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Department of Chemistry

Insights into bacterial protection and survival

A study of three enzymes from cold adapted bacteria

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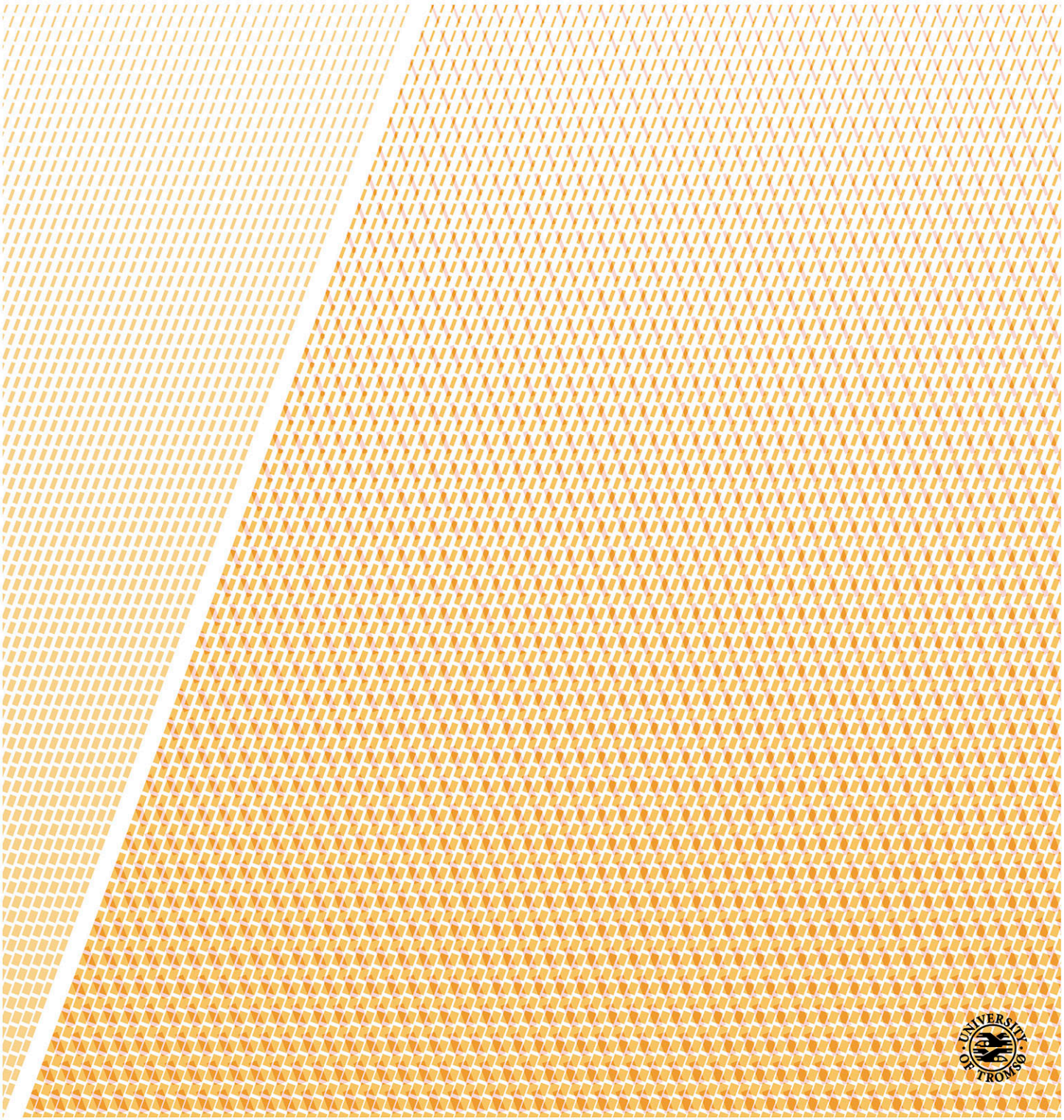
Miriam Grgic

A dissertation for the degree of Philosophiae Doctor – September 2015



Insights into bacterial protection and survival.

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Don´t panic

To my Mom, sister and nieces
In loving memory of my Dad

Acknowledgments

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Miriam Grgic,

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TABLE OF CONTENTS

Summary

Bacteria are the most abundant organisms and can be found in different habitats, from polar regions, deserts and volcanoes, deep ocean trenches to the upper atmosphere. In all these environments, they are exposed to various chemical hazards, such as antibacterial chemicals (antibiotics) produced by other organisms that are found in the same habitat. In addition, they are exposed to threats from infection by bacteriophages. In order to protect themselves, bacteria have evolved several mechanisms towards avoiding antibiotics and phage infection. In this work, enzymes involved in these protection mechanisms have been attempted characterized both functionally and structurally. Specifically two DNA modifying enzymes have been studied. They are involved in bacterial protection from phages, namely the type II restriction enzyme DpnI and the C5-DNA-MTase ParI, both originating from the psychrophilic bacterium *Psychrobacter arcticus.* The third enzyme that has been investigated is a metallo-β-lactamase (ALI-1) from the cold adapted marine bacterium *Aliivibrio salmonicida.* Since all these enzymes originate from organisms adapted to a cold environment, the presence of cold adapted enzyme features like heat lability and high activity at low temperature, were investigated.

In Manuscript I and in Manuscript III the type II restriction enzyme DpnI and the C5-DNA-MTase ParI were partially characterized. In addition to being important for the bacterial cell, these types of enzymes are valuable in several molecular biology techniques and the development of more efficient enzymes is necessary in terms of reducing cost and increasing efficiency. Due to their characteristics such as higher activity at lower temperatures and heat lability cold adapted enzymes became very attractive targets. Both DpnI and ParI were recombinantly expressed, but with some difficulties, that were overcome by usage of specialized *E. coli* strains. Both enzymes were partially characterize with regards to function, DpnI showed activity *in vitro,* while MTase was shown to have methylating activity *in vivo.*

One of the mechanisms in antibiotic resistance is the acquirement and development of β-lactamases, enzymes that can cleave the amide bond in the β-lactam ring, a common structure in all β-lactam antibiotics, and thus inactivate it. This presents a huge problem in usage of β-lactam antibiotics, which are widely used against bacterial infections in both humans and animals. In Paper II, the metallo-β-lactamase ALI-1, from the cold adapted marine bacterium *Aliivibrio salmonicida*, was characterised. When compared to a mesophilic counterpart (VIM-

2 from *Pseudomonas aeruginosa*) it was shown to be adapted to its natural habitat, in terms of being more active at lower temperatures and in higher salt concentrations. In addition, in this work it was found that the gene encoding ALI-1 is present in environmental bacterial samples. This could point towards a broader role for ALI-1, such as in regulating quorum sensing signalling in bacteria.

LIST OF PAPERS

Manuscript I - A study of a type II restriction endonuclease from the cold-adapted organism *Psychrobacter arcticus.* Manuscript.
Grgic Miriam, Altermark Bjørn, Leiros Ingar

Paper II - *Properties and distribution of a metallo-β-lactamase (ALI-1) from the fish pathogen Aliivibrio salmonicida LFI1238.* Published in J. Antimicrob. Chemother.; Oct 31, 2014.
Kristiansen Anders; Grgic Miriam; Altermark Bjørn; Leiros Ingar.

Manuscript III - Initial characterization of ParI, an orphan C5-DNA methyltransferase from *Psychrobacter arcticus* 273-4. Manuscript. To be submitted to BMC Biochemistry

Grgic Miriam, Bjerga Gro Elin Kjæreng, Williamson Adele Kim, Altermark Bjørn, Leiros Ingar

ABBREVIATIONS

DNA - deoxyribonucleic acid

Sie system- super infection exclusion system

RM system - restriction modification system

REase - restricition endonuclease

MTase - methyltransferase

Abi system - abortive infection systems

MBL -metallo-β-lactamase

dsDNA - double stranded deoxyribonucleic acid

ΔG - Gibbs free energy

ΔS - enthalpy

ΔH - entropy

G, C, A, T - guanine, adenine, cytosine, thymine

bp - base pair

SAM (AdoMet) - S-adenosyl methionine

SAH - S-adenosyl-L-homocysteine

C5-DNA MTase - cytosine specific DNA methyltransferase

GTP - guanosine-5'-triphosphate

kb - kilo base

TRD - target recognition domain

RNA - ribonucleic acid

AP – alkaline phosphatase

DSC – Differential scanning calorimetry

TF – Thermofluor

CD – circular dichroism

I. INTRODUCTION

1 Host defence in bacteria

Bacteria are exposed to various hazards from the environment they live in; chemical as well as threats from infection by bacteriophages. One of the biggest threats bacteria have to deal with are various antibacterial chemicals (antibiotics) produced by other organisms that are found in the same habitat (1). In order to protect themselves, bacteria have evolved several mechanisms towards avoiding antibiotics (Figure 1). As one of four main mechanisms, β-lactamases have been very efficient in the bacterial protection against antibiotics (Figure 1). The problem with antibiotic resistance has steadily increased since the 1950s when the β-lactam antibiotic penicillin was discovered and became widely used against bacterial infections in both humans and animals (2).

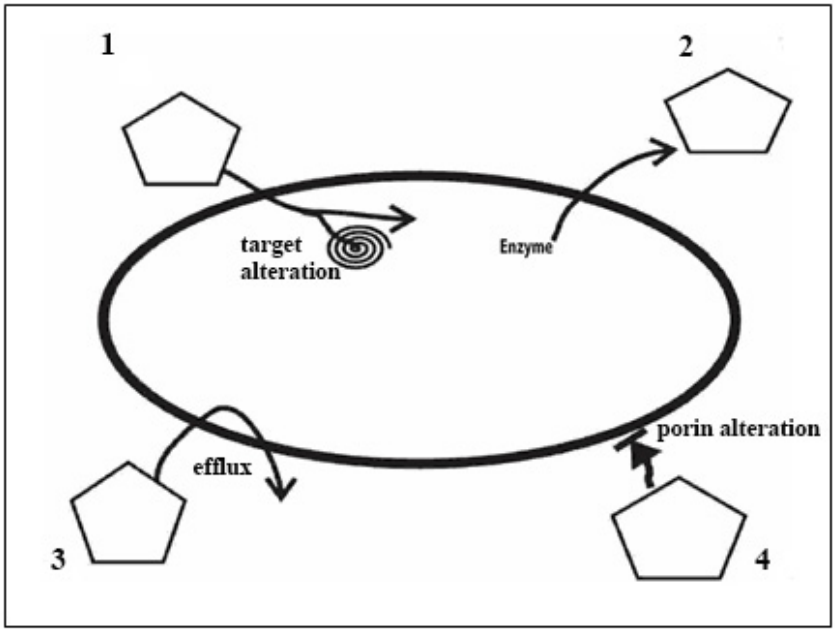

Figure 1. Schematic representation of the major antibiotics resistance mechanisms in bacteria. 1. Alteration of specific targets (here presented as spiral), which have a necessary role in microbial growth that leads to loss or decrease in drug affinity. 2. Production of enzymes by the host that can inactivate or modify the antibiotic chemical, such as β-lactamases. This is a major mechanism of resistance to antibiotics. 3. The development of energy-dependent pumps and removal of the drug by active efflux, which limits intracellular accumulation of toxic compounds. It is mediated by membrane-based efflux proteins acting as pumps. 4. Impermeability by mutation of a porin channel (pore in the external membrane involved in selective transport) causing very low permeability of antibiotics. Pentagon in all steps presents antibiotic molecule. The figure is made in ChemDoodle based on (3) .

In addition to antibiotics, bacteria have to deal with continued threats of bacteriophage (bacterial virus) infections. In order to protect themselves, bacteria have developed several antiviral mechanisms. These mechanisms can be divided into four groups:

1. Prevention of phage adsorption, where bacteria can either change the three-dimensional conformations of cell surface receptors, produce extracellular polymers that serve as protectants of the bacterial cell or produce competitive inhibitors that bind specifically to phage receptors (4).
2. Prevention of phage DNA entry by super infection exclusion (Sie) systems. This system comprises a group of proteins that are anchored in the membrane and block the entry of phage DNA into the bacterial cell (5, 6).
3. Digestion of phage nucleic acids by the restriction-modification systems (RM systems), which are comprised of a restriction endonuclease (REase) and a methyltransferase (DNA-MTase). REase cleaves non-self (phage) DNA, while DNA-MTase modifies self-DNA at the same site as the REase recognizes and thus protects it from restriction (7, 8).
4. Usage of abortive infection systems (Abi systems), which are a group of proteins that provide resistance to phages by acting on replication, transcription or translation of the phage. This system leads to death of the bacterial cell (9, 10).

In this thesis, three case studies with focus on metallo-β-lactamase and enzymes from RM-systems will be presented.

2 Bacterial defence against antibiotics

Based on their chemical structure, substrate and target preferences, antibiotics are classified into several groups, *e.g.* the ß-lactams, the aminoglycosides and the tetracyclines (3). A common feature of all ß-lactam antibiotics is the 4-membered ß-lactam ring (Figure 2). In the bacterial cell wall, the β-lactam antibiotics, such as penicillins and cephalosporins, block cell wall synthesis. The cell wall is a mechanical protection from the environment and preserves cell shape and rigidity. Synthesis of the cell wall occurs in several steps in the cytoplasm. First, the muramyl pentapeptide is synthesized and translocated to the outside of the cell membrane. The synthesis of the cell wall is then completed by cross-linking of peptidoglycan units by transglycosylases and transpeptidases. It is this second step that is targeted by the ß-lactam antibiotics and causes cell death (11, 12). Antibiotics can also affect prokaryote development and propagation through the inhibition of protein biosynthesis or inhibition of DNA replication (3).

