DIPLOMARBEIT

Bacterial Ghosts as Carrier of Her-2/neu Subunit Vaccines

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Contents

Abbreviations

aa	amino acid
AB	antibiotics
Ag	antigen
Amp	ampicillin resistance cassette
APC(s)	professional antigen presenting cell(s)
BCCP	biotin carboxyl carrier protein
BG(s)	bacterial ghost(s)
bp	base pair
cfu	colony forming units
CM	cytoplasmic membrane
CPS	cytoplasmic space
CTLs	cytotoxic T-cells
dH2O	deionized water
E. coli	*Escherichia coli*
Her-2/neu	Human epidermal growth factor receptor 2
HLA	human leukocyte antigen
hm	herceptin mimotope
Ig	immunoglobulin
IL	interleukin
IMP	inner membrane protein
IM	inner membrane
kb	kilo base pairs
kDa	kilo Dalton
l	liter
LacIq	repressor of lac operon
LacZα	gene encoding the lacZα protein
LB	lysogeny broth
LE	Lysis Efficiency
LI	lysis induction
LPS	lipopolysaccharide

M	molar
mM	milli molar
M13	intergenic region, origin of replication (+ -)
malE	gene encoding the maltose binding protein
MCS	multiple cloning site
MBP	maltose binding protein
MBP-467	maltose binding protein – peptide 4, 6, 7 fusion protein
MBP-467ivb	maltose binding protein – peptide 4, 6, 7 fusion protein with an in vivo biotinylation sequence
MBP-hm	maltose binding protein - herceptin mimotope fusion protein
MBP-hmivb	maltose binding protein - herceptin mimotope fusion protein with an in vivo biotinylation sequence
mg	milligram
µg	microgram
MHC	major histocompatibility complex
min	minutes
µl	micro liter
nm	nanometer
ng	nanogram
OD_{600}	optical density at $\lambda = 600$ nm
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
PAGE	polyacrylamid gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pMB1	origin of replication
PPS	periplasmic space
pSIP	self immobilizing plasmid
P_{TAC}	promotor of *malE*
rop	regulation of plasmid DNA replication by antisense RNA
sdH2O	sterile deionized water

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TA	target antigen
TT	tetanus toxin
WB	western blot

1. Zusammenfassung

1.1 Zielsetzung

Her-2/neu (human epidermal growth factor receptor 2) steht als Onkogen im Mittelpunkt vieler Bestrebungen ein therapeutisches Mittel gegen Brustkrebs zu entwickeln. Als Mitglied der Familie der epidermalen Wachstumsfaktorrezeptoren (EGFR) ist es in 30% der Brust-und Prostatakarzinomen überexprimiert und ist mit einem progressiven Verlauf der Krankheit und einer hohen Rückfallquote verbunden [1-3]

An der Medizinischen Universität Wien wurden Peptide, die Teilstrukturen des Oberflächenrezeptors darstellen, sogenannte Mimotope, auf deren Immunogenität untersucht. Einige molekulare Mimikripeptide führten in Mäuseversuchen zu Induktion von anti Her-2/neu Antikörper, die eine Inhibition des Tumorwachstums zur Folge hatte [4]. Ziel laufender Studien ist, die Qualität der Immunantwort zu verbessern und mit geeigneten Adjuvanzien sowohl eine B- also auch T-Zell Antwort auszulösen.

Dafür sollen in dieser Arbeit die erfolgreichsten Peptide dieser Studien für das Delivery mittels der Bacterial Ghost Platform Technology ausgewählt werden. Die Peptidsequenzen P4, P6 und P7, lineare Epitope von Her-2/neu, sollen gekoppelt, als Multiepitop in den Plasmidvektor pMal-p2x kloniert und in *Escherichia coli* (*E. coli*) transformiert werden. Der resultierende Vektor pMal-467 erzeugt durch IPTG-Induktion eine Maltose-Bindeprotein (MBP) Fusion mit den Peptiden p467. Die Signalsequenz von MBP leitet das MBP-467 Fusionsprotein in das Periplasma des Bakteriums. Ebenso soll ein Konformationsepitop von Her-2/neu, Herzeptin Mimotop (hm), in den Vektor kloniert und als MBP-hm exprimiert werden. Das Periplasma sollte vor allem diesem über Schwefelbrücken zirkularisierten Mimotop durch die oxydative Umgebung gute Voraussetzungen bieten, um die vorgesehene Konformation, die ja nur als solche immunogen ist, einzunehmen. Zur Produktion von Bacterial Ghosts (BGs) soll die Expression dieser Fusionsproteine mit nachgeschalteter Protein E vermittelter Lyse durchgeführt werden. Resultat ist eine nicht lebensfähige Bakterienhülle, deren periplasmatischer Raum mit dem Fusionsprotein gefüllt ist. Diese mit Tumorantigenen bestückten BGs sind als Vakzin konzipiert, das durch aktive Immunisierung eine präventive Langzeitimmunität gegen Brutkrebs erzeugen soll.

1.2 Ergebnisse

In Vorversuchen mit MBP-467 Fusionsproteinen produzierenden *E. coli* wurde die MBP-467 Expression zuerst indirekt mit αMBP, und anschließend direkt mit p467 spezifischen Antiseren mittels Western Blot Analyse nachgewiesen. Nach der Kotransformation von *E. coli* mit dem Lyseplasmid pGLysivb und dem Expressionsplasmid pMal-467 wurden Vorversuche zur Expression mit nachfolgender Protein E vermittelten Lyse erfolgreich abgeschlossen. Eine Lyseeffizienz von 99,9% wurde erreicht und *E. coli* NM522 (pGLysivb, pMal-467) ging daraufhin in die Produktion. In einem 20l Fermenter wurden BGs produziert, sie wurden dann geerntet, gereinigt und lyophilisiert. Das fertige Produkt wurde an der Medizinischen Universität von Wien in einer Immunisierungstudie an Balb-c Mäusen getestet, und es konnte eine Induktion peptidspezifische Antikörper nachgewiesen werden.

Vorversuche für das zyklische Herzeptin Mimotop verliefen im Rahmen dieser Arbeit nicht erfolgreich. Die Expression wurde in verschiedenen Bakterienstämmen ausgetestet, unterschiedliche Methoden des Zellaufbruchs wurden untersucht, ebenso chemische Renaturierung zur Konformationserkennung und die Expressionsdauer und Zusammensetzung des Lysepuffers wurden variiert. DNA Sequenzierung des klonierten Vektors pMal-hm zeigte zwar, dass das Mimotop im korrekten Leserahmen war und somit als Fusionsprotein erzeugt werden sollte, aber ein eindeutiger Nachweis über den Her-2/neu spezifischen Antikörper Trastuzumab (Handelsname Herzeptin) gelang nicht. Der Nachweis über den MBP-Teil zeigte in der Western Blot Analyse vermutlich überwiegend ein Abbauprodukt des Fusionsproteins, nur bei einzelnen Klonen war eine schwache Bande für die zu erwartende Peptidgröße sichtbar. Das Ausmaß der Expression, des Transports und der korrekten Faltung des Proteins war also entweder für einen Nachweis zu gering, oder das Fusionsprotein war instabil und wurde abgebaut.

2. Summary

2.1 Objectives

In the development of therapeutic agents for treatment of breast cancer, many studies focus on Her-2/neu (human epidermal growth factor receptor 2). This oncogen that is a member of the human epidermal growth factor- receptor- family is over expressed in ~30% of breast and prostate carcinoma and linked to poor prognosis and a high cancer relapse [1-3].

Studies conducted at the Medical University of Vienna evaluate the anti-tumor potential of several mimotopes, which are peptide structures displaying parts of the extracellular domain of Her-2/neu in this case. Immunization of mice with the peptide mimics elicits Her-2/neu-specific antibodies inhibiting tumor progression [4]. Ongoing studies aim to further increase the efficacy of immune response by the use of adjuvants, which are capable of eliciting both B cell and T cell immunity.

To do this, the most promising peptides out of their studies should be used for delivery with the Bacterial Ghost Platform Technology. Peptides 4, 6 and 7 - linear epitopes of Her-2/neu - should be coupled as multiepitope, cloned into the vector pMal-p2x and transformed into *E. coli*. By induction of IPTG a fusion protein of the maltose binding protein (MBP) and p467 is produced (MBP-467). The signal sequence of MBP directs the fusion protein to the periplasmic space (PPS). In addition a conformational epitope of Her-2/neu, herceptin mimotope (hm), should be cloned into the vector. The oxidative surrounding of the periplasmic space should enable disulfide bridging especially for the circularized mimotope - which is very important since the natural conformation is essential for immunogenicity. Production of BGs should be accomplished by expression of these fusion proteins - followed by E-mediated lysis resulting in non-living bacterial cell envelopes carrying the desired fusion proteins in the PPS. Active immunization with tumor antigen loaded BGs should generate preventive long term immunity against breast cancer.

2.2   Results

Preliminary growth and expression studies showed positive evidence of MBP-467. The fusion protein was first detected indirectly by αMBP and then via p467 specific immune sera in western blot analysis. Cotransformation of *E. coli* with the lysis plasmid pGLysivb and expression plasmid pMal-467 and subsequent expression and lysis tests were successfully completed. Lysis efficiency resulted in 99.9% and *E. coli* NM522 (pGLysivb, pMal-467) was used for BG production in a 20l Techfors fermenter. After harvesting, the BGs were purified - and lyophilized. The final product was tested at the Medical University of Vienna. Immunizations of Balb-c mice showed the induction of p467 specific antibodies.

Preliminary experiments for detection of the circularized herceptin mimotope were not successful. Expression was tested in different bacterial strain, different methods of cell disruption were tried out, expression time and composition of lysis buffer were varied, also attempts for chemical renaturation were made. Although DNA-sequencing of the vector showed the mimotope in the correct reading frame, detection with the monoclonal antibody Trastuzumab (trademark Herceptin) was not possible. Detection of the MBP portion by western blot analysis showed mainly a degradation product of the fusion protein and only for individual clones a faint band at the anticipated molecular weight of the target protein. The degree of expression, transport or correct folding of the protein was either too low to be detected or due to instability the fusion protein was degraded.

3.   Introduction

3.1   Cancer and the need for therapeutic vaccines

According to the latest statistics, cancer is responsible for 7.6 million deaths each year worldwide. The number of new cases per year is 10 million, with 60% of those cases occurring in developing countries, mainly liver-, stomach- and cervical cancer. Prostate-, colon- and breast cancer prevail in developed countries [5].

Although substantial improvements have been made in mortality due to cancer in the last 30 years with the use of surgery, combined with radiation, chemotherapy and endocrine manipulation, these improvements are limited to developed countries having a functional health care system [6]. Furthermore, these therapies are aiming to gain control of the disease, but as they are not specifically targeting tumor cells only, they are damaging as well normal tissues to a certain extent. Moreover these therapies often go along with appearance of resistant tumor cells escaping the treatment, resulting in cancer relapse [7-9]. The invention of molecularly targeted therapy provides the opportunity to specifically interact with regulatory pathways of malignant proliferation. For example antibody treatment with Trastuzumab (Herceptin), currently a standard monotherapy of advanced breast cancer overexpressing the human epidermal growth receptor 2 (Her-2/neu) oncoprotein [10], is inhibiting malignant cell proliferation by binding the extracellular domain of Her-2/neu and blocking the downstream signaling [11]. But it is very cost intensive, needs repeated application and is limited by inadequate tissue distribution [12]. In summary, the limited access to affordable health care together with the mechanisms of therapeutical resistance of tumors to conventional therapies requires new cancer therapies and ways for cancer prevention. Using cancer vaccines would be a highly attractive approach for cancer management.

One of modern medicines greatest achievement so far is immunization against infectious diseases. Preventive vaccine administration erased small pox and polio and decreased mortality of several other infectious diseases.

Why is it so difficult to develop cancer vaccines?

When the role of cytotoxic T-cells in destroying cancer cells was recognized the focus in cancer vaccine research was directed on primarily tumor antigens recognized by T-cells. While humoral immune response (antibody response) is essential for fighting infectious disease by eliciting antibodies against foreign antigens with limited immunoregulation, cancer is controlled by cellular immunity (T-cell response) but T-cell response is often involved in immunoregulatory pathways that contribute to continued tumor persistence and proliferation [13]. Even tumor cells develop from normal cells and have self signals, the immune system is able to recognize them as a threat. But with time tumor cells acquire characteristics which allow them to escape immune surveillance. "Immune escape" is one back up mechanism for tumor cells, in which they alter their surface structure (e.g. MHCI), masking them from the immune system. Tumor cells are also capable of mobilizing regulatory T-cells and are thereby suppressing the immune response [14]. Several escape mechanisms have been described [15] indicating the importance of context and time of tumor antigen detection by the immune system and also for cancer vaccine based strategies.

Developing anti-tumor vaccines based on active immunization was often aiming to generate a strong anti-tumor T-cell reactivity. Cytotoxic T-cells (CTLs) are important effector cells against cancer, but the high frequency of human leukocyte antigen Class I down regulation in primary breast cancer and other malignancies [16-20] and thereby leading to immune escape redirected the attention of several research groups to successes seen with anti-cancer antibodies that may be important for tumor rejection [21]. Considering the limitations of passive antibody application mentioned above for Trastuzumab, an active immunization against cancer eliciting antibodies of the desired type might be biologically important to develop a potent anti-tumor vaccine.

3.2   Her-2/neu, a target for immunological attack

Breast cancer is with 17.2% of all tumor cases the most common cancer in women in the eastern mediterranean region and the leading cause of cancer mortality worldwide. The proto-oncogene Her-2/neu is a member of the ErbB protein family, also known as the epidermal growth factor receptor family. Her-2/neu is modestly expressed in normal

adult tissues [22] and over expressed in ~30% of primary breast- and prostate cancer [23, 24] leading to increased responsiveness to growth factors of the EGF family and resulting in malignant proliferation [25]. Overexpression is related to a high cancer relapse and to a more aggressive course of the disease in general, resulting in poor survival rates. These findings made Her-2/neu a target for developing anti cancer therapeutic agents.

As the anti Her-2/neu antibody 4D5 showed inhibitory effects in mice [26], a humanized form , termed Trastuzumab, was tested in clinical trials showing an increased survival rate of patients with Her-2/neu over expressing breast tumors [11]. Besides Trastuzumab a large number of antibodies against Her-2/neu were developed which show different properties. Depending on the receptor binding site of the antibodies, they showed inhibitory or enhancing properties [27]. Therefore immunization with whole antigen can lead to opposing biological effects, whereas epitope specific vaccination may induce only wanted humoral immune responses, effective to treat cancer.

3.3   Selection of predicted B cell epitopes

Two different strategies to obtain peptide antigens of Her-2/neu for immunization experiments were conducted at the Medical University of Vienna, Department of Pathophysiology, Prof. Wiedermann. One gaining linear epitopes consisting of three peptides (p4, p6, p7) and the other obtaining a circularized epitope (QMW, Herceptin mimotope), which were later on used as peptide antigens in Bacterial Ghost (BG) delivery in this work.

Computer aided analysis

This analysis used algorithms locating surface exposed regions that are most likely involved in antibody binding. The epitopes were scanned for hydrophilicity, accessibility, flexibility, charge distribution or secondary structure propensities [28]. Seven putative B cell epitopes of the human extracellular domain of Her-2/neu were selected and tested in proliferation assays and in complement dependent- and antibody dependent- cell lysis (ADCC) experiments [4]. The tested epitopes were not equally immunogenic; p4 as well as a combination of p6 mixed with p7 were able to induce peptide specific antibody levels, primarily of the IgG1 isotype. In cell proliferation assays epitopes p4, p6 and p7 led to a 9–

16% inhibition of the tumor cell proliferation in vitro and in ADCC experiments to a 31–46% lysis of Her-2/neu over expressing tumor cells.

Phage display technique

For eliciting a specific humoral and cellular immune response peptides from 6 to 20 amino acid length can be used. Peptides that mimic the structure of an epitope are referred to as mimotopes. Using the phage display technique mimotopes of the extracellular domain of Her-2/neu were selected. A phage library can contain up to 10^9 different peptides bound to the phages minor coat protein pIII or the major coat protein pVIII. Biopanning the libraries with the inhibitory monoclonal antibody Trastuzumab selects the mimicking peptides and they can further be enriched [29, 30]. Three rounds of biopanning were performed, using a phage library expressing cystein-flanked dekapeptides circularized by disulfide bridging. Five insert sequences were deduced from 94 positive phage clones selected by the criteria of the highest frequency of detection found in colony screenings, the highest intensity of recognition by Trastuzumab and satisfying mimicry test results [31]. The most promising candidate was the herceptin mimotope C-QMWAPQWGPD-C proved to elicit antibodies recognizing the original antigen Her-2/neu.

3.4 Bacterial Ghost System

Bacterial Ghosts (BGs) are the product of a genetically controlled process called E-lysis. During the infectious cycle of bacteriophage ΦX174 gene E is expressed to release phage particles from the infected E. coli [32]. By cloning and expression of gene E in E. coli was proved that the gene product, protein E, is sufficient to cause lysis [33]. Protein E is highly hydrophobic, 91aa long, with a mass of 10.5kDa [34]. It tends to assemble at potential division zones and at the polar sites, oligomerizes into a transmembrane tunnel structure [35], and thereby fuses the inner and outer membrane of the Gram$^-$ bacteria. The formation of the lysis tunnel (40-200nm) was described to go through three phases [36]. In phase one protein E integrates into the inner membrane, with the C-terminal part directing to the cytoplasm. Phase two is marked by a conformational change, the C-terminal part migrates across the inner membrane, most probably due to a cis-trans isomerization of protein E in an α-helical region of domain 1 [37]. In phase three the inner

and outer membrane fuse, whereby the C-terminal part is reaching the surface of the outer membrane. Thus BGs are formed sealing the periplasmic space and releasing all the cytoplasmic content into the medium due to the change of osmotic pressure between cell interior and the surrounding media. Pictures of the efflux of bacterial cytoplasm and the fusion of inner and outer membrane are shown in Fig 3-1 and Fig 3-2.
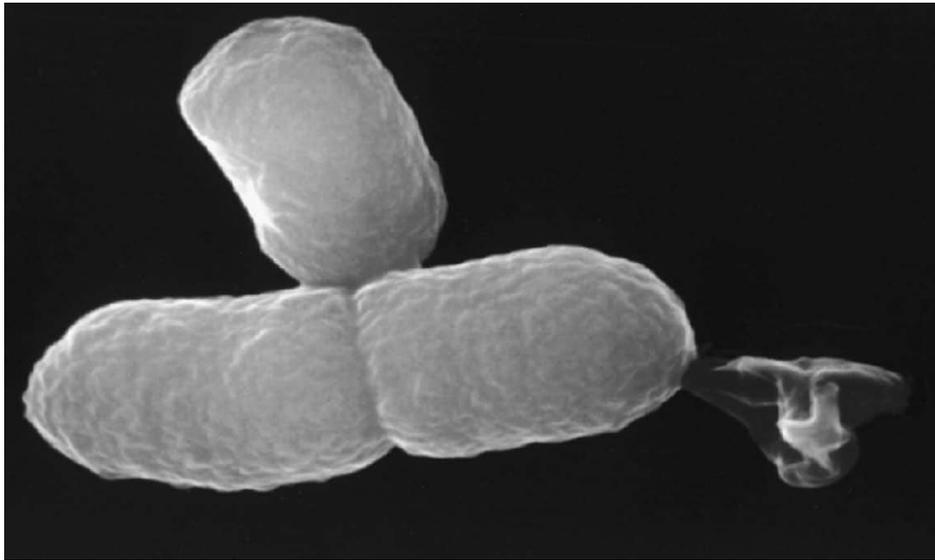

Fig 3-1 High resolution field emission scanning electron micrograph of the expulsion of the cytoplasmic content during BG formation[38]
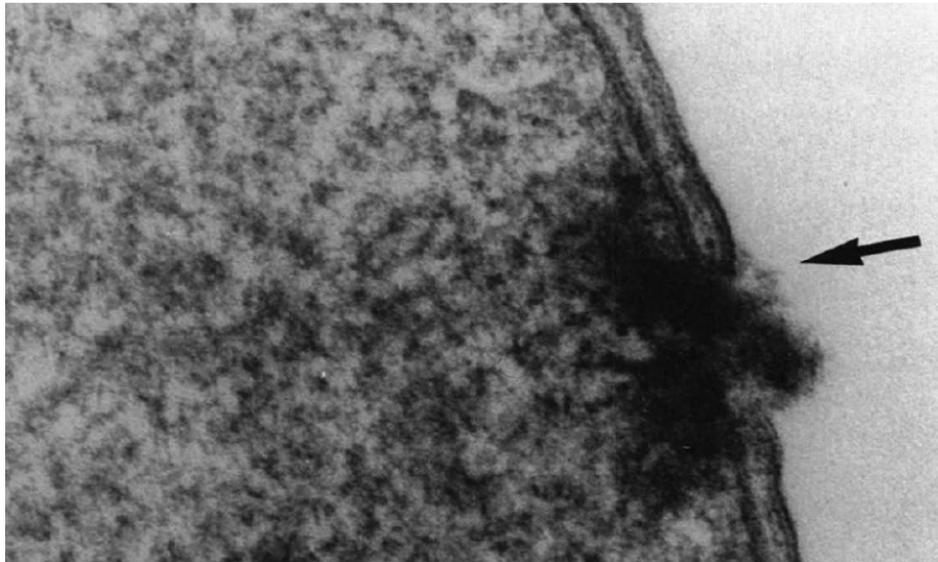

Fig 3-2 Transmission electron micrograph of lysis tunnel formation [39]

3.4.1 Lysis induction on a molecular basis

The earliest lysis studies were performed with the toxic gene *E* under the control of a lacI repressor/promotor system (lac PO-lacI[q]). Further development proved the temperature-sensitive λ-system (λpL/λpR-cI857) to be more quick and efficient with no need for adding chemical substances for lysis induction (LI) by temperature upshift above +30°. The next step forward was to reach more favorable growth rates at temperatures at about +35°C before E-mediated LI. Therefore mutations of the λpL/λpR-cI857 repressor/promotor system were analyzed for altered temperature conditions. A mutation in the O_R2 operator region of the λpR was detected resulting in tight repression of gene *E* up to +38°C [40, 41]. LI is currently done by a temperature upshift of the culture to +40 and +42°C, respectively. This lysis plasmid is also available with an in vivo biotinylation sequence (ivb) at the C-terminus of gene *E* and can therefore serve as a tool for detection of protein E. The molecular weight of protein Eivb is 12.7kDa. A scheme of this lysis plasmid pGLysivb (6.1.3) used in this work is shown in Fig 3-3.
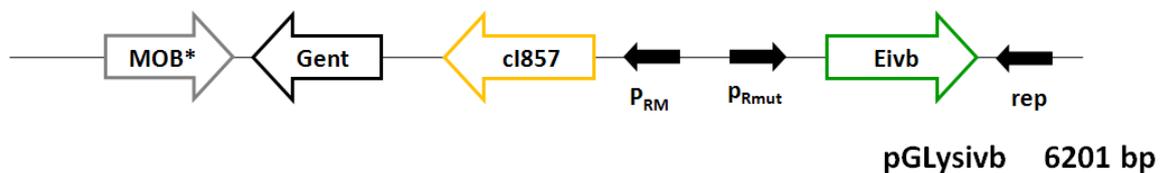
Fig 3-3 Scheme of lysis plasmid pGLysivb.

MOB*: mutated mobilization sequence (mobilization frequency: $3x10^{-2}$) Gent: gentamycin resistance cassette cI875: thermo sensitive phage λ repressor cI P_{RM}: „maintenance"promoter of the phage λ P_{Rmut}: mutated promoter of the phage λ P_R Eivb: gene *E* with ivb sequence fusion rep: broad-host-range origin of replication

3.4.2 BGs for vaccine application

During production of BGs all their natural surface structures are preserved. Major immune stimulating elements like lipopolysaccharides, monophosphoryl lipid A, peptidoglycan or flagella, known as pathogen-associated molecular patterns are recognized by antigen presenting cells triggering the innate immune system. Recognition and phagocytosis by antigen presenting cells through various surface receptors like complement receptors and toll like receptors has been shown [42]. Their intrinsic

adjuvant properties enables BGs also to induce humoral and cellular immune responses, shown in animal experiments [43].

This opens up a broad range of applications. A wide range of Gram⁻ bacteria can be used for BG production and can be applicated as vaccines directly [44]. Using recombinant DNA technology BGs can be used as carrier of foreign antigens in various ways. Antigens can be attached to the inner or outer membrane via membrane anchors, transported to the periplasm as maltose binding protein fusions (MBP-fusion) or coupled to other PPS transport signal sequences. Antigens can also be fused to S-layer sequences [45] forming sheet-like self assembling superstructures in the cytoplasm, resisting the expulsion during E-lysis. When coupled to a PPS signal sequence or to MBP, recombinant S-layers can also be transported to the periplasm. BGs can be loaded with active substances [46], either as liquid or absorbed to the lipid compartment, or specifically attached by using for example membrane anchored streptavidin binding a biotinylated substance or Ag to the inside of the cytoplasmic membrane. And they can also be loaded with DNA either by unspecific binding by simple resuspension of BGs in DNA solution or via specific membrane attachment using self immobilizing plasmids (pSIP) anchored by DNA-binding molecules (lacI) [47]. A schematic overview is given in Fig 3-4.
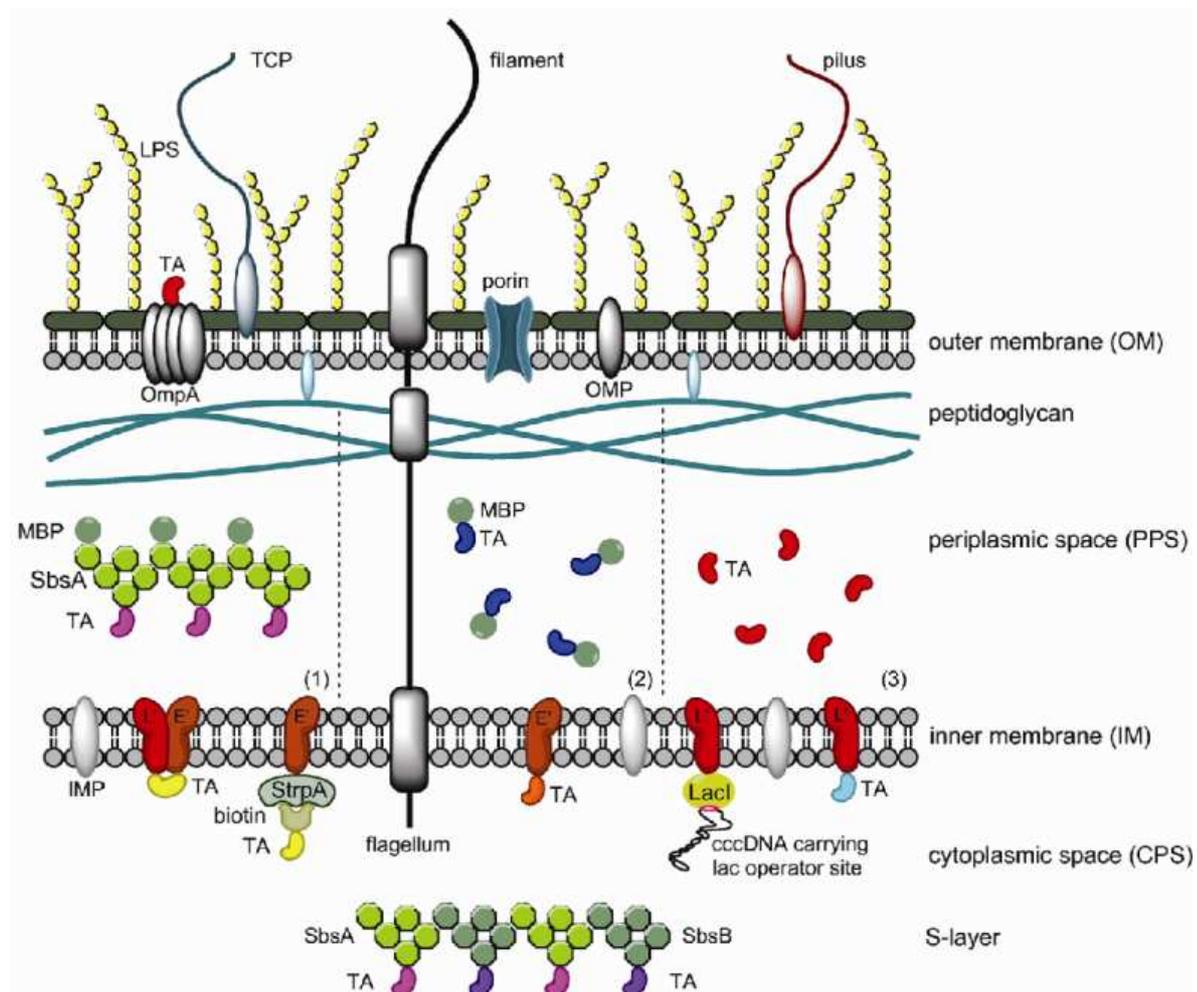
Fig 3-4 Different methods for AG presentation in the BG envelope complex—BG themselves carry native Ag (AG: LPS, OMP, IMP, TCP, flagella, pili)— target Ag (TA) may be presented on the cell surface via fusion with OmpA(1)—the PPS can be loaded with TA via MBP-SbsA-fusion proteins (1), by fusion of the TA with MBP (2) or as sole TA using the gene III signal sequence (3) Protein TA may be incorporated into the IM via E', L' or E'/L'-anchoring (1-3), biotinylated AG can be attached to E'-FXa-StrpA membrane anchors (1), DNA carrying the lac operator site can be attached to L'-anchored lacI repressor molecules (3); whole figure from [48]

Subunit vaccines are normally poor immunogenic and need adjuvants to potentiate the immune response to target antigens. The diverse capacity of BGs carrying foreign antigens combined with their intrinsic adjuvant properties can be used to design new vaccine candidates without the need of additional adjuvants.

