Purification and characterization of a salt tolerant metallo-beta-lactamase from *Aliivibrio salmonicida*

KJE-3900

Anders Kristiansen

Master's Thesis in Chemistry

Faculty of Science and Technology

University of Tromsø

July, 2011

Acknowledgements

I wish to thank the staff at Norstruct for being helpful and patient with me. Especially I wish to thank my supervisor Ingar Leiros and co-supervisor Bjørn Altermark for making this thesis possible, and also wish to thank Miriam Grgic for introducing me to the world of biochemistry laboratory work. Last, but not the least, I wish to thank my parents, my brother, my grandparents, friends, fellow students at the office and Eili for keeping me sane during this insane time.

Tromsø, July 2011

Anders Kristiansen

Abbreviations

Δε	: Extinction coefficient
6-APA	: 6-aminopenicillanic acid
7-ACA	: 7-Aminocephalosporanic acid
A. salmonicida	: *Aliivibrio salmonicida*
asMBL	: *Aliivibrio Salmonicida* metallo-beta-lactamase
AU	: Absorption Unit
B. subtilis	: *Bacillus subtilis*
BBL	: Standard numbering scheme for class B beta-lactamases
BLAST	: Basic Local Alignment Search Tool
BSA	: Bovine Serum Albumin
CV	: Column Volume
DHF	: Dihydrofolate
DLS	: Dynamic Light Scattering
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide
DTT	: Dithiothreitol
E. coli	: *Escherichia coli*
EARSS	: European Antimicrobial Resistance Surveillance System
EDTA	: Ethylenediaminetetraacetic acid
EEA	: European Economic Area
EFTA	: European Free Trade Association
ESBL	: Extended-spectrum beta-lactamase
EU	: European Union
ExPASy	: Expert Protein Analysis System
Hepes	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
k_{cat}	: Turnover number used in Michaelis-Menten kinetics
K_m	: Dissociation constant for enzyme-substrate (ES) complex used in Michaelis-Menten kinetics
LB	: Lysogeny Broth
LD	: Loading Dye
MBL	: Metallo-beta-lactamase
Mes	: 2-(*N*-morpholino)ethanesulfonic acid
MIC	: Minimum inhibitory concentration
MTEN	: Buffer consisting of Tris, ethanolamine, Mes and NaCl
MW	: Molecular Weight
nt	: Nucleotide
NOK	: Norwegian Kroner
OD	: Optical density
P. aeruginosa	: *Pseudomonas aeruginosa*
PABA	: Para-aminobenzoic acid

PBP	: Penicillin Binding Protein
PCR	: Polymerase Chain Reaction
PDB	: Protein Data Bank
pK_a	: Acid dissociation constant
ppMBL	: *Photobacterium profundum*
RNA	: Ribonucleic acid
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	: Standard deviation
SI	: Sub-inhibitory
ssMBL	: *Shewanella sediminis*
TAE	: Tris-acetate-EDTA
TCEP	: Tris(2-carboxyethyl)phosphine
THF	: Tetrahydrofolate
Tris	: Tris(hydroxymethyl)aminoethane
vfMBL	: *Vibrio fischeri*
VIM	: Verona integrin-encoded metallo-beta-lactamase

Abstract

Beta-lactamases are enzymes that inactivate beta-lactam antibiotics by hydrolyzing the amide bond that exists in the beta-lactam ring, disrupting the ring structure and make the antibiotics nonfunctional against bacteria. Metallo-beta-lactamases (MBLs) are a group of beta-lactamases which needs metal ions bound to its active site in order to function and are an important factor in many bacteria in order to be resistant to antibiotics. Resistance to antibiotics is a serious health problem which increases with excessive use of antibiotics. This study is based on the purification and characterization of MBL from the psychrophilic organism *Aliivibrio salmonicida* which is the causative agent for the Hitra disease, or cold-water vibrosis, in seawater-farmed Atlantic salmon (*Salmo salar*). Characterization of asMBL has revealed interesting properties in regards of temperature-, salt-, pH optimum and enzyme kinetics. asMBL properties have been compared to VIM-7, a MBL isolated from hospital which thrives in mesophilic conditions. Compared to VIM-7, asMBL proves to be adapted to the colder and saltier environment of the seawater. asMBL enzyme kinetics have also been compared to MBLs from human pathogenic bacteria and asMBL showed a general lower enzymatic efficiency in terms of lower k_{cat} and higher K_m at the same experimental conditions as the other MBLs. Nitrocefin, the substrate used for the characterization of asMBL, had its stability in temperature, pH and Tris investigated. From these investigations it seems plausible that nitrocefin is auto-hydrolyzed in a synergetic matter when present at high pH and in Tris buffer. An attempt to crystallize the protein and retrieve a structure was unsuccessful as it turns out that the protein seems to be aggregating at high concentrations.

Keywords

Marine environment adaption; salt tolerance; nitrocefin stability; enzyme characterization; purification; metallo-beta-lactamase; temperature stability; experimental parameters; enzyme kinetics; *Aliivibrio salmonicida.*

Contents

Aim of study

First and foremost the goal of this study was to produce pure asMBL in high enough quantity to crystallize it and ultimately solve the structure of the protein.

As asMBL originates from a marine organism living in colder and saltier condition compared to organisms inhabiting human environment, it was interesting to see how enzymes from these two environments would be different. To clarify these apparent differences, the aim was to characterize asMBL optimal conditions in terms of pH, temperature, salt and kinetic studies. As the study went on, an interest of how different experimental parameters affect each other arose.

Introduction

Beta-lactamases – classification

Metallo-beta-lactamases belongs to the protein family of beta-lactamases, a group of enzymes which breaks open the ring structure of beta-lactam antibiotic and deactivates it. There are approximately 900 sequences of beta lactamases which have been reported, and of these, about 80 sequences of metallo-beta-lactamases have been reported (*1, 2*). From figure 1 we can observe how the discovery of unique beta-lactamases has been throughout the years.

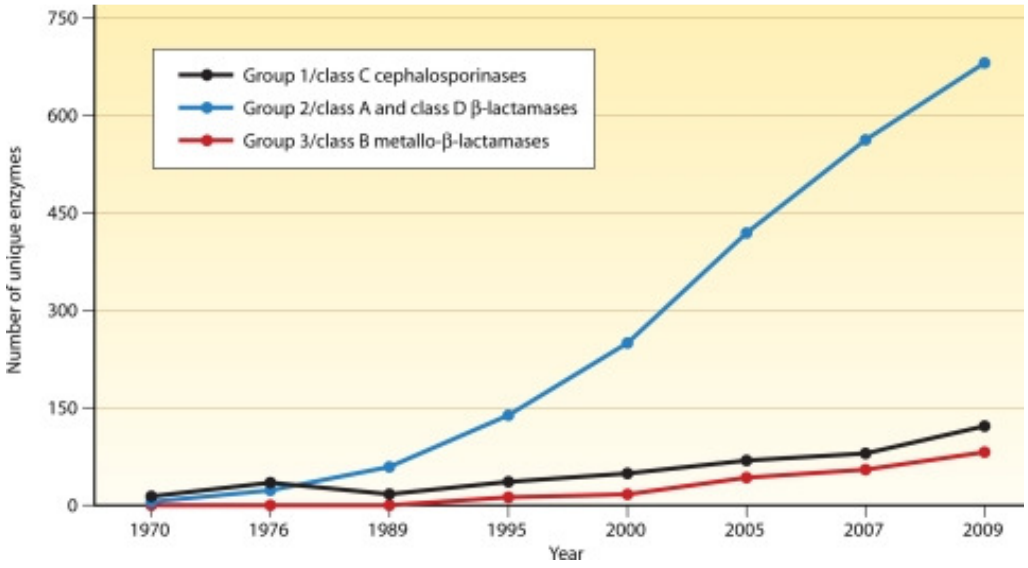

Figure 1 The discovery of different beta-lactamases over the years. Figure retrieved from Bush et al (*1*).

Beta-lactamases are being classified in two different ways; functional classification, which emphasizes on the substrates and inhibitors of the protein, and molecular classification, which is based on the amino acid sequence of the proteins.

Functional classification

All beta-lactamases are divided into three different functional groups by Bush et al (*1*).

Group 1 beta-lactamases is termed cephalosporinases. These beta-lactamases prefers cephalosporin substrates and is not inhibited by EDTA or clavulanic acid and/or tazobactam. Representatives for this group are ACT-1 (*3*), FOX-5 (*4*) and AmpC ADC-33 (*5*).

Group 2 is by far the largest group of beta-lactamases, consisting of serine beta-lactamases. This group of beta-lactamases consists of many subgroups (2a, 2b, 2be, 2br…) and their preferred substrates range from penicillins, cephalosporins, monobactams, carbenicillin, cefepime, cloxacillin and carbapenems. These enzymes will not be inhibited by EDTA, but most of them are inhibited by clavulanic acid and/or tazobactam. Examples of group 2 enzymes are TEM-135 (*6*), OXA-1 (*7*), and cepA (*8*).

Group 3 consists of MBLs which is the group that the beta-lactamase from *A. salmonicida*, asMBL, belongs to. This group's generally preferred substrate is carbapenems. MBLs will not be inhibited by clavulanic acid and/or tazobactam, but will be inhibited by EDTA, because of the chelating properties of EDTA.

The group 3 beta-lactamases is divided into two subgroups, 3a and 3b, where 3a consists of MBLs having a broad range of different substrate which it can hydrolyze, while 3b MBLs mainly hydrolyze carbapenems. Enzymes belonging to the group 3a are the likes of IMP-19 (*9*), IND-5 (*10*) and VIM-19 (*11*). Subgroup 3b is smaller than 3a, and consists of for instance CphA from *Aermonas hydrophilia (12)* and Sfh-1 (*13*).

Molecular classification

Ambler (*14*) proposed a molecular classification of beta-lactamases based on sequence similarity in 1980. This classification divides the beta-lactamases into 4 groups; A, B, C and D. This classification has similarities with the functional classification, as the molecular groups A and D are the same as the functional group 2 (serine beta-lactamases), the molecular group B corresponds to the functional group 3 (metallo-beta-lactamases) and the molecular group C corresponds to the functional group 1 (cephalosporinases).

However, when in the functional classification, the metallo-beta-lactamases are divided into 2 subgroups, in the molecular classification the metallo-beta-lactamases are divided into 3 subgroups; B1, B2 and B3. Even though B1 and B3 belong to two different subgroups and have weak sequence similarity, they have a high degree of structural similarity (*15*). Both B1- and B3-enzymes need two zincs bound for maximum activity, while B2-enzymes will be inhibited if two zincs are bound to it (*16*).

MBL structure

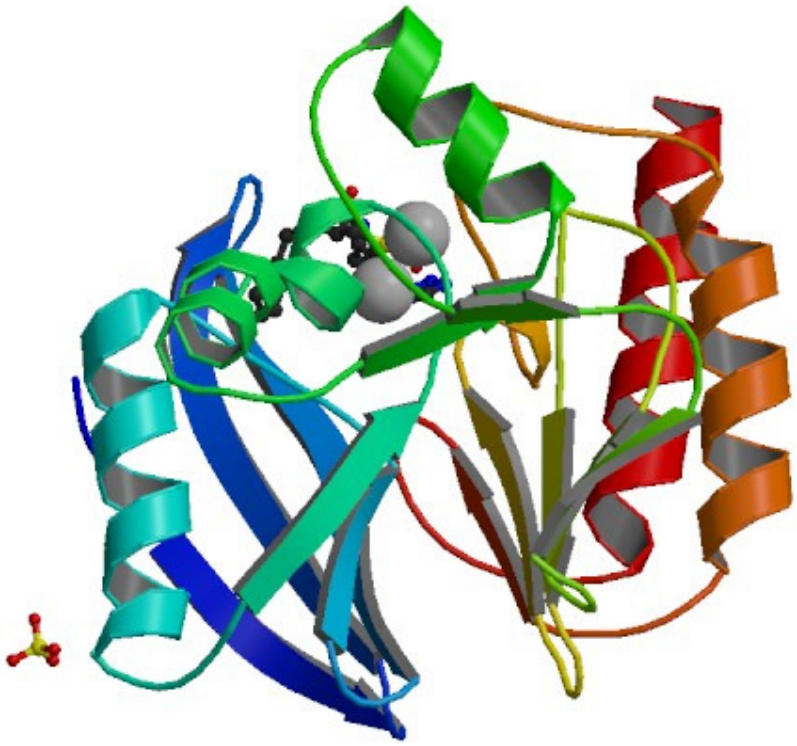

Figure 2 The structure of IMP-1, a similar MBL belonging to the molecular class B1. The enzyme is in complex with a mercaptocarboxylate inhibitor. The inhibitor and the two zinc ions indicate the whereabouts of the active site of MBL. This figure is retrieved from RCSB of PDB ID: 1DD6 (*17*).

Metallo-beta-lactamases have fairly similar structures which consists of an αββα structure composed of two central beta-sheets and 5 solvent-exposed alfa-helices (see figure 2) (*18*). From observations of active site orientation of all known structures it is concurrent that the active site of MBLs is to be residing in the external edge of the ββ-sandwich. It has been suggested that he structure of metallo-beta-lactamases arose from a duplication of a gene (*19*), since the N-terminal and C-terminal parts of the molecule can be superposed by a 180 rotation around a central axis. The N-terminal of B1 metallo-beta-lactamases includes a loop (residues 61-65) that is not present in subclasses B2 or B3 (*18*), and is important for the activity of the enzyme (*20*).

The active site consists of two zinc-binding regions which are conserved among the class B1, B2 and B3 metallo-beta-lactamases (table 1).

Table 1 Numbering of the ligands binding Zn2+ ions in the different metallo-beta-lactamases molecular groups based on the work of Garau et al 2004. BBL = Standard numbering scheme for class B beta-lactamases.

Functional group	Zn1 ligand			Zn2 ligand		
Consensus BBL B1	His116	His118	His196	Asp120	Cys221	His263
Consensus BBL B2	Asn116	His118	His196	Asp120	Cys221	His263
Consensus BBL B3	His/Gln116	His118	His196	Asp120	His121	His263

Most MBLs are functional active as dizinc compounds, but enzymes belonging to the B2 subgroup are active as a monozinc compound and will be inhibited if two zinc ions are bound to it (*21*). The characteristics and importance of binding of zinc to the two zinc binding regions vary among the different MBLs, where the dizinc and monozinc component are discussed (*22-24*).

In B1 enzymes the binding of zinc is believed to be similar to how it is in BcII from *Bacillus Cereus (25)*. The Zn1 metal is tetracoordinated by the three histidines (His116, His118 and His196) and a water molecule (figure 3). The Zn2 metal is pentacoordinated by His263, Asp120, Cys221 and a water molecule which is bridging the two metals. The fifth ligand to the Zn2 metal is a carbonate (*25*) or a water molecule (*26*). The water molecule bridging the two zinc ions is believed to be a hydroxide molecule which is a strong nucleophile (*26*).

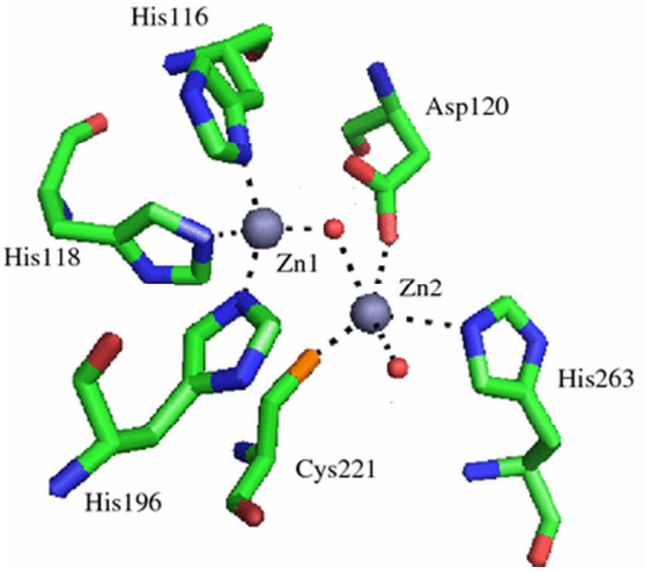

Figure 3 Active site of dizinc BcII, illustrating electrostatic interactions between zinc ions and MBL. The zinc ions are the big grey spheres while the small, red spheres are water molecules. The hydroxide bridging the two zinc ions is available at a pK_a value of less than 5.25 (*27*). Figure is retrieved from Baderau et al (*28*).

The mechanism on how MBL performs its hydrolysis has not been agreed upon. A suggestion on how a dizinc MBL performs its catalytic activity proposed by Wang et al follows (*27*) (figure 4):

The reaction's first step is that the nitrogen bound on the substrate interacts with Zn2 and displaces the water previously bound. Zn1 together with Asn-193 acts like an oxyanion hole for the carbonyl group of the beta-lactam compound. The hydroxide bound to Zn1 acts as a nucleophile and disrupts the C-N bond. Zn2 ion stabilizes the intermediate by electrostatic interactions on the charged nitrogen. The intermediate is being transformed to product by protonation of the negatively charged nitrogen and the enzyme is regenerated by ligand exchange of Zn1 metal ion in which a water molecule replaces the acyl group.

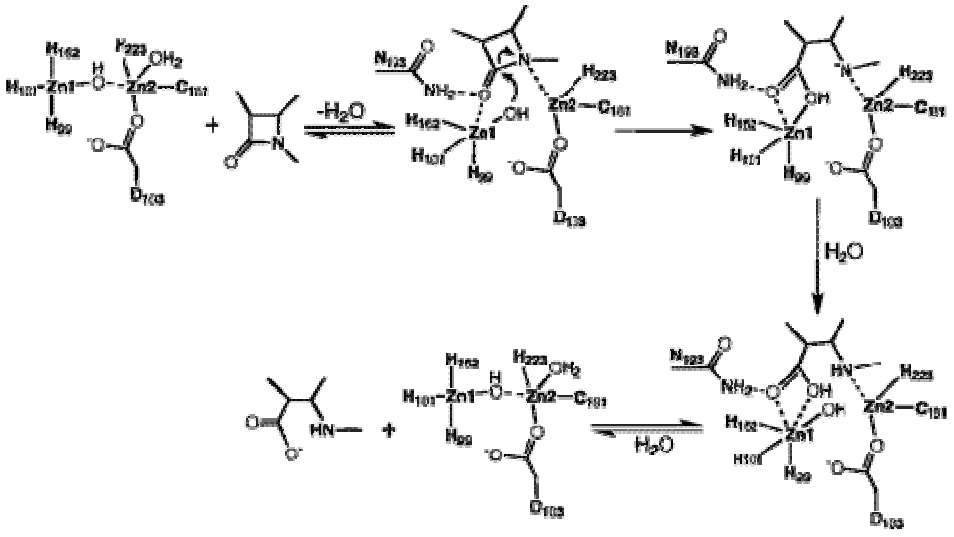

Figure 4 Mechanism of dizinc MBL as in the case of BcII from *Bacteroides fragilis*. Figure retrieved from Wang et al (*27*).

Aliivibrio salmonicida

The MBL studied in this report is originating from *Aliivibrio salmonicida* which is a gram-negative, motile and rod-shaped bacterium, previously known as *Vibrio salmonicida* (*29*). It is found in marine environment and is often associated with fish; some species have been regarded as pathogens of marine animals as for instance the causative agent of Hitra disease or cold-water vibrosis. This is a condition that appeared in the late 1970s in Norwegian fish farms and has most exclusively been recognized in farmed Atlantic salmon (*Salmo salar L.*), but this serious problem was solved through vaccination (*30*).

Enzyme activity, cold adaption and salinity

Enzyme activity is influenced by many factors. Salt concentration, temperature, pH and substrate saturation are some of the most important parameters and these change according to the type of protein and what environment it belongs to. In the case of MBL from *A. salmonicida*, the environment which surrounds this protein is seawater because this protein resides in the periplasm of the cell or is secreted further to the extracellular environment. It is reasonable to think that such secreted enzymes are very much affected by the surroundings of the bacteria and have through evolution adapted to function optimally here.

Coastal seawater has a salinity of about 3.5% and contains about 470 mM Na^+ and 540 mM Cl^- (*31*). Another important component in seawater related to MBL is the content of Zn^{2+},

which is 0,005 ppm or 76 nM (*31*). The pH in seawater is around 8.0 to 8.2 (*32*), but could vary according to special local conditions. Based on numbers presented by *Institute of Marine Research* from their hydrographic station in Eggum, the coastal average temperature in the Norwegian Sea can be estimated to be between 5 to 6 ^{0}C in January and between 10 and 11^{0}C in July (*33*).

Tolerance to salt and low temperature is features of the exposed marine proteins, which show how they are adapted to their environment (*34*). Halophilic enzymes, enzymes adapted to high concentrations of salt are, for instance characterized by an abundance of acidic amino acids which is believed to increase the proteins aqueous solubility and protect it from the salting out effect (*35*). Another feature of most halophilic proteins is that they are inactivated by salt concentrations of less than 2 M NaCl or KCl (*36, 37*). Temperature adaption is seen as a higher flexibility of structure seen in cold adapted enzymes. Higher flexibility leads to an unfolding of the protein at lower temperatures and higher specific activity at low temperatures compared to mesophilic enzymes. In terms of enzyme kinetics, cold adapted enzymes have a high k_{cat} and high K_m (*36*).

Salt influences the protein by neutralizing charges on the protein surface, and as a consequence of this, stabilize the protein (*38*). The stabilization of the protein is also a result of a gain in hydrophobic effect, which strengthens the folded state of the protein (*38*). Even though a decrease of positive electric interactions also occurs when charges are neutralized, this seems not to affect the positive net effect the salt has on the stability of the protein.

Michaelis-Menten kinetics

In order to study enzyme kinetics in regards of asMBL's hydrolysis of beta-lactam substrates, the Michaelis-Menten model is used to describe the enzymatic reactions. The model relates reaction rate of the enzyme (V_0) to the concentration of substrate [S] according to this formula:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

V_{max} and K_m are kinetic constants, in which V_{max} is defined as the maximum reaction rate of the enzyme and K_m is defined as the substrate concentration in which the enzyme has a reaction rate of half V_{max}. K_m indicates how efficiently an enzyme selects its substrate and

converts it to product. V_{max} is reached as the substrate concentration reaches infinity, which means that the reaction rate (V_0) asymptotically reaches V_{max} (figure 5). This can be easily visualized when letting [S] go to infinity, the value of K_m could be neglected, as it is a constant. [S] above and below the fraction line cancel each other out, which leaves us with $V_0=V_{max}$. This relation between [S] and reaction rate tells us about how the efficiency of the enzyme increases as the enzyme is saturated with substrate.

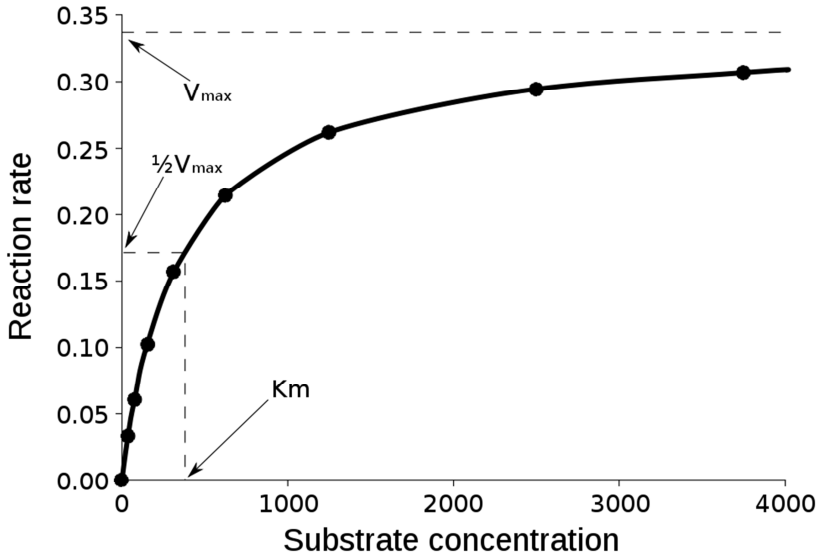

Figure 5 V_{max} and K_m is illustrated according to the Michalis-Menten model; the numbers on the x-axis and y-axis are examples of substrate concentrations and reaction rates. The figure is retrieved from Wikipedia (*39*).