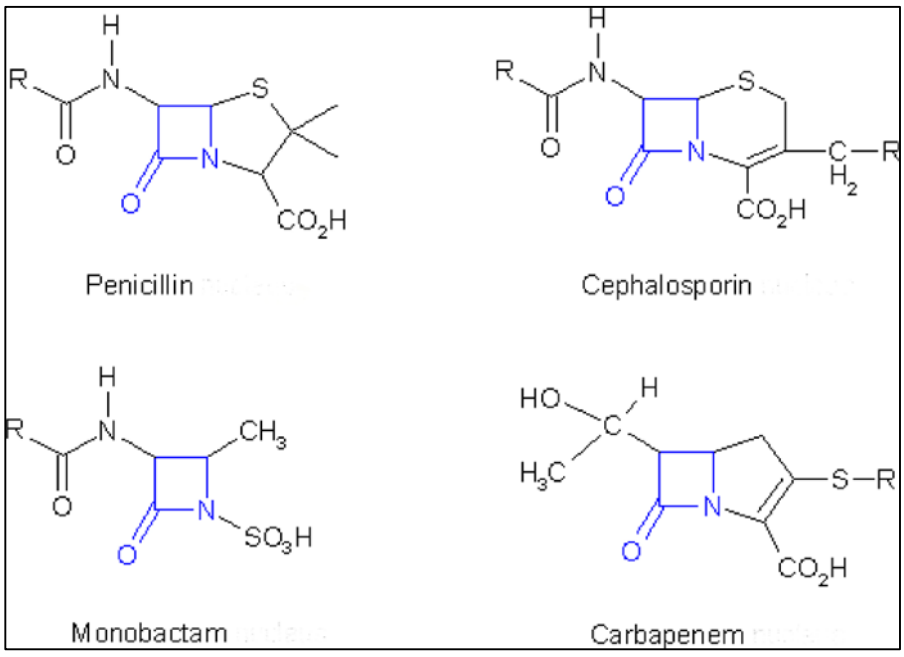

Figure 2. Structures of β-lactam antibiotics. The β-lactam antibiotics, such as penicillins, cephalosporins, monobactams and carbapenems, are grouped together based upon a shared structural feature, the beta-lactam ring a four-membered cyclic amide (in blue). The classes of β-lactams are distinguished by the variation in the ring adjoining the β-lactam ring and the side chain (R in the figure) at the α position**.** The figure has been adapted from (13).

2.1 β-lactamases

The increased occurrence of mobile, plasmid-borne β-lactamases among human pathogens in the recent decades has become a huge problem in health care (14, 15). β-lactamases cleave the amide bond of the β-lactam ring and thereby inactivate the antibiotic (Figure 3). The production of β-lactamases is thought to have evolved as a defence against antibiotics produced by other organisms in their natural habitat (1, 16, 17). Allen *et al* conducted research in the Alaskan environment and found many different freshwater species of *Enterobacteria* with broad spectrum of antibiotic resistance as well as a diverse set of lactamases (1). This area has been free from human influence, and demonstrates the existence of β-lactamases before human antibiotic overuse, which indicates that β-lactamases also have other roles. For example, production of low concentrations of antibiotics might be used in communication between bacteria, and in that case, β-lactamases may have a role in the adjustment of such quorum sensing signals (16, 18).

There are several classification schemes for the β-lactamases. Richard P. Ambler suggested the first complete classification in the beginning of the 1980s, based on sequence similarity (19). According to his classification, the β-lactamases are divided into the four classes A, B, C, and D. Classes A, C and D are all serine β-lactamases having serine in the active site, while class B contains the metallo-β-lactamases (MBL), requiring divalent cations (zinc), for activity (19, 20). This classification was later complemented by Karen Bush to include functional and mechanistic criteria such as the β-lactamases' ability to hydrolyse specific β-lactam antibiotics and their inhibition. Based on these criteria, β-lactamases are divided into groups 1, 2, 3, and 4. Group 1 consists of cephalosporinases that are weakly inhibited by the β-lactamase inhibitor clavulanic acid. Group 2 consists of penicillinases and extended spectrum β-lactamases, which are sensitive to clavulanic acid. Group 3 contains the metallo-β-lactamases, and group 4 includes other β-lactamases that are not sensitive to clavulanic acid (21, 22).

The genes encoding β-lactamases can be found on bacterial chromosomes, plasmids and transposons. Their appearance on transposons makes them highly mobile and can explain the increasing number of β-lactamases that appear in bacterial strains (12). Additionally, β-lactamase-encoding genes have been discovered on integrons (23, 24), genetic elements that vary in length and are able to acquire and rearrange open reading frames embedded in gene cassette units and convert them to functional genes by ensuring their correct expression.

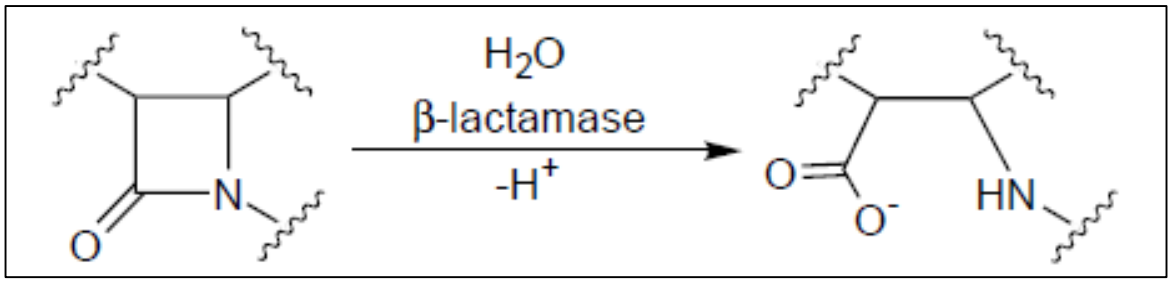

Figure 3. Hydrolysis of the β-lactam ring by β-lactamases. Following hydrolysis by the β-lactamase the β-lactam ring is opened and thus the molecule's antibacterial properties are deactivated. For simplicity, residual groups on the antibiotic structure have been removed and are here represented by wavy lines. Republished with permission from (25).

2.2 Metallo β-lactamases

The first MBL enzyme was described in the mid-1960s and originated from the non-pathogenic bacterium *Bacillus cereus* (26). Shortly after, other MBLs from *Stenotrophomonas* (*Pseudomonas*) *maltophilia, Serratia marcescens, Aeromonas hydrophila* and *Bacteroides fragilis* were described (27-30). All of these enzymes were localized extracellularly or in the periplasm and encoded from chromosomal genes, and were not considered harmful. In the beginning of the 1990s the first plasmid-borne MBL was detected from *Pseudomonas aeruginosa* (31). Not long after that, an MBL was then isolated from *B. fragilis* found in clinical isolates in Japan and Italy, which pointed to the possibility of horizontal gene transfer from one bacteria to another and to the potential spread of MBL-encoding genes, carried on mobile DNA elements among major Gram-negative pathogens (32, 33). An additional risk of the spreading of these enzymes comes from their ability to degrade almost all β-lactam antibiotics while being resistant to therapeutic β-lactamase inhibitors (34).

In the Bush classification, group 3 of the β-lactamases are metallo-enzymes that require Zn^{2+} cations for catalysis and were additionally divided into three functional subgroups, a-c (Table 1). Most of the MBLs have a broad substrate profile (table 1) and can be expressed in combination with other β-lactamases (32).

Table 1. Bush's classification of class B MBLs. Data is based on (22, 35, 36).

Functional group	Spectrum	Zn1 site	Zn2 site	Comment	Examples
Group 3a	Broad spectrum	3 His	Asp-Cys-His	Two Zn atoms for optimal hydrolysis	Bc II, IMP-I, CcrA, VIM, GIM, SPM-1
Group 3b	Carbapenems	2 His 1Asn	Asp-Cys-His	Second Zn atom is inhibitory	CphA, Sfn-1
Group 3c	Cephalosporins	3 His	Asp-His-His	Two Zn atoms for optimal hydrolysis	LI, FEZ-1, Gob-1, CAU-1

Structurally these enzymes have a similar fold (Figure 4A). They all possess an αββα-structure composed of two central β-sheets and five α-helices, with the active site placed on the external edge of the ββ-sandwich near the N-terminal end of a helix (Figure 4) (25, 34, 37). Due to the fact that the N- and C-terminal parts of the molecule can be superposed by a 180° rotation around a central axis, it has been suggested that the structure of MBLs arose from a gene duplication event (25). Almost all MBLs are monomers (25, 37, 38), with only the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* known as a tetramer (39). All MBLs require zinc ions for their activity (Table 1). The active site consists of two zinc-binding regions, the Zn1 and Zn2 sites, which are conserved among all groups (Table 1, Figure 4B). Most MBLs are active as di-zinc compounds (as shown in Figure 4), with the exception of enzymes belonging to the 3b group, which are active as mono-zinc compounds and are inhibited by a second zinc ion (32).

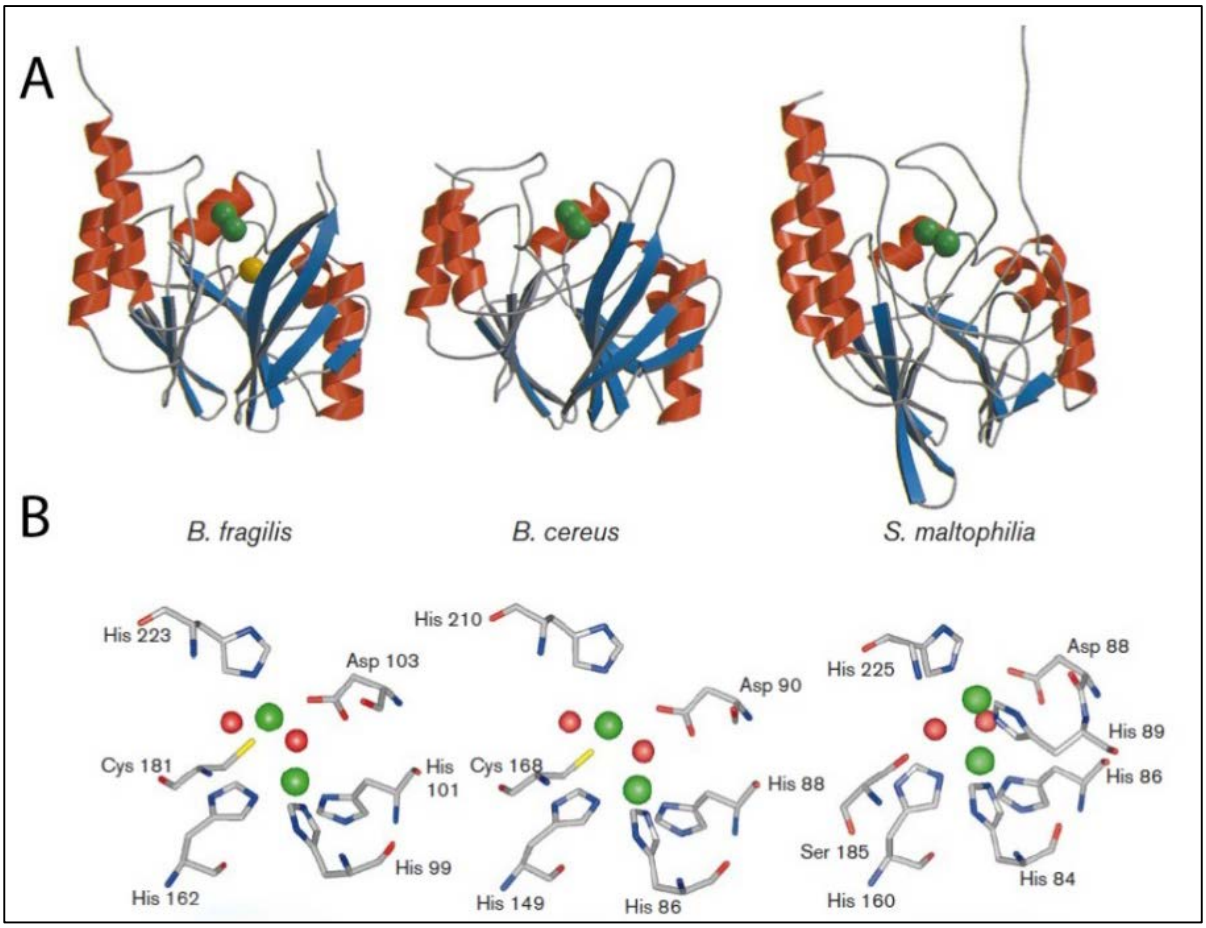

Figure 4. Three-dimensional structures of three homologous metallo-β-lactamases of *B. fragilis* (left), *B. cereus* (center), and *S. maltophilia* (right). **A**. **Comparison of overall structures.** All homologs share an αββα-structure composed of two central β-sheets (blue) and five α-helices (red), with the active site placed on the external edge of the ββ-sandwich near the N-terminal end of a helix The green spheres represent the two bound Zn-ions in the active site. The yellow sphere in the *B. fragilis* structure represents a Na-ion.

B. **Comparison of the dinuclear Zn (II) centers of the MBLs**. Relevant side chain residues are shown in stick-representation. The conserved histidine triad and a bridging water molecule in the histidine site tetrahedrally coordinate Zn1 ion. The Zn2 site has a trigonal-bipyramidal orientation, which includes two water molecules and conserved His or Cys. The water molecule from Zn2 site is believed to behave as a strong nucleophile. The green spheres represent Zn-ions. The red spheres represent oxygen atoms of the bridging water/hydroxide molecule and the apical water molecule. The figure is adapted from (25).

2.2.1 The catalytic mechanism of metallo-β-lactamases

In the catalytic mechanism proposed for the mono-zinc form of the BCII enzyme from *B. cereus* (Figure 5A), zinc acts as a nucleophile attacking the carbonyl group of the β-lactam ring (25, 34). This forms a tetrahedral intermediate, which is stabilized by interactions with Zn^{2+}. Asp120 deprotonates OH^- and creates a second tetrahedral intermediate stabilized by Zn^{2+}. Following

this, Asp120 protonates the nitrogen of the β-lactam ring and, thus, opens it. The proposed mechanism for di-zinc enzymes is based on the structure of the CcrA enzyme from *B. fragilis* (Figure 5B) (25). In this mechanism, the OH^- ion between the two Zn^{2+} ions is responsible for the nucleophilic attack on the amide bond of the β-lactam ring. A negatively charged intermediate is formed that is stabilized by the enzyme's oxyanion hole. The nitrogen from the amide bond in the β-lactam ring is protonated by an apical water molecule bound to Zn^{2+} (25, 34, 37, 40, 41).

