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Bacterial ghosts presenting fertility proteins for induction of
immunocontraception in possum

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Abbreviations and Symbols



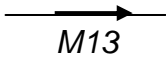

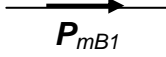
µg	microgram
µl	micro liter
aa	amino acid
AB	antibiotics
BG(s)	bacterial ghost(s)
bp	base pair
CAP	cyclic adenosine phosphate
cAMP	cyclic adenosine monophosphate
cfu	colony forming units
Cp4	coat protein 4
dH₂O	deionized water
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
Fwd	forward
IL	interleukin
kb	kilo base pairs
kDa	kilo Dalton
LGR	Landcare research, New Zealand
LPS	lipopolysaccharide
MCS	multiple cloning site
min	minutes
mg	milligram
ml	milliliter
nm	nanometer
ng	nanogram

nt	nucleotide
OD₆₀₀	optical density at 600 nm
ori	origin of replication
piii	minor capsid protein from filamentous phage fd
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPS	periplasmic space
Rev	reverse
rpm	rounds per minute
sdH₂O	sterile deionized water
TH1	T-helper cells 1
Tm	annealing temperature
U	unknown sample
Vap1	vesicle associated protein 1
WB	western blot
Zp1, Zp2, Zp3	zona pellucida protein 1, 2, 3
ZP2C	C-terminal part of zona pellucida protein
ZP2Copt	C-terminal part of zona pellucida protein optimized for expression in E. coli

Genes

Amp	Ampicillin resistance cassette
Kan	Kanamycin resistance cassette
Gent	Gentamycin resistance cassette
cl875	Thermosensitive allele of the phage λ -repressor gene
Eivb	C-terminal fusion of the gene encoding the lysis protein E of the bacteriophage phiX174 and the in vivo-biotinylation sequence
Mob	gene encoding a mobility protein
GIII-MCS-myc-His	<i>gIII</i> -signal sequence from the bacteriophage fd gene III protein, multiple cloning site, myc-epitope and his-epitope
araC	Gene encoding the repressor of the <i>ara</i> -operon of <i>E. coli</i>
MalE	Maltose binding protein sequence for protein transport in the periplasmic space
lacIq	lacI gene under control of a mutated high expression lacI promoter
ZP2C	C-terminal part of the zona pellucida 2 gene
ZP2Copt	Sequence optimized (seq.opt.) C-terminal part of the zona pellucida 2 gene
GST Cp4 fusion protein	Glutathione-S-transferase (GST) Cp4 fusion protein
GST Vap1 fusion protein	Glutathione-S-transferase (GST) Vap1 fusion protein

Regulatory elements and origins of replication

	<p>promoter of the <i>ara</i>-operon</p>
	<p>mutated promoter of P_R promoter in bacteriophage λ and repressor maintenance promoter of bacteriophage λ</p>
	<p>M13 intergenic region, origin of replication</p>
	<p>Broad host range origin derived from the <i>Bordetella bronchioseptica</i> plasmid pBBR1</p>
	<p>origin of high-copy number plasmids derived from the pUC-vector series</p>

1 Zusammenfassung

1.1 Zielsetzung

Ziel dieser vorliegenden Arbeit war die Produktion eines immunocontrazeptiven Impfstoffes auf Basis des Bacterial Ghost Systems, um die Opossum (*Trichosurus vulpecula*) Wildpopulation Neuseelands einzudämmen. Für die Herstellung eines fertilitätskontrollierenden Impfstoffes wurden zunächst vier unterschiedliche Ziel-Sequenzen ausgewählt:

- ZP2C, Zona Pellucida Protein 2, C-terminale Sequenz
- ZP2Copt, Zona Pellucida Protein, mit optimiertem Codon für E. coli Expression
- Cp4, Coat Protein 4 des Conceptus
- Vap1, Vesicle Associated Protein 1 der frühen Oogenese

Die vier oben genannten immunologischen Sequenzen wurden in E. coli produziert, wobei die jeweilige Ziel-Sequenz in den periplasmatischen Raum transportiert wurde. Anschließend wurden die Bakterien durch E-gesteuerte Lyse in Bacterial Ghosts überführt. BG-Impfstoffe, stimulieren auf natürliche Weise das Immunsystem, ohne dass weitere Adjuvanten benötigt werden. Durch die Anwendung der sogenannten Impfstoffe in Opossums soll das Immunsystem der Tiere angeregt werden um eine humorale sowie zelluläre Immunantwort gegen die verwendeten Ziel-Proteine zu induzieren.

1.2 Resultate

Um die Fertilität von Opossums zu kontrollieren, wurden zunächst vier unterschiedliche immunologische Target-Sequenzen ausgewählt um eine Immunantwort in Opossums zu erzeugen. Dafür wurden die vier Sequenzen anschließend in den pBGKB Vektor kloniert und die daraus entstandenen vier Plasmide: pBGKBZP2C, pBGKBZP2Copt, pBGKBCp4, pBGKBVap1, beinhalten Opossum spezifische Sequenzen die für eine normale Entwicklung des Tieres verantwortlich sind. Protein Expression des pBGKB Vektors unterliegt dem pBAD Promoter und wird induziert durch die Zugabe von L-Arabinose. Die immunologischen Ziel-Sequenzen werden als Fusionsproteine zusammen mit der gIII-Signalsequenz exprimiert, welche für den Transport der Proteine ins Periplasma maßgeblich sind, wo sie anschließend abgespalten wird. Dadurch befinden sich im periplasmatischen Raum ausschließlich die exprimierten Ziel-Proteine. Im ersten Klonierungsschritt wurden die immunologischen Ziel-

Sequenzen in den pBGKB Vektor kloniert und Proteinexpression in E. coli getestet. Anschließend wurden die verschiedenen rekombinanten E. coli Stämme mit dem Lyse-Plasmid pGLysivb co-transformiert. Mehrere Vorversuche mit unterschiedlichen Expressions- und Lysezeiten wurden durchgeführt, um jene Klone mit optimaler Proteinexpression und hoher Lyseeffizienz für die Fermentation im 30L Fermenter auszuwählen. Die Produktion von pBGKBZP2C und pBGKBZP2Copt Bacterial Ghosts im Fermenter wurde durchgeführt und die Konzentration der exprimierten Antigene wurde bestimmt. Anschließend wurden die getrockneten Bacterial Ghosts nach Neuseeland verschickt, um dort Fertilitätsstudien am Opossum durchzuführen. Aufgrund von fehlerhafter Sequenz in den Plasmiden pGEX2TCp4 und pGEX2TVap1 konnte mit diesen nicht weiter gearbeitet werden.

2 Summary

2.1 Objective

The ambition of this work was the construction of a bacterial ghost system which presents proteins for brushtail possum (*Trichosurus vulpecula*) breeding. This immunological based fertility control involves the production of a bacterial ghost vaccine which induces possum's immune system and makes antibodies against its own reproductive system that will block reproduction. For this reason four different possum-immunocontraceptive targets were chosen:

- ZP2C, zona pellucida protein 2, C-terminal sequence
- ZP2Copt, zona pellucida protein 2, C-terminal sequence, with a codon optimized for expression in *E. coli*
- Cp4, coat protein 4 of the possum's conceptus
- Vap1, vesicle associated protein 1 of early oogenesis

These four immunological gene sequences were cloned into the pBADGIII-derived vector pBGKB. In this work contraceptive antigens were produced in non-living bacterial ghosts that are able to express high levels of recombinant protein by the export of proteins into the periplasmic space. The transport of the proteins into the periplasmic space is due to E-mediated lysis of the bacteria, resulting in the release of the cytoplasmic cell content and providing a sealed periplasmic space. Bacterial ghosts therefore function as a vaccine and are able to stimulate cellular and humoral immune responses due to recognition of the bacterial cell surface by antigen presenting cells. Additionally the possum immunocontraceptive antigens are released and are used to produce antibodies against the possum's reproductive system.