The basic model (figure 6) which the Michaelis-Menten kinetics is based on is explained as; the enzyme (E) binds reversibly to substrate (S) to form a complex (ES) which eventually converts into product P and regeneration of the enzyme. Two assumptions are made in order to convert this model into an analysis of K_m and V_{max}; the concentration of ES is steady during the time interval of the kinetic reaction and the formation of ES from E+P is negligible, because the concentrations of product is low at the start of the reaction.

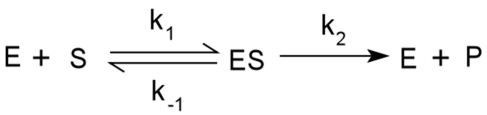

Figure 6 Basic model for enzyme kinetics

k_1, k_{-1} and k_2 are rate constants that respectively tell the rate of complex (ES) formation, the reverse formation of substrate from complex and product formation. For this type of simple

reaction $k_{cat}=k_2$, and can be termed as the turnover number as it indicates how many number of cycles that each active site undergoes per unit time. K_{cat} is related to V_{max} and enzyme concentration (E) by this formula:

$$k_{cat} = \frac{V_{max}}{E}$$

When analyzing the result of Michaelis-Menten kinetics an indication for a better performing enzyme is raised k_{cat} and decreased K_m. In order to estimate the enzyme's overall performance k_{cat}/K_m, in which a raised value is positive indicator for the enzyme's overall kinetic capability.

Antibiotics

A definition of antibiotics as suggested by Waksman (*40*), is as follows; An antibiotic is a chemical substance which has an antagonistic effect on an microbial environment. Antibiotics are often divided into two groups depending on their impact on the microbial target; i) bacteriostatic antibiotics – which halts bacterial growth and ii) bactericidal antibiotics – which kills the bacteria. The efficacy of antibiotics is often measured as MIC (minimal inhibitory concentration), which is the lowest concentration of antibiotics which inhibits the growth of a microorganism after overnight incubation.

Antibiotics are being classified according to three main properties; i) The target on the bacteria cell for its antibacterial effect (figure 7) ii) The specificity of the antibiotics (spectrum) iii) The chemical structure of the antibiotic.

A broad spectrum antibiotic works against many different bacteria, which makes this antibiotic precious considering resistance to antibiotics. Therefore the broad spectrum antibiotic is not administered as a first line of medication against bacterial infection. A narrow spectrum antibiotic is rather given as a first-line treatment, while the broad spectrum antibiotic is not given unless if it is absolutely necessary.

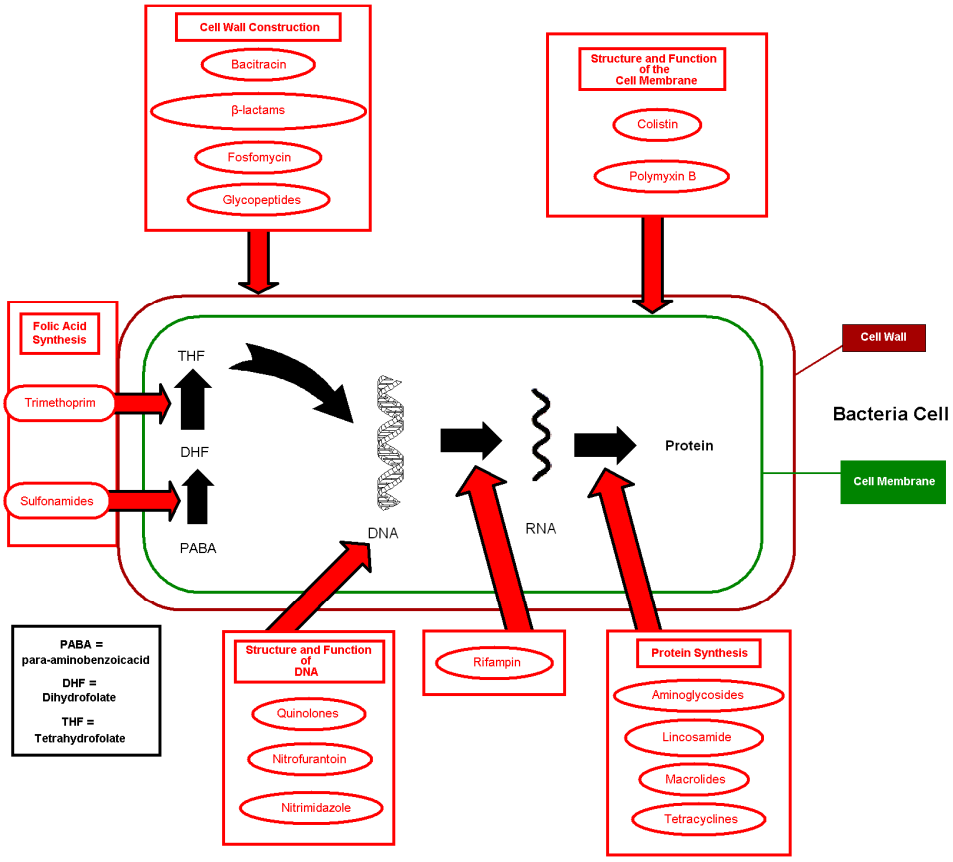

Figure 7 Different antibiotics and their antibiotic function. Retrieved from Wikipedia (*41*).

As seen from figure 7, there are a lot of different types of antibiotics. Some of the most used antibiotics are the beta-lactams, macrolides and quinolones.

Quinolones

Quinolones are recognized by their heterobicyclic aromatic compound which often has a fluoride substitute (see figure 8), which makes it a fluoroquinolone. Quinolones work by blocking DNA replication leading to cell death, by forming complexes with DNA and either DNA gyrase or Topoisomerase IV (*42*). Compounds have, in addition to their antimicrobial effects, shown potential effect in treatment of cancer (*43*).

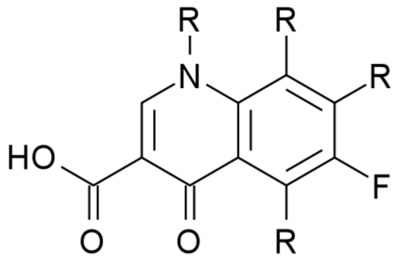

Figure 8 The backbone of quinolones.

Macrolides

Macrolides are a structurally diverse group of antibiotics which are recognizable by their macrocyclic lactam ring (figure 9).The most important macrolide antibiotics are 14, 15 and 16 membered ring compounds (*44*). The mechanism for the macrolides' antibacterial effect is by inhibiting the protein synthesis by binding to the bacterial 50S ribosome subunit. Macrolides are used in the treatment of upper and lower respiratory tract infections caused by bacteria (*45*) and have also been seen as potential anti-inflammatory medication (*46*).

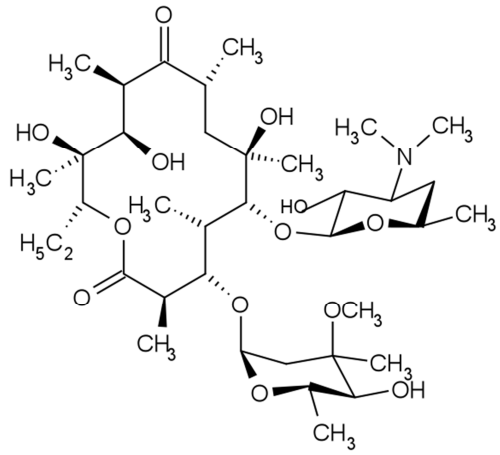

Figure 9 Erythromycin, an example of a macrolide. The macrolide ring is the 14-membered ring at the upper-left corner.

Beta-lactams

Beta-lactam antibiotics are a large group of antibiotics known by their beta-lactam ring (see figure 10). These antibiotics target the cell wall biosynthesis pathway which causes an inhibition of the bacterial growth. This is done by inactivating PBP (penicillin binding protein), which causes irregularities in the peptidoglycan structure. Peptidoglycan is an

important structure in the bacterial cell wall, and irregularities in this component in the cell wall could lead to a hampering of its reproduction and eventually lead to cell death (*47*).

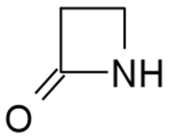

Figure 10 The beta-lactam ring.

Penicillin

Penicillin is the best known beta-lactam antibiotic and it was discovered by Alexander Fleming in 1928 (*48*), which eventually lead to the Nobel prize in physiology/medicine in 1945 for Alexander Fleming, Ernst Boris Chain and Howard Walter Florey because of penicillin's discovery and its use in infectious diseases (*49*). The basic structure of penicillins consists of a thiazolidine ring (A) connected to a beta-lactam ring (B) (see figure 11). A side chain (R) is attached to the basic structure through a peptide bond, and this side chain displays the specific effect of the individual penicillin. When introducing a penicillin nucleus, 6-aminopenicillanic acid (6-APA), examples of many penicillins we know today emerged, like methicillin, ampicillin and cloxacillin (*50, 51*).

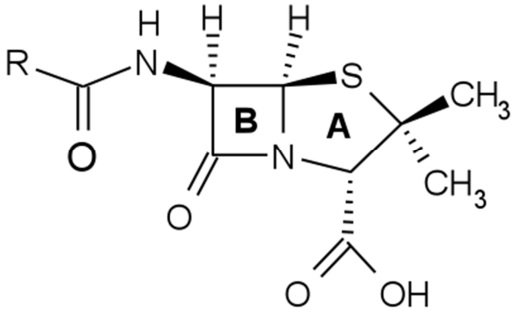

Figure 11 The backbone structure of penicillin, 6-APA. A= thiazolidine ring, B= beta-lactam ring.

Cephems; cephalosporins and cephamycins

Cephems is a subgroup of antibiotics which includes cephamycins and the more explored cephalosporins. Cephamycins are very much alike cephalosporins and are sometimes being termed as cephalosporins too. When synthesizing cephamycins, these compounds were believed to be more resilient towards beta-lactamase than their similar compounds

cephalosporins (*52*). An example of a cephamycin is cefoxitin (*53, 54*). The exploration of cephalosporins initiated when 7-ACA (7-Amino-Cephalosporanic Acid) (figure 12) was synthetized (*50, 55*), which is a cephalosporin scaffold in the same way as 6-APA is the scaffold when synthesizing different penicillins. Cephalosporins are usually classified into four different generations based on their antimicrobial activity, and for each generation its spectrum is more concentrated towards gram negative bacteria and resistance to beta-lactamases (*56*). Examples of cephalosporins are; Cefuroxime (second generation) (*57*) Ceftadizime (third generation) (*58*), and Cefepime (*59, 60*).

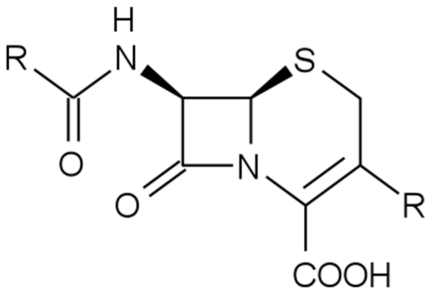

Figure 12 The backbone of cephalosporins, 7-ACA (*61*).

Carbapenems

This branch of beta-lactams was explored when thiamycine was synthetized, and this antibacterial showed good effect towards a broad spectrum of bacteria (*62*). But thiamycine was an unstable compound and therefore the synthesis of the more stable compound imipeneme (MK0787) was initiated and showed promising antibacterial activities (*63*). This initiated the exploration of the beta-lactam antibiotics group of carbapenems. An interesting feature of carbapenems is their *trans* configuration of their side chain (see figure 13), penicillins and cephalosporins have *cis* configuration of their side chain. This is believed to be the reason for their resistance against beta-lactamase activity (*64*). Examples of other carbapenems are Meropenem (*65*) and Ertapenem (*66*).

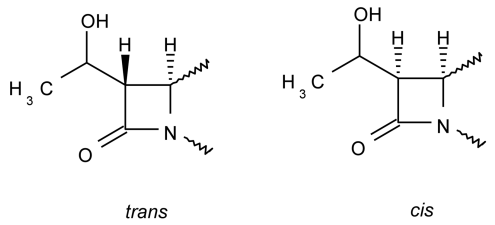

Figure 13 The left structure illustrates a *trans* conformation which is typical for most beta-lactams, and the right structure illustrates a *cis* conformation which is typical for carbapenems. Figure based on the work of Birnbaum et al (64).

Monobactams

As the name implies they have a single beta-lactam ring structure (figure 14). The compounds of monobactams found in nature have no special antibacterial activity, but derivatives which are based on these natural monobactams have shown antibiotic activity (*67*). Aztreonam is the only clinically available monobactam, a compound with good activity against gram negative bacteria, but no activity against gram positive or anaerobic bacteria (*68*).

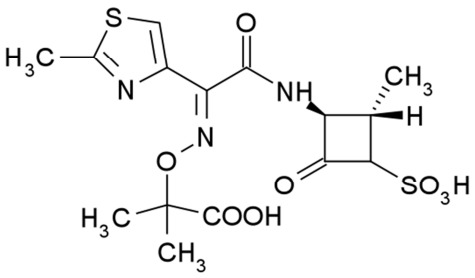

Figure 14 Structure of Aztreonam.

Nitrocefin

Nitrocefin is a chromogenic compound which is used to detect the activity of lactamases (*69*). This substrate belongs to the cephalosporin group, a group which normally has absorption of substrate and product in the ultraviolet spectrum. Nitrocefin separates itself from the usual cephalosporins, as measuring the absorption of substrate and product is done by using wavelengths of visible light. This is thought to be a consequence of an unusually highly conjugation with the substituent at the third position at the β-lactam ring and the double bond in the dihydrothiazine ring (A) (se figure 15) (*69*). This unusual structure

is also a possible explanation for its reactivity, which has revealed this substrate's usefulness as a reporter substance (*70*).

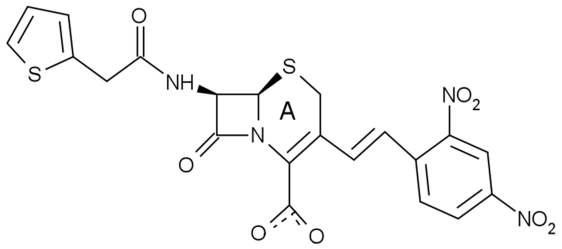

Figure 15 Structure of nitrocefin A=dihydrothiazine ring

Nitrocefin is often being used as a substrate for MBL. Fields of application are for instance using it as a detector of extended-spectrum beta-lactamases (ESBL) (*71, 72*), detecting the presence of beta-lactamase activity in bacteria (*73-75*) and also in kinetic analysis (*76-78*).

Why beta-lactamases? - Where do beta-lactamases come from?

Antibiotic resistance is by many thought of as a mechanism driven forward by man's thoughtless use of antibiotics. The human behavior which causes and enhances the antibiotic resistance in bacteria is (*79*); i) overuse of antibiotics in medical relations. ii) The change of demographic parameters - the modern lifestyle of humans implicates a lot of traveling which causes spreading of antibiotic resistance. iii) The increased commercialization and effectiveness pressurizes food producers into a liberal use of antibiotics in the chase for a larger profit.

The mechanism for transfer of antibacterial resistance In bacteria lies in plasmids which could be horizontally transferred between the different bacteria (*79*). Production of beta-lactamases, and especially the production of Extended-spectrum beta-lactamases (ESBLs), in bacteria is seen as a serious threat to people's health as beta-lactam antibiotics are frequently used as a treatment for pathogen bacterial infections. And metallo-beta-lactamases have been seen as a foreseeable threat, since these proteins often provide a broad spectrum of resistance against different types of antibiotics.

But where does the production of beta-lactamases originate from? First and foremost it is natural to think that the beta-lactamases was originally used in the fighting for the natural habitat amongst bacteria. Hypothetically, beta-lactamase production can be seen as a

response to a different organism's capability to produce antibiotic. The production of toxins, or antibiotic, and the production of beta-lactamases could be seen as a part of a competitive evolution (*80*).

In Alaskan environment where no human antibiotic pressure has been applied, a diverse set of lactamases have been found and also other substrates than the beta-lactam ring has been suggested for the beta-lactamases (*81*). In addition to the research in Alaskan environment, there have also been found many different species of Entero bacteria with a broad spectrum of resistance in freshwater with no connection to human activities (*82*). This indicates that human antibiotic overuse is perhaps not the only reason for multi antibiotic resistance found in bacteria. The reason for finding these "anomalies" is not very well known.

But a possible hypothesis for finding resistance to antibiotics in natural bacterial colonies could be that the resistance functions as a regulator of quorum sensing (*83*). It is suggested that antibiotics are used to communicate between bacteria, which could mean that, for instance, beta-lactamase's role is to adjust this quorum sensing signals.

Quorum sensing is a way for bacteria to make a response when reaching some sort of a threshold, for instance a certain size of a bacteria colony. The response could be to make a biofilm, as seen in *P. aeruginosa* colonies (*84*). Other examples of quorum sensing are found in *Vibrio fischeri* giving a response of bioluminescence (*85*), *Escherichia coli* regulating its virulence factors (*86*), *Acinetobacter baumannii* in regards of biofilm production (*87*) and *Aeromonas hydrophilia* by affecting its virulence capabilities (*88*).

In the natural environment it is likely that the production of antibiotics is at the level of sub inhibitory (SI) concentrations, and microorganisms have been shown to respond to antibiotics in a certain way called *hormesis* (*89*). Hormesis is defined as a dose-response relationship where a low concentration gives stimulating processes until higher doses give toxic effects (see figure 16), which is thought to be a way for an organism to allocate its resources for optimal use (*90*). For instance, it is suggested that SI concentration of imipenem induce production of alginate, which compromises the effect of this antibiotic treatment (*91*), and SI concentrations of aminoglycoside induce biofilm formation in *P. aeruginosa* and *Escherichia coli* (*92*). Biofilm formation has the ability to protect bacteria from antibiotics (*93*). Sub inhibitory inflicts other responses in bacteria; induction of heat

shock genes and increased synthesis activity of purine nucleotide in *Streptococcus pneumoniae* (*94*), induction of a general stress response in *Bacillus subtilis* (*95*) and expression of genes involved in osmotic stress in *Escherichia coli* (*96*).

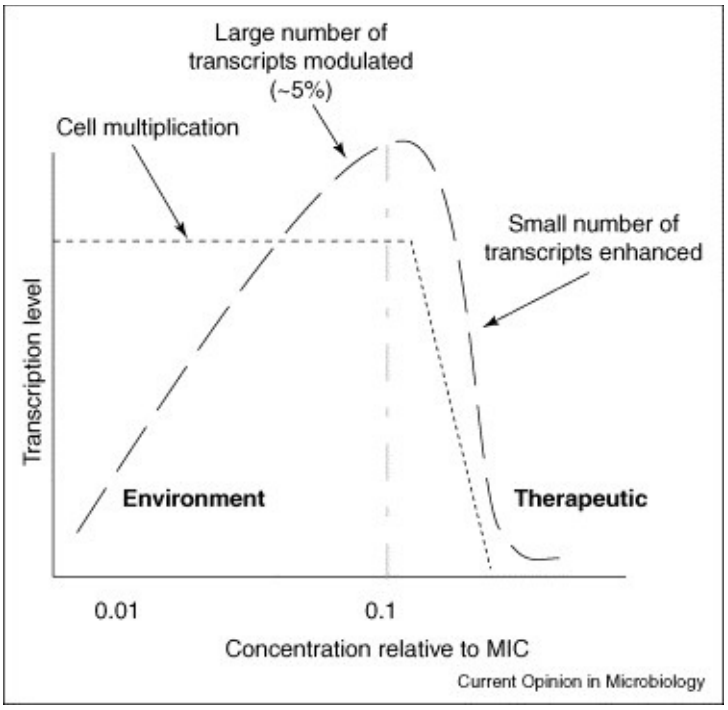

Figure 16 An illustration of how hormesis could work in regards of antibiotic. Low doses of antibiotics will not affect the bacteria, but could in a certain range enhance the number of transcripts modulated. Higher doses of antibiotics disrupt cell multiplication and will not enhance the number of transcripts modulated. MIC = Minimum inhibitory concentration. Retrieved from Davies et al (*89*).

Antimicrobial resistance impact on society

Antibiotic resistance, including resistance to multiple antibiotics, is seen as a serious threat to human health. And judging from the report by the European Centre for Disease Prevention and Control (*97*), it seems like the resistance is increasing. For instance, it has been observed that *Escherichia coli* showed an increase of resistance to all antibiotic classes all across Europe (figure 17). *E. coli* is the most common Gram-negative bacteria which is responsible for urinary tract infections and bacteraemia (bacteria in the bloodstream) (*97*). Other resistant human pathogens are showing increasing resistance as well (*97*); i) *Klebsiella pneumoniae* show high levels of resistance to third-generation cephalosporins, fluoroquinolons and aminoglycosides. ii) *Pseudomonas aeruginosa* obtain resistance readily during antibiotic treatment and resistance to the five antimicrobial antibiotics recorded by EARSS was common (6%). iii) *Enterococcus faecalis* shows a high-level aminoglycoside

resistance and among most of the European countries aminoglycoside-resistant isolates varied from 25% and 50%. The increase of these resistances decreases the number of options for treating infections caused by bacteria.

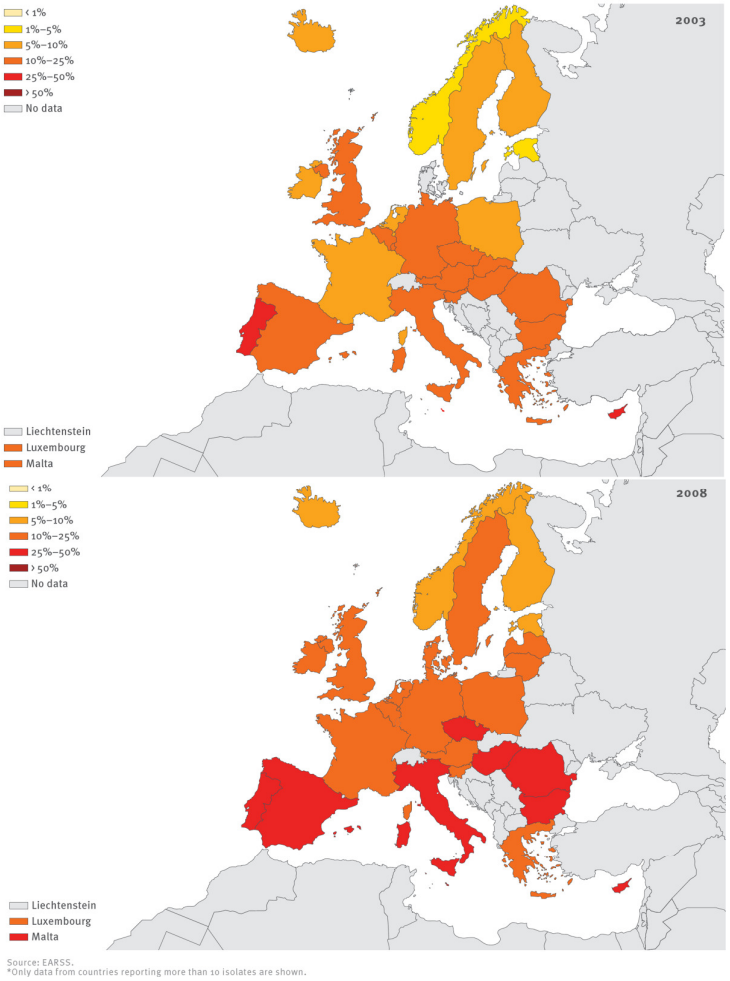

Figure 17 The increase of resistance to fluoroquinolons in *E. coli* from cerebrospinal fluid isolates in EU and EEA/EFTA countries from 2003 and 2008. Retrieved from EARSS (*97*).

