, searches in the literature revealed that it was already known that members of one group of cyclic lipopeptides, the viscosins, are active against *M. smegmatis*. Together with the molecular mass of streptovoracin, which is close to that of known viscosins but not identical, the fact that only viscosins among the cyclic-lipopeptides are known to be active against mycobacteria strongly indicates that streptovoracin belongs to the viscosin group (Geudens et al., 2017). In the literature, the MICs reported for of various viscosins range from 8 – 16 µg/ml against *M. smegmatis*, while they range from 1-32 µg/ml against *S. pneumoniae* (Geudens et al., 2017). On the background of these data, my estimates of the MICs of streptovoracin against *S. pneumoniae* and *M. smegmatis* seem reasonable. However, as stated above, the MICs should be re-calculated when larger amounts of pure streptovoracin have been produced.

Streptovoracin 1 and 2 have very similar properties and differs only by 28 Da, which corresponds to the mass of two carbon and four hydrogen atoms. This can be explained by assuming that the acyl chain of streptovoracin 2 is two carbon atoms longer than that of streptovoracin 1. As the acyl chains of cyclic-lipopeptides are known to vary in length, this is a reasonable assumption. Interestingly, a small peak designated peak 3, eluted between peak 1 and peak 2. The size of peak 3 increased in step with the other two in an experiment measuring streptovoracin production over an eight-day period. If peak 3 is solely a contaminant, this should not happen. Hence it is possible that peak 3 represents a third variant of streptovoracin, possibly with an acyl chain that is only one

carbon and two hydrogen atoms longer than streptovoracin 1. Streptovoracin 2 was according to the microtiter plate screening, the most active compound. This is in accordance with the findings of Falanga et al. (2017), who observed that a slightly longer acyl chain could lower the MIC for cyclic lipopeptides (Falanga et al., 2017). However, the finding that streptovoracin 2 is more active than streptovoracin 1 against *S. pneumoniae* needs to be confirmed, as the experiment comparing their activities was only performed once. Moreover, calculations of their concentrations were based on the areas of peak 1 and 2 in the chromatogram. The calculations showed that peak 1 contained approximately five times more material than peak 2. To perform the calculation, the best fitting curves corresponding to peak 1 and 2 had to be drawn manually. As it was difficult to do this 100% accurately, small calculation errors could have been made.

4.5 Mode of action of streptovoracin

One of the goals of this thesis was to gain information about the mode of action of streptovoracin against *S. pneumoniae*. The initial strategy used was to screen for spontaneous mutants that displayed increased resistance against the antibiotic. As I did not have access to large amounts of pure streptovoracin, the screening for resistant mutants was based on the overlay assay. The plan was to sequence the whole genome of spontaneously resistant mutants and to compare their genome sequences to that of their sensitive parental strain. By identifying the mutations that gave rise to the resistant phenotype it might be possible to deduce how streptovoracin kills susceptible pneumococci. Unfortunately, no colonies of resistant mutants appeared inside the rim of the inhibition zone even after prolonged incubation of the plates. A few colonies were picked along the edge of the inhibition zone, but they did not display smaller inhibition zones when re-screened in an overlay assay. Although the failure to generate spontaneously resistant mutants was disappointing in the respect that this approach did not lead to a better understanding of the mode of action of streptovoracin, it was also encouraging as it demonstrated that resistance will not emerge easily if streptovoracin should be approved for clinical use sometimes in the future. In the case of daptomycin, which already is in clinical use, resistance is not easily induced (Hover et al., 2018; Kleijn & Martin, 2018). However, it appears sporadically during treatment, most often when patients have been previously treated with vancomycin (Kleijn & Martin, 2018). Presumably, a more prolonged period of selection with sublethal doses of streptovoracin is required to generate

resistant mutants. Yet, even this approach might fail, as it was tested by Hover et. al. (2018) on malacidins without success.

Somewhat surprisingly, *S. mutans* was completely resistant against streptovoracin. If streptovoracin recognizes a membrane protein or another membrane component, it appears that this docking molecule is substantially altered or lacking in *S. mutans*. It follows from this that the genetic trait responsible for streptovoracin resistance in *S. mutans* might be transferable to *S. pneumoniae*. Hence, I purified genomic DNA from two fully resistant species (*S. mutans* UA130 and *S. anginosus* NCTC 10713) and two low-sensitivity species (*S. sanguinis* SK1, and *S. gordonii* Challis), and used this DNA to transform *S. pneumoniae* through the mechanism called natural genetic transformation. During natural transformation externally added DNA is taken up and incorporated into the genome of competent pneumococci by homologous recombination (Straume et al., 2015). In theory, a sensitive pneumococcus might acquire the trait associated with resistance and become sensitive or even fully resistant. Transformed pneumococcal cultures were used as indicator bacteria in overlay assays where a colony of the streptovoracin producing *Pseudomonas* strain had been growing and producing the drug for four days. No colonies appeared inside the rim of the inhibition zone, demonstrating that I had not succeed in transferring the genetic determinant(s) associated with resistance in the four species described above. A possible explanation for this failure is that the genetic distance between *S. pneumoniae* and the four donor strains is too large. A large genetic distance will reduce the efficiency of homologous recombination and might also give rise to non-functional genes and gene product. In an attempt to overcome such problems, the plan was to transform the hypersensitive strain *S. mitis* SK564 with DNA from low-sensitivity strains belonging to the same species. However, even though the native CSP of *S. mitis* SK564 was used, it was not possible to induce the competent state in this strain.

To test whether streptovoracin damages the cytoplasmic membrane of *S. pneumoniae*, sytox green was added during cultivation of wild-type, RH14 (Δ*lytA*) and RH16 (Δ*lytA*, Δ*lytB*) strains. Sytox green, which fluoresce strongly upon binding DNA, cannot pass through an intact cytoplasmic membrane. However, if the integrity of the membrane is disturbed or if pores are created, sytox green should be able to diffuse into the cytoplasm, bind DNA and fluoresce. A strong fluorescence signal was observed when the wild-type strain was subjected to streptovoracin(Håvarstein, 2018). In addition, the culture lysed rapidly. The latter effect was due

to activation of the autolysin *lytA*, since no lysis was observed when RH14 and RH16 cultures were treated with corresponding concentrations of streptovoracin. All cultures, however, produced a strong fluorescence signal when treated with streptovoracin. These findings strongly indicate that streptovoracin kills *S. pneumoniae* and other susceptible streptococci by making their membranes leaky. This finding represents an important step towards the identification of the mode of action of streptovoracin against *S. pneumoniae*. The missing piece in the puzzle, and the hardest to find, is the receptor or docking molecule used by streptovoracin to penetrate inti the cytoplasmic membrane.

As mentioned previously, it is unlikely that streptovoracin kills *S. pneumoniae* and *M. smegmatis* by the same mechanism. The main reason for this is the large phylogenetic distance between the two species. It is almost certain that streptovoracin needs a docking molecule to work. The chances that this molecule should be conserved in mycobacteria is very low, but cannot be ruled out. In contrast to pneumococci, mycobacteria have a dense glycolipid layer outside the peptidoglycan sacculus. This layer protects mycobacteria against toxins, including antibiotics. However, the glycolipid layer also contains porins, and it is possible that streptovoracin can diffuse through these pores and reach the cytoplasmic membrane. Another possibility, which I favour, is that streptovoracin interferes with the synthesis of the glycolipid layer. *M. smegmatis* bacteria subjected to streptovoracin changed their appearance when imaged by phase contrast microscopy. Dark spots appeared within each bacterium, while the rest of the bacterium looked almost translucent. Nothing like this was seen when streptovoracin-treated *S. pneumoniae* was examined by phase contrast microscopy.

4.6 Identification of *Pseudomonas* isolate no. 33

Before I started my thesis, the 16S rRNA gene of isolate no. 33 had been sequenced. BLAST searches with this sequence against the NCBI database showed that the streptovoracin producer strain belongs to the *P. fluorescence* group within the genus *Pseudomonas*. Furthermore, the closest hit was a species named *P. brenneri*. However, the resolution obtained with a 16S rRNA gene sequence is usually not good enough to determine whether an unknown isolate should be assigned to an established species or if it should be given status as a new species. In an attempt to distinguish between these alternatives, I used multilocus sequence typing, MLST, to further characterize

isolate no. 33. MLST is often used in epidemiological studies for characterizing isolates of bacterial species using the sequences of internal fragments of (usually) seven housekeeping genes. All unique sequences for a given housekeeping gene are assigned an allele number in the order of discovery. For a given isolate, the seven genes are combined into an allelic profile and assigned a sequence type (ST). Each isolate of a species is therefore unambiguously characterized by a series of seven integers which correspond to the alleles at the seven housekeeping loci (Maiden et al., 1998). I only succeeded in obtaining the sequence of six of the seven housekeeping genes used for MLST in the *P. fluorescence* group. Nevertheless, the allelic profile obtained with these genes did not match any previously registered profiles. Furthermore, when the six housekeeping gene sequences were blasted individually against the NCBI database, the best hit was not *P. brenneri* but various other *Pseudomonas* species from the *P. fluorescence* group. Hence, the MLST data indicate that isolate no. 33 might be a new species. However, the fact that the 16S rRNA gene sequence of isolate no. 33 is more than 97% identical to the 16S rRNA gene sequence of *P. brenneri* points to the opposite conclusion; namely that they should be classified as the same species (Håvarstein, 2018). If I would have had more time, I would have concatenated the sequences of the six housekeeping genes and the 16S rRNA gene to form a single sequence, and used this sequence to perform a phylogenetic analysis. Phylogenetic analyses are commonly used to determine whether an unknown isolate belongs to a particular species. Nevertheless, the gold standard and the most recommended method to discriminate between bacterial species is still DNA-DNA-hybridization. The problem with this method is that it is laborious, requires special laboratory equipment and relies on specialized expertise (Vasquez-Ponce et al., 2018). It is therefore only performed by a few laboratories worldwide.