2.2 Results

Depending on the bacterial ghost system, an immunocontraceptive vaccine was produced to control possum's fertility. Therefore four diverse target molecule sequences (ZP2C, ZP2Copt, Cp4, and Vap1) were cloned into the pBGKB vector for protein expression. These four different immunogenic target sequences are all essential for normal possum development and act at different developmental stages. The pBGKB plasmid is a pBR322 derived vector and protein expression is under the control of the pBAD promoter, induced

due to the addition of L-arabinose. Proteins are expressed as fusion proteins together with the gIII signal sequence which transports the protein into the periplasmic space and is cleaved off afterwards; therefore only the protein of interest results in the periplasmic space. The first cloning steps included the production of the vector plasmid pBGKB with one of the immunogenic target sequences, resulting in the production of four different plasmids. After protein expression was determined, the plasmids were co-transformed with the lysis plasmid pGLysivb which is responsible for E-mediated lysis and the formation of the transmembrane tunnel. Lysis studies as well as expression studies were performed and the best clones were taken for bacterial ghost production in the 30L fermenter. The obtained bacterial ghosts with the expressed immunogenic proteins were harvested and protein expression was determined using western blot technique. Furthermore the quantity of the expressed antigens in the bacterial ghost was measured and the lyophilized bacterial ghosts were shipped to New Zealand for further fertility studies in the brushtail possum (*Trichosurus Vulpecula*). Due to incorrect sequences of the plasmids pGEX2TCp4 and pGEX2TVap1 no further studies concerning Cp4 and Vap1 as immunological targets, could be made.

3 Introduction

3.1 Brushtail possum (*Trichosurus Vulpecula*) fertility control

The brushtail possum (*Trichosurus Vulpecula*) is not native to New Zealand; it was brought from Australia for starting a fur industry [1-2]. Since these animals have no natural enemies in New Zealand they became one of the most significant vertebrate pests [3-5]: they harm forests and trees and destroy bird's natural habitat, they are often carriers for bovine's tuberculosis which can spread to cattle and deer [8] and furthermore they are quite efficient reproducers [6]. In consideration of these facts it is important to control possum's fertility by achieving a cost-effective and long-term solution [7]. Target molecules acting during embryonic development of possums had to be found and were selected [4] because they are:

- important for normal development
- have possum-specific components
- act at different stages of development

These immunocontraceptive targets are used for vaccine production to maintain immunological based fertility control by inducing the possum's immune system and by making antibodies against its own reproductive system [9].

3.2 Immunocontraception

Immunologically based fertility control, also known as immunocontraception, is an important method for the regulation of human and animal population numbers [10]. The process involves vaccination against sperm, eggs, or reproductive hormones to prevent either fertilization or production of gametes [11]. Immunocontraceptives for wild animals have a different objective than those for humans. For animals such a technique may provide a long-term and cost-efficient solution to check population growth rather than to contracept particular individuals [9-11]. Furthermore immunocontraception turns out to be more "humane" than traditional wildlife population control methods like shooting, trapping or poisoning. Despite the necessity of finding the appropriate target, easy application plays a major role, outlining the usage of immunocontraception in baits for wildlife administration [12]. Bacterial Ghosts function as a promising delivery system as they are environmentally

safe and field deliverable. Research progress was achieved in the field of immunocontraceptive vaccine development, focusing in this work on the topic of brushtail possum immunocontraception, by using different target molecules in combination with the bacterial ghost system.

3.3 Brushtail possum immunocontraceptive targets

3.3.1 Zona pellucida proteins

One of the most important immunocontraceptive targets is the zona pellucida protein ZP [13]. The zona pellucida consists of three glycosylated proteins ZP1, ZP2, ZP3 [13-19] and is the extracellular coat around all mammalian eggs. ZP proteins are strongly immunogenic and immunization with zona pellucida proteins inhibits fertilization whereas antibodies against zona pellucida are ovary-specific and prevent sperms from binding [15-16] and penetrating the ova or disrupt the development of follicles in the ovary or the embryo.

Investigations of the ZP2 protein have identified two immunogenic peptide sequences that appear to be both: correlated with infertility and species specific. Therefore the ZP2 C-terminal gene sequence (ZP2C) was used in this study [18].

Additionally the ZP2Copt which contains the brushtail possum zona pellucida 2 (ZP2) C-terminal sequence gene with an optimized (opt.) codon for the protein expression in *Escherichia coli* was used as immunocontraceptive target.

3.3.2 Coat proteins

The coat proteins from the mucoid and shell coat of the possum's conceptus are secreted by the oviduct and uterus and had also turned out to be an immunogenic target molecule [20]. Experiments were done with the coat protein 4 (Cp4) which plays an important role in the late cleavage for normal blastocyte development and epithelial maintenance [21-22]. As the shell coat in particular is unique in all marsupials, it is a very suitable target for immunocontraception.

3.3.3 Vesicle associated proteins

The family of the vesicle associated proteins plays a major role in early development and is formed in the oocyte during oogenesis. The vesicles accumulate in the oocyte and become located at one pole of the oocyte after fertilization [22-23]. Sequence analysis showed that

especially the Vap1 protein has unique features in molecular constitution and is therefore used as a possum fertility control target protein in this study.

3.4 Bacterial ghost system

Bacterial ghosts are empty cell envelopes originating from Gram-negative bacteria based on the expression of the lysis gene E from the bacteriophage PhiX174 [24]. E-mediated lysis of the bacteria results in formation of an E-specific transmembrane tunnel structure through which the cytoplasmic content is expelled, resulting in empty bacterial cell envelopes [25-26]. Therefore bacterial ghosts have a natural outer surface which provides them with the original targeting functions of the bacteria they are derived from [27].

3.4.1 E-mediated lysis

Bacterial lysis is maintained by the gene E which encodes for a highly hydrophobic, 91 amino acid protein [28-29]. Protein E expression can be induced by a chemical promoter system (e.g. araBAD or LacPO) or by a heat inducible promoter system (e.g. λ pL/pR-cl857). As there is only a small amount of protein E needed to start lysis, the induction time is kept short and a strong repressor system is necessary to avoid premature lysis [30]. E-mediated lysis of the bacteria results in the formation of a transmembrane tunnel structure by the fusion between the inner and outer membrane. Through the E-mediated lysis tunnel, with its diameter between 40 to 200 nm, the cytoplasmic content is expelled, resulting in an empty bacterial cell envelope with a sealed periplasmic space, suitable to carry foreign proteins [30, 33].