In Norway the defined daily dosage of antibiotics for systemic usage per 1000 inhabitants per day was 19.59 in 2010, which is an increase of 3% from 2009 (*98*). Defined daily dosage is an average maintenance dose per day for a drug (*99*). Norwegians used 450 million NOK on antibacterial medication and about a quarter of the Norwegian population picked up a prescription on antibacterial drugs in 2010 (*98*).

In fish farms the organisms with antibiotic resistance are favored when antibiotics are introduced to the environment. For instance a study by Petersen et al showed that where integrated fish farming was performed an increase of antibiotic resistance bacteria was

observed (*100*). Integrated fish farming is a practice in which animal manure is introduced to fish farms to support growth of photosynthetic organisms. The animal manure originates from livestock which often were given antibiotics.

Fish farming is an important industry in Norway - 840,343 tons of fish were produced in 2008 and 742,976 tons of these were Atlantic salmon (*Data provided by the Norwegian Directorate of fisheries)* (*101*). The usage of antibiotics in fish farming is a way to ensure that as many farmed animals as possible can be healthy until they are slaughtered. In 1987 48 tons of antibiotics were used. From 1987 to 1996 it was observed a decline of 98% antibiotic usage in fish farms and simultaneously the production of farmed fish increased with more than sixty times to 321,257 tons (*101*). Mainly efficient vaccination is attributed as the factor that decreased the antibiotic usage. In the year of 2008 a total of 941 kg of antimicrobial agents were prescribed for therapeutic use in farmed fish in Norway. But 65% of the prescribed amounts of antimicrobial agents were intended for use on Atlantic cod, a fish that holds about 2% of the total production of farmed fish (*101*). How all this antibiotic usage in farmed animals has affected and will affect the microbial environment of fish farms is not fully known.

Materials and methods

Cloning of MBL from *A. salmonicida* LFI1238

The gene encoding *A. salmonicida* metallo-beta-lactamase had previously been cloned into the expression vector pET-26b(+) (Novagen) by researcher Bjørn Altermark. In short the restriction sites for NdeI and XhoI were utilized and the gene was cloned without its periplasmic leader sequence. Six histidines were added in the N-terminus to facilitate purification. The plasmid confers kanamycin resistance and contains a T7 promoter in front of the multi-cloning site. The plasmid was transformed into the expression strain *Escherichia coli* Rosetta2 pLysS (Novagen) and kept as a glycerol stock at -80°C.

Expression of MBL from *A. salmonicida*

10 ml LB (Lysogeny Broth) media was inoculated from a glycerol stock of *E. coli* Rosetta pLysS containing the cloned MBL gene. The culture was grown with agitation over night at 37^0C.

The overnight culture was used to inoculate 4x250 ml of LB media. The cultures were grown at 37^0C, and their OD 600 nm was measured every hour until it reached 0.5-0.7. Then the cultures were induced by adding IPTG (Takara Bio, Otsu Japan) to a final concentration of 0.5 mM. Induced cultures were grown overnight at 20^0C with agitation.

The cells were harvested at 6000 rpm for 25 minutes at 4^0C. The supernatant was discarded and each pellet was resuspended in 30 ml of extraction buffer (50 mM Tris, 250mM NaCl, 5 mM beta-mercaptoethanol, 10 mM imidazole and 0.1 mM $ZnCl_2$ at pH 7.5) also containing 1 tablet of protease inhibitor cocktail (Roche, Germany). The cells were transferred to 4 different 50 ml Sarstedt tubes and frozen at -20^0C for later use.

Cell disruption

DNase (Sigma-Aldrich) and lysozyme (Sigma-Aldrich) were added to the thawed cell suspension before sonication. Sonication was performed using a Vibra-Cell VCX 500 (Sonics, Connecticut USA), using the following settings; temperature below 20^0C, amplitude of 25% and sonication period 10 seconds on and 10 seconds off for a total of 30 minutes. The sonicated cells were then centrifuged in SS34 tubes at 9000g for 30min at 4^0C and the supernatant was kept for further use.

Protein purification

HisTrap FF column

As the MBL from *A. salmonicida* was expressed with a Histidine-tag, this will increase the affinity to a Histrap column (Pharmacia Biotech, Uppsala Sweden) compared to other native proteins. Before putting the protein solution on the HisTrap FF column, the solution was filtered using a Millex-GP Filter unit (Millipore, Cork Ireland) with a pore size of 0.22 µm. Buffer A contained 200 mM Tris, 0.1 mM $ZnCl_2$, 250 mM NaCl and 10 mM imidazole at a pH 7.5. Buffer B contained 200 mM Tris, 0.1mM $ZnCl_2$, 250mM NaCl and 500 mM imidazole at a pH 7.5. At first, this purification was done with a 5%-100% imidazole gradient over 10 CV, and the protein was collected in 1ml fractions. The column size was 5 ml.

Later the procedure was optimized using a 10%-100% imidazole gradient over 15 CV.

Fractions were collected based on what seemed to contain most MBL and small amount of contamination.

Dialysis

The selected fractions were dialyzed using a SnakeSkin Pleated Dialysis tubing (Thermo Scientific, Rockford USA) with a cutoff of 3 000 Da overnight in a buffer containing 50 mM Tris pH 7.5, 0.1 mM $ZnCl_2$, 250 mM NaCl. This step was used in order to maintain the protein in an appropriate and stable solution, especially since imidazole is prone to destabilize proteins. As well as purifying the protein, a dilution of the protein is also experienced.

Increasing the concentration of a protein sample

After the dialyzing step it was desired to have a higher concentration of the protein before doing the next purification step. The protein concentration was increased using an Amicon Ultra Centrifugal Filter Unit (Millipore, Cork Ireland) with a cutoff of 10,000 Da, according to the Millipore user specifications.

Concentration measurement

The Bradford reagent (Bio-Rad, California USA) is diluted 1:5 with MilliQ water. The sample with protein and diluted Bradford reagent was then made so the concentration of protein was in the range 10-100 µg/ml which was the limits of standard curve of Bovine serum albumin (BSA). The measurement was done on the spectrophotometer GeneQuant Pro

(Amersham Bioscience, Minnesota USA) at an absorption of 595nm. The protein solution was diluted in Bradford Reagent and measured and compared according to the BSA standard graph to reveal the protein concentration.

The protein concentration was also measured using NanoDrop 2000c (Thermo Scientific, Rockford USA). The MBL extinction coefficient was determined using the ProtPram tool at ExPASy (*102*), a web based tool which calculates the extinction coefficient using the protein sequence.

Gel filtration

Gel filtration was carried out using a buffer containing 50 mM Tris pH 7.5, 0.1 mM $ZnCl_2$, 250 mM NaCl. The motivation for using this purification method was to get as pure protein as possible for crystallization and characterizing the molecular mass of MBL and eventually other contaminants. The gel filtration was done using a Superdex 75 gel filtation column (10/300, Pharmacia Biotech, Uppsala) at a flow rate of 1 ml/min and 1 ml fractions were collected.

Dynamic light scattering

A Dynamic light scattering (DLS) experiment was done on the DynaPro Dynamic Light Scattering system MS/X (Protein solutions, Charlottesville, Virgina USA) with dialyzed protein which had been concentrated to 10 mg/ml. Before performing the experiment, the protein was diluted to 5 mg/ml. The measurements were done at temperatures of 4, 9, 14, 19, 24, 29, 34 and 39^0C. At each temperature 20 measurements were done.

SDS-PAGE

SDS-PAGE or sodium dodecyl sulfate polyacrylamide gel electrophoresis is a technique which separate proteins according to their electrophoretic mobility using an electric field.

After each purification step, SDS-PAGE was performed on various fractions which were loaded on a 4-20% Pierce Precise Protein Gels (Thermo scientific, Rockford USA) and run according to manufacturer's protocol and Laemmli 1970 (*103*).

A Loading Dye (LD) consisting of (1ml); 750µl NuPage LDS sample buffer (Invitrogen, California USA), 100µl MilliQ water and 150µl beta-Mercaptoethanol was used. Each sample was made with 20µl protein solution, which was diluted in case of high protein

concentration, and 5µl LD. Before the samples were applied on the gel they were heated at 95^0C for 5 minutes. The instrument (X-Cell SureLock Electrophoresis Cell) was assembled and Tris-Hepes-SDS buffer (Thermo scientific, Rockford USA) was used. The different samples, and one reference lane consisting of 10µl molecular weight marker Mark12 Unstained Standard (Invitrogen, California USA), were loaded in the wells. The gel was run at 140V for 45 minutes. Afterwards, the gel was rinsed in MilliQ water and heated in a microwave oven for one minute and put on an orbital shaker for one minute. The water was then discarded, and the water rinsing step was repeated twice. SimplyBlue Safe-Stain (Invitrogen, California USA) was poured on the gel and heated in the microwave oven for one minute and put on an orbital shaker for approximately 7 minutes. The stain was discarded and the gel was again put in MilliQ water.

Crystallization trials

Crystallization trials were set up using protein which had been concentrated to about 10 mg/ml. These trials were done both manually and with a crystallization robot.

11 different screens made by researcher Kenneth Johnson were set up with a Phoenix DT crystallization robot (Rigaku, Sendagaya Japan); using sitting drops containing 200 nl of concentrated protein solution and 200 nl reservoir solution or 100 nl of concentrated protein and 100 nl reservoir solution.

Crystal screening was also conducted manually with the Crystal screen and Crystal screen 2 (Hampton Research, Aliso Viejo, California USA) and JCSG-*plus* screen (Molecular dimensions, Suffolk United Kingdom) with hanging drops containing 1 µl protein solution and 1µl reservoir solution with a reservoir volume of 500 µl.

Alignment

A BLAST (*104*) search was performed using *A. salmonicida* MBL as a search sequence. The four most similar sequences were downloaded together with IMP-1 from *Pseudomonas aeruginosa* which was the most similar MBL with known crystal structure. The different sequences were aligned using the T-Coffee Multiple sequence alignment tool (*105*). Also a prediction of asMBL secondary structure was done using the PSIPRED web server (*106*). Secondary structure of IMP-1 was retrieved from Protein Data Bank (*107*) using the entry

PDB ID: 1DD6 (*17*). The sequence alignment and the structure predictions were visualized using the ESPript web server (*108*). The numbering of the different amino acid position was done according to Garau et al (*109*) proposition of standard numbering for class B beta-lactamases. The presence of an N-terminal signal peptide was analyzed using Signal P 3.0 web tool (*110*).

Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique which amplifies a target gene sequence using primers complementary to the target region and a DNA polymerase that assembles new DNA strands from nucleotides. Repeated heating and cooling, or thermal cycles, are used to respectively separate the DNA strands and amplify the target DNA by DNA polymerase.

The degenerate primers MBL-F1 (CAYTTTCATGAAGAYCAAAC) and MBL-R1 (GCAYCACCWGTCCASCCAAT), which were constructed by Bjørn Altermark based on selected MBL nucleotide sequences, were used to screen various *Aliivibrio* isolates from both the aquaculture industry and from wild fish caught in the Barents Sea. The letters represent the different nucleotides, see table 2. If positive, the PCR should give a 300 nt product.

Table 2 The IUPAC standard code for nucleotides

Nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
Y	C or T
S	G or C
W	A or T

A typical amount of reagents for one reaction mixture made for PCR:

Water	36.7 µl
dNTP mix	1 µl
Taq Polymerase	0.3 µl (1.25 units)
Buffer (10X)	5 µl
$MgCl_2$ (25mM)	3 µl
Template (genomic 100 ng/ml)	1 µl
Primer (10 µM)	1.5 µl forward, 1.5 µl reverse
TOTAL:	50 µl

The chemicals used to make the reaction mixes were; dNTP mix F-560 (Thermo Scientific, Rockford USA), Taq polymerase (VWR, Dublin Ireland), Thermo Pol buffer (New England biolabs, Ipswich USA).

All solutions, except Taq polymerase, were vortexed and centrifuged before being added to the reaction mixture and each test tube was mixed and centrifuged before being loaded on the PCR machine PTC 200 DNA Engine (Ramsey, USA), which had the following settings:

- 3 minutes at 94^0C for initial denaturation
- 30 cycles which includes:
    - 30 s at 94^0C for denaturation
    - 30 s at 52^0C for primer annealing
    - 2 min at 72^0C for extension process
- Ending the program with 7 minutes at 72^0C.

The test tubes were put on ice after the program had finished.

The agarose gel was made of a solution containing 1% agarose and 0.01% GelGreen nucleic acid stain (Biotium, Hayward California USA) in Milli-Q water. The solution was heated in a microwave oven until it was homogeneous. The homogeneous solution was put in an agarose gel container with a well comb for an hour to solidify. The samples which were applied to the agarose gel were made of 50 µl samples from the PCR test tubes and 7 µl TAE (Tris-acetate-EDTA) loading buffer. And together with the different samples also a 1 Kb DNA Ladder (Invitrogen, Carlsbad California USA) was applied. The agarose gel was then run at 90 V for an hour.

Activity test using nitrocefin

To identify purification fractions containing MBL, a nitrocefin (Merck, Darmstadt Germany) test was performed on the fractions of interest. A test solution was made with 980µl buffer

(250mM NaCl, 50mM Tris pH 7.5, 0,1mM $ZnCl_2$), 10µl nitrocefin and 10µl of protein sample. After approximately 5 minutes it could be concluded if the MBL activity was present in the specific fraction. As the native color of nitrocefin is yellow, a test solution which after 5 minutes still would look yellow was concluded to not contain MBL. While developing a red color during the 5 minutes, this would indicate the presence of MBL in the fraction.

Assay for MBL with nitrocefin

The appropriate wavelengths for measuring quantity of the substrate, nitrocefin, and product were found by comparing wavelengths found in literature from Hu et al (*111*) and Lisa et al (*112*), and making experimental measurements of their absorbance spectrum. From experiments the wavelength for measuring substrate was at 395 nm and the wavelength for measuring product was at 485 nm. All measurements were done using a Spectramax $M2^e$ (Molecular devices, Sunnyvale California USA).

Preparation of the substrate nitrocefin

As suggested by the manufacturer, nitrocefin was dissolved in DMSO solution. The concentration of nitrocefin in solution was 2 mM.

Zinc-influence

The reaction mixture used when testing Zinc-influence was 0.5 M NaCl, 10 mM Tris, 0.2 mM nitrocefin at pH 7.5 at 37^0C, where the enzyme was incubated for 5 minutes in reaction mixture at 37^0C.

Reducing agents

To observe the influence of reducing agents on the enzyme, 5mM of beta-mercaptoethanol, DTT (Dithiothreitol) or TCEP (*Tris*(2-carboxyethyl)phosphine) was added to a reaction mixture containing 0.5 M NaCl, 10mM Tris, 25 µM $ZnCl_2$ and 10 µl 20 µg/ml MBL at pH 7.5 in 37^0C. The reaction mixture was equilibrated for 5 min at 37^0C before starting the reaction by adding 0.2 mM nitrocefin.

pH optimum

The optimal pH for MBL was identified using only Hepes buffer in the pH range 6.5-8.5 because of the interactions of between buffer molecules found on the substrate, which will

be discussed in more detail later. The experiment was done in a reaction mixture containing 0.5 M NaCl, 0.2 mM nitrocefin, 50 mM Hepes, 100 µM $ZnCl_2$ at room temperature (22^0C).

pH and Tris influence on substrate

Reaction mixture was as follows; 0.5 M NaCl, 100 µM ZnCl2, 0.2 mM nitrocefin and 50 mM buffer (Tris and Hepes). Experiment was done at room temperature (22^0C).

NaCl optimum

The search for the optimal concentration of NaCl for MBL activity was done in a reaction mixture with 25 µM $ZnCl_2$, 10 mM Tris, 0.2 mM nitrocefin at pH 7.5 at 37^0C. The enzyme was incubated in reaction mixture at 37^0C for 5 minutes before adding substrate.

Temperature optimum

The reaction mixture was made of these components: 0.2 mM nitrocefin, 25 mM Hepes pH 7.5, 200 µM $ZnCl_2$ with concentrations of NaCl at 0 M, 0.2 M and 0.5 M.

The experiment was carried out having the reaction mixture set to the intended temperature before adding enzyme. After 10 minutes the reaction was stopped by adding EDTA and putting the mixture on ice before measuring its endpoint activity by the spectrophotometer.

The blanks for this experiment were made without added enzyme. The blank solution had the same components as the reaction mixture for finding temperature optimum, including the same amount of EDTA. From these measurements an assumption of the stability of the substrate was found.

Refolding of protein

The refolding capability of the protein was analyzed in a reaction mixture containing 0.5 M NaCl, 100 µM $ZnCl_2$, 50 mM Hepes and 0.2 mM nitrocefin at pH 7.5 with 10 µl 0.2 mg/ml of asMBL. The protein (0.2 mg/ml) aliquoted into 0.2 ml tubes, were incubated at 60^0C and samples were taken out after 5 minutes, 10 minutes and 30 minutes. After incubation the tubes were put on ice for 30 minutes before being assayed using nitrocefin. A solution not being incubated was used as a reference.

Kinetics

The different substrates were dissolved in solution of 50 mM Hepes pH 7.2 and 100 µM $ZnCl_2$. The substrate concentration of this stock solution was decided using the spectrophotometer and extinction coefficient of each substrate. Based on the concentration of stock solution the solutions for the kinetic analysis were made in the range of 2-1,000 µM.

The kinetic analysis was performed in a reaction mixture containing 50 mM Hepes pH 7.2 and 100 µM $ZnCl_2$ and measured on a SpectraMax using 96 well falcon UV microplates (BD Biosciences, USA) on all substrates, except nitrocefin in which case the 96 well flat bottom non-binding surface (Corning, Edison, New Jersey USA) was used. Incubation of the reaction mixture was done for 5 minutes at 30^0C. The extinction coefficients, wavelengths and also plate specific extinction coefficients were kindly provided by researcher Ørjan Samuelsen, and those used during this experiment are mentioned in table 3.

Table 3 Extinction coefficients for substrate concentration determination, plate specific coefficient for determination of V_{max} and k_{cat} and wavelengths for the different beta-lactams. *= Extinction coefficient not calculated. Numbers were provided by researcher Ørjan Samuelsen.

	Extinction coefficient (Δε,OD/M)	Plate specific coefficient (Δε, OD/µM)	Wavelength (nm)
Meropenem	-6,500	0.00306	300
Imipenem	-9,000	0.00347	300
Ertapenem	-6,920	0.00710	300
Nitrocefin	17,400	0.00645	482
Cefuroxime	-7,600	0.00252	260
Cefepime	-10,000	0.00392	260
Ceftadizime	-9,000	0.00431	260
Cefoxitin	-7,700	0.00231	260
Piperacillin	-820	0.000344	235
Aztreonam	-700	-*	320

The results of the kinetic analysis were processed in Microsoft Excel using solver, an algorithm that can be used for non-linear regression. This method which was used is based on a general description on how to solve non-linear regression in Microsoft Excel (*113*).

Screening of inhibitors

Inhibitors in solid state were kindly provided by researcher Hanna-Kirsti Schrøder Leiros. These were solved in MilliQ water, and some of the less water soluble inhibitors were ionized using NaOH. The reaction mixture of each inhibitor contained: 0.5 M NaCl, 100 µM

$ZnCl_2$, 50 mM Hepes at pH 7.5 and 1 mM of the inhibitor. The reaction mixture was incubated at 5 min at 30^0C, before enzyme was added. The pH of each reaction mixture was measured with litmus paper after the activity measurements were done.

Results

Purification

The protein was first purified using His trap column at 5-100% imidazole gradient which later was optimized into a 10-100% imidazole gradient. As seen from the chromatograms before (figure 18) and after the optimization (figure 19) two peaks have become one peak, indicating that more impurities have been separated. The peak representing MBL has an absorption of about 250 mAU and has a ledge present in the beginning of the peak, indicating a presence of impurities in the first fractions of the peak. It seems like the optimization has positive influence on the ledge, the peak representing the ledge has an absorption of about 200 mAu before optimization and about 30 mAu after optimization (absorption at 280 nm), but it seems like the ledge is stretched out. Based on the protein sequence the mass of asMBL is predicted to be about 26 kDa. From the SDS PAGE gel picture (figure 20) the strong band is in the expected area between 21.5 and 31 kDa. MBL activity of these fractions was tested using nitrocefin as substrate and proved to be positive. Some impurities can be spotted on SDS PAGE gel picture (figure 20), but the gel was overloaded with protein sample and impurities will be more visible. The most troublesome impurities are those with higher molecular weight than 10,000 Da, since these will not be dispatched when accomplishing dialysis, because of dialysis cutoff limit of 10,000 Da. The impurities are most present in the earliest fractions and fades away as the gradient increases.

Activity of fraction 16 and 25 was tested. Fraction 16 showed some MBL activity and fraction 25 showed high degree of MBL activity.

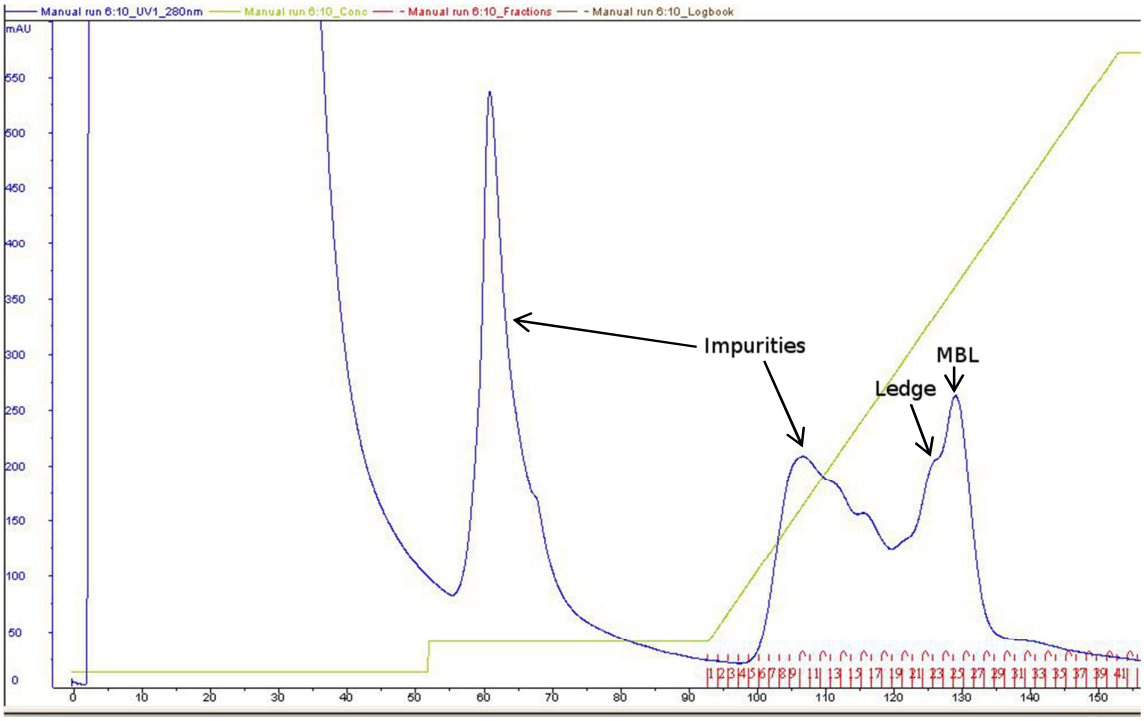

Figure 18 HisTrap purification with a 5%-100% imidazole gradient using a HisTrap FFcolumn (5 ml) over 10 CV with a flow rate of 1 ml/min and collection of 1 ml fractions. The brown line indicates the gradient and the blue line indicates the absorption at 280 nm. Different peaks are marked, those peaks with impurities display impurities which were washed away at the 5% step and which impurities that were washed away during the gradient. Collected fractions are marked in red along the x-axis. Absorption in mAu is marked along the y-axis and eluted volume in ml is marked along the x-axis.