3.5   pMal-p2x Protein Fusions

The pMal-p2x vector provides the prerequisite for expression of proteins from cloned genes in the PPS. Insertion of the gene downstream from the *malE* gene, encoding the maltose binding protein (MBP), results in the expression of a MBP fusion protein [49, 50].

The vector expresses the *malE* gene with its signal sequence fused to the *lacZα* gene. A polylinker site between *malE* and *lacZα* provides restriction sites for inserting the gene of interest. By using an α-complementing host insertion results in a blue to white color change of the colonies when using Xgal plates. The system uses the strong "P$_{TAC}$" promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences after induction with IPTG [51, 52]. The vector is also carrying the lacIq gene coding for overexpression of the Lac repressor, keeping the expression in absence of IPTG low.

pMal vectors are available in different versions [53]. For this work pMal-p2x was used which includes the signal sequence for transport to the periplasmic space. Successful export to the periplasm allows folding and disulfide bond formation of the protein. A schematic drawing of pMal-p2x is shown in Fig 3-5, a more detailed illustration of the MCS with marked restriction sites used in this work is given in Fig 3-6.
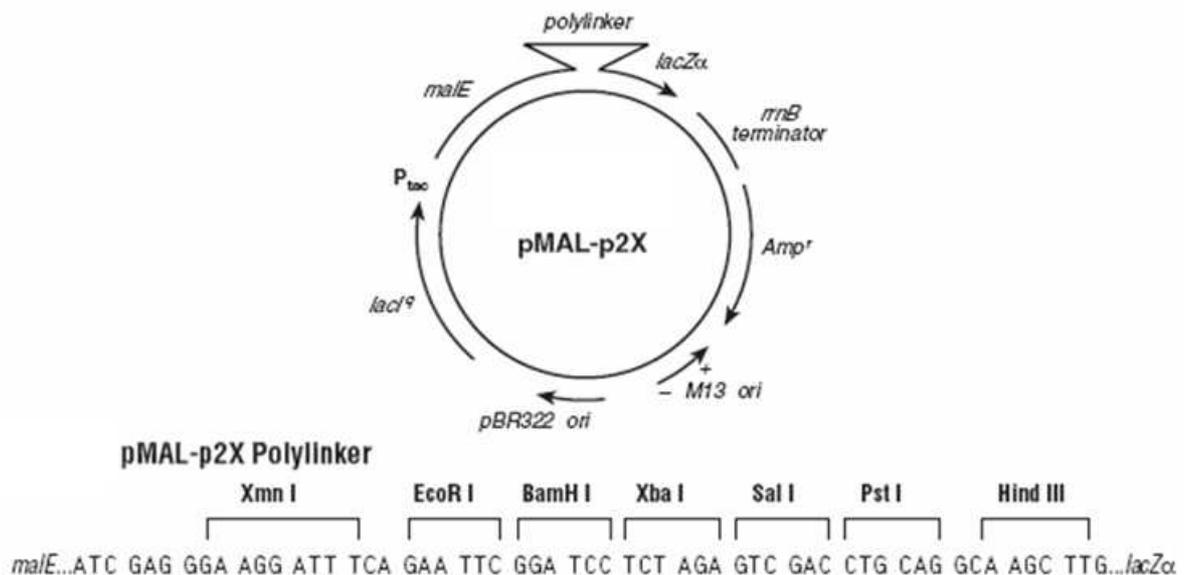
Fig 3-5 pMal-p2x vector. Arrows indicate the direction of transcription. Unique restriction sites in the polylinker are listed in the line at the bottom [53].
malE: gene coding for MBP lacZα: gene encoding the lacZα protein rrnB terminator: a portion from the rrnB operon, containing two terminators derived from the vector pKK233-2, prevents transcription originating from P$_{TAC}$ from interfering with plasmid functions Amp^r: ampicillin resistance cassette M13 ori: intergenic region, origin of replication (+ -) pBR322 ori: origin of replication (pMB1) lacIq: repressor of lac operator P$_{TAC}$: promotor of malE
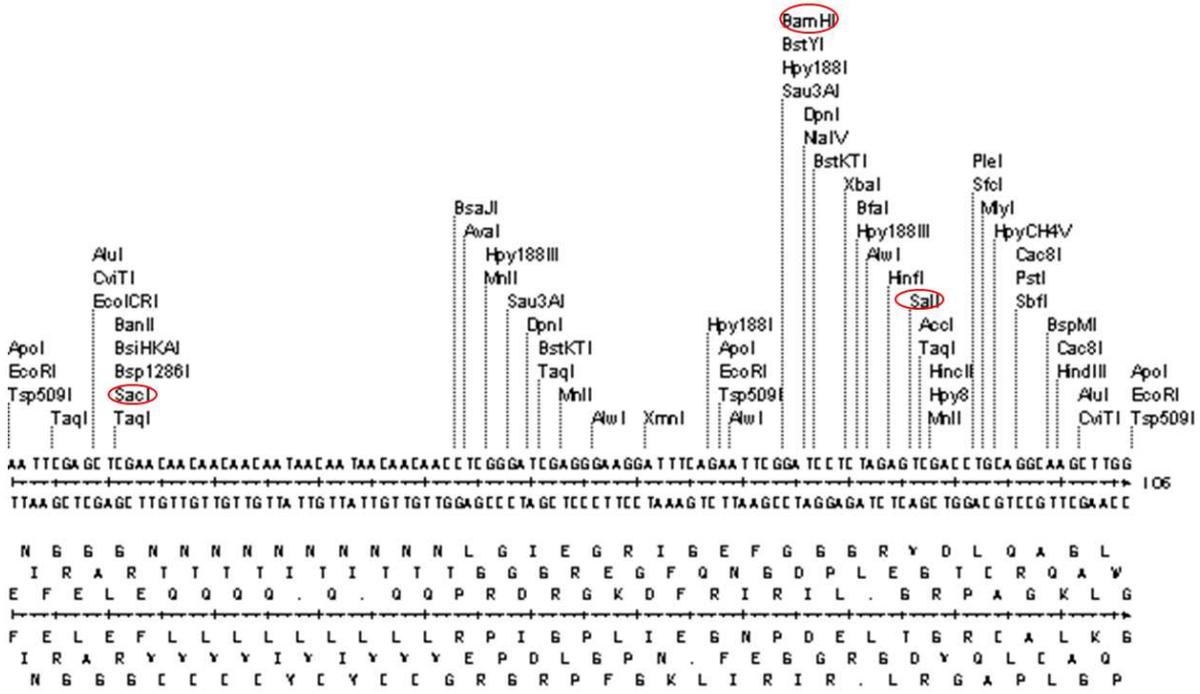
Fig 3-6 Multiple cloning site of the vector pMal-p2x. Restriction enzymes indicated by red frames were used in this work for insertion of DNA coding for epitopes of the tumor oncogen Her-2/neu. SacI and SalI were used to insert p467; BamHI and SalI to insert hm.

4.   Results

4.1   Theoretical design of new expression plasmids carrying peptide epitopes of the oncogenic protein Her-2/neu: pMal-467and pMal-467ivb

DNA fragments coding for extracellular domains of the human tumor antigen Her-2/neu were cloned into the ORF of *malE* of two vectors, pMal-p2x and pMal-ivb [54], targeting expression of MBP fusion proteins. Preliminary studies at the Medical University of Vienna suggested three peptide epitopes which induced humoral immune response with anti tumor activity in their study [55]. Sequences of these peptides 4, 6 and 7 (p467) were coupled and linker sequences GGGGGC and GGG were inserted for effective epitope exposure (Fig 4-1).

Peptide 4: SSPESFDGDPASNTAPLQPGGGGGCGS

Peptide 6: RVLQGLPREYVNARHCSR

Peptide 7: GGGYMPIWKFPDEEGACVD
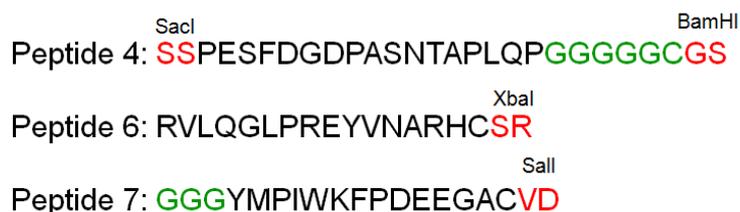
Fig 4-1 Peptide sequences of p4, p6 and p7. Peptide sequences are shown in black, green letters show the linker sequences and red letters the sequences of the restriction sites SacI, BamHI, XbaI and SalI, which were inserted for this work.

The sequence was codon optimized for *E. coli*, using the software GeneRunner. Additional restriction sites (SacI, BamHI, XbaI and SalI) were inserted together with a cystein after SacI to bring the Her-2/neu fragment into the *malE* reading frame to allow transport of the fusion protein into the PPS. The range of Her-2/neu peptide sequences plus the DNA sequences (194bp) used in the following work is shown in Fig 4-2. Backbone vectors pMal-p2x and pMal-ivb and resulting final expression vectors pMal-467-lacZα and pMal-467ivb-lacZα are shown in Fig 4-3 and Fig 4-4. They lack a stop codon before *lacZα* and therefore the recombinant proteins contain the lacZα at the C-terminus. To simplify matters the pMal- fusions will hence be indicated without lacZα in the text.

Fig 4-2 p467: Amino acid and DNA sequence of peptides 4, 6 and 7. The sequence was translated into *E. coli* codon usage, linked with appropriate restriction sites and a cystein after the SacI restriction site was inserted for cloning it into the correct ORF of *malE*. The length of the multiepitope insert p467 is 194bp.

Fig 4-3 Cloning of pMal-467. Insertion of the DNA sequence of 467 into the MCS of the backbone vector pMal-p2x was done using the restriction enzymes SacI and SalI. Gene *malE* is under the control of the IPTG inducible P_{TAC} promotor. Addition of 2mM IPTG leads to expression of the gene product MBP-467-lacZα

LacIq: repressor P_{TAC}: promotor of malE: gene encoding the N-terminal part of Maltose Binding Protein LacZα: gene encoding the lacZα protein Amp: ampicillin resistance cassette M13: intergenic region, origin of replication (+ -) pMB1: origin of replication rop: regulation of plasmid DNA replication by antisense RNA.

Fig 4-4 Cloning of pMal-467ivb. Insertion of the DNA sequence 467 into the MCS of the backbone vector pMal-ivb was done using the restriction enzymes SacI and SalI. Gene *malE* is under the control of the IPTG inducible P_{TAC} promotor. Addition of 2mM IPTG leads to expression of the gene product MBP-467ivb-lacZα.
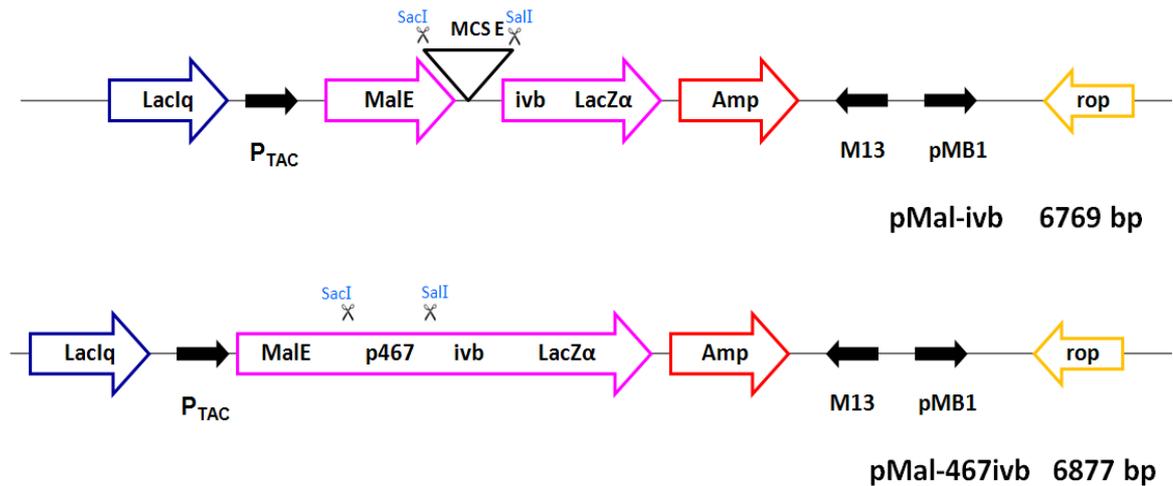
LacIq: repressor P_{TAC}: promotor of malE: gene encoding the N-terminal part of Maltose Binding Protein LacZα: gene encoding the lacZα protein Amp: ampicillin resistance cassette M13: intergenic region, origin of replication (+ -) pMB1: origin of replication rop: regulation of plasmid DNA replication by antisense RNA.

Fusion proteins MBP-467 and MBP-467ivb expression is driven by the IPTG inducible P_{TAC} promotor that is repressed in the absence of IPTG by the lacI repressor. Fusion proteins will be transported to the PPS by the signal sequence (SS) of MBP. Unified atomic mass units in Dalton of MBP, MBP-467 and MBP-467ivb and length in bp are listed in Tab 4-1. Sequence of vector pMal-p2x is provided by NEB [56] but no sequence specification of the SS for the PPS transport. According to the manual of the pMAL™ Protein Fusion and Purification System the pMal-p2x includes the "normal" SS of the periplasmic MBP of *E. coli*. Transport to the PPS leads to a truncated mature peptide devoid of the SS. Database of UniProtKB/Swiss-Prot provides the whole peptide sequence including the SS [57], which was used for calculations of the mature periplasmic protein mass in this work. Complete sequences are stored at the plasmid data base at BIRD-C. An overview of all plasmids constructed in this work is given in 7.

Peptide	Mass incl. SS [kDa]	Mass – SS [kDa]	Length incl. SS [bp]	Length –SS [bp]
Recombinant MBP- lacZα	53.4	50.6	1464	1386
E. coli **MBP**	43.4	40.7	1188	1110
MBP-467-lacZα	57.0	54.2	1572	1494
MBP-467ivb-lacZα	58.8	56.0	1620	1542
MBP*		42.5[x)]		

Tab 4-1 Atomic mass units of *E. coli* MBP and MBP fusion proteins in kDa. Mature peptides in the PPS lack SS. Recombinant MBP: maltose binding protein from the expression vector pMal-p2x, *E. coli* MBP: chromosomal encoded maltose binding protein of *E. coli*, MBP-467: MBP-467 fusion protein, MBP-467ivb: p467 fusion protein coupled to ivb, still fused to lacZα, MBP*: breakdown product of fusion protein

[x)]protein size is given in literature [53]

4.1.1 Cloning of pMal-467and pMal-467ivb

Two possibilities for acquiring the 194bp DNA of p467 were taken into consideration for cloning. One was the ordering of a synthetic ds DNA from GenArt (Regensburg, Germany). The other possibility was ordering ss DNA molecules which were completed using PCR. The latter possibility was followed first and as it was told by the customer service of VBC Genomics (Vienna, Austria) that synthesis of correct sequences up to 120bp can be guaranteed. The p467 sequence was ordered in two parts, the N-terminal part sense p467 and the C-terminal part antisense p467. A long primer for each construct was used to fill up in a first PCR reaction and two short primers were used for each construct to amplify in the second PCR reaction. Sequences of sense, antisense and primers are given in Fig 4-5 and a scheme of the PCR reactions in Fig 4-6. Restriction digest of amplified fragments p46 and p7 with XbaI was followed by ligation. The whole multiepitope p467 was then inserted in the MCS of pMal-p2x using the flanking restriction sites SacI and SalI and transformed into *E. coli* NM522. Several attempts produced constructs which showed base deletions and mismatch regions by sequencing of *malE-p467* region from "positive" clones. To overcome this problem it was decided to go for the second strategy and to order the p467 fragment synthesized by GenArt.

a)

GA GC TCC CC GGA AA GC T TT GA TGGC GA TCC GGC GA GC AAC AC CGC GC CGC TGC AGCC GGGC GGC GGC GGC GGC TGC GGA TCCC GTG TGC TGC A GGGCC TGCC GCG TGA A TA TG TGAAC GC GCG TCA TT GC

C TCGAGGGGCC T TTC GA AA C TA CC GC TAGGCC GC TCG TTG TGGC GC GGC GAC GTC GGCCC GC CGC CGCC GCC GAC GC C TA GGGCA CAC GA CGTCC CGGAC GGC GC AC T TA TAC AC TTGC GC GC AG TAA CG

SacI   P4   BamHI   P6

TC TA GA GGC GGC GGC TA TA TGC CGA TT TGGAA A TT TC CGGA TGAA GA AGGC GC GTGC GTC GA C

AGA TC TC CGCC GCC GA TA TACGGC TAAA CC TT TAA A GGCC TA C TTC T TCC GC GCA CGCAGC TG   193

XbaI   P7   SalI

b)

sense 46
5'-CCCCGGAAAGCTTTGATGGCGATCCGGCGAGCAACACCGCGCCGCTGCAGCCGGGCGGCGGCGGCGGCTGCGGATCCCGTCTGCTGCAGGGC-3'

P6 antisense
5'-GCCGCCTCTAGAGCAATGACGGCGGTTCACATATTCACGCGGCAGGCCCTGCAGCACACGGGATCCGCAGC-3'

P46 primer
5'-GCCGCCTCTAGAGCAATGACG-3'

P7 primer short
5'-ATTGCTCTAGAGGCGGCG-3'

P7 primer long
5'-TTGCTCTAGAGGCGGCGGCTATAT-3'

antisense 467
5'-TCGACGCACGCGCCTTCTTCATCCGGAAATTTCCAAATCGGCATATAGCCGCCGCCTCTAGAGCAATGACGCGCGTTCACATATTCACGCGGCAG-3'

5' 467
5'-GACGACGAGCTCCCCGGAAAGCTTTGAT-3'

3' 467
5'-ATAACAGTCGACGCAGCGGCCTT-3'

Fig 4-5 Sequence of the multiepitope p467 and sequences used for construction of the multiepitope. a) shows the whole multiepitope with restriction sites which was aimed to construct with the sense, antisense and corresponding primers shown in b)

a)

5'467 primer

SacI   sense 467   BamHI

BamHI   P6 antisense   XbaI

p46 primer

b)

P7 primer short

P7 primer long

XbaI   antisense 467   SalI

3'467 primer

c)

SacI   P4   BamHI   P6   XbaI   P7   SalI

Fig 4-6 Construction of the multiepitope p467 by PCR. a) In the first PCR reaction the sense467 and overlapping P6antisense was filled up indicated by the pink dotted line generating the p46 fragment. The second PCR was performed with 5'467primer and p46primer and amplified the p46 fragment. b) in the first PCR antisense 467 and the long overlapping P7primer was filled up indicated by the pink dotted line generating the P7 fragment. The second PCR reaction amplified the P7 fragment. Restriction of amplified fragments from a) and b) with XbaI and following ligation was expected to give the whole multiepitope p467 shown in c)

The synthetic 194bp DNA was assembled upon demand (Fig 4-2) from synthetic oligonucleotides by GenArt. The fragment was cloned by GenArt into pPCR-Script using SacI and KpnI restriction sites flanking the p467 sequence. The plasmid 053935pPCR-Script was purified from transformed bacteria and the final construct verified by sequencing. 10µg of the plasmid were lyophilized and delivered by GeneArt. Fig 4-7 shows the plasmid map of the delivered synthetic DNA.
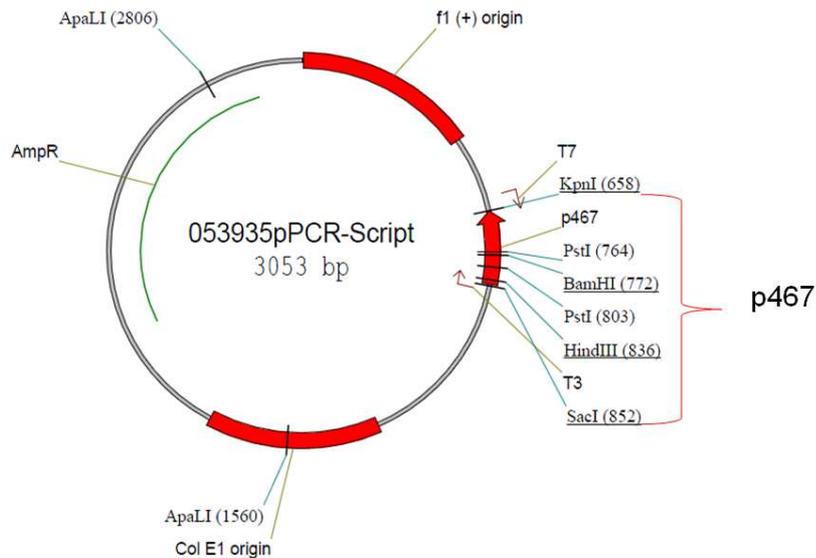

Fig 4-7 Synthetic gene p467 cloned into pPCR-Script using KpnI and SacI

10µg of the lyophilized pPCR-Script vector were resuspended in 50µl sterile H_2O, left for one hour at room temperature and stored at -20°C for further use. 5µl of the solution were used for MOPS transformation into *E. coli* NM522. Positive clones were selected on LB agar plates with ampicillin. The p467 fragment was obtained by double digest of the pPCR-Script vector using the enzymes SacI and SalI, which were included in the sequence for cloning reason. The vectors pMal-p2x and pMal-ivb were also prepared for cloning by cutting with SacI and SalI. The unique restriction sites in the MCS of both vectors ensure orientated cloning of p467 into the ORF of *malE*. Separation of fragment and vectors was done by gel electrophoresis (Fig 4-8 and Fig 4-9) and purification by subsequent extraction using PCR Purification Kit from Quiagen (Duesseldorf, Germany).
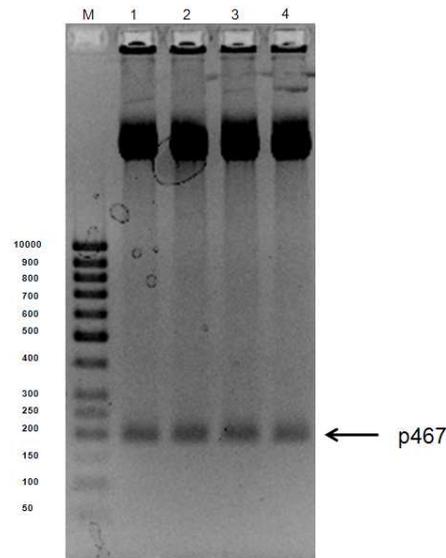
Fig 4-8 Restriction analysis of pPCR-Script vector on 2% agarose gel. The product of the double digest of pPCR-Script vector was loaded on lane 1-4. For DNA sizing a 50bp DNA ladder was loaded. The 194bp fragment p467 is visible at the right size of ~ 200bp. M: 50bp ladder
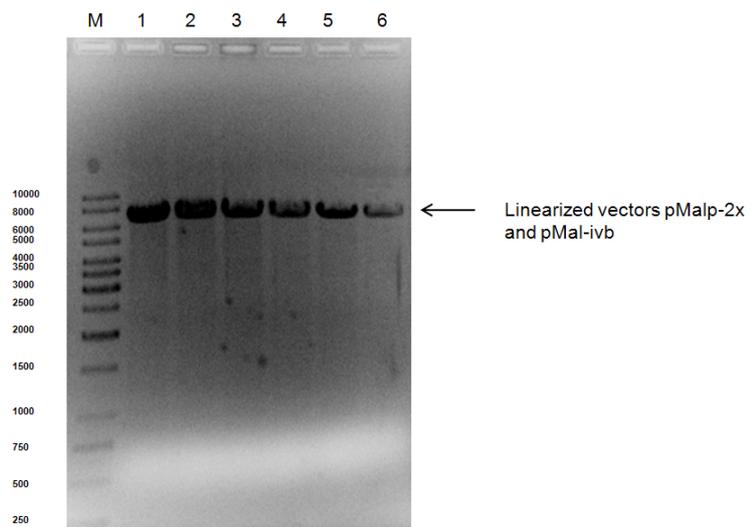

Fig 4-9 Restriction analysis of pMal-p2x and pMal-ivb vectors on 1% agarose gel. The product of the double digest with SacI and SalI of vectors pMal-p2x (lane1 – 3) and pMal-ivb (lane 4 – 6) are visible as linearized vectors at the correct size. Vectors pMalp2x (6646bp) and pMal-ivb (6694bp) show the expected size and are reduced in size by a 78bp fragment lying between SacI and SalI . M: 1kb ladder

Ligation of vectors pMal-p2x and pMal-ivb with the fragment p467 was done with the molar ratio of vector: insert = 1: 10. Both ligation products, vector pMal-467 and pMal-467ivb, were then transformed into *E. coli* NM522, purified by minipreps and afterwards tested for the correct size by restriction digest. Cutting with the restriction enzyme Eco01009I indicates a positive clone by giving a special restriction fragment pattern for

the vectors. Vector pMal-467 cut with Eco01009I should result in 3 fragments, 3964/2821/42, whereas the backbone vector pMal-p2x without p467 would result in two fragments, 6679/42. Vector pMal-467ivb cut with Eco01009I should result in 3 fragments, 4012/2821/21, whereas the backbone vector pMal-ivb without p467 would result in two fragments, 6727/42. Another gel electrophoresis confirmed positive sized clones for these ligations (Fig 4-10 and Fig 4-11); digest of backbone vectors is not shown. As the p467 fragment was supplied with a sequence protocol positive clones were not sequenced once again.
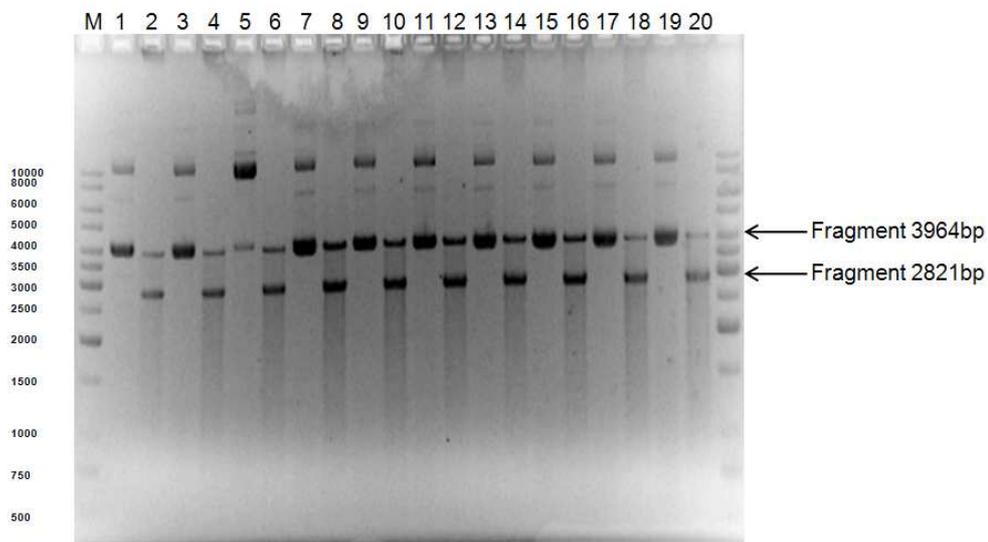
Fig 4-10 Restriction analysis of pMal-467 on 1% agarose gel. 10 clones were cut with Eco01009I to confirm correct size of plasmids. Lane 1 – 20 show clone 1 – 10 consecutively loaded: odd numbers are uncut and following even numbers are the cut version of the same clone. (M 1kb ladder)
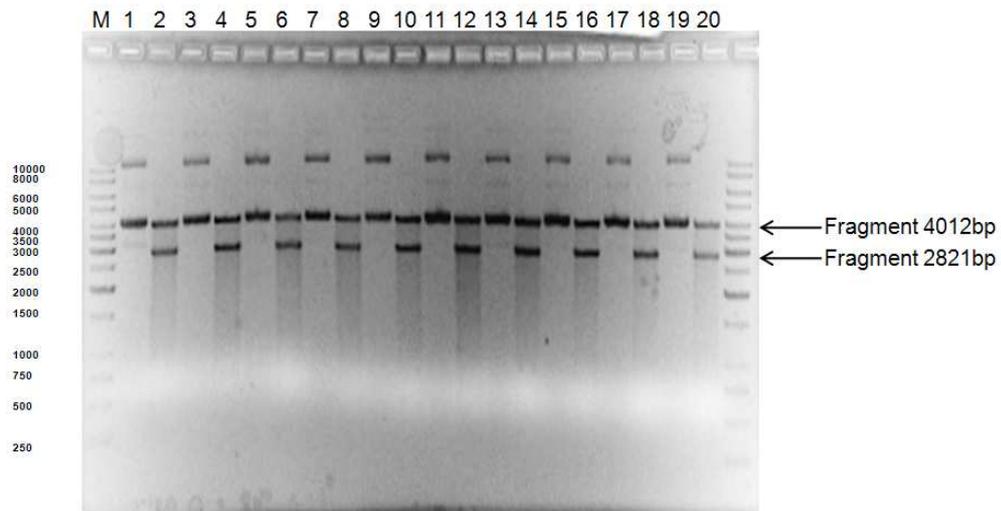
Fig 4-11 Restriction analysis of pMal-467ivb 1% agarose gel. 10 clones were cut with Eco01009I to confirm correct size of plasmids. Lane 1 – 20 show clone 1 - 10 consecutively loaded: odd numbers are uncut and following even numbers are the cut version of the same clone. (M 1kb ladder)

Plasmids isolated from *E. coli* NM522 (pMal-467) c1 and *E. coli* NM522 (pMal-467ivb) c5 were isolated, freshly transformed into *E. coli* NM522 and five clones of each stored as glycerin stocks at -80°C. Growth and expression was analyzed in noseflask experiments.