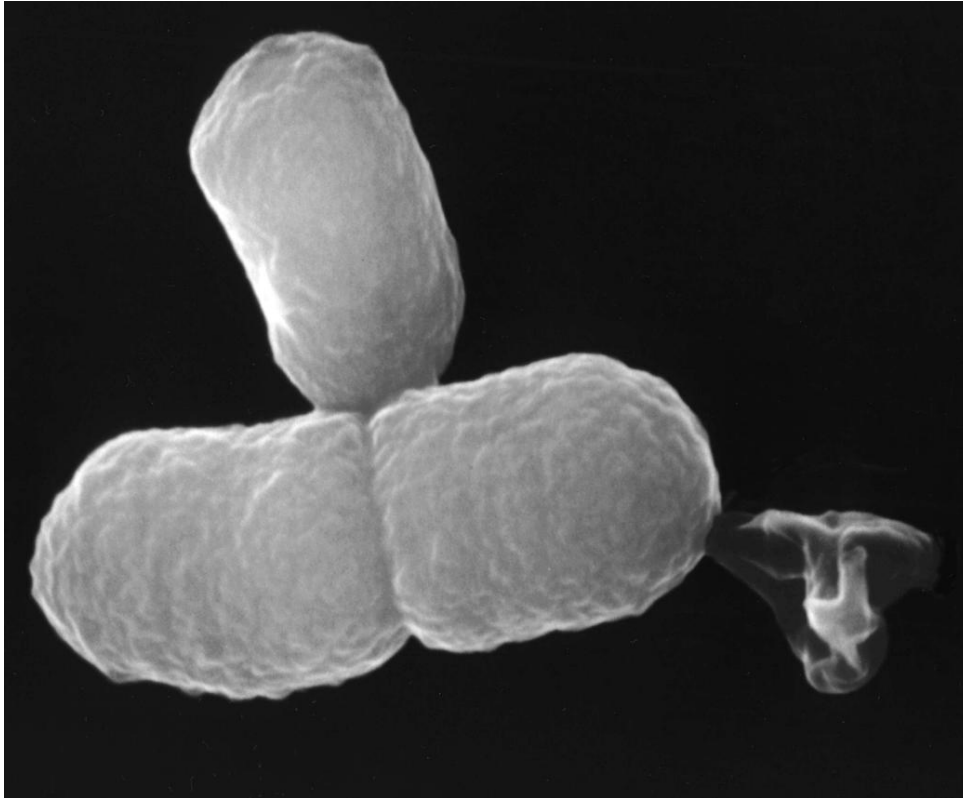


Fig. 3.1: E-mediated lysis in *E. coli*. Cytoplasmic content of the bacterium is expelled through E-mediated transmembrane tunnel structure. [34]

3.4.2 Lysis plasmid pGLysivb

In this work the lysis plasmid pGLysivb was used for bacterial ghost production. The plasmid consists of the pBBR1 derived backbone plasmid and the lysis cassette, which carries the gene E [35]. The bacteriophage PhiX174 gene E is under the transcriptional control of the mutated heat-inducible λ pL/pR-cl857 promoter – repressor system, allowing the expression of the protein E at temperatures above 37°C and represses gene E expression at temperatures below 37°C [35-37]. Additionally the pGLysivb lysis plasmid contains a gentamycin resistance cassette and a mutated mobilization gene sequence with mobilization activity.



Fig. 3.2: pGLysivb plasmid. Mob: mobilization gene; Gent: gentamycin resistance cassette; cl857: thermo-sensitive phage λ -repressor gene; Eivb: protein fused to in-vivo biotinylation sequence.

3.4.3 Recombinant bacterial ghost vaccine

As bacterial ghosts retain all morphological, structural and antigenic features of the cell wall they are proposed to be an innovative vaccine delivery system. Bacterial ghosts have been produced from a variety of bacteria always combining their natural intrinsic adjuvant properties with the versatile carrier functions for foreign antigens, immunomodulators or other substances [38, 39]. In recombinant ghosts, foreign proteins can be inserted into the inner membrane prior to E-mediated lysis, via specific N-, or C-, or N-and C-terminal anchor sequences. [40]. Proteins can be carried in the sealed periplasmic space of bacterial ghosts. Moreover S-layer proteins can be expressed in bacterial strains prior to E-mediated lysis, carrying inserts of foreign epitopes [41, 42]. The bacterial ghost outer membrane contains lipopolysaccharides (LPS), pili and proteins which are able to recognize receptors on target cells and are furthermore recognized by the innate immune system [42]. Therefore bacterial ghosts offer a broad spectrum for anchoring foreign antigens [32].

It has been shown in former studies that bacterial ghosts are taken up by dendritic cells and macrophages. Experiments in THP-1 human macrophage cell lines confirmed a significant activation of IL-12, an interleukin which is of special importance in the activation of cellular TH1 immune responses. Moreover a rapid uptake of bacterial ghosts by macrophages could be confirmed, resulting in the suggestion that bacterial ghosts effectively stimulate monocytes and macrophages [42]. Despite the benefit of cellular and humoral immune response generation, the bacterial ghost system provides other relevant advantages such as

- bacterial ghosts are non living and represent an alternative to heat or chemically inactivated bacteria [38]
- bacterial ghosts are safe as they do not contain pathogenic host DNA
- the production process does not denature bacterial ghost envelopes and is quick, easy and cheap [40]
- recombinant proteins are inserted into a highly immune stimulatory environment [38] and there is no limitation in size of foreign antigens to be inserted
- the carrier capacity of bacterial ghost's membranes, periplasma and internal lumen can fully be utilized
- bacterial ghosts are stable for long periods of time and do not require the cold chain storage system [38]
- application can be done by several mucosal routes such as oral, conjunctival, rectal or aerogenic.

3.5 pBADGIII derived vector plasmid system

The pBADGIII vectors are pBR322-derived plasmids from Invitrogen which are available in three versions, for cloning into different reading frames. In this work the pBADGIIIB vector was used for further cloning.

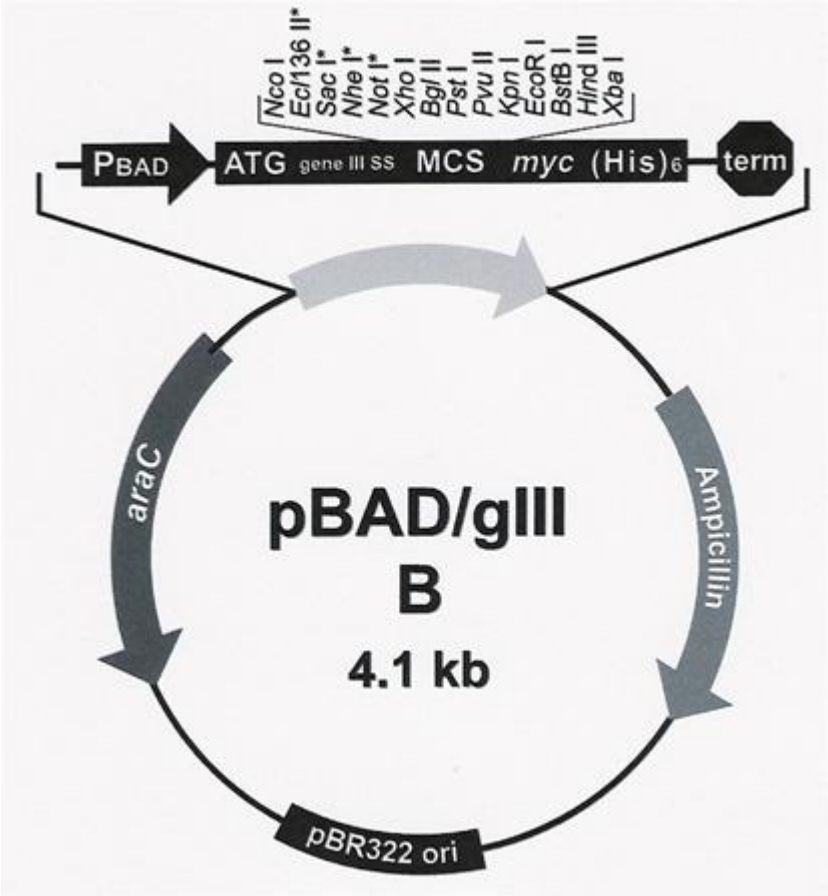


Fig. 3.3: Plasmid pBADGIIIB. Invitrogen, [50]