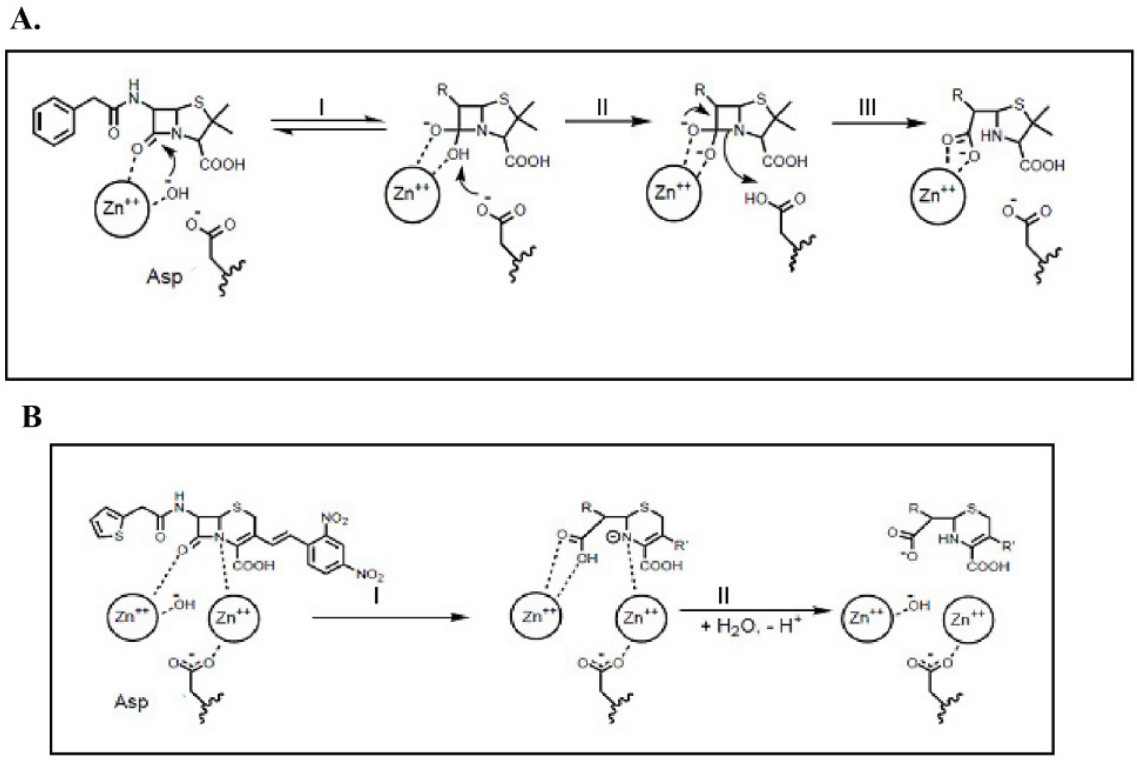

Figure 5. Proposed catalytic mechanisms for mono-zinc and di-zinc MBLs. A) Mono-zinc enzymes. The zinc behaves as a Lewis acid by decreasing the pKa of the water and thus generating an OH^- ion that acts as a nucleophile attacking the carbonyl group of the β-lactam ring (step I). A tetrahedral intermediate is formed and is stabilized by interactions with Zn^{2+}, while Asp120, acts as a base and deprotonates OH^-. A second tetrahedral intermediate, stabilized by Zn^{2+} is formed (step II). Following this Asp120 protonates the nitrogen of the β-lactam ring causing it to open (step III). **B) Di-zinc enzymes.** The OH^- ion between the two Zn^{2+} ions makes a nucleophilic attack on the amide bond of the β-lactam ring (step I). The newly formed negatively charged intermediate is stabilized by the enzyme´s oxyanion hole. The apical water molecule bound to Zn^{2+} protonates nitrogen from the amide bond in the β-lactam ring (step II). Only the functional groups of the amino acids involved are presented, while the rest of the molecule is represented with wavy line. The figure has been adapted from (25).

3 Restriction of phage nucleic acids by bacterial RM systems

3.1 DNA-interacting proteins

Deoxyribonucleic acid (DNA) is a carrier of the genetic instructions in all known living organisms and many viruses. DNA is a chemically stable molecule that can be inert for long periods (42). Recognition and binding of proteins to specific sites on DNA is important for many cellular processes in both eukaryotes and prokaryotes, such as regulation of transcription, gene expression, DNA repair, recombination and replication. In order to bind double stranded DNA (dsDNA), relevant proteins harbour specific structures that are complementary to the DNA double helix, both structurally and electrostatically. The binding process starts with the formation of a tight complex between DNA and protein, involving distortions to both molecules. DNA-binding proteins utilize two main interacting modes, specific or non-specific. In the specific interaction mode, bases from the major groove of DNA provide hydrogen bond donors and acceptors, as well as non-polar groups that are recognized and "read" by specific amino acids side chains on the protein. The non-specific interaction mode is purely electrostatic and involves base pairs forming specific structures that can be recognized by the protein (43, 44). In regulation of gene expression, covalent modifications of DNA bases such as phosphorylation, acetylation and methylation are very important (45, 46). These base modifications can form favourable interactions between proteins and DNA and thereby strengthen the recognition.

3.1.1 Structure of DNA

In 1953, Francis Crick and James Watson published the structure of the DNA molecule, based on the x-ray image of DNA taken by Rosalind Franklin (47). The Watson-Cricks DNA model is a double helix with two strands coiled around the same axis (47). Basic DNA units are deoxyribonucleotides, which are built from 2-deoxyribose and phosphate groups linked with phosphodiester bonds in forming the DNA backbone. Deoxyribose is in turn covalently linked to one of four bases, thymine, cytosine, adenine and guanine (Figure 6) (48).

The two DNA strands are oriented in opposite directions to each other, *i.e.* antiparallel, and are held together by hydrogen bonds between opposing bases and base-stacking. The bases are paired depending on their structures and their abilities to form hydrogen bonds. Adenine is paired with thymine by two hydrogen bonds, while guanine and cytosine share three hydrogen bonds (Figure 6). The bases can be aligned in any order along one DNA strand and these

sequences represent the genetic information. Due to this base pairing, the sequence of bases on one strand determines the sequence on the complementary strand. The bases are planar and almost completely perpendicular to the helix axis spaced at a distance of 3.4 Å. The two strands form a right handed helix with a helical repeat every 34 Å and 10 bases per turn (42). Due to the stacking of the bases and the twisting of the helix there are two grooves that can be distinguished by their size, a wider major groove and narrower minor groove (Figure 6). Both grooves may be involved in protein binding, with the major groove usually having a more important role. The width of the major groove is similar to the width of an α helix in proteins, which allows for a tight fit between them. The major groove also contains both polar and nonpolar groups that are easy accessible and can be recognized by DNA-binding proteins. The minor groove is narrower than the major groove and contains fewer functional groups from the bases, which decreases its potential for interaction with other molecules, such as proteins. To achieve successful binding through the minor groove of DNA, structural distortions have to take place (42, 48, 49).

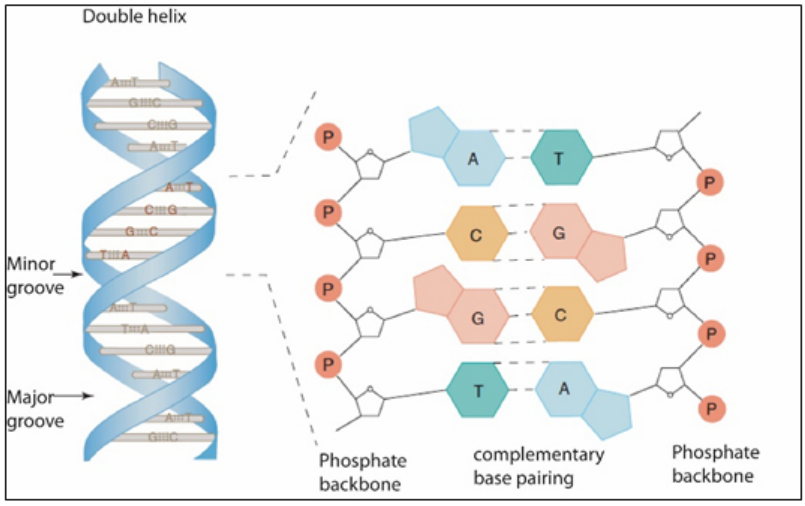

Figure 6. A simplified model of the DNA double helix. To the left a DNA double helix model is shown, followed by a schematic representation of complementary base pairing in DNA. In the structure of DNA, the sticks represent base pairs, and the ribbons represent the deoxyribose phosphate backbones of the antiparallel strands. In the schematic representation of the base pairing, dotted lines represent hydrogen bonds between bases. The bases are denoted with the letters A for adenine; T for thymine; C for cytosine and G for guanine; phosphate is denoted with the letter P. The figure has been adapted from (50).

3.1.2 Protein-DNA recognition and binding

One of the first steps in protein-DNA recognition is direct contact of the protein with exposed edges of the DNA bases, usually those located at the major groove of DNA. These contacts typically involve hydrogen bonds and van der Waals interactions between the DNA bases, and the amino acid residues of the protein. Small molecules that are bound to either the DNA or the protein, such as water molecules, can provide additional contacts (51, 52). This non-specific protein-DNA complex then allows diffusion of the protein along the DNA as the protein "scans" the DNA in search for a specific recognition site. The protein translocates along the DNA while it is loosely bound to the DNA with its catalytic centre distant from the phosphate backbone. There are several hypotheses for how proteins move along the DNA molecule. In a mechanism called one-dimensional diffusion, the enzyme is constantly bound to DNA and slides in a helical movement along a groove of the DNA until it reaches a specificity site. Since the enzyme is constantly bound to DNA no sites are ignored, however, ligands or other proteins interacting with both minor and major grooves can be obstacles for this movement. The second mechanism of DNA movement is called three-dimensional (3D) diffusion, where the enzyme dissociates and re-associates with the DNA. A problem with the latter mechanism is that when the enzyme is dissociated from DNA, some specificity sites may be ignored. On the other hand, small ligands bound to DNA would not cause hindrance. A third mechanism of moving is called intersegment transfer. This movement requires two DNA binding sites on the enzyme. While the DNA is bound to one site on the enzyme, the other binding site of the enzyme can dissociate from the DNA and bind to the same DNA molecule on the more distant site (53-55).

When an interaction between the DNA molecule and a protein occurs, there will be a change in the Gibbs free energy (ΔG). The interaction involves conformational changes of both the protein and the DNA, as well as a release of water and counter-ions from the protein-DNA interface (56). This result in a favourable entropic change (ΔS) that compensates for unfavourable entropy contributions that originates from immobilization of amino acid side chains at the protein-DNA interface. The enthalpy change (ΔH) from direct non-covalent protein-DNA interaction is additionally decreased by distortions on the DNA due to the base pair destacking (57).

Some proteins, such as restriction endonucleases (REases) and DNA methyltransferases (DNA-MTases), recognize and interact with a specific base sequence in DNA. Other proteins have structural features that are important for recognition, but are non-specific with regard to the

DNA sequence. The most important role in interaction is played by hydrogen bonds between the protein and the DNA base pairs. Side chains of certain amino acids such as Ser, Thr, Tyr, Asn, Gln and His are considered the best hydrogen bond donors and/or acceptors. In addition, the positively charged amino acids can make hydrogen bonds and salt bridges with the phosphate backbone of the DNA (43, 58, 59). Hydrogen bonds also have an important role in the selectivity that amino acids have towards bases, which is very important when it comes to specific recognition (Figure 7). Besides the hydrogen bonds in these interactions, van der Waals interactions, hydrophobic interactions and electrostatic interactions between functional groups in bases and proteins also play important roles (57, 60).

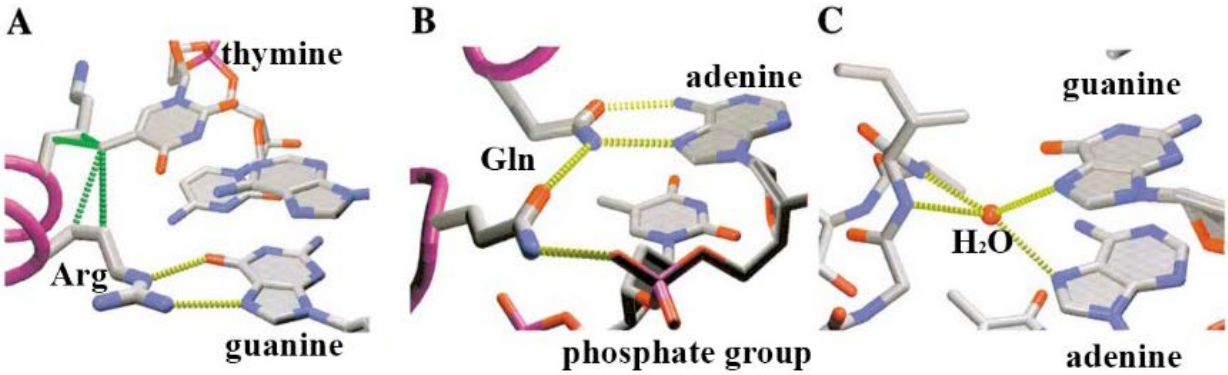

Figure 7. Examples of protein-DNA contacts. **A) Bidentate contacts between arginine side chain and guanine base** (yellow dashed lines) and hydrophobic contacts to a thymine methyl (green dashed lines). **B) Bidentate contact between glutamine and adenine.** In addition to contacting the adenine, this side chain hydrogen bonds to a second glutamine side chain, which in turn contacts a phosphate group. **C) Water-mediated hydrogen bonds** at the protein-DNA interface of the Trp repressor-DNA complex. The figure has been adapted from (58)

The α-helix is the most frequent structural element involved in protein-DNA interactions. It fits into the major groove, as it has a similar diameter (12Å), while the side chains on the helix can interact with bases on DNA. Small β-sheets can pack against the major groove, and side chains from exposed amino acids can interact with neighbouring bases. Since larger β-sheets are more rigid and often twisted, DNA has to be distorted in order for the β-sheet to insert into either of the grooves (*e.g.* TATA-box binding proteins). Loops are more flexible and can take up a variety of conformations. They can also take up various positions towards DNA in order to be placed in the best way to make favourable interactions to the DNA helix. Loops are common elements in DNA binding motifs (*e.g.* in helix-loop-helix motifs) (46, 49). Various combinations of α-helices, β-sheets and loops constitute different binding motifs. So far, several different structural motifs are known, with the helix-turn-helix-, leucine zipper- and zinc-finger- motifs being the best studied (49). These motifs may appear alone or be part of larger domains.