4.1.2   Growth and expression of *E. coli* NM522 (pMal-467) and *E. coli* NM522 (pMal-467ivb)

The experiment was carried out in noseflasks containing 30ml LB and ampicillin (100µg/ml). Three clones of each construct were tested. To evaluate possible effects of recombinant protein expression on the cell viability and to verify expression of the recombinant protein, periodic sample were taken for western blot analysis, cfu determination and OD measurements according to 6.3.3.

Expression of the recombinant protein MBP-467/MBP-467ivb of the vector pMal-467/pMal-467ivb was induced by addition of 0.2mM IPTG when the cultures reached an OD_{600} of about 0.2. Growth diagram in Fig 4-12 shows that expression of the fusion protein had no negative effect on cell growth in OD as well as in cfu. Western blot samples were separated by a 15% SDS – PAGE. WBs analyses in Fig 4-13 and Fig 4-14 proof expression of the MBP-467 and MBP-467ivb fusion proteins. The ivb-sequence is biotinylated in *E. coli* in vivo and this biotin can be detected in WB using streptavidin. Natural occurrence of the carboxyl carrier protein (BCCP) in *E. coli* is used for internal

control for ivb detection. BCCP is encoded by the *accB* gene and is a component of acetyl CoA carboxylase. Samples exhibit protein bands at ~58kDa, which reacted in western blot analysis with αMBP and α rabbit IgG-HRP, respectively streptavidin-HRP. The WB also shows that the P_{TAC} promotor is not tightly repressed before IPTG induction to reduce preinduced expression of MBP-467 and MBP-467ivb. This suggested addition of glucose to the overnight culture for following studies.
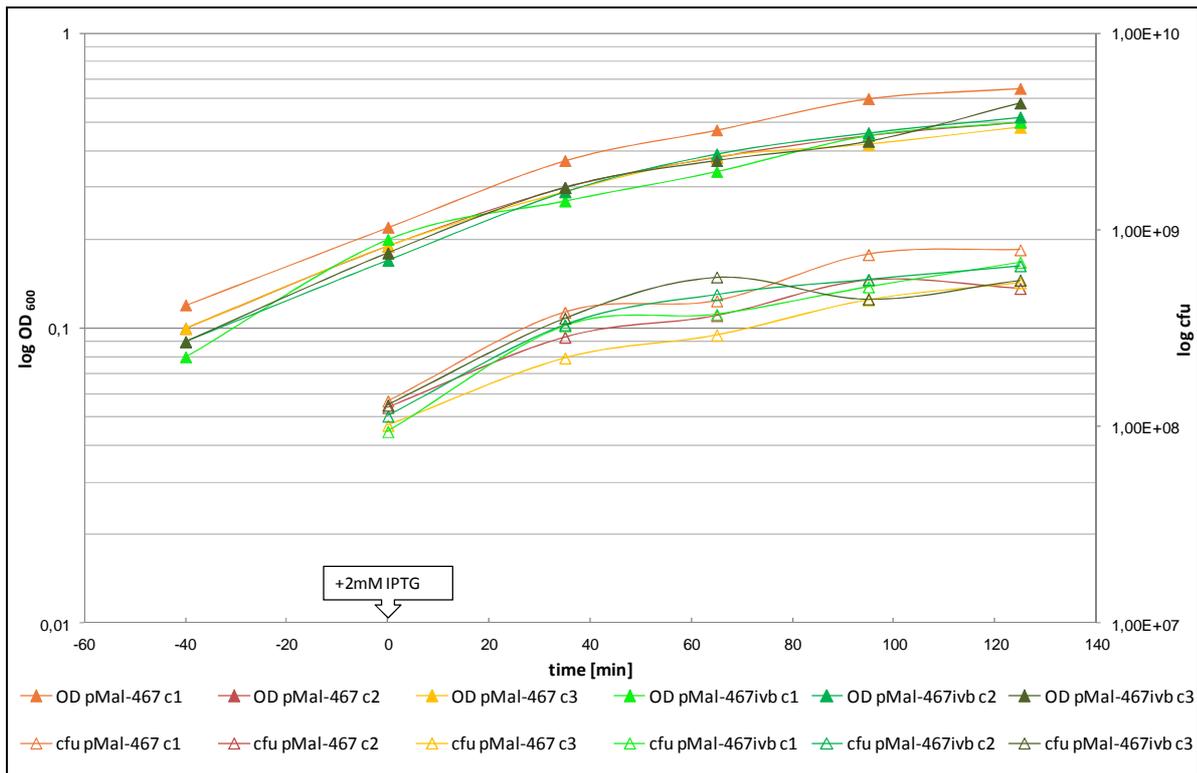

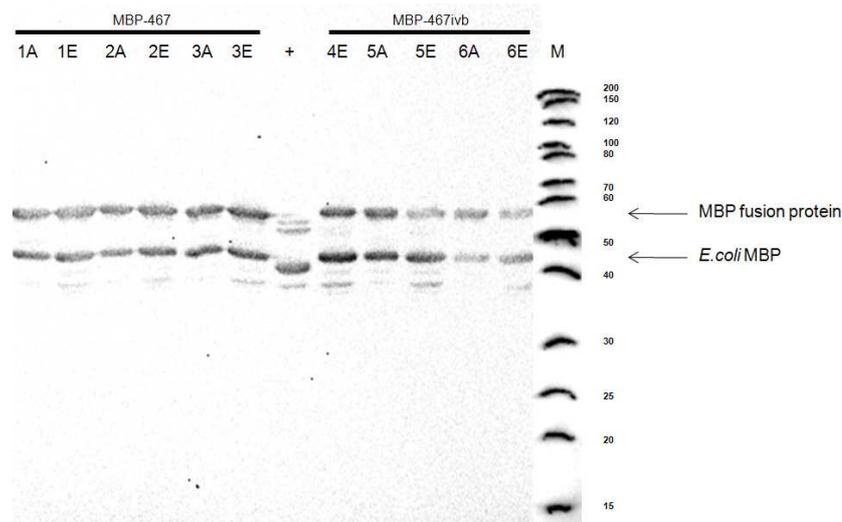Fig 4-12 Growth curve of *E. coli* NM522 (pMal-467) and *E. coli* NM522 (pMal-467ivb).

Fig 4-13 Western blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-467) and *E. coli* NM522 (pMal-467ivb). Cells expressing the MBP-467 fusion protein (~58kDa), 1, 2 and 3 prior (A) and after (E) induction. Cells expressing the MBP-467ivb fusion protein (~59kDa), 4, 5 and 6 prior (A) and after (E) induction. Positive control (+) expressing the MBP (~54kDa) of the backbone plasmid pMalp2x. The presence of the MBP portion of the fusion protein and the naturally occurring MBP (~43kDa) was examined with αMBP and α rabbit IgG-HRP.
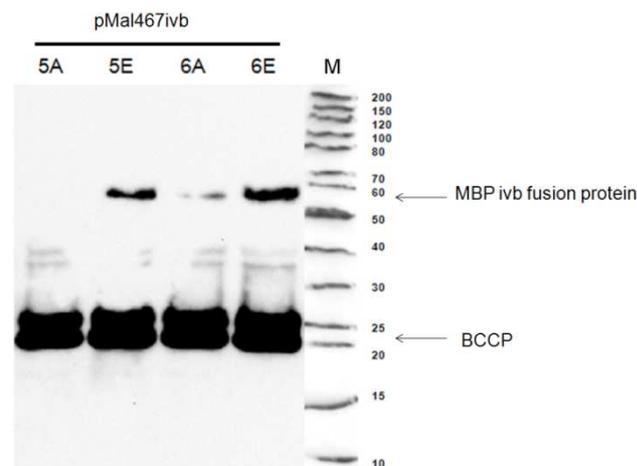

Fig 4-14 Western blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-467ivb). Cells expressing the MBP-467ivb fusion protein (~59kDa), 5 and 6 prior (A) and after (E) lysis induction. The presence of the biotin portion of the fusion protein was examined with streptavidin-HRP. The biotin carboxyl carrier protein (BCCP) of *E. coli* can be detected at 22,5kDa.

4.1.3   Recombinant protein expression and lysis of *E. coli* NM522 (pMal-467)

Electro competent *E. coli* NM522 were transformed with pMal-467 and pGLysivb. Five positive clones plus *E. coli* NM522 (pMalp2x, pGLysivb) as control were tested for lysis efficiency and recombinant protein expression in this study. The experiment was carried out in noseflasks containing 30ml LB, ampicillin (100µg/ml) and gentamycin (20µg/ml).

Periodic samples were taken (according to 6.3.3) to calculate the lysis efficiency (LE) and to detect the expression of the fusion protein MBP-467on WB.

Expression was induced by adding 2mM IPTG when OD_{600} was about 0.2. Lysis was induced by temperature up shift from +36°C to +42°C 60 min after induction of recombinant protein expression (time point 0 min). The growth and lysis diagram in Fig 4-15 illustrates ongoing growth until lysis induction, followed by a cfu decay and drop of optical density, indicating lysis. The LE was determined as follows and calculations are shown in Tab 4-2.

$$LE = \left(1 - \frac{cfu_{(t)}}{cfu_{(t_0)}}\right) \cdot 100$$

t_0...time point of lysis induction
t ...time point after lysis induction used for determination of LE; for this work t =120 min after induction

Clone	LE [%]
E. coli NM522 (pMal-467, pGLysivb) c1	99,84
E. coli NM522 (pMal-467, pGLysivb) c2	99,05
E. coli NM522 (pMal-467, pGLysivb) c3	99,90
E. coli NM522 (pMal-467, pGLysivb) c4	97,07
E. coli NM522 (pMal-467, pGLysivb) c5	99,66
E. coli NM522 (pMalp2x, pGLysivb)	99,73

Tab 4-2 Lysis efficiencies (LE) calculated with cfu counts from samples before (0 min) and 120 min after LI
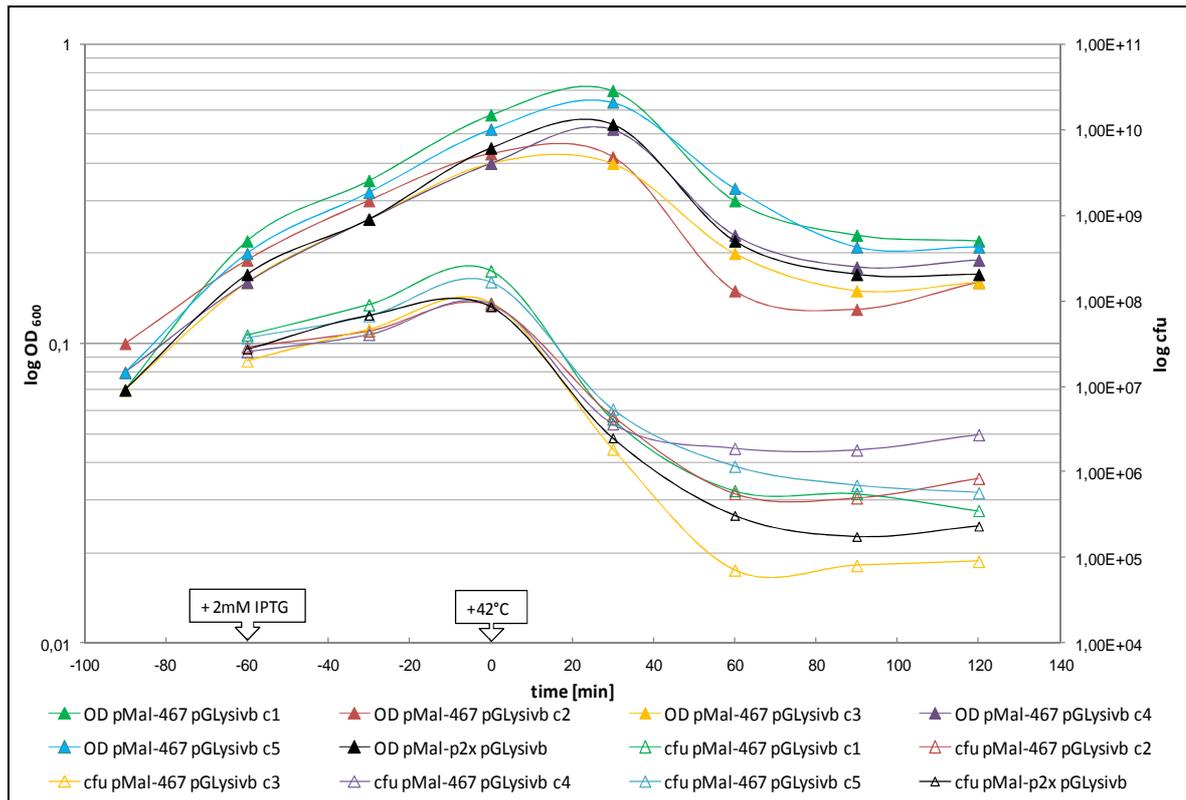
Fig 4-15 Growth and lysis of *E. coli* NM522 (pMal-467, pGLysivb) c1 – c5 and control *E. coli* NM522 (pMalp2x, pGLysivb). Addition of 2mM IPTG was done at time point -60 min and temperature up shift from +36°C to +42°C at time point 0 min. The drop of cfu counts and optical density indicates lysis.

A pre-cast 12% NuPAGE® Bis-Tris Gel was used for protein separation. The nitrocellulose membrane was stripped (6.2.5.3) allowing detection of the fusion protein with different anti sera on the same membrane. The fusion protein reacted in western blot analysis with rabbit anti p467 serum (Fig 4-16), mouse anti p467 serum (Fig 4-17) and purified rabbit anti p467 serum (Fig 4-18). *E. coli* NM522 (pMalp2x, pGLysivb) served as a control for growth and lysis and samples were not analyzed by WB. The strongest signal showed clone 2 by detection with the mouse serum, whereas the same clone showed the weakest signal by detection with rabbit serum. The mouse serum showed the least amount of unspecific bands and therefore lowest background signals. Samples taken after induction of expression exhibited a protein band at ~58kDa (and its degradation product below at ~50kDa) for all sera which was absent in samples taken prior to induction.
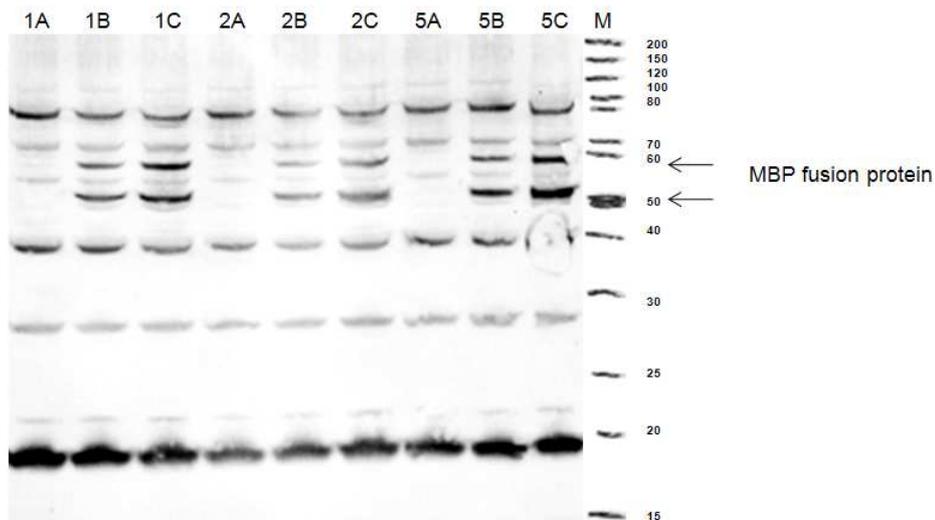
Fig 4-16 Western blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-467, pGLysivb). Bacteria expressing the MBP-467 fusion protein (~58kDa), clone 1, 2 and 5 prior (A), 30 min after (B) and 60 min after (C) induction of expression. The presence of the 467 portion of the fusion protein was examined with rabbit anti p467 serum (1:100) and anti rabbit IgG-HRP (1:20000).
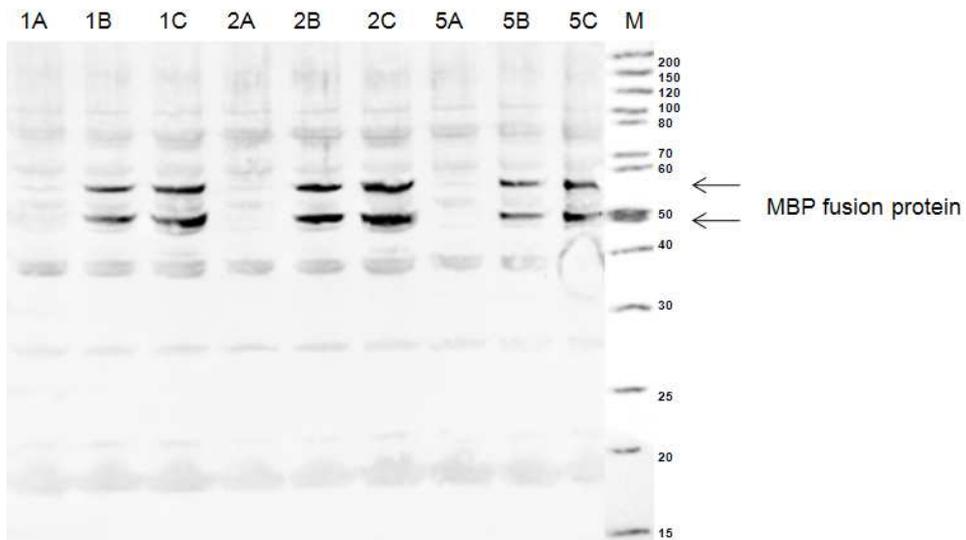

Fig 4-17 Western blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-467, pGLysivb). Bacteria expressing the MBP-467 fusion protein (~58kDa) clone 1, 2 and 5 prior (A), 30 min after (B) and 60 min after (C) induction of expression. The presence of the 467 portion of the fusion protein was examined with mouse anti p467 serum (1:500) and anti mouse IgG-HRP (1:3000).
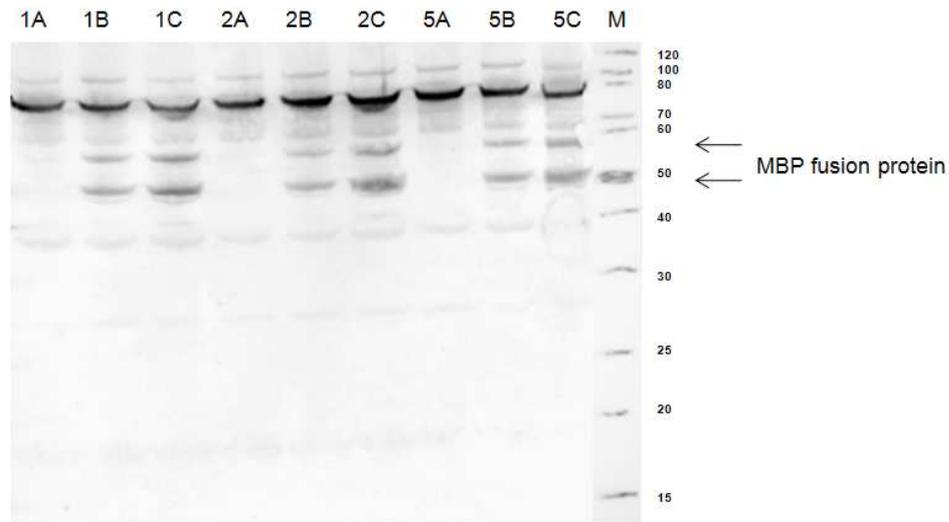
Fig 4-18 Western blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-467, pGLysivb). Bacteria expressing the MBP-467 fusion protein (~58kDa) clone 1, 2 and 5 prior (A), 30 min after (B) and 60 min after (C) induction of expression. The presence of the 467 portion of the fusion protein was examined with purified rabbit anti p467 serum (1:50) and anti rabbit IgG-HRP (1:20000).

4.1.4   Production of *E. coli* NM522 (pMal-467, pGLysivb) BGs

E. coli NM522 (pMal-467, pGLysivb) BGs were produced in 20.0l low density batch fermentation using a 30l Techfors-S fermenter (6.3.4, 6.3.5). Fermentation was performed in LB medium, supplemented with gentamycin (20µg/ml) and ampicillin (100µg/ml). Pre-cultures were grown in 4x500ml LB medium overnight, supplemented with gentamycin (20µg/ml), ampicillin (100µg/ml) and glucose (1% w/v for repression of recombinant protein expression). Inoculation of the fermenter was done with 870ml overnight culture reaching an optical density of ~ 0.1. Bacteria were grown at +35°C, at constant air flow of 5l/min and increased stirring rates, 100 to 300 rpm. Recombinant protein expression was induced by addition of 2mM IPTG 60 min prior LI. Lysis was induced by temperature up shift to +42°C. Final killing was done 120 min after LI by adding streptomycin and tetracycline (200ug/ml and 20ug/ml end concentration, respectively, and incubated for another 120 min. Online process data are monitored by the IRIS software and displayed in Fig 4-19.

Generally the increase of oxygen in of the media during cultivation goes along with cell death and is expected after LI, as bacteria are no longer consuming oxygen. Although earlier studies proved no negative effect for this fusion protein expression on cell viability, oxygen increased when protein expression was induced at point -60min. The loss of

viability prior and after LI was calculated and is shown in Fig 4-20 and Tab 4-3. Reduction of viability during 60 min of recombinant protein expression was 42.08%. This value needs to be viewed critically since it is received from agar plates that were incubated over night and could therefore be attributed to a long term effect on bacteria. Thirty min of LI resulted in a significant decrease of living cell counts gaining a total killing of 98.69%. After 60 min lysis the concentration of cfu dropped below the counting range which gives reliable data to make extrapolations for the used sample dilutions. According to the manufacturer's deposition parameters (Don Whitley Scientific), the counting range lies between 4.10^2 and $4x10^5$ cfu/ml. For the 10^2 dilution, used for plating after LI, the range lies between $4x10^4$ and $4x10^7$cfu/ml. From this it follows that no detectable cfu values at 60, 90 and 120 min after lysis correspond to actual values below $4x10^4$ cfu/ml. Predominantly BGs were seen when investigating the sample from 120 min after LI through light microscopy (Fig 4-21).
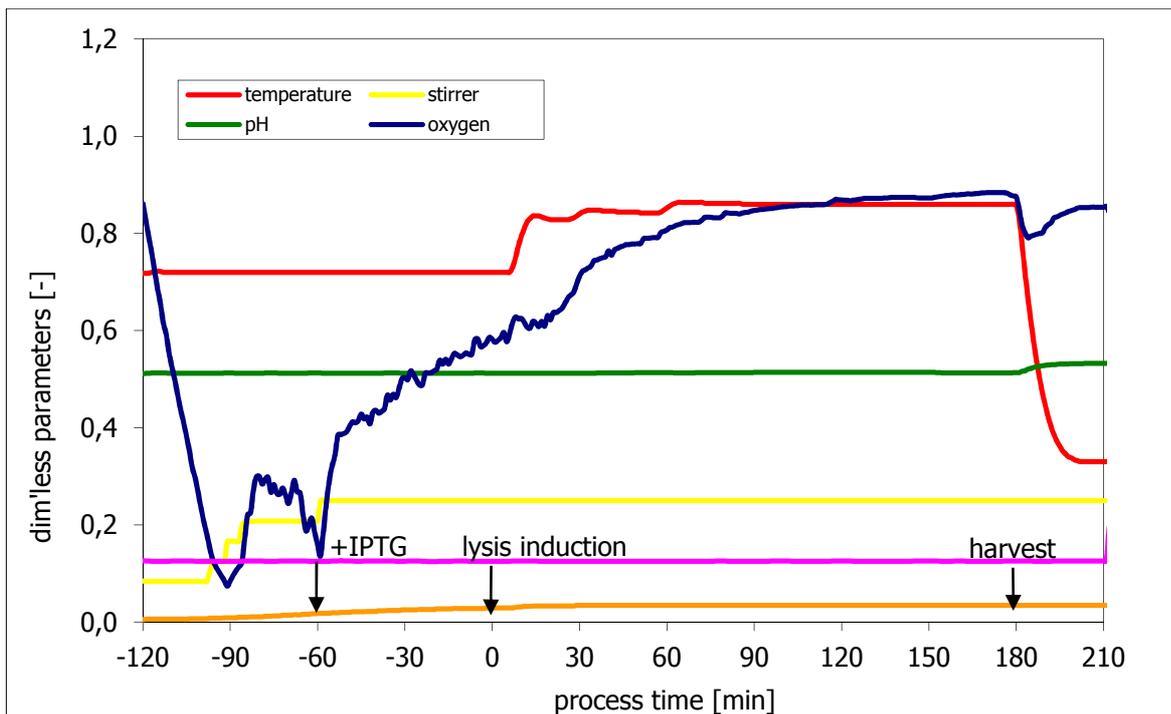

Fig 4-19 IRIS data curve of *E. coli* NM522 (pMal-467, pGLysivb) fermentation.
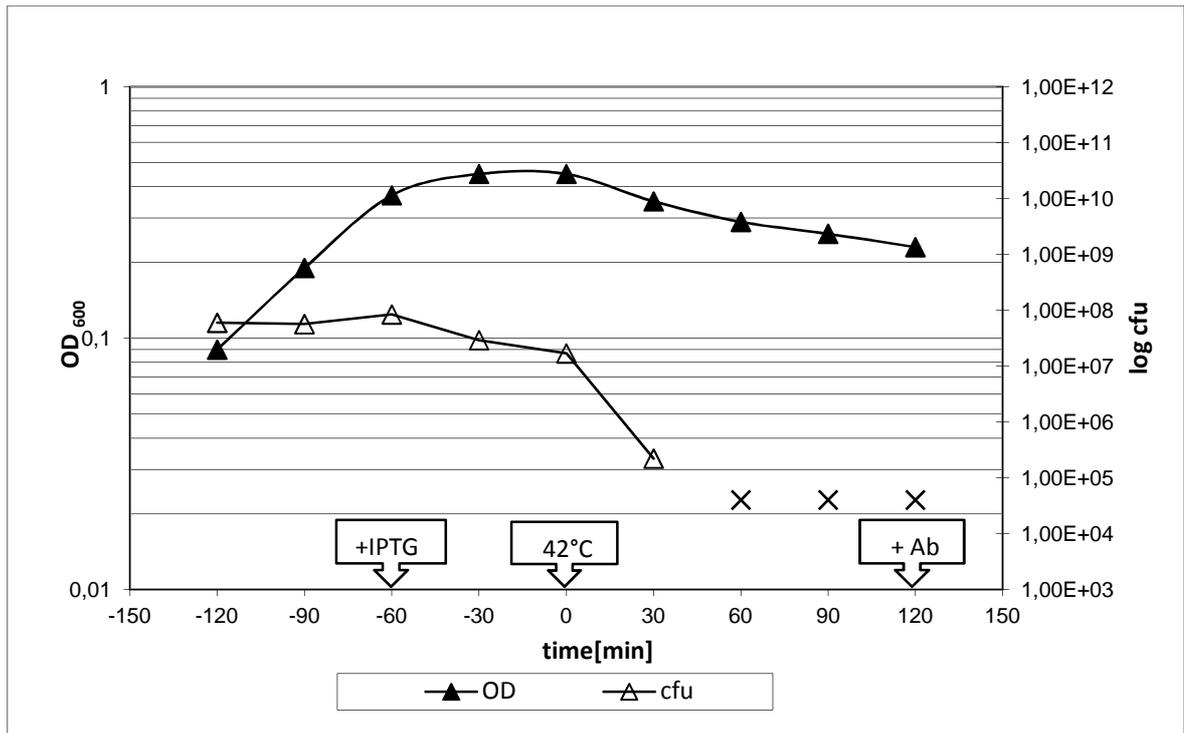
Fig 4-20 Growth and lysis curve of large scale production of *E. coli* NM522 (pMal-467, pGLysivb) BGs by fermentation.

time point	activity	cfu
-60 min	+IPTG	8,39E+07
-30 min		2,91E+07
0 min	T up shift to +42°C	1,68E+07
30 min		2,20E+05
60 – 120 min		≤ 4,00E+04 *

Tab 4-3 Cfu values during large scale BG production. Cfu dropped already before LI and continues with a significant drop of cfu 30 min after LI. The * marked value is a virtual value and defines the lowest cfu which would be detectable for a 10^2 dilution (commonly used dilution for samples after LI) lying in the counting range.