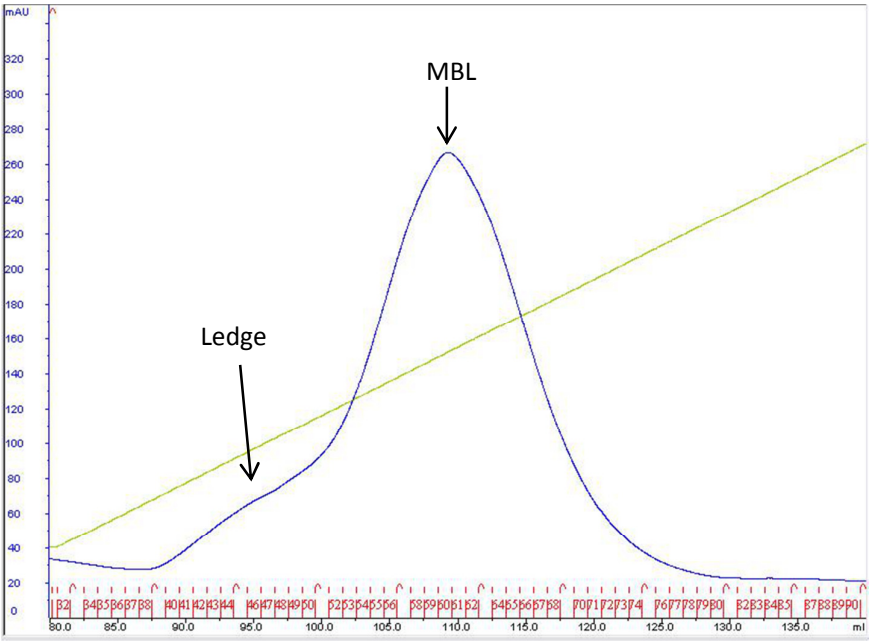

Figure 19 His-trap purification with 10%-100% imidazole gradient, using a HisTrap FFcolumn (5 ml) over 15 CV with a flow rate of 1 ml/min and collection of 1 ml fractions. The brown line indicates the gradient and the blue line indicates the absorption at 280 nm. Peaks with ledge and MBL are marked. Collected fractions are marked in red along the x-axis. Absorption in mAu is marked along the y-axis and eluted volume in ml is marked along the x-axis.

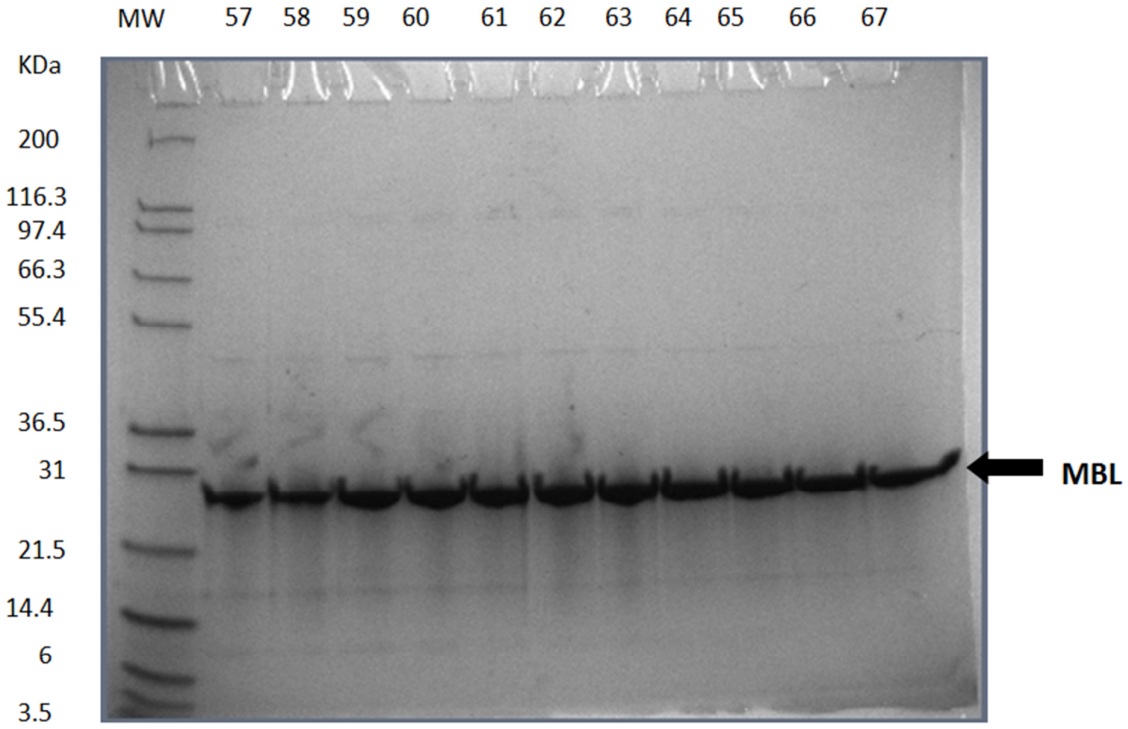

Figure 20 Gel picture from His Trap purification with 10%-100% gradient.

Gel filtration

For the column used for gel filtration (Superdex 75 gel filtation column (10/300) a protein weighing 67 kDa would elute at about 9-10 ml (*114*). From the chromatographs an elution of MBL at around 8 ml can be observed (figure 21). Heavy material elutes faster than lighter material, which indicates that the protein is unstable and most likely is in an aggregated state. This was confirmed by a DLS experiment.

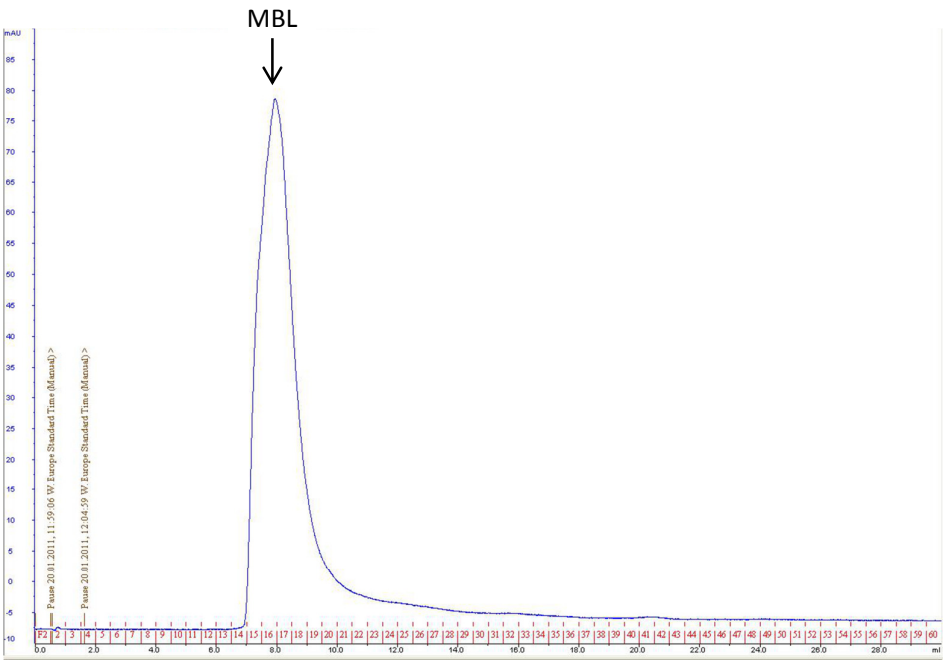

Figure 21 Chromatograms from gel filtration of asMBL using a Superdex 75 gel filtration column (10/300) at a flow rate of 1 ml/min and 1 ml fractions were collected. Collected fractions are marked in red along the x-axis. Absorption in mAu is marked along the y-axis and eluated volume in ml is marked along the x-axis.

Crystallization

To obtain a structure of asMBL crystallization trials were set up, both manually and with robotic help. The proteins used for these trials were from after the dialysis step, not the gel filtration step because of apparent problems with soluble aggregation. From present studies no crystals from these trials is observed.

PCR

The molecular weight standard used for the preparation of this agarose gel do not show a good separation of the different bands, but give an general impression of the size of the PCR products (figure 22). The PCR products of all MBLs from *Aliivibrio* species associated with fish industry (A) and two *Aliivibrio* species found in environment untouched by human influence seems to be in proximity with molecular weight band of 298 nt, which coincides with the expected product of 300 nt. The band from the positive control also supports that the product from the *Aliivibrio* species is MBL.

Some unspecific bands of higher nt is present in those *Aliivibrio* species that do not seem to have MBL in its genome.

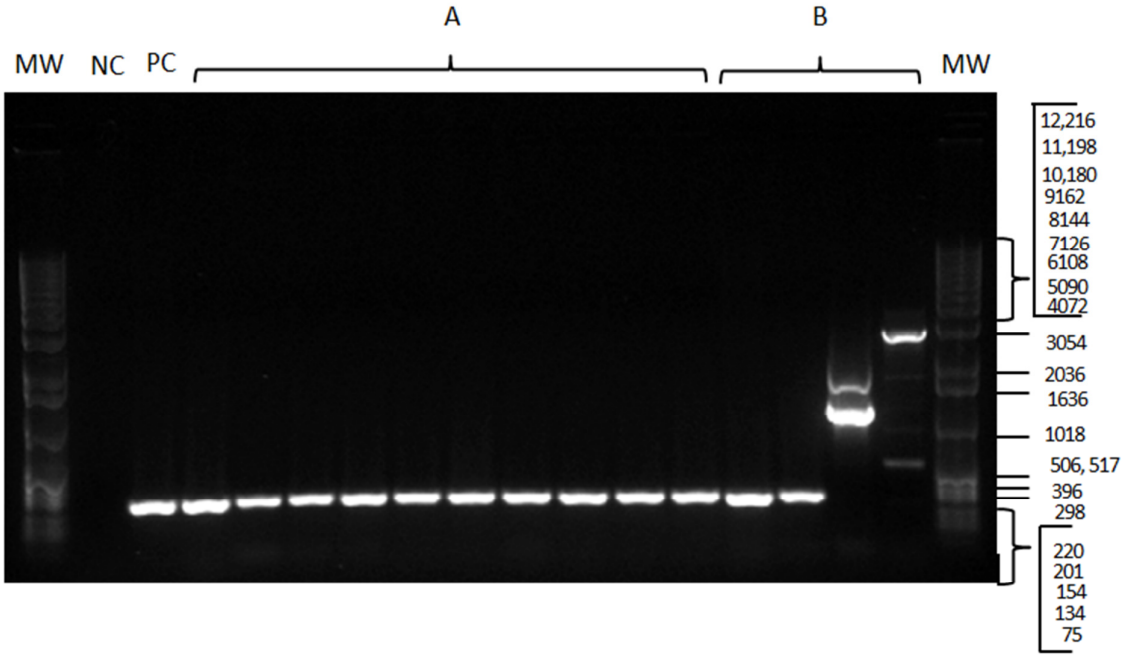

Figure 22 PCR result. MW= molecular weight marker, NC = Negative control, PC = Positive control A =*Aliivibrio salmonicida* from marine aquaculture, B = *Aliivibrio* species from Barents Sea fish.

Sequence alignment

5 different sequences, which included MBLs from *Aliivibrio salmonicida*, *Shewanella sediminis*, *Pseudomonas Aeruginosa*, *Photobacterium profundum* and *Vibrio fischeri*, were downloaded and aligned. The sequence numbering is based on the BBL classification scheme and some gaps are introduced in the aligned sequence to make it fit to the classification scheme (colored triangles in figure 23). Some residues were surplus compared to the BBL classification and is marked with letters a, b, c, d and so on.

From the overall look at the sequence alignment in figure 23 it is rather easy to spot the conserved amino acids (residues in red blocks). The most interesting and highly conserved amino acids are those participating in the catalysis of the beta-lactam ring. Those conserved amino acids marked in figure 23 are amino acids responsible for the binding of the two Zn^{2+} ions; His106, His118 and His196 coordinate Zn1 and Asp120, Cys221 and His263 coordinate Zn2.

When comparing the prediction of the secondary structure of *A. salmonicida* and the secondary structure of IMP-1 the predicted secondary structure has a high degree of resemblance to the structure of IMP-1. All alpha-helices are predicted in the same area, and

more or less all beta-sheets are found in the same regions. This indicates that the structure of MBL from *A. salmonicida* would look similar to the structure of IMP-1.

In the sequence of asMBL the signal sequence is included, as this protein is transported to the periplasmic space of the bacteria (*115*). Using a prediction tool, it was found that the signal peptide probably includes the amino acids from Met0*a* to Ala36, as indicated in figure 23. An interesting point is that an alpha helix is predicted in the same region as the predicted signal peptide. As seen in the other MBLs they all are predicted to have a signal peptide. IMP-1's signal peptide is not shown as it is not part of the downloaded sequence, as the sequence originates from a structure of IMP-1. The cleavage site of the different MBLs are not conserved, for asMBL it was predicted to be between Ala28 and Ala36, for ssMBL it was predicted to be between Ala29 and Glu30 or Leu12 and Arg13, for ppMBL it was predicted to be between Ala36 and Glu37 and for vfMBL it was predicted to be between Ser36 and Ala37.

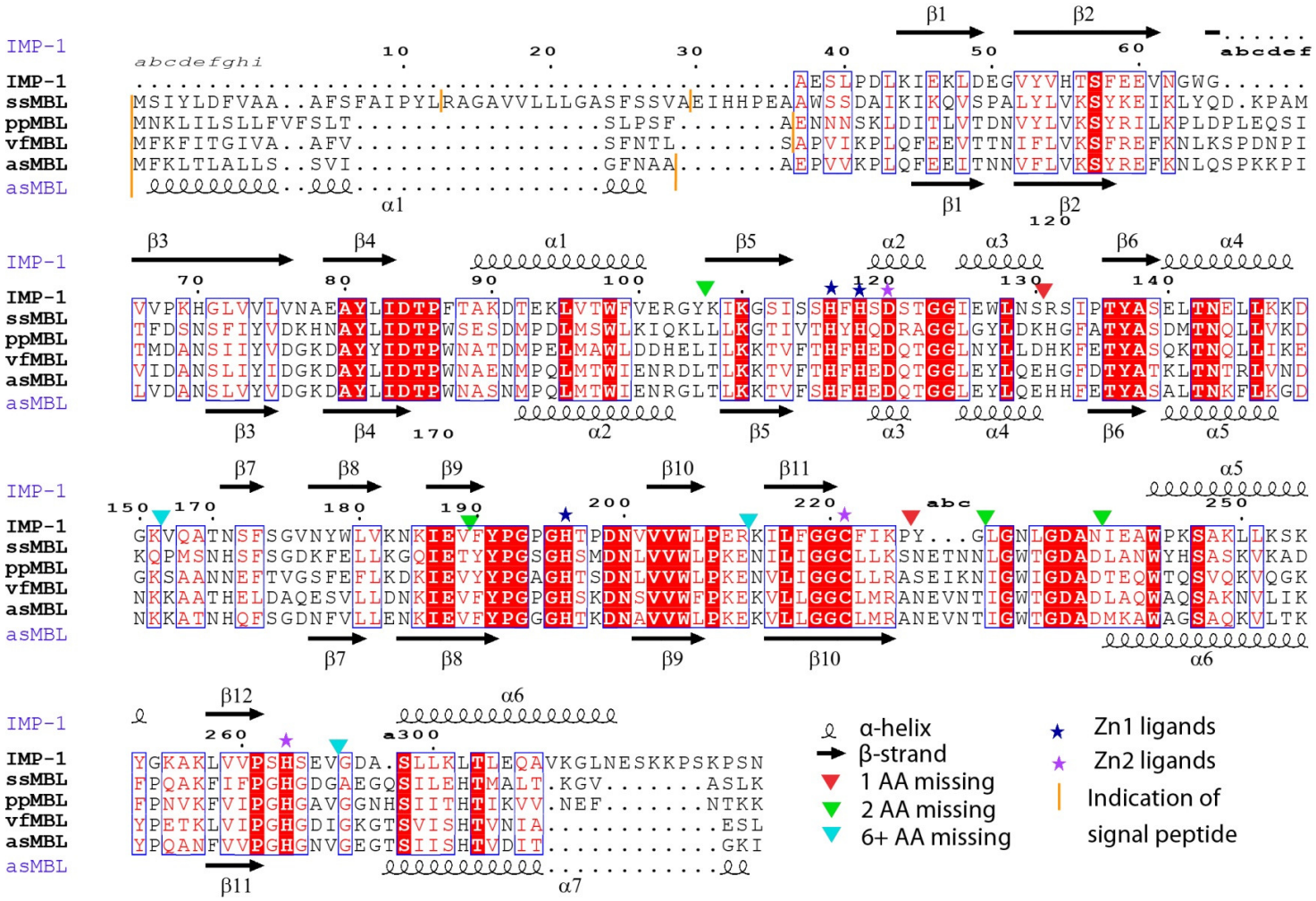

Figure 23 Sequence alignment of different MBL numbered according to BBL classification of numbering. Secondary structure of IMP-1 and predicted secondary structure of asMBL are included.

Zinc influence

Zinc is important to MBL as the enzyme is inactive without Zn^{2+} bound to it (figure 24). This is shown in the temperature experiments when EDTA is added to the reaction mixture and stops the hydrolysis reaction, which have been shown for other MBLs as well (*116*). It seems that the concentrations of zinc have no impact as long as the enzyme has its minimum concentration of zinc (*28*).

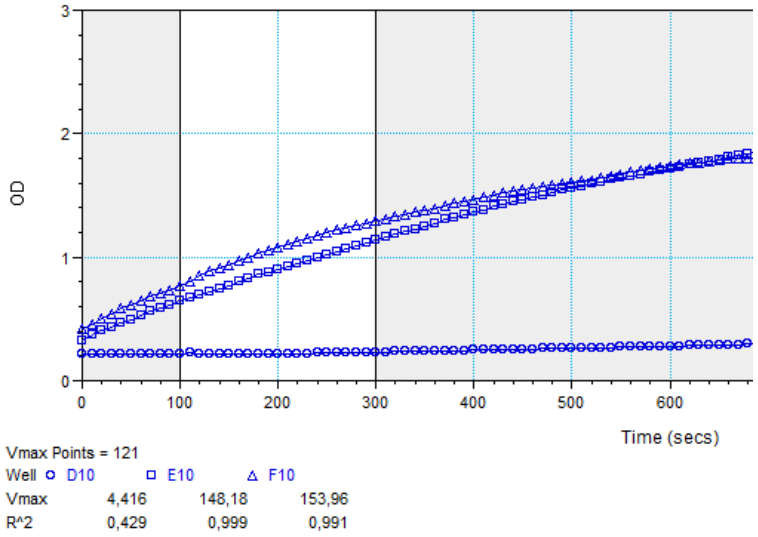

Figure 24 D10 is blank (no enzyme added), while E10 has 100 µM ZnCl2 and F10 has 5mM ZnCl2.

Reducing agents

In the presence of the reducing agents DTT, beta-mercaptoethanol and TCEP the activity of MBL is lowered (figure 25). In the case of TCEP, the activity of the enzyme is entirely halted.

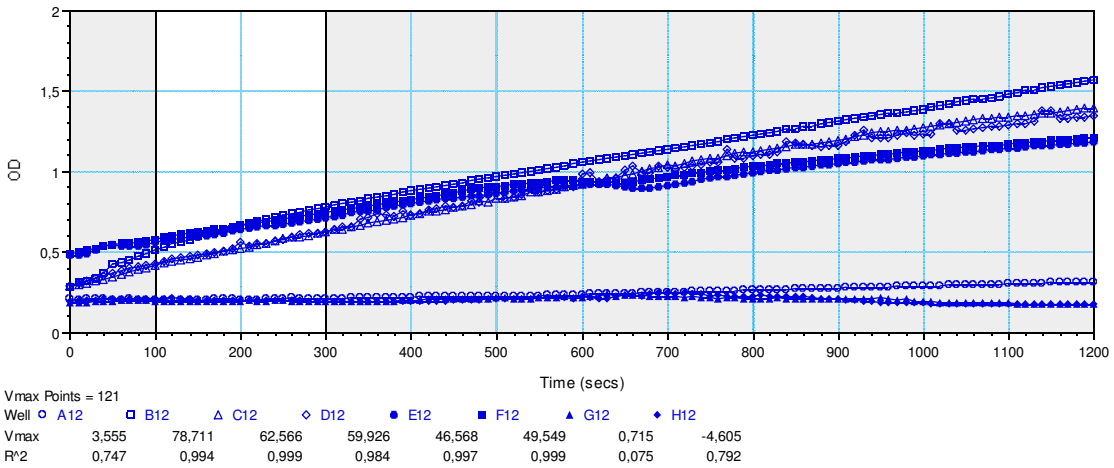

Figure 25 A12 is a blank, while B12 has no reducing agent added to the reaction mixture. To the other reaction mixtures a reducing agent has been added; C12 and D12 contain beta-mercaptoethanol, E12 and F12 contain DTT and G12 and H12 contain TCEP.

pH optimum

asMBL has a broad pH range where the activity is at its best (figure 26). Compared to the pH in coastal water, pH 8.0-8.3, it is expected of asMBL to have optimal activity in this range. It was the intention of this experiment to test the activity at higher pH than done in this study, but as mentioned in *Nitrocefin - Tris reactivity and pH stability*, nitrocefin is not stable at high pH and some buffers seem to destabilize nitrocefin more than others.

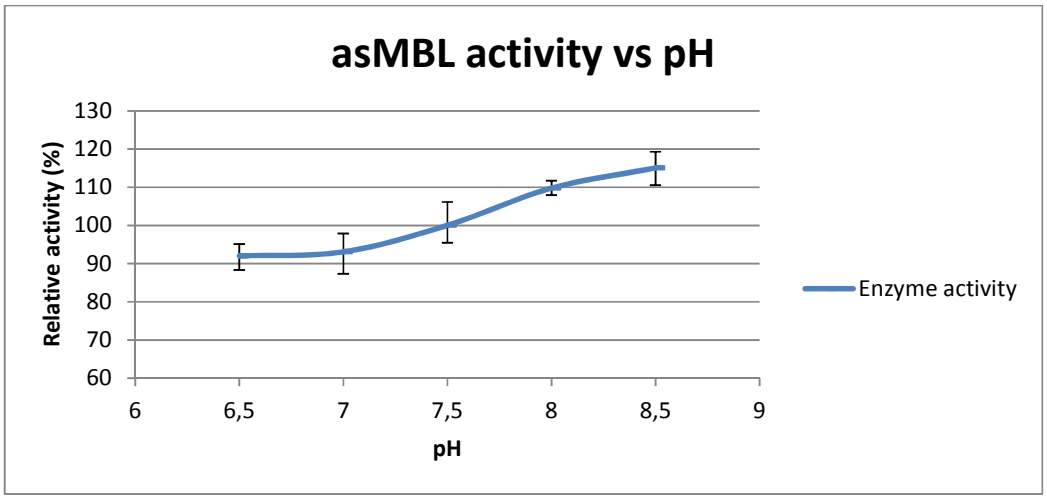

Figure 26 Activity vs. pH for asMBL in Hepes buffer in the range of pH 6.5 to 8.5. All points are relative to the average activity measured at pH 7.5 (100%). The antenna at each measured pH displays the range of enzyme activity measured.