The recognition of specific sequences in DNA is the basis of the recognition and interaction of the proteins structural motifs with the DNA. For example, those enzymes that are involved in DNA replication, transcription and recombination depend on sequence specific DNA interactions. The best known example is the Lac repressor, the first regulatory system discovered whose binding site on DNA consists of a 17 bp palindromic sequence (TTGTGAGCSGCTCACAA; where S corresponds to either G or C) (61). Some proteins consist of several domains that have individual, but related functions, such as recognition domains that recognize a specific DNA sequence and catalytic domains. Examples in this group are DNA-MTases, type II REases, DNA polymerases, deoxyribonucleases and DNA repair enzymes (60).

3.2 Restriction-modification systems

Restriction-modification (RM) systems are widespread among microorganisms, but are predominantly found in bacteria (7). Additionally, certain cyanobacterial viruses possess RM systems that degrade host DNA and provide free deoxyribonucleotides that the virus can incorporate into its own DNA (62-64). RM systems consist of two components: restriction endonucleases (REases) that cleave foreign DNA (*e.g.* phage DNA) at specific recognition sites, and the DNA-MTases that modify adenosine or cytosine of the host DNA preventing host DNA cleavage (65). Recently, Kobayashi and colleagues introduced yet another role of RM systems in bacteria, defining them as selfish elements with the ability to maintain themselves

in a cell population regardless of the consequences for their host cell. If the cell loses the RM system, for example through a recombination with a competitive gene, the cell would undergo a process resembling post-segregation killing resulting in its death, while the RM system would be preserved in neighbouring cells that do not have such interactions (66-68).

RM systems were first suggested by Luria and Bertani in the early 50s when they noticed that some bacteria could limit growth of bacteriophage λ (69, 70). Since their discovery, in the 60s many different types of restriction and modification enzymes have been discovered and characterized (71). Initially, for classification purposes, newly discovered enzymes were named according to the genus and species from which they were discovered, followed by the existing strain designation. Multiple RM systems identified from the same organism are distinguished by roman numbers. For example, HindII is the second out of four restriction enzymes isolated to date from *Haemophilus influenzae* serotype d (72, 73).

3.3 Types of Restriction-modification systems

All RM systems have the same overall function, that is, protection of host DNA. Based on the differences in composition, co-factor requirements and specificity, they are divided into types I, II, III and IV (detailed in Table 2) (7, 72). A key point of this division is whether the endonuclease function, responsible for cutting the DNA, and the methyltransferase function, responsible for protecting the DNA, are carried out by the same enzyme (as in types I and III), or whether these functionalities are encoded by separate proteins (as in types II and IV). In this thesis, enzymes of the type II RM systems have been studied. As single enzymes from type IV RM systems have been applied in laboratory techniques, these will also be briefly outlined in the following subsections.

Table 2. Characteristics of the four types of restriction modification systems. Modified from (74) and (75-77).

Features	Type I	Type II	Type III	Type IV
Nature of enzyme	Single, multifunctional enzyme	Separate endonuclease and methylase	Single, multifunctional enzyme	Single enzyme
Protein structure	3 different subunits (R2M2S1)[1]	2 distinct proteins (RM)[1]	2 different subunits (R2M2)[1]	3 different subunits
Numbers of genes	3	2	2	2
Location of genes	Chromosomal	Chromosomal or plasmid	Chromosomal or plasmid	Chromosomal
Cofactor	SAM, ATP, Mg^{2+}	Mg^{2}	ATP, Mg^{2+}, SAM stimulates restriction, but not required	Mg^{2}, GTP
Cleavage sites	Random, ~1000bp from recognition site	Within recognition site	24-26 bp to the 3´of recognition site	C5 or N4 methylated cytosine following a purine
DNA translocation	Yes	No	No	Yes
Site of methylation	Recognition site	Recognition site	Recognition site	Does not possess methyltransferase activity
Restriction versus methylation	Mutually exclusive	Separate reactions	Simultaneous	Possess only restriction activity
Examples	EcoAI, EcoKI	R.EcoRI /M.EcoRI R.HhaI/M.HhaI	EcoP1I, EcoP15I	Mrr, McrBC

[1] R indicates restriction subunit; M modification subunit and S specificity subunits of the enzyme complexes.

3.3.1 Type II RM systems

The type II RM systems are the most common and most studied RM systems (62). According to REBASE[1], an online database that contains information about restriction enzymes and DNA methyltransferases, there has been an increase in the discovery and characterization of type II restriction enzymes and methyltransferases in the last decades (78). In type II RM systems individual genes encode the MTase and the REase that can act dependently or independently of each other. The recognition sequences are defined, and are often symmetric, palindromic and usually 4-8 bp long (for example, EcoRI recognizes the sequence G↓AATTC, where ↓ indicates

[1] http://rebase.neb.com/rebase/rebase.html

the restriction site). The recognition sequence can also be asymmetric or degenerate. Some enzymes, termed isoschizomers, recognize the same sequence and cleave at the same site. Neoschizomers are enzymes that cut at different positions within the same recognition sequence (79, 80). Cleavage occurs either, as just described, at the recognition site, or at a neighbouring site. As cofactors, REases usually require Mg^{2+} (or other divalent metal ions) while DNA-MTases require S-adenosyl methionine (SAM). The DNA-MTases catalyse the transfer of a methyl group from SAM to an adenine or cytosine in the recognition sequence on both DNA strands, which subsequently leads to the formation of N6-methyladenine, N4-methylcytosine or C5-methylcytosine (Figure 8).

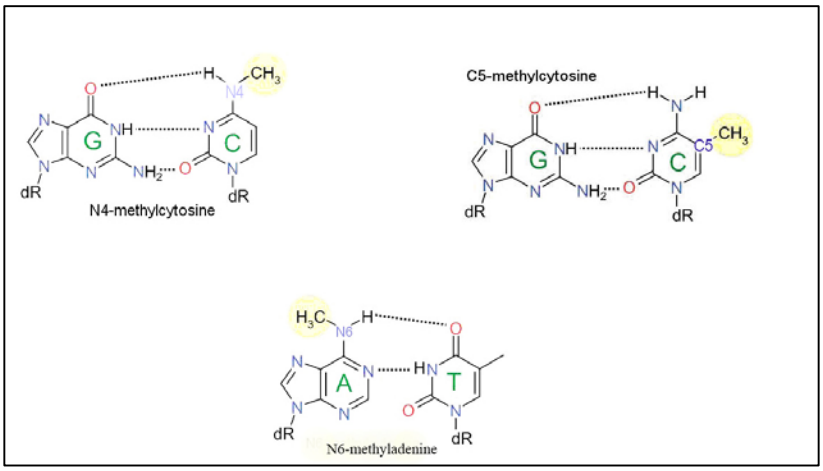

Figure 8. Structures of methylated DNA bases. The structure of each of the modified bases, N6-methyladenine, C5-methylcytosine and N4-methylcytosine, is presented in the context of AT and GC base pairs, respectively. In all cases, the methyl group is located in the major groove of the DNA and does not interfere with the Watson–Crick base pairing. The figure has been adapted from (81).

Non-modified DNA is the preferred substrate for restriction enzymes that cleave DNA within or close to the recognition sequence (82, 83). The vast numbers of enzymes that belong to this group differ in their preferred recognition site, their need for a cofactor, or their subunit structure. Due to their diversity, the type II RM enzymes are further divided into several subtypes (Table 3, section 3.4.1) (84). In addition to these regular types, some DNA-MTases and REases occur as so-called orphans or solitaires, *i.e.* unaccompanied by the counterpart enzymes. Orphan DNA-MTases are more common than orphan REases and have been found to be involved in mismatch repair, regulation of initiation of DNA replication, regulation of transcription of housekeeping and virulence genes and cell cycle control (63). All orphan REases have specificity towards methylated substrate (85, 86).

In this thesis, we have explored an orphan type II REase, DpnI, which restricts DNA once adenines are methylated in DNA, and the orphan C5-DNA MTase ParI. Enzymes that belong to type II RM systems are discussed in detail in Sections 3.4 and 3.5.

3.3.2 Type IV RM systems

Type IV RM systems consist only of restriction enzymes and lack an accompanying methyltransferase. For this reason, they only cleave modified DNA, such as methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA and cannot be strictly considered as members of RM systems. The best studied type IV restriction enzyme is the McrBC from *E. coli* (K12), where *mcr* stands for "modified cytosine restriction" and BC stands for the two subunits encoded by the genes *mcr*B and *mcr*C, which are required for a functional enzyme (77, 85, 87). The enzyme consists of three proteins, two of which are encoded by the *mcr*B gene and both contain a conserved motif required for GTP binding, while one protein is encoded by the *mcr*C gene and contains the motif involved in protein-protein interactions. The McrBC enzymes require Mg^{2+} and GTP hydrolysis for restriction and translocation of the enzyme along DNA. The requirement of GTP for translocation makes them unique among nucleases (75). The GTP binds first to the McrB subunit that leads to its stabilization, and then subsequent DNA binding and the initial formation of the McrBC interaction (76, 88, 89). These enzymes recognize C5- or N4-methylated cytosines following a purine, for example in the DNA sequence 5`-GC-3`. For cleavage, these enzymes require two modified sites typically separated by 40-80 bp, but can also be separated up to 3 kb. The restriction occurs at a random position between these two sites. During translocation along the DNA the enzyme binds to the recognition site and stalling of the enzyme initiates cleavage. Due to the unspecific cleavage

pattern, type IV enzymes have not been commercialized and applied in biotechnology, but they have recently been found to be useful for detection of methylation patterns in eukaryotes. In prokaryotes, the *E. coli* McrBC system is considered a technical problem in experimentation during the study of novel DNA-MTases due to the restriction of cloning or expression plasmids carrying functional methylase genes (90). Once DNA-MTases are translated they will methylate the recognition sequence leading to cleavage of self-DNA and subsequent cell death. To overcome this, several strains have been engineered by deletion of these genes, such as the Mcr-negative *E. coli* T7 Express strain (from New England Biolabs).

3.4 Restriction enzymes that belong to the type II RM systems

3.4.1 The structure of type II restriction enzymes

According to REBASE more than 4000 type II restriction enzymes, the nuclease component of the type II RM systems, are characterized so far with ~300 different specificities, which illustrates the diversity of this group of enzymes (78). On a sequence level there is no sequence identity, but homology can be identified when proteins are compared on a structural level (84). Canonical type II REases are homodimers that recognize a palindromic DNA sequence of 4-8 bp and cleave within or next to the recognition sequence leaving 5´-phosphate and 3´-hydroxyl ends (62). All enzymes are composed of three subdomains including a conserved catalytic domain, and two domains that are more diverse; the DNA binding domain and the dimerization domain. In addition to these structural features, many enzymes have additional unique characteristics that divide these enzymes into subgroups (Table 3). However, this division is not definite since some enzymes have characteristics that can be attributed to more than one subgroup (72, 84). As mentioned above, common structural features can be observed when comparing the structures of type II enzymes, despite their low sequence similarities. A common core contains the active site and a stabilization center, which can also be found in various other DNA interacting enzymes that participate in DNA replication, repair and recombination (62). The common structural core is composed of four mixed β-strands flanked by α-helices where a Pro-Asp-X_{10-20}-(Asp/Glu)-X-Lys motif, in short termed PD…(D/E)XK, where X denotes any amino acid, is generally found (Figure 9) (91-93). The acidic and basic residues from Asp, Glu and Lys are involved in cofactor binding and restriction.

Table 3. Subtypes of type II REases. Characteristics of the subgroups are based on the review by Roberts, R.J., *et al* (72).

Subtype	Characteristics	Examples	Recognition sequence[1]	Ref.
A	Asymmetric recognition sequence	FokI	GGATG (9/13)	(94)
B	Cleaves both sides of target on both DNA strands	BplI	(8/13)GAG(N)5CTC (13/8)	(95)
C	Have both restriction and modification domains within a single polypeptide, which has quaternary structure A2B, with both domains in A subunit and target recognition domain (TRD) located in the B subunit. Recognize symmetric or asymmetric target.	BcgI	(10/12) CGANNNNNNTGC (12/10)	(96)
E	Needs to interact with two copies of the recognition sequence; one copy of the recognition sequence serves only as allosteric effector while the other one is the actual target.	EcoRII	↓CCWGG	(97)
		NaeI	GCC↓GGC	(98)
F	Homotetrameric enzymes, cleave both copies of the recognition sequence cooperatively.	Cfr10I	R↓CCGGY	(99)
		NgoMIV	G↓CCGGC	(100)
G	Modification and restriction domains are combined into one polypeptide. Stimulated by SAM. Recognize symmetric or asymmetric target.	BpuSI	(10/14) GGGAC	(101)
H	Combine behavior of type II enzymes with genetic organization of type I enzymes. The active enzyme complex is composed of one restriction subunit and two modification and specificity subunits. Recognize symmetric or asymmetric target.	AhdI	GACNNN↓NNGTC	(102)
M	Require methylated target. Appear alone without a modification counterpart. Solitary endonucleases	DpnI	Gm6 A↓TC	(86)
S	Homodimers; each monomer is composed of two domains. One is responsible for identifying the recognition sequence and the other is responsible for restriction and dimerization. They cleave at least one strand of targeted DNA outside of the recognition sequence.	MmeI	TCCRAC	(103)
T	Symmetric or asymmetric target. R genes are heterodimers	BslI	CCNNNNN↓NNGG	(104)

[1] ↓ represents cleavage site; in numbers in brackets *e.g.* (8/13) the one preceding the recognition sequence indicates number of bases in front of the sequence where cleavage occurs while the second number indicates number of bases before the sequence on the complementary strand. The number in brackets following the recognition sequence indicates cleavage number of bases after the recognition sequence on both strands.