References

Baida, N., Yazourh, A., Singer, E., & Izard, D. (2001). Pseudomonas brenneri sp nov., a new species isolated from natural mineral waters. *Research in Microbiology, 152*(5), 493-502. doi:Doi 10.1016/S0923-2508(01)01223-2

Butler, M. S., Blaskovich, M. A. T., Owen, J. G., & Cooper, M. A. (2016). Old dogs and new tricks in antimicrobial discovery. *Current Opinion in Microbiology, 33*, 25-34. doi:10.1016/j.mib.2016.05.011

Chen, H. L., & Juang, R. S. (2008). Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction. *Biochemical Engineering Journal, 38*(1), 39-46. doi:10.1016/j.bej.2007.06.003

Cherazard, R., Epstein, M., Doan, T. L., Salim, T., Bharti, S., & Smith, M. A. (2017). Antimicrobial Resistant Streptococcus pneumoniae: Prevalence, Mechanisms, and Clinical Implications. *American Journal of Therapeutics, 24*(3), E361-E369. doi:Doi 10.1097/Mjt.0000000000000551

Cochrane, S. A., & Vederas, J. C. (2016). Lipopeptides from Bacillus and Paenibacillus spp.: A Gold Mine of Antibiotic Candidates. *Medicinal Research Reviews, 36*(1), 4-31. doi:10.1002/med.21321

Collaborators, G. L., Troeger, C., Forouzanfar, M., Rao, P. C., Khalil, I., Brown, A., . . . Mokdad, A. H. (2017). Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infectious Diseases, 17*(11), 1133-1161. doi:10.1016/S1473-3099(17)30396-1

Craig, W. A., Andes, D. R., & Stamstad, T. (2010). In Vivo Pharmacodynamics of New Lipopeptide MX-2401. *Antimicrobial Agents and Chemotherapy, 54*(12), 5092-5098. doi:10.1128/Aac.00238-10

Cui, X., Harling, R., Mutch, P., & Darling, D. (2005). Identification of N-3-hydroxyoctanoyl-homoserine lactone production in Pseudomonas fluorescens 5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing. *European Journal of Plant Pathology, 111*(4), 297-308. doi:10.1007/s10658-004-4171-z

de Bruijn, I., & Raaijmakers, J. M. (2009). Diversity and Functional Analysis of LuxR-Type Transcriptional Regulators of Cyclic Lipopeptide Biosynthesis in Pseudomonas fluorescens. *Applied and Environmental Microbiology, 75*(14), 4753-4761. doi:10.1128/Aem.00575-09

Dubern, J. F., Lugtenberg, B. J. J., & Bloemberg, G. V. (2006). The ppuI-rsaL-ppuR quorum-sensing system regulates biofilm formation of Pseudomonas putida PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. *Journal of Bacteriology, 188*(8), 2898-2906. doi:10.1128/Jb.188.8.2898-2906.2006

Dugourd, D., Yang, H. Y., Elliott, M., Siu, R., Clement, J. J., Straus, S. K., . . . Rubinchik, E. (2011). Antimicrobial Properties of MX-2401, an Expanded-Spectrum Lipopeptide Active in the Presence of Lung Surfactant. *Antimicrobial Agents and Chemotherapy, 55*(8), 3720-3728. doi:10.1128/Aac.00322-11

Epand, R. M., Walker, C., Epand, R. F., & Magarvey, N. A. (2016). Molecular mechanisms of membrane targeting antibiotics. *Biochimica Et Biophysica Acta-Biomembranes, 1858*(5), 980-987. doi:10.1016/j.bbamem.2015.10.018

Eurosurveillance-Editorial-Team. (2013). CDC publishes report on antibiotic resistance threats in the United States for the first time. *Eurosurveillance, 18*(38), 28-28.

Falanga, A., Nigro, E., De Biasi, M. G., Daniele, A., Morelli, G., Galdiero, S., & Scudiero, O. (2017). Cyclic Peptides as Novel Therapeutic Microbicides: Engineering of Human Defensin Mimetics. *Molecules, 22*(7). doi:ARTN 121710.3390/molecules22071217

Furin, J., Cox, H., & Pai, M. (2019). Tuberculosis. *Lancet, 393*(10181), 1642-1656. doi:10.1016/S0140-6736(19)30308-3

Geudens, N., & Martins, J. C. (2018). Cyclic Lipodepsipeptides From Pseudomonas spp. - Biological Swiss-Army Knives. *Frontiers in Microbiology, 9*. doi:ARTN 186710.3389/fmicb.2018.01867

Geudens, N., Nasir, M. N., Crowet, J. M., Raaijmakers, J. M., Feher, K., Coenye, T., . . . Deleu, M. (2017). Membrane Interactions of Natural Cyclic Lipodepsipeptides of the Viscosin Group. *Biochimica Et Biophysica Acta-Biomembranes, 1859*(3), 331-339. doi:10.1016/j.bbamem.2016.12.013

Guez, J. S., Muller, C. H., Danze, P. M., Buchs, J., & Jacques, P. (2008). Respiration activity monitoring system (RAMOS), an efficient tool to study the influence of the oxygen transfer rate on the synthesis of lipopeptide by Bacillus subtilis ATCC6633. *Journal of Biotechnology, 134*(1-2), 121-126. doi:10.1016/j.jbiotec.2008.01.003

Hassan, K. A., Johnson, A., Shaffer, B. T., Ren, Q. H., Kidarsa, T. A., Elbourne, L. D. H., . . . Loper, J. E. (2010). Inactivation of the GacA response regulator in Pseudomonas fluorescens Pf-5 has far-reaching transcriptomic consequences. *Environmental Microbiology, 12*(4), 899-915. doi:10.1111/j.1462-2920.2009.02134.x

Hennessy, R. C., Phippen, C. B. W., Nielsen, K. F., Olsson, S., & Stougaard, P. (2017). Biosynthesis of the antimicrobial cyclic lipopeptides nunamycin and nunapeptin by Pseudomonas fluorescens strain In5 is regulated by the LuxR-type transcriptional regulator NunF. *Microbiologyopen, 6*(6). doi:ARTN e51610.1002/mbo3.516

Ho, S. W., Jung, D., Calhoun, J. R., Lear, J. D., Okon, M., Scott, W. R. P., . . . Straus, S. K. (2008). Effect of divalent cations on the structure of the antibiotic daptomycin. *European Biophysics Journal with Biophysics Letters, 37*(4), 421-433. doi:10.1007/s00249-007-0227-2

Hover, B. M., Kim, S. H., Katz, M., Charlop-Powers, Z., Owen, J. G., Ternei, M. A., . . . Brady, S. F. (2018). Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nature Microbiology, 3*(4), 415-422. doi:10.1038/s41564-018-0110-1

Huang, Z. H., Hu, Y., Shou, L. F., & Song, M. X. (2013). Isolation and partial characterization of cyclic lipopeptide antibiotics produced by Paenibacillus ehimensis B7. *Bmc Microbiology, 13*. doi:Artn 8710.1186/1471-2180-13-87

Håvarstein, L. S. (2018). *Purification, characterization and structure determination of a novel antibiotic highly active against Streptococcus pneumoniae- Unpublished manuscript*. Norwegian University of Life Sciences.

Ines, M., & Dhouha, G. (2015). Lipopeptide surfactants: Production, recovery and pore forming capacity. *Peptides, 71*, 100-112. doi:10.1016/j.peptides.2015.07.006

Jahanshah, G., Yang, Q., Gerhardt, H., Pataj, Z., Lammerhofer, M., Pianet, I., . . . Gross, H. (2019). Discovery of the Cyclic Lipopeptide Gacamide A by Genome Mining and Repair of the

Defective GacA Regulator in Pseudomonas fluorescens Pf0-1. *Journal of Natural Products, 82*(2), 301-308. doi:10.1021/acs.jnatprod.8b00747

Janek, T., Lukaszewicz, M., Rezanka, T., & Krasowska, A. (2010). Isolation and characterization of two new lipopeptide biosurfactants produced by Pseudomonas fluorescens BD5 isolated from water from the Arctic Archipelago of Svalbard. *Bioresource Technology, 101*(15), 6118-6123. doi:10.1016/j.biortech.2010.02.109

Kleijn, L. H. J., & Martin, N. I. (2018). The Cyclic Lipopeptide Antibiotics. In J. F. Fisher, S. Mobashery, & M. J. Miller (Eds.), *Antibacterials: Volume II* (pp. 27-53). Cham: Springer International Publishing.

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., . . . Shen, J. Z. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infectious Diseases, 16*(2), 161-168. doi:10.1016/S1473-3099(15)00424-7

Magetdana, R., Lakey, J. H., & Ptak, M. (1988). A Comparative Monomolecular Film Study of Antibiotic A21978c Homologs of Various Lipid Chain-Length. *Biochimica Et Biophysica Acta, 962*(2), 201-207. doi:Doi 10.1016/0005-2760(88)90160-9

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., . . . Spratt, B. G. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America, 95*(6), 3140-3145. doi:DOI 10.1073/pnas.95.6.3140

Martens, E., & Demain, A. L. (2017). The antibiotic resistance crisis, with a focus on the United States. *Journal of Antibiotics, 70*(5), 520-526. doi:10.1038/ja.2017.30

Miller, W. R., Bayer, A. S., & Arias, C. A. (2016). Mechanism of Action and Resistance to Daptomycin in Staphylococcus aureus and Enterococci. *Cold Spring Harbor Perspectives in Medicine, 6*(11). doi:ARTN a02699710.1101/cshperspect.a026997

Morikawa, M., Hirata, Y., & Imanaka, T. (2000). A study on the structure-function relationship of lipopeptide biosurfactants. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 1488*(3), 211-218. doi:Doi 10.1016/S1388-1981(00)00124-4

Muraih, J. K., Pearson, A., Silverman, J., & Palmer, M. (2011). Oligomerization of daptomycin on membranes. *Biochimica Et Biophysica Acta-Biomembranes, 1808*(4), 1154-1160. doi:10.1016/j.bbamem.2011.01.001

Nguyen, T. N. A., Anton-Le Berra, V., Banuls, A. L., & Nguyen, T. V. A. (2019). Molecular Diagnosis of Drug-Resistant Tuberculosis; A Literature Review. *Frontiers in Microbiology, 10*. doi:ARTN 79410.3389/fmicb.2019.00794

O'Brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., McCall, N., . . . Dis, H. P. G. B. (2009). Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet, 374*(9693), 893-902. doi:Doi 10.1016/S0140-6736(09)61204-6

Olaitan, A. O., Morand, S., & Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology, 5*. doi:ARTN 64310.3389/fmicb.2014.00643

Organization, W. H. (2018). *Global tuberculosis report 2018*. Retrieved from file:///C:/Users/Bruker/Downloads/9789241565646-eng.pdf

Patel, S., Ahmed, S., & Eswari, J. S. (2015). Therapeutic cyclic lipopeptides mining from microbes: latest strides and hurdles. *World Journal of Microbiology & Biotechnology, 31*(8), 1177-1193. doi:10.1007/s11274-015-1880-8

Perry, M. R., McClean, D., Simonet, C., Woolhouse, M., & McNally, L. (2018). Focussing on resistance to front-line drugs is the most effective way to combat the antimicrobial resistant crisis. *Preprint Des. 2018*. doi:https://doi.org/10.1101/498329

Philippe Desjardins, D. C. (2010). NanoDrop Microvolume Quantitation of nuclei Acids. *Journal of visualized experiments, 45*(2565). doi:doi:10.3791/2565

Raaijmakers, J. M., de Bruijn, I., & de Kock, M. J. D. (2006). Cyclic lipopeptide production by plant-associated Pseudomonas spp.: Diversity, activity, biosynthesis, and regulation. *Molecular Plant-Microbe Interactions, 19*(7), 699-710. doi:10.1094/Mpmi-19-0699