Reduction of viability [%] prior lysis from time point -60 min to 0 min:

$$\left(1 - \frac{\text{cfu}_{(t_{-60})}}{\text{cfu}_{(t_0)}}\right) \cdot 100 = 42,08$$

LE [%] within 30 min from time point 0 min to 30 min:

$$\left(1 - \frac{cfu_{(t_0)}}{cfu_{(t_{30})}}\right) \cdot 100 = 98,69$$

LE [%], including the virtual value from time point 0 min to 60 min:

$$\left(1 - \frac{cfu_{(t_0)}}{cfu_{(t_{60})}}\right) \cdot 100 = 99,76$$
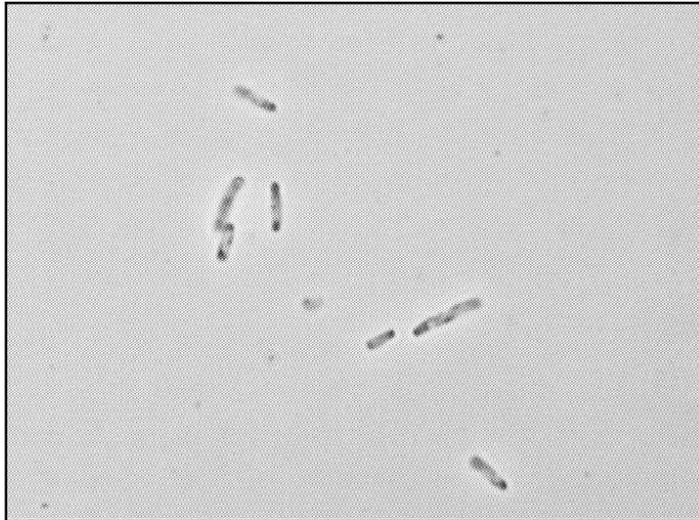

Fig 4-21 Light microscopy picture of BGs 120 min after LI.

Samples taken during fermentation were separated on a NuPAGE® 4-12%-Bis-Tris-Gel for MBP-467 detection, and on a NuPAGE® 12% Tris-Tricine-Gel for Eivb detection. Incubation of the membrane with mouse anti p467 serum and anti mouse IgG-HRP exhibits the expected band of MBP-467 at about 58kDa (Fig 4-22). The sample at time point -60 min (induction of protein expression) was taken by accident after addition of IPTG and therefore a band can be seen already at this time point. At +120 min the MBP-467 band is missing. To find out whether transfer for this membrane portion failed or the fusion protein was degraded the blot was repeated with lyophilized BG samples. Fig 4-23 shows a strong signal at ~58kDa which proves the presence of MBP-467. Incubation of the second membrane with streptavidin-HRP exhibits biotinylated E at about 13kDa. The lysis

protein accumulates after LI whereas the cytosolic BCCP (~23kDa) is released through the lysis tunnel (Fig 4-24), mainly visible after 120 min of E-lysis.
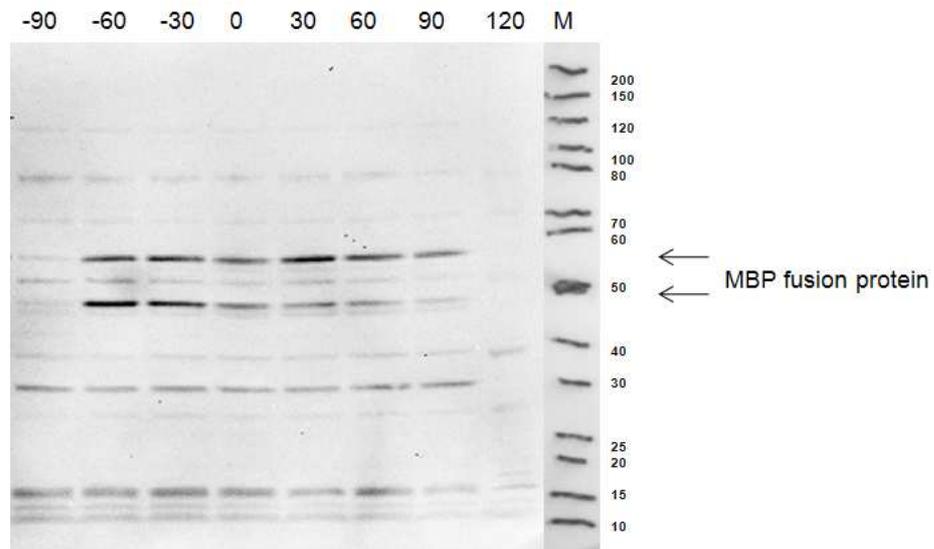

Fig 4-22 Western blot analysis of whole cell protein extracts of *E. coli* NM522 (pMal-467, pGLysivb) fermentation. Bacteria express the MBP-467 fusion protein (~58kDa) upon induction at -60 min. Time points from 90 min prior (-90 min) to 120 min after LI (120 min) are shown. The presence of the 467 portion of the fusion protein was examined with mouse anti p467 serum (1:500) and anti mouse IgG-HRP (1:3000).

Fig 4-23 Western blot analysis of whole cell protein extracts of lyophilized *E. coli* NM522 (pMal-467, pGLysivb) BGs. BGs carry the MBP-467 fusion protein (~58kDa). Lyophilized BGs were resuspended in sample buffer and loaded in different concentrations: 12µg/ml (lane 1), 1, 2µg/ml (lane 2), 0, 12µg/ml (lane 3). The presence of the 467 portion of the fusion protein was examined with mouse anti p467 serum (1:500) and anti mouse IgG-HRP (1:3000).
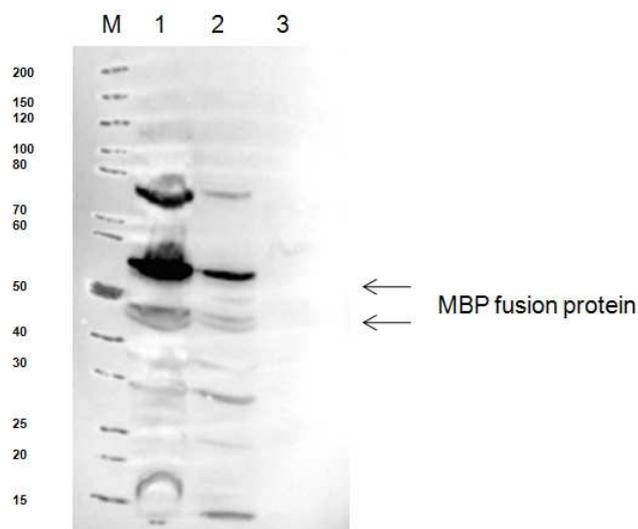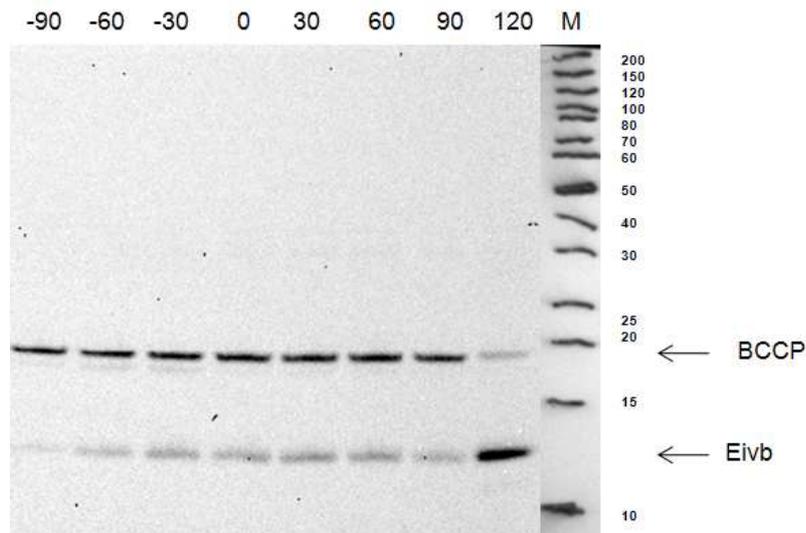
Fig 4-24 Western blot analysis of whole cell protein extracts of *E. coli* NM522 (pMal-467, pGLysivb) fermentation. Bacteria express the lysis protein Eivb (~13kDa) upon induction by temperature up shift to +42°C at time point 0 min. The presence of the biotinylation of Eivb was examined with streptavidin-HRP. Eivb is accumulating and the cytosolic protein BCCP (~23kDa) is released during lysis.

The final killing with antibiotics was successful as no survivors were detected on the agar plates inoculated with undiluted samples. Harvest of the total volume of 20l followed by washing and lyophilization yielded in the amount of 1388mg BGs dry weight. The material was proved to be sterile in sterility testing. The calculated amount of particles per mg was 1.20×10^{09}.

4.1.5 Immunization of BALB/c mice with *E. coli* NM522 (pMal-467, pGLysivb) BGs

Mice experiments were performed at the Medical University of Vienna, Department of Pathophysiology. Eight week old BALB/c mice (n=6/group), purchased from Charles River (Sulzfeld, Germany) were immunized with BGs comprising the coupled peptides 4, 6 and 7 of Her-2/neu. BGs were injected 4 times subcutaneously in intervals of 2-3 weeks using different concentrations in group A (3.0mg BG-467), group B (1.0mg BG-467), group C (0.5mg BG-467) and control group D (3mg empty BGs) per injection. Blood samples were taken after the third and fourth immunization. Seven days after the second blood sample mice were killed and spleen was removed and cells were further analyzed in cell culture.

4.1.5.1 Humoral immune response

After the third immunization a blood sample was taken and analyzed. Peptide-specific antibodies could be detected within a 1:250 diluted serum. A significant higher titer of the

experimental groups versus control group shows the effective delivery of Her-2/neu epitopes P4, 6 and 7 by BGs (Fig 4-25, Fig 4-26, Fig 4-27). The titer was too low to compile a subclass profile.

Fig 4-25 Peptide specific anti P4 antibodies (IgG) onto 3 subcutaneous applications of BGs. Applied concentration for the experimental groups was 3.0 mg (A), 1.0mg (B) and 0.5mg (C) per injection. Immunization of the control group (D) was done with 3mg empty BGs.

Fig 4-26 Peptide specific anti P6 antibodies (IgG) onto 3 subcutaneous applications of BGs. Applied concentration for the experimental groups was 3.0 mg (A), 1.0mg (B) and 0.5mg (C) per injection. Immunization of the control group (D) was done with 3mg empty BGs.
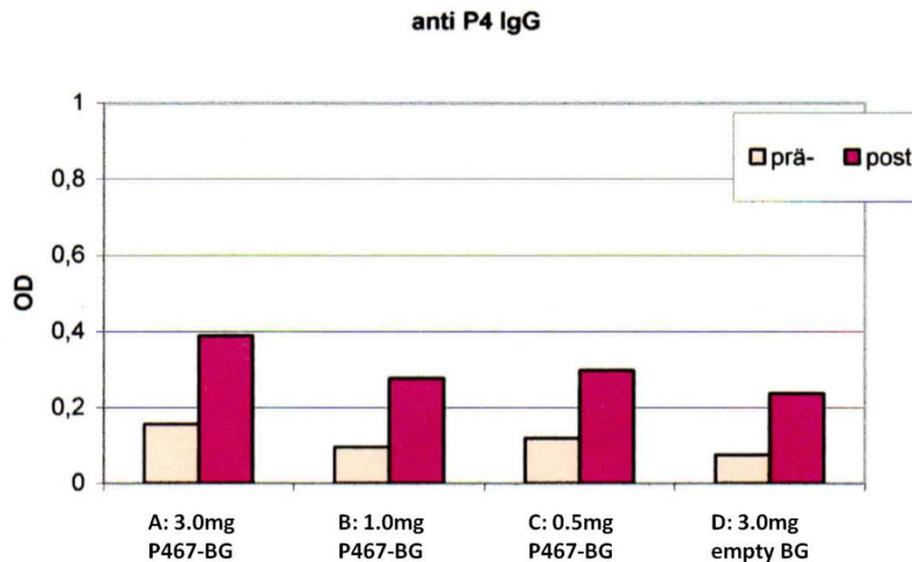
Fig 4-27 Peptide specific anti P7 antibodies (IgG) onto 3 subcutaneous applications of BGs. Applied concentration for the experimental groups was 3.0 mg (A), 1.0mg (B) and 0.5mg (C) per injection. Immunization of the control group (D) was done with 3mg empty BGs.
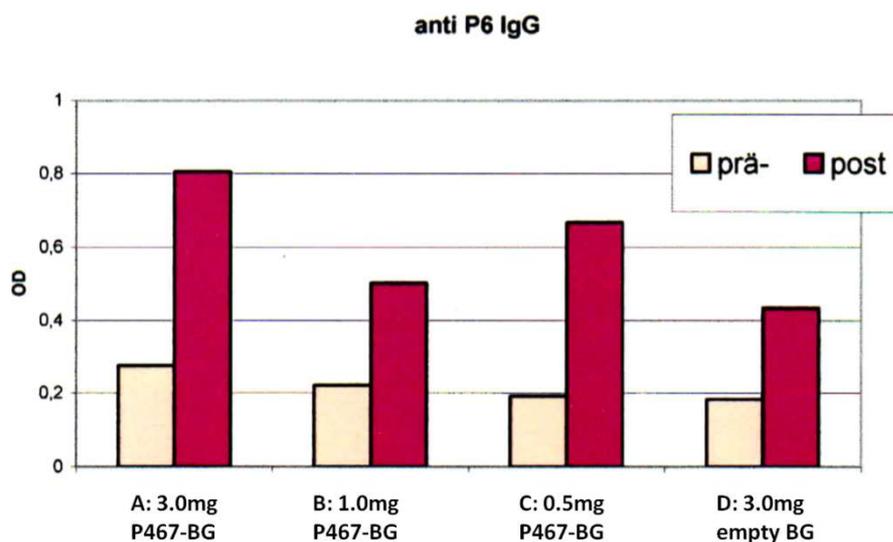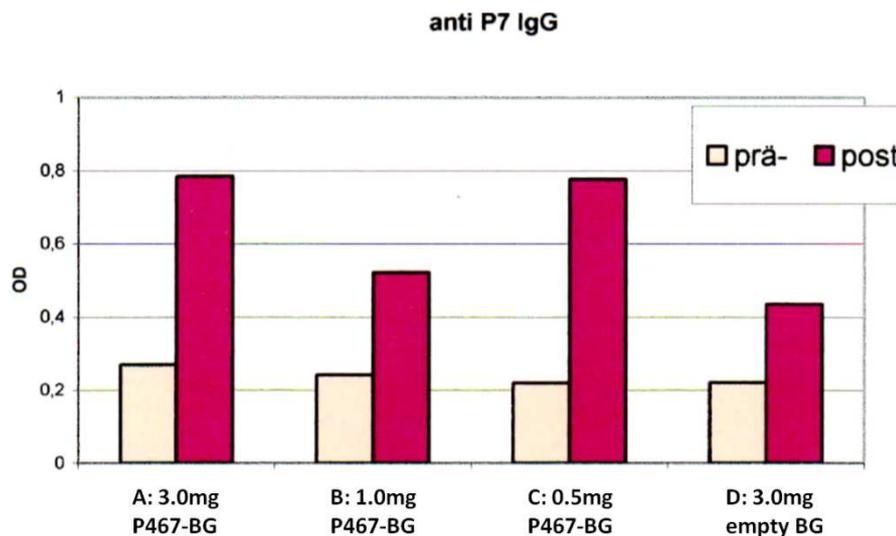
4.1.5.2 Cellular immune response

Seven days after the fourth immunization spleen was removed and tested in cell culture. Proliferation of spleen cells was measured by H^3 thymidin incorporation. In vitro stimulation with BGs-P467 did not lead to increased proliferation. IFN-gamma production by spleen cells was measured in the supernatants by ELISA. IFN-gamma production did not increase after in vitro stimulation with BGs-P467. The results correspond to earlier data and confirm the hypothesis, that the used Her-2/neu sequences display B-cell epitopes and therefore do not trigger T-cell response themselves.

4.2 Theoretical design of new expression plasmids carrying mimotopes of the oncogenic protein Her-2/neu: pMal-hm and pMal-hmivb

The DNA sequence coding for a herceptin mimotope (hm) recognized by Trastuzumab, was cloned into the *malE* ORF of vectors pMalp2x and pMal-ivb, targeting expression of MBP fusion proteins. Hms were generated using the phage display technique and selected by specifically binding to Trastuzumab at the Medical University of Vienna [31]. In this work the herceptin mimotope QMW (Fig 4-28a), further referred to as hm, circularized by disulfide bridging was supplemented with restriction sites for cloning into the expression plasmids. Linker sequences which should assist in circularization by separating the epitope from the residual fusion partner were also included. Design of hm

sequences for cloning into pMal-p2x (Fig 4-28b) and into pMal-ivb (Fig 4-28c and d) is shown below. Sequencing clones carrying the construct displayed in Fig 4-28c showed an unwanted stop mutation. Therefore the hm sequence displayed in Fig 4-28b was used for amplification via PCR (Primer sequences see Fig 4-29), allowing to exclude the stop, yielding to hm sequence as shown in Fig 4-28d. PCR product was used for cloning into the vector pMal-ivb.

a) **C-QMWAPQWGPD-C (1-12 cyclo)**

b) BamHI   SalI
GGGPGPGCQMWAPQWGPDC . . VD

c) BamHI   SalI
GGGPGPGCQMWAPQWGPDCGPGPGVD

d) BamHI   SalI
GGGPGPGCQMWAPQWGPDCVD

Fig 4-28 a) Peptide sequence of the herceptin mimotope (hm) b) hm supplemented with restriction sites BamHI and SalI (red letters), a linker sequence (green letters) and two stop codons, indicated by red dots, for cloning into vector pMalp2x c) hm supplemented with restriction sites BamHI and SalI (red letters), and linker sequences (green letters) for cloning into the vector pMal-ivb d) sequence for cloning hm into the vector pMal-ivb using PCR of the hm sequence displayed in b)

hm 5' primer: cagaattcggatccggccc

hm 3' primer: tatggtcgacgcaatccgggcccact

Fig 4-29 Primer designed for amplification of hm figured in Fig 4-28b to exclude the stop codon for coupling MBP-hm to ivb of the vector pMal-ivb. Sequence verified vector pMal-hm was used as target.

By designing the sequence theoretically using the software GeneRunner, the sequence was codon optimized for *E. coli*. The hm sequences were inserted into the ORF of *malE* to allow transport of the fusion protein into the PPS. The range of the hm peptide sequence plus the DNA sequence of the hm fusion peptide used in the following work is shown in Fig 4-30. Backbone vectors and final expression plasmids (pMal-hm, pMal-hmivb) are illustrated in Fig 4-31 and Fig 4-32.
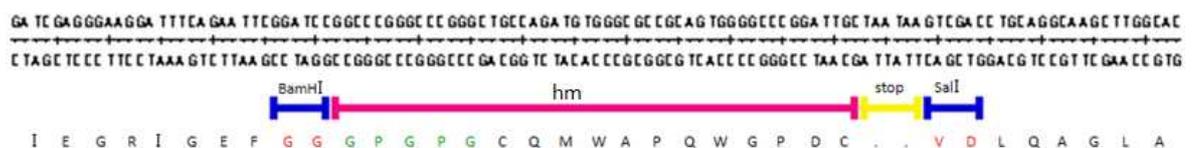

Fig 4-30 Amino acid and DNA sequence of hm. Sequence was translated into *E. coli* codon usage and inserted into the ORF of *malE* via MCS (BamHI, SalI) of the vector pMalp2x. Sequence for cloning into the vector pMal-ivb lacks the two stop codons (not shown)
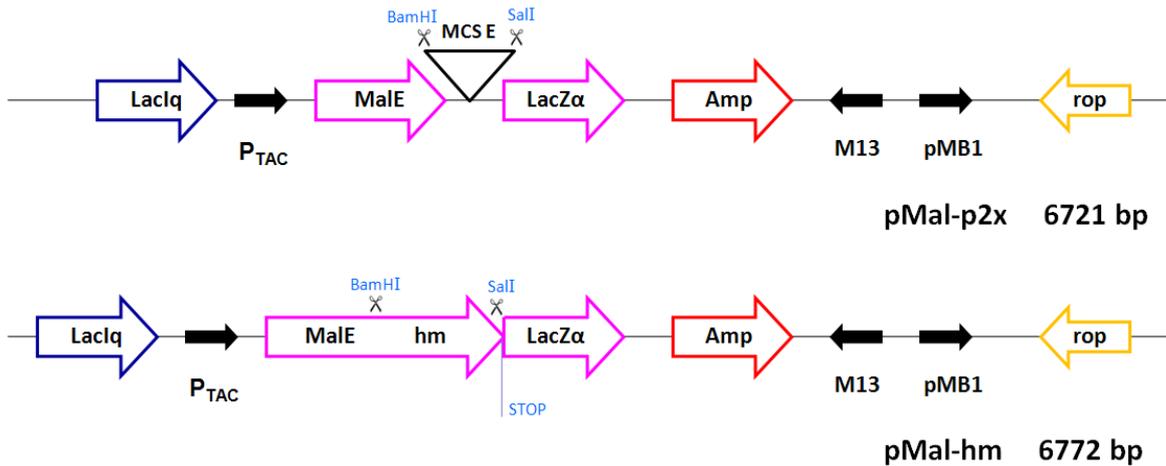
Fig 4-31 Cloning of pMal-hm by inserting the hm DNA sequence into the MCS of the backbone vector pMalp2x using BamHI and SalI. Gene *malE* is under the control of the IPTG inducible P_{TAC} promotor. Addition of 2mM IPTG leads to expression of the fusion protein MBP-hm.

LacIq: repressor P_{TAC}: promotor of *malE malE*: gene encoding the N-terminal part of Maltose Binding Protein *LacZα*: gene encoding the lacZα protein Amp: ampicillin resistance cassette M13: intergenic region, origin of replication (+ -) pMB1: origin of replication rop: regulation of plasmid DNA replication by antisense RNA.
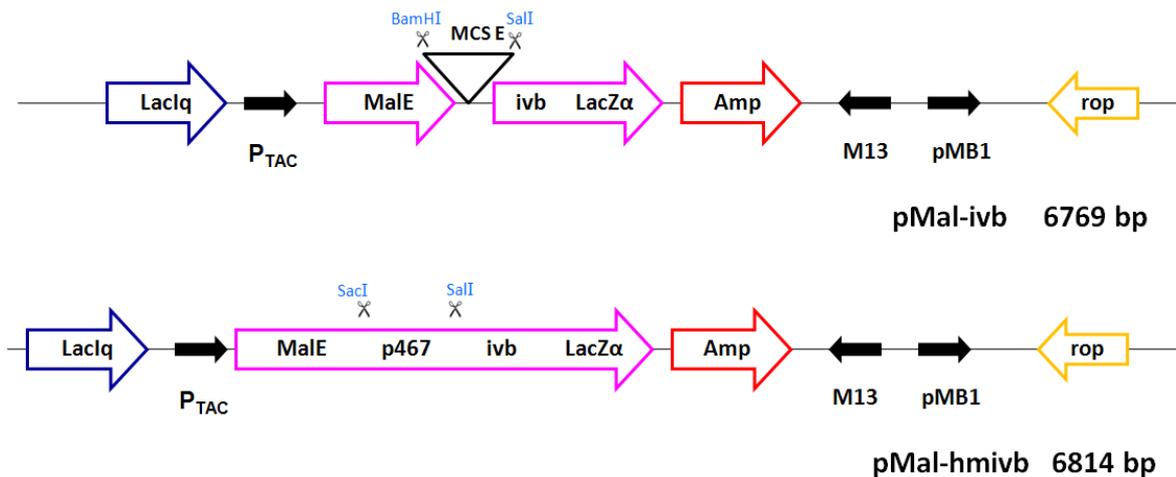
Fig 4-32 Cloning of pMal-hmivb by inserting the hm DNA sequence into the MCS of the backbone vector pMal-ivb using BamHI and SalI. Gene *malE* is under the control of the IPTG inducible P_{TAC} promotor. Addition of 2mM IPTG leads to expression of the fusion protein MBP-hmivb.

LacIq: repressor P_{TAC}: promotor of *malE malE*: gene encoding the N-terminal part of Maltose Binding Protein *LacZα*: gene encoding the lacZα protein Amp: ampicillin resistance cassette M13: intergenic region, origin of replication (+ -) pMB1: origin of replication rop: regulation of plasmid DNA replication by antisense RNA.

Fusion proteins MBP-hm and MBP-hmivb expression is driven by the IPTG inducible P_{TAC} promotor that is repressed in the absence of IPTG by the lacIq repressor and the fusion proteins are transported to the PPS by the SS of MBP. Unified atomic mass units in Dalton of MBP, MBP-467 and MBP-467ivb and length in bp are listed in Tab 4-2. The DNA sequence of the fusion protein MBP-hmivb lacks the stop codons after the hm sequence and is therefore expressed with lacZα giving a MBP-hmivb-lacZα fusion, whereas the DNA

sequence of MBP-hm includes stop codons after the hm sequence and is expressed without lacZα. In the further text pMal-hmivb-lacZα is referred to as pMal-hmivb. Sequence of vector pMal-p2x is provided by NEB [56] but no sequence specification of the SS for the PPS transport . The manual of the pMAL™ Protein Fusion and Purification System says that the pMal-p2x includes the "normal" SS of the periplasmic MBP of *E. coli*. Transport to the PPS leads to a truncated mature peptide devoid of the SS. Database of UniProtKB/Swiss-Prot provides the whole peptide sequence including the SS [57], which was used for calculations of the mature periplasmic protein mass in this work. Complete sequences are stored at the plasmid data base at BIRD-C. An overview of all plasmids constructed in this work is given in 7.

Peptide	Mass incl. SS [kDa]	Mass – SS [kDa]	Length incl. SS [bp]	Length - SS [bp]
Recombinant MBP-lacZα	53.4	50.6	1464	1386
E. coli **MBP**	43.4	40.7	1188	1110
MBP-hm	47.4	44.6	1308	1230
MBP-hmivb-lacZα	56.8	53.9	1557	1479
MBP*	42.5[x)			

Tab 4-4 Atomic mass units of *E. coli* MBP and MBP fusion proteins in kDa. Mature peptides in the PPS lack the SS. Recombinant MBP: maltose binding protein from the expression vector pMal-p2x *E. coli* MBP: chromosomal encoded maltose binding protein of *E. coli* MBP-hm: MBP- herceptin mimotope fusion protein MBP-hmivb: herceptin mimotope fusion protein coupled to ivb, still fused to lacZα MBP*: breakdown product of fusion protein

[x)protein size according to literature [53]

4.2.1 Cloning of pMal-hm and pMal-hmivb

Hm sequences from Fig 4-28b and c were produced at VBC genomics (Vienna, Austria). DNA was ordered as sense and antisense DNA so that cloning into appropriate vectors was possible without cutting with BamHI and SalI (ordered sequence shown in black letters in Fig 4-33). DNA for cloning into pMal-ivb was amplified by PCR from pMal-hm using primers shown in Fig 4-29 to give the hm construct displayed in Fig 4-28d.

```
hm sense:
5'-GGATCCGGCCCGGGCCCGGGCTGCCAGATGTGGGCGCCGCAGTGGGGCCCGGATTGCTAATAAGTCGAC-3'
hm antisense:
5'-CTCGACTTATTAGCAATCCGGGCCCCACTGCGGCGCCCACATCTGGCAGCCCGGGCCCGGGCCGGATC-3'
hmivb sense:
5'-GGATCCGGCCCGGGCCCGGGCTGCCAGATGTGGGCGCCGCAGTGGGGCCCGGATTGCGTCGAC-3'
hmivb antisense:
5'-CTCGAAGCAATCCGGGCCCCACTGCGGCGCCCACATCTGGCAGCCCGGGCCCGGGCCGGATC-3'
```

Fig 4-33 Sense and antisense DNA of hm. DNA in black letters was ordered so that cloning into appropriate vectors was possible without cutting with BamHI and SalI. Red letters display the complementary restriction sites present in the plasmids cut by BamHI and SalI.