NaCl optimum

As this protein is collected from a bacterium which thrives in the sea, an interesting point is to see how the protein is adapted to salt. Since the sea water has a salinity of about 0.5 M NaCl it was expected that the optimal salt conditions for asMBL would be somewhere around this value. As can be seen from figure 27 the salt optimum for asMBL is 0,5 M NaCl, as expected, but the range of its optimal salt condition for its activity is very broad, having a range of 0,5-2 M NaCl. Even at salt concentrations of around 2.5 M NaCl the activity is about 90% of its optimal NaCl activity.

The optimal salt conditions for asMBL were compared to the optimal salt conditions for VIM-2, a MBL originating from *P. Aeruginosa* a bacteria which has shown to be pathogenic in humans (*117*). As this organism thrives in blood stream conditions, an optimal level of salinity would be 0.6% (*118*) or about 0.1 mM NaCl. From figure 27 it can be stated that VIM-2 has an optimal NaCl concentration of 0.2 M NaCl for its activity, which is lower compared to asMBL. VIM-2 does not show the same degree of salt tolerance as asMBL, as the activity drops more quickly in a nearly linear fashion after reaching its optimal salt concentration.

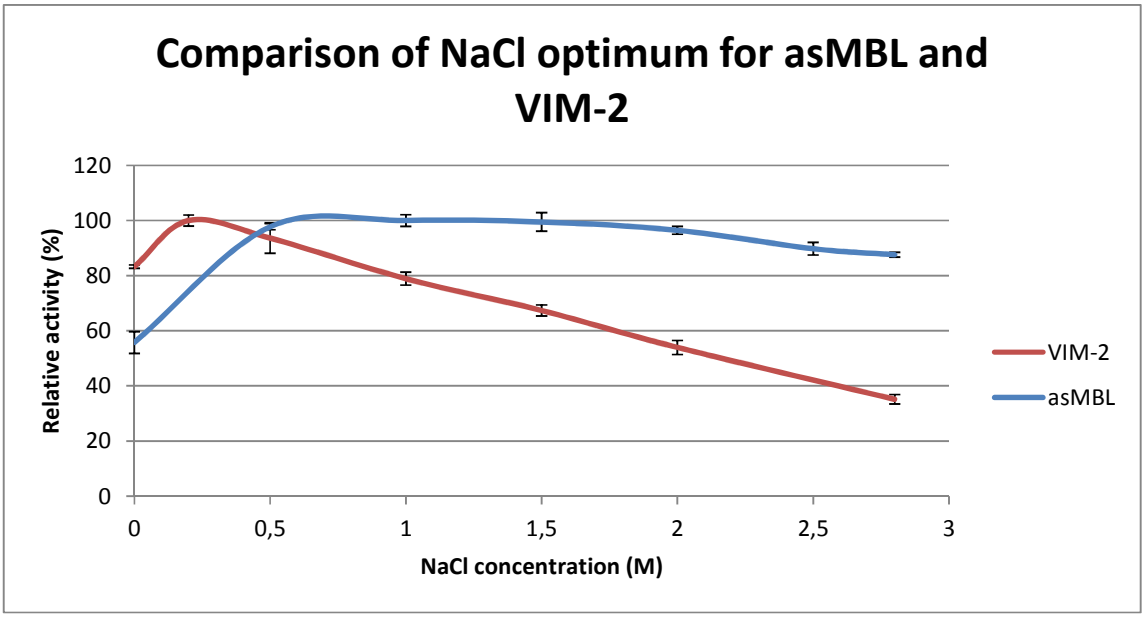

Figure 27 The salt optimum for asMBL and VIM-2. All residual activities are relative to the highest average activity for its separate enzyme. For asMBL the highest activity is at 0.5 M NaCl, while for VIM-2 it is at 0.2 M NaCl. The other measured NaCl concentrations are 0, 1, 1.5, 2, 2.5, and 2.8 M. At each salt concentration, the uncertainty is given as the range of activity measured. Other salt optimum trials for asMBL showed a good salt tolerance (approximately 80%) up to 3.5 M NaCl (not shown). It is a clear difference between the psychrophilic, marine MBL and mesophilic, terrestrial MBL. The antenna at each measured NaCl concentration displays the range of enzyme activity measured.

Temperature optimum

In these experiments the goal was to find the optimal temperature for the asMBL and VIM-2, compare the values and see how different concentrations of salt affect the temperature optimum.

The highest activity shown for asMBL and VIM-2 is at 0.5 M and 0.2 M respectively. At both of these salt concentrations asMBL has a lower optimum temperature than VIM-2 (figure 28 and figure 29); at 0.2 M NaCl asMBL and VIM-2 have an optimal temperature of 30^0C and 40^0C respectively and for 0.5 M NaCl the values are shifted to 35^0C and 45-50^0C respectively. asMBL originates from an organism which is a psychrophile while VIM-2 originates from a mesophile organism.

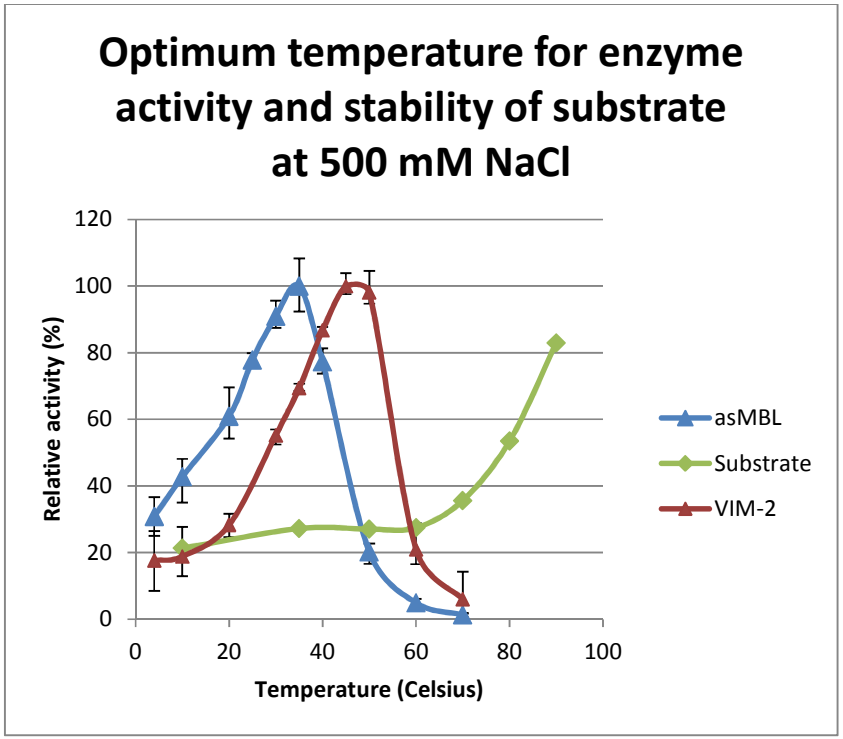

Figure 28 Comparison of temperature optimum for activity at 500 mM for asMBL and VIM-2 and also the stability of nitrocefin depending on the temperature. Activity was measured at the temperatures; 4, 10, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 and 900C. All the measurements in a series were set relative to its series maximum value. For asMBL it was at 350C and for VIM-2 it was at 450C. The values of substrate stability measurements were relative to the maximum value of the asMBL series. The range of measured enzyme activities is displayed by the antennas.

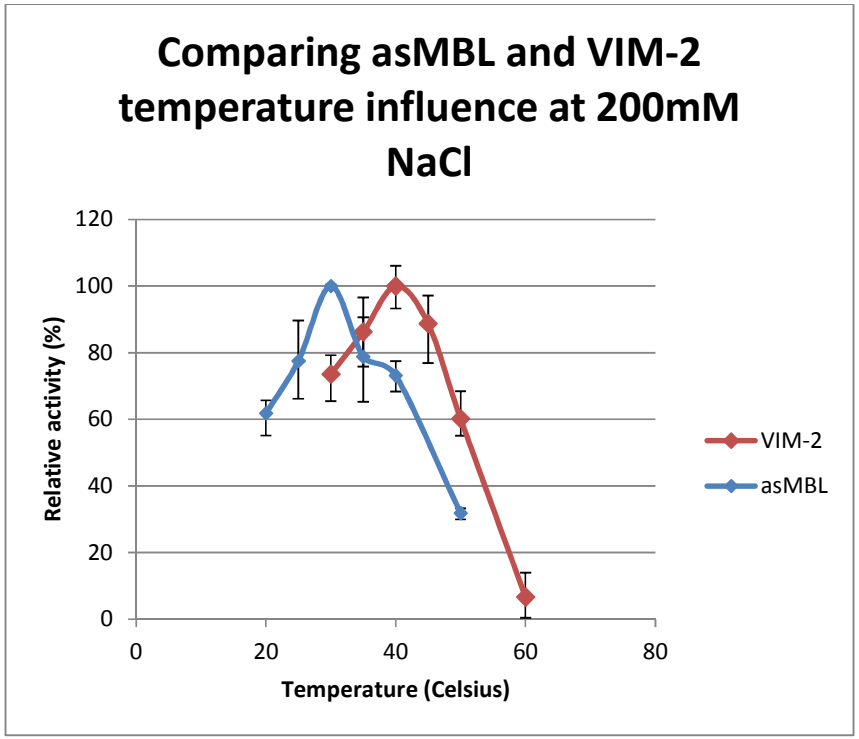

Figure 29 Comparison for temperature optimum for asMBL and VIM-2 at 200 mM NaCl. The measured temperatures were; 20, 25, 30, 35, 40, 45, 50 and 60 degrees Celsius. All measurements were made relative according to the maximum value of its series. The range of measured enzyme activities is displayed by the antennas.

The amount of salt present affects the optimal temperature for the enzyme activity. This is imminent in both asMBL and VIM-2 (figure 30 and figure 31). For both of the enzymes the optimal temperature is lowered by approximately 5-10 degrees when lowering the salt concentration from 0.5 M to 0.2 M. From figure 30 it can be spotted a recess of overall activity when lowering the salt concentration, which correlates to the NaCl optimum of asMBL (figure 27). VIM-2 has its highest measured activity at 40^0C and 200 mM NaCl (figure 31), which also correlates to the NaCl optimum of VIM-7 (figure 27).

Another point of interest is the stability of the substrate nitrocefin at higher temperatures. As seen in figure 28, nitrocefin decays at higher temperatures as it is being turned into product. The instability of nitrocefin is not a problem at temperatures ranging from 10^0C to 60^0C, while at higher temperatures it could have impact on the result, if the blank value is not subtracted from the measured value. For instance, at 70^0C the blank value is OD of 0.174, while the other blanks at lower temperature have values of 0.13. Without this correction of blanks, the average measured enzyme activity at 70^0C for asMBL would seem to be unexpectedly higher than the average measured enzyme activity at 60^0C for asMBL. At the temperature of 90^0C the instability of nitrocefin is quite high with a value of OD of 0.405, as the average measured enzyme activity at 35^0C for asMBL is OD of 0.621.

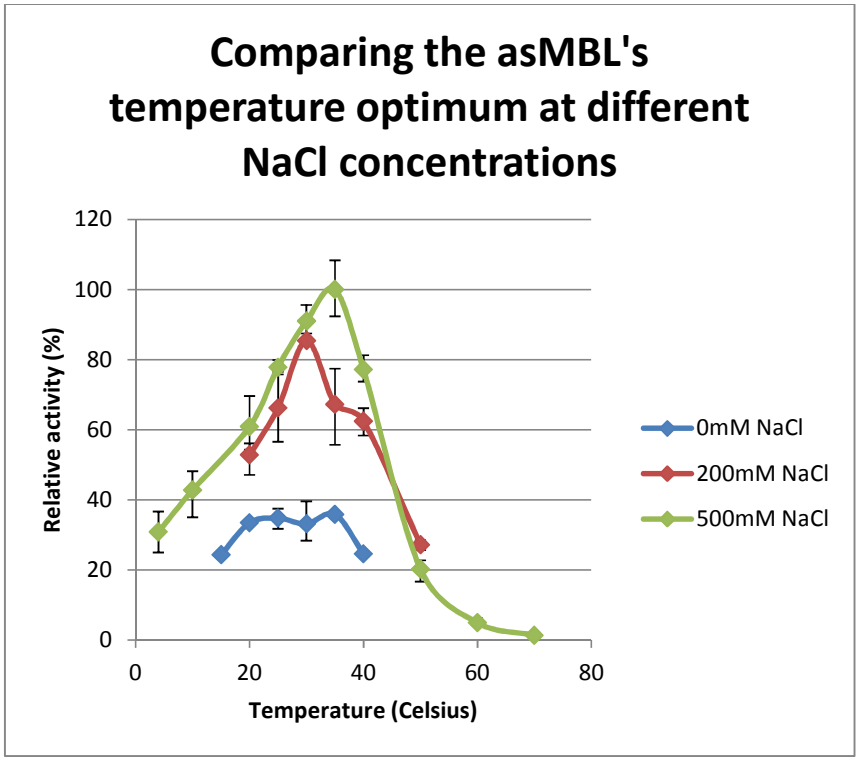

Figure 30 Presentation of how asMBL's optimal temperature for activity differs according to NaCl concentration. Activity was measured at the temperatures; 4, 10, 15, 20, 25, 30, 35, 40, 50, 60 and 70^{0}C. All measurements were made relative according to optimal temperature at 500 mM NaCl. The range of measured enzyme activities is displayed by the antennas.

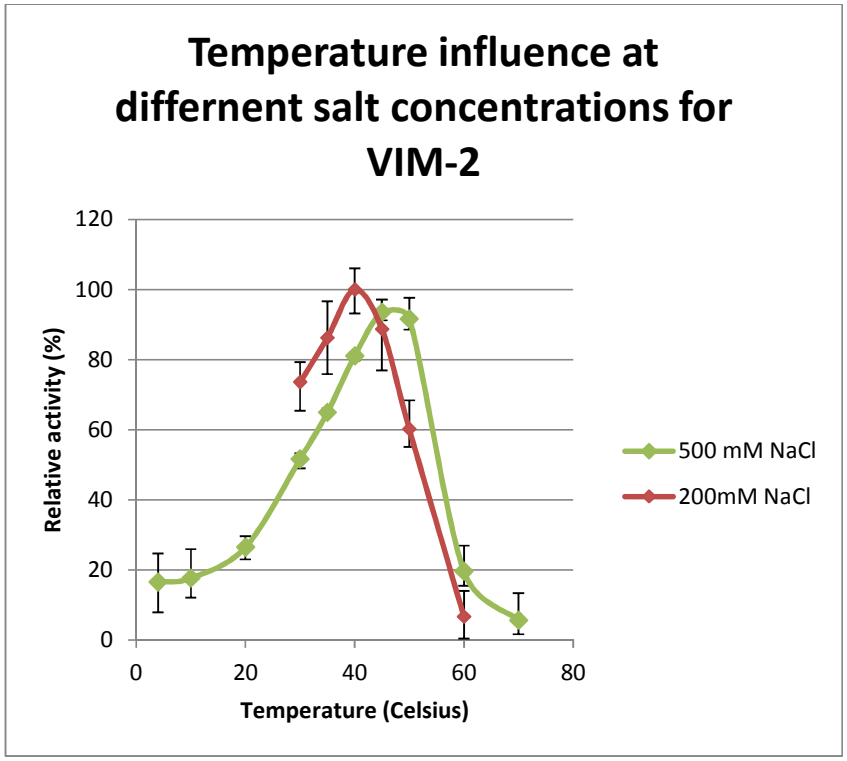

Figure 31 The optimal temperature for VIM-2 activity at different concentrations of NaCl. The activity was measured at the temperatures; 4, 10, 20, 30, 35, 40, 45, 50, 60 and 70^{0}C. All measurements were made relative according to optimal temperature at 200 mM NaCl. The antenna at each measured temperature displays the range of enzyme activity measured.

Nitrocefin - Tris reactivity and pH stability

Nitrocefin stability was tested of various pH, temperature and Tris concentrations. Its stability at different temperature has already been shown.

From figure 32 a relation between pH and auto-hydrolysis is seen, higher pH increases the auto-hydrolysis, but only seen together with the buffer Tris. When nitrocefin is present in Hepes buffer no auto-hydrolysis is observed at any pH, the exception is some auto-hydrolysis at pH >8 after two hours.

A comparison of pH and concentration of Tris was made at figure 33. The amount of Tris present has influence on the rate of nitrocefin auto-hydrolysis to a certain degree. At concentrations of Tris from 0.08 M and beyond do not affect the rate of nitrocefin auto-hydrolysis as much.

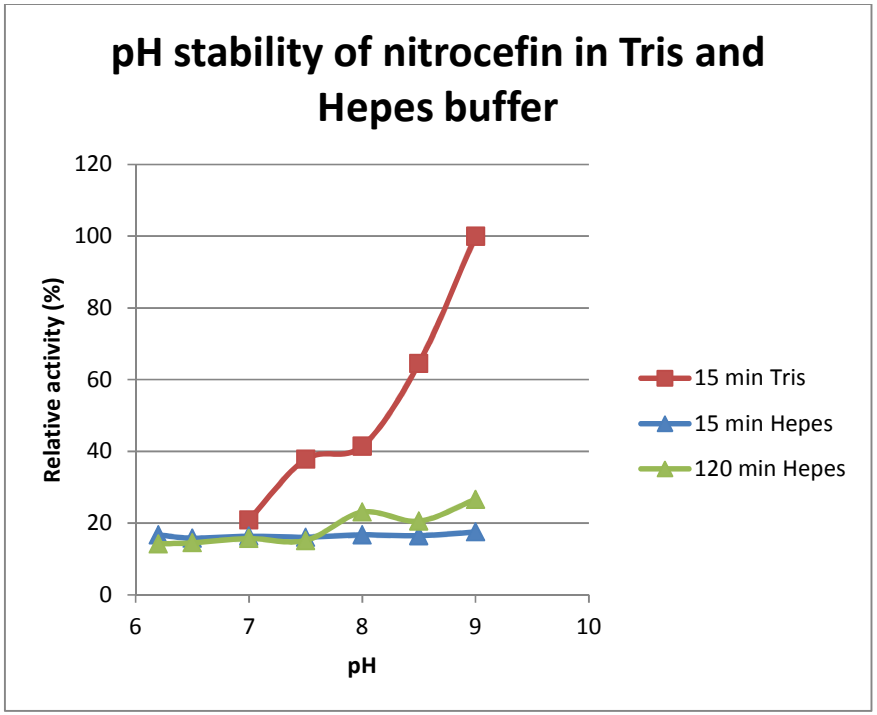

Figure 32 The difference of stability of nitrocefin in Tris and Hepes buffer. All measurements were made relative to measurement made with Tris at pH 9.

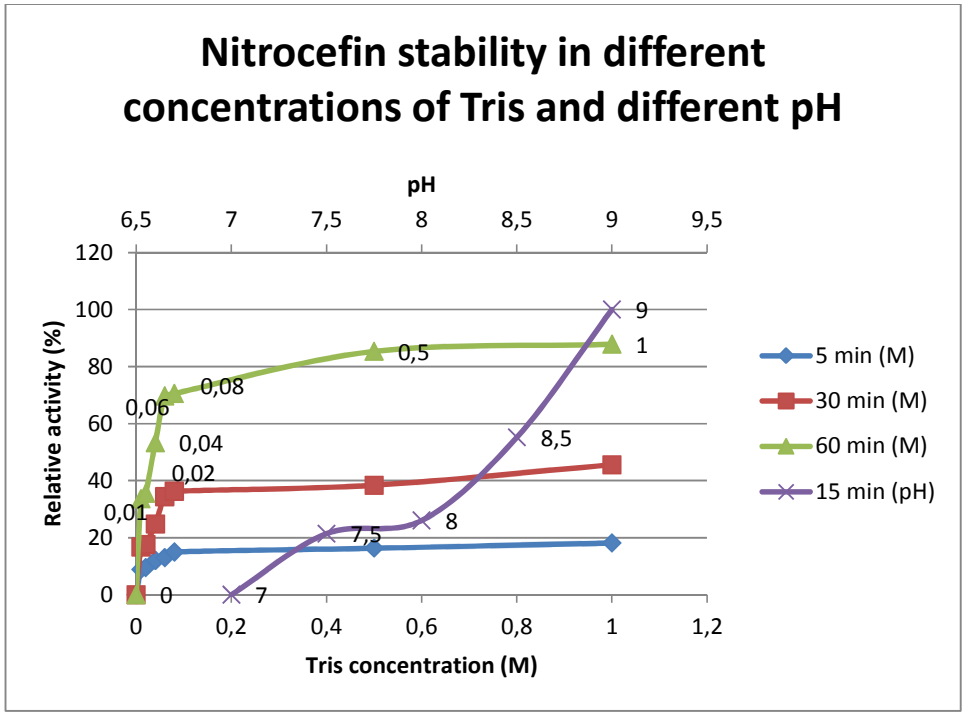

Figure 33 Tris concentration and different pH influence the stability of nitrocefin. All points are relative to the highest measurement done at pH 9 with 25 mM Tris. The activity measurement done at pH 7 was set as blank for the experiment on pH influence on nitrocefin, since it is about the same level as the other blanks for the experiments with different Tris concentrations. pH and Tris concentrations are marked at the different points. The pH series in this figure is the same as the one showed in figure 32.

Refolding

Results from these experiments showed no apparent refolding of asMBL. Solutions incubated at 60^0C had no activity compared to the solution which was not incubated.

Kinetics

Results from the kinetic study in this report were compared to similar MBLs from functional group B1 (table 4). The MBLs which were focused on was IMP-1 from *Pseudomonas aeruginosa (116)* because of its sequence similarity and VIM-2 from *Pseudomonas aeruginosa* (*119*) because of the characterization done in this study. Also a study involving VIM-7 was included (*120*), since its kinetic characterization is done at the most similar conditions as the kinetic characterization done for asMBL.

asMBL hydrolyzed more or less all beta-lactam antibiotics, except from the monobactam aztreonam and the penicillin piperacillin (see table 4 and figure 34-42).

The performance constant of asMBL is generally poorer than the other enzymes, ranging from 10^6 and 10^3 $M^{-1}*s^{-1}$, while for instance IMP-1 has a performance constant ranging from about 10^6 and 10^5 $M^{-1}*s^{-1}$ for the same substrates.

IMP-1 is the MBL solved structure which has the most similar amino acid sequence as asMBL, but their kinetic constants are very different. Generally asMBL has both higher K_m and lower k_{cat} than IMP-1.

When comparing the performance constants for the different substrates, asMBL seems to have a better affinity for carbapenems than cephalosporins and cephamycins, in which cefuroxime is an exception. Nitrocefin is secluded from this comparison as it is made to be readily hydrolyzed. Piperacillin is a penicillin which is used as an extended spectrum antibiotic, which could explain asMBLs apparent inability to hydrolyze penicillins in this study. As long as this is the only penicillin substrate in this study there is no definite conclusion about asMBL's capability of hydrolyzing penicillins in general.

The presence of 0.5 M NaCl in the reaction mixture raised asMBL's performance constant for the hydrolysis of ertapenem. The improvement in the performance constant, when having

salt present, is not caused by a rise in k_{cat}, but a lowering of the enzyme affinity, K_m (table 4 and figure 43)

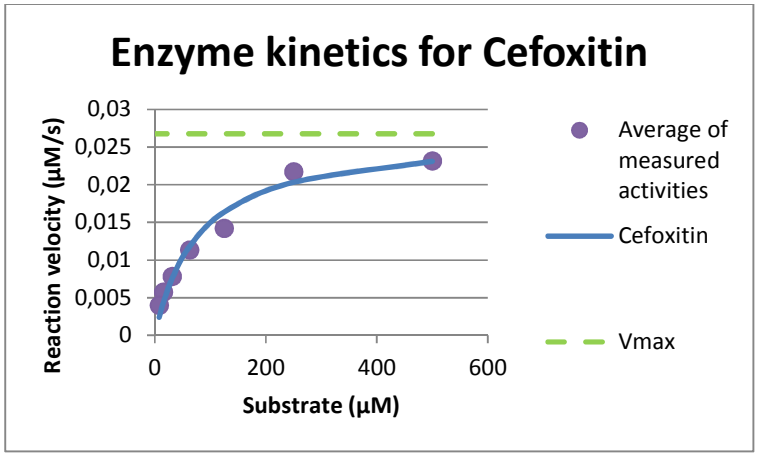

Figure 34 Enzyme kinetics for Cefoxitin using 100 nM enzyme.