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., & Ongena, M. (2010). Natural functions of lipopeptides from Bacillus and Pseudomonas: more than surfactants and antibiotics. *Fems Microbiology Reviews, 34*(6), 1037-1062. doi:10.1111/j.1574-6976.2010.00221.x

Ramos-Sevillano, E., Ercoli, G., & Brown, J. S. (2019). Mechanisms of Naturally Acquired Immunity to Streptococcus pneumoniae. *Frontiers in Immunology, 10*. doi:ARTN 35810.3389/fimmu.2019.00358

Rangarajan, V., & Clarke, K. G. (2016). Towards bacterial lipopeptide products for specific applications - a review of appropriate downstream processing schemes. *Process Biochemistry, 51*(12), 2176-2185. doi:10.1016/j.procbio.2016.08.026

Reder-Christ, K., Schmidt, Y., Dorr, M., Sahl, H. G., Josten, M., Raaijmakers, J. M., . . . Bendas, G. (2012). Model membrane studies for characterization of different antibiotic activities of lipopeptides from Pseudomonas. *Biochimica Et Biophysica Acta-Biomembranes, 1818*(3), 566-573. doi:10.1016/j.bbamem.2011.08.007

Sauermann, R., Rothenburger, M., Graninger, W., & Joukhadar, C. (2008). Daptomycin: A review 4 years after first approval. *Pharmacology, 81*(2), 79-91. doi:10.1159/000109868

Savulescu, C., Krizova, P., Lepoutre, A., Mereckiene, J., Vestrheim, D. F., Ciruela, P., . . . Grp, S. (2017). Effect of high-valency pneumococcal conjugate vaccines on invasive pneumococcal disease in children in SpIDnet countries: an observational multicentre study. *Lancet Respiratory Medicine, 5*(8), 648-656. doi:10.1016/S2213-2600(17)30110-8

Schneider, T., Mullera, A., Miess, H., & Gross, H. (2014). Cyclic lipopeptides as antibacterial agents - Potent antibiotic activity mediated by intriguing mode of actions. *International Journal of Medical Microbiology, 304*(1), 37-43. doi:10.1016/j.ijmm.2013.08.009

Schobert, M., & Jahn, D. (2010). Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung. *International Journal of Medical Microbiology, 300*(8), 549-556. doi:10.1016/j.ijmm.2010.08.007

Silverman, J. A., Mortin, L. I., VanPraagh, A. D. G., Li, T. C., & Alder, J. (2005). Inhibition of daptomycin by pulmonary surfactant: In vitro modeling and clinical impact. *Journal of Infectious Diseases, 191*(12), 2149-2152. doi:Doi 10.1086/430352

Song, C. X., Aundy, K., van de Mortel, J., & Raaijmakers, J. M. (2014). Discovery of new regulatory genes of lipopeptide biosynthesis in Pseudomonas fluorescens. *Fems Microbiology Letters, 356*(2), 166-175. doi:10.1111/1574-6968.12404

Spaulding, C. N., Klein, R. D., Schreiber, H. L., Janetka, J. W., & Hultgren, S. J. (2018). Precision antimicrobial therapeutics: the path of least resistance? *Npj Biofilms and Microbiomes, 4*. doi:UNSP 410.1038/s41522-018-0048-3

Straume, D., Stamsas, G. A., & Havarstein, L. S. (2015). Natural transformation and genome evolution in Streptococcus pneumoniae. *Infection Genetics and Evolution, 33*, 371-380. doi:10.1016/j.meegid.2014.10.020

Straus, S. K., & Hancock, R. E. W. (2006). Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: Comparison with cationic antimicrobial peptides and lipopeptides. *Biochimica Et Biophysica Acta-Biomembranes, 1758*(9), 1215-1223. doi:10.1016/j.bbamem.2006.02.009

Sutcliffe, I. C. (2000). Characterisation of a lipomannan lipoglycan from the mycolic acid containing actinomycete Dietzia maris. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 78*(2), 195-201. doi:Doi 10.1023/A:1026562610490

Taylor, S. D., & Palmer, M. (2016). The action mechanism of daptomycin. *Bioorganic & Medicinal Chemistry, 24*(24), 6253-6268. doi:10.1016/j.bmc.2016.05.052

Tedesco, K. L., & Rybak, M. J. (2004). Daptomycin. *Pharmacotherapy, 24*(1), 41-57. doi:DOI 10.1592/phco.24.1.41.34802

van der Poll, T., & Opal, S. M. (2009). Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet, 374*(9700), 1543-1556. doi:Doi 10.1016/S0140-6736(09)61114-4

Vasquez-Ponce, F., Higuera-Llanten, S., Pavlov, M. S., Marshall, S. H., & Olivares-Pacheco, J. (2018). Phylogenetic MLSA and phenotypic analysis identification of three probable novel Pseudomonas species isolated on King George Island, South Shetland, Antarctica. *Brazilian Journal of Microbiology, 49*(4), 695-702. doi:10.1016/j.bjm.2018.02.005

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P & T a peer-reviewes jornal for formulary management, 44*(4), 277-283.

Victora, C. G., Requejo, J. H., Barros, A. J. D., Berman, P., Bhutta, Z., Boerma, T., . . . Bryce, J. (2016). Countdown to 2015: a decade of tracking progress for maternal, newborn, and child survival. *Lancet, 387*(10032), 2049-2059. doi:10.1016/S0140-6736(15)00519-X

Vilhena, C., & Bettencourt, A. (2012). Daptomycin: A Review of Properties, Clinical Use, Drug Delivery and Resistance. *Mini-Reviews in Medicinal Chemistry, 12*(3), 202-209. doi:Doi 10.2174/1389557511209030202

Watson, D. A., Musher, D. M., Jacobson, J. W., & Verhoef, J. (1993). A Brief-History of the Pneumococcus in Biomedical-Research - a Panoply of Scientific Discovery. *Clinical Infectious Diseases, 17*(5), 913-924. doi:DOI 10.1093/clinids/17.5.913

Xue, Y., Wang, M. Y., Zhao, P. C., Quan, C. S., Li, X., Wang, L. N., . . . Li, P. (2018). Gram-negative bacilli-derived peptide antibiotics developed since 2000. *Biotechnology Letters, 40*(9-10), 1271-1287. doi:10.1007/s10529-018-2589-1

Zavascki, A. P., Goldani, L. Z., Li, J., & Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of Antimicrobial Chemotherapy, 60*(6), 1206-1215. doi:10.1093/jac/dkm357

Appendix

Table of content:

Appendix A- Media

Media were added 15 g per 1l when agar plates were made, and 8 g per 1l when soft agar was made. Chemicals and reagents used to make media are listed in appendix B.

A.1 Brain Heart infusion (BHI) – medium

Table A.1. Brain Heart infusion medium (BHI). Reagents were mixed accordingly, and pH adjusted to pH 7.4 ± 0.2 at 25°C.

Reagents	**Quantities**
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Protease peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Di-sodium phosphate	2.5
Total volume adjusted with dH_2O	1 l

A.2 Bacto ™ Todd Hewitt Broth

Table A.2. Todd Hewitt broth (TH). Reagents were mixed accordingly, and pH adjusted to pH 7.8 ± 0.2 at 25°C.

Reagents	**Quantities**
Heart infusion from 500g	3.1 g
Neopeptone	20.0 g
Dextose	2.0 g
Sodium chloride	2.0 g
Disodium phosphate	0.4 g
Sodium carbonate	2.5 g
Total volume adjusted with dH_2O	**1 l**

A.3 C-medium

In the following tables are all components required to make C-medium (table A.3-A.8). C-medium were made fresh every day it was required (table A.8) but all other component could be stored. The finished C-medium was stored at 4°C throughout that given day.

Table A.3. Yeast extract. The solution was mixed for 10 minutes before incubated 2-4 hours at 4°C. The coal started to precipitate to some degree. After incubation the mixture was filtered through glass wool and celite overnight, gravity pulling the mixture through. The filtered mixture was adjusted to pH 7,8 using high molarity NaOH. The finished mixture was sterile filtered and sectioned into 4 ml segments in 15 ml falcon tubes. Stored in -80 °C freezer.

Reagents	**Quantities**
Yeast extract	40 g
dH_2O	36 ml
37% HCl	6 ml
Active coal	16 g
Total volume later adjusted with dH_2O	400 ml

Table A.4. ADAMS I- Solution was adjusted to pH 7 by HCl and sterile filtered before storage at 4°C

Reagents	**Quantity**
Biotine 0.5 mg/ml	150 µl
Acide nicotiniqe	75 mg
Pyridoxine hydrochloride (4°C)	87.5 mg
Calcium phanthothenate (4°C)	300 mg
Thiamine hydrochloride	80 mg
Riboflavine	35mg
dH_2O	To 500 ml

Table A.5. ADAMS II. Solution was sterile filtered and stored at 4°C

Reagent	**Quantity**
Iron (II)sulphate heptahydrate	500 mg
Copper sulphate pentahydrate	500 mg
Zincsulphate heptahydrate	500 mg
Mangan (II)chloride tetrahydrate	200 mg
HCl concentrate	10 ml
Total volume adjusted with dH_2O	100 ml

Table A.6. ADAMS III. The solutions corrected to pH 7,6 and sterile filtered

Reagents	**Quantities**
ADAMS I	128 ml
10X ADAMS II	3,2 ml
Asparagine monohydrate	1,6g
Choline	160 mg
Calcium chlorin anhydride	0,4 g
Magnesium chlorin heksahydrat	16 g
Total volume adjusted with dH_2O	800 ml

Table A.7. Pre C-medium

Reagents	**Quantity**
L-cyctein HCl	22,5 mg
Sodiumacetate	4 g
Casitone	10 g
L-tryptophan	12 mg
Di-calciumphosphate	17 g
Total volume adjusted with dH_2O	2 l

Table A.8. C- medium. Solution sterile filtered and stored at 4°C.