Lyophilized hm DNA was resuspended in ultrapure water (100pmol/ml) and was left on the shaker for 30 min at room temperature. 15μl of corresponding sense and antisense DNA each were unified and diluted 1:2 with ultrapure water. The mixture was then heated to 95°C for 10 min and let cool down to room temperature for strand annealing. Hm DNA was checked on an 8% PAGE (Fig 4-34), cut out from the gel and purified by DNA elution according to 6.2.8. Vectors pMal-p2x and pMal-ivb were prepared for cloning by cutting with BamHI and SalI. The unique restriction sites in the MCS of both vectors ensure orientated cloning of hm into the ORF of *malE*. Separation of the vectors was done by gel electrophoresis and purification by subsequent extraction using Quiagen PCR Purification Kit. Ligation of hm sequence and pMalp2x was done at +4°C over night, followed by MOPS transformation into *E. coli* NM522. Isolated plasmid DNA pMal-hm from a positive clone, checked by sequencing, was used for PCR to generate DNA construct hmivb. The PCR product was checked on a 2% agarose gel (Fig 4-35) the correct sized band was cut out from the gel and was purified using the Promega Gel Extraction Kit. After digest of the hmivb construct with BamHI and SalI, the DNA was purified with the Promega Wizard SV Gel and PCR Clean up System. The hmivb construct was ligated with vector pMal-ivb at 4°C over night and the ligation mix was transformed into *E. coli* NM522. Sequencing showed the correct sequence in the reading frame.
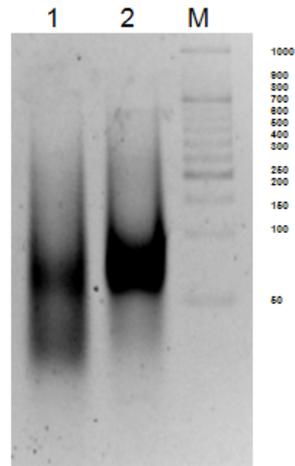
Fig 4-34 Strand annealing of hmivb (lane 1) and hm (lane 2) checked on an 8% PAGE.
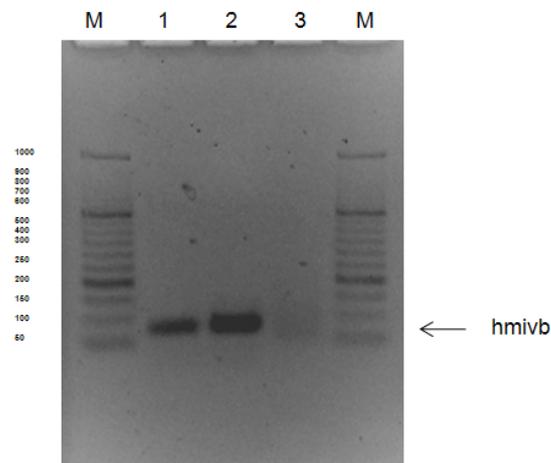
Fig 4-35 PCR product of hmivb amplification. 3µl on lane 1, 12µl on lane 2. PCR 0-control on lane 3.

Plasmids from transformants were purified by minipreps and afterwards tested for correct size by restriction digest. Screening of positive clones was done with Eco0109I for pMal-hm and with KasI for pMal-hmivb. The fragment size after digest of pMal-hm for positive clones is 42/3889/2840bp, whereas the backbone vector pMal-p2x without hm would result in two fragments 42/6679bp. The fragment size after digest of pMal-hmivb for positive clones is 1738/5076bp, whereas the backbone vector pMal-ivb without hmivb would be linearized giving a band at 6769bp. Plasmid preparation pMal-hm from clone 5 on lane 4 shows the correct restriction pattern at ~3900 and ~2900bp and was selected for transformation (Fig 4-36). For pMal-hmivb the plasmid on lane 1 (cut) from clone 12 shows the correct restriction pattern at ~1700 and ~5000bp and was selected for transformation (Fig 4-37).
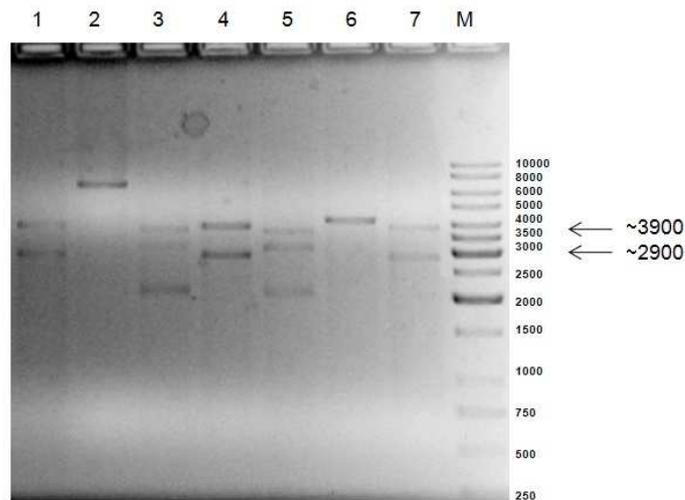
Fig 4-36 Restriction analysis of putative pMal-hm plasmids on 1% agarose after digest with Eco0109I.
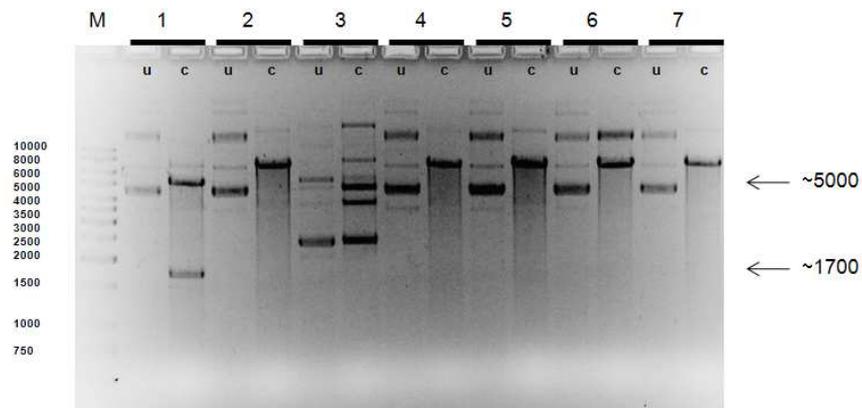
Fig 4-37 Restriction analysis of putative pMal-hmivb plasmids on 1% agarose after digest with KasI. Each plasmid preparation was loaded on two lanes, displaying uncut (u) and cut (c).

Correct plasmids pMal-hm and pMal-hmivb, verified by sequencing, were freshly transformed into *E. coli* NM522 and stored as glycerin cultures at -80°C. Growth and expression was analyzed in noseflask experiments

4.2.2 Growth and expression of *E. coli* NM522 (pMal-hm)

The experiment was carried out in noseflasks containing 30ml LB and ampicillin (100µg/ml). Four clones of *E. coli* NM522 (pMal-hm) plus one with the backbone plasmid pMalp2x were tested. To evaluate possible effects of recombinant protein expression on the cell viability and to verify expression of the recombinant protein, periodic sample were taken for western blot analysis, cfu determination and OD measurements according to 6.3.3.

Expression of the recombinant protein MBP-hm of the vector pMal-hm was induced by addition of 0.2mM IPTG when the cultures reached an OD_{600} of about 0.15-0.2. Growth diagram in Fig 4-38 shows that expression of the fusion protein had no negative effect on cell growth in OD as well as in cfu. Sample preparation for WB was done under non reducing conditions to conserve the disulfide bridging of hm. Breakage of bacteria was done via freeze and thaw treatment and resuspension in non reducing lysis bufferI (6.2.4.3.2) . Successful breakage was first confirmed by detection of cytoplasmic MBP proteins with αMBP and α rabbit IgG-HRP (not shown). The prepared samples were then dotted on two nitrocellulose membranes, 1µl of supernatant and a 1:10 dilution in triplets each. For the positive control 0.5µl purified QMW (1µg/µl) were applied (derived from the Medical University of Vienna). Membranes were incubated with the monoclonal antibody herceptin (Trastuzumab) and α human IgG-HRP, in parallel with the isotype control Rituximab and αhuman IgG-HRP. Dot blot analysis showed binding of herceptin with the positive control but no reaction with the *E. coli* NM522 (pMal-hm) samples (Fig 4-39). Analysis with the isotype control Rituximab (no positive control was available) showed a weak signal for clone 3 (Fig 4-40 3F). For clone 4 one dot of 6 seemed to react weakly positive (Fig 4-39 4F). The dot blot was repeated at the Medical University of Vienna but again herceptin showed no specific binding to MBP-hm (not shown).
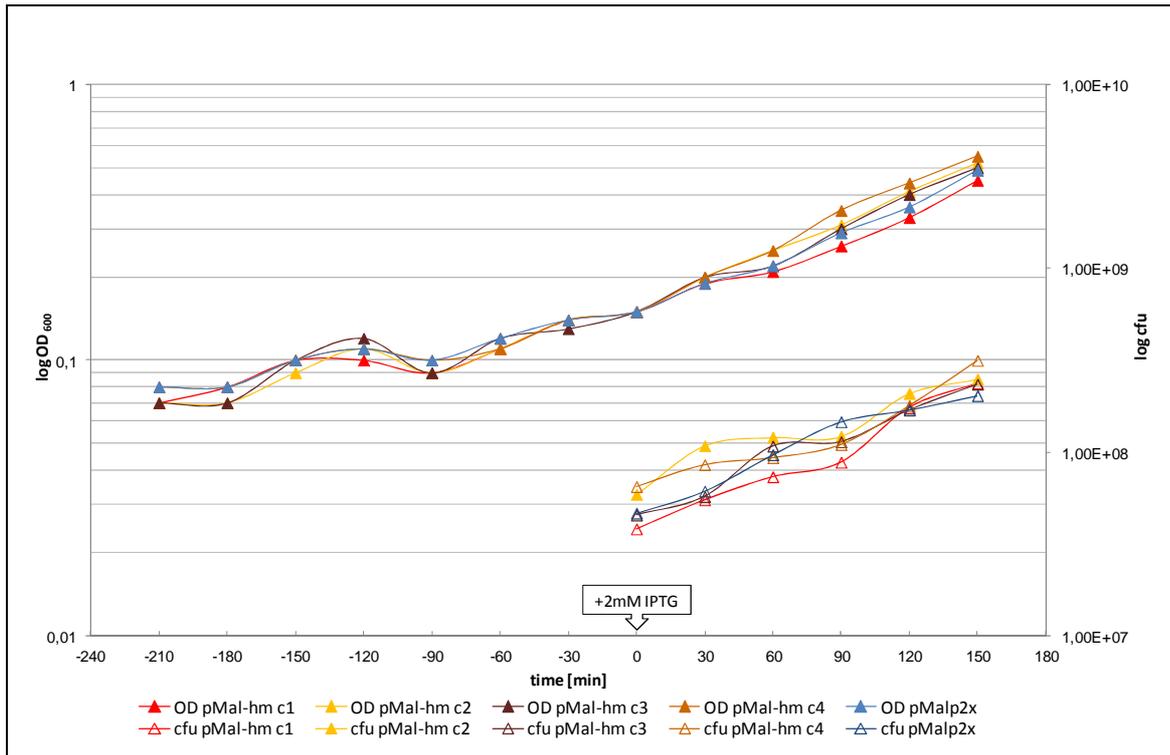
Fig 4-38 Growth curve of *E. coli* NM522 (pMal-hm) and control *E. coli* NM522 (pMalp2x). Recombinant protein expression induced by 2mM IPTG shows no negative effect on growth.
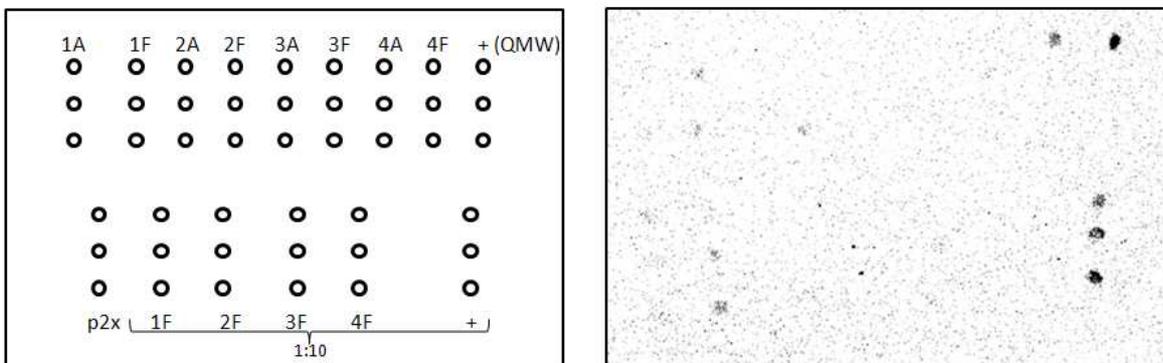
Fig 4-39 Dot blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-hm). Cone 1, 2, 3 and 4 before induction of recombinant MBP-hm expression (A) and after 150 min (F). p2x: NM522 (pMalp2x) negative control. QMW: positive control. The presence of the hm portion of the fusion protein was examined with the monoclonal antibody herceptin and αhuman IgG-HRP.
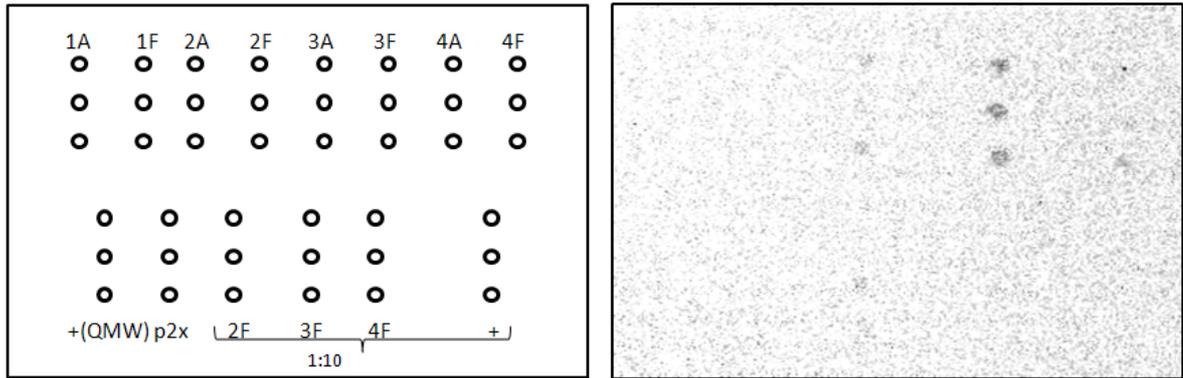
Fig 4-40 Dot blot analysis of whole cell protein extract of *E. coli* NM522 (pMal-hm). Clone 1, 2, 3 and 4 before induction of recombinant MBP-hm expression (A) and after 150 min (F). p2x: NM522 (pMalp2x) negative control. QMW: isotype control. The presence of the hm portion of the fusion protein was examined with the isotype control Rituximab and αhuman IgG-HRP.

As dot blot analysis failed to clearly show hm expression it was decided to go for a native PAGE. Electrophoretic separation could facilitate greater accessibility to the mimotope, which could be hidden in the whole cell protein extract in dot blot analysis. In native PAGE proteins are not covered by negative charges, provided from the dye, which are proportional to their protein mass like in SDS PAGE. Mobility of proteins in native PAGE depends on the intrinsic charge of the proteins. Proteins are under non-reducing and non-denaturing conditions retaining their native structure. Samples from *E. coli* NM522 (pMal-hm) c 3 and c2 were run on a gel, blotted onto a nitrocellulose membrane and detected with αMBP and αrabbit IgG-HRP. As shown in Fig 4-41 MBP is negatively charged and can be detected via native PAGE. From this it was assumed that MBP-hm is also detectable with the specific antibody herceptin at the size of the naturally occurring *E. coli* MBP, as the small hm-fusion portion should not add a big difference in charge. But a native PAGE and subsequent examination of the membrane with herceptin and αhuman IgG-HRP showed no signal. Also incubation of the membranes of native and SDS-Pages with ROTI-REFOLD (Carl Roth GmbH, Karlsruhe, Germany) trying to renature the protein before incubation with Trastuzumab, showed no hm detection. A standard WB and detection with αMBP and αrabbit IgG-HRP was performed, to prove if the size difference between *E. coli* MBP (~41kDa) and the recombinant MBP-hm (~45kDa) of about 4kDa can be detected on WB. Just for clone 3, 120 min after induction of expression a band emerges at about the expected size (Fig 4-42 3E). It is nearly overlapping with the *E. coli* MBP and could as well be a breakdown product of the fusion protein (Fig 4-42). Therefore, detection of MBP-hm using αMBP antibodies is not applicable for standard WBs.
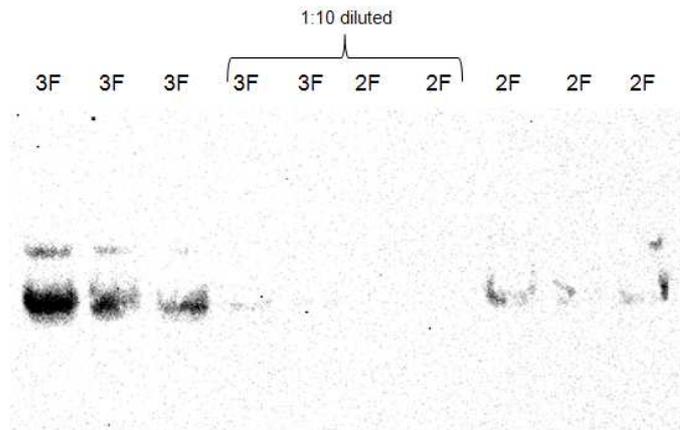
Fig 4-41 WB analysis (10% native PAGE) of whole cell protein extract of *E. coli* NM522 (pMal-hm) c2 and c3. F: 150 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP and αrabbit IgG-HRP.
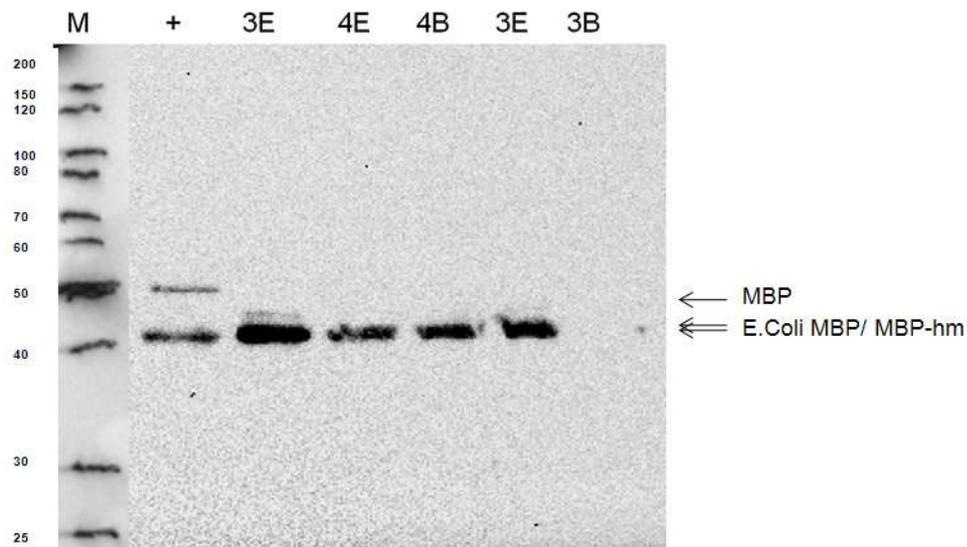
Fig 4-42 WB analysis (10% SDS PAGE) of whole cell protein extract of *E. coli* NM522 (pMal-hm) c3 and c4. +: *E. coli* NM522 (pMalp2x) E: 120 min and B: 30min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP and α rabbit IgG-HRP.

4.2.3 Growth and expression of *E. coli* PC2133 (pMal-p2x), *E. coli* NM522 (pMal-hm), *E. coli* W3110 (pMal-hm) and *E. coli* PC1363 (pMal-hm)

With new transformed strains of *E. coli* PC2133 (pMal-p2x), *E. coli* NM522 (pMal-hm), *E. coli* W3110 (pMal-hm) and *E. coli* PC1363 (pMal-hm) noseflask experiments were performed. The intension was to check whether the expression varies in different strains leading to better hm expression, and to test more clones in general. Further hm expression should be monitored in course of time.

The experiments were carried out in noseflasks containing 30ml LB and ampicillin (100µg/ml). *E. coli* strains with the plasmid pMal-hm and with the backbone plasmid pMalp2x as control were tested. Periodic samples were taken for western blot analysis, cfu determination and OD measurements according to 6.3.3. Expression of the recombinant proteins MBP-hm/recombinant MBP of the vector pMal-hm/pMalp2x was induced by addition of 0.2mM IPTG when the cultures reached an OD_{600} of about 0.15-0.2.

4.2.3.1 *E. coli* PC2133

E. coli PC2133 was supposed to be Δ*malE*. A strain devoided of *E. coli* MBP would offer valuable clues to recombinant MBP protein expression and detection. PC2133 was transformed with the backbone plasmid pMal-p2x to see if the western blot analysis would allow clear identification of MBP-hm without an interfering band of *E. coli* MBP at about 40-42kDa. A growth study was performed (Fig 4-43) and periodic sample were processed for SDS-PAGE and subsequent western blot analysis (Fig 4-44). As expected the addition of IPTG had no negative effect on cell growth in OD as well as in cfu. *E. coli* strain PC2133 exhibits besides the recombinant MBP (~51kDa) the *E. coli* MBP band at about 40kDa, seen in samples before IPTG addition (Fig 4-44 A), and therefore shows no advantage over the standard lab strains for detection of recombinant MBP fusion proteins.

Fig 4-43 Growth curve of *E. coli* PC2133 (pMalp2x). Recombinant protein expression was induced by addition of 2mM IPTG.

Fig 4-44 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* PC2133 (pMalp2x) c1, c2 and c3. +: *E. coli* NM522 (pMalp2x) A: time point of induction, B: 30min and C: 60 min after induction of protein expression. The presence of the MBP portion was examined with αMBP and α rabbit IgG-HRP. *E. coli* MBP appears at about 41 kDa.

4.2.3.2 Growth curve of *E. coli* PC1363 (pMal-hm), *E. coli* NM522 (pMal-hm) and *E. coli* W3110 (pMal-hm)

Growth and expression experiments of different *E. coli* strains harboring plasmid pMal-hm were performed showing similar growth behavior for all tested strains (Fig 4-45, Fig

4-46, Fig 4-47). Addition of IPTG had no negative effect on cell growth in OD as well as in cfu.
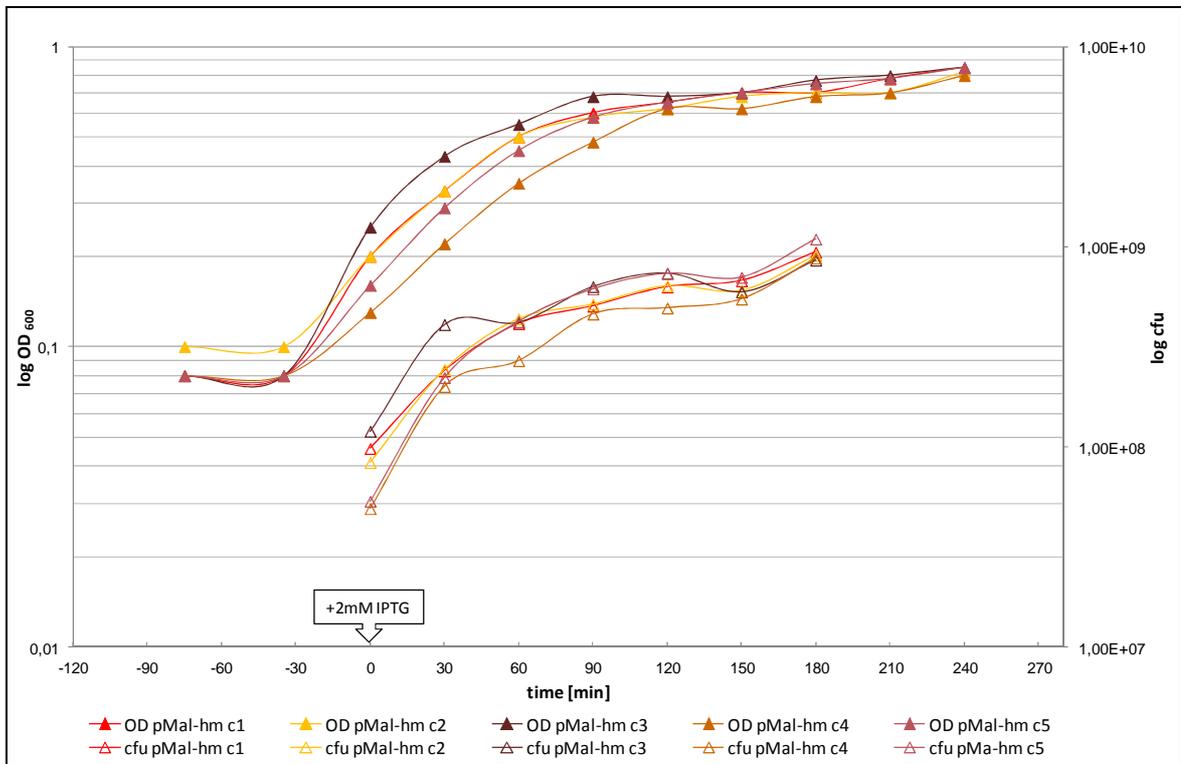
Fig 4-45 Growth curve of *E. coli* NM522 (pMal-hm). Recombinant protein expression was induced by addition of 2mM IPTG.
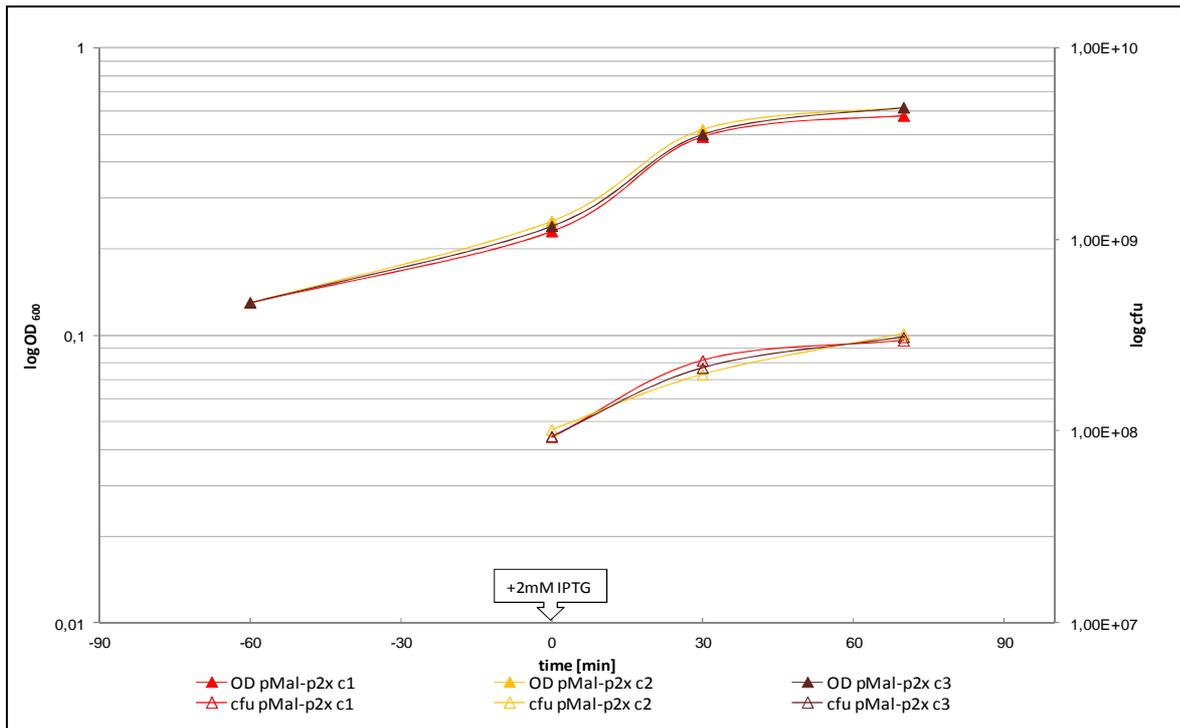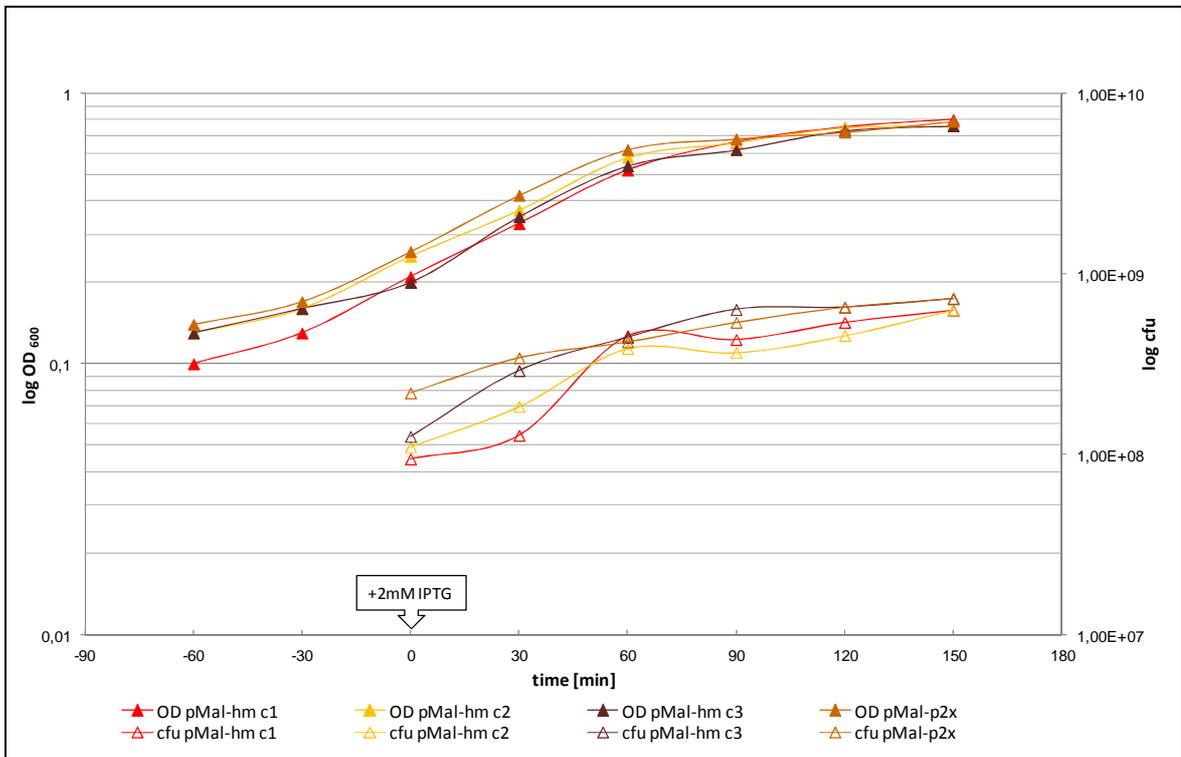

Fig 4-46 Growth curve of *E. coli* PC1363 (pMal-hm). Recombinant protein expression was induced by addition of 2mM IPTG.
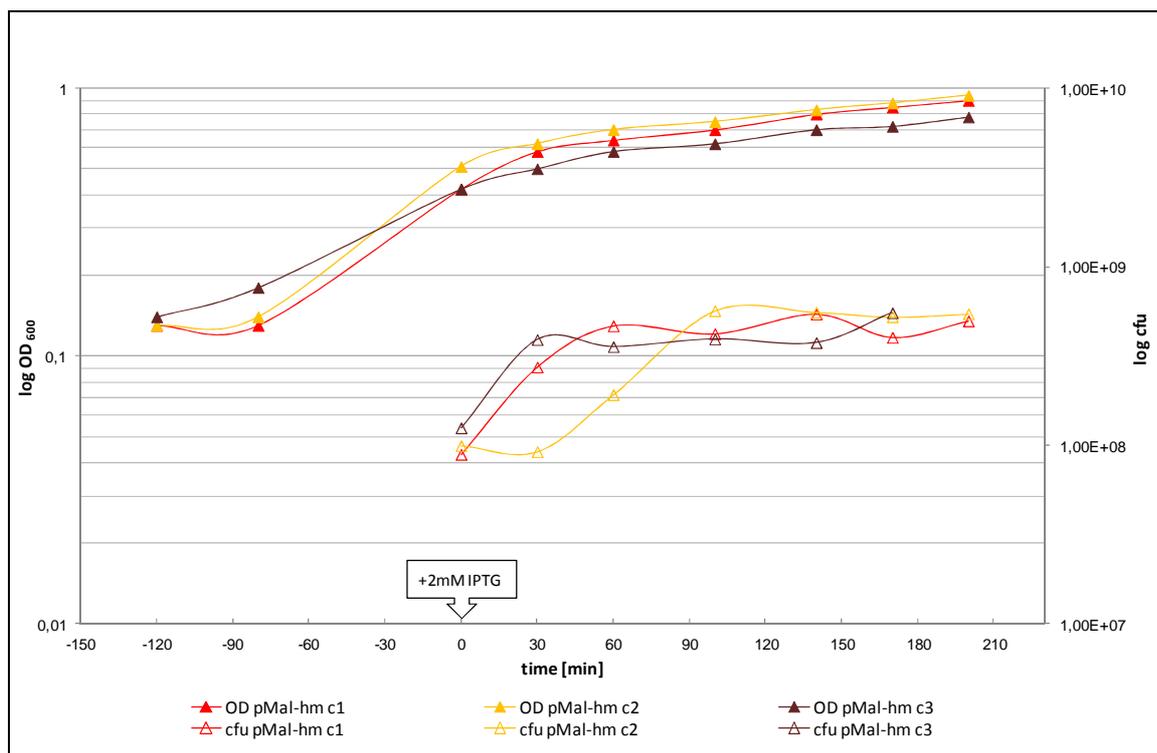
Fig 4-47 Growth curve of *E. coli* W3110 (pMal-hm). Recombinant protein expression was induced by addition of 2mM IPTG.