Based on the mother plasmid pBADGIIIB (Invitrogen) the plasmid pBGKB was cloned by removing the ampicillin resistance cassette and replacing it with a kanamycin resistance cassette. Plasmid pBGKB provides all necessary features to express recombinant proteins:

- *gIII*-signal sequence from the bacteriophage fd gene III protein for protein transport into the periplasmic space
- *araBAD* promoter for tightly regulated expression [43]
- Translation initiation signals for optimized E. coli expression
- C-terminal *c-myc* epitope for detection and analysis with an Anti-*myc* antibody
- C-terminal polyhistidine (6xHis) tag for purification

3.5.1 pBAD promoter

Protein expression in pBGKB is under the control of the *araBAD* promoter which is derived from the *ara* operon. The *ara* operon codes for three different enzymes that are required to catalyze the metabolism of arabinose [43-48]:

- *araA* (arabinose isomerase)
- *araB* (ribulokinase)
- *araD* (Ribulose-5-phosphate epimerase)

These three structural genes are arranged in an operon that is positively and negatively regulated by the product of the *araC* gene [43] which forms a complex with L-arabinose. In the absence of arabinose the *araC* dimer inhibits transcription of the three structural genes [45-47]; *araC* also prevents self expression and therefore is an autoregulator of its own expression. When arabinose is present, it binds to *araC* and allows transcription to begin.

Expression levels can be repressed by introducing glucose to the growth medium. Glucose acts by lowering the cAMP levels which in turn decreases the binding of CAP following by transcriptional decrease.

For optimized protein expression the L-arabinose concentration can be varied and for expression of toxic or essential genes tight regulation of pBAD via *araC* is quite useful [46].

The production of an immunocontraceptive vaccine to inhibit reproduction of brushtail possum based on the Bacterial Ghost system involved the design of an appropriate vector plasmid and the choice of possum immunological-target sequences. The working steps for construction of the vector plasmid pBGKB as well as the production of immunological based fertility control proteins are described in the following chapter.

4 Results

The four different immunological target sequences ZP2C, ZP2Copt, Cp4 and Vap1 were cloned into the multiple cloning site of pBKGB providing all of the necessary features for protein expression in the periplasmic space:

- *gIII*-signal sequence from the bacteriophage fd gene III protein for protein transport into the periplasmic space
- *araBAD* promoter for tightly regulated expression
- Translation initiation signals for optimized *E. coli* expression
- C-terminal *c-myc* epitope for detection and analysis with an Anti-*myc* antibody
- C-terminal polyhistidine (6xHis) tag for purification

4.1 pBGKB cloning strategy

The ampicillin resistance cassettes from plasmids pBADGIIIA, B, C were removed by restriction digest with the restriction enzyme *BspHI* (New England Biolabs) and replaced by the kanamycin resistance cassette from plasmid pBHR1.

Cloning procedure for the pBGKB plasmid is shown in figure 4.1. The *BspHI* enzyme cuts before and after the ampicillin resistance cassette (Fig.4.1) and results in two fragments of 3139bp and 1008bp (ampicillin resistance cassette). The 3139bp fragment was ligated with the PCR amplified kanamycin resistance cassette from the plasmid pBHR1 (Fig.4.1). By PCR *NcoI* restriction sites were introduced which are compatible with the *BspHI* restriction sites of the vector fragment.

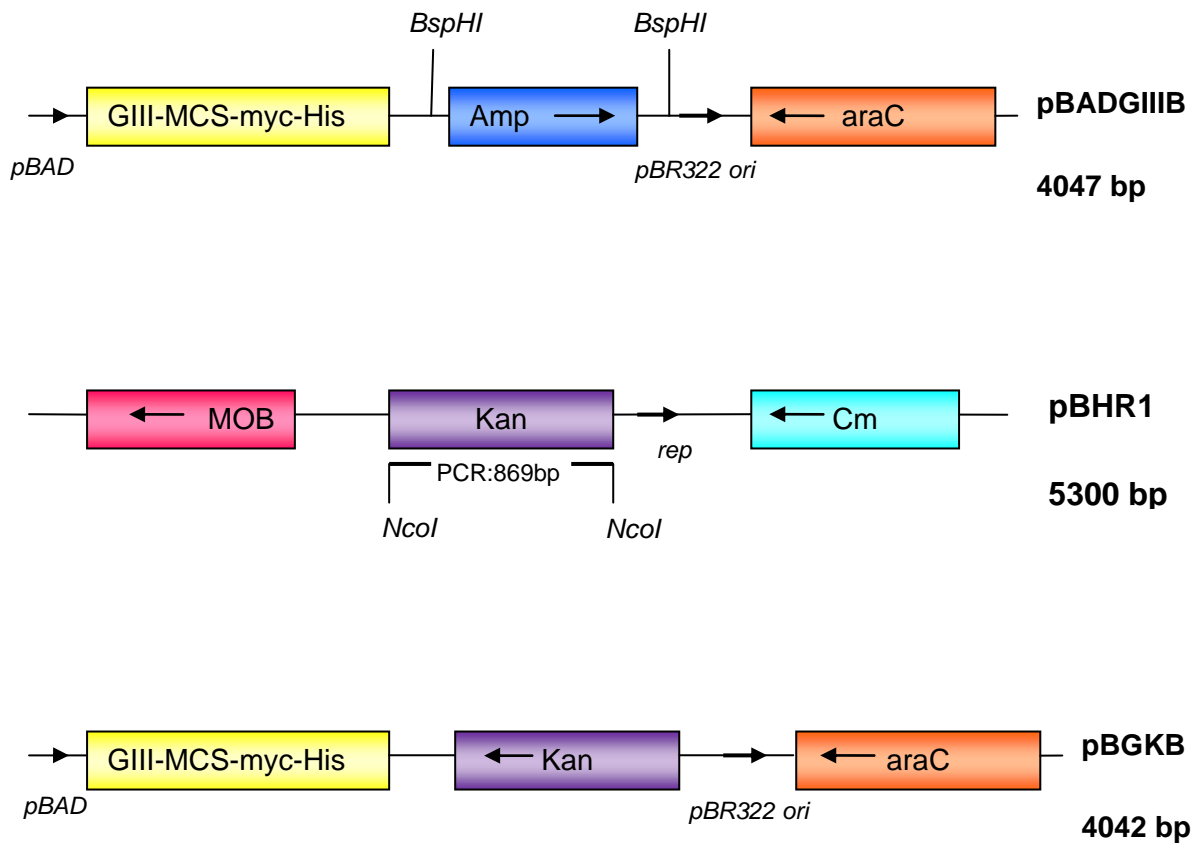


Fig. 4.1: Cloning strategy for pBGKB. Restriction digest of pBADGIIIB with *BspHI* results in loss of ampicillin resistance. Amplification of the kanamycin resistance cassette via PCR introduces *NcoI* restriction sites. Ligation of the kanamycin cassette with the pBADGIIIB fragment results in the production of the plasmid pBGKB.

Control digests of pBADGIIIB plasmid (Invitrogen) were performed with different restriction enzymes (fast digest enzymes from Fermentas) to proof its correctness (Fig. 4.2). By ligation of *BspHI* and *NcoI* overhangs both restriction recognition sites were lost.