Reagents	**Quantities**
0,4 mM Mangan (II) chloride	150 µl
20 % Glucose	1,5 ml
ADAMS III	3,75 ml
3 % Glutamine	110 µl
2% Sodium pyruvate	2,5 ml
1,5 M Sucrose	95 µl
2 mg/ml Uridine adenosine	1,5 ml
8% Albumine/BSA	1,5 ml
Yeast extract	3,75 ml
All reagents above is added to 150 ml pre-C-medium	

A.4 M17 Broth

Table A.9. M17 broth. Solution was adjusted to pH 6,9 ±0,2 at 25°C

Reagents	Quantities
Tryptone	5 g
Soya peptone	5 g
Meat digest	5 g
Yeast extract	2,5 g
Ascorbic acid	0,5 g
Magnesium sulphate	0,25 g
Di-sodium-glycerophosphate	19 g
Total volume adjusted with dH_2O	1 l

A.5 M.R.S. Broth (de man, Rogosa, Sharpe)

Table A.10. MRS broth. Solution adjusted to pH 6,2 ± 0,2 at 25°C. Finished medium is autoclaved and 10X Glucose is added

Reagents	Quantities
Peptone	10 g
'Lab-Lemco' Powder	8 g
Yeast extract	4 g
Glucose	20 g
Sorbitan mono-oleate	1 ml
Di-potassium hydrogen phosphate	2 g
Sodium acetate $3H_2O$	5 g
Tri-ammonium citrate	2 g
Magnesium sulphate $7H_2O$	0,2 g
Manganese sulphate $4H_2O$	0,05 g
Total volume adjusted with dH_2O	1 l

A.6 Blood agar

Table A.11. Blood agar. Horse blood is added to the solution after mixing.

Reagents	Quantities
Protease peptone	15 g
Liver digest	2,5 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	12 g
Total volume adjusted with dH_2O	1 l

A.7 M9 Minimal medium

Components for M9 medium listed in table 14 and 15.

Table A.12. 5X M9 salts

Reagents	Quantities
Di sodium phosphate · $7H_2O$	64 g
Potassium dihydrogen phosphate	15 g
Sodium chloride	2,5 g
Ammonium chloride	5 g
Total volume adjusted with dH_2O	1 l

Table A.13. M9 minimal medium

Reagents	Quantities
5x M9 salts	200 ml
1 M Magnesium sulphate	2 ml
20% glucose	20 ml
1 M Calcium chloride	0,1 ml
Total volume adjusted with dH_2O	1 l

A.8 Pseudomonas broth

Table A.14. Pseudomonas broth. Reagents are mixed accordingly and autoclaved.

Reagents	Quantities
Peptone	16 g
Casein hydrolysate	10 g
Potassium sulphate	10 g
Magnesium chloride	1,4 g
Total volume adjusted with dH_2O	**1 l**

Appendix B- Chemicals

Table B.1. Chemical and media used in this thesis. Most chemicals were used to make nutrient media.

Names	Formula	Producer
Acetate buffer	CH_3COOH	
Acetonitrile	CH_3CN	
Acid nicotine	$C_{10}H_{14}N_2$	
Agar		VWR
Albumin/BSA	$C_{123}H_{193}N_{35}O_{37}$	
Ammonium chloride	NH_4Cl	
Asparagine monohydrate	$C_4H_{10}N_2O_4$	
Bacto TM Todd Hewitt Broth		BD bioscience
Biotine	$C_{10}H_{16}N_2O_3S$	
Blood agar		OXOID
Brain Heart Infusion Broth		OXOID
Calcium chloride	$CaCl_2$	
Calcium pantothenate	$C_{18}H_{32}CaN_2O_{10}$	
Casein hydrolysate		Merck
Choline	$C_5H_{14}NO$	
Coppersulphate pentahydrate	CuSO4 ·5H2O	
Dimethyl sulfoxide	C_2H_6OS	
Di-potassium phosphate	K_2HPO_4	
Di-sodium phosphate	Na_2HPO_4	
Di sodium phosphate · $7H_2O$	$Na_2HPO_4 \cdot 7H_2O$	
Gel loading dye purple		BioLabs
Glucose	$C_6H_{12}O_6$	
Glutamine	$C_5H_{10}N_2O_3$	
Hydrogen chloride	HCl	
Iron (III)sulphate heptahydrate	$FeSO_4, 7H_2O$	
L-cysteine HCl	$C_3H_7NO_2S$	
L-tryptophan	$C_{11}H_{12}N_2O_2$	

M17 Broth		FORMEDIUM
Magnesium chloride	$MgCl_2$	
Magnesium sulphate	$MgSO_4$	
Mangan (II)chloride	$MnCl_2$	
Mangan (II)chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	
Methanol	CH_3OH	
MRS Broth		OXOID
peqGREEN dye		Peqlab (a VWR company)
Phosphate-buffer saline (PBS)	K_2HPO_4, KH_2PO_4, NaCl	
Phusion DNA polymerase		BioLabs
Phusion reaction buffer		BioLabs
Potassium dihydrogen phosphate	KH_2PO_4	
Potassium sulphate	K_2SO_4	
Pyridoxine hydrochloride	$C_8H_{12}ClNO_3$	
Riboflavin	$C_{17}H_{20}N_4O_6$	
Sodium acetate	$C_2H_3NaO_2$	
Sodium acetate trihydrate	$C_2H_3NaO_2 \cdot 3H_2O$	
Sodium carbonate	Na_2CO_3	
Sodium chloride	NaCl	
Sodium pyruvate	$C_3H_3NaO_3$	
Sucrose	$C_{12}H_{22}O_{11}$	
Sytox nuclei acid green stain		Thermofisher
Thiamine hydrochloride	$C_{12}H_{18}Cl_2N_4OS$	
Trifluoroacetic acid	CF_3COOH	
Uridine adenosine	$C_9H_{12}N_2O_6$	
Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	

Appendix C- Strains

Table C.1. All species used in this thesis. All species were testes against streptovoracin either on microplate or as overlay with the except of *Pseudomonas* isolate no. 33, since this was the producer strain of streptovoracin. Strains highlighted in read was only tested on blood agar. All SK strains were gifted to the research team from Professor Mogens Kilian, and NCTC, ATCC and NCDO strains have the group ordered from respective species banks over several years. All other species have been gifted from diverse groups and research teams. Only *M. smegmatis* was specifically ordered for this thesis. The table is organized downward based on relations to *S. pneumoniae* on the phylogenetic tree, however, this does not apply to non-streptococcus species.

Code	**Species**
Pseudomonas isolate no. 33	
RH425 (Control strain)	*S. pneumoniae*
RH14	*S. pneumoniae* lacking Lyt A
RH16	*S. pneumoniae* lacking lyt A and Lyt C
SK851	*S. pneumoniae*
SK865	*S. pneumoniae*
A66 capsule	*S. pneumoniae* *
A66 non-capsulated	*S. pneumoniae*
TIGR4	*S. pneumoniae* *
B14	*S. pneumoniae* *
Type 4	*S. pneumoniae* *
CP1200	*S. pneumoniae*
D39	*S. pneumoniae* *
SK95	*S. mitis*
SK149	*S. mitis*
SK272	*S. mitis*
SK148	*S. mitis*
SK145	*S. mitis*
SK102	*S. mitis*
SK21	*S. mitis*
SK24	*S. mitis*

SK608	*S. mitis*
SK601	*S. mitis*
SK602	*S. mitis*
SK596	*S. mitis*
SK667	*S. mitis*
SK137	*S. mitis*
SK564	*S. mitis*
SK142	*S. mitis*
Sk599	*S. mitis*
SK598	*S. mitis*
ATCC 10557	*S. oralis*
SK79	*S. oralis*
SK305	*S. oralis*
SK610	*S. oralis*
SK286	*S. oralis*
SK39	*S. oralis*
SK100	*S. oralis*
SK571	*S. oralis*
NCTC 11427	*S. oralis*
SK155	*S. oralis*
SK304	*S. oralis*
SK153	*S. oralis*
SK958	*S. perori*
SK140	*S. infantis*
SK282	*S. infantis*
SK283	*S. infantis*
SK605	*S. infantis*
SK603	*S. infantis*
SK350	*S. infantis*
SK959	*S. infantis*
SK348	*S. infantis*

SK236	*S. parasanguinis*
SK438	*S. parasanguinis*
ATCC 15912	*S. parasanguinis*
NCTC 7869	*S. gordonii*
SK120	*S. gordonii*
SK6	*S. gordonii (ATCC12396)*
SK3	*S. gordonii (ATCC 10558)*
NCTC 7865	*S. gordonii*
Challis	*S. gordonii* ^{t}
Wicky villt	*S. gordonii*
ATCC 10558	*S. gordonii (SK3)*
NCTC 3165	*S. gordonii*
NCTC 12479	*S. cristatus*
SK230	*S. cristatus*
SK975	*S. cristatus*
SK1	*S. sanguinis* ^{t}
SK90	*S. sanguinis*
NCTC 10231	*S. sanguinis*
NCTC 7863	*S. sanguinis*
NCTC 11062	*S. anginosus subsp. Milleri*
NCTC 10708	*S. anginosus subsp. Milleri*
SK87	*S. anginosus*
NCTC 10713	*S. anginosus*^{t}
NCDO 2226	*S. constellatus*
NCTC 11325	*S. constellatus*
ATCC 13419	*S. salivarius subsp. Salivarius*
NCTC 12166	*S. vestibularis*
NCTC 12167	*S. vestibularis*
NCTC 8177	*S. bovis*
NCTC 8181	*S. agalactiae**
ATCC 29128	*S. phocae*

ATCC 9812	*S. equinus*
ATCC 19642	*S. criceti*
NCTC 8198	*S. pyogenes* *
NCTC 4676	*S. equi subsp. Zooepidemicus*
NCTC 10999	*S. porcinus*
NCTC 10449	*S. mutans*
UA159	*S. mutans*
UA130	*S. mutans*[t]
LM7	*S. mutans*
ATCC 19645	*S. ratti*
NCTC 10919	*S. sobrinus*
NCDO 2714	*Lactobacillus sake*
IL 1403	*Lactococcus lactis*
MG 1363	*Lactococcus lactis subsp. Cremoris*
EF 2708	*Enterococcus faecalis EF BRIDGE (B)*
LMG 2787	*Enterococcus faecium*
ATCC 4516	*Bacillus cereus* *
ATCC 6051	*Bacillus subtilis* *
DSM 20600	*Listeria monocytogenes* *
D5Hα	*E. coli*
SPH 492	*Dietzia*
NCTC 8159	*M. smegmatis* *

* Pathogenic strain

t Strains used for transformation experiment.

Appendix D- species and inhibition zones

Table D.1. Screened species with measured inhibition zones. Table is organized downward based on relations to *S. pneumoniae* on the phylogenetic tree, this does not apply to the non-streptococcus species. This table also show the inhibition zones measured from the overlay assay. The zones were measured by using a ruler.