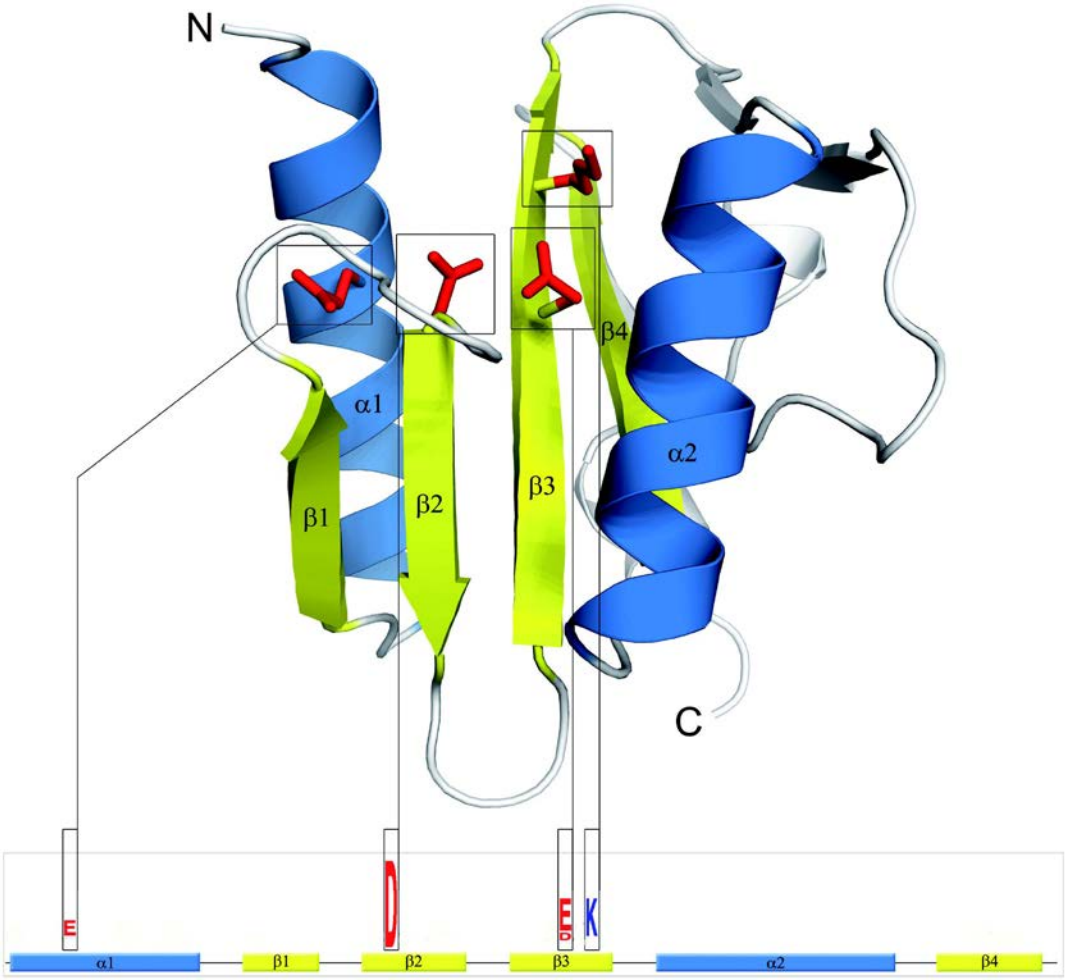

Figure 9. The commonly conserved core of the PD…(D/E)XK motif. Active site residues that are involved in cofactor binding and restriction (Asp, Glu and Lys) are shown as red sticks and marked in the corresponding sequence. α-helices are in blue while β-strands are in green. The figure has been adapted from (93).

In addition to the PD…(D/E)XK family some REases belong to other families, such as the H-N-H family and the GIY-YIG family (105, 106). For all type II REases, Mg^{2+} is essential for cleavage, but it can be substituted by other divalent cations that severely reduces activity, most often by Mn^{2+}, but also by Co^{2+}, Zn^{2+}, Fe^{2+}, and Ni^{2+} (82). In such situations, where Mg^{2+} is replaced, the enzyme cleaves sequences that are similar but not identical to their defined recognition sequence. This is known as "star activity" of the enzyme and is found under suboptimal *in vitro* conditions, such as the abovementioned substitution of Mg^{2+}, high concentrations of the enzyme, non-optimal buffer, prolonged reaction time, or presence of organic solvents (*e.g.* DMSO, ethanol) (107). Based on their structural similarities, type II REases can be divided into two families, the α-subfamily (*Eco*RI family) and the β-subfamily (*Eco*RV family) (Figure 10).

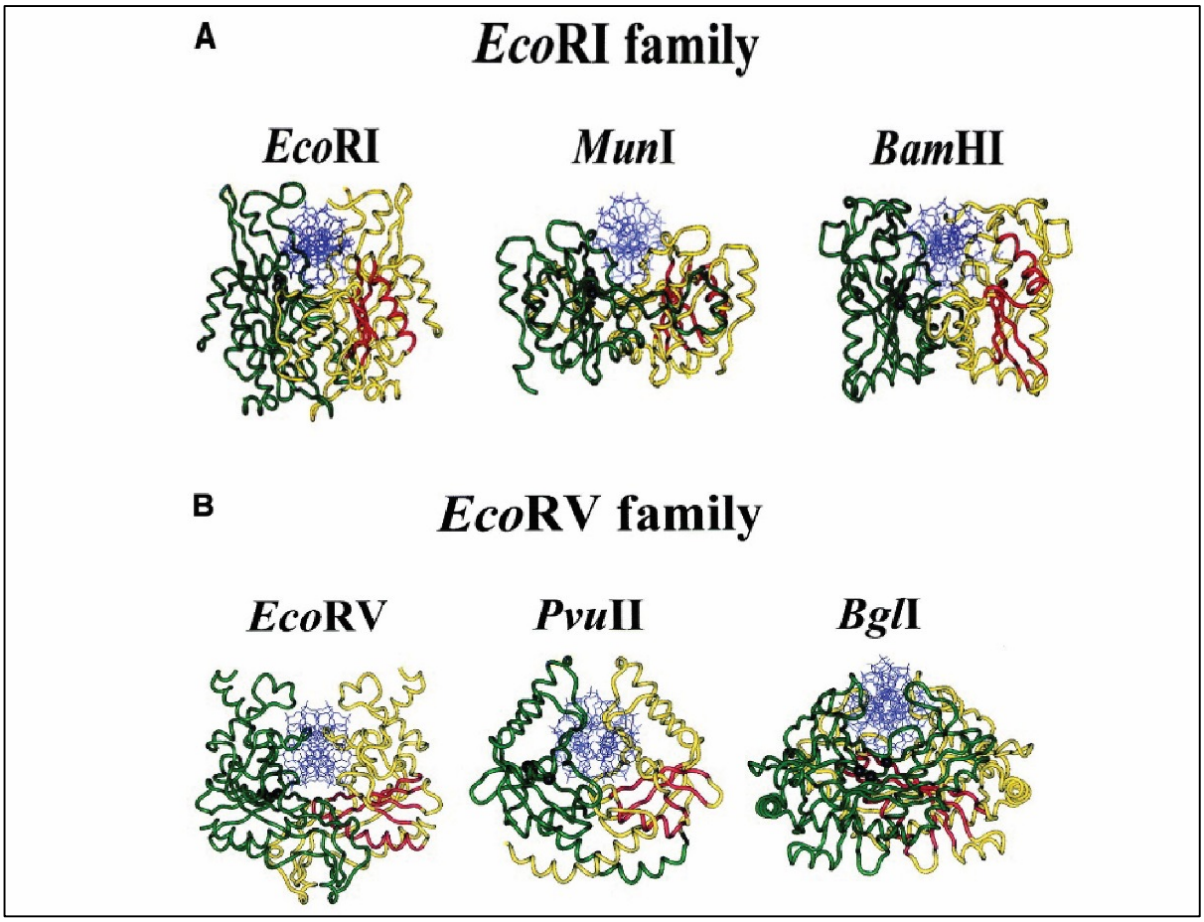

Figure 6. Crystal structures of specific REase in complex with DNA. A. EcoRI-like (α subfamily). Enzymes belonging to the α-subfamily, such as EcoRI, BamHI and MunI, bind the major groove of the DNA and use an α-helix and loop in recognition of DNA and leave 5´overhangs on the DNA after cleavage. **B. EcoRV-like** (β subfamily). Members of this family, such as EcoRV, PvuII, and BglI, bind DNA through the minor groove and generate blunt ends or 3´ overhang DNA products. For recognition and interaction with the specificity site these enzymes use a β-strand (56, 108). In all structures, the two subunits of the enzymes are shown in yellow and green. The strictly conserved β-strands and α-helix of the common core are red. DNA is coloured in blue. The figure has been adapted from (84).

3.4.2 Sequence recognition by type II REases - binding and cleavage

The first step in DNA cleavage by the REase is binding of the enzyme to DNA. It is assumed that the enzyme binds to DNA in either a specific- or a non-specific manner (section 3.1). In non-specific binding, water molecules from the DNA surface are lost and the protein undergoes conformational changes that facilitate DNA-binding. Here the protein-DNA complexes are less compact, there are no contacts between the protein and the DNA phosphate backbone and the overall contact surface is smaller than in the specific complex (56). When it comes to recognition of specific sites on DNA by REases, some common events based on available

crystallographic data of REase-DNA complexes can be observed (84). In specific binding, DNA undergoes structural distortions, which bring key residues from the REase into proximity with the specificity site on DNA and its phosphates, allowing hydrolysis of the phosphodiester bond. Additionally, some structural changes occur in the protein upon DNA binding. In a fully formed REase-DNA complex, DNA is completely encircled by the enzyme (62). Upon the assembly of a specific complex, a highly cooperative hydrogen bond network is created. It is composed of direct contacts between the protein and the DNA bases and contacts to the sugar-phosphate backbone of the DNA. Additionally, van der Waals interactions are formed with the bases of the recognition sequence (62). Among REases all types of movement along DNA previously introduced, one dimensional, three dimensional moving and intersegment transfer, can be observed (Section 3.1.2) (56, 62). Which of these movements prevails depends mostly on the conditions in the surroundings, especially on ion strength and concentration of Mg^{2+} ions, as well as on the structures of both the enzyme and the DNA (62, 109).

3.4.3 The mechanism of DNA cleavage by REases

Cleavage of the phosphodiester bond in DNA by type II REases follows an SN_2-type mechanism once all base-specific contacts have been established. The cleavage mechanism is characterized by the inversion of the stereochemical configuration at the phosphorous atom in the DNA backbone. The general mechanism is divided into three steps (Figure 11).

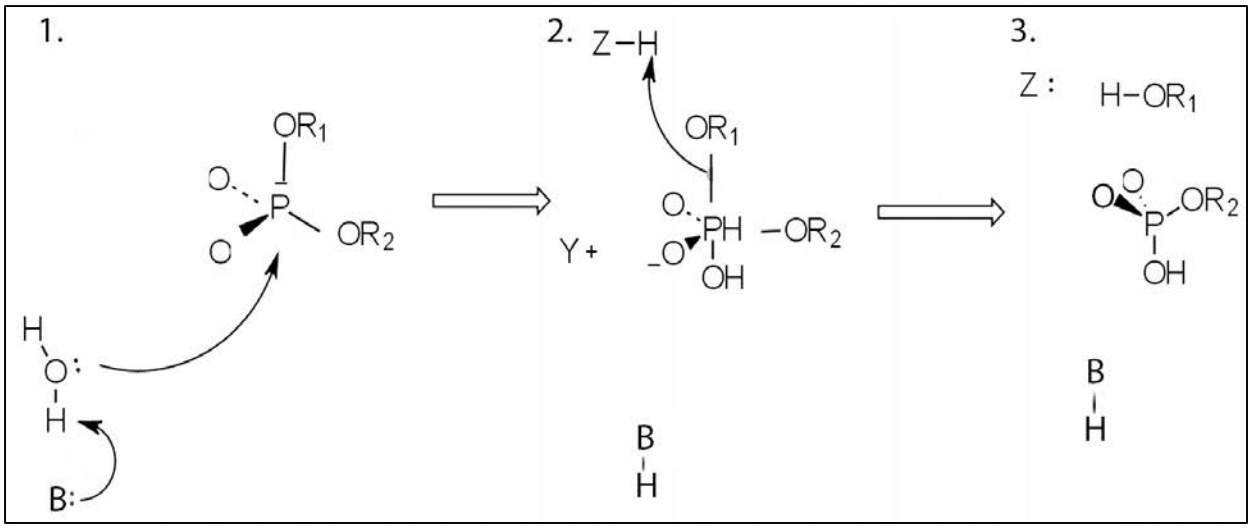

Figure 7. The mechanism of DNA cleavage by REase. In the first step (1) the attacking nucleophile is deprotonated by a base (B) from the PD-(D/E)XK motif . The second step (2) is characterized by the formation of a pentavalent transition state by a nucleophilic attack of the hydroxide ion on the phosphorous. The pentavalent transition state is stabilized by a Lewis acid with two negative charges. In the third (3) and final step of the mechanism the 3´ hydroxyl group is released after protonation by the acid (62, 110). B, Y and Z-H are a general base, Lewis acid and general acid, respectively. The figure is made in ChemDoodle based on (56).

The negatively charged side-chains of Glu (E) or Asp (D) in the PD-(D/E)XK motif coordinate the divalent cation (Mg^{2+}, Section 3.3.1). The main role of the cations in REases is to stabilize the pentavalent transition state. The number and exact position of the cations involved in the cleavage is still unknown, but three alternative catalytic mechanisms have been proposed (62). It is still unknown which base is involved in stabilization of the attacking nucleophile. The most likely candidate for the water deprotonation event is a second molecule of water, which, with the help of metal ions, lowers the pKa of the neighbouring atoms. This assumption is supported by molecular dynamics simulations performed on EcoRI and EcoRV (111) and based on experimental data (62, 84).