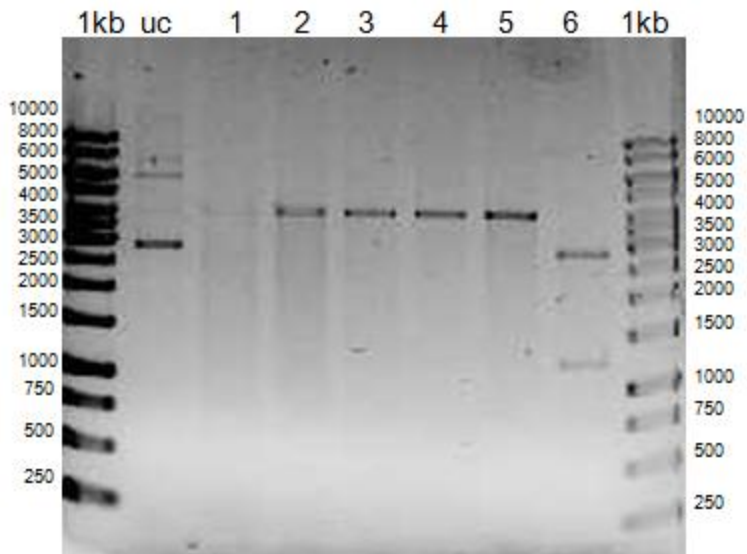


Fig. 4.2: pBADGIIIB restriction digest. Lane 1, 2: *NheI* 4147bp correct, lane 3: *EcoRI* 4147bp correct; lane 4: *XbaI* 4147bp correct, lane 5: *HindIII* 4147bp correct, lane 6: *AvaI* 1230/2917bp correct. Marker: 1kb DNA ladder (Fermentas).

Plasmid pBADGIIIB was furthermore digested with the *BspHI* restriction enzyme (New England Biolabs) according to the manufacturer's instructions. The bigger fragment (3139bp) was purified using the Promega Purification Kit as can be seen in figure 4.3.

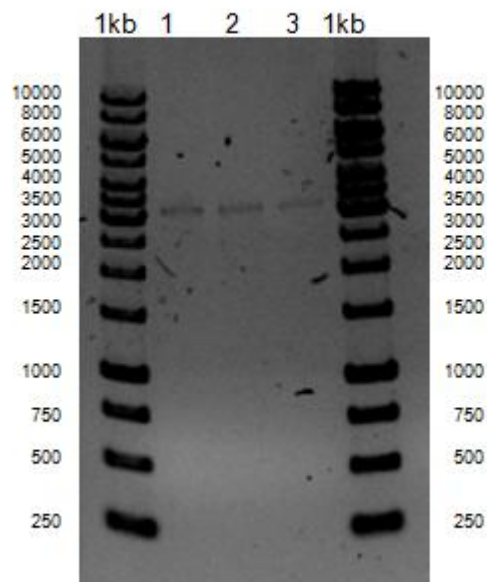


Fig. 4.3: pBADGIIIB purified. Purified vector fragment without ampicillin resistance cassette. Lane 1-3 purified pBADGIIIB fragment: 3139 bp correct. Marker: 1kb DNA ladder (Fermentas).

The kanamycin cassette was amplified from pBHR1 plasmid using PCR with primers containing *NcoI* restriction sites:

KanFWD(<i>NcoI</i>)	5' tta cca tgg tgt tac att gca caa gat aa 3'
KanREV(<i>NcoI</i>)	5' att cca tgg tta gaa aaa ctc atc gag cat 3'

PCR was carried out according to standard protocol using:

- Pfu-Polymerase (Fermentas)
- 10x pfu-buffer (+MgCl₂)
- dNTPs Mix (2mM)
- annealing temperature calculated according to primers melting temperature: 50°C
- elongation time: 2h 40min, 72°C

After PCR the fragment (869bp) was eluted from a 2% agarose gel and purified with the Promega Purification Kit as described in materials and methods.

The purified kanamycin fragment was checked on a 2% agarose gel (Fig.4.4).

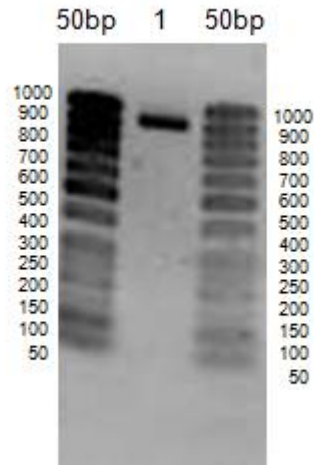


Fig. 4.4: Kanamycin PCR product. The gel shows the purified kanamycin fragment (869bp) after amplification from plasmid pBHR1. Lane 1: kanamycin fragment 869bp correct. Marker: 50bp ladder (Fermentas).

Ligation of the purified kanamycin fragment with the vector fragment (Fig. 4.3.) was done over night at 16°C using the T4 DNA ligase as described in materials and methods. After transformation and picking of clones, the plasmids were checked for correctness with different enzymes to identify a correct pBGKB clone (Fig. 4.5.)

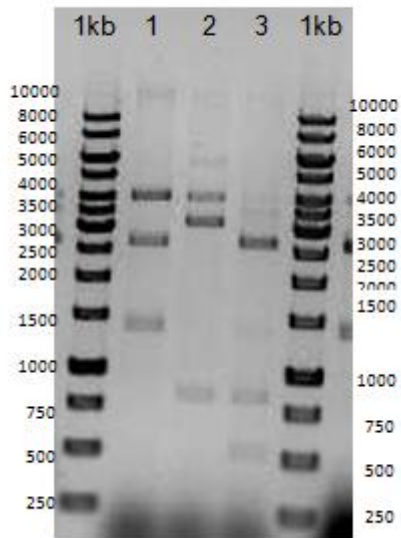


Fig. 4.5: pBGKB restriction digest. Plasmid check using different restriction enzymes. Lane 1: *XhoI* 2658/1384bp; lane 2: *HindIII* 3213/829bp; lane 3: *HindIII* & *XbaI* 2658/829/520/35bp. Marker: 1k DNA ladder (Fermentas).

Due to the band patterns obtained from the restriction digests (Fig. 4.5) the correctness of the pBGKB clone could be proofed.

4.2 Construction of possum protein expressing plasmids

4.2.1 Cloning pBGKBZP2C

The pBGKBZP2C plasmid contains the brushtail possum zona pellucida 2 (ZP2) C-terminal sequence which was cloned into the pBGKB vector according to the cloning strategy shown in figure 4.6.