Code	Species	Inhibition zone, diameter in cm. Colonies were on average 0,5-0,8 cm in diameter
RH425 (control strain)	*S. pneumoniae*	3
SK851	*S. pneumoniae*	3,1
SK865	*S. pneumoniae*	2,8
A66 non-capsule	*S. pneumoniae*	3,2
TIGR4	*S. pneumoniae* *	2,4
B14	*S. pneumoniae* *	2,2
A66 capsule	*S. pneumoniae* *	3,0
Type 4	*S. pneumoniae* *	2,7
CP1200	*S. pneumoniae*	3,5
TIGR4	*S. pneumoniae* *	2,2
D36	*S. pneumoniae* *	2,2
A66 capsule wildtype	*S. pneumoniae* *	2,4
A66 non-capsuleated mutant	*S. pneumoniae*	1,9
SK95	*S. mitis*	2,4
SK149	*S. mitis*	1,5
SK272	*S. mitis*	3,0
SK148	*S. mitis*	1,7
SK145	*S. mitis*	3,0
SK102	*S. mitis*	1,1
SK21	*S. mitis*	1,6
SK24	*S. mitis*	1,1

SK608	*S. mitis*	3,0
SK601 ?	*S. mitis*	1,7
SK602 ?	*S. mitis*	1,8
SK596	*S. mitis*	2,1
SK667	*S. mitis*	2,1
SK137	*S. mitis*	1,8
SK564	*S. mitis*	4,5
SK142	*S. mitis*	2,0
SK599	*S. mitis*	3,7
SK598	*S. mitis*	2,3
ATCC 10557	*S. oralis*	1,7
SK79	*S. oralis*	2,8
SK305	*S. oralis*	2,1
SK610	*S. oralis*	2,3
SK286	*S. oralis*	2,1
SK39	*S. oralis*	2,2
SK100	*S. oralis*	2,4
SK571	*S. oralis*	3,4
NCTC 11427	*S. oralis*	1,7
SK155	*S. oralis*	2,5
SK304	*S. oralis*	2,2
SK153	*S. oralis*	1,7
SK958	*S. perori*	1,7
SK140	*S. infantis*	2,5
SK282	*S. infantis*	2,0
SK283	*S. infantis*	2,5
SK605	*S. infantis*	2,1
SK603	*S. infantis*	2,4
SK350	*S. infantis*	2,7
SK959	*S. infantis*	2,2
SK348	*S. infantis*	3,0

SK236	*S. parasanguinis*	2,0
SK438	*S. parasanguinis*	1,4
ATCC 15912	*S. parasanguinis*	1,5
NCTC 7869 (SG69)	*S. gordonii*	1,6
SK120	*S. gordonii*	1,6
SK6	*S. gordonii* (ATCC 12396)	2,4
SK3	*S. gordonii* (ATCC 10558)	2,3
NCTC 7865	*S. gordonii*	1,3
Challis	*S. gordonii* [t]	1,1
Wicky villt	*S. gordonii*	1,3
ATCC 10558	*S. gordonii* (SK3)	1,5
NCTC 3165	*S. gordonii*	1,5
NCTC 12479 (SCr79)	*S. cristatus*	1,9
SK230	*S. cristatus*	2,5
SK975	*S. cristatus*	2,3
SK1	*S. sanguinis* [t]	1,0
SK90	*S. sanguinis*	1,7
NCTC 10231	*S. sanguinis*	1,0
NCTC 7863	*S. sanguinis*	1,1
NCTC 11062	*S. anginosus subsp. milleri*	1,2
NCTC 10708	*S. anginosus subsp. milleri*	1,1
SK87	*S. anginosus*	1,2
NCTC 10713	*S. anginosus* [t]	NA
NCDO 2226	*S. constellatus*	NA
NCTC 11325	*S. constellatus*	1,7
ATCC 13419	*S. salivarius subsp. Salivarius*	1,4
NCTC 12166	*S. vestibularis*	1,5
NCTC 12167	*S. vestibularis*	1,2
NCTC 8177	*S. bovis*	1,0
NCTC 8181	*S. agalactiae* *	1,5
ATCC 29128	*S. phocae*	2,7

ATCC 9812	*S. equinus*	1,0
ATCC 19642	*S. criceti*	-
NCTC 8198	*S. pyogenes* *	2,0
NCTC 8198	*S. pyogenes* *	2,0
NCTC 4676	*S. equi subsp. zooepidemicus*	2,0
NCTC 10999	*S. porcinus*	1,4
NCTC 10449 (SM49)	*S. mutans*	-
UA159	*S. mutans*	-
UA130	*S. mutans* [t]	-
LM7	*S. mutans*	NA
ATCC 19645	*S. ratti*	1,0
NCTC 10919	*S. sobrinus*	-
NCDO 2714	*Lactobacillus sake*	-
IL 1403	*Lactococcus lactis*	-
MG 1363	*L. lactis subsp. Cremoris*	-
EF 2708	*E. faecalis EF BRIDGE (B)*	1,8
LMG 2787	*E. faecium*	-
ATCC 4516	*Bacillus cereus* *	-
ATCC 6051	*Bacillus subtilis* *	-
DSM 20600	*Listeria monocytogenes* *	-
D5Hα	*E. coli*	-
SPH 492	*Dietzia*	2,3
NCTC 8159	*M. smegmatis* *	2,2

Strains coloured in red was grown on blood agar. Star (*) indicate pathogenic strains, some of these are less dangerous than others. [t] indicates stains used for transformation

Appendix E- Sytox staining of RH16 culture

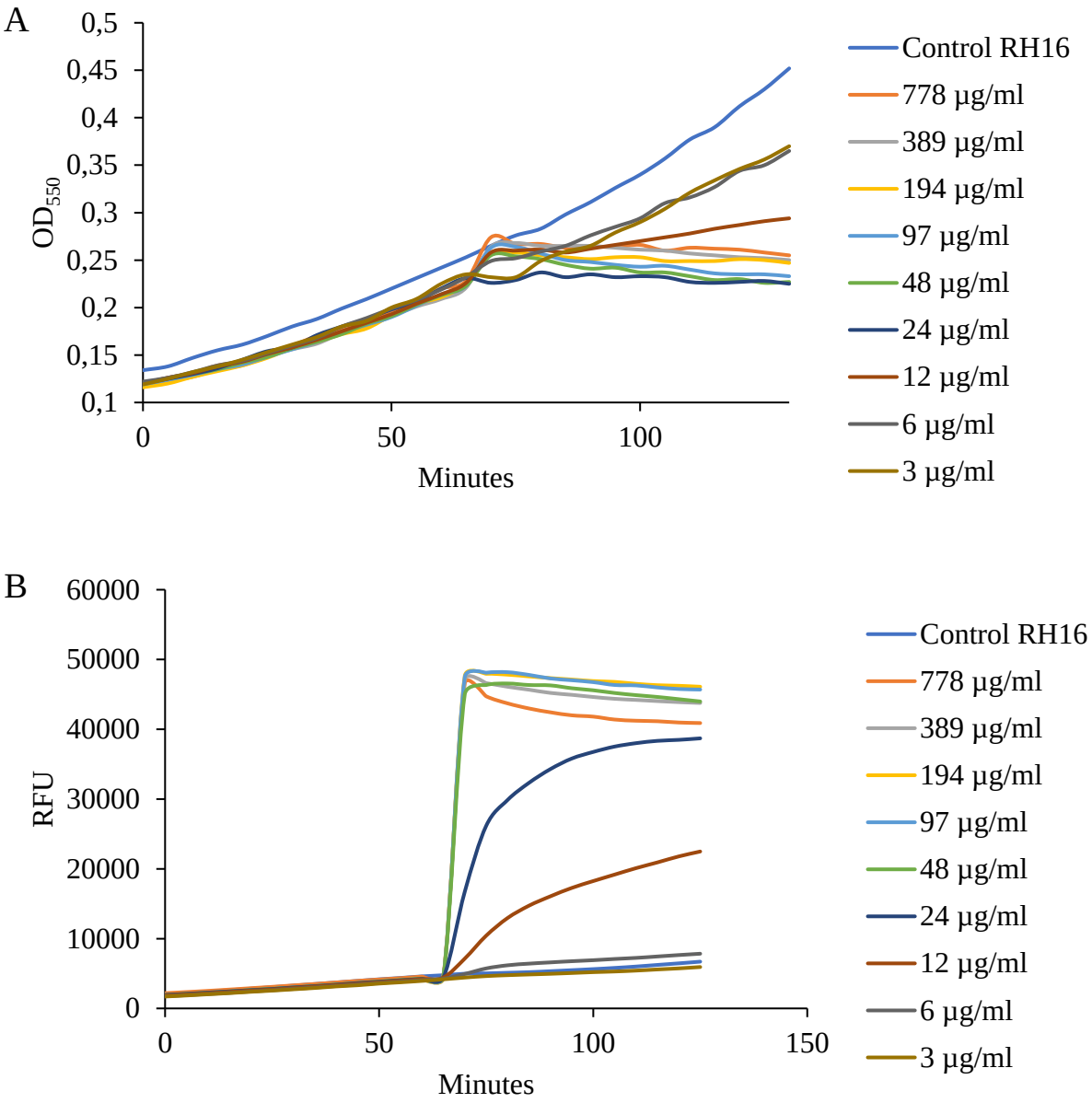


Figure 1 Detection of streptovoracin-induced damage to the cytoplasmic membrane in a pneumococcal strain (RH16) lacking LytA. Streptovoracin was added to the cultures as indicated by the arrow at the concentrations listed on the right-hand side. Panel A shows fluorescence caused by the binding of Sytox green to intracellular DNA. The cytoplasmic membrane is normally impermeable to Sytox green, but when its integrity is damaged, Sytox green is able to penetrate, bind intracellular DNA and fluoresce. Panel B depicts the growth curves of the cultures whose fluorescence signals are measured in panel A. For every time point, optical density (OD550) as well as fluorescence are measure in each culture.

Appendix F- Sequence matches

The following figures shows the best match from blast of the nucleotide sequenced from genes from MLST test. Both nucleotide and protein sequence is showed from the best match on nucleotide sequence.