3.5 The methyltransferases of type II RM systems

The DNA-MTases are the second member of bacterial type II RM systems and are responsible for methylation of the DNA recognition sites, thereby protecting the host DNA from cleavage by the accompanying REases. The DNA-MTases are translated and acting independently of the restriction enzyme component. In rare cases, the DNA-MTase can occur without a REase counterpart and these are known as orphan DNA-MTases.

Some DNA-MTases recognize and modify only non-methylated DNA as substrate and are known as *de novo* DNA-MTases. DNA-MTases that recognize hemimethylated DNA (where only one strand of the DNA duplex is methylated) and modify it are known as maintenance DNA-MTases (112).

The C5-methylcytosine is considered as an additional base of the genetic code that carries heritable information that is not encoded in the DNA sequence itself and is present in all domains of life with only a few exceptions. Generally it is accepted that with increased genome size, the regulatory role of methylation becomes increasingly important (113, 114). In prokaryotes most DNA-MTases are members of an RM system and have a role in discrimination between self- and non-self DNA. Additionally, DNA-MTases have roles in controlling gene expression, DNA replication, cell cycle and DNA post replicative mismatch repair (81, 115, 116). The latter activities are mostly coordinated by N-DNA-MTases (117, 118) and will not be further discussed here. In most higher eukaryotes, DNA methylation is involved in controlling gene expression, maintenance of genome integrity, parental imprinting, chromatin condensation, silencing of genes, controlling cellular differentiation and development (114, 119-122). It is believed that DNA methylation has an important role in protein-DNA interaction, either by enhancing or disrupting the binding of proteins to DNA (123). Changes in methylation patterns are closely related to the development of various diseases, such as cancer, but also neurological and genetic disorders in humans, which underlines their biological importance (115, 124-127).

In the following sections, prokaryotic C5-DNA-MTases will be detailed.

3.5.1 The structure of C5-DNA-MTases

There is an overall low sequence similarity between C5 DNA-MTases, both prokaryotic and eukaryotic. Ten highly conserved motifs (I-X) are, however, present among all DNA-MTases (Figure 12) (81, 115, 128). Whereas prokaryotic DNA-MTases contain one catalytic domain, eukaryotic DNA-MTases are multidomain proteins wherein the C-terminal catalytic domain has DNA-MTase activity and contains the ten conserved motifs mentioned above (128). The most conserved motifs are important for catalysis or are involved in binding of the cofactor SAM and DNA. Motifs that are identified in all DNA-MTases are motif I (FGG), which is involved in SAM binding, motif IV (PCQ), a catalytic motif involved in the transfer of the methyl group, and motif VI (ENV), involved in DNA binding (Figure 12). The variable target

recognition domain (TRD) region is located between motifs VIII and IX (116). All catalytic DNA-MTase domains studied so far have the same structural organization; they are comprised of two sub-domains, one large domain and one small domain (Figure 12). The large domain is composed of a central, mixed seven-stranded β-sheet flanked by α-helices, known as the AdoMet-dependent fold, which is conserved among different SAM-dependent enzymes (129). A topological switch point is located between strands one and four, which loosely divides the SAM binding domain into two parts, one that is involved in binding of the cofactor SAM and a second that is involved in forming the catalytic part of the enzymes (Figure 12A). The SAM binding part of the large domain is composed of conserved amino acid motifs, including the highly conserved motif I, and residues from motifs II, III, V and IX. The structure of the catalytic part is composed mostly of motif IV, but includes also motifs VII and VIII (112, 115, 117, 129).

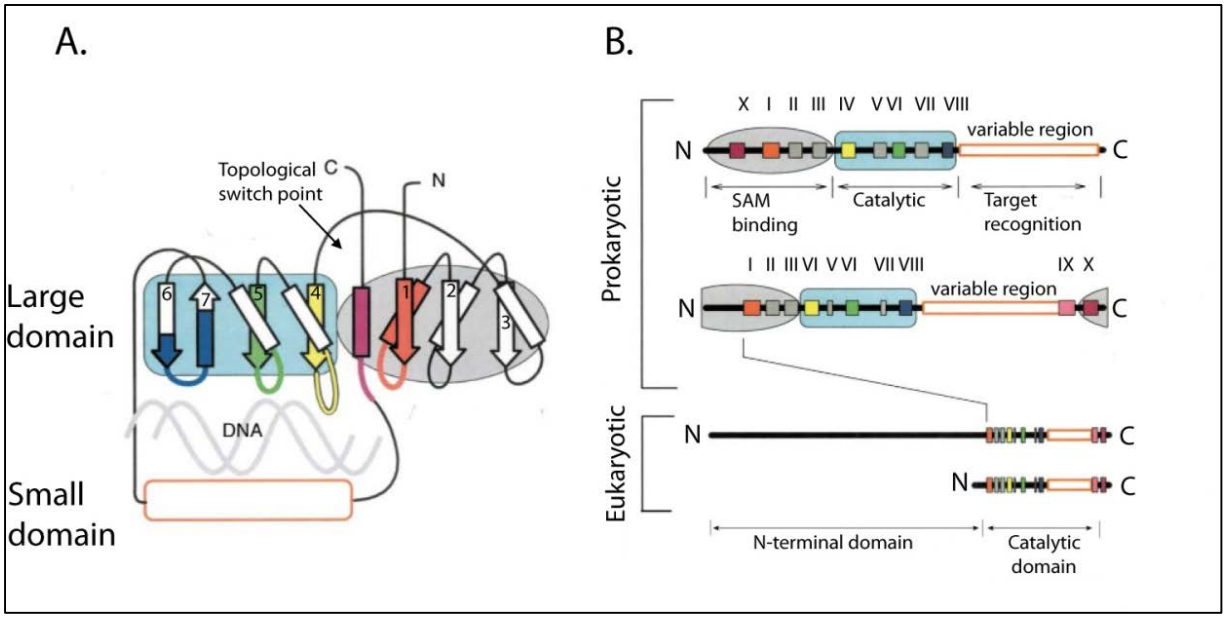

Figure 12. Schematic representation of both prokaryotic and eukaryotic DNA-MTase motifs and domains. A. Structure of MTases. An idealized representation of the structure of a C5-MTase. β-strands are represented by arrows and are numbered 6, 7, 5, 4, 1, 2, and 3, from left to right, α-helices are presented by rectangles and loops by curved lines. Grey wavy double lines indicate DNA. **B. Schematic representation of MTase motifs and domains.** The prokaryotic C5-DNA-MTases, and the C-terminal catalytic domain of eukaryotic enzyme families all share the same motif and domain arrangement. The DNA-MTase structure is divided into three domains, the SAM binding domain (filled grey oval), the catalytic domain (filled cyan rectangle), and the target recognition domain (open brown rectangle). Motifs I through X are shown and are represented by the same colour in both parts (A and B) of the figure. The figure has been adapted from (116).

The small domain of various DNA-MTases is significantly less conserved in size, sequence and structure. It contains the variable TRD domain that is involved in the sequence-specific DNA recognition, which explains its diversity (115). The DNA is bound in a cleft between the large and small domain such that the minor groove faces the small domain, while the major groove faces the large domain (112). A similar structural organization can be observed also in other DNA-MTases that methylates proteins and RNA molecules (129).

3.5.2 DNA recognition by C5-DNA-MTases

In order for the transfer of the methyl group from SAM to DNA to occur, the enzyme needs to recognize and bind to a specific DNA sequence. This sequence specific recognition arises from contacts between the enzyme and specific bases in the DNA, which are located in the minor and major grooves and the phosphodiester backbone. As previously stated in section 3.5.1, the small domain of the DNA-MTase interacts with the minor groove of DNA, while the large domain interacts with the major groove, and the complex formation can lead to strong bending of the DNA. The degree of sequence specificity varies between different the DNA-MTases with some having similar specificities as the REases, while others are more promiscuous when it comes to DNA interaction (81). DNA-MTases bind DNA in two steps. The enzyme binds first to any site on the DNA in a non-specific manner, after which it slides along the DNA by facilitated diffusion in a one-dimensional movement in search of a target sequence (section 3.1.2). In cases where there is more than one recognition sequence on the same DNA molecule, this movement can either be in a processive manner similar to a one-dimensional diffusion, where the DNA-MTase remains bound to the DNA until all target sites are modified. It can also move in a distributive manner in which case the enzyme dissociates from the DNA after every turnover, a movement resembling three-dimensional movements of the protein on DNA (Section 3.1.2). Solitary DNA-MTases (M.SssI, CcrM, and Dam) modify the DNA in a processive manner, while DNA-MTases that are members of RM systems (*e.g.* M.Hpal, M.Hhal) move in a distributive manner. This difference in the mode of movement between solitary and RM-related DNA-MTases might be explained by their biological roles. DNA-MTases that are members of the RM systems protect host DNA as their main role and should not act too fast in order to prevent protection of phage DNA, while rapid methylation of newly replicated DNA, which is done by solitaire MTases, is more desirable. (81).

3.5.3 Catalytic mechanism of C5-DNA-MTases and target base-flipping

For a successful transfer of the methyl group from SAM to the aromatic ring of the DNA base, proximal contact between the catalytic site of the enzyme and the base is required. These contacts are impossible to achieve while the target base is located inside in the double helix and paired by Watson-Crick base pairing. To overcome this problem, DNA-MTases have developed the special mode of action to expose the base to be methylated (Figure 13). According to this mechanism, the base, together with the sugar phosphate backbone, is rotated by 180° from the DNA helix into the catalytic pocket of the enzyme. During this rotation, the DNA helix is not distorted, whereas the hydrogen bonds between the base pairs and the stacking π interactions with neighbouring base pairs are disrupted. This mechanism was first identified in M.HhaI, the DNA-MTase from *Haemophilus haemolyticus* (130).

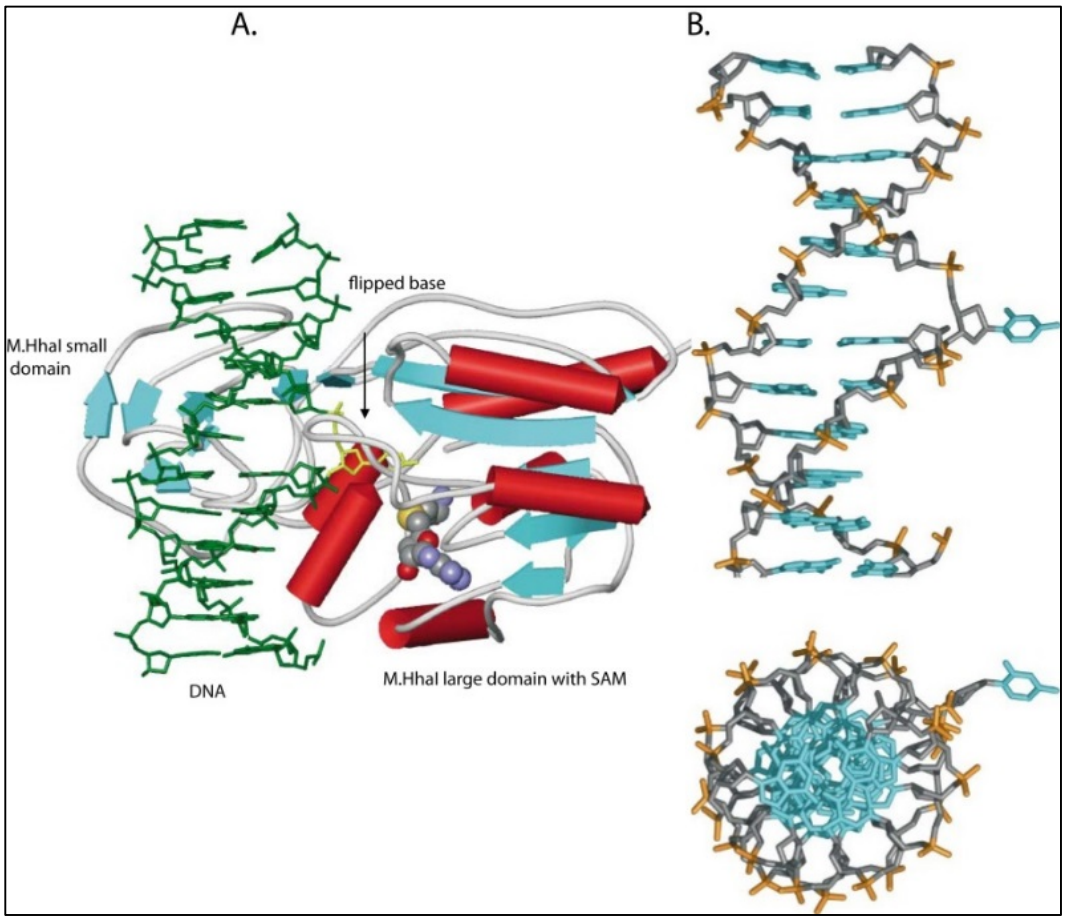

Figure 13. Base flipping mechanism. A. Structure of M.HhaI (grey, blue, and red) showing the target base (yellow) flipped out of the DNA helix (green). **B. DNA base flipping**. The DNA (shown from the side and end-on) backbone is grey with orange phosphates and the bases are cyan. With the exception of the flipped cytosine, the structure is a classical B-form DNA. The figure has been adapted from (81).

In M.HhaI the cavity created by the flipped-out base is filled by a Gln residue from the small domain that is further stabilized by hydrogen bonds to a Ser from the catalytic loop (112, 130). Other C5-DNA-MTases have been shown to use different amino acid residues for stabilization, for example in M.HaeIII, the DNA-MTase from *Haemophilus aegypticus* (112, 131).