4.2.3.3 Western blot analysis of *E. coli* NM522 (pMal-hm), *E. coli* PC 1363 (pMal-hm), and *E. coli* W3110 (pMal-hm)

Analysis of protein samples from growth and expression studies of different strains harboring plasmid pMal-hm did not result in an ambiguously detection of MBP-hm in WB analysis. A bulky band that shows up at ~40-43kDa throughout all western blot analyses might be *E. coli* MBP as well as the breakdown product MBP*. WB analysis of protein samples from *E. coli* NM522 (pMal-hm) in Fig 4-48, *E. coli* W3110 (pMal-hm) in Fig 4-49, *E. coli* PC1363 (pMal-hm) in Fig 4-50 and combined in one blot in Fig 4-51 show for individual clones a faint band at the anticipated molecular weight of the target protein. This is for *E. coli* PC1363 (pMal-hm) c2 in Fig 4-50, *E. coli* PC1363 (pMal-hm) c1 in Fig 4-51 and after 180 min expression *E. coli* NM522 (pMal-hm) c3 in Fig 4-52. In WB analysis shown below (Fig 4-48, Fig 4-49, Fig 4-50, Fig 4-51, Fig 4-52) sample A, taken before IPTG induction, shows neither a band for the *E. coli* MBP at ~ 41kDa nor for the recombinant MBPs at ~45kDa. This is attributed to addition of glucose to the overnight cultures which

represses not only recombinant MBP expression but also blocks the transcription *malT* and therefore the *malT* dependent gene *malE.*
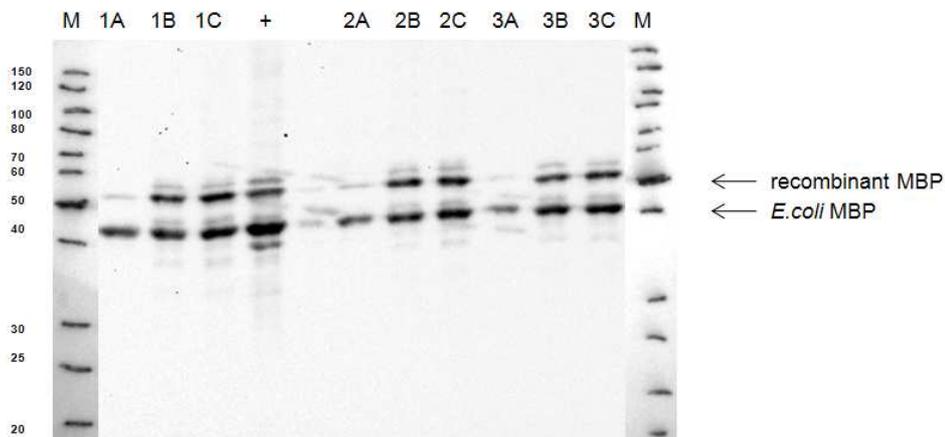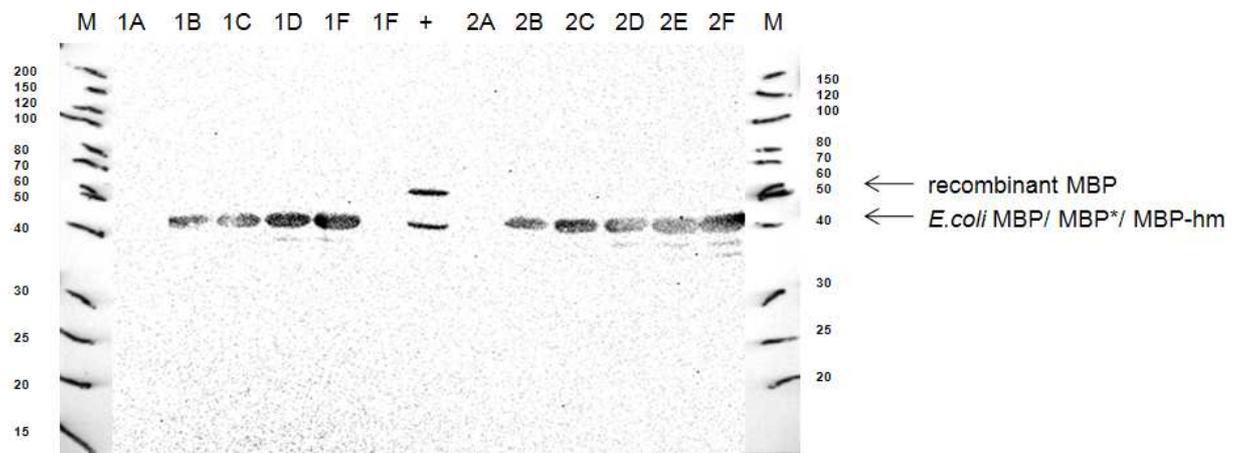

Fig 4-48 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* NM522 (pMal-hm) c1 and c2. +: *E. coli* NM522 (pMalp2x) A: time point before induction; B: 30 min, C: 60 min, D: 90 min, E 120 min and F: 150 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP-HRP and α rabbit IgG-HRP.
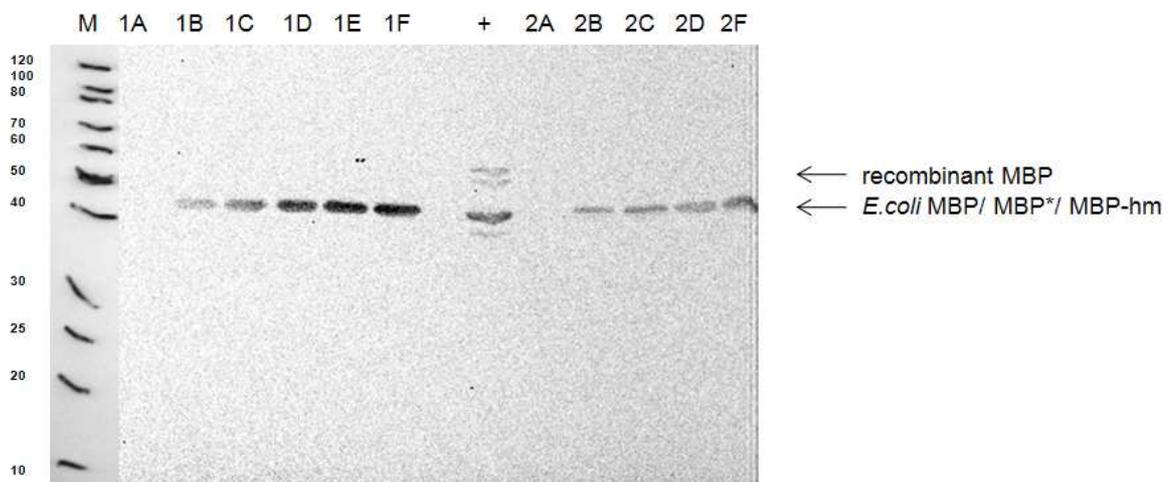
Fig 4-49 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* W3110 (pMal-hm) c1 and c2. +: *E. coli* NM522 (pMalp2x) A: time point before induction; B: 30 min, C: 60 min, D: 90 min, E 120 min and F: 150 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP and α rabbit IgG-HRP.
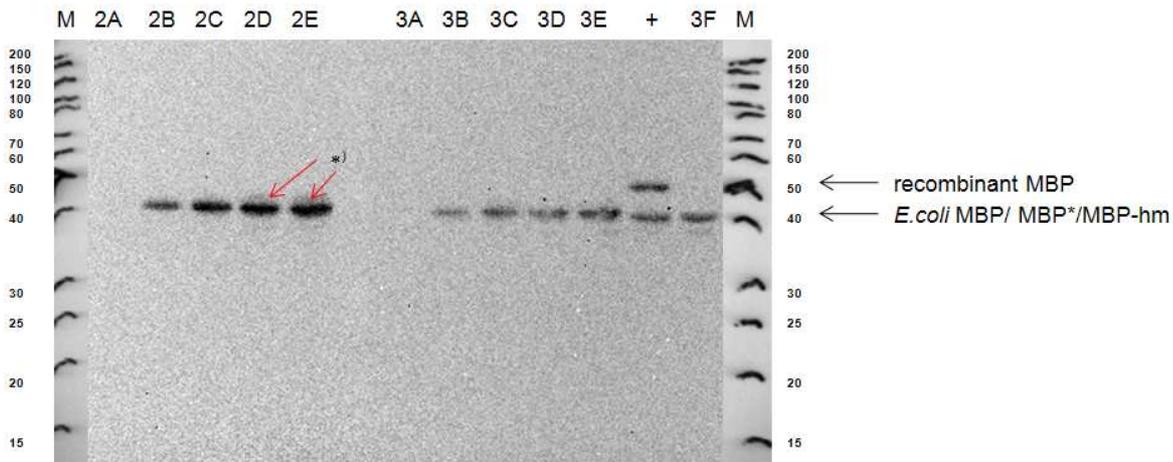
Fig 4-50 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* PC1363 (pMal-hm) c2 and c3. +: *E. coli* NM522 (pMalp2x) A: time point before induction; B: 30 min, C: 60 min, D: 90 min, E 120 min and F: 150 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP and α rabbit IgG-HRP. ∗) c2 shows a weak band appearing at the expected size of MBP-hm (~45kDa)
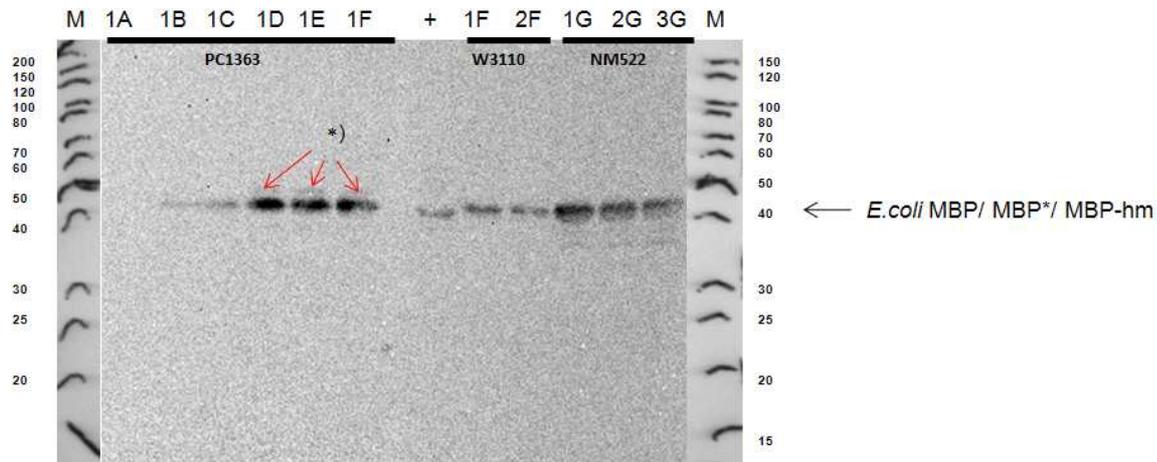
Fig 4-51 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* PC1363 (pMal-hm) c1, *E. coli* W3110 (pMal-hm) c1 and *E. coli* NM522 (pMal-hm) c1, c2 and c3. +: *E. coli* NM522 (pMalp2x) A: time point before induction; B: 30 min, C: 60 min, D: 90 min, E 120 min, F: 150 and G: 180 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP-HRP and α rabbit IgG-HRP. ∗) c1 shows a weak band appearing at the expected size of MBP-hm (~45kDa).
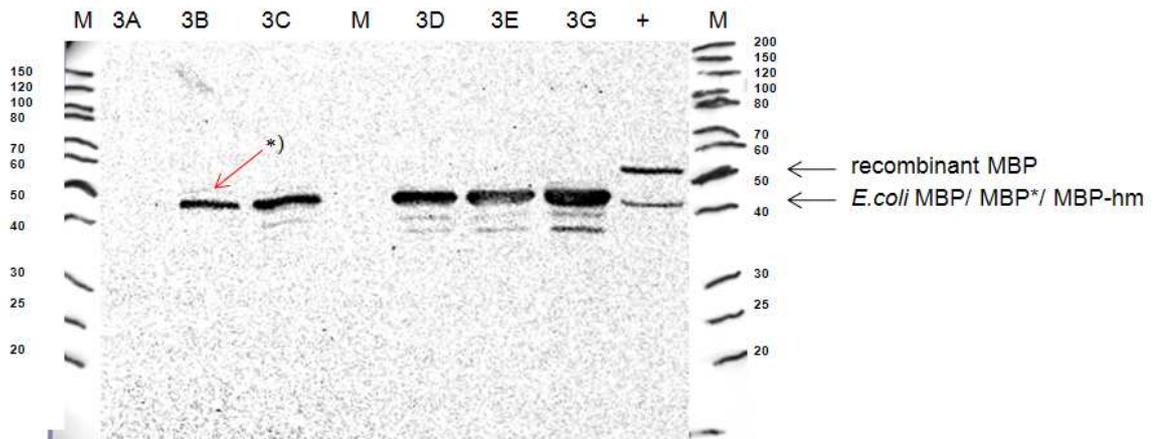
Fig 4-52 WB analysis (15% SDS PAGE) of whole cell protein extract of *E. coli* NM522 (pMal-hm) c3. +: *E. coli* NM522 (pMalp2x) with MBP at about 51kDa A: time point before induction; B: 30 min, C: 60 min, D: 90 min, E 120 min, F: 150 min and G 180 min after induction of recombinant protein expression. The presence of the MBP portion was examined with αMBP and α rabbit IgG-HRP. ∗) c3 shows a weak band appearing at the expected size of MBP-hm (~45kDa

4.2.3.4 Growth and expression of *E. coli* NM522 (pMal-hm) and NM522 (pMal-hmivb)

Additionally new transformed clones NM522 (pMal-hm) and NM522 (pMal-hmivb) were included and the experiment was carried out as given in 4.2.2. Growth diagram shows that expression induction of the fusion protein had no negative effect on cell growth in OD (Fig 4-53) (no cfu values were determined). In this experiment cell breakage for WB samples was done via osmotic shock treatment and resuspension in lysis bufferIII (6.2.4.3.2), including EDTA to inhibit proteases and lysozyme. In addition the ivb-portion might guard the peptide from degradation. No signal was detected in WB analysis after membrane development with herceptin and anti human IgG-HRP (not shown).
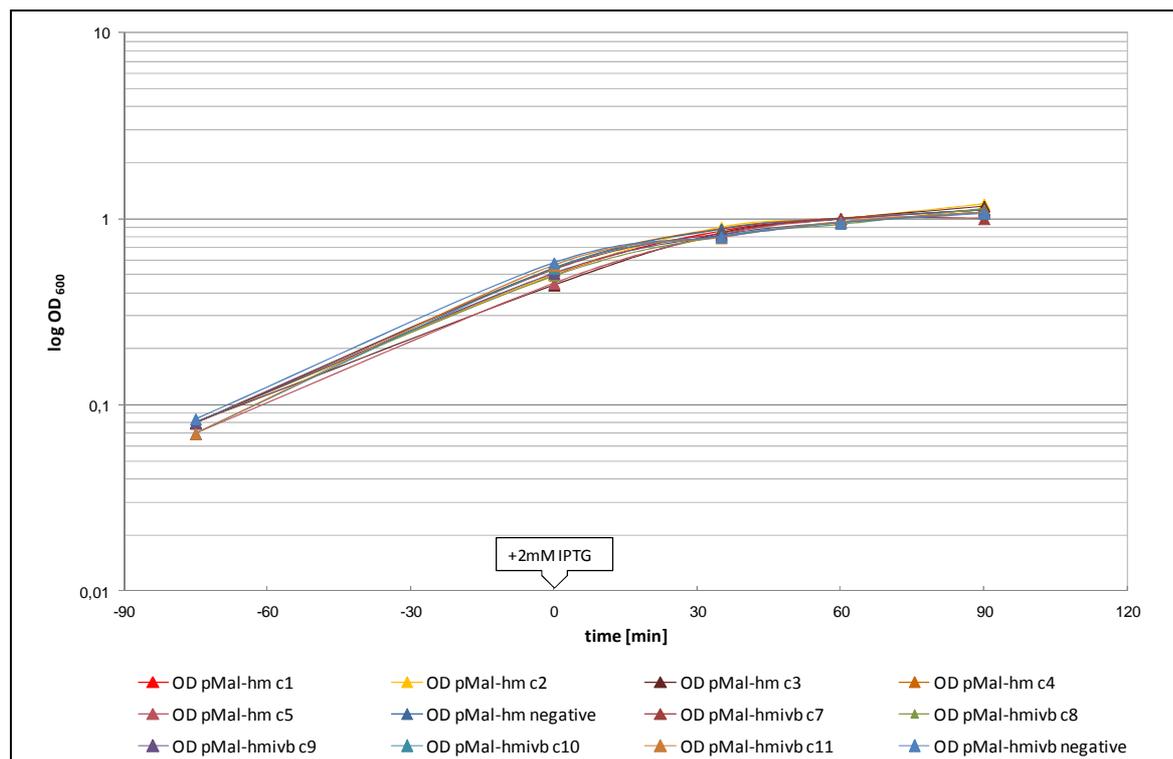
Fig 4-53 Growth curve of *E. coli* NM522 (pMal-hm) and *E. coli* NM522 (pMal-hmivb). Recombinant protein expression was induced by addition of 2mM IPTG.

4.2.4   Cell fractionation of *E. coli* NM522 (pMal-hm)

The cellular location and solubility of the target protein was analyzed in different cell fractions: total cell protein (TCP), media sample (M), periplasmic fraction (P) and soluble cytoplasmic fraction (C). An insoluble fraction was not further processed.

The experiment was carried out in an Erlenmeyer flask containing 60 ml LB and ampicillin (100µg/ml), inoculated with *E. coli* NM522 (pMal-hm). As the culture reached an OD_{600} of 0.15, it was split into two flasks, each 30 ml. One flask was induced by adding IPTG (2mM), the other flask served as a control (uninduced). After reaching an OD_{600} of 0.7, cultures were spinned down in SS34 tubes at 4°C, 6500g 15 min and further treated as given in 6.2.7 to get different cell fractions.

Normalized volume of cell fractions for loading a 15% SDS PAGE is given in Tab 4-5. Fig 4-54 shows the cell fraction proteins of induced (i) and uninduced (ui) culture samples separated on the SDS PAGE and developed using αMBP and αrabbit IgG-HRP. Ui samples exhibit a band at about 40kDa which is expected for *E. coli* MBP. No big difference is visible in ui and i samples. No bands were detected in the M fraction. The TCP samples

show two bands, one at ~40kDa and a second at ~43kDa. The same bands are visible in P fraction. The C samples show the same bands in lower concentrations and additional bigger bands are visible in the C fraction of i samples. The band at about 43kDa was not expected, since it would either be related to unprocessed *E. coli* MBP, which would not show up in PP, or to MBP*, which should just appear in induced samples. One possible explanation could be that the fusion protein is produced when not repressed by glucose during growth, and therefore a breakdown product can be detected. A faint band at about 47kDa shows up, which could be related to the unprocessed MBP-hm, and bands above 80 and 150 kDa. The 80 kDa band is also slightly visible in P fraction of i sample. These bands are supposed to be multimers of MBP-hm proteins. Occasionally multimers of a fusion protein can be observed, mostly due to disulfide bonds that were not adequately reduced before loading the gel. This experiment was performed to gain insight into the recombinant protein localization but has to be repeated to deduce reliable information. The TCP fraction should display all bands visible in P, C and M, which was not observed.

	Sample conc. factor	OD_{600} at harvest	Z (conc. Factor × OD_{600})	Volume to Load 15-well mini-gel 180 µl ÷ Z
Induced Samples				
TCP	20	0.7	14	6.4
Media	5	0.7	3.5	12.8
Periplasmic	6.25	0.7	4.4	10.2
Soluble Cytoplasmic	16.6	0.7	11.8	7.8
Uninduced Samples				
TCP	20	0.7	1.4	6.4
Periplasmic	6.25	0.7	4.4	12.8
Media	5	0.7	3.5	10.2
Soluble Cytoplasmic	16.6	0.7	11.8	7.8

Tab 4-5 Determination of the normalized sample volumes to load on a standard 15-well mini SDS-PAGE gel.
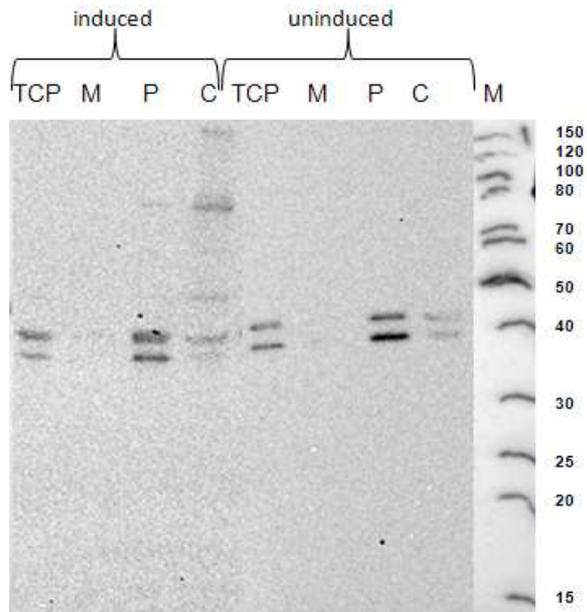
Fig 4-54 15% SDS-PAGE of induced (i) and uninduced (ui) culture of *E. coli* NM522 (pMal-hm). Different cell fractions were analyzed: TCP total cell protein, M media sample, P periplasmic fraction, C soluble cytoplasmic fraction

5. Discussion

The aim of this work was to adopt the Bacterial Ghosts system as candidate for delivery of tumor peptide antigens. Therefore putative B-cell epitope mimics of the tumor antigen Her-2/neu, which already showed tumor growth inhibition [31, 55], were introduced in the system, intending to design a therapeutic anti cancer vaccine with preventive long term immunity against breast cancer.

One part of this work dealt with three linear epitopes, p4, p6 and p7, which were fused and as a multiepitope successfully cloned into the pMal-p2x expression system, directing the epitopes as a MBP fusion protein to the periplasm of *E. coli*. The results in this work demonstrate detection of the epitopes with p467 specific immune sera in western blot analysis, and showed that produced BGs are carrying p467. In mice immunization experiments a significant higher titer of elicited antibodies of the experimental groups versus control group showed the effective delivery of p4, 6 and 7 by BGs.

The second part of this work was to express a circularized conformational mimotope, herceptin mimotope (hm), in the same expression system pMal-p2x. Especially for the hm, transport and subsequent disulfide bridging in the PPS is essential, as conformational accuracy is required for immunogenicity. Although cloning succeeded and sequencing of the vector showed the hm in the correct reading frame, detection with the monoclonal antibody Trastuzumab was not successful.

5.1 Multiepitope p467

Initial attempts in cloning p467 presented some difficulties. The 194 bp p467 sequence had to be constructed by ourselves, as no DNA template was provided. Peptide sequences of the single linear epitopes, which were successfully tested in preliminary experiments at the Medical University of Vienna, were translated into DNA, considering the *E. coli* codon usage. When designing the multiepitope, the three single epitope sequences were coupled with restriction sites to remain flexible for using just one or two parts of it, or using it in different expression systems later on. Linker sequences were inserted, consisting of glycine amino acids, so that the protein domains are free to move and do not sterically interfere with one another.

The sequence was ordered, like primers, as ssDNA at VBC Genomics (Vienna, Austria). DNA accuracy is guaranteed up to 120bp maximum, so the whole 194 bp sequence was planned to be produced by PCR in two parts in multistep reactions. An overview is given in Fig 4-5 and Fig 4-6. Several attempts to produce the p467 fragment fail presumably due to formation of secondary structures during PCR. Sequencing of "positive" clones always indicated some mismatch regions and base deletions. It could have been tried to solve these problems by using single strand binding proteins like T4 Gen 32 protein, which have been reported to increase the specificity of PCR reactions [58], but as a lot of time had been spent on PCR and clone testing, it was decided to order the sequence at GenArt (Regensburg, Germany). The synthetic gene was delivered incorporated into a cloning vector with an attached sequence protocol; the p467 sequence was cut out, cloned into vector pMal-p2x and the vector pMal-467 was amplified in *E. coli* NM522 within a short period of time.

The first growth and expression experiments in *E. coli* NM522 showed that expression of the MBP-467 fusion protein did not impair growth (Fig 4-12). Western blot analysis of the whole cell protein extract and detection with αMBP antibodies revealed that the fusion protein is stable at least for 120 min without degradation (Fig 4-13). Cotransformation of pMAL-467 and the lysis plasmid pGLysivb and subsequent lysis studies of 5 clones achieved LEs of up to 99.9%. The whole cell protein extract of the BGs was checked with p467 specific mouse, rabbit and purified rabbit antiserum in WB analysis exhibiting a clearly recognizable fusion protein band at about 57kDa for all sera, which is absent prior expression induction (Fig 4-16, Fig 4-17, Fig 4-18). The same pattern shows up at about 50kDa, which is most likely a truncated form of the fusion protein produced by proteolysis, but still recognized by p467 antiserum. In several WBs the mouse serum has shown the least amount of unspecific bands and therefore lowest background signals.

The final BG-p467 production step was done in a 20.0l low density batch fermentation using a 30l Techfors-S fermenter (Infors-HT). Online process data were monitored by the IRIS software (Infors-HT) and displayed an oxygen increase immediately after induction of expression (Fig 4-19). Generally the increase of oxygen in the media during cultivation goes along with cell death and is expected after LI, as bacteria are no longer consuming oxygen. Although earlier studies proved no negative effect for this fusion protein

expression on cell viability, oxygen increased when protein expression was induced 60 min before lysis induction. Reduction of viability during 60 min of recombinant protein expression was 42, 08%. This value needs to be viewed critically since it is received from agar plates that were incubated over night and could therefore be attributed to a long term effect of MBP-467 on bacteria. Ideal fermentation conditions may lead to a higher rate of recombinant protein production having a toxic long term effect on bacteria not seen for bacteria grown in noseflasks under suboptimal growth condition without pH and oxygen level regulation. Stagnation in growth due to increased recombinant protein production together with simultaneously increased stirring rate could both contribute to the accumulation of dissolved oxygen in the culture broth after IPTG induction. Thirty min of LI resulted in a significant decrease in cfu giving a total killing of 98.69%. After 60 min lysis the cfu values dropped below the counting range which gives reliable data to make extrapolations for the used sample dilutions. When using the lowest detection limit as a virtual value calculation of LE results in >99.76%. The fact that microscopy showed almost exclusively BGs after LI confirms the presumption that E-mediated lysis produced successfully BGs, and cell death observed before LI occurred due to a long term effect on plates, as E-mediated lysis depends on specific functions of the cell division machinery of a viable culture [59]. Western blot analysis of lyophilized BG-p467 samples using p467 mouse antiserum showed a strong band at about 57kDa (Fig 4-23) and samples taken during fermentation present the accumulation of protein E after LI and the decrease of cytosolic BCCP, indicating E-mediated lysis.

No quantification of the pMal-467 fusion protein was performed in this study. Recent studies using PPS expression of foreign proteins in BGs reached 200ng recombinant PorB and 340ng recombinant MOMP/µg lyophilized BGs [60]. Sterility of lyophilized p467-BGs was confirmed and the final product was handed over to the Department of Pathophysiology at the Medical University of Vienna (Austria) for animal experiments. Immunization of BALB/c mice was done through subcutaneous injection with different p467-BG concentrations and resulted in induction of peptide specific antibodies (Fig 4-25, Fig 4-26, Fig 4-27). The slight increase in titers of the control group could be due to the unspecific binding of antibodies to BG components, but the significant higher titers of the test groups show that BG mediated delivery of p467 induces successfully peptide specific IgG antibodies (Fig 4-25, Fig 4-26, Fig 4-27). But compared to other systems, like TT- and

virosom-conjugates, the titer was rather low according to the first partial report (just limited information was available, therefore no precise numbers can be offered). The proliferation of spleen cells was measured by H^3 thymidin incorporation. In vitro stimulation with BG-467 peptides did not lead to increased proliferation. IFN-gamma measurements of the supernatants using ELISA did not show an increase of IFN-gamma production after in vitro stimulation with BG-467 peptides. These results were similar to the TT-and virosom-conjugate data. Even only limited results are available at the moment in general it can be concluded that the results correspond to earlier data and confirm the hypothesis, that the used Her-2/neu sequences display B-cell epitopes and therefore do not trigger T-cell response themselves. Also a combination of B-cell and T-cell epitopes could be advantageous, which could be easily combined in the BG system. This should allow determining the advantages of BG-467 as a therapeutic cancer vaccine in comparison to TT- or virosom-conjugates delivered p4, p6 and p7 antigens.

5.2   Herceptin mimotope

Cloning of the herceptin mimotope (hm) into pMal-p2x involved *E. coli* codon optimization and insertion of a linker peptide between MBP and hm. Therefore a glycine-proline linker was used to separate the epitope from the residual fusion partner and to allow protein tertiary structure formation by disulfide bridging in the periplasm. The 69bp fragment was ordered as ssDNA sense and antisense at VBC Genomics (Vienna, Austria).

Sequence verified clones of *E. coli* NM522 (pMal-hm) were analyzed in growth and expression experiments. The production of the MBP-hm fusion protein did not impede growth (Fig 4-38). Cell disruption as well as detection of the fusion protein by the monoclonal antibody Trastuzumab had to be done under non denaturing conditions to preserve the native structure to ensure antibody binding. Cell disruption was done using the freeze/thaw method [61] to release recombinant proteins. Effective cell breakage was first confirmed by αMBP detection and afterwards specific detection of the native conformation with Trastuzumab was performed, but could not be proved in dot blots (Fig 4-39). Therefore it was decided to conduct protein separation by native page, which could gain access to the mimotope, that otherwise might be hidden in the whole cell protein extract. As in native PAGE proteins are not covered by negative charges the migration is

therefore not corresponding to protein mass like in SDS PAGE. The intrinsic charge of MBP/MBP-hm had to be explored first by detection of the MBP portion of the fusion protein with αMBP. WB analysis showed that MBP was negatively charged and could be detected on nitrocellulose membranes after native PAGE runs. A weaker band, probably MBP-hm, is seen next to a dominant band, which was supposed to be the *E. coli* MBP or/and a breakdown product, respectively (Fig 4-41). Assuming that the small hm portion is not adding a big difference in charge (molecular weight of hm is a tenth of MBP) suggests that the fusion protein should be detectable next to the *E. coli* MBP and may therefore be visible as the weaker band in Fig 4-41. But a native PAGE performed under the same conditions, blotted to a nitrocellulose membrane and detected by Trastuzumab produced no positive signal for the recombinant MBP-hm.

Growth and expression tests were extended to additional *E. coli* strains. Different clones of newly transformed *E. coli* NM522 (pMal-hm), *E. coli* W3110 (pMal-hm) and *E. coli* PC1363 (pMal-hm) were checked to see whether the expression varies in different strains and may lead to better expression in one of them. Protein samples were analyzed by SDS-PAGE separation and subsequent western blot analysis detecting the MBP portion of the fusion protein. The intension was to estimate the time point of detectable protein expression start on the blot, the amount of full length protein compared to degradation products production and the time frame in which the fusion protein is stable and detectable. For only two clones of *E. coli* PC1363 and one clone of *E. coli* NM522 a faint band could be assigned to MBP-hm, located slightly above the dominant band, which is supposed to be the *E. coli* MBP at about 40-42kDa (Fig 4-50, Fig 4-51, Fig 4-52). This weak band MBP-hm shows up at about 90 min after protein induction and is thereafter stable, or at least in a balance between production and proteolysis. A dot blot analysis for these studies was performed to explore the native conformation of MBP-hm. Cell breakage was done by osmotic shock this time, because it might be "more gentle" than freezing and thawing, but incubating the membrane with Trastuzumab revealed no signal specific for hm.

According to the protocol for "expression of recombinant proteins by fusion to the maltose binding protein" [53] there could be two possible explanations for a breakdown product of the fusion protein. One is translation termination, if the protein of interest is in

the wrong translational reading frame, the other is proteolytic cleavage due to protein instability, whereby in this case mostly a small amount of full length protein can be seen. As sequencing confirmed the fusion protein to be in the right reading frame, the accumulation of *E. coli* MBP and the breakdown product of the MBP-hm fusion protein at 40-42kDa giving together the detected bulky band is most likely. The fact that this dominant band mostly does not show up before induction of protein expression does not necessarily mean that it is solely the recombinant protein but may be due to the addition of glucose to the overnight cultures. When cells are growing under high glucose concentrations the transport of glucose via the phosphotranferase system exerts strong catabolite repression which blocks the transcription of *malT* and the *malT*-dependent genes of the *malK lamb malM* and *malE malF malG* operons of the *E. coli* chromosome [62]. In expression analysis of this work missing the addition of glucose to the overnight culture (Fig 4-44), *E. coli* MBP was detected before IPTG induction resulting from endogenous synthesis of the inducer. The uninduced level of *E. coli mal* gene expression seems to be a preparation for the arrival of the "true" substrate by endogenous induction, geared for the scavenging of low levels of maltose and maltodextrins [62, 63].

The oxidizing environment of the PP contributes to the formation and rearrangement of disulfide bonds [64] and is therefore an attractive destination for recombinant eukaryotic proteins. For transport to the PP the MBP was found to display an effective solubilizing partner inhibiting misfolding and exerting benefits during and after folding [65]. Transport to the periplasm often leads to proper folding and thus to a more stable fusion protein, but this was not observed in this work for pMal-hm. Mostly proteins are unstable because they do not fold properly, this is particularly true for proteins with disulfide bonds [53]. Continuative work should therefore go along with using different protease negative strains as well as trying several methods for non denaturing cell disruption as for many unstable proteins most of the degradation happens during harvest and cell breakage. In addition, using *malE* negative strains would give more precise information about the dominant band at 40-42kDa; to which extent it is really related to a breakdown product of the MBP-hm fusion protein or if it is solely *E. coli* MBP. It would also be useful to try co-overproduction of protein disulfide isomerases, pratically DsbC, which has been shown to greatly improve proper disulfide bond formation [66].

In general it has to be noted that some inaccuracies regarding the detected protein sizes in WB analysis were observed in this work. In Tab 4-1 and Tab 4-4 the protein sizes of *E. coli* MBP and all MBP-fusions are listed with and without signal peptide. Usually, the signal peptide is cleaved off by the cell's secretion apparatus [67] during transport action through the cytoplasmic membrane. In WB analysis of this work often protein bands at the size of the precursor fusion protein including the SS were detected, giving in case of MBP-467 a band at ~57kDa instead of ~54kDa. This could be due to imprecise size separation of proteins in SDS-PAGE, but also due to the high rate of recombinant protein production. Overproduction often leads to only partial processing of the recombinant protein, retaining the leader peptide after transport to the PPS [68]. Therefore detection of a precursor protein does not have to mean that transport to the PP was not successful. By increasing the expression of the signal peptidase I, which is responsible for cleaving the SS of MBP, the rate of processed proteins might be increased. However, cleavage is not necessary to accomplish transport to the PP and BGs devoiding their cytoplasmic content after lysis have been shown to comprise the MBP-467 fusion protein proving thereby the periplasmic location of the MBP-467.

6.   Material and methods

6.1   Material

6.1.1   Chemicals

Chemicals, media, solutions and buffers are purchased from Merck (Darmstadt, Germany) and Roth (Carl ROTH, Karlsruhe, Germany).

6.1.2   Buffers and solutions

0,5M EDTA	186g EDTA
	800mL dH2O
	pH 8 with NaOH

Ethidium Bromide 10mg/ml	0,2g Ethidium bromide
	to 20mL with dH2O

1M Tris	1M Tris
	121,1g Tris
	to 800mL with H2O
	cool to room temperature
	desired pH with HCl

MOPS I	100mM MOPS
	10mM CaCl2
	10mM RbCl2
	with KOH to pH 7

MOPS II	100mM MOPS
	70mM CaCl2
	with KOH to pH 6,5

1% and 2 % Agarose	3g (or 6g) Agarose
	6mL 50x TAE buffer
	294mL dH2O

50x TAE	2M Tris
	1M CH3COOH
	0,1M EDTA pH 8,2

10x PBS	137mM NaCl
	2.7mM KCl
	10 mM Na2HPO4
	2 mM KH2PO4
	800mL dH2O
	pH 7,4 with HCl
	fill up to 1L with dH2O

50% Glycerol	25ml 100% glycerol
	25ml ddH2O
	mix and autoclave

0,85% Saline Medium	8,5g NaCl
	1l ddH2O
	filled into eprouvettes 9 ml and 9,9 ml using a dispenser and autoclave

1xNuPage® Sample Buffer	6,5ml PBS
	2,5ml NuPage® LDS Sample Buffer (4x)
	1ml NuPage® Reducing Agent (10x)

1xNuPage® MES Running Buffer	50ml NuPage® MES Buffer (20x)
	950ml deionized water

1x Transfer Buffer	50ml NuPage® Transfer Buffer (20x)
	100ml Methanol
	850ml deionized water

1x Blocking Solution	3ml Roth Roti-Block (10x)
	27ml deionized water

1x TBS	100ml TBS (10x, ROTH)
	900ml deionized water

1x TBST	100ml TBST (10x, ROTH)
	900ml deionized water

PonceauS	0,2g PonceauS
	3,0g trichloric acetic acid
	100ml ddH2O

Laemmli buffer	4% SDS
	20% glycerin
	10% mercaptoethanol
	0.004% bromophenol blue
	0.125 MTris HCl

6.1.3 Plasmids

Name	Size(nt)	Origin	Resistance	Reference	Specification
pMalp2x	6721	MB1	amp	New England Biolabs *Inc*	Backbone Plasmid
pMal-ivb	6769	MB1	amp	[54]	Backbone Plasmid
pGLysivb	6201	Rep	gent	[69]	Lysis Plasmid
pMal-467	6829	MB1	amp	this work	Expression Plasmid
pMal-467ivb	6877	MB1	amp	this work	Expression Plasmid
pMal-hm	6772	MB1	amp	this work	Expression Plasmid
pMal-hmivb	6814	MB1	amp	this work	Expression Plasmid

6.1.4 Bacterial Strains

Escherichia coli K12 NM522 sup E thi-1Δ(Lac-proAB)Δ(mcrB-hsdSM) 5(rK⁻mK⁻) (F'proABlacIqZΔM15) (source: Stratagene, Heidelberg, Germany)

Escherichia coli K12 W3110 Ion⁻ lΔ⁻F⁻mcrA mcrB IN(rrnD – rrnE) 1; (source: Lab Stock)

Escherichia coli K12 PC1363 – (pheA) (source: Phabagen Collection, University of Utrecht, The Netherlands)

Escherichia coli K12 PC2133 (source: Angela Witte, Department of Microbiology and Genetics, University of Vienna, Austria)

6.1.5 Markers

PAGE Ruler™ Unstained Protein Ladder, Fermentas (St. Leon-Rot, Germany)

Protein Molecular Weight Marker, Fermentas (St. Leon-Rot, Germany)

GeneRuler™ 50bp DNA Ladder, Fermentas (St. Leon-Rot, Germany)

GeneRuler™ 1kb DNA Ladder, Fermentas (St. Leon-Rot, Germany)

6.1.6 Enzymes

All enzymes are purchased from New England Biolabs (Hitchin, UK) and Fermentas Life Sciences (St. Leon-Rot, Germany). Double digests are performed according to the manufacturer's instructions.

6.1.7 Antibodies

Antibodies are diluted in 1xTBS/0.3%BSA/0.05%NaN$_3$, can be reused and stored at +4°C, except Streptavidin HRP, which is diluted in just 1xTSB and is not reused. .

Primary antibody	Species type	Application	Specificity	Source
α-MBP	rabbit	0.1-1µg ml^{-1}	MBP	NEB
Mouse α p467 immune serum	mouse	1:100-1:500	P467	MUW
Rabbit α p467 immune serum	rabbit	1:100	P467	MUW
Purified rabbit α p467 serum	rabbit	1:20-1:50	P467	MUW
Streptavidin HRP	n/a	0.2- 0.05µg ml^{-1}	Eivb, BCCP	Invitrogen
Herceptin/Trastuzumab	n/a	0.1µg/ml	Herceptin mimotope/QMW	MUW
Rituximab	n/a	0.1µg/ml	QYN	MUW

NEB (New England Biolabs, Hitchin, UK)

MUW (Medical University of Vienna, Vienna, Austria, Department of Pathophysiology)

Invitrogen (Carlsbad, California)

secondary antibody	Species type	Application	Specificity	Source
α human IgG HRP	n/a	1:8000	herceptin	MUW
α-rabbit IgG HRP	n/a	1:20000	rabbit IgG	n/a
α-mouse IgG HRP	rabbit	1:3000	mouse IgG	n/a

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6.1.8 Antibiotics

Antibiotics stocks are prepared and stored for long term at -20°C, when in use they are kept on 4°C

Name	Stock [mg/ml]	Final concentration [µg/ml]
Gentamycin	10	20
Ampicillin	50	100

6.2 Microbiological methods

6.2.1 Isolation of plasmid DNA

Isolation of plasmid DNA is done with Plasmid Miniprep Kit I from PEQLAB (Erlangen, Germany)

6.2.2 Restriction digest and ligation

Sequence specific cleavage

All restriction digests are performed according to the manufacturer's instructions.

Ligation

Ligations are performed with T4 ligase and corresponding buffer from New England Biolabs (Hitchin, UK) according to the manufacturer's instruction. Ligation was done over night at 16°C.

6.2.3 Agarose gel electrophoresis

DNA fragments are separated on 1% and 2% agarose gels [70] depending on their size, using voltage from 120 – 180 and 1xTAE for running buffer. Staining of gels is done with ethidium bromide (1µg/ml), visualization via UV light (250nm) by ChemiDoc™ XRS (Bio-Rad, Hempstead, UK) using the software QuantityOne (Bio-Rad, Hempstead, UK).

6.2.4 Polyacrylamid Gel Electrophoresis (PAGE)

6.2.4.1 Standard SDS Acrylamidgel

6.2.4.1.1 Gel preparation

Preparation of SDS – gels are done according to [71]. Stacking gel contains 4% and separation gel 8 – 15 % acrylamid, according to protein size. Polymerization is done with TEMED and 10% (w/v) APS.

6.2.4.1.2 Sample preparation

Pellets of 1ml culture samples were resuspended in 1xTBS according to their OD_{600} (OD600 x 250 = volume of 1xTBS in µl used for resuspension + same volume of 2x Laemmli-buffer. After lysis the highest OD_{600} value before LI is used for calculation. Samples are heated at +96°C for 15 min. before loading on the gel the samples have to be spinned down for 3 min at maximum speed. 15-20µl of the supernatant is loaded on the gel, 5µl of molecular weight protein marker are loaded.

6.2.4.1.3 Gel run

For migration into the stacking gel an initial voltage of 30V is set for 10 min and then increased to 100V at a maximum of 30mA.

6.2.4.2 Precast Bis Tris Acrylamidgels

6.2.4.2.1 Gel preparation

Pre-Cast gels from Invitrogen (Carlsbad, California) are used (NuPAGE® Novex 12, 15%Bis-Tris Gels) according to the manufacturer's instructions. Equipment for electrophoresis as well as the buffers (1xMES running buffer) are also from Invitrogen.

6.2.4.2.2 Sample preparation

Pellets of 1ml culture samples were resuspended in 1x NuPage sample buffer according to their OD_{600} (OD_{600} value x 250 = volume of 1x NuPage sample buffer) and it is mixed well. After that samples are is incubated at +99°C for 10 min. The protein sample can be stored at -20°C or used directly. Before loading on the gel the samples have to be spinned down for 3 min at maximum speed. 15µl to 20µl of the supernatant is loaded on the gel, 5µl of molecular weight protein marker are loaded.

6.2.4.2.3 Gel run

The gel is run at 180 Volt for ~60 min.

6.2.4.3 Native Acrylamidgel

For native PAGE reducing and denaturizing agents were excluded as possible.

6.2.4.3.1 Gel preparation

10% Gel, 5ml	H$_2$O	1.9ml
	30% Acryl amid mix	1.7ml
	1.5M Tris (pH 8.8)	1.3ml
	APS	0.05ml
	Temed	0.002ml

Gel loading buffer	50mM Tris-Cl (pH 6.8)
	0.1% Bromephenol blue
	10% Glycerol

1xTris glycine electrophoresis buffer

25mM Tris

250mM Glycin (pH 8.3)

6.2.4.3.2 Sample preparation

Freeze and thaw

Disruption of bacterial cells for detection of proteins under non reducing conditions was done by spinning down 1ml culture sample and resuspending the pellet in 20µl lysisbuffer. Three cycles of freeze and thaw were performed: 10 min at -20°C and let thaw at room temperature.

| LysisbufferI: | 50mM Tris HCl | pH 7.5 |
| | 150mM NaCl | |

Lysisbuffer II:	50mMTrisHCl	pH 7.5
	150mMNaCl	
	20mM EDTA	

Osmotic shock

Pellet of 1ml culture sample was resuspended in 1ml Lysisbuffer III and left waving at room temperature for 10 min. After centrifugation for 10min, 10 000g at 4°C the supernatant was decanted. For osmotic shock the pellet was resuspended in ice-cold 5mM $MgSO_4$, waving on ice for 10 min. Periplasmic proteins end up in the supernatant by spinning down for 10min, 10 000g at +4°C.

LysisbufferIII:	10mg Lysozyme	
	20%w/v Saccharose	
	30mM Tris HCl	pH 8
	1mM EDTA	

5xSample buffer	15.5 ml of 1M Tris-HCl (pH=6.8)
	2.5ml of a 1% solution of bromophenol blue;
	7.0ml of water
	25ml of glycerol

6.2.4.3.3 Gel run

The gel was run at 125V and 12mA for 6 hours.

6.2.5   Western Blot Analysis

6.2.5.1   Transfer of proteins

Transfer from proteins of SDS – gels to nitrocellulose membranes is done by a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hempstead, UK) according to the manufacturer's instruction. Transfer of pre – cast gels is done by wet blot using XCell II™ Blot Module from Invitrogen (Carlsbad, California) according to the manufacturer's instructions. The protein transfer is verified by staining with PonceauS and the band of the molecular weight marker is marked with a ball pen. Blocking is carried out for 2 hours at room temperature or over night at +4°C, using 5% "fat-free milk powder" in 1xTBST.

6.2.5.2 Antibody incubation and development

The membrane is washed 3x5 min in 1xTBST and is then incubated with the first antibody for 2 hours at room temperature. After incubation the membrane is washed 3x5 min in 1xTBS. Then the second antibody (HRP-coupled) is applied for one hour and the membrane is washed again three times in 1xTBS. Then membranes are incubated with 5ml SuperSignal West Pico Chemiluminescent Substrate from Pierce (now Thermo Scientific, Rockford, USA) for 3 min according to the manufacturer's instructions. All steps are performed under agitation. Detection is done with ChemiDoc™ XRS (Bio-Rad, Hempstead, UK) using the software QuantityOne (Bio-Rad, Hempstead, UK).

6.2.5.3 Stripping of PVDF membranes

Roti®-Free Stripping-Puffer (Carl Roth, Karlsruhe, Germany) provides the opportunity to remove antibodies and chemiluminescence staining from WB membranes and allows therefore multiple detections on one membrane. All steps are performed under agitation:

a) Used membrane is washed with 1xTBST for 20 min

b) Incubation of 30 min with Roti®-Free Stripping-Puffer at ~ 60°C

c) 2x20 min washed with 1xTBST

d) 1 hour blocking in 1xTBS/ 5% fat-free milk powder

e) Continue with antibody incubation and development 6.2.5.2

6.2.6 Dot blot analysis

Dilute protein samples in buffer (lysisbufferI - III was used) to final protein concentrations of ~1-100ng/µl. Apply 1µl samples directly onto nitrocellulose membrane. After applying the samples, the membrane is dried for at least 2 hours at room temperature before proceeding with the detection process. Immune-detection is done according to 6.2.5.2.

6.2.7 Preparation of separated cell fractions and detection of target proteins

Preparation of cell fractions was done according to the protocol of target protein verification from the pET System manual, Novagen, 8[th]edition, page 35 – 38. Following fractions were analyzed: total cell protein (TCP), media sample (M), periplasmic fraction

(P) and soluble cytoplasmic fraction (C). Fractions of culture samples were kept on ice and processed and analyzed in parallel. A culture sample for TCP was taken prior harvest. The culture was harvested and the supernatant after centrifugation yielded in the M sample which was TCA precipitated. The pellet was resuspended in Tris-HCl and after addition of EDTA gently stirred at room temperature. Centrifugation and subsequent resuspension in ice cold $MgSO_4$ and stirring at room temperature released the periplasmic proteins. The P fraction was TCA precipitated from the supernatant after centrifugation. The pellet was treated with BugBuster Protein Extraction Reagent, formulated for gentle disruption of the cell wall, resulting in the liberation of soluble protein, the C fraction. Samples were normalized according to the following worksheet and loaded onto a 15% polyacrylamid gel.

Worksheet 1: Determination of the culture OD_{600} at harvest.

	Dilution Factor (DF)	OD_{600} of diluted sample	OD_{600} at harvest (DF × OD_{600} of diluted sample)
Induced Culture			
Uninduced Culture			

Worksheet 2: Determination of the normalized volume of sample to load on a standard 10-well or 15-well mini SDS-PAGE gel.

	Sample conc. factor	OD_{600} at harvest	Z (conc. Factor × OD_{600})	Volume to Load 15-well mini-gel 180 µl ÷ Z	Volume to Load 10-well mini-gel 270 µl ÷ Z
Induced Samples TCP					
Media					
Periplasmic					
Soluble Cytoplasmic					
Insoluble Fraction					
Uninduced Samples TCP					
Periplasmic					
Media					
Soluble Cytoplasmic					
Insoluble Fraction					

6.2.8 Purification of DNA fragments after gel separation

6.2.8.1 Elution of bands from agarose gels

DNA purification when excised from an agarose gel is done using Gel Extraction Kit from Quiagen (Duesseldorf, Germany), according to the manufacturer's instruction.

6.2.8.2 Elution of bands from polyacrylamid gels

a) Complete electrophoresis, staining of the gel and gel documentation (according to 6.2.3)

b) Excise intensely stained DNA band out of the gel and put into one eppendorf tube

c) Incubate for 3 hours at 37°C in 500µl elution buffer to elute DNA

d) Centrifuge tubes at maximum speed (benchtop centrifuge) and transfer supernatant to a clean tube

e) Supernatant is precipitated in 1ml ethanol over night

f) Centrifuge at maximum speed (benchtop centrifuge) remove ethanol and dry DNA for ~20min at 65°C

g) DNA is resuspended in 15-20µl ultrapure water for further use

Elution buffer	NaCl	0.3mM
	EDTA	3mM
	Tris(pH 7.6)	30mM

6.2.9 Preparation of $CaCl_2$/$RbCl_2$ competent cells

1 ml overnight culture of the desired bacterial strain is taken for inoculation of 30ml LB. The culture is grown under constant stirring in a tempered water bath at +36°C to an OD_{600} of 0.5. Cells are collected by centrifugation (10 min at +4°C and 1700g) and kept ice cold for all further steps. The supernatant is decanted and the cells are resuspended in 20 ml ice cold MOPS I solution and held on ice for 10 min. Centrifugation is repeated and cells are resuspended in 6 ml MOPS II solution and hold on ice for 30 min. After the final centrifugation cells are resuspended in 480 µl MOPS II and 180 µl 50% glycerol. 100 µl aliquots are stored at -80°C.

6.2.10 Transformation of CaCl$_2$/RbCl$_2$ competent cells

Competent cells are thawed on ice (~10 min). 2µl DNA is added to 100µl of competent cells and they are kept on ice for 30 min. Heat shock at +42°C for 2min is performed (+36°C for transformation with plasmids carrying a temperature inducible promotor), followed by addition of 700 µl LB medium and shaking for 1 hour at +36°C. After that cells are stroke on agar plates, containing selective antibiotics.

6.2.11 Electro competent cells

1 ml overnight culture of the desired bacterial strain is taken for inoculation of 30ml LB. The culture is grown under constant stirring in a tempered water bath at +36°C to an OD$_{600}$ of 0.5. Cells are kept on ice for 30min and then collected by centrifugation (10min at +4°C and 1700g). Cells are resuspended in 30 ml ice cold 10% glycerol and kept ice cold for all further steps. Centrifugation is repeated, cells are resuspended in 15 ml 10% glycerol. Centrifugation is repeated, cells are resuspended in 600µl 10% glycerol. After the final centrifugation for 5min cells are resuspended in 300 µl 10% glycerol. For 0.1/0.2 cm electroporation cuvettes 50/100 µl aliquots are stored at -80°C, respectively.

6.2.12 Transformation of electro competent cells

Electroporation cuvettes and carriage are pre-cooled at -20°C. Electro competent cells are thawed on ice (~10min). 2 µl DNA is added and the mix is transferred into electroporation cuvette. Pulse settings for *E. coli* in 0.1 cm cuvettes: 25 µF/1.8kV/200Ω (0.2cm cuvettes: 25µF/2.5kV/200Ω) using the BioRad Gene Pulser II Electroporation System (BioRad, Munich, Germany). After the pulse is given cells are immediately mixed with 600 µl LB medium and kept shaking on +36°C for 1 hour. After that cells are stroke on agar plates containing selective antibiotics.

6.3   Cultivation, growth and lysis in bacterial ghost production

6.3.1   Cultivation medium for *Escherichia coli*

E. coli are grown in LB medium which consists of 10g Peptone, 5g Yeast extract and 5g NaCl, adjusted to a pH of 7.4. For agar plates, 15 g/l agar is added to the media. All media

are autoclaved for 30 min at +121°C and 2bar. All chemicals were obtained from Carl Roth, Karlsruhe, Germany.

6.3.2 Cultivation and storage of *Escherichia coli*

E. coli are incubated in eprouvettes in 5ml LB at +36°C in a rotating incubator (Heraeus BK5060E, Frankfurt, Germany) over night. These precultures are further used for noseflask experiments or plasmid isolations. For long time storage glycerin stocks of the preculture are produced and stored at -80°C in 25% glycerol..

6.3.3 Growth, expression and lysis studies

Clones are inoculated in 5ml LB medium each, supplemented with appropriate antibiotics and 2% glucose (when using IPTG inducible promotor). Eprouvettes are incubated in an incubation wheel at +36°C over night. 100ml noseflasks containing 30ml LB medium supplemented with antibiotics without glucose are prepared for inoculation with the overnight culture. At inoculation, the starting culture should reach an optical density (OD_{600}) of roughly 0.10–0.15. Flasks are transferred to a 36°C water bath and stirred on a magnetic plate at 300 rpm. For induction of protein expression 2mM IPTG (60µl of 1M IPTG stock) is added when OD_{600} reaches 0.2–0.3. For LI cultures are transferred to a +42°C water bath when an OD_{600} 0.4–0.5 is reached. For these studies throughout the whole experiment growth and protein expression of cultures is monitored.

➔ by measuring the optical density at 600 nm (OD_{600}) using Spectronic 20 Milton Roy Spectrophotometer (Milton Roy, Ivyland, USA) every 30 min, starting at time point of inoculation. OD values are illustrated as a graph in a logarithmic manner together with the cfu values in one diagram.

➔ by determining the viable cell counts (colony forming units = cfu)

The cfu are determined using a spiral plater (Don Whitley Scientific, Shipley, UK). Prior to plating bacterial samples are serially diluted in 0.85% NaCl (saline). Eprouvettes containing 9 ml saline are used for 1:10 dilutions (10^1): 9ml saline + 1ml culture. Eprouvettes containing 9.9 ml saline are used for 1:100 dilutions (10^2): 9.9 ml saline + 0.1ml culture

Dilutions according to the OD_{600} of growing and lysing bacterial cultures are given as a reference in Tab 6-1:

Growth		Lysis	
OD_{600}	Dilution	OD_{600}	Dilution
0.05 – 0.5	10^4	1.5 – 1.0	10^5
0.5 – 1.0	10^5	1.0 – 0.5	10^4
		0.5 – 0.2	10^3
1.0 – 2.0	10^6	0.2 – 0.01	10^2

Tab 6-1 Dilutions for plating of bacterial cultures according to OD_{600} prior and after LI

50 µl and 100 µl of the final dilution are plated in logarithmic manner on separate LB agar plates without any antibiotics. LB plates are incubated at +36°C over night. LB plates are counted using the ProtoCOL SR 92000 colony counter (Synoptics Ltd, Cambridge, UK). Cfu values are illustrated as a graph in a logarithmic manner together with the OD_{600} values in one diagram.

Sample points for cfu analysis of growth and expression:

- o 0 min/ expression induction
- o 30 min
- o 60 min
- o 90 min
- o 120 min
- o 150 min
- o 180 min

Sample points for cfu analysis of growth, expression and lysis:
(time point of expression induction has to be evaluated)

- o -90 min
- o -60 min/ expression induction
- o -30 min
- o 0 min/lysis induction
- o 30 min
- o 60 min

- o 90 min
- o 120 min

→ by light microscopy using Leitz DM-RB microscope (Wetzlar, Germany)

Growth and lysis is observed by light microscopy. Growing and dividing cells are contrasted and are expected to become translucent after LI.

Sample points for analysis of growth and expression:

- o 60 min
- o 120 min
- o 180 min

Sample points for analysis of growth, expression and lysis:

(time point of expression induction has to be evaluated, first microscopy sample is taken prior induction of expression)

- o -90 min/60 min/30 min
- o 0 min/lysis induction
- o 60 min
- o 120 min

→ By confirming the protein E and the recombinant protein expression by western blot analysis (6.2.5).

1ml culture of each sample is spinned down and centrifuged in a bench top centrifuge at full speed for 3 min and the pellet is stored at -20°C for further analysis.

Sample points for western blot analysis:
(time point of expression induction has to be evaluated)

- o -90 min
- o -60 min
- o -30 min
- o 0 min/LI

- o 30 min
- o 60 min
- o 90 min
- o 120 min

6.3.4 Fermentation

Fermentation is performed in a 30l stainless steel fermenter Techfors-S (Infors HT, Bottmingen, Switzerland) with a maximal working volume of 20l. Temperature is controlled by a Pt-100 temperature probe, acid and base pumps are used for pH-control and an InPro 6800 120mm Clarke electrode measures the oxygen level. Aeration is done with compressed air, the flow is controlled by a mass flow controller (Bronkhorst, Ruurlo, NL). The fermenter was rebuilt by changing the heating mechanism from hot water to steam, allowing a rapid temperature shift during the process. Steam is provided by a 10kW steam generator (Infors-HT, Bottmingen, Switzerland), which allows in situ sterilization. A sterilizable sample port is located on the bottom of the vessel. The whole process is controlled using the software IRIS V.5.2 (Infors-HT, Bottmingen, Switzerland).

Preparation

Sterilization (+121°C, 2bar, 30 min) of the vessel containing 20l LB is done the day before fermentation. A sterile run over night ensures sterility of the unit. For preparation of the pre culture 4x500ml LB in 2 l Erlenmeyer flasks are autoclaved. At 5pm the day before fermentation appropriate antibiotics and glucose are added and the flasks are inoculated.

Fermentation

Prior to inoculation samples are taken before and after addition of antibiotics, and plated undiluted onto LB plates without antibiotics to determine the sterility of the medium. Fermenter is inoculated with preculture according to following equation:

Volume of pre-culture (ml) necessary for inoculation =

$$\frac{desired\ starting\ OD600\ x\ Volume\ of\ medium\ in\ fermenter\ (ml)}{measured\ OD600\ of\ the\ pre\mbox{-}culture}$$

Immediately after inoculation the first culture sample is taken. Sampling (~40ml) continues every 30 min. Processing of the samples is done as described for OD_{600}

measurements, cfu determination, western blot analysis and microscopy in 6.3.3. During fermentation process several parameters (temperature, oxygen flow, pO2, pH, stirring) are observed and controlled by the IRIS program. Temperature up shift is done manually.

Killing

Streptomycin and Tetracycline (200µg/ml and 20µg/ml end concentration, respectively) are added to the culture at the end of the lysis process and incubated for 1 hour.

6.3.5 Harvesting and lyophilization

Excerpted from BIRD-C SOP "Fermentation in Total" from 13.09.2005

Sterile harvesting via separator
− Enter the **Param** screen of fermenter and select **Temp** on
− Set the Temp to e.g. 17°C
− Switch OFF the Regulation for pH, antifoam and pO$_2$
− Make sure the tube for the culture supernatant (Klarphasenablauf) is fixed into a big bottle (big enough for 20l) that can be autoclaved afterwards
− Plug in the heavy current
− Switch on the separator (green switch I)
− Check the direction of rotation (red-white arrow)
− Let the rotor warm-up
− Make sure the movement of the oil is visible
− Close valve 525, 527 and 029
− Open valve 046 and 527
− Set the stirring to 400 rpm and keep the flow still at the value of fermentation
− Open the green rotary knob of the harvest outlet (turn to the left side)
− Valve 029 (inlet controller) is closed and is opened slowly and carefully to a flow rate of ~ 30-50l / hours
− Valve 046 (responsible for the counter pressure against the inlet flow) is open and is closed slowly and carefully to reach a pressure of ~ 1 bar
Harvest the total fermenter

- The flow could be increased carefully to harvest the total fermenter (up to 10 or 15l/min)

- Close the green rotary knob of the harvest outlet (turn to the right side)

- Bring the flow in the fermenter to 1 (devoid pressure in the fermenter)

- Close valve, 029, 027, 046, 525 and 526

- Fill 5 liters of autoclaved water into the fermenter

- Open the green rotary knob of the harvest outlet (turn to the left side) again

- Increase the flow again (up to 10 or 15 l / M)

- Carefully open the valve 029

- Let the water run through the separator

- Close the green rotary knob of the harvest outlet (turn to the right side) again

- Close the valve 029 completely

- Switch off the separator and disconnect the power supply

Washing of bacterial ghosts (BG) I

- The BGs are collected into a sterile centrifuge tube.

- The BG pellet is resuspended into sterile water and divided into 6 centrifuge flasks under the laminar

   Washing step:

- The 6 flasks are filled up to ~400ml

- The centrifuge flasks are weighted in the laminar and brought to an identical weight

- One centrifuge flask can be filled with 400ml

- Using 6 centrifuge flasks ~ 2400ml water will be used for washing at once

- BGs are harvested by centrifugation in Hermle ZK 401 centrifuge at 8000rpm for 15min and 4°C.

- The supernatant is decanted carefully

- Pellets can be stored now at 4°C for further washings on the next day

Washing of bacterial ghosts (BG) II

   Washing step:

- The 6 flasks are filled with ~100ml and the pellets are resuspended

- properly

- The centrifuge flasks are weighted in the laminar and brought to an identical weight
- One centrifuge flask can be filled with 400ml
- Using 6 centrifuge flasks ~ 2400ml water will be used for washing at once
- BG are harvested by centrifugation in Hermle ZK 401 centrifuge at 8000rpm for 15min and 4°C.
- The supernatant is decanted carefully

    Washing step:

- The 6 flasks are filled with ~100ml and the pellets are resuspended properly
- The centrifuge flasks are weighted in the laminar and brought to an identical weight
- One centrifuge flask can be filled with 400ml
- Using 6 centrifuge flasks ~ 2400ml water will be used for washing at once
- BGs are harvested by centrifugation in Hermle ZK 401 centrifuge at 8000rpm for 15min and 4°C.
- The supernatant is decanted carefully

    Washing step:

- The 6 flasks are filled with ~50l sterile water and the pellets are resuspended properly
- The suspension of the 6 flasks are combined into one centrifuge flask
- BGs are harvested by centrifugation in Hermle ZK 401 centrifuge at 8000rpm for 15min and 4°C.
- The supernatant is decanted carefully

Lyophilization of the BGs
- 11 lyophilization bottles are labeled and weighted on the analytical balance
- Write down the weight of each bottle into the lab book
- BG are resuspended in 200ml sterile distilled water
- 10 lyophilization bottles are filled with 20ml of the BG suspension each (in total 200ml)
- The remaining BG suspension is transferred into bottle 11 for sterility testing
- The tubes are stored at –80°C until lyophilization

6.3.6 Calculation of lysis efficiency and particles per mg

<u>Lysis Efficiency</u>

$$LE = \left(1 - \frac{cfu_{(t)}}{cfu_{(t_0)}}\right) \cdot 100$$

t_0...*time point of lysis induction*

t ...time point after lysis induction used for determination of LE; for this work t =120 min after induction

<u>Particles per mg</u>

$$\frac{particles}{mg} = \frac{cfu_{highest} \, volume_{total\,harvested\,ml}}{yield_{total\,mg}}$$
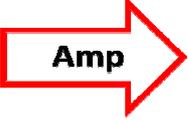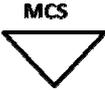
6.3.7 Sterility testing of the material

~10mg lyophilized material is resuspended into 5ml LB medium and used for:

- o "Koch'sches Plattengußverfahren"(1ml, 2ml)
- o plating on agar plates (100μl, 400μl)
- o liquid enrichment cultivation: 1ml inoculated into 5ml LB medium and incubated for 7 days at +28°C followed by plating of 100 and 400μl on agar plates

7. Appendix

7.1 Symbols used in cloning strategies

Symbol	meaning
LacZα	Gene encoding the lacZα protein
ivb LacZα	Gene encoding the lacZα protein, coupled to an in vivo biotinylation sequence
Amp	Ampicillin resistance cassette
MalE	Gene encoding the N-terminal part of the maltose binding protein
LacIq	repressor of lac operon
rop	regulation of plasmid DNA replication by antisense RNA
pMB1	origin of replication
M13	intergenic region, origin of replication (+ -)
MCS	multiple cloning site
P_{TAC}	Promotor of *malE*

7.2 Construction of pMal-467

Sequence p467 was cloned in the MCS of the backbone vector pMal-p2x resulting in pMal-467:
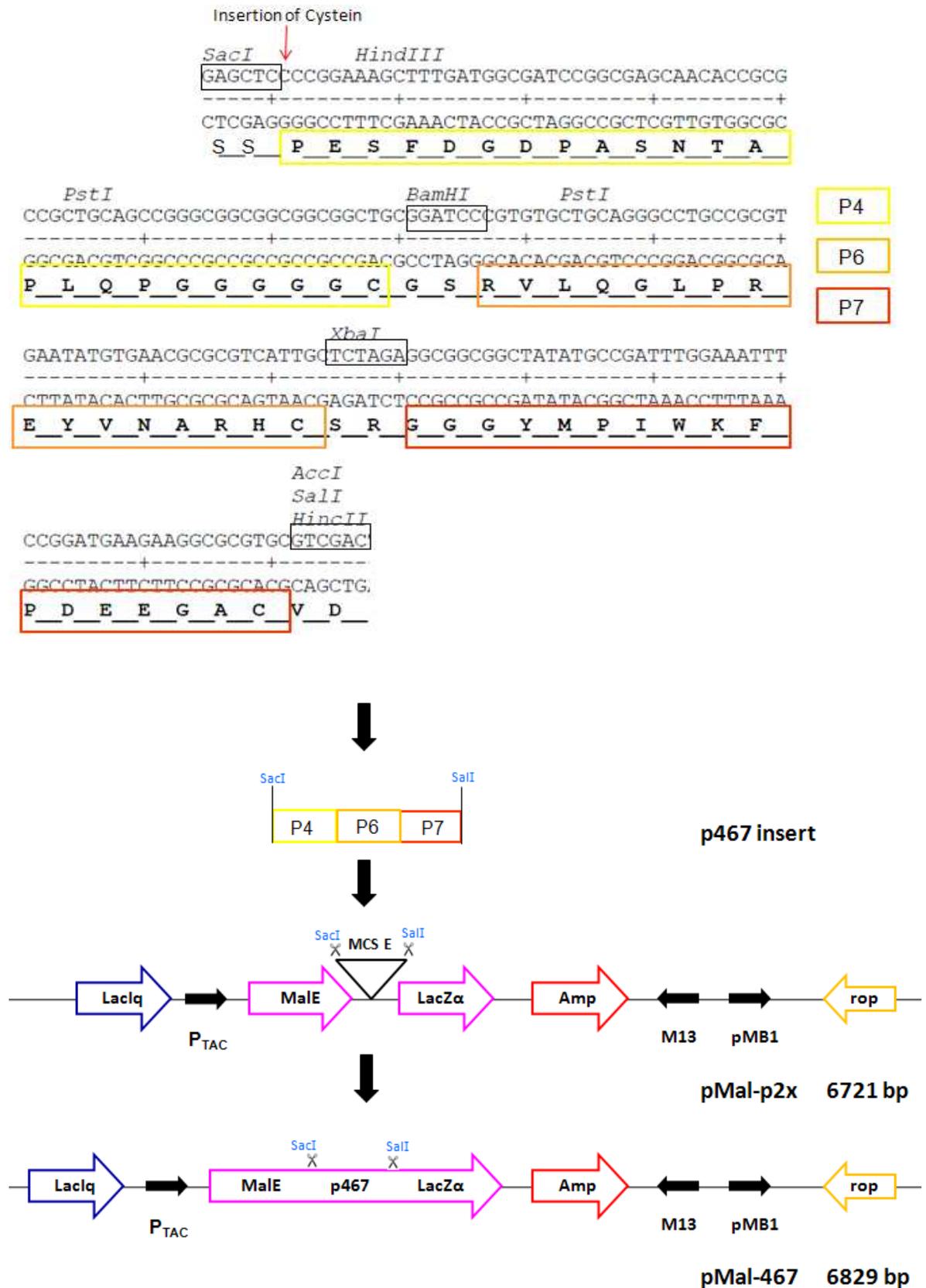
7.3 Construction of pMal-467ivb

Sequence p467 was cloned in the MCS of the backbone vector pMal-ivb resulting in pMal-467ivb:

Insertion of Cystein

```
  SacI        HindIII
 GAGCTCCCCGGAAAGCTTTGATGGCGATCCGGCGAGCAACACCGCG
 -----+---------+---------+---------+---------+
 CTCGAGGGGCCTTTCGAAACTACCGCTAGGCCGCTCGTTGTGGCGC
  S  S   P  E  S  F  D  G  D  P  A  S  N  T  A
```
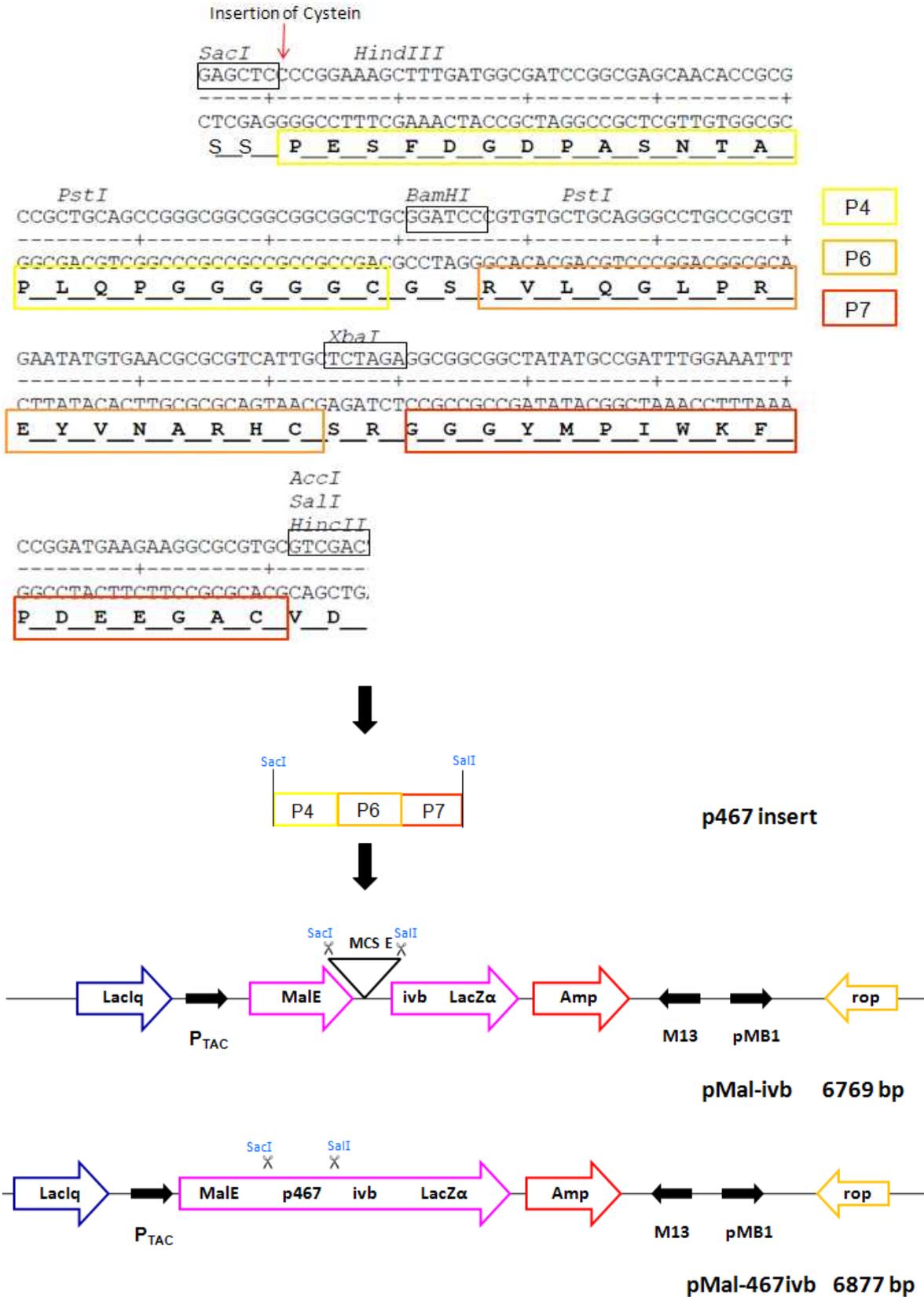
```
    PstI                           BamHI        PstI
 CCGCTGCAGCCGGGCGGCGGCGGCGGCTGCGGATCCCGTGTGCTGCAGGGCCTGCCGCGT
 ---------+---------+---------+---------+---------+---------+
 GGCGACGTCGGCCCGCCGCCGCCGCCGACGCCTAGGGCACACGACGTCCCGGACGGCGCA
  P  L  Q  P  G  G  G  G  G  C  G  S  R  V  L  Q  G  L  P  R
```

| P4 |
| P6 |
| P7 |

```
              XbaI
 GAATATGTGAACGCGCGTCATTGCTCTAGAGGCGGCGGCTATATGCCGATTTGGAAATTT
 ---------+---------+---------+---------+---------+---------+
 CTTATACACTTGCGCGCAGTAACGAGATCTCCGCCGCCGATATACGGCTAAACCTTTAAA
  E  Y  V  N  A  R  H  C  S  R  G  G  G  Y  M  P  I  W  K  F
```

```
   AccI
   SalI
   HincII
 CCGGATGAAGAAGGCGCGTGCGTCGAC
 ---------+---------+-------
 GGCCTACTTCTTCCGCGCACGCAGCTG
  P  D  E  E  G  A  C  V  D
```

SacI ——— SalI

P4 P6 P7

p467 insert

SacI ✕ MCS E ✕ SalI

LacIq — P_TAC — MalE — ivb LacZα — Amp — M13 pMB1 — rop

pMal-ivb 6769 bp

SacI ✕ ——— SalI ✕

LacIq — P_TAC — MalE p467 ivb LacZα — Amp — M13 pMB1 — rop

pMal-467ivb 6877 bp

7.4   Construction of pMal-hm

Sequence hm was cloned in the MCS of the backbone vector pMal-p2x resulting in pMal-hm:
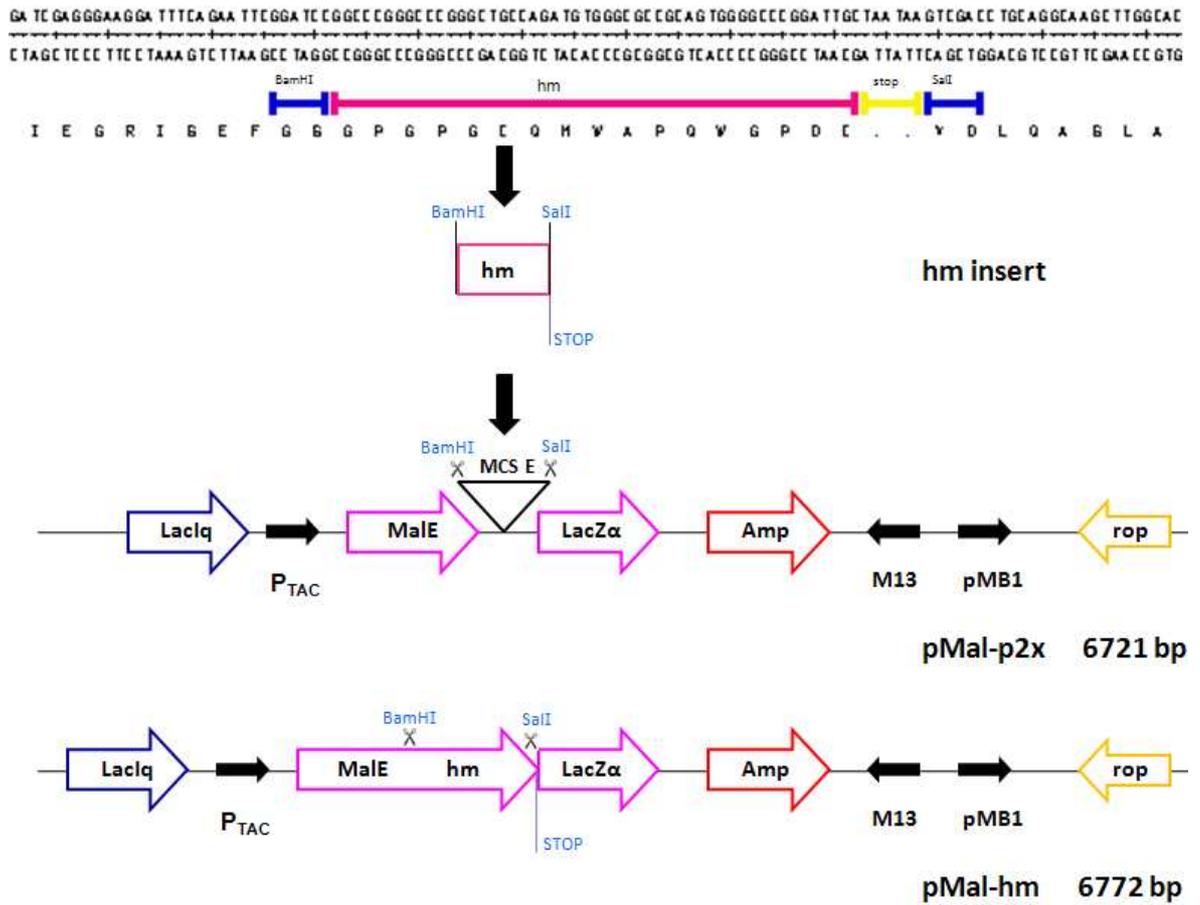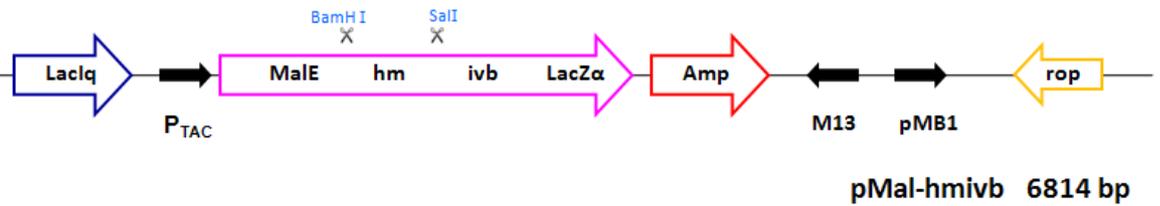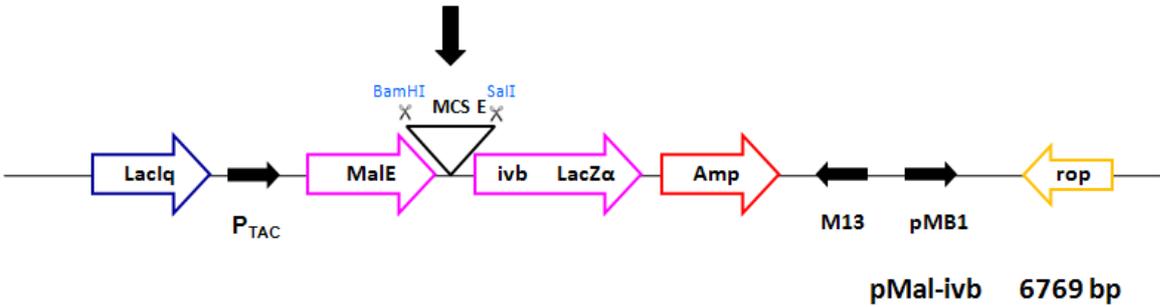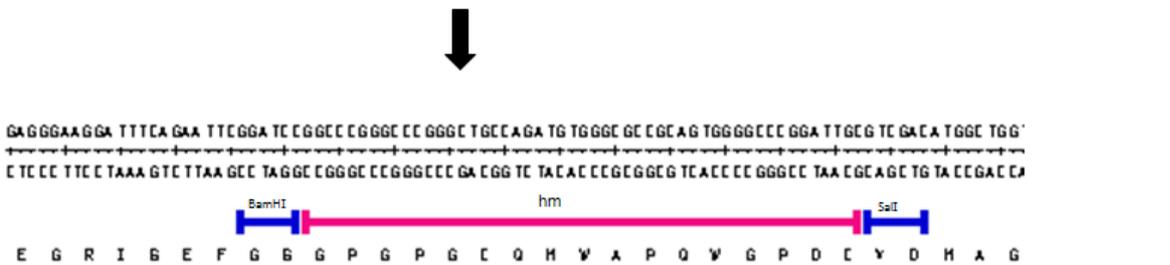
7.5 Construction of pMal-hmivb

Sequence hm from 7.4 serves as template. Primer were designed to exclude the stop for cloning into pMal-ivb. PCR amplified hm sequence was cloned in the MCS of the backbone vector pMal-ivb resulting in pMal-hmivb:
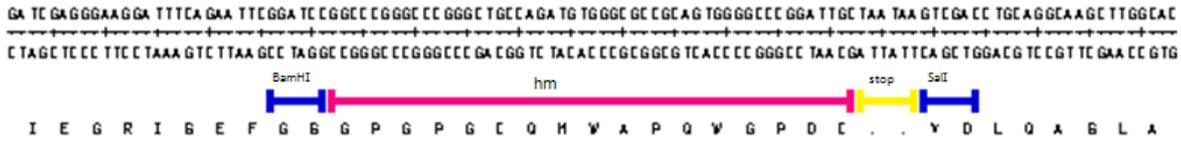
hm 5' primer: cagaattcggatccggccc

hm 3' primer: tatggtcgacgcaatccgggccccact

pMal-ivb   6769 bp

pMal-hmivb   6814 bp

8.   References

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