F.1 Gyr B

Pseudomonas sp. R76 DNA gyrase subunit beta (gyrB) gene, complete cds

Sequence ID: KT890289.1 **Length:** 2418 **Number of Matches:** 1

Range 1: 178 to 830 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1057 bits(572)	0.0	627/654(96%)	1/654(0%)	Plus/Plus

```
Query  1    GACATCAGCATTATCATCCACCCGGATGAGTCCATCACCGTGCGCGACAACGGTCGCGGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178  GACATCAGCATTATCATCCACCCGGATGAGTCCATCACCGTGCGCGACAACGGTCGCGGC  237

Query  61   ATTCCGGTCGATGTGCACAAAGAAGAAGGCGTATCGGCGGCAGAGGTCATCATGACCGTG  120
            |||||||| ||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  238  ATTCCGGTAGATGTGCACAAAGAAGAAGGCGTTTCGGCGGCAGAGGTCATCATGACCGTG  297

Query  121  CTTCACGCCGGCGGTAAGTTCGACGACAACTCCTATAAAGTCTCCGGCGGTTTGCACGGT  180
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298  CTCCACGCCGGCGGTAAGTTCGACGACAACTCCTATAAAGTCTCCGGCGGTTTGCACGGT  357

Query  181  GTAGGTGTGTCGGTGGTGAACGCTCTGTCCGAAGAGCTTATCCTGACTGTTCGCCGTAGC  240
            ||||||||||||| |||||||| ||||| |||||||| ||||||||||||||||||||||
Sbjct  358  GTAGGTGTGTCGGTAGTGAACGCCCTGTCTGAAGAGCTGATCCTGACTGTTCGCCGTAGC  417

Query  241  GGCAAGATCTGGGAACAGACCTACGTGCATGGTGTTCCACAAGAACCGATGAAAATCGTT  300
            ||||| ||||||||||||| |||||||| ||||| |||||||| ||||||||||||||||
Sbjct  418  GGCAAAATCTGGGAACAGACTTACGTGCACGGTGTGCCACAAGAGCCGATGAAAATCGTT  477

Query  301  GGTGACAGTGAATCCACCGGTACGCAGATCCATTTCAAGCCTTCGGCAGAAACCTTCAAG  360
            || |||||||||||||||||||||||||||| |||||||| ||||| ||||||||||||
Sbjct  478  GGCGACAGTGAATCCACCGGTACGCAGATCCACTTCAAGCCATCGGCTGAAACCTTCAAG  537

Query  361  AATATCCACTTCAGCTGGGACATCCTGGCCAAGCGTATTCGTGAACTGTCGTTCCTTAAC  420
            |||||||||||||||||||||||||||||||||||||||||| |||||||| ||||| |||
Sbjct  538  AATATCCACTTCAGCTGGGACATCCTGGCCAAGCGTATTCGCGAACTGTCCTTCCTCAAC  597

Query  421  TCCGGTGTGGGTATCGTCCTCAAGGACGAGCGCAGCGGCAAGGAAGAGTTGTTCAAGTAC  480
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  598  TCCGGTGTGGGTATCGTCCTCAAGGACGAGCGCAGCGGCAAGGAAGAGCTGTTCAAGTAC  657

Query  481  GAAGGCGGCTTGCGTGCGTTCGTTGAATACCTGAACACCAACAAGACTGCGGTCAACCAG  540
            ||||||||| |||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  658  GAAGGCGGCCTGCGTGCGTTCGTTGAATACCTGAACACCAACAAGACGGCGGTCAACCAG  717

Query  541  GTGTTCCACTTCAACATCCAGCGTGAAGACGGCATCGGCGTTGAAATCGCCCTGCAGTGG  600
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  718  GTGTTCCACTTCAACATCCAGCGTGAAGACGGCATCGGCGTGGAAATCGCCCTGCAGTGG  777

Query  601  AACGACAGCTTCAACGAGAACCTGTTGTGCTTCACCAACAACTTTCCCTCAGCG  654
            |||||||||||||||||||||||||||||||||||||||||| ||||  |||||
Sbjct  778  AACGACAGCTTCAACGAGAACCTGTTGTGCTTCACCAACAACATTCCA-CAGCG  830
```

Figure F.1.1 Screen-print: Blastn search using the forward strain for sequenced Gyr B

DNA gyrase subunit B, partial [Pseudomonas putida]

Sequence ID: CCO02545.1 **Length:** 363 **Number of Matches:** 1

Range 1: 21 to 236 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
415 bits(1067)	5e-144	Compositional matrix adjust.	215/216(99%)	215/216(99%)	0/216(0%)	+1

```
Query  1    DISIIIHPDESITVRDNGRGIPVDVHKEEGVSAAEVIMTVLHAGGKFDDNSYKvsgglhg  180
            DISIIIHPDESITVRDNGRGIPVDVHKEEGVSAAEVIMTVLHAGGKFDDNSYKVSGGLHG
Sbjct  21   DISIIIHPDESITVRDNGRGIPVDVHKEEGVSAAEVIMTVLHAGGKFDDNSYKVSGGLHG  80

Query  181  vgvsvvNALSEELILTVRRSGKIWEQTYVHGVPQEPMKIVGDSESTGTQIHFKPSAETFK  360
            VGVSVVNALSEELILTVRRSGKIWEQTYVHGVPQEPMKIVGDSESTGTQIHFKPSAETFK
Sbjct  81   VGVSVVNALSEELILTVRRSGKIWEQTYVHGVPQEPMKIVGDSESTGTQIHFKPSAETFK  140

Query  361  NIHFSWDILAKRIRELSFLNSGVGIVLKDERSGKEELFKYEGGLRAFVEYLNTNKTAVNQ  540
            NIHFSWDILAKRIRELSFLNSGVGIVLKDERSGKEELFKYEGGLRAFVEYLNTNKTAVNQ
Sbjct  141  NIHFSWDILAKRIRELSFLNSGVGIVLKDERSGKEELFKYEGGLRAFVEYLNTNKTAVNQ  200

Query  541  VFHFNIQREDGIGVEIALQWNDSFNENLLCFTNNFP  648
            VFHFNIQREDGIGVEIALQWNDSFNENLLCFTNN P
Sbjct  201  VFHFNIQREDGIGVEIALQWNDSFNENLLCFTNNIP  236
```

Figure F.1.2 Screen-print: Blastx search using the forward strain for sequenced GyrB.

F.2 GlnS

Pseudomonas sp. LBUM920 chromosome, complete genome
Sequence ID: CP027762.1 **Length:** 6533866 **Number of Matches:** 1

Range 1: 4111574 to 4112152 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
970 bits(525)	0.0	562/580(97%)	1/580(0%)	Plus/Minus

Features: Glutaminyl-tRNA synthetase

```
Query  1        AGCGACATCAAGTGGCTGGGCTTCGAATGGTCCGGTGAAGTGCGCTATGCGTCCAAGTAT  60
                |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  4112152  AGCGACATCAAGTGGCTGGGCTTCGAATGGTCCGGTGAAGTGCGCTATGCCTCCAAGTAT  4112093

Query  61       TTCGACCAGTTGTTCGACTGGGCCGTCGAGCTGATCAAGGCCGGCAAGGCCTACGTCGAC  120
                |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  4112092  TTCGACCAGTTGTTCGACTGGGCCGTCGAGCTGATCAAGGCCGGCAAGGCCTATGTCGAC  4112033

Query  121      GACCTGACCCCGGAGCAGGCCAAGGAATACCGTGGCACGCTGACCGAGCCGGGCAAGAAC  180
                |||||||||||||||| ||||||||||||| ||||  || ||||||||||||||||||||
Sbjct  4112032  GACCTGACCCCGGAGCAAGCCAAGGAATACCGCGGCAGCCTCACCGAGCCGGGCAAGAAC  4111973

Query  181      AGCCCGTTCCGTGACCGTTCGGTAGAAGAGAACCTCGACTGGTTCAACCGCATGCGCGCC  240
                |||||||||| |||||||||| |||||||||| ||||||||||||||||||||||||||
Sbjct  4111972  AGCCCGTTCCGCGACCGTTCGGTCGAAGAGAACCTGGACTGGTTCAACCGCATGCGCGCC  4111913

Query  241      GGTGAGTTCCCGGACGGCGCCCGCGTGCTGCGCGCCAAGATCGACATGGCCTCGCCGAAC  300
                |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  4111912  GGTGAGTTCCCGGACGGCGCCCGCGTGCTGCGCGCCAAAATCGACATGGCCTCGCCGAAC  4111853

Query  301      ATGAACCTGCGCGACCCGATCATGTACCGCATCCGCCACGCCCATCACCACCAGACCGGT  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4111852  ATGAACCTGCGCGACCCGATCATGTACCGCATCCGCCACGCCCATCACCACCAGACCGGT  4111793

Query  361      GACAAGTGGTGCATCTACCCGAACTATGACTTCACCCACGGTCAGTCGGACGCCATCGAA  420
                |||||||||||||||||||||||||| |||||||||||||||||||||||||| ||||||
Sbjct  4111792  GACAAGTGGTGCATCTACCCGAACTACGACTTCACCCACGGTCAGTCGGACGCTATCGAA  4111733

Query  421      GGCATCACCCACTCCATCTGCACCCTGGAGTTCGAAAGCCATCGCCCGCTGTATGAGTGG  480
                ||||||||||||| |||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  4111732  GGCATCACCCACTCGATCTGCACCCTGGAGTTCGAAAGCCATCGCCCGCTGTACGAGTGG  4111673

Query  481      TTCCTCGACAGCCTGCCGGTTCCGGCGCACCCGCGTCAGTACGAGTTCAGCCGCCTGAAC  540
                |||||||||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  4111672  TTCCTCGACAGCCTGCCGGTGCCGGCGCACCCGCGTCAGTACGAATTCAGCCGCCTGAAC  4111613

Query  541      CTGAACTACACCATCACCAGCAAGCGCAAGCCTCAAGCAA  580
                |||||||||||||||||||||||||||||| ||||||||
Sbjct  4111612  CTGAACTACACCATCACCAGCAAGCGCAAGC-TCAAGCAA  4111574
```

Figure F.2.1 Screen-print: Blastn search using the forward strain for sequenced GlnS

glutamine--tRNA ligase, partial [Pseudomonadales bacterium RIFCSPLOWO2_12_FULL_59_450]
Sequence ID: OHC41822.1 **Length:** 536 **Number of Matches:** 1

Range 1: 65 to 254 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
404 bits(1039)	9e-138	Compositional matrix adjust.	190/190(100%)	190/190(100%)	0/190(0%)	+1

```
Query  1    SDIKWLGFEWSGEVRYASKYFDQLFDWAVELIKAGKAYVDDLTPEQAKEYRGTLTEPGKN  180
            SDIKWLGFEWSGEVRYASKYFDQLFDWAVELIKAGKAYVDDLTPEQAKEYRGTLTEPGKN
Sbjct  65   SDIKWLGFEWSGEVRYASKYFDQLFDWAVELIKAGKAYVDDLTPEQAKEYRGTLTEPGKN  124

Query  181  SPFRDRSVEENLDWFNRMRAGEFPDGARVLRAKIDMASPNMNLRDPIMYRIRHAHHHQTG  360
            SPFRDRSVEENLDWFNRMRAGEFPDGARVLRAKIDMASPNMNLRDPIMYRIRHAHHHQTG
Sbjct  125  SPFRDRSVEENLDWFNRMRAGEFPDGARVLRAKIDMASPNMNLRDPIMYRIRHAHHHQTG  184

Query  361  DKWCIYPNYDFTHGQSDAIEGITHSICTLEFESHRPLYEWFLDSLPVPAHPRQYEFSRLN  540
            DKWCIYPNYDFTHGQSDAIEGITHSICTLEFESHRPLYEWFLDSLPVPAHPRQYEFSRLN
Sbjct  185  DKWCIYPNYDFTHGQSDAIEGITHSICTLEFESHRPLYEWFLDSLPVPAHPRQYEFSRLN  244

Query  541  LNYTITSKRK  570
            LNYTITSKRK
Sbjct  245  LNYTITSKRK  254
```

Figure F.2.2 Screen-print: Blastx search using the forward strain for sequenced GlnS.