The exact mechanism of base flipping is still unknown, but it is assumed to follow three steps. In the first step, the MTase locates and binds to DNA. The second step involves MTases "invasion" of the DNA and flipping out the base. The third step involves trapping of the flipped DNA base and its stabilization by interactions between the flipped base and the residues from motifs IV, V and VIII (129, 132). This mechanism is also observed in other enzymes that need access to bases buried in the DNA helix, such as DNA glycosylases and AP endonucleases (112, 115, 129, 130, 133, 134).

In C5-DNA-MTases, the C5 atom of cytosine is not reactive enough to perform the attack on the methyl group of SAM itself, so in order to obtain an effective transfer, the methyl group is first activated then involved in a nucleophilic addition, a process called the Michael addition (Figure 14). The key point in this reaction is the formation of a transient covalent complex between the enzyme and the DNA (120, 135). After the transfer of the methyl group from SAM to the DNA, C5-methylcytosine and S-adenosyl-L-homocysteine (SAH) are produced and released. SAH acts as an inhibitor of the reaction both *in vivo* and *in vitro* (45, 136, 137).

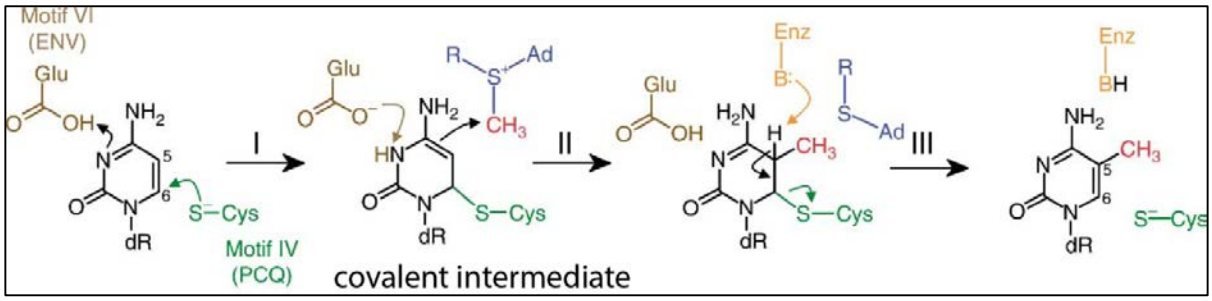

Figure 14. Schematic representation of the catalytic mechanisms of DNA C5-methylation. The conserved motifs VI and IV are indicated. In step I the C5 atom of cytosine is activated by nucleophilic addition, thus forming a transient covalent complex between the enzyme and the DNA. Here a thiol group from cysteine (shown in green) from motif IV starts with nucleophilic attack on the C6 carbon of the cytosine pyrimidine ring, forming a covalent DNA-protein intermediate. In step II, Glu in motif VI (shown in brown) protonates the N3 nitrogen of the pyrimidine ring, while an Arg residue from motif VIII additionally aids the process. Activated C5 carbon attacks the methyl group (shown in red) of SAM (blue). After methyl transfer is accomplished, SAH (shown in blue) is released, and by additional β-elimination of proton from position 5, the protein-DNA complex dissociates (step III). The figure has been adapted from (81, 115).

4 Adaptations to prosper in a cold environment

On Earth, we find life in many different habitats, for example places with extremely high or low temperature or pressure; high or low content of available oxygen or carbon dioxide; high levels of radiation, acidity, or alkalinity; absence of water or water containing a high concentration of salt; presence of sulphur, and other toxic substances. Examples of extreme environments include the geographical poles, extremely dry deserts, volcanoes, deep ocean trenches, the upper atmosphere, or even outer space. Organisms are classified intro groups depending on which habitat they occupy, and their optimal growth temperature. Thermophiles grow and survive at high temperatures up to 113ºC (138), mesophiles thrive in moderate temperature in range between 20 and 45 ºC, and psychrophilic organisms are able to grow and live below this temperature range (139-141). Within these categories, the psychrophiles are the most abundant group, due the fact that permanently cold environments dominate more than 80% of Earth's surface, including the Arctic and Antarctic regions, mountain regions, and deep-sea waters and glaciers (142, 143). In these areas, a wide range of species is found, from Gram-positive and Gram-negative bacteria to multicellular organisms such as yeast, algae, various invertebrates and polar fish (144-146). To survive in such environments psychrophiles have evolved a variety of different structural and physiological adaptations, including regulation of membrane fluidity and ion channel permeability. Additionally, many of these organisms express cold-shock proteins that regulate synthesis of cellular proteins, especially at the transcription level and at the initiation of translation. Many species synthesize cryoprotectors and antifreeze molecules that stabilize proteins and prevent formation of ice crystals inside cells (142). Psychrophilic organisms have evolved enzymes that tolerate and act at low temperatures (147, 148).

4.1 Cold adaptation of enzymes

One of the biggest challenges to organisms that are living in cold environments, is dealing with the slow reactions rates caused by the low temperature (142). Cold-adapted enzymes have a high catalytic activity at low temperatures, but are less thermally stable (147). It is generally believed that the high activity of cold-adapted enzymes is achieved through destabilizing of the active site allowing it to be more flexible at lower temperatures than for mesophilic and thermophilic enzymes homologs (149). This increased flexibility also contributes to decrease in activation enthalpy and more negative entropy (150, 151). For most psychrophilic enzymes, the increased flexibility is localized only in the active site, while other parts of the proteins are

more stable (147, 152). Additionally, psychrophilic enzymes have a larger catalytic site, that makes binding of substrates and the release of products easier (142). This can be achieved by replacing bulky amino acids with amino acids that have smaller side chains or by changing conformations of loops surrounding the active site. A consequence of this adaptation is that these changes in the active site dynamics reduce the strength of substrate binding in the active site (151, 153). Other adaptations to low temperatures include a lower frequency of Pro residues and higher occurrence of Gly residue clusters, a decrease in the number of disulfide bonds, salt bridges and hydrogen bonds (147). In addition, a change in the electrostatic distribution of the protein is observed. A plausible explanation for this is that by a change in the electrostatics, the binding of protein to its substrate is increased (148, 153-155).

4.2 *Psychrobacter arcticus*

This thesis focuses on two enzymes from a member of the gamma proteobacteria genus *Psychrobacter*. Members of this genus are Gram-negative, aerobic, spherical or rod-shaped bacteria, they are chemo- and osmotolerant, non-motile, grow at neutral pH and are almost all cold-adapted (156). They are primarily found in cold, saline habitats such as glacials, sea ice, but can also be found in chilled meat and clinical samples. Because many members of the genus *Psychrobacter* are living in constantly cold environments they are considered as good model organisms for investigation of cold adaptation of enzymes (157). In our work, we focused predominantly on *P. arcticus,* a species isolated from permafrost sediment cores in the Kolyma region in Siberia (158, 159). Some isolates of *P. arcticus* have been shown to grow at temperatures ranging from -10°C to 28°C and can survive up to 12% NaCl (158). One isolate was also shown to have a generation time of 3.5 days at -2.5°C and can survive for a long time under freezing conditions (160). By comparison to homologues proteins from mesophilic counterparts, some general strategies for cold adaptation can be elucidated, such as reduction in Pro and Arg residues and increase in Lys, and a generally lower number of hydrophobic and acidic residues (160, 161). In addition, enzymes involved in DNA double strand break repair systems that are active down to -15°C have been identified (160, 162-164).

In two independent case stories in this thesis, we have recombinantly expressed a type II REase (DpnI) and a C5-DNA-MTase (ParI) from *P. arcticus*.

4.3 *Aliivibrio salmonicida*

Aliivibrio salmonicida (previously known as *Vibrio salmonicida* (165)) is a marine, pathogenic, Gram-negative bacterium discovered in the late 1970s in Norwegian salmon fish farms (166). This bacterium is curved, rod-shaped and motile, possesses nine polar flagella and is salt tolerant. Members of this genus can grow on NaCl concentrations from 0.5-4% w/v (166). It causes the disease cold-winter vibriosis (Hitra disease) in salmon and cod (166, 167). *A. salmonicida* grows at temperatures between 1-22°C with optimum growth occurring between 6-15°C. As with *P. arcticus*, the enzymes of *A. salmonicida* have been used as a model system to study cold adaptation mechanisms (153, 168, 169). The enzymes of *A. salmonicida* are shown to be more flexible than their mesophilic homologues; they have a decreased number of hydrophobic residues and show an increase in charged and uncharged residues and increase of volume of the core of the protein (170).

As part of this thesis, we have recombinantly expressed and characterized a metallo-β-lactamase (MBL) from *A. salmonicida*, ALI-1.

5 Biotechnological application of enzymes from cold adapted bacteria

Due to their specificity and precision, both type II REases and DNA-MTases have potential application areas in biotechnology. Since their discovery, REases have been extensively used in molecular biology, most notably in DNA cloning and recombinant DNA technology. Commonly used REases for molecular cloning includes EcoRI, SalI, NotI and HindIII (171, 172). The orphan DpnI is frequently used in downstream reactions following mutagenesis PCR to remove parental DNA templates (173). More recently, the type IIS restriction enzymes, such as SapI, are used in high-throughput gene cloning methods like ‘Golden gate cloning’ or the ‘FX-cloning’ approaches (174-176). Type II REases have also been applied in Restriction Fragment Length Polymorphism analyses, a technique used in determination of differences in gene sequences between individuals, based on the restriction cleavage pattern on specific areas in the genome (177).

Based on their function in transferring a methyl group to specific sequence on DNA, DNA-MTases are used as a tool in studying the binding of proteins to DNA as well as in regulation of gene expression (178). DNA-MTases are used in many applications, such as *in vivo* and *in vitro* foot printing (179), targeted DNA methylation, targeted gene silencing and studying of protein-DNA interaction(180, 181). In all these application areas a DNA-MTases with known

specificity is fused to a targeting module, which can be a small molecule, polynucleotides or protein domains (*e.g.* zinc-finger) that direct the DNA-MTase to the target on the DNA (182, 183).

Lately, cold-adapted enzymes became more used in biotechnology. The biggest advantage is their susceptibility to heat compared to their mesophilic and thermophilic counterparts, as seen for example in alkaline phosphatase (AP). *E. coli* AP is resistant to heat inactivation, while AP from Arctic shrimp or from Antarctic bacteria can be irreversibly inactivated at 65°C or 55°C, respectively, after a shorter period of heating (184-186). In addition, cold adapted enzymes have higher catalytic activity at lower temperatures compared to their mesophilic counterparts (187).

II. Aims of the project

REases and DNA-MTases are sold on the market and are highly used in molecular biology research. We were interested in investigating the applied potential for two of the putative cold-active enzymes we were working with, namely the DpnI and ParI.

Because of the wide impact bacteria have on environment, it is important to thoroughly understand how they adapt to the environments they are exposed to. In this project, we have investigated three independent cases that contribute to our understanding of some of the defence mechanisms bacteria possess against both extrinsically applied chemical threats as well as infection by bacteriophages. The three independent cases are:

1. Protection against antibiotics: Resistance by metallo-β-lactamases
2. Protection against phage infections: DpnI restriction of non-self adenine-methylated DNA
3. Protection against phage infections: DNA-MTase-directed cytosine methylation of self-DNA

The common aim of these case studies was to functionally and structurally characterize the enzymes and to elucidate the role they have in the bacteria. In addition, as all three enzymes originate from cold-adapted organisms we were interested in describing structural determinants for cold-adaptation.

III. Description of the work

Manuscript I - A study of a type II restriction endonuclease from the cold-adapted organism *Psychrobacter arcticus.* Manuscript. In prep.
Grgic Miriam, Altermark Bjørn, Leiros Ingar

Cold-active restriction endonucleases are attractive reagents in molecular biology due to their higher activity at lower temperatures. A great advantage of such cold-active enzymes is that many can be irreversibly heat-inactivated by a moderate heat step. In this work, we attempted to functionally and structurally characterize the restriction endonuclease DpnI from the cold-adapted *P. arcticus*. Thorough understanding of this enzyme by solving its 3-dimensional structure and describing the catalytically mechanism, as well as the features responsible for its cold-adaptation would provide better insight and also improve molecular biology techniques where this enzyme type is currently applied. Several expression trials were performed using different expression conditions and modified *E. coli* host strains. In addition, purification of recombinant DpnI was pursued, but despite all attempts to characterize the the enzyme, this part of the project was terminated before publishable results were obtained. The main problem was achieving sufficient yield and purity of the enzyme. Therefore, to describe the work that was conducted as part of this thesis, we have chosen to present it in a manuscript form.

Paper II - *Properties and distribution of a metallo-β-lactamase (ALI-1) from the fish pathogen Aliivibrio salmonicida LFI1238.* Published in J. Antimicrob. Chemother.; Oct 31, 2014.
Kristiansen Anders; Grgic Miriam; Altermark Bjørn; Leiros Ingar.

The increase in occurrence of antibiotic resistant bacteria among hospitalized patients is a global problem and a serious threat to human welfare. As antibiotics are the most important treatment we have against bacterial infections, understanding the mechanisms of bacterial resistance to antibiotics is necessary. One way to achieve this is thorough characterization of the enzymes involved in resistance, such as the metallo-β-lactamases (MBLs). In Paper II we functionally characterized the MBL from *A. salmonicida*. The protein was recombinantly expressed and kinetic parameters, NaCl dependence, pH optimum and temperature optimum were determined using purified enzyme. Our data expands the current knowledge regarding

these important enzymes and may be useful in future inhibitor development towards homologous enzymes found in human and animal pathogens.

Manuscript III - Initial characterization of ParI, an orphan C5-DNA methyltransferase from *Psychrobacter arcticus* 273-4. Manuscript. To be submitted to BMC Biochemistry

Grgic Miriam, Bjerga Gro Elin Kjæreng, Williamson Adele Kim, Altermark Bjørn, Leiros Ingar

DNA methylation is important for healthy growth and development in both prokaryotes and eukaryotes. In prokaryotes, in addition to having roles in various cellular events, they are part of a host protection system as members of the restriction modification (RM) systems where their function is protection of self-DNA from cleavage. DNA methylation also has an important role in protein-DNA interaction, either by enhancing or disrupting binding of proteins to DNA. By better understanding the function of these enzymes, we can gain knowledge of the processes they are involved in. This manuscript describes a preliminary study of a C5-DNA methyltransferase (C5 DNA-MTase) from the bacterium *P. arcticus*. The protein was recombinantly expressed and characterized in terms of thermal stability and activity, both *in vivo* and *in vitro.* In addition, experiments to determine the sequence specificity of the ParI protein has been performed.