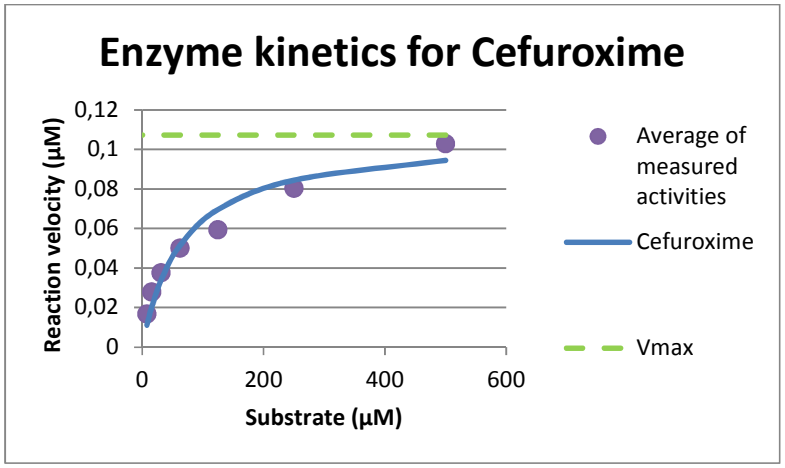

Figure 35 Enzyme kinetics for Cefuroxime using 10 nM enzyme.

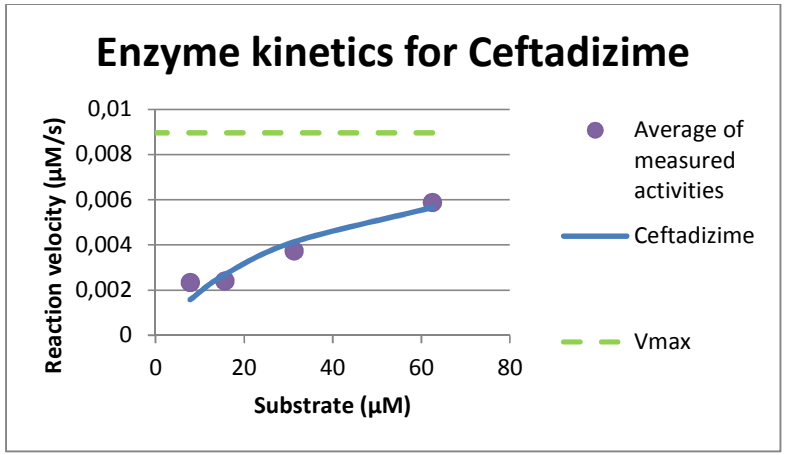

Figure 36 Enzyme kinetics for Cefuroxime using 100 nM enzyme.

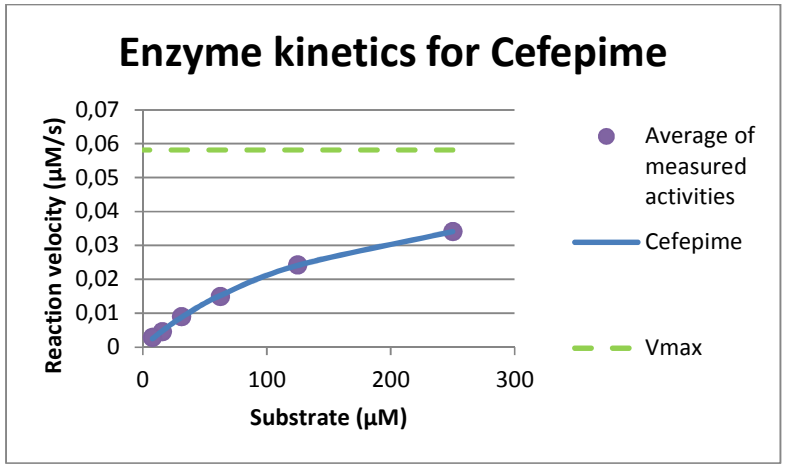

Figure 37 Enzyme kinetics for Cefuroxime using 100 nM enzyme.

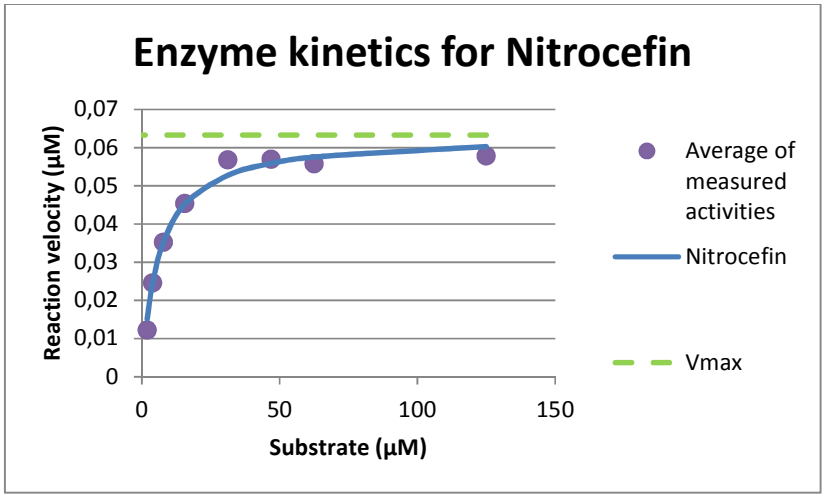

Figure 38 Enzyme kinetics for Nitrocefin using 10 nM enzyme.

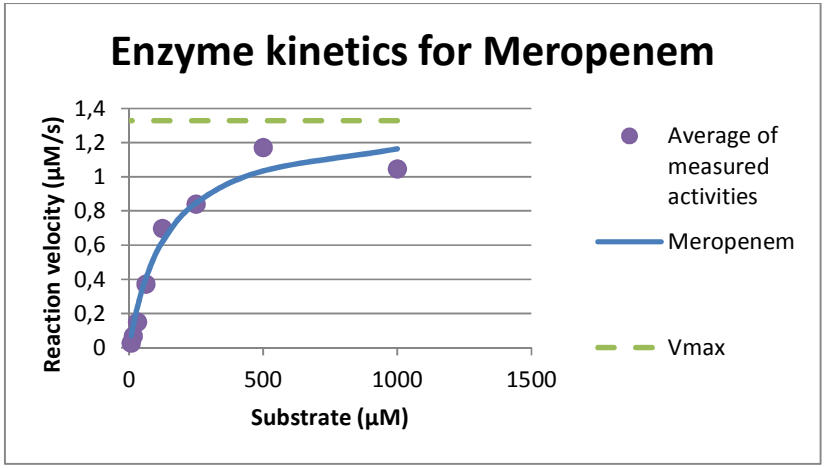

Figure 39 Enzyme kinetics for Meropenem using 100 nM enzyme.

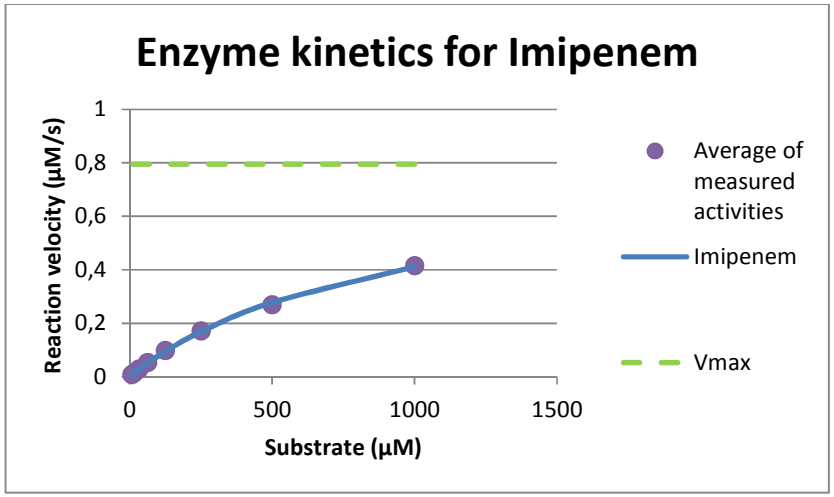

Figure 40 Enzyme kinetics for imipenem using 100 nM enzyme.

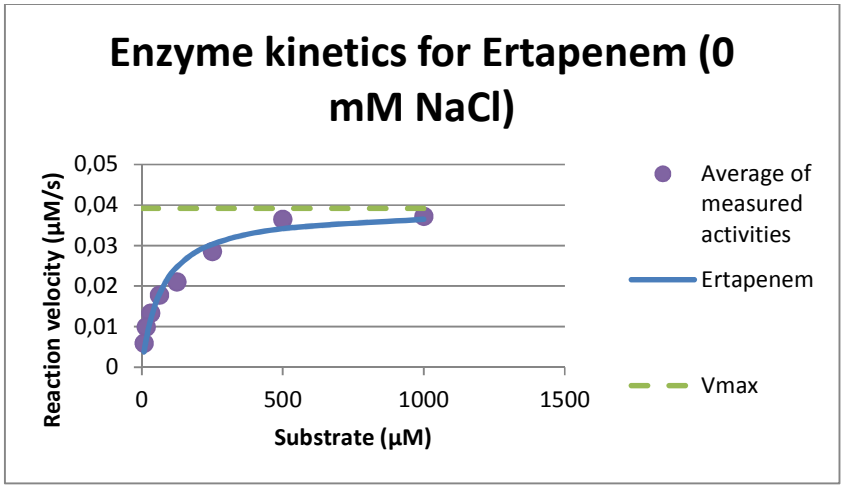

Figure 41 Enzyme kinetics for ertapenem at 0 mM NaCl using 10 nM enzyme.

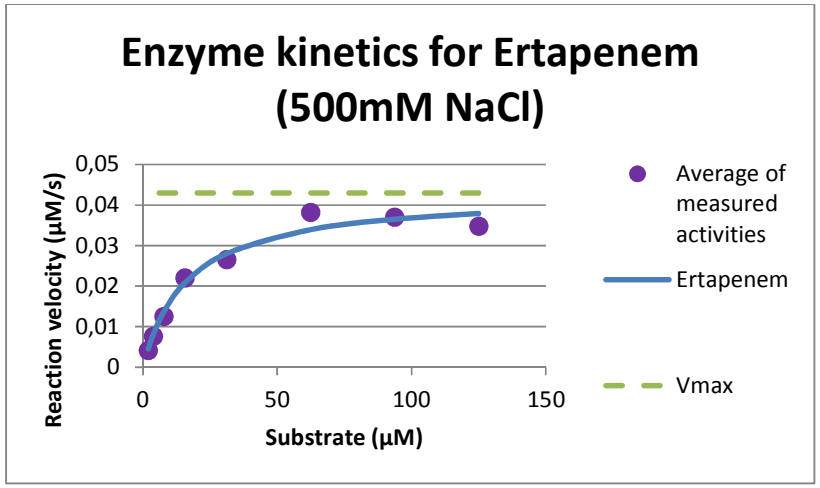

Figure 42 Enzyme kinetics for ertapenem at 500 mM NaCl using 10 nM enzyme.

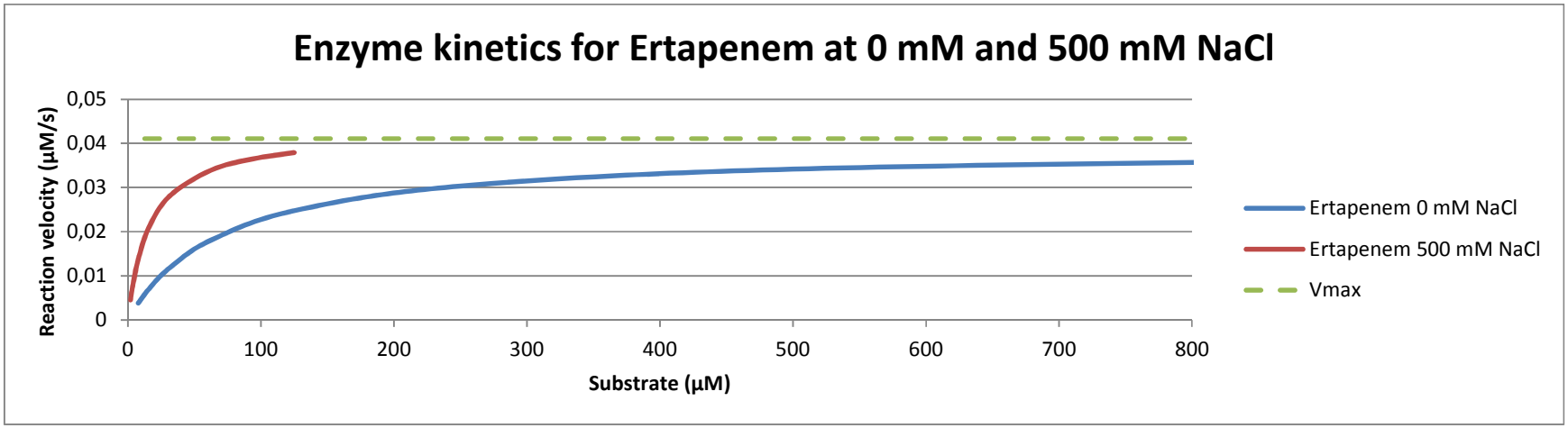

Figure 43 Difference in enzyme kinetics with various NaCl concentrations. The enzyme concentration was 10 nM. V_{max} when having 0 mM NaCl and 500 mM NaCl is regarded as the same and therefore V_{max} in this diagram is the mean between those two approximately equal values.

Table 4 Comparison of k_{cat}, K_m and performance constant for asMBL (in bold, values from current work), IMP-1, VIM-2 and VIM-7 (values from literature). The kinetic parameters for VIM-2 had standard deviations that always were <10%. NA=Results not available, NM=No activity measured, ND=Not determined.

	k_{cat} (s-1)				K_m (µM)				k_{cat}/K_m (1/µM*s)			
Substrate	**asMBL**	IMP-1	VIM-2	VIM-7	**asMBL**	IMP-1	VIM-2	VIM-7	**asMBL**	IMP-1	VIM-2	VIM-7
Meropenem	**13.3 ± 5**	50 ± 5	5	42 ± 0.8	**142 ± 100**	10 ± 2	2	38 ± 4	**0.94**	0.12	2.5	1.1
Imipenem	**7.95 ± 0.6**	46 ± 3	9	100 ± 2	**933 ± 70**	39 ± 4	34	27 ± 2	**0.0085**	1.2	3.8	3.7
Ertapenem	**3.9 ± 1**	NA	NA	8 ± 0.2	**73 ± 30**	NA	NA	28 ± 3	**0.054**	NA	NA	0.29
Ertapenem (NaCl)	**4.3 ± 0.4**	NA	NA	NA	**17 ± 7**	NA	NA	NA	**0.25**	NA	NA	NA
Nitrocefin	**6.3 ± 0.6**	63 ± 10	18	1,500 ± 29	**6.2 ± 3**	27 ± 3	770	58 ± 3	**1.0**	2.3	43	26
Cefuroxime	**10.7 ± 3**	8 ± 1	20	16 ± 0.5	**68 ± 30**	37 ± 3	8	29 ± 4	**0.16**	0.22	0.40	0.55
Cefepime	**0.581 ± 0.005**	7 ± 0.5	>400	5.3 ± 0.2	**177 ± 14**	11 ± 1	>40	580 ± 61	**0.0033**	0.66	0.10	0.0091
Ceftadizime	**0.089 ± 0.03**	8 ± 1	72	1.4 ± 0.1	**37 ± 12**	44 ± 3	3.6	120 ± 25	**0.0024**	0.18	0.0500	0.012
Cefoxitin	**0.27 ± 0.09**	16 ± 1	13	10 ± 0.3	**79 ± 30**	8 ± 1	15	68 ± 7	**0.0034**	2	1.2	0.15
Piperacillin	**NM**	ND	125	140 ± 13	**NM**	ND	300	26 ± 3	-	0.72	2.4	5.4
Aztreonam	**NM**	>0.01	>1,000	NM	**NM**	>1,000	>0.01	2,700 ± 630	-	<0.0001	<0.00040	ND

Inhibitory studies

Potential inhibitors belonging to the molecular groups of thiophenes, pyrroles and pyridines and also the known beta-lactamase inhibitor clavulanic acid (figure 44), were screened. The definition of an inhibitor in this study is substance that induces a 50% lesser activity compared to normal activity. From table 5, it can be observed that some of the potential inhibitors have effect on the MBL; 2-picolinc acid, 2,6-pyridine-di-carboxylic acid, pyridine-2,5-dicarboxylic acid and 2-pyridinecarboxylic acid. But many of these molecules are known to chelate divalent cations, therefore it is doubtful that they have any inhibitor effect besides their chelating capabilities.

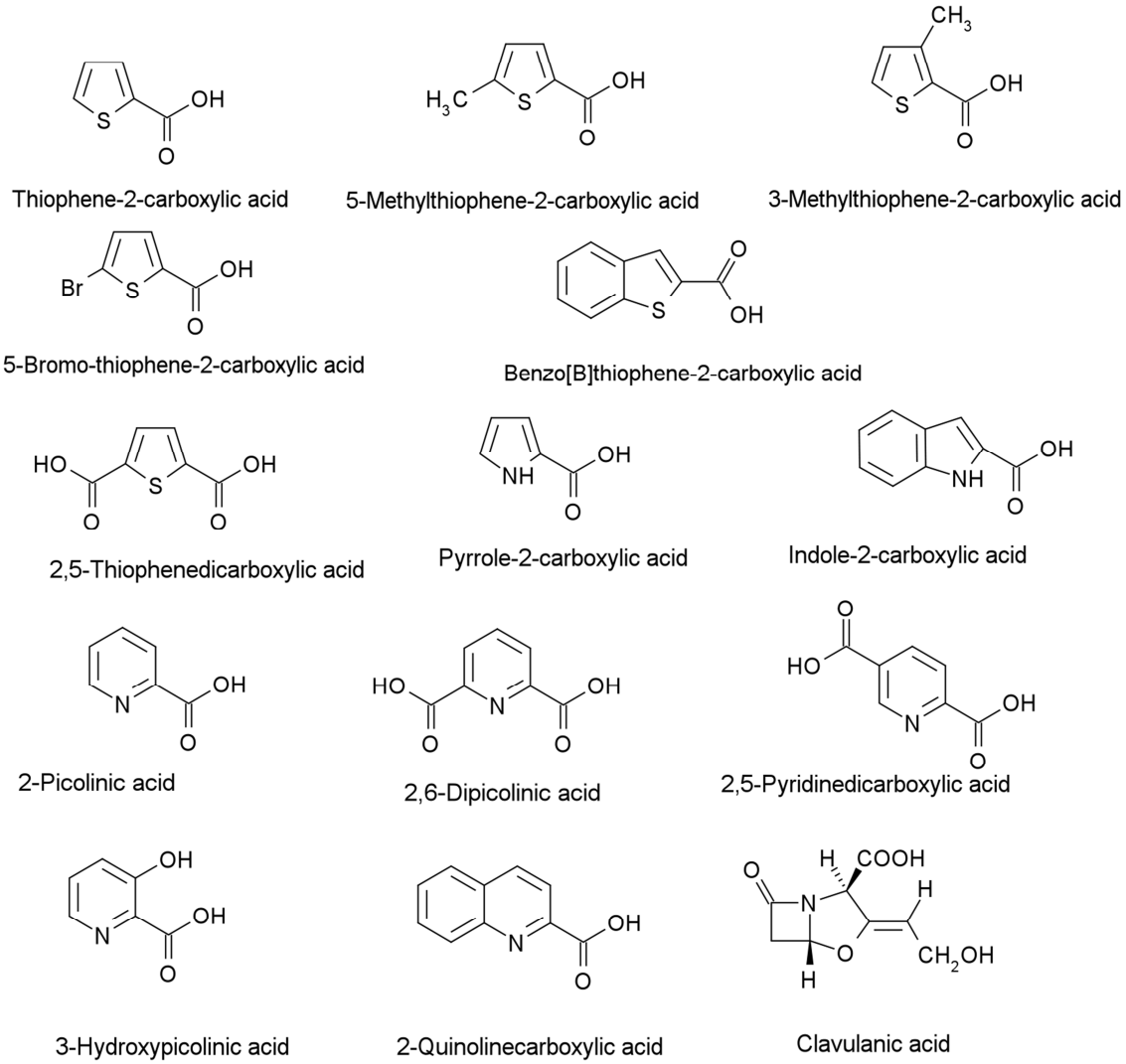

Figure 44 Structures of all inhibitors tested against asMBL.

Table 5 List of all inhibitors tested and their inhibitory effect. * = Inhibitory effect was measured, but there is uncertainty if it is interacting with enzyme or just acting as chelating agents.

Inhibitor	Inhibitory effect
Thiophene-2-caboxylic acid	No
5-Methylthiophene-2-carboxylic acid	No
3-Methylthiophene-2-carboxylic acid	No
5-Bromo-2-thiophenecarboxylic acid	No
Benzo[B]thiophene-2-carboxylic acid	No
2,5-Thiophenedicarboxylic acid	No
Pyrrole-2-carboxylic acid	No
Indole-2-carboxylic acid	No
2-Picolinic acid	Yes*
2,6-Dipicolinic acid	Yes*
2,5-Pyridinedicarboxylic acid	Yes*
3-Hydroxypicolinic acid	Yes*
2-Quinolinecarboxylic acid	No
Clavulanic acid	No

Discussion

Classification of asMBL

Based on the sequence alignment performed in this study (figure 23) the conserved amino acids being ligands for the two zinc ions are identical with respective amino acids found in molecular class B1. Additional characterization information supports this view as the amount of zinc present does not affect the hydrolyzing capabilities of the enzyme as long as there presumably is a minimum concentration of zinc present. This indicate that the enzyme truly is a metallo-beta-lactamase which belongs to the molecular class 3 and it does not belong to the molecular class B2 which is inhibited by two zinc bound to the active site.

From the results already mentioned above regarding molecular classification it is strongly indicated that the enzyme belongs to the functional group 3a, since most MBLs molecular classified as B1 or B3 belongs to this class. asMBL is not inhibited clavulanic acid, but is inhibited by EDTA, which proofs that this enzyme belongs to the functional group 3. The enzyme kinetics indicates that asMBL readily hydrolyses all beta-lactam substrates, except monobactams, which supports that this enzyme belongs to the functional class 3a, even though this study does not show if this enzyme hydrolyze penicillin substrates.

Reductive environment

The reducing agents DTT, beta-mercaptoethanol and especially TCEP are presumably affecting asMBL capability to hydrolyze. A reason for this behavior could be that they reduce the catalytic important residue Cys221. Their potential to reduce a disulfide bond is in increasing order of reduction potential; beta-mercaptoethanol has reduction potential of -0.26 volts (*121*), DTT has a reduction potential of -0.33 volts (*122*) and TCEP has no reduction potential as posphines reduce disulfide bonds via an irreversible pathway (*123*), but TCEP has been found to be more reductive than DTT and BME (*124*). As the reduction potential of these compounds increases so do their inhibition of asMBL activity. Since all these reducing agents are known to have its influence on cysteine, this strongly indicates that Cys221 is affected so it cannot coordinate Zn2 and inhibit the activity of asMBL. Another possibility is that these reduction agents do not affect asMBL, but affect nitrocefin in such way that the enzyme faces difficulties when trying to hydrolyze this substrate. Also the zinc ions could be

affected in such way that they experience a reduction from Zn^{2+} to the solid Zn. When zinc is in solid form it will be unavailable for asMBL and a decline of hydrolysis would occur. But during the experiment there was no apparent sign of solid material in the reaction mixture, which would be expected in the case of formation of solid zinc.