F.3 IleS

Pseudomonas antarctica strain PAMC 27494, complete genome

Sequence ID: CP015600.1 **Length:** 6441449 **Number of Matches:** 1

Range 1: 924805 to 925442 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1051 bits(569)	0.0	616/639(96%)	2/639(0%)	Plus/Plus

```
Query  3       CTGCAGCGCTGGGACAGTATTGGCCTGTACGGAAAGTTGCGCGAAATTGGCAAGGATCGT  62
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924805  CTGCAGCGCTGGGACAGTATTGGCCTGTACGGAAAGTTGCGCGAAATTGGCAAGGATCGT  924864

Query  63      CCGAAGTTCGTCCTGCACGACGGCCCTCCTTATGCCAACGGCACGATTCACATCGGTCAT  122
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924865  CCGAAGTTCGTCCTGCACGACGGCCCTCCTTATGCCAACGGCACGATTCACATCGGTCAT  924924

Query  123     GCGCTGAACAAAATTCTCAAGGACATGATCCTGCGTTCGAAAACCCTGTCGGGCTTCGAC  182
               ||||||||  |||||||||||||||||||||||||  ||||||||||||||||  ||||||
Sbjct  924925  GCGCTGAATAAAATTCTCAAGGACATGATCCTGCGCTCGAAAACCCTGTCGGGTTTCGAC  924984

Query  183     GCGCCTTATGTTCCGGGCTGGGACTGCCACGGCCTGCCGATCGAACACAAAGTCGAAGTG  242
               |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924985  GCGCCGTATGTTCCGGGCTGGGACTGCCACGGCCTGCCGATCGAACACAAAGTCGAAGTG  925044

Query  243     ACCTACGGCAAGAACCTGGGCGCGGATAAAACCCGCGAACTGTGCCGTGCCTACGCCACC  302
               ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  925045  ACCTACGGCAAGAACCTGGGCGCGGATAAGACCCGCGAACTGTGCCGTGCCTACGCCACC  925104

Query  303     GAGCAGATCGAAGGGCAGAAGTCCGAATTCATCCGCCTGGGCGTGCTGGGCGAGTGGGAC  362
               |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  925105  GAGCAGATCGAAGGGCAGAAGTCCGAATTCATCCGCCTGGGCGTGTTGGGCGAGTGGGAC  925164

Query  363     AACCCGTACAAGACCATGAACTTCAAGAACGAGGCCGGTGAAATCCGTGCCTTGGCCGAG  422
               |||||||||||  ||||||||||||||||||||||||||||||||||  ||||||||||||
Sbjct  925165  AACCCGTACAAAACCATGAACTTCAAGAACGAGGCCGGTGAAATCCGCGCCTTGGCCGAA  925224

Query  423     ATCGTCAAAGGCGGTTTCGTGTTCAAGGGCCTCAAGCCCGTGAACTGGTGCTTCGACTGC  482
               ||||||||  ||||||||||||||||||||||||||||||||||||||  |||||||||
Sbjct  925225  ATCGTCAAGGGCGGTTTCGTGTTCAAGGGCCTCAAGCCCGTGAACTGGTGTTTCGACTGC  925284

Query  483     GGTTCGGCCCTGGCTGAAGCGGAAGTCGAGTACGAAGACAAGAAGTCCTCGACCATCGAC  542
               |||||||||||||  ||||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  925285  GGTTCGGCCCTGGCCGAAGCGGAAGTCGAGTACGAAGACAAAAAGTCCTCGACCATCGAC  925344

Query  543     GTGGCCTTCCCGATCGCCGACGACGACAAGCTGGCCCAAGCCTTTGGCCTGTCCAGCCTG  602
               |||||||||||||||||||||||||  ||||||||||  | ||||||||||||  | ||||||
Sbjct  925345  GTGGCCTTCCCGATCGCCGACGACGCCAAGCTGGCCGAGGCCTTTGGCCTGGCGAGCCTG  925404

Query  603     C-CAAAGCCTGCAGCCATCGTGATCTGGACCACCACCCC  640
                 ||||  ||  ||  ||||||||||||||||||||||||||
Sbjct  925405  AGCAAA-CCGGCTGCCATCGTGATCTGGACCACCACCCC  925442
```

Figure F.3.1 Screen-print: Blastn search using the forward strain for sequenced IleS.

MULTISPECIES: isoleucine--tRNA ligase [Pseudomonas]

Sequence ID: WP_017137608.1 **Length:** 943 **Number of Matches:** 1

▶ See 1 more title(s)

Range 1: 29 to 241 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
445 bits(1144)	3e-148	Compositional matrix adjust.	213/213(100%)	213/213(100%)	0/213(0%)	+3

```
Query  3    LQRWDSIGLYGKLREIGKDRPKFVLHDGPPYANGTIHIGHALNKILKDMILRSKTLSGFD  182
            LQRWDSIGLYGKLREIGKDRPKFVLHDGPPYANGTIHIGHALNKILKDMILRSKTLSGFD
Sbjct  29   LQRWDSIGLYGKLREIGKDRPKFVLHDGPPYANGTIHIGHALNKILKDMILRSKTLSGFD  88

Query  183  APYVPGWDCHGLPIEHKVEVTYGKNLGADKTRELCRAYATEQIEGQKSEFIRLGVLGEWD  362
            APYVPGWDCHGLPIEHKVEVTYGKNLGADKTRELCRAYATEQIEGQKSEFIRLGVLGEWD
Sbjct  89   APYVPGWDCHGLPIEHKVEVTYGKNLGADKTRELCRAYATEQIEGQKSEFIRLGVLGEWD  148

Query  363  NPYKTMNFKNEAGEIRALAEIVKGGFVFKGLKPVNWCFDCGSALAEAEVEYEDKKSSTID  542
            NPYKTMNFKNEAGEIRALAEIVKGGFVFKGLKPVNWCFDCGSALAEAEVEYEDKKSSTID
Sbjct  149  NPYKTMNFKNEAGEIRALAEIVKGGFVFKGLKPVNWCFDCGSALAEAEVEYEDKKSSTID  208

Query  543  VAFPIADDDKLAQAFGLSSLPKPAAIVIWTTTP  641
            VAFPIADDDKLAQAFGLSSLPKPAAIVIWTTTP
Sbjct  209  VAFPIADDDKLAQAFGLSSLPKPAAIVIWTTTP  241
```

Figure F.3.2 Screen-print: Blastx search using the forward strain for sequenced IleS.

F.4 RecA

Pseudomonas fluorescens (clone pFAJ2006) recombination enzyme A (recA) gene, complete cds

Sequence ID: M96558.1 **Length:** 1444 **Number of Matches:** 1

Range 1: 222 to 718 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
894 bits(484)	0.0	493/497(99%)	1/497(0%)	Plus/Plus

```
Query  3    GGTGCCGT-ATGCGTATGGGCGATCACGACCGTCAGGCGATCCCGGCTATTTCCACTGGC  61
            |||||||| |||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  222  GGTGCCGTAATGCGTATGGGCGATCACGACCGTCAAGCGATCCCGGCTATTTCCACTGGC  281

Query  62   TCTCTGGGTCTGGACATCGCACTCGGCATTGGCGGCCTGCCAAAAGGCCGTATCGTTGAA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  TCTCTGGGTCTGGACATCGCACTCGGCATTGGCGGCCTGCCAAAAGGCCGTATCGTTGAA  341

Query  122  ATCTACGGTCCTGAATCTTCCGGTAAAACCACCCTGACCCTGTCGGTGATTGCCCAGGCG  181
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  342  ATCTACGGTCCTGAATCTTCCGGTAAAACCACCCTGACCCTGTCGGTGATTGCCCAAGCG  401

Query  182  CAAAAAATGGGCGCCACCTGTGCGTTCGTCGACGCCGAGCACGCCCTGGACCCGGAATAC  241
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  402  CAAAAAATGGGCGCCACCTGTGCGTTCGTCGACGCCGAGCACGCCCTTGACCCGGAATAC  461

Query  242  GCCGGTAAGCTGGGCGTCAACGTTGACGACCTGCTGGTTTCCCAGCCGGACACCGGTGAG  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  GCCGGTAAGCTGGGCGTCAACGTTGACGACCTGCTGGTTTCCCAGCCGGACACCGGTGAG  521

Query  302  CAAGCCCTGGAAATCACCGACATGCTGGTGCGCTCCAACGCCATCGACGTGATCGTGGTC  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522  CAAGCCCTGGAAATCACCGACATGCTGGTGCGCTCCAACGCCATCGACGTGATCGTGGTC  581

Query  362  GACTCCGTGGCTGCCCTGGTACCGAAAGCTGAAATCGAAGGCGAAATGGGCGACATGCAC  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  582  GACTCCGTGGCTGCCCTGGTACCGAAAGCTGAAATCGAAGGCGAAATGGGCGACATGCAC  641

Query  422  GTGGGCCTGCAAGCCCGCCTGATGTCCCAGGCGCTGCGTAAAATTACCGGTAACATCAAG  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642  GTGGGCCTGCAAGCCCGCCTGATGTCCCAGGCGCTGCGTAAAATTACCGGTAACATCAAG  701

Query  482  AACGCCAACTGCCTGGT  498
            |||||||||||||||||
Sbjct  702  AACGCCAACTGCCTGGT  718
```

Figure F.4.1 Screen-print: Blastn search using the forward strain for sequenced RecA

RecA, partial [Pseudomonas sp. R11-23-07]

Sequence ID: ACT64201.1 **Length:** 186 **Number of Matches:** 1

▶ See 9 more title(s)

Range 1: 10 to 174 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
268 bits(686)	1e-89	Compositional matrix adjust.	163/165(99%)	163/165(98%)	0/165(0%)	+2

```
Query  11   MRMGDHDRQAIPAISTgslgldialgigglPKGRIVEIYGPESSGKTTLTLSVIAQAQKM  190
            MRMGDHDRQAIPAISTGSLGLDIALGIGGLPKGRIVEIYGPESSGKTTLTLSVIAQAQKM
Sbjct  10   MRMGDHDRQAIPAISTGSLGLDIALGIGGLPKGRIVEIYGPESSGKTTLTLSVIAQAQKM  69

Query  191  GATCAFVDAEHALDPEYAGKLGVNVDDLLVSQPDTGEQALEITDMLVRSNaidvivvdsv  370
            GATCAFVDAEHALDPEYAGKLGVNVDDLLVSQPDTGEQALEITDMLVRSNAIDVIVVDSV
Sbjct  70   GATCAFVDAEHALDPEYAGKLGVNVDDLLVSQPDTGEQALEITDMLVRSNAIDVIVVDSV  129

Query  371  aalvPKAEIEGEMGDMHVGLQARLMSQALRKITGNIKNANCLVTH  505
            AALVPKAEIEGEMGDMHVGLQARLMSQALRKITGNIKNANCLV
Sbjct  130  AALVPKAEIEGEMGDMHVGLQARLMSQALRKITGNIKNANCLVIF  174
```

Figure F.4.2 Screen-print: Blastx search using the forward strain for sequenced RecA.