IV. Discussion and Conclusion

6 Discussion

Bacteria can be found in all habitats, either as free-living organisms, or in different symbiotic relationships. Many are pathogenic towards humans, animals and plants. In their environment, bacteria are exposed to various chemical threats (antibiotics) produced by nearby microorganisms as well as the threat of being infected by bacteriophages.
In order to protect themselves, bacteria have evolved several resistance mechanisms.

6.1 Environmental adaptation

Depending on the environment where they thrive, organisms have adapted differently, making them successful in surviving in the surrounding conditions, such as a cold environment (142, 143, 145, 188). To survive in such environments bacteria have evolved a variety of different structural and physiological adaptations, such as regulation of membrane fluidity and ion channel permeability, expression of cold-shock proteins that regulate synthesis of cellular proteins, or synthesizing cryoprotectors and antifreeze molecules that stabilize proteins and prevent formation of ice crystals inside cells (142). In addition, psychrophilic organisms have evolved enzymes that tolerate and act at low temperatures. One of the adaptations is increased molecular flexibility of the enzymes that compensates for the higher activity at low temperatures. However, the increase in molecular flexibility is causing intrinsic molecular instability, which makes these enzymes more temperature labile (147, 152, 189). In this work, we characterized three enzymes originating from cold adapted organisms. The MBL ALI-1 from the marine bacterium *A. salmonicida,* and a DpnI-like REase and a C5-DNA MTase, both from *P. arcticus*. These two bacteria have an active metabolism in their respective environments (158, 160, 166, 190) and it would be reasonable to assume that they both possess proteins that are cold adapted as well. As it can be seen in Paper II, Figure 3, ALI-1 has a lower temperature optimum when compared to VIM-2 that originates from the human pathogen *Pseudomonas aeruginosa*. In addition, it showed higher tolerance with respect to NaCl concentration, retaining high activity in the concentration range 500 mM up to 2 M, while the optimal NaCl concentration for VIM-2 is 200 mM. These results are fully understandable when the origin of the organisms is considered as well as the fact that ALI-1 is secreted into the periplasm of a marine bacterium and thus faces higher salinity.

6.2 The DpnI-like enzyme from *Psychrobacter arcticus*

REases also have very important roles in molecular biology and are being used in many different types of experiments (*i.e.* cloning, mutagenesis). Enzymes that have higher activity at lower temperature (*i.e.* RT or even 4 ºC) and display heat lability (i.e. they would not have to be exposed to high temperatures (> 60 ºC) to inactivate), would be very beneficial as well as cost reducing (187). Due to its specificity towards only methylated DNA, DpnI has many applications in molecular biology. Since the DpnI-like enzyme we worked on (Manuscript I) is from a psychrophilic organism, we started with the assumption that the enzyme itself could be cold adapted, and due to that possess some of the very attractive characteristics and thus can have further application in molecular biology.

REases have a very important role in bacterial cells where they are considered as the immune system of bacteria and are protecting bacteria against bacteriophage attack by restricting its DNA before it has a chance to replicate. By inhibiting the bacterial REase, the phage could be efficient in killing bacteria. This method, where bacteriophages are used for treatment against bacterial infections, is completely safe for humans or animals, is called phage therapy and is nowadays seen as a possible replacement for antibiotics (191-195).
By combining inhibitors for REases with a phage cocktail (196), it could serve as an efficient treatment against bacteria and in order to achieve that, better characterization of REases should be done. In our work, we attempted to characterize a DpnI-like protein from *P. arcticus*, a REase that specifically cleaves methylated DNA.

Recombinant protein was produced and purified form the original organism (Manuscript I, Figures 3, 4 and 7). When purified from a *P. arcticus* culture and as a native construct (without any additional tags) DpnI showed activity towards methylated substrate (Manuscript I, Figures 9 A and B).
In all cases, the amounts of protein acquired were very low and in addition, protein was highly unstable and lost activity after a short time at 4 ºC. In addition to a low amount, contamination from other proteins was posing a problem. Unfortunately, due to the difficulties in producing protein in decent amounts, we could not proceed with experiments that could help in better structural and functional understanding of this protein.

Recent publications on *S. pneumoniae* DpnI (197, 198) also showed difficulties in production of the recombinant protein. The authors managed to overcome their problems with expression by adding a cleavage site after the N-terminal his-tag for tag-removal as well as adding more steps in protein purification (198). This enabled them to produce protein in sufficient amounts for crystallization studies (197, 198). Based on their success, we believe that in order to increase protein expression and produce stable recombinant protein some changes should be done. One such change is improving the expression by using removable solubility tags *e.g.* maltose binding protein (MBP),the sumo fusion partner, CPD tag (199-201) or cloning of the *dpnC* gene downstream a cold-shock promoter for low-temperature expression (202). Alternative expression systems, such as yeast or cell-free protein expression, may also be used to further explore the recombinant expression of DpnI because eukaryotes do not possess adenine specific methyltransferases and thus DpnI would not have a substrate to act upon while being produced.

6.3 Characterization of the Metallo-β-lactamase ALI-1 from *Aliivibrio salmonicida*

Investigation on environmental DNA samples can give us insight into how enzymes have developed and what their original function was. In Paper II the chromosome-encoded marine MBL from *A. salmonicida* (ALI-1) was characterized and compared with the plasmid-borne MBL (VIM-2) encoded by a human pathogen (*P. aeruginosa*). Both enzymes showed activity towards degradation of β-lactam antibiotics, but they exhibit different adaptations, which correlates with the origination of enzymes from organisms adapted to different environments. ALI-1 is secreted in periplasm of the marine bacteria living in a cold and salty environment, while VIM-2 originates from a human pathogen living in warmer and less salty conditions in body fluids. When the effect of NaCl on activity is compared, ALI-1 had its highest activity at NaCl concentrations up to 500 mM, which is approximately the same as seawater. VIM-2 has a less salt-dependent activity, with optimum NaCl concentration at 200 mM. Both K_M and kcat are affected at higher NaCl concentration. In the presence of NaCl, K_M decreases dramatically while the kcat increases slightly (Paper II, Figure 2). NaCl probably favours the substrate binding by increasing the hydrophobic interactions between substrate and enzyme, thereby lowering the K_M, but the detailed mechanism for an increased catalytic efficiency with increased concentration of NaCl remains yet to be fully explored.

The two enzymes also showed significant differences in optimum temperature for activity. As expected, the ALI-1 enzyme is more cold-active compared with VIM-2, showing increased activity at lower temperatures and a lower optimum temperature (Paper II, Figure 3). This can be explained by decreased temperature stability, leading to a more rapid temperature-induced denaturation. In addition, with increased NaCl concentration in the reaction buffer, a shift in optimum temperature for activity was observed, as can be seen in Paper II, Figure 4 which indicates increase in protein stability. This stabilizing effect may come from NaCl´s ability to reduce repulsive interactions between charges on the surface of the protein and by strengthening the hydrophobic effect.

Testing the effect of different reducing agents showed loss of activity when using 5 mM TCEP in the reaction buffer. This is likely due to the strong redox potential of TCEP that was enough to reduce the cysteines thiolate (S-) to the sulfhydryl form (SH). Since cysteine coordinates the second zinc ion in the active site, reducing it can in turn disturb the Zn coordination, which may disrupt the activity of the enzyme. Recently, TCEP has been proposed as a treatment for botulinum toxin due to its ability to reduce key disulfide bonds (203). Since at a concentration of 1 mM, TCEP is not cytotoxic, genotoxic or mutagenic (204) it could be used in some cases as a combined treatment together with β-lactam antibiotics to abolish the MBL activity of a pathogen, but this remains to be tested.

The screening of different environmental isolates, as well as strains originating from the fish-farming industry, also suggests that the gene is common in both habitats and could indicate an additional role of MBLs such as their involvement in quorum sensing and communication among bacteria (16).

6.4 Investigating DNA Methylation in *Psychrobacter arcticus*

In Manuscript III, the C5-DNA MTase ParI from the psychrophilic bacterium *P. arcticus* was characterized. Despite low yields of protein (2.5 mg/l culture), we performed some characterization experiments.

a set of experiments was completed, where we investigated the thermal stability of ParI, using DSC, TF and CD. In the CD measurements, no secondary structures were observed at 65 ºC meaning that the protein is unfolded at that temperature (Manuscript III, Figure 7). The DSC

and TF results confirmed this, and showed that the protein unfolds at temperatures of 54 ºC and 53 ºC, respectively. In both cases, the melting curves were gradual and stretched over a long temperature range, which indicates that the protein is unstable (Manuscript III, Figures 5 and 6).

In the *in vivo* activity assays, we determined that ParI is active inside the bacterial cell as well as being active when expressed recombinantly in *E.coli* (Manuscript III, Figure 8). This raised a question regarding the possible biological role of ParI in *P. arcticus.* When compared with other species from the genus *Psychrobacter* that have a sequenced genome, *P. arcticus* is the only one that possesses an orphan C5-DNA MTase (Manuscript III, table 1 and additional data from Rebase (78)). This could indicate that ParI does not have an important role in the bacterial cell, but evolutionary, ParI could have been part of a functional type II RM system that over time has lost its REase member as it became redundant (205). The reason why ParI has survived through evolution may be that it bares the same specificity as other members of type II RM system found in *P. arcticus*, thus, complementing it, and by that providing more efficient protection of the host DNA. A similar system exists in *E. coli*, where Dcm, an orphan C5-DNA MTase, has the same recognition sequence as the EcoRII RM system (CCWGG). One of the hypothesis that might explain this is that Dcm serves as a backup for methylation of *E. coli* DNA (205, 206) and in case the methylation member of the host RM system is lost, it may protect the host from being restricted by the remaining REase (207).

Surrounding genes to *parI* are showing strong similarity to phage proteins, which may indicate that *parI*, as well as surrounding genes, may have been horizontally transferred, and are of phage origin, although they are no longer part of an intact temperate phage.

To determine the specific sequence that ParI recognizes and modifies on DNA we performed bisulfite conversion (208). For this we used a commercially available kit from ZYMO Research as well as substrate they provided. Despite various try-outs we did not manage to get conclusive results in this case (Manuscript III, figure 9). One of reasons for the lack of success of this experiment might be that the substrate used was not optimal. It being only 362 bp long means that the specific site that ParI recognizes might have been omitted among this short DNA stretch. In addition, there is a possibility that ParI does not have a specific site, but it is rather

more promiscuous when it comes to DNA methylation, and methylates most of the available cytosines as seen in (209). This would explain our results as well (Manuscript III, Figures 8 and 9). It is also possible that the reaction conditions were suboptimal, causing ParI to act in a nonspecific manner. In order to determine the exact site that ParI is methylating, more experiments should be done, possibly including radioactive labeling or detection of a methyl group on DNA by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (210). Single molecule real time sequencing (SMRT sequencing) of the *E. coli* genome cultured in the presence and absence of the ParI encoding plasmid could also provide data needed for elucidation of methylated cytosines in genome (211, 212).

When comparing the protein sequence of ParI to other MTases we noticed one curiosity. In catalytic motif IV, which is highly conserved among MTases, Asp replaces Pro. It is assumed that Pro in this motif has a role in the orientation of the activated cytosine and SAM so that the methyl group can be transferred successfully (213). By doing this the ProCys dipeptide stabilizes a transient tertiary complex and therefore has a very important role in catalysis. Substitutions of Pro with Ala, Ser, Cys, Phe, Val, Arg, His and Leu in this motif caused a destabilization of the intermediate, which in turn decreases the catalysis rate (213). Pro is generally characterized as a rigid amino acid, and in protein structures, it imposes rigidity. It also has less conformational freedom, especially in unfolded structures. Cold adapted enzymes are characterized by increased flexibility in the catalytic site, which is in order to overcome challenges of low temperature. Pro, due to its characteristics cannot provide this much-needed flexibility and is usually substituted with a more flexible amino acid, which might be the reason why ParI has Asp in catalytic site instead of Pro (147, 152, 214).

7 Conclusion

In Manuscript I and Manuscript III, we characterized two DNA interacting and modifying enzymes from the psychrophilic bacterium *P. arcticus*. In both cases, we had problems with stability of the proteins. Due to that, it was difficult to obtain reasonable amounts of protein to work with and therefore the project described in Manuscript I was terminated. In Manuscript III, we characterized C5-DNA MTase ParI in terms of thermal and structural stability and detected activity.

Both these enzymes are considered to play a role in bacterial protection against invading bacteriophages, either by disrupting invading DNA (DpnI) or by protecting its own DNA (ParI). We managed to produce both enzymes and detect their activity, based on which we can conclude that they do still have a role in *P. arcticus.*

Elucidating the specificity of the ParI enzyme proved to be difficult and we could detect only very broad specificity. To obtain results that are more conclusive additional experiments should be run.

Paper II deals with MBL from the marine bacterium *A. salmonicida.* The enzyme was characterized in terms of kinetic parameters, NaCl dependence, pH optimum and temperature optimum and compared with the MBL VIM-2 from a human pathogen. From our data we conclude that ALI-1 shows similar *in vitro* optima in terms of NaCl and temperature to that of *A. salmonicida* in its natural cold and marine environment.

The existence of bacteria encoding ALI-1 in environmental samples that had no contact with fish farms and thus have not been exposed to synthesized antibiotics, can suggest a “protective” role of this enzyme in the bacterium. It is assumed that MBLs serve as protection against antibacterial chemicals that some organisms, living in the same environment, are producing (for example penicillin from the mold *Penicillium notatum).* In addition, these enzymes can have a role in adjustment of quorum sensing signals and be part of bacterial communication.

We conclude that all three enzymes, DpnI, ParI and ALI-1, are active enzymes that can be used by bacteria in order to protect themselves from enviromental threats. In order to get better

insight into all of them and get better understanding of their function and exact role in the bacterial cell, additional research should be done, in both structural and functional studies.

V. References

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