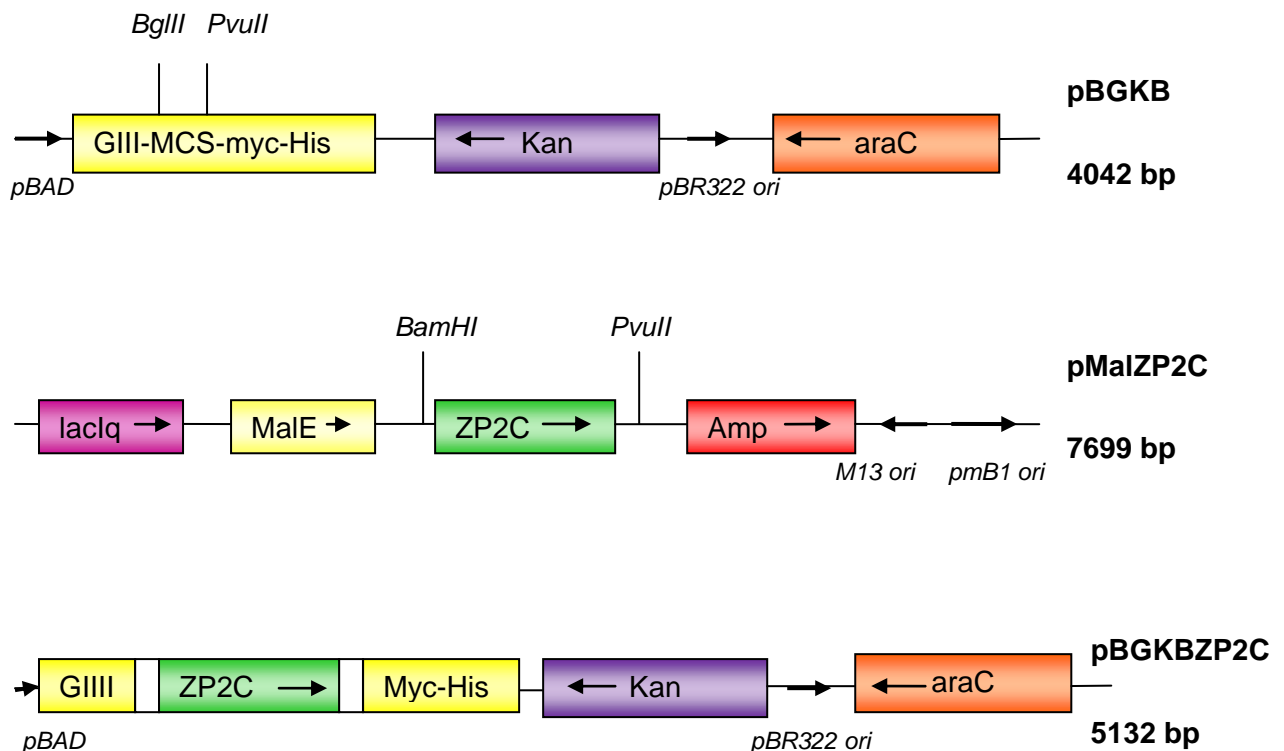


Fig. 4.6: pBGKBZP2C cloning strategy. Double digest with the restriction enzymes *BglII* and *PvuII* in the multiple cloning site of pBGKB, as well as double digest with *BamHI* and *PvuII* in pMalZP2C to obtain the ZP2C gene sequence were performed. The final ligation of ZP2C sequence and vector fragment of pBGKB results in the new pBGKBZP2C plasmid (5132bp), with ZP2C in frame with the leader peptide (gIII) which directs the recombinant protein into the periplasmic space. Myc detection is possible in this plasmid

To obtain the ZP2C sequence the plasmid pMalZP2C [49] was double digested with the enzymes *BamHI* and *PvuII* (Fermentas) resulting in three fragments, where the 1099bp fragment contains the ZP2C gene sequence. Plasmid pBGKB was opened in the multiple cloning site with the enzymes *BglII* and *PvuII*. Both, vector fragment (Fig. 4.8) as well as the ZP2C fragment (Fig. 4.7) were extracted from the agarose gel and purified using GeneXpress purification kit as described in materials and methods.

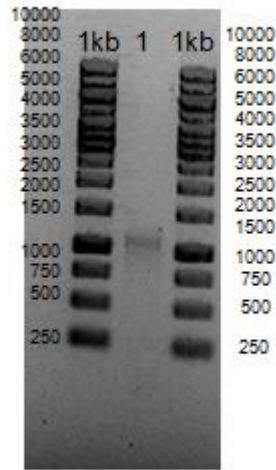


Fig. 4.7: ZP2C fragment purified. The agarose gel picture shows the purified ZP2C fragment (1099bp) which was obtained from *pMalZP2C* using *Bam*HI and *Pvu*II restriction enzymes. Marker: 1kb DNA ladder (Fermentas)

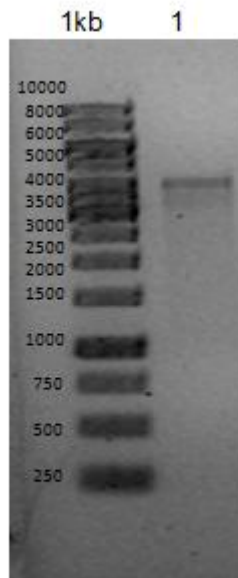


Fig. 4.8: pBGKB digest. The *pBGKB* plasmid was double digested using *Bg*III and *Pvu*II restriction enzymes. Lane 1: purified *pBGKB* fragment 4035bp, correct. Marker: 1kb DNA ladder (Fermentas)

Due to the problem of previously occurred double-ligation of the *pBGKB* fragment dephosphorylation of the plasmid was performed under following conditions:

- BAP Buffer 10x (Fermentas)
- BAP Dephosphorylase (Fermentas)
- 60°C, 60min

Additionally purification of the dephosphorylated pBGKB fragment was done using GeneXpress purification kit and ligation was performed at 16°C over night using T4 DNA ligase (New England Biolabs). After transformation into MOPS-competent *E. coli* NM522 cells, clones were picked and the correctness was checked using different restriction enzymes (Fig. 4.9). By ligation of *Bgl*II and *Bam*HI overhangs both restriction recognition sites were lost.

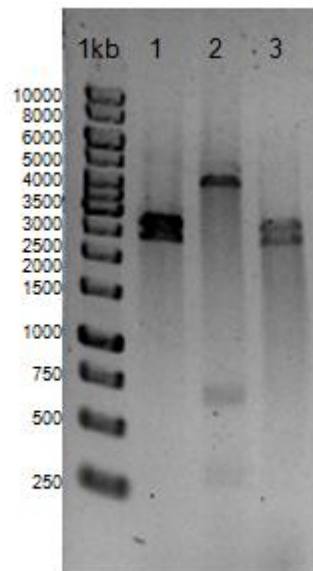


Fig. 4.9: pBGKBZP2C restriction digests using different restriction enzymes. Lane 1 and lane 3: *Dra*I 2723/2409bp and lane 2: *Eco*RI 4052/667/300/113bp). *Dra*I as well as *Eco*RI cut in the ZP2C insert of the pBGKBZP2C plasmid. Marker: 1kb DNA ladder (Fermentas)

Due to the band patterns obtained from the restriction digests (Fig. 4.9) the correctness of the pBGKBZP2C clone could be proofed.

4.2.2 Cloning pBGKBZP2Copt

The pBGKBZP2Copt plasmid contains the brushtail possum zona pellucida 2 (ZP2) C-terminal sequence with a codon optimization (opt.) for the protein expression in Escherichia Coli. The ZP2Copt gene sequence was cloned into the pBGKB vector according to the cloning strategy shown in figure 4.10.

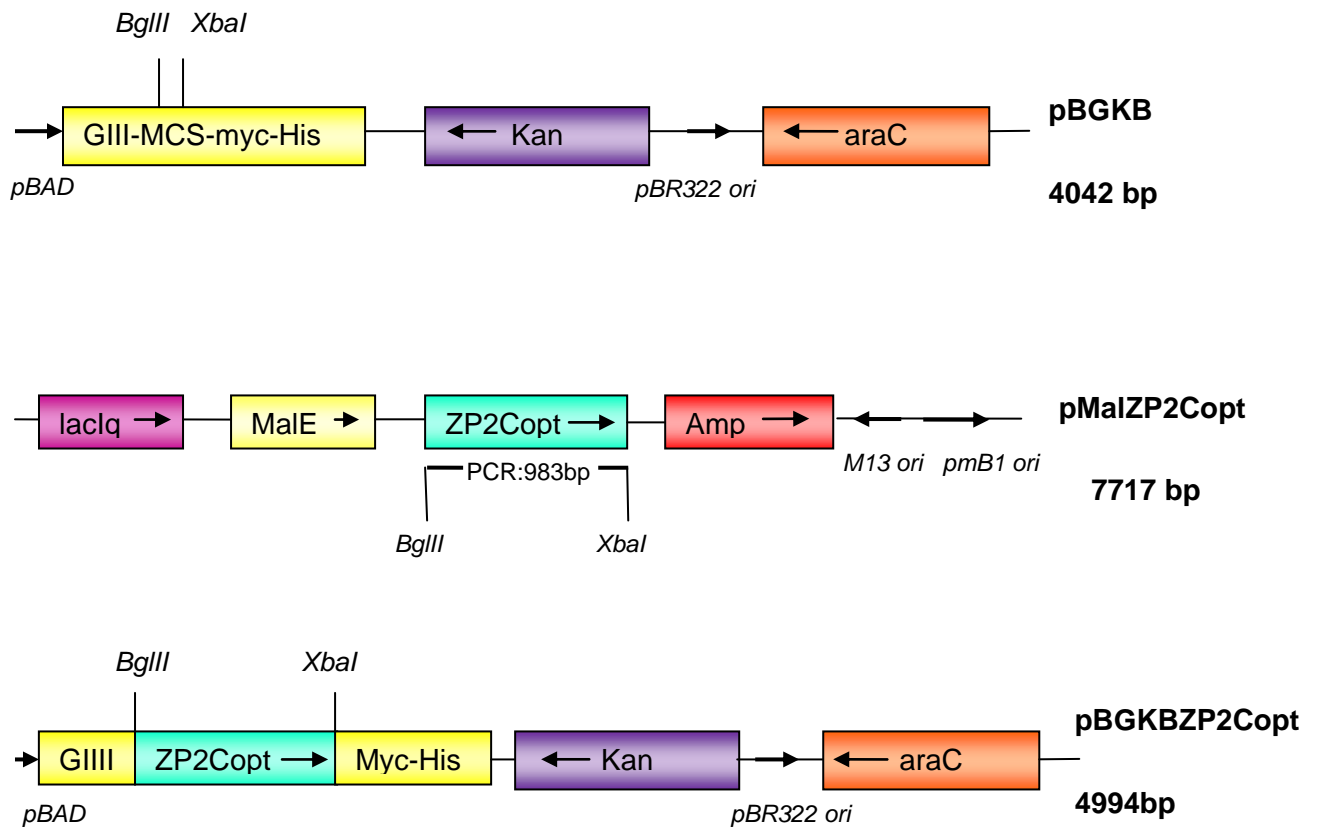


Fig. 4.10: pBGKBZP2Copt cloning strategy. Restriction digests of the vector plasmid with *Bgl*III and *Xba*I in the multiple cloning site. PCR amplification of the ZP2Copt gene sequence from *pMalZP2Copt*. Ligation of the ZP2Copt fragment into the multiple cloning site of the pBGKB vector results in the formation of pBKBZP2Copt (4994bp) plasmid with ZP2Copt under the direction of the *gIII*-signal sequence for transport into the periplasmic space.

The ZP2Copt gene sequence was amplified from plasmid pMalZP2Copt [49] using PCR with appropriate primers introducing *Bgl*III and *Xba*I sites:

```
FWD [BglIII] 5'pBGKBZP2Copt: ACA AGATCT AAT GGC AGC CGT CTGC 3'
REV [XbaI] 5'pBGKBZP2Copt: TAA TCTAGA CTG CTA CCC GGG CAG 3'
```

The PCR was carried out according to standard protocol, using:

- High-fidelity-Polymerase (Fermentas)
- 10x high-fidelity-buffer (+MgCl₂)
- dNTPs Mix (2mM)
- annealing temperature calculated according to primers melting temperature: 50°C
- elongation time: 2h 40min, 72°C

PCR of pMalZP2Copt resulted in a 983bp fragment which was additionally digested with the restriction enzymes *Bgl*III and *Xba*I (Fig. 4.11). Afterwards the fragment was eluted from a 2% agarose gel and purified using GE-healthcare purification Kit (GE Healthcare). The purified fragment was checked on a 2% agarose gel as shown in figure 4.12.

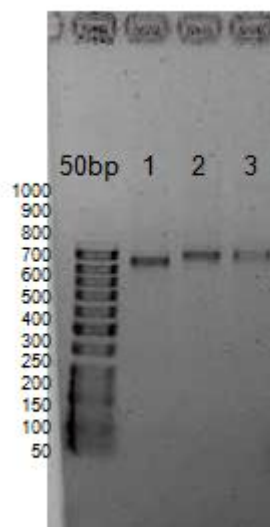


Fig. 4.11: ZP2Copt restriction digest. Lane 1: Cp4 fragment after PCR and restriction digest with *Bgl*III and *Xba*I (953bp), lane 2: ZP2C fragment after PCR and restriction digest with *Kpn*I and *Xba*I (1001bp), lane 3: ZP2Copt fragment after PCR and restriction digest with *Bgl*III and *Xba*I (983bp). Marker: 50bp DNA ladder (Fermentas)

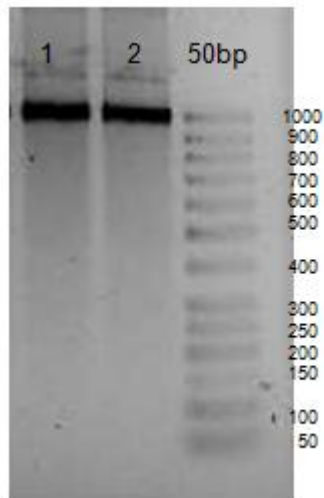


Fig. 4.12: ZP2Copt fragment purified. The agarose gel picture shows the purified ZP2Copt fragment (983bp) which was obtained after PCR and digest with *Bgl*III and *Xba*I. Marker: 50bp DNA ladder (Fermentas)

The vector pBGKB was double digested with *Bgl*III and *Xba*I restriction enzymes which cut in the multiple cloning site; resulting in a 4005bp fragment which was purified using Zymo Clean and Concentrator Kit as described in materials and methods (Fig. 4.13).

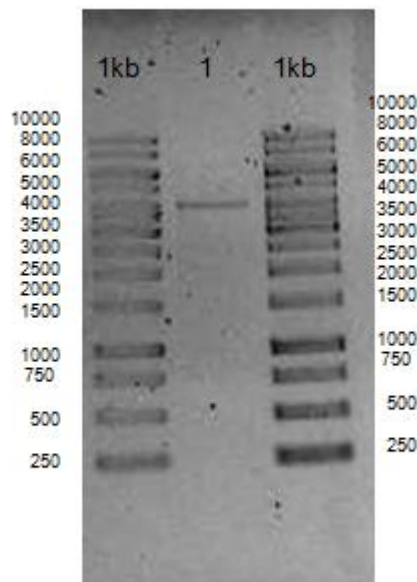


Fig. 4.13: pBGKB digest. The pBGKB plasmid was double digested with *Bgl*III and *Xba*I, resulting in a fragment of 4005bp. The agarose gel picture shows the purified pBGKB fragment (4005bp). Marker: 1kb DNA ladder (Fermentas).

Ligation was done over night at 16°C using T4 DNA ligase (New England Biolabs). After transformation into MOPS-competent E. coli NM522 cells clones were picked and digested with different enzymes to identify a correct pBGKBZP2Copt plasmid (Fig. 4.14).