F.5 RpoB

Pseudomonas tolaasii strain 2192T genome
Sequence ID: CP020369.1 **Length:** 6856683 **Number of Matches:** 1

Range 1: 6691609 to 6692177 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1011 bits(547)	0.0	562/569(99%)	1/569(0%)	Plus/Minus

Features: DNA-directed RNA polymerase subunit beta

```
Query  3        ATGCCGGTTG-AGACATGCCGCACGATGCCAATGGCACCCCGGTCGACGTCGTCCTCAAC  61
                |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6692177  ATGCCGGTTGAAGACATGCCGCACGATGCCAATGGCACCCCGGTCGACGTCGTCCTCAAC  6692118

Query  62       CCGTTGGGCGTACCTTCGCGTATGAACGTTGGTCAGATCCTCGAAACCCACCTGGGCCTC  121
                |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  6692117  CCGTTGGGCGTACCTTCGCGTATGAACGTTGGTCAGATCCTTGAAACCCACCTGGGCCTC  6692058

Query  122      GCGGCCAAAGGTCTGGGCGAGAAGATCAACCGTATGATCGAAGAGCAGCGCAAGGTTGCT  181
                |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  6692057  GCGGCCAAAGGTCTGGGCGAGAAGATCAACCGCATGATCGAAGAGCAGCGCAAGGTTGCT  6691998

Query  182      GACCTGCGTAAGTTCCTGCACGAGATCTACAACGAGATCGGCGGTCGCAACGAAGAGCTG  241
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6691997  GACCTGCGTAAGTTCCTGCACGAGATCTACAACGAGATCGGCGGTCGCAACGAAGAGCTG  6691938

Query  242      GACACCTTCTCCGACCAGGAAATCCTGGACCTGGCGAAGAACCTGCGCGGCGGCGTTCCA  301
                |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  6691937  GACACCTTCTCCGACCAGGAAATCCTGGACCTGGCGAAAAACCTGCGCGGCGGCGTTCCA  6691878

Query  302      ATGGCTACCCCGGTGTTCGACGGTGCCAAGGAAAGCGAAATCAAGGCCATGCTGAAACTG  361
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6691877  ATGGCTACCCCGGTGTTCGACGGTGCCAAGGAAAGCGAAATCAAGGCCATGCTGAAACTG  6691818

Query  362      GCAGACCTGCCGGAAAGCGGCCAGATGCAGCTGTTCGACGGCCGTACCGGCAACAAGTTT  421
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6691817  GCAGACCTGCCGGAAAGCGGCCAGATGCAGCTGTTCGACGGCCGTACCGGCAACAAGTTC  6691758

Query  422      GAGCGCCCGGTTACTGTTGGCTACATGTACATGCTGAAGCTGAACCACTTGGTAGACGAC  481
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6691757  GAGCGCCCGGTTACTGTTGGCTACATGTACATGCTGAAGCTGAACCACTTGGTAGACGAC  6691698

Query  482      AAGATGCACGCTCGTTCTACCGGTTCGTACAGCCTGGTTACCCAGCAGCCGCTGGGTGGT  541
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6691697  AAGATGCACGCTCGTTCTACCGGTTCGTACAGCCTGGTTACCCAGCAGCCGCTGGGTGGT  6691638

Query  542      AAGGCTCAGTTCGGTGGTAGGCGTTTCGG  570
                |||||||||||||||||  |||||||||
Sbjct  6691637  AAGGCTCAGTTCGGTGGTCAGCGTTTCGG  6691609
```

Figure F.5.1 Screen-print: Blastn search using the forward strain for sequenced RpoB.

DNA-directed RNA polymerase beta chain, partial [Pseudomonas mediterranea]
Sequence ID: ATD84645.1 **Length:** 481 **Number of Matches:** 1

Range 1: 245 to 430 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
375 bits(962)	7e-127	Compositional matrix adjust.	180/186(97%)	185/186(99%)	0/186(0%)	+2

```
Query  14   DMPHDANGTPVDVVLNPLGVPSRMNVGQILETHLGLAAKGLGEKINRMIEEQRKVADLRK  193
            DMPHDANGTPVDVVLNPLGVPSRMNVGQILETHLGLAAKGLGEKINRM+EEQRKVADLRK
Sbjct  245  DMPHDANGTPVDVVLNPLGVPSRMNVGQILETHLGLAAKGLGEKINRMLEEQRKVADLRK  304

Query  194  FLHEIYNEIGGRNEELDTFSDQEILDLAKNLRGGVPMATPVFDGAKESEIKAMLKLADLP  373
            FLHEIYNEIGGRNE+LD+FSDQEILDLAKNL+GGVPMATPVFDGAKE EIKAMLKLADLP
Sbjct  305  FLHEIYNEIGGRNEDLDSFSDQEILDLAKNLKGGVPMATPVFDGAKEVEIKAMLKLADLP  364

Query  374  ESGQMQLFDGRTGNKFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQQPLGGKAQF  553
            ESGQMQLFDGRTGNKFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQQPLGGKAQF
Sbjct  365  ESGQMQLFDGRTGNKFERPVTVGYMYMLKLNHLVDDKMHARSTGSYSLVTQQPLGGKAQF  424

Query  554  GGRRFG  571
            GG+RFG
Sbjct  425  GGQRFG  430
```

Figure F.5.2 Screen-print: Blastx search using the forward strain for sequenced RpoB.

F.6 RpoD

Pseudomonas sp. R76 RNA polymerase sigma factor (rpoD) gene, complete cds
Sequence ID: KT890334.1 **Length:** 1851 **Number of Matches:** 1

Range 1: 1262 to 1849 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1040 bits(563)	0.0	580/588(99%)	1/588(0%)	Plus/Plus

```
Query  1     AGTTCGAATACCGTCGCGGCTAC-AGTTCTCGACTTATGCCACCTGGTGGATCCGTCAGG  59
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1262  AGTTCGAATACCGTCGCGGCTACAAGTTCTCGACTTATGCCACCTGGTGGATCCGTCAGG  1321

Query  60    CGATCACTCGCTCGATCGCCGACCAGGCTCGCACCATCCGTATTCCGGTGCACATGATCG  119
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1322  CGATCACTCGCTCGATCGCCGACCAGGCCCGCACCATCCGTATTCCGGTGCACATGATCG  1381

Query  120   AGACGATCAACAAGCTCAACCGTATTTCCCGGCAGATGTTGCAGGAAATGGGTCGCGAAC  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1382  AGACGATCAACAAGCTCAACCGTATTTCCCGGCAGATGTTGCAGGAAATGGGTCGCGAAC  1441

Query  180   CGACCCCGGAAGAGCTGGGCGAACGCATGGAAATGCCTGAGGATAAAATCCGCAAGGTAT  239
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1442  CGACCCCGGAAGAGTTGGGCGAACGCATGGAAATGCCTGAGGATAAAATCCGCAAGGTAT  1501

Query  240   TGAAGATCGCTAAAGAGCCGATCTCCATGGAAACGCCGATTGGTGATGACGAAGACTCCC  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1502  TGAAGATCGCTAAAGAGCCGATCTCCATGGAAACGCCGATTGGTGATGACGAAGACTCCC  1561

Query  300   ACCTGGGTGACTTCATCGAAGACTCGACCATGCAGTCGCCAATCGATGTCGCCACTGTTG  359
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1562  ATCTGGGTGACTTCATCGAAGACTCGACCATGCAGTCGCCAATCGATGTCGCCACTGTTG  1621

Query  360   AGAGCCTTAAAGAAGCGACTCGCGACGTACTGTCCGGCCTCACTGCCCGTGAAGCCAAGG  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1622  AGAGCCTTAAAGAAGCGACTCGCGACGTACTGTCCGGCCTCACTGCCCGTGAAGCCAAGG  1681

Query  420   TACTGCGCATGCGTTTCGGCATCGACATGAATACCGACCACACCCTTGAGGAAGTCGGTA  479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1682  TACTGCGCATGCGTTTCGGCATCGACATGAATACCGACCACACCCTTGAGGAAGTCGGTA  1741

Query  480   AGCAGTTTGACGTGACCCGCGAGCGGATCCGTCAGATCGAAGCCAAGGCACTGCGCAAGT  539
             |||||||||||||||| || ||||||||||||||||||||||||| ||||||||||
Sbjct  1742  AGCAGTTTGACGTGACGCGTGAGCGGATCCGTCAGATCGAAGCCAAGGCGCTGCGCAAGT  1801

Query  540   TGCGCCACCCGACGCGAAGCGAGCATCTGCGCTCCTTCTTCGACGAGT  587
             ||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  1802  TGCGCCACCCGACGCGAAGCGAGCATCTGCGCTCCTTCCTCGACGAGT  1849
```

Figure F.6.1 Screen-print: Blastn search using the forward strain for sequenced RpoD

Sigma-70 [Pseudomonas sp. 22 E 5]
Sequence ID: CRM93685.1 **Length:** 249 **Number of Matches:** 1
▶ See 1 more title(s)

Range 1: 62 to 249 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
381 bits(979)	1e-132	Compositional matrix adjust.	186/188(99%)	187/188(99%)	0/188(0%)	+2

```
Query  23   QFSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLNRISRQMLQEMGREPTPEELGE  202
            +FSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLNRISRQMLQEMGREPTPEELGE
Sbjct  62   KFSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLNRISRQMLQEMGREPTPEELGE  121

Query  203  RMEMPEDKIRKVLKIAKEPISMETPIGDDEDSHLGDFIEDSTMQSPIDVATVESLKEATR  382
            RMEMPEDKIRKVLKIAKEPISMETPIGDDEDSHLGDFIEDSTMQSPIDVATVESLKEATR
Sbjct  122  RMEMPEDKIRKVLKIAKEPISMETPIGDDEDSHLGDFIEDSTMQSPIDVATVESLKEATR  181

Query  383  DVLSGLTAREAKVLRMRFGIDMNTDHTLEEVGKQFDVTRERIRQIEAKALRKLRHPTRSE  562
            DVLSGLTAREAKVLRMRFGIDMNTDHTLEEVGKQFDVTRERIRQIEAKALRKLRHPTRSE
Sbjct  182  DVLSGLTAREAKVLRMRFGIDMNTDHTLEEVGKQFDVTRERIRQIEAKALRKLRHPTRSE  241

Query  563  HLRSFFDE  586
            HLRSF DE
Sbjct  242  HLRSFLDE  249
```

Figure F.6.2 Screen-print: Blastx search using the forward strain for sequenced RpoD.

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway