Towards a Macrolide Biosensor:

Mutation and Production of MphR(E) Repressor Protein

Master´s Thesis
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Master´s Thesis

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Abstract

Background and aims: Among other antibiotics macrolides have been used in a large scale for decades which leads into an increase of antibiotics in food, feed and environment and results in antibiotic resistance in bacteria. Therefore, a publicly available and cost-efficient macrolide antibiotic detection method by a whole cell biosensor is developed. The whole cell biosensor in process is constructed in *Estericia coli* cells containing a self luminescent luciferase plasmid regulated by macrolide 2'-phosphotransferase regulating protein (MphR(E)). The model molecule of MphR(E) is 2'-phosphotransferase I regulating protein MphR(A) which binds to its promoter with the dissociation constant of 574 ± 29 nM. The aim of this study was to require information of the regulatory protein MphR(E) and its affinity towards promoter DNA by making mutations to the DNA binding HTH motif of MphR(E).

Methods: Six different mutations were designed for the DNA binding helix-turn-helix motif of MphR(E) to increase the affinity towards the promoter DNA. The mutations were made by splicing by overlap extension PCR (SOE-PCR), inserted into a Pac400c plasmid and transformed into *Escherichia Coli* XL-1 Blue. Five of the mutations, MphR(E) and MphR(A) were produced and purified using affinity chromatography. MphR(E) was measured by mass spectrometry. Furthermore, the Dissociation constant (Kd) was calculated for MphR(E) and two mutations both containing histidine tags after fluorescence polarization.

Results: The five mutations were successfully produced in *E. Coli* and purified along with MphR(E) and MphR(A). In fluorescence polarization the undigested MphR(E) had a Kd of 204 nM, MphR(E)_CG had a Kd of 576 nM and MphR(E)_K had a Kd of 652 nM. Showing that the more MphR(E) becomes like MphR(A) the weaker the affinity towards the promoter DNA becomes. The mass spectrometry showed that MphR(E) was well purified and that the histidine tag was cleaved from the specific cleavage site and from two other sites.

Conclusions: Most of mutations designed were produced and purified. The purification was successful but the cleavage needed further optimization. The aims of this study were met by gaining information of the DNA binding affinity of MphR(E) which was higher than the one of MphR(A) unlike presumed. The mutations made MphR(E) more like MphR(A) and on the contrary did not succeeded in lowering the DNA affinity. All of the proteins can be further analyzed by mass spectrometry and by fluorescence polarization to determine the Kd and to evaluate the cleavage of the histidine tag.

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Bakteerien antibioottiresistenttiys lisääntyy muun muassa makrolidiantibioottien laajan käytön seurauksena. Antibioottien määrä on lisääntynyt elintarvikkeissa, rehuissa ja ympäristössä. Edullista ja julkisesti saatavilla olevaa makrolidiantibiootteja havaitsevaa kokosolubiosensoria kehitetään, jotta makrolidien havaitseminen helpottuisi. Tarkoituksena on kehittää kokosolubiosensori, jossa on valoa tuottava plasmidi makrolidi-2´fosfotransferaasi-säätelijäproteiinin (MphR(E)) säätelyn alaisena. MphR(E):n mallimolekyylinä toimii makrolidi-2´fosfotransferaasi-säätelijäproteiini I (MphR(A)), jonka dissosiaatiovakio promoottoriaan kohden on 574 ± 29 nM. Tavoitteena on kerätä tietoa MphR(E):stä ja sen affiniteetistä DNA-promoottoriaan kohtaan tekemällä mutaatiota MphR(E)-säätelijäproteiiniin.

Tutkimusmenetelmät: Kuusi mutaatiota suunniteltiin MphR(E):n DNA:ta sitovaan kierre-käännös-kierre (HTH) motiiviin. Mutaatiot tehtiin ”liittäminen limittäisesti pidentämällä” *PCR*:llä (Splicing by overlap extencion PCR). Mutaatiot liitettiin Pac400c-plasmidiin ja transformoitiin *Escherichia coli* – bakteerikantaan. Mutaatioista viisi tuotettiin ja puhdistettiin affiniteettikromatografialla. Lisäksi, MphR(E) analysoitiin massaspektrometrilla. Fluoresenssipolarisaation jälkeen dissosiaatiovakio laskettiin MphR(E):lle ja kahdelle histidiinikahvan sisältävälle proteiinille.

Tutkimustulokset: Viisi MphR(E):n mutanttia, MphR(E) ja MphR(A) tuotettiin *E. colissa* ja puhdistettiin onnistuneesti. Fluoresenssipolarisaatiosta saatiin dissosiaatiovakioksi MphR(E):lle 204 nM, mutanteille MphR(E)_CG:lle 576 nM ja MphR(E)_K:lle 635 nM. Tulokset osoittavat affiniteetin heikkenevän kun MphR(E):tä muutetaan enemmän MphR(A):n kaltaiseksi. Massaspektrometrillä nähtiin MphR(E):n puhdistuksen onnistuneen ja histidiinikahvan pilkkoutuneen kolmesta eri kohdasta.

Johtopäätökset: Tutkimuksen tavoite saavutettiin saamalla tietoa MphR(E):stä ja sen sitoutumisesta DNA-promoottoriin. Suurin osa suunnitelluista mutaatioista tuotettiin ja puhdistettiin. Puhdistus oli onnistunut, mutta histidiinikahvan leikkaus vaatii vielä optimointia. Toisin kuin oletettiin, MphR(E) sitoutuu voimakkaammin promoottoriinsa kuin MphR(A). Mutaatioilla, jotka tekivät MphR(E):stä enemmän MphR(A):n kaltaisen, oli heikompi affiniteetti DNA:han. Kaikki tuotettujen ja puhdistettujen proteiinien dissosiaatiovakio ja digestio voidaan jatkossa mitata massaspektrometrillä ja fluoresenssipolarisaatiolla.

Contents

Abbreviations

Cm	cloramfenicol
ESI	external electronspray ionization
FP	fluorescence polarization
HTH	helix-turn-helix DNA binding motif
Kd	dissociation constant
MphR(A)	macrolide 2'-phosphotransferase I regulating protein
MphR(E)	macrolide 2'-phosphotransferase regulating protein
MphR(E)_ CG	MphR(E) mutation CG insertion between R22 and P23
MphR(E)_K	MphR(E) mutation T35K
MphR(E)_L	MphR(E) mutation V33L
MphR(E)_N	MphR(E) mutation D32N
MphR(E)_H	MphR(E) mutation D32H
MphR(E)_Y	MphR(E) mutation I45Y
MS	mass spectrometer
Q-FT-ICR	hybrid quadrupole Fourier transform ion cyclotron resonance
SOE-PCR	splicing by overlap extension PCR
TD	touchdown PCR program
TetR	tetracycline repressor protein
Tn10	tetracycline responsive elements of transposon

1. Introduction

The increasing use and misuse of antibiotics in medicine and animal feed has lead into an increase in the rate of antimicrobial resistance among clinical and environmental bacteria. Furthermore these resistance genes are transferred intergenetically among bacteria. (Hu, Sillaots et al. 2007) In bacteria the resistance genes can be easily moved between ecosystems: from humans and animals to soil and water. It is shown that antimicrobial drug resistance in bacteria correlates with increased use of antimicrobial agents. (Nwosu 2001) A significant amount of macrolides has been used because macrolides are one of the most clinically important antibiotics (Gaynor, Mankin 2003). Therefore, it is important to find new easier and more cost efficient ways for measuring low macrolide levels in feed, food, soil and water. Whole cell biosensors have high cost effectiveness because they are cheap and relative fast in comparison to expensive and fast immunological tests (Okerman, Croubels et al. 2004). A working whole cell biosensor has been developed for tetracycline by Nina Virolainen (Tampere University of Technology, Tampere, Finland) in *Escherichia coli* cells with a plasmid containing *Photorhabdus luminescent* –derived bacterial self-luminescent luciferase operon under the control of tetracycline responsive elements of transposon (Tn10) which is regulated by tetracycline repressor protein (TetR). The binding of TetR is reduces by tetracycline binding allowing transcription of the promoter. (Virolainen, Pikkemaat et al. 2008) The same idea has been followed in developing whole cell biosensors for macrolides. The regulator in these cells is negatively regulating macrolide 2'-phosphotransferase regulating protein (MphR(E)). The problem is that the biosensor does not work well and it is taught to be of a low DNA binding affinity.(Virolainen 2009) A working and patented version of macrolide biosensor has been already developed with the macrolide 2'-phosphotransferase I regulating protein (MphR(A)). (Eberz, Mohrle 2004) The low DNA binding affinity of MphR(E) might be improved by mutating the DNA binding HTH-motif of MphR(E).

2. Review of the literature

2.1. Repressor Proteins MphR(A) and MphR(E) of the TetR Family

The TetR family's repressor proteins have a similar 47 amino acid residues that form a helix-turn-helix DNA binding motif (HTH) (Ramos, Martinez-Bueno et al. 2005). The amino acid sequence and the 3D-structure of MphR(A) (PDB coordinate 3G56) show that it has a similar HTH-motif than the repressors in the TetR family (Zheng, Sagar et al. 2009). MphR(E) (GenBank AM260957) is a transcriptional regulator of the TetR-family and has 40 % identity with MphR(A). (Szczepanowski, Krahn et al. 2007). Therefore making MphR(A) the only model protein for MphR(E) available at the moment.

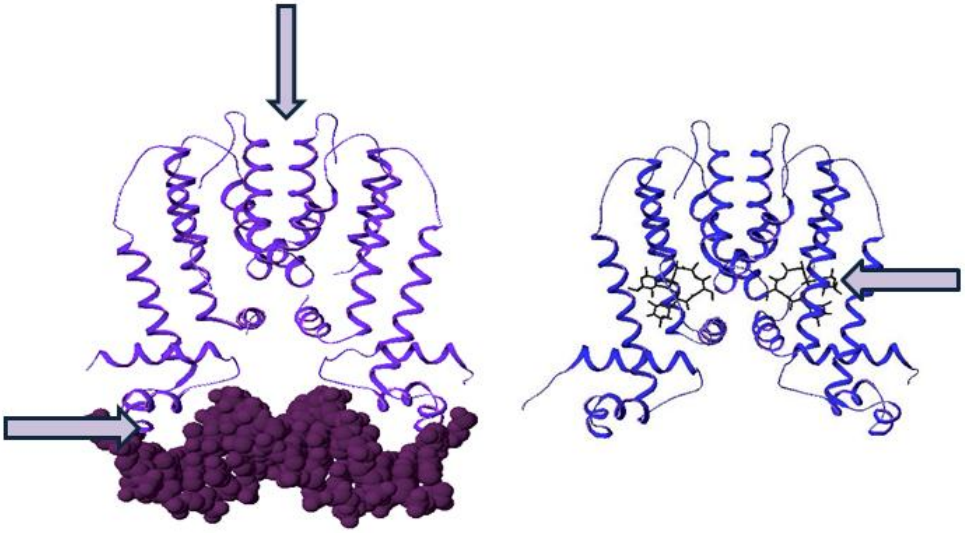
MphR(A) negatively regulates the expression of a macrolide 2´phosphotransferase I resistance gene (mphA) binding to its operator 35-bp upstream of the start codon. The MphR(A) forms a homodimer which de-represses by the presence of erythromycin. Two erythromycin antibiotics bind to the ligand binding pocket one for each monomer. The 189 residues long MphR(A) monomer is built of nine α-helices (Picture 1). The residues from 8 to 50 form a DNA binding HTH-motif to the N-terminal end where the most important aminoacids which interact with the negative DNA backbone are positive lysine (K35) and arginines (R41 and R51). The rest of the protein from helixes four to eight donate residues to a hydrophobic macrolide binding pocket and to the dimeric interface. (Zheng, Sagar et al. 2009)

The MphR(E) monomer model can be used in predicting mutation possibilities. These suggestions made based on a model and sequence analysis are most likely to show better results than just random mutagenesis (Viitamäki 2010). The mutation suggestions can be made to affect the binding of MphR(E) to DNA, the binding of the macrolide and the dimerization (Picture 1). The mutations affecting MphR(E)´s binding affinity towards should be focused to the amino acids in the DNA binding HTH-motif. If the mutations are wanted affect the macrolide binding affinity the mutations should be aimed to the amino acids of the macrolide binding pocket. On the other hand, MphR(E) most likely needs the dimeric structure in order to function as a repressor protein. The mutations made can affect the dimerization of the two monomers. A low

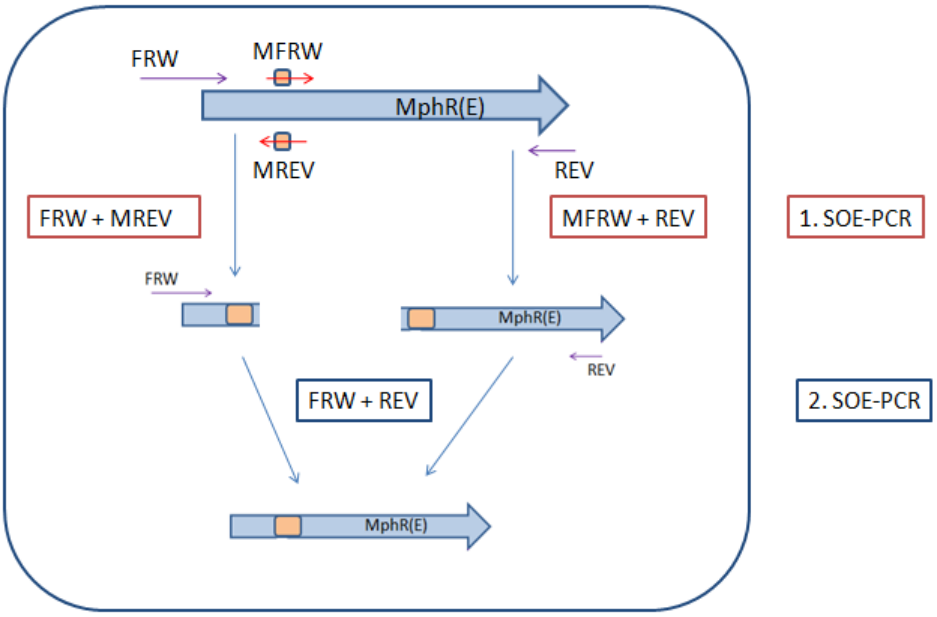
dimerization degree would weaken MphR(E)´s function as a repressor protein. By mutating the amino acids at the dimerization site could give more stable dimers. The best way to evaluate the functionality of the mutated MphR(E)´s is to see weather or not they can form a dimer. Furthermore, native ESI mass spectrometry can analyze the protein in its native state and observe whether or not it can form a dimer accurately and reliably (Winston, Fitzgerald 1997).

2.2. Mutation Method Splicing by Overlap Extension PCR

Splicing by overlap extension PCR (SOE-PCR) allows for making insertions deletions or other changes in a DNA sequence (Ho, Horton 1991). SOE-PCR is relatively straightforward, efficient and reliable (Bryksin, Matsumura 2010) because PCR methods is based on method described forty years ago (Kleppe, Ohtsuka et al. 1971). Nowadays it has become a routine laboratory tool. The mutagenic SOE-PCR needs four kinds of primers: two flanking primers, which are the upstream (FRW) and the downstream (REV); and two mutagenic primers, forward (MFRW) and reverse (MREV). The flanking primers FRW could contain a cleavage site and a possible tag and other improvements to the protein. In the first SOE-PCR step for every mutation two reactions are made separately. One reaction contains the FRW and the MREV primes and the other reaction contains MFRW and REV primers. (Simionatto, Marchioro et al. 2009) After the first SOE-PCR the product should be fractioned and purified preferably after gel electrophoresis so that maximum yield can be obtained (Urban, Neukirchen et al. 1997). In the second SOE-PCR step the two products forms the first step are mixed forming the required template for the second PCR reaction. (Picture 2) (Simionatto, Marchioro et al. 2009)



Picture 1. MphR(A) regulates negatively: in the left picture MphR(A) is bound to its promoter DNA sequence. The lower arrow shows the DNA binding site of MphR(E) and the upper arrow shows the dimerization location. MphR(A) releases the DNA in the presence of macrolides shown in the right picture where MphR(A) homodimer is bound to two macrolides. The arrow shows one of the bound macrolide in its macrolide binding pocket.



Picture 2. SOE-PCR. In the first reaction of SOE-PCR (1. SOE-PCR) the primers FRW and MREV are used in one PCR tube and in another tube the primers MFRW and REV are used both with the MphR(E) template. MFRW and MREV contain a mutation (orange box). In the second SOE-PCR (2. SOE-PCR) the products of the 1. SOE-PCR are used as a template and the primers FRW and REV are used. The product is a mutated MphR(E) protein.

The SOE-PCR patent and an article was compared for their second SOE-PCR reaction mixture and program to have a better understanding of this method (Table 1). In all of them the Taq polymerase was used. The overlapping part of the mutating primers was between 15-25 bp. If not designed properly primers might from inter or intermolecular annealing, little or no amplification products or nonspecific amplification and smearing (Mergulhao, Kelly et al. 1999). This problem can be resolved by designing primers with a program such as Vector NTI 10 Software (Invitrogen) (Simionatto, Marchioro et al. 2009). The reaction volumes ranged from 25 µl to 100 µl. The primer amount varied from 10 to 25 pmol per 25 µl of reaction mixture. In the first PCR reaction the template amount was 25 - 250 ng possibly depending if the template was a plasmid or a shorter DNA strand. In the second SOE-PCR the template amount was 25 % of the first SOE-PCR meaning 25 µl or of a equimolar concentration between 0.4 - 0.8 pmol. The mutating primers had 15 – 22 bp overlap and they contained either single of multiple mutations.

Table 1. The comparison of different SOE-PCR reaction mixtures.

	VTOT µl	Primer	1. SOE Template	2. SOE Template	Polymerase	Overlap bp	Mutations / Primer
(Ho, Horton 1991)	100 (25)	100 pmol (25 pmol)	100 - 1000 ng (25 - 250 ng)	25 %	Taq Perkin-Elmer Cetus 0.5 µl	15	Single or M
(Simionatto, Marchioro et al. 2009)	25	10 pmol	(50 ng)	Equimolar 0.4 - 0.8 pmol	Taq 2 U	15 - 22	Multiple

The PCR program for SOE-PCR is most likely the one suggested for the Taq polymerase by the manufacturer (Table 2). In SOE-PCR it is good to use the PCR mixture and PCR program suggested by the manufacturer of the polymerase. In first SOE-PCR it would be good to use the amount of DNA normally used in PCR for that polymerase or suggested by the manufacturer. The only changing part is how much of template is used in second SOE-PCR reaction and what is the annealing temperature in the PCR program. An equimolar concentration 0.4-0.8 pm per 25 µl of reaction mixture of templates in the second SOE-PCR and a lowered annealing temperature

in the PCR program is suggestible. (Peng, Xiong et al. 2006) The melting temperature (Tm) of primer-template can be calculated using the Finnzymes Tm calculator (https:// .finnzymes.fi/tm_determination.html : 02/2011). It is good to use relatively low annealing temperature in the reaction 5 – 10 ˚C below the calculated Tm of the primer-plasmid complex (Bryksin, Matsumura 2010).

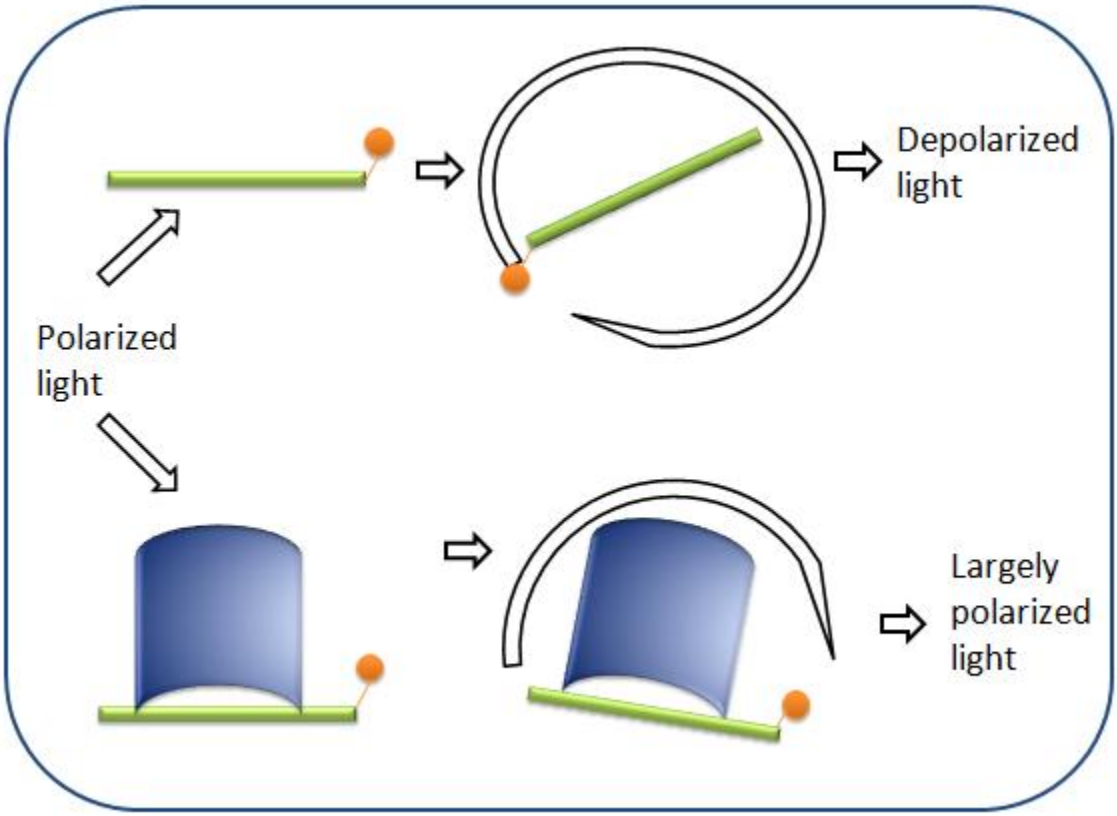
Table 2. Comparison of different SOE-PCR programs in second SOE-PCR reactions.

	Cycles	Initial Denaturation	Denaturation	Annealing	Extension	Incubation
(Ho, Horton 1991)	25	not mentioned	94 ˚C 1 min	50 ˚C 2 min	72 ˚C 3 min	72 ˚C 10 min
(Simionatto, Marchioro et al. 2009)	30	95 ˚C 7 min	95 ˚C 1 min	50 ˚C 1 min	72 ˚C 1 min	72 ˚C 7 min

2.3. Fluorescence Polarization

2.3.1. Fluorescence Polarization in Measuring Protein-DNA Affinity

Fluorescence polarization (FP) or fluorescence anisotropy is a fast, sensitive and robust method for measuring the relationship between protein and DNA interactions (Roehrl, Wang et al. 2004). The basic idea of fluorescence polarization is when a small fluorescently labeled molecule is exited by plane-polarized light; it emits polarized light that is inversely proportional to the molecular rotation. When a small nucleic acid is covalently attached to a fluorescent label such as a fluorophore it can be excited by polarized light at a certain excitation wavelength. The ligand reorients significantly due to molecular tumbling during the excited fluorophores lifetime. In this case the emitted light is largely depolarized. If the nucleic acid is bound to a large protein, the resulting complex tumbles much slower due to the significantly reduced rotational speed. Thus the emitted light maintains its polarization to a significant degree (Picture 3). (Moerke 2010)



Picture 2. In fluorescence polarization in polarized light a large particle leaves the light largely polarized when a small particle depolarizes the light. The smaller particle should have a fluorophore which can be excited and the emitted light can be measured. A protein bound to DNA tumbles less than a sole DNA with a fluorophore which undergoes significant tumbling motion and therefore emits depolarized light. In the picture: DNA (green) with a fluorophore (yellow) attaches to the protein (blue).

Historically, protein-DNA interactions have been measured using the gel mobility shift assay (GMSA) which is time consuming, uses radioactivity and where the gel interferes with the true equilibrium measurement (Owen., McMurray 2009). FP allows true equilibrium analysis because DNA-protein interaction are measured directly in solution without solid support (Yamagata, Masui et al. 2000) FP has some additional advantages which make it well suited for high-throughput screening applications: it is carried out in a nonradioactive solution, it does not need any separation for bound or free ligands, and it is adaptable to low volumes. (Moerke 2010) FP is a user friendly method and furthermore it detects low molarities. The changing in anisotropy can be detected from a binding constant (Owen., McMurray 2009). Many interactions between a repressor protein of the TetR family and a ligand or promoter are analyzed by FP. The DNA

binding affinity of MphR(A) was measured utilizing fluorescence polarization (Zheng, Sagar et al. 2009) In addition, the DNA binding affinity of TetR and its mutants were observed by fluorescence polarization (Kamionka, Bogdanska-Urbaniak et al. 2004). The DNA binding affinities of AcrR to its promoter and ligand binding affinities were determined with fluorescence polarization (Su, Rutherford et al. 2007). The ligand binding affinities of the mutant AcrR regulator were determined by FP (Li, Gu et al. 2007).

2.3.2. Fluorescence Polarization in Calculating Disassociation Constant

The polarization values are exchanged into anisotropy (A) because the binding curve should be plotted from an additive molecular parameter. Fluorescence polarization is not an additive molecular parameter. (Active Motif 2009).

$$\mathrm{mA} = \frac{2\,x\,\frac{mP}{1000}}{\left(3 - \frac{mP}{1000}\right)}$$

The equation above shows the relationship between mA and mP, where A is a unitless value. (Zhang, Chen et al. 2007)

$$\mathrm{A} = (Amax - Amin)\,\frac{Eo}{Kd + Eo} + Amin$$

The dissociation constant (Kd) of the protein/fluorophore complex can be calculated from the equation above when the binding reaction between a fluorophore (F) and protein (E) is 1:1 and [F]<<[E]. Where, Amin is the anisotropy value of the free fluorophore, Amax is the anisotropy of protein/fluorophore complex, Eo is the total protein concentration and Kd is the dissociation constant. (Zhang, Chen et al. 2007)

$$Kd = -Eo - \frac{(Amax - Amin) * Eo}{(Amin - A)}$$

The equation above gives straight the dissociation constant. The dissociation constant (Kd) of a protein-DNA affinity is sensitive to the concentration of salts and in some cases also to oligodeoxynucleotides (Su, Rutherford et al. 2007). The Kd for TetR is 0.2 nM (Kamionka, Bogdanska-Urbaniak et al. 2004, Su, Rutherford et al. 2007) while the Kd for MphR(A) has a low affinity interaction 574 ± 29 nM (Zheng, Sagar et al. 2009). It is presumable that MphR(E) has a dissociation constant of a nanomolar concentration.

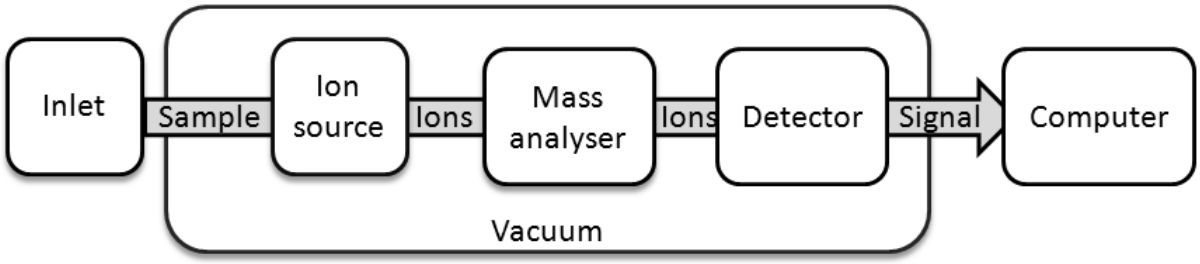
In numerous examples in literature FP has been fitted into an incorrect relationship using a linear superposition principle polarization. Anisotropy or a correct nonlinear superposition principle should have been used instead. (Roehrl, Wang et al. 2004)

2.4. Measuring Proteins with Mass Spectrometry

The mass spectrometer (MS) available in the Eastern University of Finland for protein analyzing is a 4.7T hybrid quadrupole Fourier transform ion cyclotron resonance (Q-FT-ICR) instrumental (APEX-Qe; Bruker Daltronics, Billerica, MA) interfaced with an external electronspray ionization (ESI) source (Apollo-II) (Viiri, Janis et al. 2009). Therefore, this chapter discusses only the mass spectrometry available.

2.4.1. General information of the mass spectrometer Q-FT-ICR

MS is a highly accurate molecular scale and it has become the gold strand for protein identification (Verrills 2006) The mass spectrometer consists of an inlet, ion source, mass analyzer, detector and computer (Picture 4). The ion source such as ESI vaporizes and ionizes the sample, the mass analyzer separates and detects the ions. Mass analyzer such as the quadrupole and Fourier transform (FT) ion cyclotron resonance (ICR) can be used alone or together as hybrids (Picture 4). The ion cyclone resonance mass spectrometer (ICR-MS) has a cubic cell inside a strong magnet. (Lane 2005)

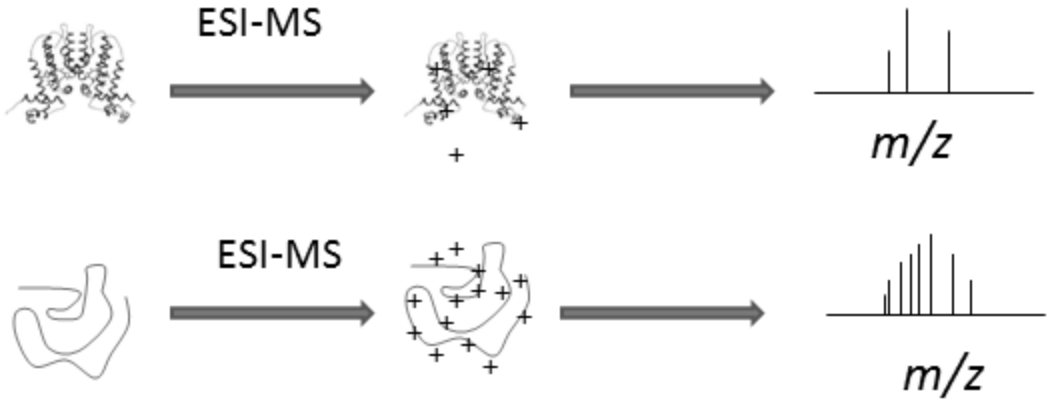


Picture 4. Basic diagram for the mass spectrometer. The ions are measured in a vacuum environment.

2.4.2. Electronspray Ionization

In the late 1990s the new ionization methods such as electronspray ionization (ESI) became the most important method in making high-precision analysis of biomolecules of very high molecular weight and started the revolution of mass spectrometry (MS) (Hoffman, Stroobant 1999). ESI detects down to femtomoles (Verrills 2006) and has the accuracy of ~±0.01 %. Furthermore, ESI can determine the analytes weight with great precision, because the masses can be calculated from several different charged states. In addition, ESI permits the analysis of high molecular weight analytes due to the multiple charging characteristics of ESI. For example, ESI can detect multiply charged ratios of 1000-2000 *m/z* of a typical 50 kDa protein retaining 30-50 differently charged states. (Fitzgerald, Siuzdak 1996)

The key feature of ESI is that it has the ability of ionizing macromolecules maintaining the noncovalent interactions intact. (Yin, Loo 2009) The ionization methods allowed the detection of multiply charged ions which are separated and detected with mass analyzers (Lane 2005). The ions are formed by creating a fine spray of highly charged droplets. The droplets are surrounded by a strong magnetic field. The ions are formed directly from the solution and even noncovalent complexes can be observed. (Fitzgerald, Siuzdak 1996) In non-denaturative solutions the proteins in its natural state for example as a homodimer and in denaturative solutions the protein denaturates into unfolded monomers. When the protein is denaturated (unfolded) the charge distribution is broader and it has a larger number of charges, i.e. larger z value, than the native (folded) one. (Hoffman, Stroobant 1999)



Picture 4. The folded and the unfolded proteins in ESI have different characteristics. Generally unfolded proteins are ionized with more charges than the unfolded proteins. Therefore, the unfolded proteins usually give multiple charged peaks corresponding to different charged states in the x-axel showing *m/z* ratio.

Because ESI mass spectrometry can keep the noncovalent interactions intact, ESI can measure protein-ligand associations. If the masses of the binding partners are known, the stoichiometry can formed complexes can be determined even for multiligand heterocomplexes using ESI. (Yin, Loo 2009) Most commonly ESI technique has been used in combination with quadrupole mass spectrometers. The conventional quadrupole mass analyzer detects up to *m/z* 3000 whereas ESI can detect *m/z* 3000 - 5000. ESI has a high capacity for detecting compactly folded native proteins which have only limited number of charges. (Fitzgerald, Siuzdak 1996) Laboratories studying proteins use hybrid combinations of MS such as a quadrupole (Eckel-Passow, Oberg et al. 2009).

Because ESI generates multiply charged molecules it can measure large proteins which exceed most of mass spectrometric analyzers. (Yin, Loo 2009) FT-ICR mass analyzer confines the molecules in a high magnetic field of a superconducting magnet where molecules orbit in an inversely proportional manner proportional to their *m/z* value (Eckel-Passow, Oberg et al. 2009). ICR-MS determines the *m/z* ration when *z* is the charge on the ion and the mass of the ion is *m* (Lane 2005). The ions with a similar *m/z* orbit together inducing an electrical current which can be detected (Eckel-Passow, Oberg et al. 2009). The number of different charged states measured depends on several factors, such as the composition and the pH of the solvent and chemical nature of the sample. (Fitzgerald, Siuzdak 1996)

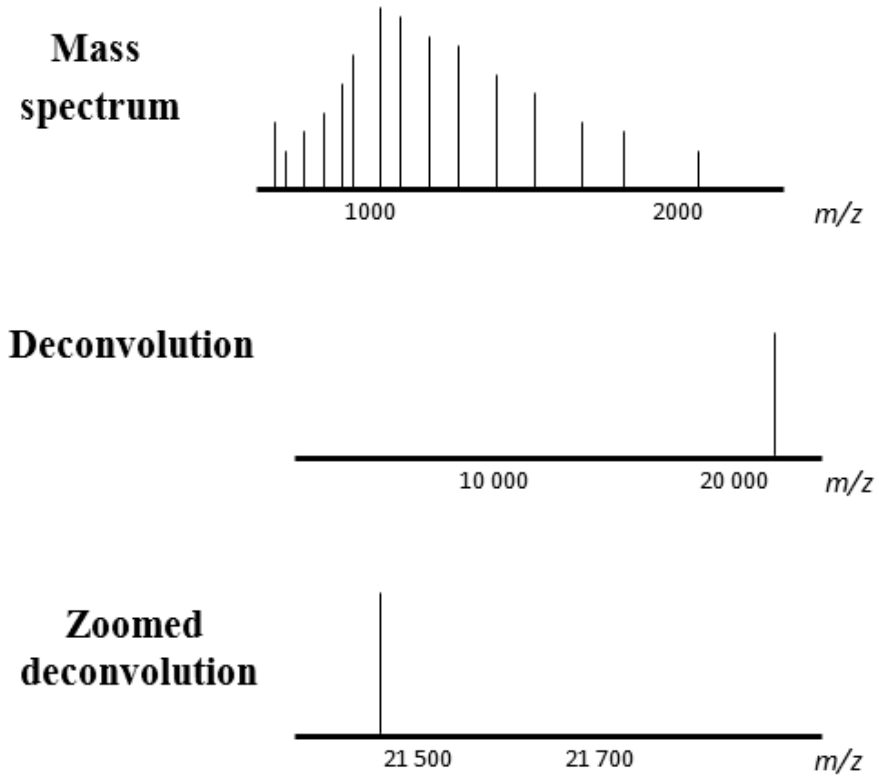
2.4.3. Native ESI mass spectrometry

The gentle nature of ESI gives the possibility for analyzing noncovalent complexes in the "native" ESI spectrometry (Fitzgerald, Siuzdak 1996). The actual detection of noncovalent interactions formed in solid phase takes place under gas phase (Winston, Fitzgerald 1997). The results given of native ESI have mostly shown consistency with the results obtained from other solution based techniques (Fitzgerald, Siuzdak 1996). At least in some cases it is evident that the noncovalent interactions are preserved when a complex is transferred from solution to the gas phase (Winston, Fitzgerald 1997)The samples analyzed are assumed to be in the same state in gas phase as in solution phase preserving their natural conformation, the ESI technique can be used in studying both noncovalent and covalent associations of biomolecules. These interactions can include protein-protein, protein-DNA, protein-ligand, enzyme-substrate, enzyme-inhibitor and DNA duplexes. (Fitzgerald, Siuzdak 1996) Protein-protein interactions can be measured even from oligomeric complexes and in some cases ESI can provide data not obtainable using other techniques. Another application of native ESI is the observations of protein-DNA interactions which can be observed sequence-specifically with one or even from a pool of related DNA sequences. The native ESI can provide precise binding stoichiometry and characterization of the relative affinities between protein and DNA. The stoichiometry of protein-ligand complexes can be established and even the relative strength of different protein-ligand complexes can be evaluated. (Winston, Fitzgerald 1997)

2.4.4. Sample Preparation for ESI

The accuracy and the sensitivity when measured with ESI are affected by the protein concentration and the contaminants of the sample. Although the amount required for measurement is very low, in microliter range, the concentration has to be high. This is problematic because generally the biological samples are low in concentration. (Hoffman, Stroobant 1999) Typically, the final concentration should be 5 μM or more (Yin, Loo 2009). The contaminants come from buffers, non-volatile salts, detergents and many compounds of unknown origin. The ESI electron sprays can tolerate only small amount of contaminants because high quantities reduce measurement quality and sensitivity. They may interfere for example in molecular weight determination. (Hoffman, Stroobant 1999) Generally noncovalent

protein complexes can be measured in physiological pH (pH 6 - 8) using a buffer suitable for ESI such as ammonium acetate of ammonium bicarbonate. When using these buffers the adduct formation is minimal (Yin, Loo 2009). Centrifugal membrane filtration devices can be used in concentrating, desalting and buffer exchange of the protein sample (Yin, Loo 2009). When measuring protein-ligand interactions small, desalted and clean ligands can be added or titrated separately into the protein solution before measuring. Although, often the ligand sample contains high concentration of salts and it is possible to mix the protein sample and ligand prior to centrifugal filtration and desalting the protein-ligand mixture. (Yin, Loo 2009)



Picture 6. The possible mass spectrometry results for MphR(E). The mass spectrum under denaturative conditions shows all of the differently charged conformations of three proteins observed between *m/z*. The proteins can be detected and turned into a deconvolution which can be zoomed and the molecular masses can be calculated for the protein.

2.4.5. Analyzing the Mass Spectrometry Data

Besides being an efficient molecular weight mass spectrometry can detect mutations, structure, purity, non-covalent protein complexes and tridimensional structural information (Hoffman, Stroobant 1999) The measured molecular weight is compared with the weight calculated from the DNA sequence. If the weight measured, and the one calculated, does not mach each other then there must be a sequence error or a modification. Non-covalent interactions of proteins such as protein-DNA interactions can be studied particularly by ESI.

The MS spectrum peaks correspond to only one peptide ion change state *m/z* (Eckel-Passow, Oberg et al. 2009). The unfolded proteins usually give multiple charged peaks corresponding to different charged states in the x-axel showing *m/z* ratio (Burkitt, Derrick et al. 2003). The ESI mass spectrum shows under denaturative conditions multiple proteins with different charges. The differently charged proteins are observed by their *m/z* ratio. The experimental molecular weight can be determined by computer software turning the data into mass spectral deconvolution. The deconvolution showing only one peak per protein can be zoomed. (Picture 6)

‘

3. Aims of the research

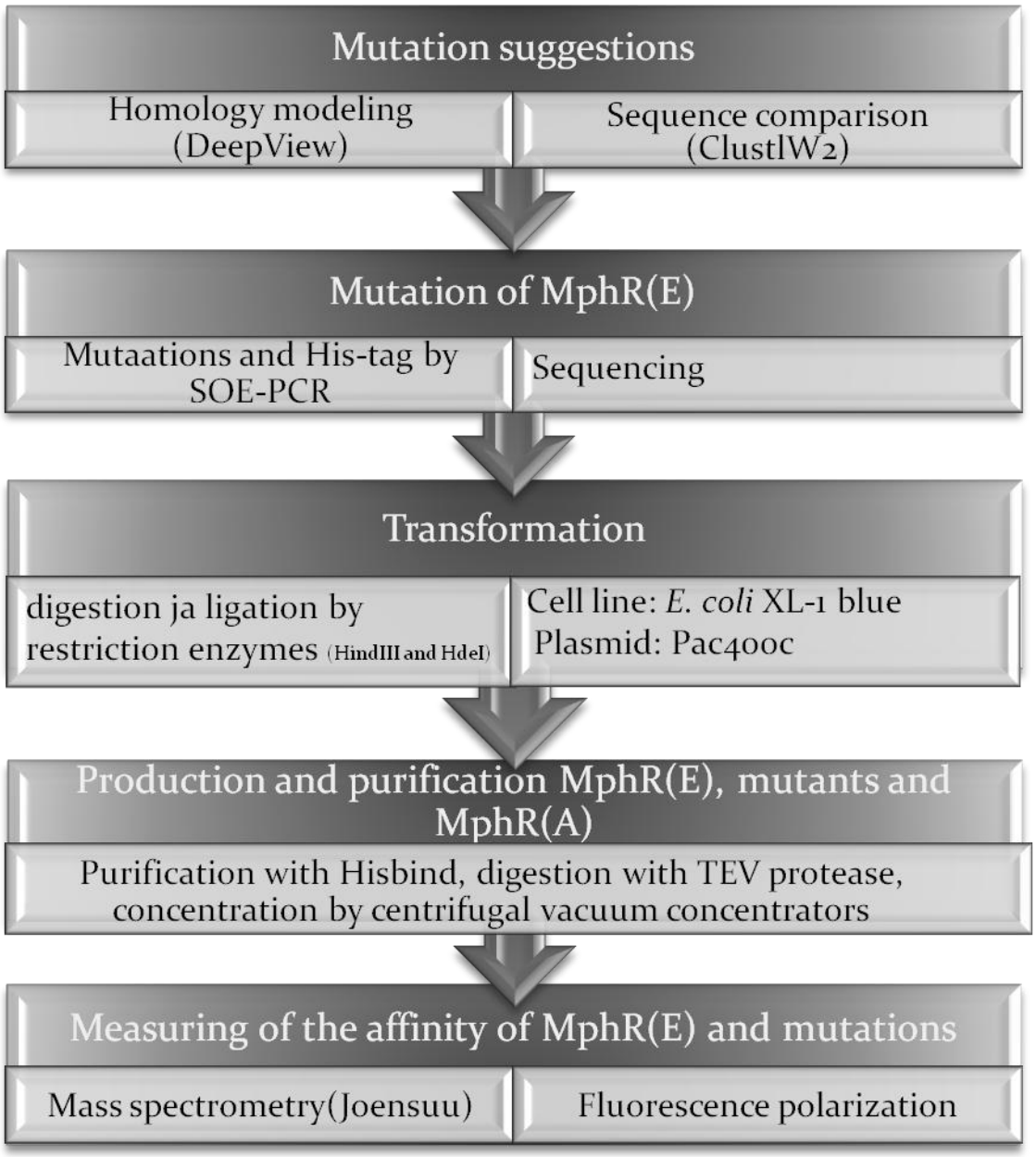
The aims of this study were to gather information concerning the development of a functional macrolide biosensor by modifying the regulator protein MphR(E) by mutating its DNA binding HTH-motif. The mutations were made to increase the binding affinity towards the promoter-DNA. In order to study the mutated MphR(E) proteins should be produced and purified. The functionality of the mutants can be tested in different ways. The most important feature for them to function properly is for the mutants to have the ability for forming homodimers. Most likely only the homodimer is able to bind to the promoter-DNA. The dimerization of the mutants can be measured by great accuracy with native ESI mass spectrometry. The functionality can be also tested with fluorescence polarization. Kd of MphR(A) is measured by fast, robust and sensitive fluorescence polarization thus making it a proficient method for measuring the Kd values of MphR(E) and mutants. Thereby, possibly making the comparison to the Kd of MphR(A) accurate.

The research questions are: Can the MphR(E) and mutants bind DNA? Are they capable of forming a homodimeric complex? What proportion of the protein is in a dimeric form? Do MphR(E) and mutants bind promoter-DNA? How does the DNA binding affinity of the mutants differ from MphR(E) and MphR(A)? How does the DNA binding affinity of MphR(E) differ from MphR(A)? Do the mutants bind the different macrolides with different affinity? Do the mutants differ in any other way from MphR(E) of MphR(A)? Does fluorescence polarization give similar results with mass spectrometry?

The information desired was to give a better understanding of MphR(E)s affinity towards DNA and during this process to give a better understanding of fluorescence polarization assay in determining biomolecular interactions. In addition, the proteins produced could be used in further investigating protein-DNA and protein-ligand interactions by mass spectrometry.

4. Methods

The work was started by making six mutation suggestions to MphR(E) during laboratory course BIKE4350 in University of Tampere, Institute of Medical Technology during Autumn 2009 (Viitamäki 2010). The mutations were designed to make MphR(E) bind with a greater affinity towards its DNA promoter. All of the mutations were designed into the DNA binding HTH-motif of MphR(E). First MphR(E) was produced and the purification with affinity chromatography (Hisbind) was optimized. Second more MphR(E) was produced and it was purified as optimized before. the histidine tag attached to MphR(E) was cleaved with a optimum amount of TEV protease. The digested protein was purified, concentrated with centrifugal vacuum concentrator and sent to mass spectrometry to the University of Eastern Finland, Department of Chemistry in Joensuu, Finland for further analysis. Third the mutations of MphR(E) were made using SOE-PCR and the mutants were ligated into Pac400c vectors with the restriction enzymes HindIII and HDeI. The plasmids were transformed into *E. coli* XL-1 Blue bacteria. After transformation all the mutants, MphR(E) and MphR(A) were produced and purified. Dissociation (Kd) constants were calculated from fluorescence polarization results for undigested MphR(E), MphR(E)_K and MphR(E)_CG. (Picture 7)



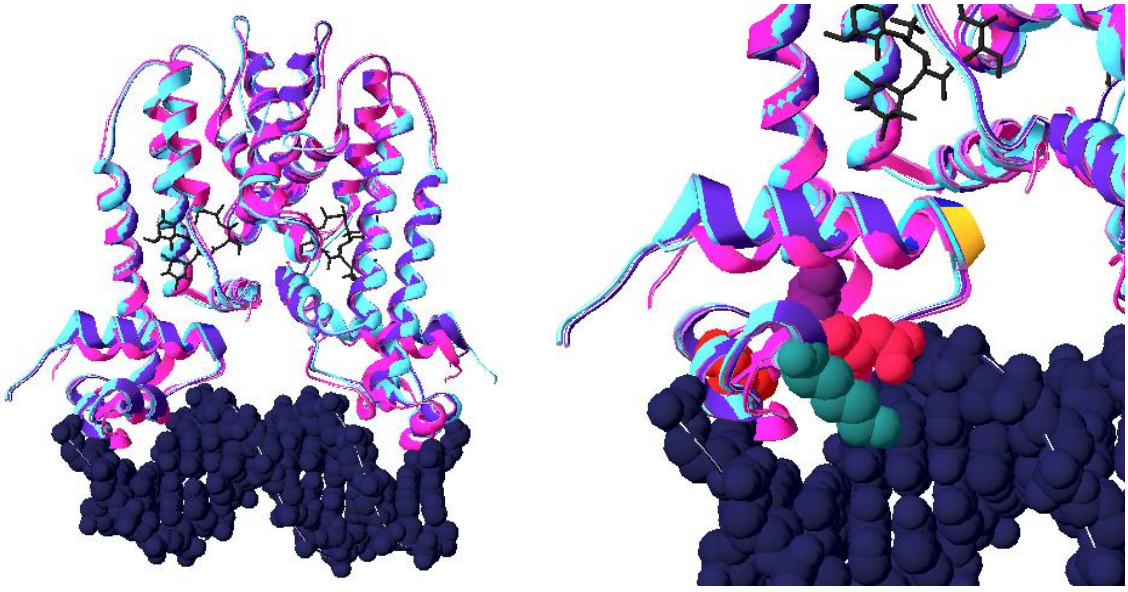
Picture 7. The work order. The work started by making mutation suggestions and continued with the making of the mutations and transformation. The transformed cells were cultured and the proteins were produced and purified. MphR(E) was produced, purified and measured before making of the mutants and MphR(A). Only the mutants MphR(E)_CG and MphR(E)_K were measured by fluorescence polarization. In addition, MphR(E) was analyzed by mass spectrometry.

4.1. Mutation Suggestion During Laboratory Rotation Course

A structural model of MphR(E) (Table 7) was made by homology modeling using Swiss port PDB Viewer software (http://au.expasy.org/sdbv : 02/2011). A DNA binding model structure was made using homologues of IcaR by PDB coordinates 2ZCN and MphR(A) by PDB coordinates 3G56. Six mutation suggestions were made (Table 3). The mutation made were: the negative aspartate 32 was mutated into histidine 32 (D32H or MphR(E)_H), the same negative aspartate 32 was mutated into polar asparagine (D32N or MphR(E)_N). These mutants were designed to reduce repulsion. The threonine 35 was mutated into lysine 35 (T35K or MphR(E)_K) and CG insertion between arginine 22 (R22) and proline 23 (P23) (MphR(E)_CG) made MphR(E) more like MphR(A). MphR(E) was made more like TetR-family when isoleusine 45 was mutated into tyrosine 45 (I45Y or MphR(E)_Y) and more like other DNA binding HTH-motifs with the mutation V33L (MphR(E)_L). Furthermore, all the possibilities were assessed by looking first at the DNA binding monomeric structural model of MphR(E). (Picture 8) (Viitamäki 2010)

Table 3. Mutation suggestions and evaluation using the MphR(E) dimer model. (Viitamäki 2010)

	Why	**Before**	**After**	**Reasoning**
Y	To bind more like the TetR-family to DNA	Ile45 (0.47%)	Tyr45 (74%)	Tyr is aromatic and possible makes a better contact with DNA and it is conserved in 74% of the TetR-family proteins. Nonpolar Ile is show only in 0.47% of TetR-proteins
K	To improve DNA binding specificity	Thr35	Lys35	Lys is important in binding MphR(A) to phosphates in DNA it may improve also MphR(E)´s specificity
L	To become more like the other HTH-motifs	Val33	Leu33	Among other HTH-motifs the Leu33 was highly conserved and may have an important function
H/N	To reduce repulsion and to facilitate DNA binding	Asp32	His /Asn	Negative Asp is close to the negative DNA and may cause repulsion. Positive His has a stiff ring structure, Polar Asn has similar size to negative Asp.
CG	To make a better loop structure	Insert between Arg22 Pro23	Cys23 and Gly24	MphR(E) lacks the GP-loop structure this may improve its folding and binding to DNA.



Picture 8. Left picture: The DNA binding MphR(A) form 3G56 (pink) and the ligand binding form 3FRQ MphR(A) (violet). The ligand binding dimer of MphR(E) (light blue). DNA (dark blue) and erythromycin (dark blue) are also present. In the proteins only the amino acid residues are shown demonstrating the helix and loop structures. **Right picture:** Mutated MphR(E). The substitutes Tyr45 (red), Lys35 (turquoise), Leu33 (violet), Asn32 (red) in red and the insertion spot for Cys23 and Gly24 (yellow). In the colored mutations all of the amino acid is shown and the colored part is the side chain of the protein. Only in the insertion CG only the residues are shown (Viitamäki, 2009)

4.2. Manufacturing a Cell Line of *E. Coli* Producing MphR(E) Mutants

The mutation suggestions (Viitamäki 2010) were done using splicing by overlap extension PCR (SOE-PCR). The PCR products were sent to Macrogen for sequencing (://dna.macrogen.com/eng/ : 02/2011). When the right mutation was conformed the PCR-products were inserted into the production vector Pac400c and transformed into *E. Coli* XL-1 Blue cells. The colonies containing the right sized PCR product and plasmid insert were again sequenced to confirm the right mutations.

4.2.1. Mutating by SOE-PCR

In SOE-PCR a PMPH1 plasmid containing MphR(E) gene with a histidine tag was first used as a template, Later on instead of the plasmid the MphR(E) PCR product made with the HIS primers was used. Mutagenic primers (Table 4) were designed to have the mutations designed earlier (Viitamäki 2010). The mutagenic primers had five triplets before and after the mutagenic triplet or the insert of two triplets. Which gave the mutagenic primers a 33-36 bp overlap in the second SOE-PCR.

Table 4. Primers for mutating MphR(E) by SOE-PCR. Mutations underlined. Histidine tag is in *italic* and the TEV-protease cuts between the amino acid in **bold**.

Primer name	Sequence (5´to 3´)
MphR HIS frw	5´TAAACATATG*CATCATCATCATCATCATCAT*TTGGAAAACCTATACTTTC A**AG**GCCCGAGACCCAAAACCGT-3´
MphR HIS rev	5´-ATTTAAGCTTCGGCGGCTACTTTAATCGTTTGGCGCAATGCC- 3´
MphR CG frw	5´-AAGATCATGTTGCGCTGCGGCCCTCAACGTGAGGCG-3´
MphR CG rev	5´-CGCCTCACGTTGAGGGCCGCAGCGCAACATGATCTT-3´
MphR Y frw	5´-AGCCGAGCCGCCTTGTATCAACGCTTCCAGAAT-3´
MphR Y rev	5´- ATTCTGGAAGCGTTGATACAAGGCGGCTCGGCT-3´
MphR K frw	5´-CTGTCTGACGTTGCCAAAGAAGTAGGCCTCAGC-3´
MphR K rev	5´-GCTGAGGCCTACTTCTTTGGCAACGTCAGACAG-3´
MphR L frw	5´- TTCACCCTGTCTGACCTGGCCACGGAAGTAGGC-3´
MphR L rev	5´-GCCTACTTCCGTGGCCAGGTCAGACAGGGTGAA-3´
MphR H frw	5´- GCGTTCACCCTGTCTCATGTTGCCACGGAAGTA-3´
MphR H rev	5´- TACTTCCGTGGCAACATGAGACAGGGTGAACGC-3´
MphR N frw	5´- GCGTTCACCCTGTCTAACGTTGCCACGGAAGTA-3´
MphR N rev	5´- TACTTCCGTGGCAACGTTAGACAGGGTGAACGC-3´

The forward flanking primer HIS FRW contained the TEV protease cleavage site, histidine tag and the mutation of the first codon and the downstream primer HIS REV had a mutation to the stop codon. The start and stop codons were changed to ones that are common in *E. Coli*

The PCR reactions in 25 µl were carried out by using 50 ng of DNA template, 0.5 µM REV primer and 0.5 µM FRW primer, 0.02 U / µl of enzyme, 0.2 mM of DNTP´s and 1 x buffer. Different conditions were tried for the first SOE-PCR reaction for the CG and Y primers (Table 5). All PCR reactions were made in the conditions recommended with the supplier (Fermentas). Two enzymes were tried Phusion hotstart and Phusion hotstart II, two different primers were used HF-buffer and GC-buffer and 5 % DMSO (Fermentas).

Table . PCR reagents and other details. TD has a Ta of 55 ˚C 10 s -0,4 ˚C / cycle.

	Enzyme	Buffer	DMSO	Ta	Cycles
1. TD	Phusion	HF	-	TD	25
2. Double TD	Phusion	HF	-	72 ˚C 10 s -1,1 ˚C / cycle and TD	15 and 15
3. 46 ˚C	Phusion II	HF and GC	0 % and 5 %	46 ˚C 20 s	35
4. Gradient	Phusion II	GC	5 %	70 ˚C-50 ˚C	35

For the mutants MphR(E)_CG and MphR(E)_Y different PCR programs were tried for the first SOE-PCR. Touchdown (TD) was the same for the HIS primers, Double TD started from a higher temperature, 46 ˚C had a long period with a low annealing temperature because DMSO decreases the annealing temperature, gradient PCR has a variety of different annealing temperatures at the same time. For the mutations MphR(E)_K, H, L and N the first SOE-PCR was made with the TD program with Phusion II and 5 % DMSO. After the first SOE-PCR was complete the second one was made TD program using equimolar amounts 0.5 pmol / 25 µl reaction of the both first SOE-PCR reactions with Phusion II polymerase and 5 % DMSO.

Table 5. PCR programs used to find the optimum conditions for the mutagenic primers in the 1. SOE-PCR for MphR(E)_CG and Y

PCR program	TD	Double TD	46 ˚C	Gradient
1. Heat	98 ˚C 30 s	98 ˚C 30 s	98 ˚C30 s	98 ˚C 30 s
2. Denaturation	98 ˚C 10 s	98 ˚C 10 s	98 ˚C 10 s	98 ˚C 10 s
3. Anneal	55 ˚C 10 s -0.4 ˚C / cycle	72 ˚C 10 s -1.1 ˚C / cycle	46 ˚C 20 s	50 ˚C – 70 ˚C 20 s
4. Extension	72 ˚C 20 s	72 ˚C 20 s	72 ˚C 15 s	72 ˚C 20 s
5. Cycles (GOTO)	2 x 25	2 x 15	2 x 35	2 x 35
6. Final heating	72 ˚C 60 s	Continues with TD program step 2. Step 3. changes to -0.6 ˚C / cycle, step 5: 6 x 15	72 ˚C 5 min	72 ˚C 5 min

4.2.2. Making the Plasmids and Transforming them into *E. Coli* XL-1 Blue

The second SOE-PCR products were purified with Genejet PCR purification Kit (Fermentas). The MphR(E)_CG mutation was quite impure and it had to be first run in agarose gel, cut and purified with Genejet gel extraction kit (Fermentas). First all of the mutations and the plasmid SP110 containing the Pac400c vector and a other insert were digested using HindIII and NdeI restriction enzymes and buffer R (Fermentas). The conditions were ~ 500 ng of DNA, 0.5 µl of HindIII, 1 µl of NdeI, buffer R 1µl MilliQ water added to the total volume of 10 µl. The total volume was doubled for the 3500 ng of SP110 plasmid. The digestion mixtures were incubated in + 37 ˚C for an hour. Afterwards the digestions were run into agarose gel. The right sized vector Pac400c ~3,7 kb the insert of 1 kb should have also been removed. The mutation should be around 650 bp. The plasmid and the mutants were purified from the gel. The volumes of the purified mutants were minimized into around 10 µl by evaporating the water by incubating them in 60 ˚C. The vector Pac400c was divided into 7 parts. One part for each mutant and one ligation control. The ligation mixture contained 5 µl of the vector, ~ 10 µl of insert, 1 µl of T4 ligase 2 µl T4 ligase buffer (Fermentas) and MilliQ water the final volume of 20 µl. The ligation control had half of volume of the inserts. The ligation mixtures were incubated overnight in room temperature The T4 ligase was deactivated by incubating for 10 min in 65 ˚C.

The ligation mixtures were inserted into electrocompetent *E. Coli* XL-1 Blue cells and the transformation was completed by electroporation. The cells were incubated for an hour in 37 ˚C in SOC medium and 100 µl and the rest of the electroporated cells were plated onto LA-plates containing 25 µg / ml cloramfenicol (Cm) and incubator overnight in 37 ˚C. Around 10 of the colonies on the LA plates plated with 100 µl of electroporated cells were run into COLONY PCR and plated. From these plates 9 - 10 colonies were chosen to COLONY PCR. The COLONY PCR shows which colonies contain sites for the HIS primer to anneal and make a PCR product. A part of the colony was transferred into 25 µl COLONY PCR mixture and run through the COLONY PCR program (Table 7). The other part was plated onto LA plates with Cm.

Table 7. Colony PCR. A small sample of the each colony was added to the PCR mix and run through the COLONY PCR program

COLONY PCR mix	COLONY PCR program
17.75 µl dd H2O 2.5 µl 10 x Buffer Dynazyme II 1.25 µl 5´HIS primer (10 µM) 1.25 µl 3´HIS primer (10 µM) 0.25 µl Dynazyme II (2U / µl) 2 µl dNTP´s (2.5 mM) V_{TOT} = 25 µl	1. 94 ˚C 4 min 2. 94 ˚C 1 min 3. 55 ˚C 1 min, - 0.4 ˚C / cycle 4. 72 ˚C 1 min 5. GOTO 2 x 25 6. 72 ˚C 10 min 7. 4 ˚C forever

The colonies which produced a PCR product could contain the right mutation insert. Some of these colonies were cultured and the plasmids were isolated by Qiaprep miniprepkit (Qiagen). These plasmids were analyzed by restriction analysis using the enzymes HindIII and NdeI with the same reaction mixtures as before. The plasmids containing a right size of insert were sent to Giagen for sequencing.

4.2.3. Producing, Purifying and Digesting MphR(E), Mutants and MphR(A)

4.2.3.1. Protein Production

The MphR(E) mutants containing the right plasmids, native MphR(E) and MphR(A) were produced using the same method. The *E. Coli* strain XL-1 Blue carrying Pac400c / his MphR(E) and MphR(A) plasmids were given by Nina Virolainen (Tampere University of Technology, Tampere, Finland). The cells were cultured in 1000 ml of SB-medium containing 2,5 mM Cm and 0.2 % glucose with the pH 8,0 in 37 ˚C and 250 rpm until the OD of 0,5. Afterwards the cells were induced to produce Mphr(E) by 100 µM of IPTG overnight in room temperature (19 ˚C to 21 ˚C) or for 5 h in 30 ˚C.

4.2.3.2. Protein Harvesting

The IPTG induced *E. Coli* cells the plasmid producing MphR(E), mutants and MphR(A) were harvested centrifuged for 15 min in 5000 x g and 4 ˚C. The harvest was lysed by 50 ml of lysis buffer (Table 8) and further centrifuged for 15 min in 5000 x g and 4 ˚C. Suspension was discarded and pellets were then suspensed into two falcon tubes with 25 ml of binding buffer each (2 x 25 ml) and then centrifuged again 15 min 5000 x g 4˚C. Suspension was discarded and wet weight was measured from the pellets. The pellets were combined into one falcon tube by 20 ml of binding buffer and frozen in -80 ˚C. The cells were melted in room temperature. 1 mg/ml of lysozyme was added and the cells were incubated on ice for 30 min afterwards they were frozen again in – 80 ˚C. The cells were melted in room temperature and 3 µl of bensonaze was added and incubated for 15 - 25 min on ice. The broken cells were centrifuged 40 – 60 min 11 500 x g 4 ˚C. The pellets were stored and the supernatant was filtered thought 0.8 and 0.45 µm filters (sample E).

4.2.3.3. Protein Purification

The MphR(E) was purified with HisBind affinity chromatography (Novagen). The colon had the volume of 1 - 2 ml and it was prepared by adding 3 column volumes of sterile MQ water, 3 volumes of 1 x charge buffer and 1 x of binding buffer (Table 8). The sample was added and after the flow trough (sample E/F) 10 volumes of wash buffer was added (sample F) and the last 1 ml of it was stored separately (sample F small). 10 volumes of elution buffer was added and the eluted protein was collected with 1 ml fractions. At last the column was purified by 10 volumes of strip buffer (sample G) and the last 1 ml was stored separately (sample G small). The column was stored with 70 % ethanol in 4 ˚C and used maximum of 3 times. The elution fractions were kept in + 4 ˚C and all the other fractions were saved in – 20 ˚C.

The purification protocol was optimized by chancing the imidazole concentration of the wash buffer. Imidazole competes with the bound histidine tagged protein and possible bound impurities. The proteins harvested from the first productions were divided into three washed with different imidazole concentrations. The concentrations were 40 mM, 50 mM and 62.5 mM of imidazole (Table 8). The next productions were purified by wash buffer containing 62.5 mM imidazole.

Table 8. Buffers needed for Protein purification in affinity chromatography.

Buffer	Imidazole (mM)	Tris-Hcl (mM)	NaCl (mM)	EDTA (mM)	NiSO4 (mM)	pH
Lysis (binding)	10	50	300	-	-	8.0
Wash 40	40	50	300	-	-	8.0
Wash 50	50	50	300	-	-	8.0
Wash 62.5	62.5	50	300	-	-	8.0
Elution	250	50	300	-	-	8.0
Strip	-	50	300	1	-	8.0
Charge	-	-	-	-	50	-

4.2.3.3. Digestion the Fusion Proteins from the Histidine Tags

The digestion was first made with TEV protease (Invitrogen) and second with a TEV protease produces by Nina Virolainen (Tampere University of Technology, Tampere, Finland). Before the digestion could be made the buffer of the elution fractions were exchanged with TEV Buffer [50 mM Tris-Hcl, 5 mM EDTA and freshly added 1 mM DDT at pH 8.00] with NAP 5 column (Amerham Biosciences). The histidine tag was digested AcTEV Protease (Invitrogen) from MphR(E) of the second production. Contrary to the recommendations protein concentration was higher 2.7 mg / ml because a high concentration of protein is needed for mass spectrometry. After digestion a SDS-PAGE gel was made to see if the digestion was successful. The TEV protease and the histidine tags were purified by HisBind column as mentioned above. With the exception that the MphR(E) will be in the flow through and wash fractions. Bind buffer was used

instead of wash buffer (Table 8) because this would minimize the amount of unwanted proteins that bind lightly to the HisBind matrix.

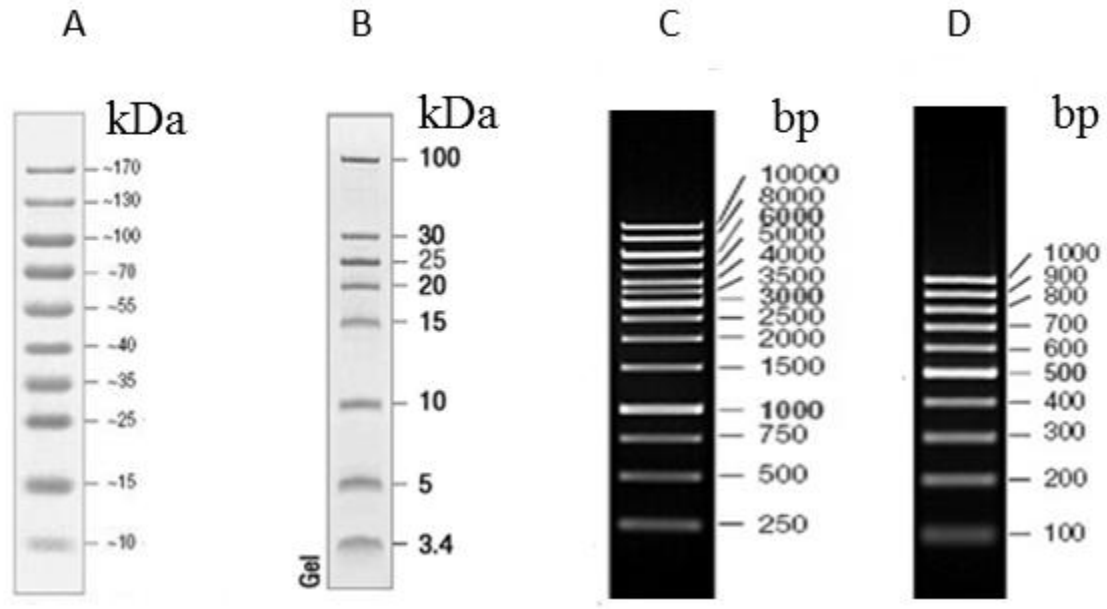
According to Invitrogens recommendations 1 Unit of TEV protease should digest 3 μg of protein in 4 h in + 4 ˚C. Even a overnight digestion in + 4 ˚C did not work so different protease concentrations were evaluated with 0.2 mg / ml of MphR(E) which is closer to Invitrogens recommendations. The concentrations 0, 0.5, 1.5, 2.5 and 5 U / μg were evaluated and the lowest concentration where all MphR(E) was digested was 1.5 U / μg. Because the TEV protease is expensive further digestions were made in room temperature overnight or up to 20 h with 1 U of TEV protease for 3 μg of MphR(E) and 1.15 mg / ml of MphR(E).

First the method was evaluated by a small amount of undigested MphR(E) ~ 0.2 mg using 1.5 U / μg TEV protease. The sample was purified once and because of the color reaction the flow trough and the wash fractions, the concentration was not measured. The purification was the same as above. The bigger amount ~ 3.5 mg of MphR(E) was digested and purified as the first smaller amount.

The next digestions were made with a TEV protease produced by Nina Virolainen (Technical University of Tampere, Tampere, Finland) from an *E. coli* expression vector pMHTDelta238 producing TEV protease (DNASU http://dnasu.asu.edu/DNASU/GetVectorDetail.do?vectorid=411 : 02/2011). The absorbance A_{280} was used to calculate the ratio of how much TEV protease there were in relation to MphR protein. Firs the digestion was optimized using TEV / MphR(E)_CG 0, 1/200, 1/100, 1/50, 1/10 and 1/5. Afterwards MphR(E) and MphR(E)_CG were digested MphR(E) with the ratio of 1/100 and MphR(E)_CG 1/50. A280 for TEV was 0.776, MphR(E) 0.44 and MphR(E)_CG 0.857. Meaning that 1μl of TEV was added to 176.4 μl of MphR(E) and 1 μl of TEV was added to 9.4 μl of MphR(E)_CG. The digestion was incubated overnight in room temperature, purified and concentrated.

4.2.3.4. SDS-PAGE and Agarose Gels

Most of the results were run into a gel to see the size of the protein or DNA. Proteins were run in SDS-PAGE gel and DNA in a agarose gel. The SDS-PAGE´s resolving gel was 13 % and the stacking gel 11 %. The samples had often 8 µl of sample buffer, 8 µl of MilliQ H_2O and 8 µl of sample. Two standards were used: PageRulerTM prestained Protein Ladder Plus and PageRulerTM unstained broad range protein ladder (Fermentas) (Picture 9). For DNA a 1 % agarose gel was used to analyze small DNA´s and 2 % agarose gel was used for longer DNA such as plasmids. Two standards were used: GeneRuler 1 kb DNA Ladder ready-to-use and Generuler 100 bp DNA ladder ready-to-use (Fermentas).



Picture 9. Protein ladders: **A)** PageRulerTM prestainded Protein Ladder Plus and **B)** PageRulerTM unstained broad range protein ladder (Fermentas). DNA ladders: **C)** GeneRuler 1kb DNA Ladder ready-to-use and **D)** Generuler 100bp DNA ladder ready-to-use (Fermentas). (www.fermentas.com 02/2011)

4.3. Concentration of the Digested MphR(E) and Mass Spectrometry

The digested MphR(E) needed concentration and buffer exchange before mass spectrometry. First the MphR(E) was concentrated and the buffer was exchanged to 10 mM ammonium acetate with Amicon Ultra-4 Centrifugal Filter 10K (Millipore) This method was not efficient because it did not concentrate MphR(E) instead it went through the filter with the flow trough. The second

concentration was made with sabant SPD111V vacuum evaporator (Thermo electronics). First the 1.5 ml of newly digested MphR(E) and the flow through from the first attempts to concentrate MphR(E) by Amicon Ultra were concentrated for 3.5 hours. The samples were pooled newly to the volume of 1.5 ml and the concentration was continued by additional 4 hours, during which all of the samples precipitated. The buffers were exchanged into 10 mM ammonium acetate with a NAP-25 column (Ge Helthcare ilustra). The samples were further concentrated by savant centrifugal vacuum concentrator in 2 ml volumes first for 3 hours and then they were pooled into 2 ml volume and concentrated again for 2.5 hours the final volume was 0.5 ml. After changing the buffer of the most concentrated sample into 10 mM ammonium acetate by NAP-5 column (Amersham Biosciences) the final concentration was 1.915 mg / ml which was ready to be sent for mass spectrometry to be measured by Professor Janne Jänis (University of Eastern Finland). The mass spectrometer was a 4.7 T hybrid quadrupole Fourier transform ion cyclotron resonance (Q-FT-ICR) instrumental (APEX-Qe; Bruker Daltronics, Billerica, MA) interfaced with an external electronspray ionization (ESI) source (Apollo-II).

4.4. Fluorescence Polarization Measurements

The fluorescence emission of MphR(E)-promoter complex was measured with a spectrofluorometer chameleon (Hidex). All measurements were taken with an excitation wavelength of 494 nm and fluorescence signal was measured at 518 nm in a black 96 welled plate at 21 ˚C. The double stranded promoter containing a conjugated fluorescein was prepared by annealing complementary oligonucleotides in 10 mM Tris - HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA by heating for 10 min in 80 ˚C. A 200 µl solution of 10 mM DNA in binding buffer containing 10 mM Mops, pH 7.0, 100 mM NaCl and 1 µg / ml of poly(dI - dC) was used for measurements. MphR(E) from the first and second production and the mutants CG and K were diluted to the binding buffer from their first elution fractions. Six wells were filled with 200 µl of each protein concentration and incubated in 21 ˚C for 30 min. The protein concentrations were 20, 40, 60, 100, 200, 400, 600, 1000, 2000, 4000 and 6000 nM.

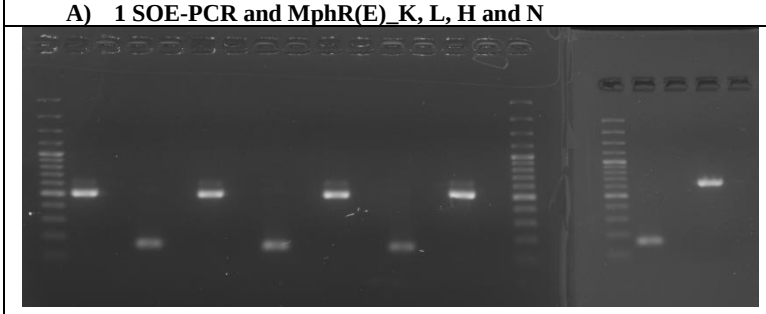
5. Results

5.1. Manufacturing a Cell Line of *E. Coli* Producing MphR(E) Mutants

In the first SOE-PCR touchdown (TD), plasmid, double TD PCR programs were unsuccessful for MphR(E)_CG and Y mutations. The gradient program showed that the annealing temperature for 50 to 55 ˚C was optimum. Later for the rest of the mutations TD PCR program did produce a right sized product. The second SOE-PCR was made with the TD program and was successful for all of the mutants. The positive control worked and the negative controls did not. (Picture 10)

The cells producing mutant proteins were made by inserting the mutants into Pac400c plasmids and transforming them to *E. Coli* XL-1 Blue cells. After which COLONY PCR, restriction analysis and sequencing were used to analyze which cell could produce the desired mutation. In COLONY PCR many cell lines had a PCR product of the desired size which could be produced with the HIS templates. These mutant cell lines were L1, 2, 5, 6, 7, 8, 10 Y1, 2, 3, 7, 9, 10, CG2, 3, 4, 5, 7, 8, 9, H3, 4, 6, 9; K2, 4, 5, 6, 7, 9; N1, 2, 3, 4, 5, 6, 8 and 9. The restriction analysis showed that L2, Y3, Y1, H4, K6, K2, N4, N1, CG 4 and CG7 had an insert of the appropriate size around 650 bp. The mutant L6 and H6 did not have an insert. All the mutant cell lines analyzed with the restriction analysis were sequenced and K2, K6, CG7, CG4, L2, H4 and N1 had the right mutation. The cells N4, Y1 and Y3 did not have the right mutation or had some extra mutations.

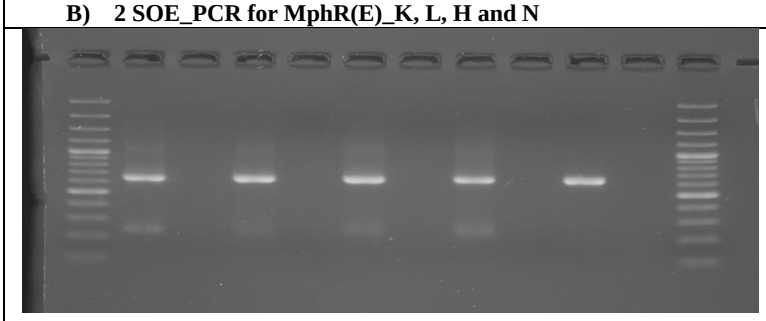
A) 1 SOE-PCR and MphR(E)_K, L, H and N



Pipetting order

1. STD 100bp	11. H/HIS-
2. K/HIS+	12. HIS/H+
3. K/HIS-	13. HIS/H-
4. HIS/K+	14. N/HIS+
5. HIS/K	15. N/HIS-
6. L/HIS+	16. STD 100BP
7. L/HIS-	17. STD 100BP
8. HIS/L+	18. HIS/N+
9. HIS/L-	19. HIS/N-
10. H/HIS+	20. HIS/HIS+
	21. HIS/HIS -

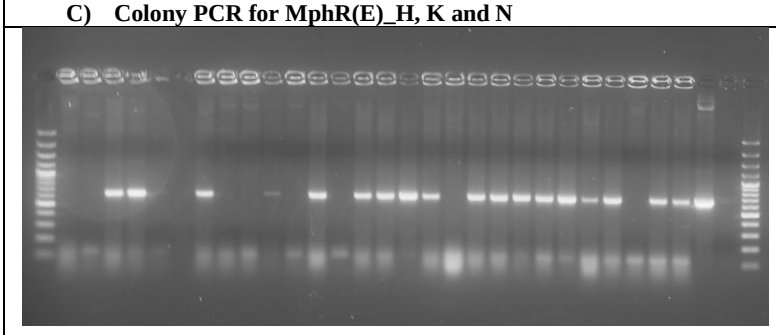
B) 2 SOE_PCR for MphR(E)_K, L, H and N



Pipetting order

1. STD 100BP
2. K+
3. K-
4. L+
5. L-
6. H+
7. H-
8. N+
9. N-
10. HIS+
11. HIS-
12. STD 100BP

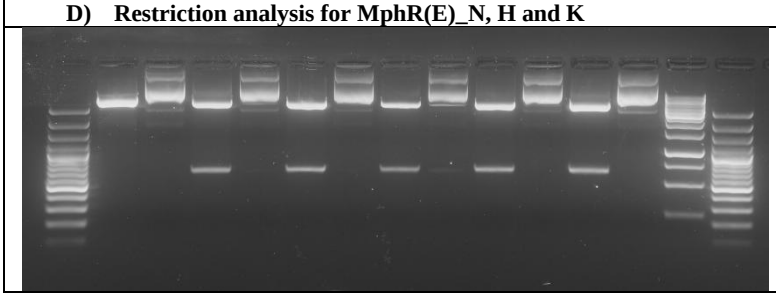
C) Colony PCR for MphR(E)_H, K and N



Pipetting order

1. STD 100bp	12. K1	24. N4
2. H1	13. K2	25. N5
3. H2	14. K3	26. N6
4. H3	15. K4	27. N7
5. H4	16. K5	28. N8
6. -	17. K6	29. N9
7. H5	18. K7	30. HIS+
8. H6	19. K8	31. HIS-
9. H7	20. K9	32.STD 100 bp
10. H8	21. N1	
11. H9	22.N2	
	23. N3	

D) Restriction analysis for MphR(E)_N, H and K



Pipetting order

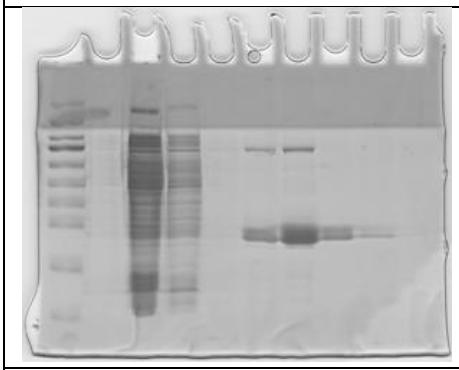
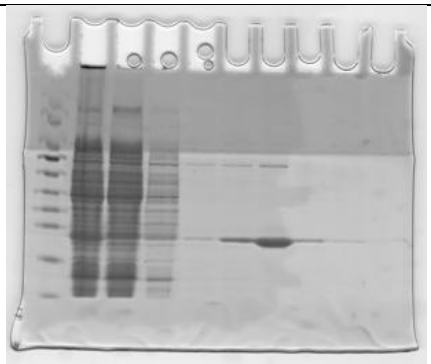
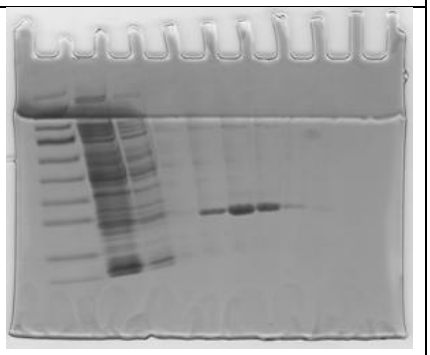
1. STD 100bp	8. K2+
2. H6+	9. K2-
3. H6-	10. N4+
4. H4+	11. N4-
5. H4-	12. N1+
6. K6+	13. N1-
7. K6-	14. STD 1Kb
	15. STD 100bp

Picture 9. The mutation process shown with the mutations MphR(E)_N, H and K. **A)** First SOE-PCR where the point mutation was made and the DNA sequence encoding protein was divided. The both parts were of the right size: The bigger ones M / HIS were ~ 500 bp and the smaller parts HIS / M were < 200 bp. The negative (-) controls did not give any product. **B)** The second SOE-PCR was made by using the first SOE-PCR products as templates in equimolar concentration. All of the positive (+) samples were of the right size ~ 650 bp and the negative (-) controls did not give any product. **C)** COLONY PCR showed which cells have the possibility of having a plasmid containing MphR(E) mutation. They were: H3, 4, 6 and 9; K2, 4, 5, 6, 7 and 9; N1, 2, 3, 4, 5, 6, 8 and 9. **D)** The restriction showed which cells have an insert in the plasmid that is the same size as it should be ~ 650 bp. Only the H6 cell colony did not have a desired insert.

5.2. Protein Production, Harvesting, Purification, Digestion and Concentration

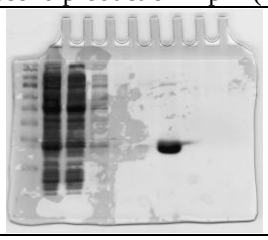
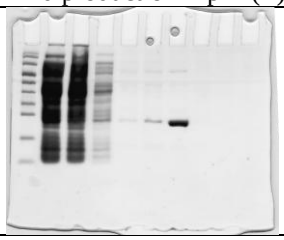
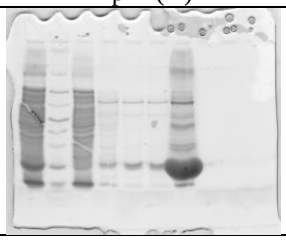
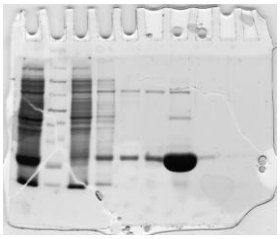
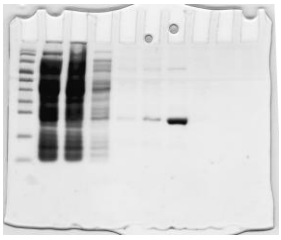
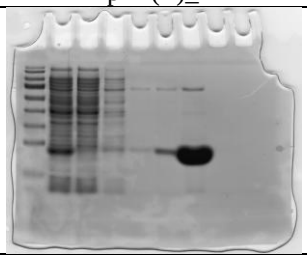
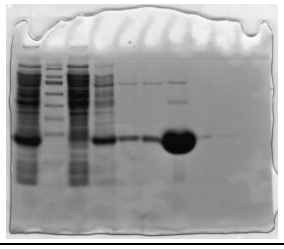
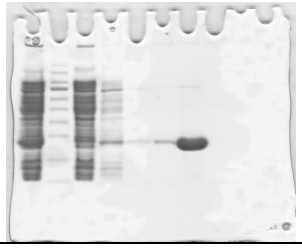
5.2.1. Protein Production, Harvesting and Purification

After being cultured the cells were lysed and the proteins were separated. The purification process was optimized by using different wash buffers (Picture 11). From the SDS-page gels it is shown that 62.5 mM imidazole concentration in wash buffer purifies MphR(E) better than lower concentrations. The next proteins are purified with the 62.5 mM imidazole wash buffer.

Wash 40 mM imidazole	Wash 50 mM imidazole	Wash 62.5 mM imidazole
		
Pipetting order 1. Standard 2. Filtered supernatant (E) 3. Flow trough(E/F) 4. Wash (F) 5. Wash last 1ml (F last) 6. Elution 1st fragment 7. Elution 2nd fragment 8. Elution 9th last fragment 9. Strip (G) 10. Strip last (G last)	Pipetting order 1. Standard 2. Filtered supernatant (E) 3. Flow trough(E/F) 4. Wash (F) 5. Wash last 1ml (F last) 6. Elution 1st fragment 7. Elution 2nd fragment 8. Elution 8th last fragment 9. Strip (G) 10. Strip last (G last)	Pipetting order 1. Standard 2. Flow trough(E/F) 3. Wash (F) 4. Wash last 1ml (F last) 5. Elution 1st fragment 6. Elution 2nd fragment 7. Elution 3rd fragment 8. Elution 10th last fragment 9. Strip (G) 10. Strip last (G last)

Picture 11. The protein purification was optimized after the first production of MphR(E). MphR(E) was purified from the other cell proteins by a HisBind chromatograph where the positively charged histidine tag stuck to the matrix while other proteins washed trough. MphR(E) was removed from the HisBind harts by imidazole. It is shown in the pictures that the 40 mM imidazole concentration gives much more impure elute fractions than the 62.5 mM concentration. It is also shown that the capacity of the HisBind matrix diminishes after one use.

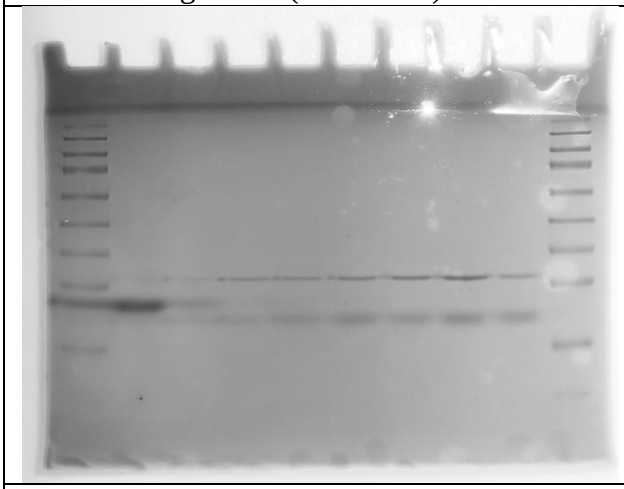
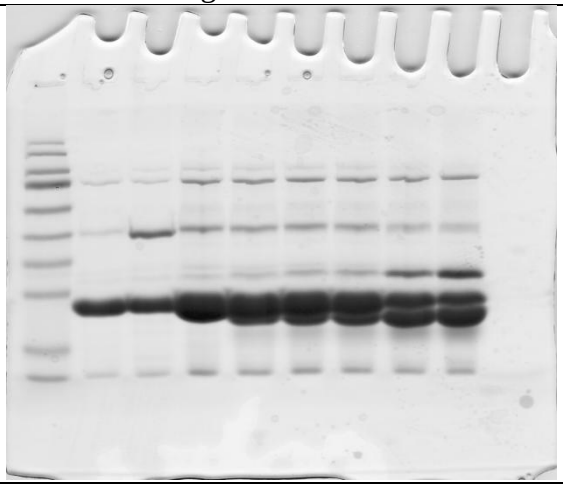
After the purification was optimized MphR(E) was produced and purified. In the third production MphR(A), MphR(E) and all the mutants excluding the MphR(E)_Y mutant were produced and purified (Picture 12) in order to further digest and analyze by mass spectrometry and fluorescence polarization. The productions were successful and all the proteins were produced and purified. MphR(A) had a bigger amount of impurities in comparison to MphR(E) and its mutants because of the purification was optimized for MphR(E). Because a large amount of MphR(A) was purified the impurities were only a small proportion in the sample. The mutants MphR(E)_K, H and N had a bigger production according to the SDS-PAGE gels than MphR(E) and the mutants CG and L.

<table>
<tr><td>Second production MphR(E)</td><td>Third production MphR(E)</td><td>MphR(A)</td></tr>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td>Pipetting order</td><td>MphR(E)_K</td><td>MphR(E)_L</td></tr>
<tr><td>1.Standard or filtered supernatant
2.Standard or filtered supernatant(E)
3. Flow trough(E/F)
4. Wash (F)
5. Wash last 1ml (F last)
6. Elution 1st fragment
7. Elution 2nd or 3rd fragment
8. Elution 9th last fragment
9. Strip (G)
10.Strip last (G last)</td><td>  </td><td>  </td></tr>
<tr><td>MphR(E)_H</td><td>MphR(E)_N</td><td>MphR(E)_CG</td></tr>
<tr><td>  </td><td>  </td><td>  </td></tr>
</table>

Picture 12. The picture shows all the purifications of the first, second and third of MphR(E) and the production of MphR(A) and the MphR(E) mutations. It would seem that the mutations MphR(E)_K, L, H and N and MphR(A) were produced more than MphR(E)_L and MphR(E).

5.2.2. The Digestion of Histidine Tag by TEV Protease from the Fusion MphR(E) Protein

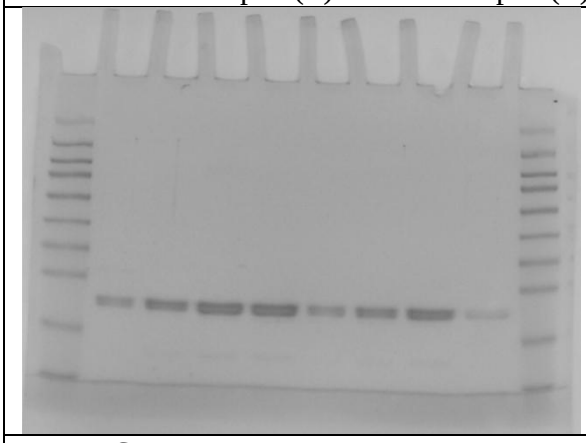
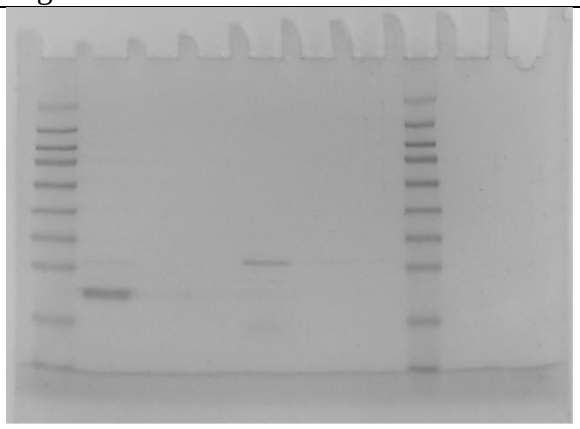
The first digestion was made for the second production of MphR(E) with the TEV protease (Fermentas). All MphR(E) was digested when 15 U of TEV protease was with 10 µg of MphR(E). In the second digestion MphR(E)_CG was digested with a TEV protease (DNASU). This TEV protease did not digest all of the protein (Picture 13).

First TEV digestion (Fermentas)	Second TEV digestion
	
1. STD 2. undigested MphR(E) in TEV buffer 3. 0U/10ug MphR(E) 4. 5U/10ug MphR(E) 5. 25U/10ug MphR(E) 6. 25/10ug MphR(E) 7. 50U/10ug MphR(E) 8. 50U/10ug MphR(E) 9. 15U/10ug MphR(E) 10. STD	1. STD Broad range 2. 3rd elution fraction of H undigested MphR(E)_CG 3. undigested MphR(E)_CG in TEV buffer 4. 0 5. 1/200 6. 1/100 7. 1/50 8. 1/10 9. 1/5 10. -

Picture 13. The TEV protease amount was optimized. **Left picture:** The TEV protease from Finnzymes digested the MphR(E) from the histidine tag where there was 15 U of protease per 10 µg of undigested MphR(E). **Right picture:** The second TEV protease (DNASU). The protease did not digest all of the MphR(E) even with the 1 / 5 ratio of TEV protease / undigested MphR(E).

5.2.3. Digestion and Concentration of MphR(E) and MphR(E)_CG

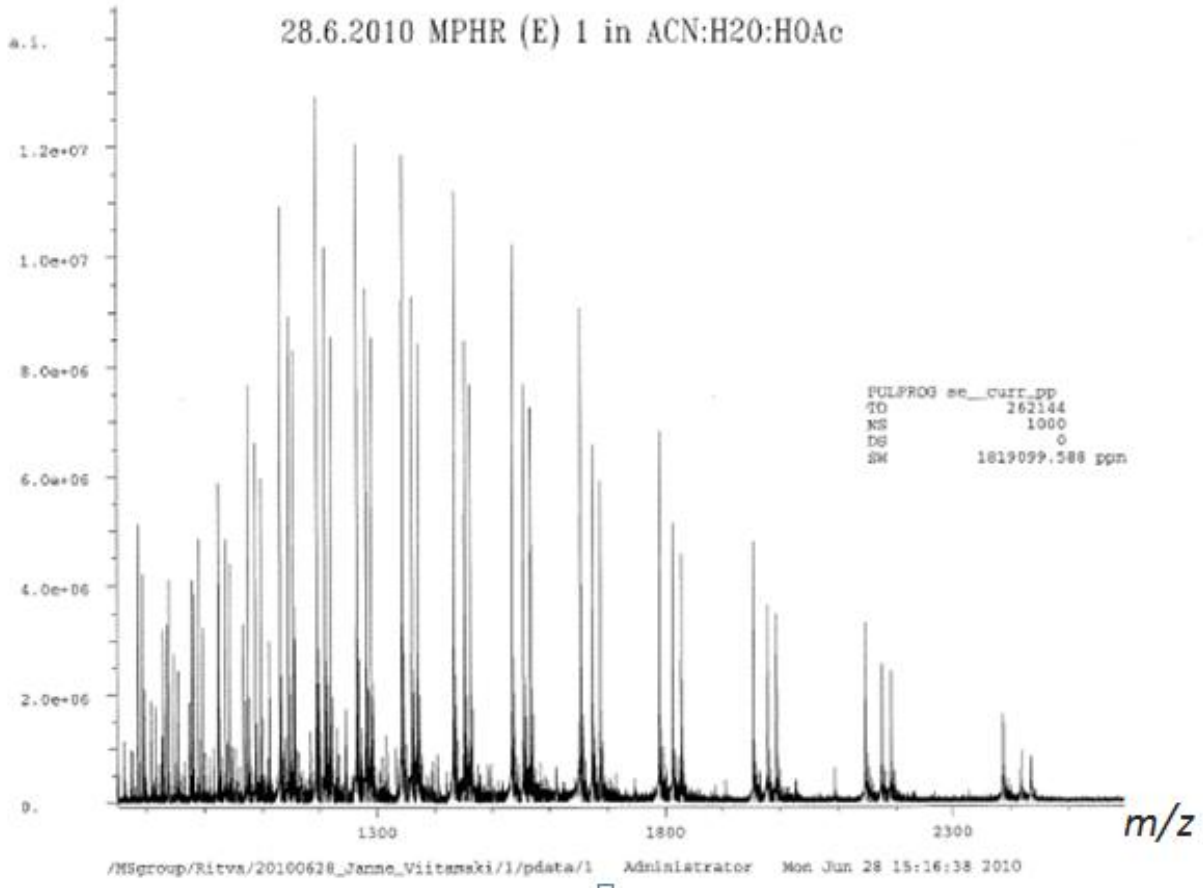
From the second production of MphR(E) around 3 mg was digested with TEV protease (Fermentas). The digestion was complete and only one band was shown in wash fraction the SDS-PAGE gel (Picture 14). The first concentration method was not efficient because it did not concentrate MphR(E) instead it went through the filter with the flow trouhgh. The precipitated protein could be diluted by adding MilliQ water. The possible conformational changes as a result of the precipitation were examined by running a native-PAGE gel. The digested MphR(E) was concentrated successfully with the saband vacuum centrifugal concentrator and the buffer was exchanged into 10 mM ammonium acetate. The concentration and the samples were sent to mass spectrometry to the University of Eastern Finland. The purification of the third production of MphR(E) and MphR(E)_CG was not as successful. The SDS-PAGE gels after purification of the third production showed that two bands of MphR(E)s size were in the elution fraction and only one in the wash fractions. The bands were fairly small and a reference sample was forgotten to ad so the wash fractions were concentrated. The concentrated samples showed that MphR(E)_CG had not been separated and MphR(E) had disappeared and had only a vague band.

Purification of MphR(E) from His-MphR(E) after digestion	
	
1. STD 2. Digested MphR(E) 3. Flow through 1 4. Flow through 2 5. Flow through 3 6. Flow through 7 7. Flow through 11 8. Wash 1 9. Wash 2 10. STD	1. STD 2. Digested MphR(E) 3. Wash 4 4. Wash 7 5. Elution 2 6. Elution 3 7. Elution 5 8. STD 9. Strip 10. Strip last

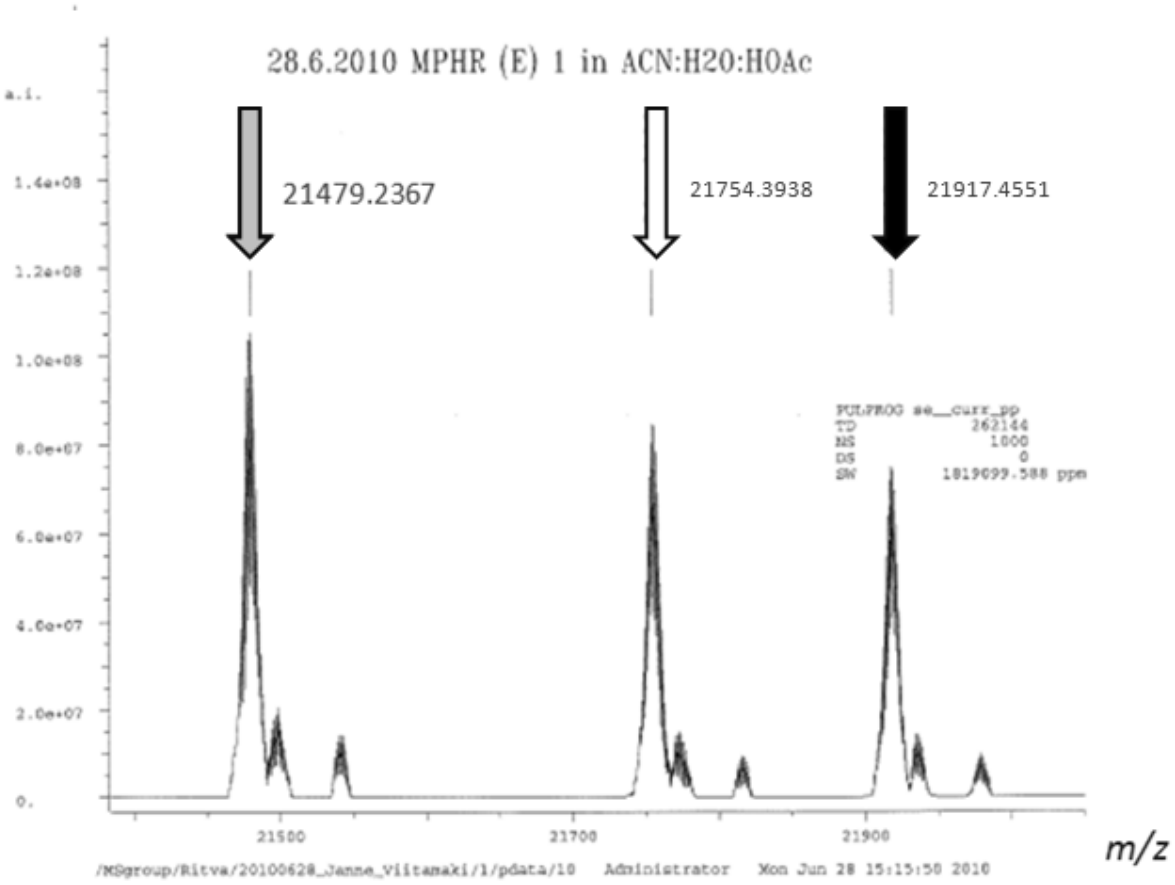
Picture 14. The digestion with the TEV protease (Invitrogen) was successful as was the purification. The flow trough and wash fractions showed only one band on the SDS-PAGE gel. The elution fractions showed dimly two fractions that could be undigested MphR(E) and MphR(E) and a bigger protein TEV protease.

5.3. Mass Spectrometry

The mass spectrometry was measured under denaturating conditions [acetone (ACN), water (H_2O) and acetic acid (HOAc)] to ensure the right amino acid composition of the monomer. The results showed that the MphR(E) was purified out of other proteins. It also shown that there were three different MphR(E) monomer variants of 21 - 22 kDa (Pictures 15). After calculating the protein masses from the sequence it was clear that the digestion of the TEV protease (Invitrogen) was not specific instead it cleaved MphR(E) from three different sites. The smallest one was 21 479.2367 Da and it was cleaved from the right site the middle one had one additional amino acid and the biggest had two additional amino acids (Picture 16).

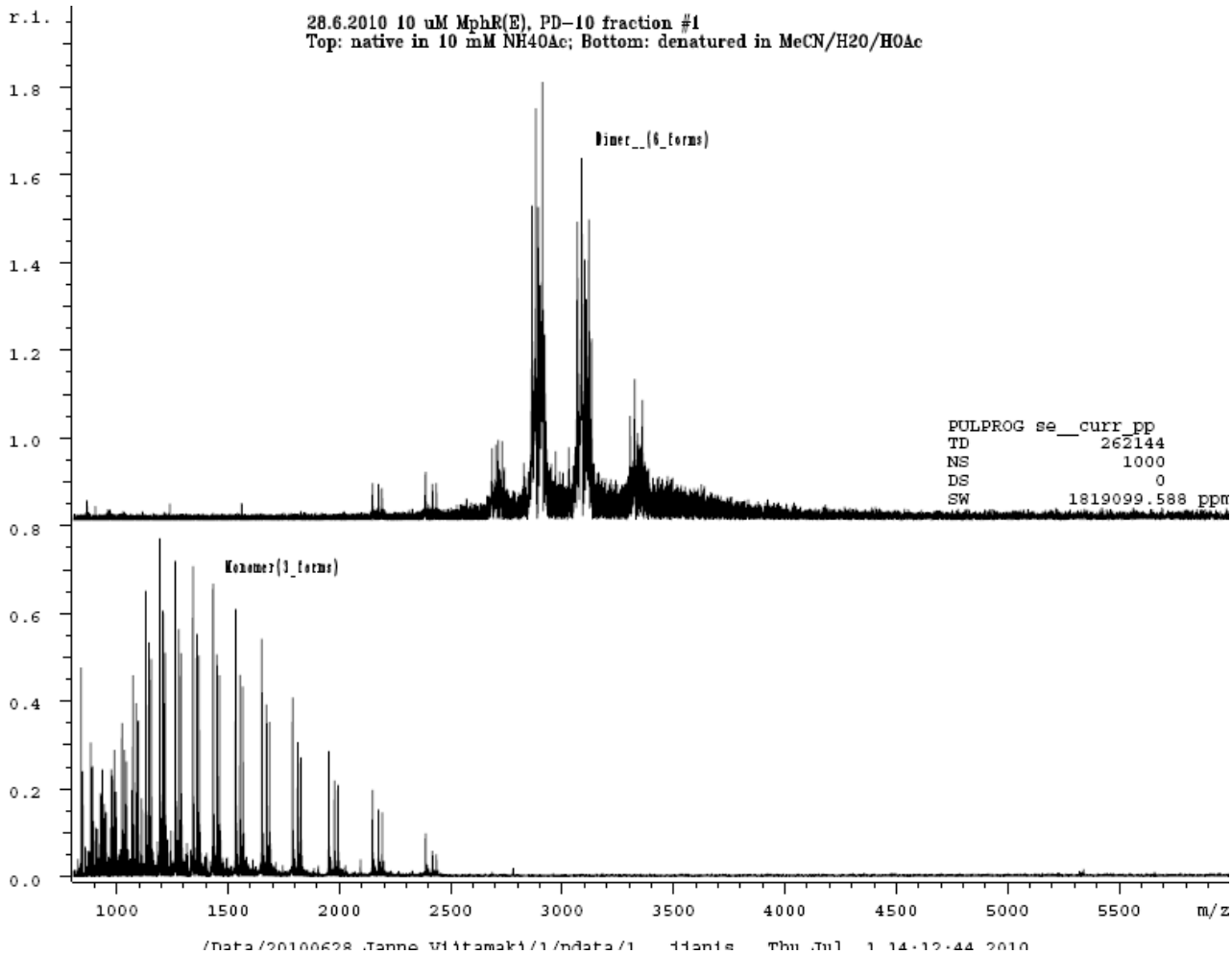


Picture 15. The mass spectrometer results showed that there was three types of MphR(E) in the digested, purified and concentrated form.



Picture 16. Zoomed deconvolution. The mass spectrometer results show that there were three protein sizes close to each other. The grey arrow points to the 21 479.2367 Da sized MphR(E) without the his tag. The white arrow points at MphR(E) monomer having one amino acid too much and the black arrow points to the MphR(E) monomer having two additional amino acids. Showing that the digestion was not totally specific.

When measured in native conditions mass spectrometry showed that 90 % of MphR(E) was in non-covalent dimeric form in 10 μM concentration. Having three different monomers in the mixture then naturally the dimmers have six different forms (Picture 17).



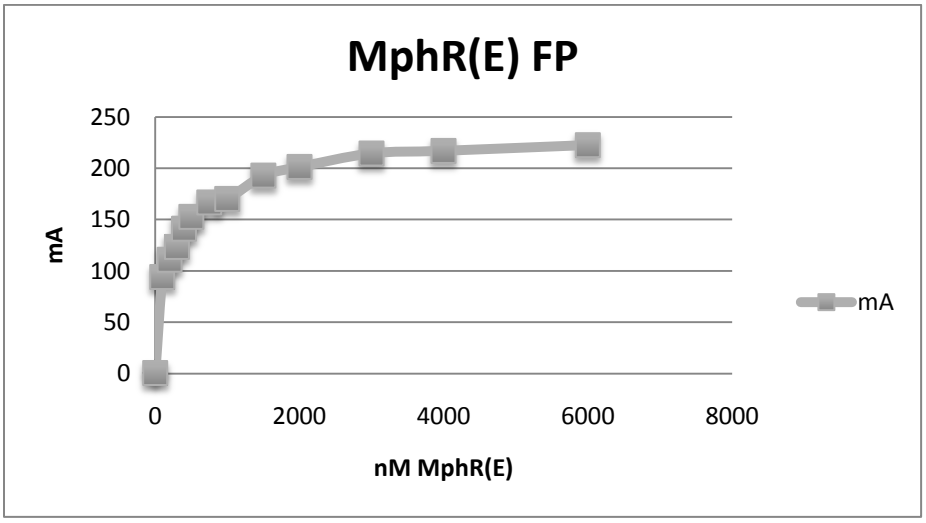
Picture 17. Mass spectrometry of MphR(E) in its native state. The picture shows it was a non-covalent dimer. It has six different charged states (six spikes), because the sample was a mixture of three different monomers. In the native spectrum also a small portion (10%) was in a monomeric formation.

5.4. Fluorescence Polarization

First the fluorescence polarization was used for MphR(E) from the second production without digestion. The mP was exchanged into mA using the equation A = 2 P / (3-P). The curve was steady suggesting that only one MphR(E) homodimer binds to the promoter (Picture 18). The curve was made into a linear one using lineweaver-burks method and it was linear with the protein concentration 100 to 2000 nM. The dissociation constant (Kd) was calculated for each of measuring points using the equation:

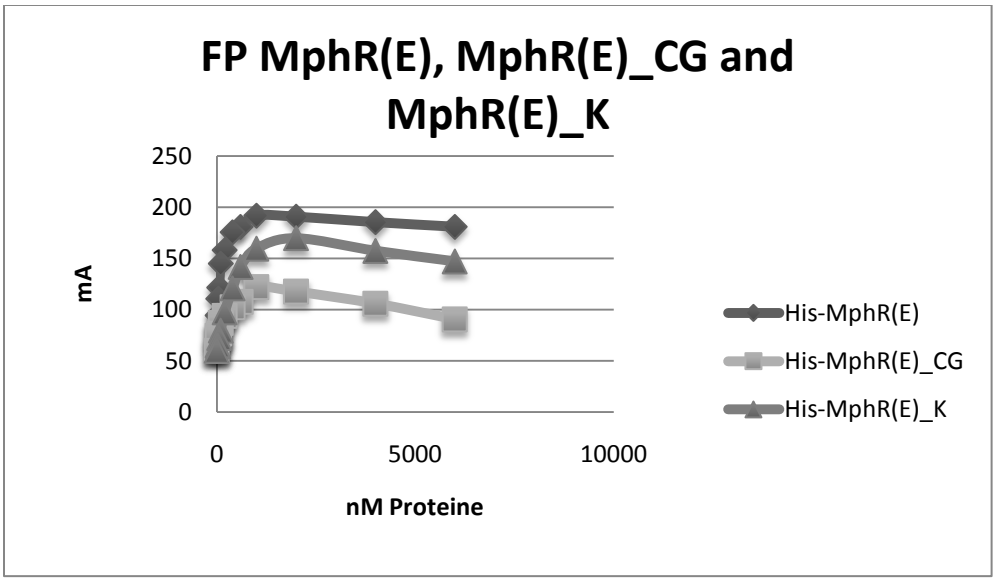
$$Kd = -Eo - \frac{(Amax - Amin) * Eo}{(Amin - A)}$$

The average was calculated from the Kd´s 300 to 2000 mM undigested MphR(E). The average Kd for MphR(E) was 227 mM.



Picture 18. The first fluorescence polarization of MphR(E) results put into anisotropy diagram.

Fluorescence polarization was measured after the third production of MphR(E) and the mutants MphR(E)_CG and K were selected. The curve did not start from 0 mM protein and mA value of the bigger concentrations decreased (Picture 19). The mP was exchanged into mA using the equation mentioned above and the average Kd values were calculated using the same logic as mentioned above. The linear line was made with the protein concentration of 40 - 600 nM. The average Kd´s were undigested MphR(E) = 204 mM from the first production undigested MphR(E)_CG = 576 nM undigested MphR(E)_K = 652 nM (Table 9).



Picture 19. The diagrams of MphR(E) and the mutants MphR(E)_CG and K. The fluorophore could be too old because the 0nM protein sample showed such a high anisotropy value.

Table 9. The dissociation values measured for MphR(E) and the mutants CG and K. MphR(E) seems to have Kd around 200 mM which is a lot lower than the mutants CG and K around 600 nM.

Protein	Concentration	Kd
Undigested MphR(E) 1st production	40 - 600 nM	204 nM
Undigested MphR(E) 2nd production	100 - 2000 nM	227 nM
Undigested MphR(E)_CG	40 - 600 nM	576 nM
Undigested MphR(E)_K	40 - 600 nM	652 nM

6. Discussion

During this study MphR(A), MphR(E) and five MphR(E) mutations out of six were produced and purified. MphR(E) was digested and sent to mass spectrometry. Furthermore, dissociation constant was calculated for MphR(E) and for two mutations from the measurements made by fluorescence polarization (Table 10.)

Table 10. This figure shows what was done during this study (x). To MphR(E) and MphR(A) a histidine tag was added and the STOP codon was changed, which was made by Nina Virolainen (Technical University of Tampere, Tampere, Finland) (n). The MphR(E)_Y was transformed but the sequencing of the plasmids showed no cell line of the right mutation. The rest of the mutations were produced and purified but they were not digested. Fluorescence polarization was measured for MphR(E) and the mutants MphR(E)_K and CG.

Protein	SOE-PCR Mutation	Sequencing of the mutant *E. Coli*	Protein production and purification	Protein digestion and purification	Fluorescence polarization	Concentration and Mass spectrometry
First production MphR(E)	n	n	x	-	204 nM	-
Second production MphR(E)	n	n	x	x	227 nM	x
Third production MphR(E)	n	n	x	-	-	-
MphR(E)_Y	x	x	-	-	-	-
MphR(E)_K	x	x	x	-	652 nM	-
MphR(E)_L	x	x	x	-		-
MphR(E)_H	x	x	x	-		-
MphR(E)_N	x	x	x	-		-
MphR(E)_CG	x	x	x	-	576 nM	-
MphR(A)	n	n	x	-	-	-

6.1. Manufacturing a Cell Line of *E. Coli* Producing MphR(E) Mutants

Making point mutations and insertions utilizing SOE-PCR method should have been a simple method but instead it turned out to be time consuming. Furthermore, the finding of an optimum annealing temperature took a lot of time for the mutating primers. In the PCR technique special attention had to be given to calculating and pipetting the right amount of reagents to the bottom of the PCR tube and to remember to change the pipette tip after every use. In addition, the same kind of precision was needed in making the plasmids by restriction and ligation. The transformation was successful after the electrocompetent *E. Coli* XL-1 Blue cells were made with the longer method. Quite many of the colonies contained a plasmid giving a PCR product with the His primers. And most of them had a plasmid with an insert of the right size (Picture 10). The sequencing revealed that most of the plasmids had the right insert with the right mutations which could be further produced. The two plasmids sequenced from the mutation MphR(E)_Y had additional mutations. The lack of time prevented from analyzing other colonies having a positive PCR product of MphR(E)_Y.

6.2. Producing, Purifying and Digesting MphR(E), Mutants and MphR(A)

The production was successful and the purification was at best when washed with the 62.5 mM imidazole wash buffer (Picture 11) It washed away most of the impurities cleaning MphR(E) and mutants better than with the lower imidazole concentrations. Although 62.5 mM imidazole wash buffer washed MphR(E) and the mutants well it was not as successful in cleaning MphR(A) from impurities. The purified MphR(A) contained much more impurities but fortunately MphR(A) was produced in a large scale (picture 12). The digestion with the TEV protease (Invitrogen) seemed specific and digested the MphR(E) fully (Picture 13) but mass spectrometry showed that the cleavage was not specific. Instead it had cut from the right spot and from two other places. The unspecific cutting places were one and two amino acids from the specific digestion site (Picture 16). The digestion site specificity could be improved by optimizing the digestion conditions. The TEV protease (DNASU) did not cleave MphR(E)_CG completely. This TEV protease might not have the same capacity to cleave or the conditions might not have been optimal. The purification or the second digestion showed one strand which could be the digested

form. From these SDS-PAGEs a reference sample containing either nondigested or digested or were forgotten. In the digestion of MphR(E)_CG the elution fraction showed two bands which would suggest that the purification was complete for MphR(E) it was not as clear. After concentration it was obvious that the purification was not complete and two bands were shown in the SDS-PAGE. Due to lack of time the purification could not be optimized but a gradient wash was would have been a way to separate the digested and undigested proteins from each other. In the gradient wash the wash buffers of different imidazole concentrations are utilized.

6.3. Concentration and Mass Spectrometry of MphR(E)

Although, MphR(E) precipitated during the concentration it could be dissolved by adding MilliQ water and it could be measured by native mass spectrometry (Picture 17). As said before the TEV protease (Invitrogen) digested MphR(E) into tree monomer forms meaning that six kings of dimers could be formed as shown in the native mass spectrometry results (Picture 17). For further analyzation with mass spectrometry it would be important to have only one monomer form. If there are multiple dimers in the analyzation of the MphR(E) binding to DNA and to macrolides will be problematic because they would give even a larger number of results. The binding of MphR(E) to a macrolide could be achievable but with DNA it would be trickier. The negatively charged DNA is normally difficult to purify totally from Na^{2+} ions which give background noise peaks to the results. In order to analyze the affinities of MphR(E) properly it would be important to have only one monomer. The expensive TEV (Invitrogen) was replaced with a cheaper one (DNASU). If all the mutants would have been cleaved with the TEV protease of Invitrogen it would have become expensive. Even with the protease (Invitrogen) the specificity of the cleavage site could not have been certain. The dissociation constant of DNA binding could be determined with mass spectrometry as could be the dissociation constant of MphR(E) towards its different macrolide ligands. The dissociation constant measured by mass spectrometry would help in optimizing fluorescence polarization as an accurate method in measuring different intermolecular interactions.

6.4. Fluorescence Polarization and Mass Spectrometry

The dissociation constant (Kd) calculations were made based on the results from fluorescence polarization of the MphR(E), MphR(E)_CG and MphR(E)_K repressor proteins which still had

their histidine tags attached. The tags were not digested because the digestion after the cleavage was not as easy to purify as at the first time with MphR(E) before mass spectrometry. The MphR(E)_CG and K were selected because they were interesting being a mutations which make MphR(E) more like MphR(A). MphR(E)_CG possibly makes a GP loop structure leading to the DNA binding HTH-motif possibly making it bind with a better affinity toward the DNA. The mutant MphR(E)_K is one of the three important DNA binding amino acids in MphR(A). MphR(E), MphR(E)_CG, K and were produced in a larger quantity and small portions could be used for a fluorescence polarization measurement. All of the proteins could have been measured but because of lack of time and material it was best to save the measurements for the digested proteins. Although fluorescence polarization is a fast method it requires a lot of pipetting. MphR(E) had a good binding affinity towards DNA Kd = 204 mM. In fact, this suggests that MphR(E) has twice the DNA binding affinity (Kd) of MphR(A). The Kd of MphR(A) is 574 ± 29 nM. The mutations MphR(E)_CG and K had bigger Kd values suggesting that the mutatis did not increase DNA binding affinity. These mutations were designed to make MphR(E) more like MphR(A) and the mutations did complete this target when Kd for CG was 576 nM and for K 652 nM. This would suggest that although the MphR(E)_K is an important amino acid for binding DNA it does not improve the DNA binding affinity of MphR(E). Acording to these results fluorescence polarization shows that MphR(E), its mutants MphR(E)_K and MphR(E)_CG can function and bind to their DNA promoter. Therefore, it is most likely that the mutants and MphR(E) can form a homodimer.

The mass spectrometry showed that the sample sent was without impurities showing that the purification protocol worked. It could be observed that MphR(E) was digested from three different sites. Fortunately, one of the sites was the desired and specific site. Native ESI mass spectrometry showed that MphR(E) can form a homodimer and that 90 % of MphR(E) was in a dimeric state although the sample was a mixture or three different monomers. Due to lack of time further measurements of DNA and macrolide binding affinities were not measured. Lack of time also prevented the measurement of the mutants. At this time it is unclear if the Kd´s measured by fluorescence polarization could give similar results with mass spectrometry. Fluorescence polarization did indicate that the produced MphR(E) could form a dimer which was later affirmed by native mass spectrometry results. Accordingly, the mutants MphR(E)_K and MphR(E)_CG most likely will show a dimeric structure in native mass spectrometry.

7. Conclusions

The aims of this study were met by finding information of MphR(E): how it can be produced and mutated and how the dissociation values can be achieved by fluorescence polarization and possibly by mass spectrometry. The fluorescence polarization method was utilized and Kd values were determined. The MphR(E) protein was analyzed by mass spectrometry.

Although, the time for this study was relatively short five out of six Mphr(E) mutations were produced and purified. The one mutation left could be found from the transformed colonies or even from the results of COLONY PCR. MphR(E) was digested and sent to mass spectrometry although it had been digested form three different places the sample showed good quality and purity. Suggesting that the production, digestion and purification were successful and the work was well done. In mass spectrometry it was shown that 90 % of was in a dimeric conformation. In the second digestion when MphR(E) and MphR(E)_CG were digested with the same protease from a different producer the purification process did not function properly. The dissociation values were measured for MphR(E), MphR(E)_CG and MphR(E)_K from the undigested proteins by fluorescence polarization. The results showed that MphR(E) has over twice higher affinity than MphR(A) towards its promoter. The mutations MphR(E)_CG and K made to imitate MphR(A) and they had a lower affinity towards the promoter. Their affinity was of the same nanomolar range with MphR(A). Suggesting that the mutations did make MphR(E) more like MphR(A).

Although the protein production and purification were successful some difficulties were found during the purification of the digested proteins. The search of annealing temperature for the mutating primers in SOE-PCR was time consuming. Even though PCR was already a familiar method accuracy in calculating and pipetting did still take special attention. In addition, time consuming was the use of fluorescence polarization method in finding the right equipment and making the calculations, finding the best way to pipette the samples and in calculating the Kd values from the polarization results.

In future a good colony of the MphR(E)_Y mutation could be found and produced. First the purification of the digestion should be optimized probably by making a gradient wash. Afterwards all of the six mutants, MphR(E) and MphR(A) can be digested and sent to mass

spectrometry for further measurements. Mass spectrometry can determine the purity and the specificity of the digestion. In addition, native mass spectrometry can show accurately if the protein has a homodimerc structure. If the samples are pure and the digestion is specific the affinity towards different macrolides can be measured and even the affinity towards the promoter. A small portion of the digested could be measured with fluorescence polarization and the Kd values could be calculated. In addition, the possible affect of the histidine tag can be seen after the digested protein is measured by fluorescence polarization.

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