The periplasm of the cell, which asMBL is directed to, is an oxidative environment. The results based on asMBL encountering reductive environment indicate that the enzyme is adapted to this.

Purification and crystallization

When performing the His trap purification step it seems like changing the gradient from 5%-100% to 10%-100% optimized the purification of asMBL. There is still a ledge of impurities present, but it would be difficult to optimize this step further. The ledge could be separated by extending the gradient by increasing the amount of column volumes this gradient goes through. It is not certain that this will separate the ledge entirely, and it would probably be easier to separate the impurities by going through a gel filtration step.

An initial goal of this study was to obtain a structure of asMBL. As crystallization trials demand a large quantity of pure protein, it was initially recommended to do a gel filtration of the protein before doing the crystallization trials. This proved to be difficult as the protein seemed to have been aggregating on the gel filtration column. Even though the protein was not entirely pure after the His-trap filtration step, it could hopefully turn out to be pure enough for crystallization trials. Results from the different trials showed no promising results as there was a majority of aggregation encountered and the DLS experiment indicated that the protein was in an aggregated form. Some aggregation was encountered when having the protein in refrigerator, but the protein was still present in solution during storage. When storing the protein it was kept in low concentration of about 0.2 mg/ml, while the protein concentration in gel filtration and DLS experiment was about 5-15 mg/ml. Even though no visual aggregation has been observed in large scale, this could indicate that the protein is in a soluble aggregated form. But the protein has shown good hydrolyzing capabilities, so it seems like the protein is in a proper condition when in low concentration.

The protein has shown to be unstable and have a potential for forming aggregates at high protein concentrations. There could be many reasons for the instability of the protein. One

of the reasons has its background in the protein construct for expression which consists of a non-cleavable histidine-tag that can render the protein unstable and cause aggregation. Another factor which can affect the protein's stability is its storage and purification buffer. This protein has proved to have a broad tolerance of salt in regards of activity and higher concentration of salt could affect the salting out effect of the protein, making it more stable for storage at higher protein concentrations. The last point, which is the crystallographer's nightmare, is that the protein itself does not maintain a proper stabilization at high protein concentration. Little is known about the general protein behavior and stability, and still much of its properties are hard to predict and understand.

NaCl vs. temperature

MBLs are directed to the periplasm or extracellular space and in the case of asMBL this means that the enzyme is exposed to marine environment and have to necessarily be differently adapted compared to enzymes existing in other environments (*34*).

Salt seems to be important for asMBL activity and because of its tolerance of high concentrations of salt this enzyme could be suggested to be a halophilic enzyme. But as most halophilic enzymes are deactivated by NaCl or KCl concentrations of less than 2 M (*36, 37*), at very least this enzyme can be classified as a very salt tolerant enzyme. VIM-7 is also quite salt-tolerant as it still has 50% relative activity, compared to optimal activity, at 1.5 M salt. It could be a general feature of MBLs to be tolerant to high concentrations of salt.

In this study the mesophilic VIM-7 and the psychrophilic asMBL were compared in regards of temperature optimum under different salt concentrations. From the studies done for asMBL at different concentrations of salt, a relation between salt concentration and temperature optimum arose; the optimal temperature and the relative activity fell as concentrations of salt fell (figure 30). This was the case with VIM-7 also in regards of optimal temperature. As asMBL is very resilient to high concentrations of salt, it would be interesting to see if the optimal temperature would increase as the salt concentration would increase even further than 0.5 M NaCl.

Other studies have observed the same relation between salt and temperature which has been experienced in this study. Niiranen et al observed an upshift in the unfolding temperature of their proteins when the salt concentration was increased (Effects of salt on

the kinetics and thermodynamic stability of endonuclease I from *Vibrio salmonicida* and *Vibrio cholera, Niiranen 2008*). However their result indicates an increase of stability of the global protein when in relation with increased salt, while the study on asMBL indicates that the stability of the active site is increased in relation with increased salt. When combining those two results it could mean that a global stabilization of the protein could lead to an increased stability of the active site.

Kinetics

First and foremost, the credibility of the kinetics results from asMBL has reasons to be questioned, when looking at the standard deviation values. It seems to be a rule of thumb that the standard deviation should not exceed 10% of the obtained results (*119*). Both values of k_{cat} and K_m suffer from high percentage of SD. If the rule of thumb is followed the k_{cat} values which can be trusted are those of imipenem, nitrocefin, ertapenem (present in 0.5 M NaCl) and cefepime, and the k_{cat} values of meropenem, ertapenem (NaCl not present), cefuroxime, ceftadizime and cefoxitin will be more questionable. For the values of K_m the standard deviation values sometimes are way beyond 10%, the worst example is meropenem with a SD of 70%. So if the rule of thumb, with a SD not exceeding 10%, is followed strictly, the K_m values for hydrolysis of meropenem, ertapenem (with and without 0.5 M NaCl present), cefuroxime, ceftadizime and cefoxitin are not to be trusted because of their wide range. Instead the K_m values for hydrolysis of imipenem, nitrocefin and cefepime can be trusted. But an SD of 10% is not enforced in all kinetic studies (*116, 120*). This means that some of the results obtained do not necessarily need to be rejected.

SD is not appropriately used since the variation is not normally distributed (*125*). Therefore many of the SDs that other studies obtain could be questionable as well. But as a better way of estimating SD is computer demanding (*113*) the linear estimation of SD could be used as approximately guideline. Having this in mind only general trends will be discussed further.

The experimental design for measuring V_{max} and K_m is suggested to be done with substrate concentrations in the range of 0.2-5 times the concentration of K_m, and equally distributed below and above K_m (*126*). Another experimental suggestion in terms of having K_m and V_{max} with as low errors as possible is done with half of the substrate concentrations near K_m and half of the observations as near V_{max} as practical possible, in which no more than 10% of the

observations being lower than K_m concentration (*127*). And it is also recommended that the spacing of substrate concentrations are geometric sequenced, an example of geometric spaced concentrations is as follows; 1 M, 2 M, 4 M, 8 M and so on. In our kinetic experiments the most optimal design was geometric spacing of substrate concentrations in the range of 0.2-5 times the concentration of K_m, in which half of observations were below K_m and the other half was above K_m.

The general picture is that asMBL is less effective in hydrolyzing the substrates compared to the other MBLs which were compared. There is one example with meropenem in which MBL perhaps has a better performance constant than one of the other MBL, but this does not change the general impression. A reason for this could be the fact that MBL is not in its optimal condition during the kinetics experiments, as there is a 40% difference in hydrolyzing capability from no NaCl present and 0.5 M NaCl present in the reaction mixture. Another reason for asMBL being a less effective MBL could be that the other MBLs which were compared are MBLs from clinical samples. MBLs found in hospital have probably encountered more antibiotics than asMBL, and have more likely specialized itself more towards the synthetic antibiotics found there. For instance, a beta-lactamase found in *Bacillus subtilis (128)*, a non-pathogenic organism, shows low beta-lactamase activity. When hydrolyzing nitrocefin, the performance constant of beta-lactamase from *B. subtilis* is 0,008054 $\mu M^{-1}x\ s^{-1}$, much lower than asMBL performance constant of 1.0 $\mu M^{-1}x\ s^{-1}$. Even though the *B. subtilis* beta-lactamase is not a metallo-beta-lactamases, it could confirm that asMBL lack of hydrolyzing properties compared to VIM-2, VIM-7 and IMP-1 is because it has not been exposed to antibiotics in the same degree as those MBLs found in organisms pathogenic to humans. Some exposure to antibiotics would asMBL possibly encounter as it is found in *A. salmonicida* from a fish farm. This could be the reason for its better hydrolyzing capabilities of nitrocefin, compared to beta-lactamases from *B. subtilis*.

In regards of kinetics an interesting point is what kind of experimental conditions should the kinetic measurements take place in. It is interesting to compare the numbers acquired from other studies, as this could reveal new knowledge, for instance about difference in binding site between different enzymes and different mutants. But how can it be compared?

Well the easy solution is to have the same standard experimental setup for each study; same pH, temperature, amount of ions and so on. If substrate and enzyme are affected by the conditions they will be affected in the same way. In this study the statement is partially correct.

This study shows that the enzyme is very much affected by the different environment surrounding it. Take the kinetic measurement of ertapenem. With 0.5 M NaCl the performance constant is rising, indicating a better performance of the enzyme. And to take it a step further this value of performance constant is similar to the value which is obtained by VIM-7. In this study the performance constants for asMBL is generally lower than for the other MBLs. It could be suggested that a better comparison of asMBL against the other MBLs would involve optimal conditions for asMBL. When comparing different MBL,s or other enzymes for that matter, it would generally be suitable to use the optimal condition for the enzyme or the condition in which it is interesting to study it under, for instance in coastal sea water or human physiological conditions.

Nitrocefin reactivity

Another thing this study has shown is how the substrate is affected by the environment. Nitrocefin proved to be unstable when in presence of Tris buffer with high pH. These results show how the composition of the reaction mixture can have impact on the behavior of the substrate. This could be seen as an argument for having equal experimental conditions when comparing properties of enzymes, since then you can rule out those differences that would affect the substrate and eventually the final results.

In this study there was no intention of characterization of nitrocefin's properties. But results from characterization of pH optimum and temperature optimum for asMBL gave some unexpected and unexplainable results. This was seen especially when measuring pH optimum, and to address this problem the different experimental parameters were tested. In the end, two experimental properties were observed to influence on the stability of nitrocefin; pH and Tris. And the interesting feature is that there seem to be a synergistic relationship between Tris and pH and the auto-hydrolysis of nitrocefin. When using Hepes as a buffer no apparent auto-hydrolysis of nitrocefin occurs at any pH, while using Tris as a buffer auto-hydrolysis occurs at pH 7 and higher (figure 32). This indicates that the buffer,

Tris, is important for auto-hydrolysis. When varying concentrations of Tris with pH 7.5, there is a relationship between the amount of Tris present and the auto-hydrolysis of nitrocefin, but only to a certain degree. There is no linear relationship between the amount of Tris and the auto-hydrolysis of nitrocefin, since at about 0.08 M Tris the rate of auto-hydrolysis declines. The fact that there is no linear relationship could result from the hypothetic synergistic relationship between Tris concentration and pH, but could very well be of some other origin.

Another interesting feature about the relationship between pH and auto-hydrolysis of nitrocefin is that at pH 8 a noticeable increase in auto-hydrolysis occurs. This corresponds to the pk_a-value of Tris which is 8.1, and could indicate that the ionic form of Tris is responsible for the auto-hydrolysis of nitrocefin (figure 45). Hepes, which was used as reaction buffer after the revelation of Tris auto-hydrolyzing effect, has a pK_a of 7.5. At higher pH than 7.5 Hepes molecule will mostly be neutral charged, while at lower pH then 7.5 the charge of the Hepes molecule will be mostly negative. So the reason for Tris auto-hydrolyzing behavior could be related to its positive charge. Therefore it is advised to be cautious when using buffers which could be positively charged in studies involving nitrocefin.

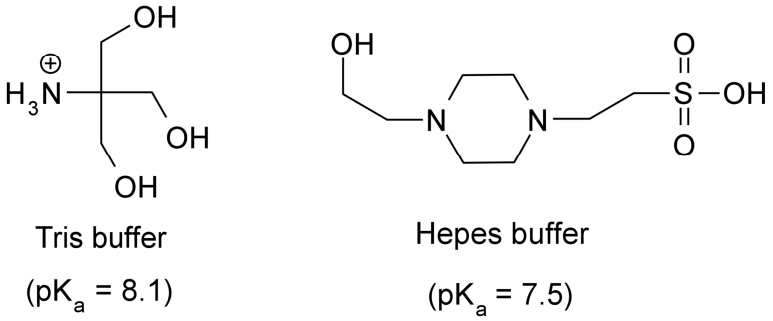

Figure 45 Structures of the buffers Tris and Hepes.

But why is it interesting to mention nitrocefin stability when the study is about MBL? Nitrocefin is widely used as substrate for beta-lactamases, and in that regard it is interesting to mention possible pitfalls when using this substrate. If it was not revealed that nitrocefin had lack of stability at high temperatures and in pH together with Tris buffer, some of the results in this study would then be more questionable. Also this information would hopefully assist others working with this substrate, helping them to avoid the same troubles which

arose during this study. For instance, making a mastermix of nitrocefin together with Tris buffer could be disastrous for the results if the components are mixed in different time intervals, which is often the case.

An example on how big impact the auto-hydrolysis may have in an experiment; A typical blank solution with 20 mM Hepes buffer pH 7.5 present have a OD of about 0.1. A blank solution of 20 mM Tris buffer at pH 7.5 which have been left for 30 minutes has an OD of 0.271. The difference in OD is 0.171. As seen on figure 24 the typical area of measured OD is in the range of 0.1 to 2, so the auto-hydrolysis of nitrocefin has a noticeable impact.

There are many examples of studies where nitrocefin is used together with a Tris buffer; steady-state kinetic studies using 50 mM Tris at pH 7.4 (*77*), steady-state kinetic studies using MTEN buffer, which consists of 50 mM Mes, 25 mM Tris, 25 mM ethanolamine and 100 mM NaCl, at pH ranging from 5.25 to 10.0 (*129*), and hydrolysis of nitrocefin using 50 mM Tris buffer at pH 8.0 (*130*). This shows that many studies using nitrocefin uses Tris as buffer. The studies are probably not incorrect but their experimental setup can be questioned. A study by Wang et al (*129*) seems to experience the same trouble as this study, because reaction rates at pH ≥7.5 were corrected for background hydrolysis. It is highly plausible that the reason for their background hydrolysis of the substrate has the same reason as what have been found during this study. This shows that a thorough examination of the experimental procedures is important in order for a better study design.

Inhibitory screening

From the inhibitory screening done in this study none of the tested molecules had any effect. Those that showed promising effects are being regarded as chelators, for instance dipicolinc acid (*116*). But a study by Horsfall et al shows that pyridine-2,4-dicarboxylate (similar to the picolinic acids in this study) interacts with the enzyme active site of MBL from the molecular subgroup B2 (*131*). Even though asMBL most likely belongs to the molecular subgroup B1, it could be plausible that the picolinic acids in this study might as well be interacting with the active site of asMBL. The inhibitory screening in this study is not able to differentiate between the picolinic acid's chelating effect and its hypothetical interaction of the active site of asMBL. In this regard a crystal structure with asMBL in complex with a

picolinic acid or experiments that could rule out the chelating effects of picolinic acids would be needed to verify the inhibitors interaction with the active site.

PCR

Results from the PCR experiment show that MBL is present in at least some *Aliivibrio* species originating from an environment which is more or less free of human influence. This could indicate that the asMBL does not necessarily need to be driven forward by antibiotic usage in fish farms. asMBL would then seem to be an integrated part of the cell's genome, and maybe have other functions than merely protecting the organism from human antibiotic usage. As already mentioned in the introduction, it is speculated that beta-lactamases could function as a modulator of quorum sensing. This is only speculation, since it may very well be a source of natural antibiotic present where the wild *Aliivibrio* species live.

Those *Aliivibrio* species from the Barents Sea that seem to not possess MBL may still have MBL present in its genome as their MBL could be connected to a larger gene. But also unspecificity present in the PCR method, for instance unspecificty of primers, could lead to PCR products present in the gel which is not related to MBL.

Conclusions

MBL from the bacterium *Aliivibrio salmonicida* has been produced, purified and characterized. At high protein concentrations the asMBL seems unstable which could be caused by the non-cleavable histidine-tag and/or the buffer components. Many properties of the marine asMBL have been discovered through the characterization. It has a high tolerance for salt and is also more adapted to cold temperatures compared to the terrestrial, mesophilic enzyme VIM-7. asMBL has optimal activity at temperatures, salinity and pH that are according to marine environment which indicates that asMBL is fine-tuned in its natural habitat. The substrates which MBL preferably hydrolyze are cephalosporins and carbapenems, and asMBL seems not to hydrolyze monobactams, which is according to what is observed for MBLs VIM-2, VIM-7 and IMP-1. The enzyme efficiency belonging to asMBL seems to be lower than that of VIM-2, VIM-7 and IMP-1, but it is strongly indicated that this is because of lack of salt in the reaction mixtures. Zinc ions are important for MBL to function as an enzyme. The inhibitors that were given for screening seemed to have no inhibitory effect, except for some of them which had a chelating effect on Zinc.

Experimental conditions have great impact on enzyme behavior, and this study shows that enzymes from different environments should be analyzed in great detail so no wrong conclusions are drawn. When comparing a marine enzyme with a terrestrial homologue their respectively environments should be taken into consideration while analyzing the results. Characterization of the substrate may be paramount, as the trials with nitrocefin show. Nitrocefin was observed to be unstable at high pH together the buffer Tris, which is a molecule that with its positive charge is believed to cause auto-hydrolysis of nitrocefin. Nitrocefin is also unstable at high temperature of about $>60^0$C.

Future work

As it seems like the protein is not stable at higher concentrations the first task to attend to would be to find a way to stabilize the protein. For instance using a cleavable histidine tag instead of the non-cleavable tag, and perhaps try to purify the protein in higher concentrations of salt. A purer and more stable protein would better suit a crystallization screening and eventually lead to solving asMBLs structure. Screening of inhibitors and cocrystallization of inhibitor-enzyme complex would be the next natural step. For instance, it would be interesting to cocrystallize the enzyme together with the inhibitor which had been cocrystallised by Horsfall et al (*131*), in order to see if it binds to the active site to confirm or reject their findings.

Other tasks which could be attended to are kinetic studies at 0.5 M NaCl and see if there is an expected increase in enzymatic efficiency for all substrates, not just for ertapenem. During this study only artificial substrates synthesized by man to be used as antibiotics (except nitrocefin) were used in kinetic studies. It would be interesting if the “natural” marine substrate of asMBL could be identified and see if it has anything to do with communication between bacteria.

References

1. Bush, K. and Jacoby, G. A. (2010) Updated functional classification of beta-lactamases, *Antimicrob Agents Chemother 54*, 969-976.
2. Jacoby, G. and Bush, K. (2011) ß-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes, http://www.lahey.org/Studies/.
3. Shimizu-Ibuka, A., Bauvois, C., Sakai, H. and Galleni, M. (2008) Structure of the plasmid-mediated class C beta-lactamase ACT-1, *Acta Crystallogr Sect F Struct Biol Cryst Commun 64*, 334-337.
4. Queenan, A. M., Jenkins, S. and Bush, K. (2001) Cloning and biochemical characterization of FOX-5, an AmpC-type plasmid-encoded beta-lactamase from a New York City Klebsiella pneumoniae clinical isolate, *Antimicrob Agents Chemother 45*, 3189-3194.
5. Rodriguez-Martinez, J. M., Nordmann, P., Ronco, E. and Poirel, L. (2010) Extended-spectrum cephalosporinase in Acinetobacter baumannii, *Antimicrob Agents Chemother 54*, 3484-3488.
6. Ohnishi, M., Ono, E., Shimuta, K., Watanabe, H. and Okamura, N. (2010) Identification of TEM-135 beta-lactamase in penicillinase-producing Neisseria gonorrhoeae strains in Japan, *Antimicrob Agents Chemother 54*, 3021-3023.
7. Schneider, K. D., Karpen, M. E., Bonomo, R. A., Leonard, D. A. and Powers, R. A. (2009) The 1.4 A crystal structure of the class D beta-lactamase OXA-1 complexed with doripenem, *Biochemistry 48*, 11840-11847.
8. Rogers, M. B., Parker, A. C. and Smith, C. J. (1993) Cloning and characterization of the endogenous cephalosporinase gene, cepA, from Bacteroides fragilis reveals a new subgroup of Ambler class A beta-lactamases, *Antimicrob Agents Chemother 37*, 2391-2400.
9. Neuwirth, C., Siebor, E., Robin, F. and Bonnet, R. (2007) First occurrence of an IMP metallo-beta-lactamase in Aeromonas caviae: IMP-19 in an isolate from France, *Antimicrob Agents Chemother 51*, 4486-4488.
10. Perilli, M., Caporale, B., Celenza, G., Pellegrini, C., Docquier, J. D., Mezzatesta, M., Rossolini, G. M., Stefani, S. and Amicosante, G. (2007) Identification and characterization of a new metallo-beta-lactamase, IND-5, from a clinical isolate of Chryseobacterium indologenes, *Antimicrob Agents Chemother 51*, 2988-2990.
11. Rodriguez-Martinez, J. M., Nordmann, P., Fortineau, N. and Poirel, L. (2010) VIM-19, a metallo-beta-lactamase with increased carbapenemase activity from Escherichia coli and Klebsiella pneumoniae, *Antimicrob Agents Chemother 54*, 471-476.
12. Sharma, N., Toney, J. H. and Fitzgerald, P. M. (2005) Expression, purification, crystallization and preliminary X-ray analysis of Aeromonas hydrophilia metallo-beta-lactamase, *Acta Crystallogr Sect F Struct Biol Cryst Commun 61*, 180-182.
13. Saavedra, M. J., Peixe, L., Sousa, J. C., Henriques, I., Alves, A. and Correia, A. (2003) Sfh-I, a subclass B2 metallo-beta-lactamase from a Serratia fonticola environmental isolate, *Antimicrob Agents Chemother 47*, 2330-2333.
14. Ambler, R. P. (1980) The structure of beta-lactamases, *Philos Trans R Soc Lond B Biol Sci 289*, 321-331.
15. Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O. and Frere, J. M. (2001) Standard numbering scheme for class B beta-lactamases, *Antimicrob Agents Chemother 45*, 660-663.
16. Hernandez Valladares, M., Felici, A., Weber, G., Adolph, H. W., Zeppezauer, M., Rossolini, G. M., Amicosante, G., Frere, J. M. and Galleni, M. (1997) Zn(II) dependence of the Aeromonas hydrophila AE036 metallo-beta-lactamase activity and stability, *Biochemistry 36*, 11534-11541.
17. Concha, N. O., Janson, C. A., Rowling, P., Pearson, S., Cheever, C. A., Clarke, B. P., Lewis, C., Galleni, M., Frere, J. M., Payne, D. J., Bateson, J. H. and Abdel-Meguid, S. S. (2000) Crystal

structure of the IMP-1 metallo beta-lactamase from Pseudomonas aeruginosa and its complex with a mercaptocarboxylate inhibitor: binding determinants of a potent, broad-spectrum inhibitor, *Biochemistry 39*, 4288-4298.
18. Bebrone, C. (2007) Metallo-beta-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily, *Biochem Pharmacol 74*, 1686-1701.
19. Carfi, A., Pares, S., Duee, E., Galleni, M., Duez, C., Frere, J. M. and Dideberg, O. (1995) The 3-D structure of a zinc metallo-beta-lactamase from Bacillus cereus reveals a new type of protein fold, *EMBO J 14*, 4914-4921.
20. Moali, C., Anne, C., Lamotte-Brasseur, J., Groslambert, S., Devreese, B., Van Beeumen, J., Galleni, M. and Frere, J. M. (2003) Analysis of the importance of the metallo-beta-lactamase active site loop in substrate binding and catalysis, *Chem Biol 10*, 319-329.
21. Costello, A. L., Sharma, N. P., Yang, K. W., Crowder, M. W. and Tierney, D. L. (2006) X-ray absorption spectroscopy of the zinc-binding sites in the class B2 metallo-beta-lactamase ImiS from Aeromonas veronii bv. sobria, *Biochemistry 45*, 13650-13658.
22. de Seny, D., Heinz, U., Wommer, S., Kiefer, M., Meyer-Klaucke, W., Galleni, M., Frere, J. M., Bauer, R. and Adolph, H. W. (2001) Metal ion binding and coordination geometry for wild type and mutants of metallo-beta -lactamase from Bacillus cereus 569/H/9 (BcII): a combined thermodynamic, kinetic, and spectroscopic approach, *J Biol Chem 276*, 45065-45078.
23. Wommer, S., Rival, S., Heinz, U., Galleni, M., Frere, J. M., Franceschini, N., Amicosante, G., Rasmussen, B., Bauer, R. and Adolph, H. W. (2002) Substrate-activated zinc binding of metallo-beta -lactamases: physiological importance of mononuclear enzymes, *J Biol Chem 277*, 24142-24147.
24. Crowder, M. W., Wang, Z., Franklin, S. L., Zovinka, E. P. and Benkovic, S. J. (1996) Characterization of the metal-binding sites of the beta-lactamase from Bacteroides fragilis, *Biochemistry 35*, 12126-12132.
25. Carfi, A., Duee, E., Galleni, M., Frere, J. M. and Dideberg, O. (1998) 1.85 A resolution structure of the zinc (II) beta-lactamase from Bacillus cereus, *Acta Crystallogr D Biol Crystallogr 54*, 313-323.
26. Fabiane, S. M., Sohi, M. K., Wan, T., Payne, D. J., Bateson, J. H., Mitchell, T. and Sutton, B. J. (1998) Crystal structure of the zinc-dependent beta-lactamase from Bacillus cereus at 1.9 A resolution: binuclear active site with features of a mononuclear enzyme, *Biochemistry 37*, 12404-12411.
27. Wang, Z., Fast, W. and Benkovic, S. J. (1999) On the mechanism of the metallo-beta-lactamase from Bacteroides fragilis, *Biochemistry 38*, 10013-10023.
28. Badarau, A. and Page, M. I. (2008) Loss of enzyme activity during turnover of the Bacillus cereus beta-lactamase catalysed hydrolysis of beta-lactams due to loss of zinc ion, *J Biol Inorg Chem 13*, 919-928.
29. Urbanczyk, H., Ast, J. C., Higgins, M. J., Carson, J. and Dunlap, P. V. (2007) Reclassification of Vibrio fischeri, Vibrio logei, Vibrio salmonicida and Vibrio wodanis as Aliivibrio fischeri gen. nov., comb. nov., Aliivibrio logei comb. nov., Aliivibrio salmonicida comb. nov. and Aliivibrio wodanis comb. nov, *Int J Syst Evol Microbiol 57*, 2823-2829.
30. Lillehaug, A. (1990) A Field Trial of Vaccination against Cold-Water Vibriosis in Atlantic Salmon (Salmo-Salar L), *Aquaculture 84*, 1-12.
31. Turekian, K. K. (1968) *Oceans*, Prentice-Hall, Englewood Cliffs, USA.
32. Ball, E. G. and Stock, C. C. (1937) The pH of sea water as measured with the glass electrode, *Biol Bull-Us 73*, 221-226.
33. Research data from the hydrographic station at Eggum. Institute of Marine Research. Retrived 15.07.2011. http://www.imr.no/forskning/forskningsdata/stasjoner/.
34. Altermark, B., Niiranen, L., Willassen, N. P., Smalas, A. O. and Moe, E. (2007) Comparative studies of endonuclease I from cold-adapted Vibrio salmonicida and mesophilic Vibrio cholerae, *FEBS J 274*, 252-263.

35. Kennedy, S. P., Ng, W. V., Salzberg, S. L., Hood, L. and DasSarma, S. (2001) Understanding the adaptation of Halobacterium species NRC-1 to its extreme environment through computational analysis of its genome sequence, *Genome Res 11*, 1641-1650.
36. Siddiqui, K. S. and Cavicchioli, R. (2006) Cold-adapted enzymes, *Annu Rev Biochem 75*, 403-433.
37. Madern, D., Ebel, C. and Zaccai, G. (2000) Halophilic adaptation of enzymes, *Extremophiles 4*, 91-98.
38. Nishimura, C., Uversky, V. N. and Fink, A. L. (2001) Effect of salts on the stability and folding of staphylococcal nuclease, *Biochemistry 40*, 2113-2128.
39. Wikipedia. Michaelis-Menten kinetics. Retrieved 12.07.2012. http://en.wikipedia.org/wiki/Michaelis-Menten_kinetics
40. Waksman, S. A. (1947) What is an antibiotic or an antibiotic substance?, *Mycologia 39*, 565-569.
41. Wikipedia. Antibacterial. Retrived 13.07.2011. http://en.wikipedia.org/wiki/Antibiotics.
42. Drlica, K. and Zhao, X. (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones, *Microbiol Mol Biol Rev 61*, 377-392.
43. Chen, H. H., Chiang, W., Chang, J. Y., Chien, Y. L., Lee, C. K., Liu, K. J., Cheng, Y. T., Chen, T. F., Kuo, Y. H. and Kuo, C. C. (2011) Antimutagenic Constituents of Adlay (Coix lachryma-jobi L. var. ma-yuen Stapf) with Potential Cancer Chemopreventive Activity, *J Agric Food Chem 59*, 6444-6452.
44. Bryskier, A. (1999) New research in macrolides and ketolides since 1997, *Expert Opin Investig Drugs 8*, 1171-1194.
45. Retsema, J. and Fu, W. (2001) Macrolides: structures and microbial targets, *Int J Antimicrob Agents 18 Suppl 1*, S3-10.
46. Guillot, L., Tabary, O., Nathan, N., Corvol, H. and Clement, A. (2011) Macrolides: New therapeutic perspectives in lung diseases, *Int J Biochem Cell Biol*.
47. Giesbrecht, P., Kersten, T., Maidhof, H. and Wecke, J. (1998) Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin, *Microbiol Mol Biol Rev 62*, 1371-1414.
48. Fleming, A. (2001) On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. 1929, *Bull World Health Organ 79*, 780-790.
49. Nobel foundation, Web Page: http://nobelprize.org/.
50. Rolinson, G. N. (1998) Forty years of beta-lactam research, *J Antimicrob Chemother 41*, 589-603.
51. Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. and Rolinson, G. N. (1959) Synthesis of Penicillin - 6-Aminopenicillanic Acid in Penicillin Fermentations, *Nature 183*, 257-258.
52. Kosmidis, J., Hamilton-Miller, J. M., Gilchrist, J. N., Kerry, D. W. and Brumfitt, W. (1973) Cefoxitin, a new semi-synthetic cephamycin: an in-vitro and in-vivo comparison with cephalothin, *Br Med J 4*, 653-655.
53. Onishi, H. R., Daoust, D. R., Zimmerman, S. B., Hendlin, D. and Stapley, E. O. (1974) Cefoxitin, a semisynthetic cephamycin antibiotic: resistance to beta-lactamase inactivation, *Antimicrob Agents Chemother 5*, 38-48.
54. Karady, S., Pines, S. H., Weinstock, L. M., Roberts, F. E., Brenner, G. S., Hoinowski, A. M., Cheng, T. Y. and Sletzinger, M. (1972) Semisynthetic cephalosporins via a novel acyl exchange reaction, *J Am Chem Soc 94*, 1410-1411.
55. Morin, R. B., Roeske, R. W., Flynn, E. H. and Jackson, B. G. (1962) Chemistry of Cephalosporin Antibiotics .1. 7-Aminocephalosporanic Acid from Cephalosporin C, *Journal of the American Chemical Society 84*, 3400-&.
56. Page, M. G. (2004) Cephalosporins in clinical development, *Expert Opin Investig Drugs 13*, 973-985.

57. O'Callaghan, C. H., Sykes, R. B., Ryan, D. M., Foord, R. D. and Muggleton, P. W. (1976) Cefuroxime - a new cephalosporin antibiotic, *J Antibiot (Tokyo) 29*, 29-37.
58. Verbist, L. and Verhaegen, J. (1980) GR-20263: a new aminothiazolyl cephalosporin with high activity against Pseudomonas and Enterobacteriaceae, *Antimicrob Agents Chemother 17*, 807-812.
59. Bodey, G. P., Ho, D. H. and LeBlanc, B. (1985) In vitro studies of BMY-28142, a new broad-spectrum cephalosporin, *Antimicrob Agents Chemother 27*, 265-269.
60. Kessler, R. E., Bies, M., Buck, R. E., Chisholm, D. R., Pursiano, T. A., Tsai, Y. H., Misiek, M., Price, K. E. and Leitner, F. (1985) Comparison of a new cephalosporin, BMY 28142, with other broad-spectrum beta-lactam antibiotics, *Antimicrob Agents Chemother 27*, 207-216.
61. Shahid, M., Sobia, F., Singh, A., Malik, A., Khan, H. M., Jonas, D. and Hawkey, P. M. (2009) Beta-lactams and beta-lactamase-inhibitors in current- or potential-clinical practice: a comprehensive update, *Crit Rev Microbiol 35*, 81-108.
62. Kesado, T., Hashizume, T. and Asahi, Y. (1980) Antibacterial activities of a new stabilized thienamycin, N-formimidoyl thienamycin, in comparison with other antibiotics, *Antimicrob Agents Chemother 17*, 912-917.
63. Kropp, H., Sundelof, J. G., Kahan, J. S., Kahan, F. M. and Birnbaum, J. (1980) MK0787 (N-formimidoyl thienamycin): evaluation of in vitro and in vivo activities, *Antimicrob Agents Chemother 17*, 993-1000.
64. Birnbaum, J., Kahan, F. M., Kropp, H. and MacDonald, J. S. (1985) Carbapenems, a new class of beta-lactam antibiotics. Discovery and development of imipenem/cilastatin, *Am J Med 78*, 3-21.
65. Sentochnik, D. E., Eliopoulos, G. M., Ferraro, M. J. and Moellering, R. C., Jr. (1989) Comparative in vitro activity of SM7338, a new carbapenem antimicrobial agent, *Antimicrob Agents Chemother 33*, 1232-1236.
66. Kohler, J., Dorso, K. L., Young, K., Hammond, G. G., Rosen, H., Kropp, H. and Silver, L. L. (1999) In vitro activities of the potent, broad-spectrum carbapenem MK-0826 (L-749,345) against broad-spectrum beta-lactamase-and extended-spectrum beta-lactamase-producing Klebsiella pneumoniae and Escherichia coli clinical isolates, *Antimicrob Agents Chemother 43*, 1170-1176.
67. Georgopapadakou, N. H., Smith, S. A. and Sykes, R. B. (1982) Mode of action of azthreonam, *Antimicrob Agents Chemother 21*, 950-956.
68. Medeiros, A. A. (1997) Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics, *Clin Infect Dis 24 Suppl 1*, S19-45.
69. O'Callaghan, C. H., Morris, A., Kirby, S. M. and Shingler, A. H. (1972) Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate, *Antimicrob Agents Chemother 1*, 283-288.
70. De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frere, J. M. and Waley, S. G. (1987) Automated analysis of enzyme inactivation phenomena. Application to beta-lactamases and DD-peptidases, *Biochem Pharmacol 36*, 2393-2403.
71. Papanicolaou, G. A. and Medeiros, A. A. (1990) Discrimination of extended-spectrum beta-lactamases by a novel nitrocefin competition assay, *Antimicrob Agents Chemother 34*, 2184-2192.
72. Marumo, K., Nagaki, T. and Nakamura, Y. (1996) Evaluation of high-level carbapenem resistance in atypical Serratia marcescens by a comparison with its revertants, *J Antimicrob Chemother 38*, 47-58.
73. Cole, M. J., Chisholm, S. A., Hoffmann, S., Stary, A., Lowndes, C. M. and Ison, C. A. (2010) European surveillance of antimicrobial resistance in Neisseria gonorrhoeae, *Sex Transm Infect 86*, 427-432.
74. Sethi, S., Sharma, D., Mehta, S. D., Singh, B., Smriti, M., Kumar, B. and Sharma, M. (2006) Emergence of ciprofloxacin resistant Neisseria gonorrhoeae in north India, *Indian J Med Res 123*, 707-710.

75. Pinheiro, E. T., Gomes, B. P., Drucker, D. B., Zaia, A. A., Ferraz, C. C. and Souza-Filho, F. J. (2004) Antimicrobial susceptibility of Enterococcus faecalis isolated from canals of root filled teeth with periapical lesions, *Int Endod J 37*, 756-763.
76. Stachyra, T., Levasseur, P., Pechereau, M. C., Girard, A. M., Claudon, M., Miossec, C. and Black, M. T. (2009) In vitro activity of the {beta}-lactamase inhibitor NXL104 against KPC-2 carbapenemase and Enterobacteriaceae expressing KPC carbapenemases, *J Antimicrob Chemother 64*, 326-329.
77. Yamaguchi, Y., Kuroki, T., Yasuzawa, H., Higashi, T., Jin, W., Kawanami, A., Yamagata, Y., Arakawa, Y., Goto, M. and Kurosaki, H. (2005) Probing the role of Asp-120(81) of metallo-beta-lactamase (IMP-1) by site-directed mutagenesis, kinetic studies, and X-ray crystallography, *J Biol Chem 280*, 20824-20832.
78. Lassaux, P., Traore, D. A., Loisel, E., Favier, A., Docquier, J. D., Sohier, J. S., Laurent, C., Bebrone, C., Frere, J. M., Ferrer, J. L. and Galleni, M. (2011) Biochemical and structural characterization of the subclass B1 metallo-beta-lactamase VIM-4, *Antimicrob Agents Chemother 55*, 1248-1255.
79. Hawkey, P. M. and Jones, A. M. (2009) The changing epidemiology of resistance, *J Antimicrob Chemother 64 Suppl 1*, i3-10.
80. Czaran, T. L., Hoekstra, R. F. and Pagie, L. (2002) Chemical warfare between microbes promotes biodiversity, *Proc Natl Acad Sci U S A 99*, 786-790.
81. Allen, H. K., Moe, L. A., Rodbumrer, J., Gaarder, A. and Handelsman, J. (2009) Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil, *ISME J 3*, 243-251.
82. Lima-Bittencourt, C. I., Cursino, L., Goncalves-Dornelas, H., Pontes, D. S., Nardi, R. M., Callisto, M., Chartone-Souza, E. and Nascimento, A. M. (2007) Multiple antimicrobial resistance in Enterobacteriaceae isolates from pristine freshwater, *Genet Mol Res 6*, 510-521.
83. Aminov, R. I. (2009) The role of antibiotics and antibiotic resistance in nature, *Environ Microbiol 11*, 2970-2988.
84. Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm, *Science 280*, 295-298.
85. Nealson, K. H. and Hastings, J. W. (1979) Bacterial bioluminescence: its control and ecological significance, *Microbiol Rev 43*, 496-518.
86. Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S. and Kaper, J. B. (1999) Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic Escherichia coli, *Proc Natl Acad Sci U S A 96*, 15196-15201.
87. Niu, C., Clemmer, K. M., Bonomo, R. A. and Rather, P. N. (2008) Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii, *J Bacteriol 190*, 3386-3392.
88. Swift, S., Lynch, M. J., Fish, L., Kirke, D. F., Tomas, J. M., Stewart, G. S. and Williams, P. (1999) Quorum sensing-dependent regulation and blockade of exoprotease production in Aeromonas hydrophila, *Infect Immun 67*, 5192-5199.
89. Davies, J., Spiegelman, G. B. and Yim, G. (2006) The world of subinhibitory antibiotic concentrations, *Curr Opin Microbiol 9*, 445-453.
90. Calabrese, E. J. and Baldwin, L. A. (2002) Defining hormesis, *Hum Exp Toxicol 21*, 91-97.
91. Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P. and Hoiby, N. (2004) Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production, *Antimicrob Agents Chemother 48*, 1175-1187.
92. Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A. and Miller, S. I. (2005) Aminoglycoside antibiotics induce bacterial biofilm formation, *Nature 436*, 1171-1175.
93. Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. and O'Toole, G. A. (2003) A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance, *Nature 426*, 306-310.

94. Ng, W. L., Kazmierczak, K. M., Robertson, G. T., Gilmour, R. and Winkler, M. E. (2003) Transcriptional regulation and signature patterns revealed by microarray analyses of Streptococcus pneumoniae R6 challenged with sublethal concentrations of translation inhibitors, *J Bacteriol 185*, 359-370.
95. Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. and Helmann, J. D. (2003) Cell wall stress responses in Bacillus subtilis: the regulatory network of the bacitracin stimulon, *Mol Microbiol 50*, 1591-1604.
96. Tomasinsig, L., Scocchi, M., Mettulio, R. and Zanetti, M. (2004) Genome-wide transcriptional profiling of the Escherichia coli response to a proline-rich antimicrobial peptide, *Antimicrob Agents Chemother 48*, 3260-3267.
97. European Centre for Disease Prevention and Control. Annual Epidemiological Report on Communicable Diseases in Europe (2010) Stockholm: ECDC; 2010, pp 167-172.
98. Department of Pharmacoepidemiology, Norwegian Institute of Public Health. Drug consumption in Norway 2006-2010 (2010), 63-65.
99. Hutchinson, J. M., Patrick, D. M., Marra, F., Ng, H., Bowie, W. R., Heule, L., Muscat, M. and Monnet, D. L. (2004) Measurement of antibiotic consumption: A practical guide to the use of the Anatomical Thgerapeutic Chemical classification and Definied Daily Dose system methodology in Canada, *Can J Infect Dis 15*, 29-35.
100. Petersen, A., Andersen, J. S., Kaewmak, T., Somsiri, T. and Dalsgaard, A. (2002) Impact of integrated fish farming on antimicrobial resistance in a pond environment, *Appl Environ Microbiol 68*, 6036-6042.
101. NORM/NORM-VET 2008. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Reasistance in Norway (2009) Tromsø/Oslo. ISSN: 1890-9965, 1-18.
102. Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D. and Hochstrasser, D. F. (1999) Protein identification and analysis tools in the ExPASy server, *Methods Mol Biol 112*, 531-552.
103. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680-685.
104. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res 25*, 3389-3402.
105. Notredame, C., Higgins, D. G. and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment, *J Mol Biol 302*, 205-217.
106. Jones, D. T. (1999) Protein secondary structure prediction based on position-specific scoring matrices, *J Mol Biol 292*, 195-202.
107. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank, *Nucleic Acids Res 28*, 235-242.
108. Gouet, P., Courcelle, E., Stuart, D. I. and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript, *Bioinformatics 15*, 305-308.
109. Garau, G., Garcia-Saez, I., Bebrone, C., Anne, C., Mercuri, P., Galleni, M., Frere, J. M. and Dideberg, O. (2004) Update of the standard numbering scheme for class B beta-lactamases, *Antimicrob Agents Chemother 48*, 2347-2349.
110. Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0, *J Mol Biol 340*, 783-795.
111. Hu, Z., Periyannan, G., Bennett, B. and Crowder, M. W. (2008) Role of the Zn1 and Zn2 sites in metallo-beta-lactamase L1, *J Am Chem Soc 130*, 14207-14216.
112. Lisa, M. N., Hemmingsen, L. and Vila, A. J. (2010) Catalytic role of the metal ion in the metallo-beta-lactamase GOB, *J Biol Chem 285*, 4570-4577.
113. Brown, A. M. (2001) A step-by-step guide to non-linear regression analysis of experimental data using a Microsoft Excel spreadsheet, *Comput Methods Programs Biomed 65*, 191-200.
114. Superdex High Performance Columns (2007) GE healthcare, 4.

115. Lattemann, C. T., Maurer, J., Gerland, E. and Meyer, T. F. (2000) Autodisplay: functional display of active beta-lactamase on the surface of Escherichia coli by the AIDA-I autotransporter, *J Bacteriol 182*, 3726-3733.
116. Laraki, N., Franceschini, N., Rossolini, G. M., Santucci, P., Meunier, C., de Pauw, E., Amicosante, G., Frere, J. M. and Galleni, M. (1999) Biochemical characterization of the Pseudomonas aeruginosa 101/1477 metallo-beta-lactamase IMP-1 produced by Escherichia coli, *Antimicrob Agents Chemother 43*, 902-906.
117. Poirel, L., Naas, T., Nicolas, D., Collet, L., Bellais, S., Cavallo, J. D. and Nordmann, P. (2000) Characterization of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and integron-borne gene from a Pseudomonas aeruginosa clinical isolate in France, *Antimicrob Agents Chemother 44*, 891-897.
118. Awad, S., Allison, S. P. and Lobo, D. N. (2008) The history of 0.9% saline, *Clin Nutr 27*, 179-188.
119. Docquier, J. D., Lamotte-Brasseur, J., Galleni, M., Amicosante, G., Frere, J. M. and Rossolini, G. M. (2003) On functional and structural heterogeneity of VIM-type metallo-beta-lactamases, *J Antimicrob Chemother 51*, 257-266.
120. Samuelsen, O., Castanheira, M., Walsh, T. R. and Spencer, J. (2008) Kinetic characterization of VIM-7, a divergent member of the VIM metallo-beta-lactamase family, *Antimicrob Agents Chemother 52*, 2905-2908.
121. Little, G. and Brocklehurst, K. (1972) Kinetics of the reversible reaction of papain with 5,5'-dithiobis-(2-nitrobenzoate) dianion: evidence for nucleophilic reactivity in the un-ionized thiol group of cysteine-25 and for general acid catalysis by histidine-159 of the reaction of the 5-mercapto-2-nitrobenzoate dianion with the papain-5-mercapto-2-nitrobenzoate mixed disulphide, *Biochem J 128*, 475-477.
122. Cleland, W. W. (1964) Dithiothreitol, a New Protective Reagent for Sh Groups, *Biochemistry 3*, 480-482.
123. Koti Ainavarapu, S. R., Wiita, A. P., Dougan, L., Uggerud, E. and Fernandez, J. M. (2008) Single-molecule force spectroscopy measurements of bond elongation during a bimolecular reaction, *J Am Chem Soc 130*, 6479-6487.
124. Han, J. C. and Han, G. Y. (1994) A Procedure for Quantitative-Determination of Tris(2-Carboxyethyl)Phosphine, an Odorless Reducing Agent More Stable and Effective Than Dithiothreitol, *Analytical Biochemistry 220*, 5-10.
125. Motulsky, H. J. and Ransnas, L. A. (1987) Fitting Curves to Data Using Nonlinear-Regression - a Practical and Nonmathematical Review, *Faseb Journal 1*, 365-374.
126. Lutz, S. and Bornscheuer, U. T. (2009) *Protein Engineering Handbook*, Vol. 1, Wiley-VCH, Weinheim, Germany.
127. Currie, D. J. (1982) Estimating Michaelis-Menten Parameters - Bias, Variance and Experimental-Design, *Biometrics 38*, 907-919.
128. Colombo, M. L., Hanique, S., Baurin, S. L., Bauvois, C., De Vriendt, K., Van Beeumen, J. J., Frere, J. M. and Joris, B. (2004) The ybxI gene of Bacillus subtilis 168 encodes a class D beta-lactamase of low activity, *Antimicrob Agents Chemother 48*, 484-490.
129. Wang, Z. and Benkovic, S. J. (1998) Purification, characterization, and kinetic studies of a soluble Bacteroides fragilis metallo-beta-lactamase that provides multiple antibiotic resistance, *J Biol Chem 273*, 22402-22408.
130. Sanschagrin, F. and Levesque, R. C. (2005) A specific peptide inhibitor of the class B metallo-beta-lactamase L-1 from Stenotrophomonas maltophilia identified using phage display, *J Antimicrob Chemother 55*, 252-255.
131. Horsfall, L. E., Garau, G., Lienard, B. M., Dideberg, O., Schofield, C. J., Frere, J. M. and Galleni, M. (2007) Competitive inhibitors of the CphA metallo-beta-lactamase from Aeromonas hydrophila, *Antimicrob Agents Chemother 51*, 2136-2142.