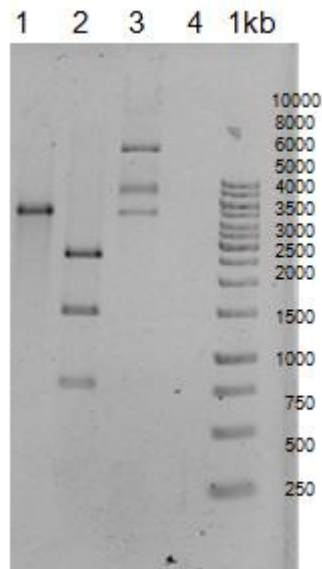


Fig. 4.14: pBGKBZP2Copt restriction digests. pBGKBZP2Copt plasmid check using different restriction enzymes. Lane 1: EcoRI 4999bp correct; lane 2: DraI 2723/1505/766bp correct, lane 3: XbaI not correct in case clone 6 instead of clone 3 was used, lane 4: no DNA was loaded. Marker: 1kb DNA ladder (Fermentas)

Due to the band patterns obtained from the restriction digests (Fig. 4.14) the correctness of the pBGKBZP2Copt clone could be proofed.

4.2.3 Cloning pBGKBCp4

The pBGKBCp4 plasmid contains the brushtail possum coat protein 4 (Cp4) of the conceptus. The Cp4 gene sequence is cloned into the pBGKB vector according to the cloning strategy shown in figure 4.15.

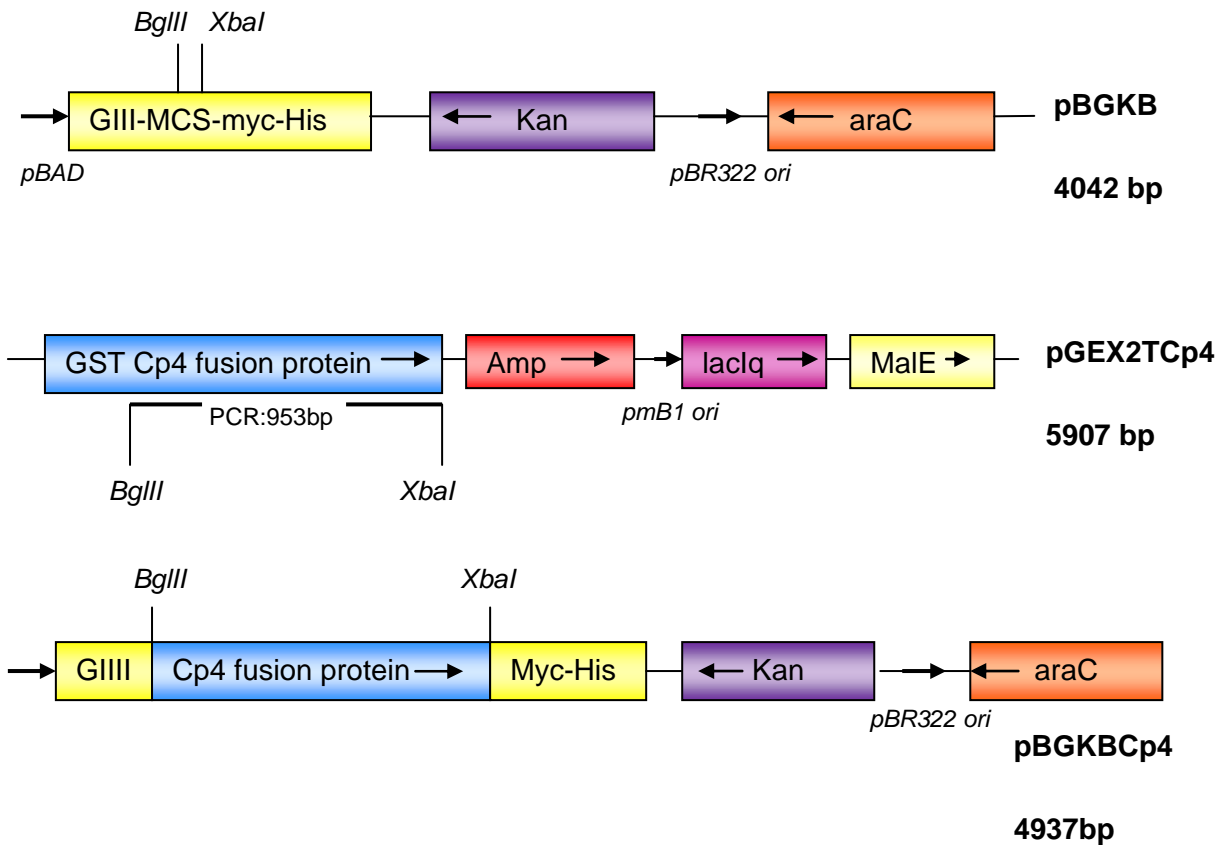


Fig. 4.15: pBGKBCp4 cloning strategy. The pBGKB vector plasmid was double digested with the restriction enzymes *BglIII* and *XbaI* which cut in the multiple cloning site. The Cp4 gene sequence was amplified using PCR with primers incorporated restriction sites *BglIII* and *XbaI* for ligation of the fragment into the vector plasmid resulting in the pBGKBCp4 plasmid with a length of 4637bp.

The Cp4 gene sequence was amplified via PCR from the plasmid pGEX2TCp4 [51] involving specific primers introducing restriction sites for *BglIII* and *XbaI*:

FWD [*BglIII*]: 5' pBGKBcp4: ACA AGATCT GAA TTC GAT AGG TAT GCT 3'
REV [*XbaI*]: 5' pBGKBcp4: TAA TCTAGA TTC TGA ATA CTT TTA TTC TGC 3'

PCR was carried out according to standard protocol using:

- High-fidelity-Polymerase (Fermentas)
- 10x high-fidelity-buffer (+MgCl₂)
- dNTPs Mix (2mM)
- annealing temperature calculated according to primers melting temperature: 50°C
- elongation time: 2h 40min, 72°C

PCR of pGEX2TCp4 resulted in a 953bp fragment which was additionally digested with the restriction enzymes *BglIII* and *XbaI* (Fig. 4.16). Afterwards the fragment was eluted from a 2% agarose gel and purified using GE-healthcare purification Kit (GE Healthcare). The purified fragment was checked on a 2% agarose gel as shown in figure 4.17.

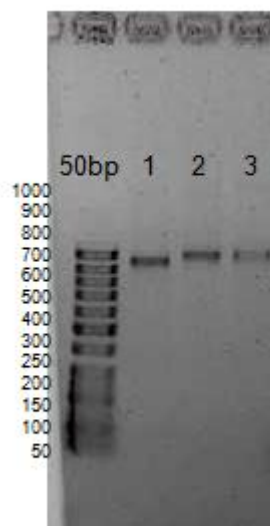


Fig. 4.16: Cp4 fragment restriction digest. Lane 1: Cp4 fragment after PCR and restriction digest with *BglIII* and *XbaI* (953bp), lane 2: ZP2C fragment after PCR and restriction digest with *KpnI* and *XbaI* (1001bp), lane 3: ZP2Copt fragment after PCR and restriction digest with *BglIII* and *XbaI* (983bp). Marker: 50bp DNA ladder (Fermentas).

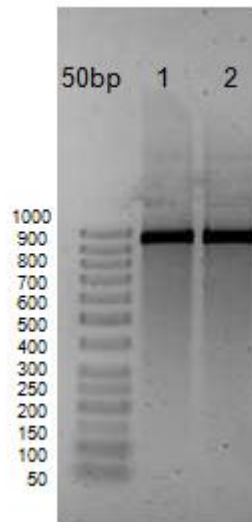


Fig. 4.17: Cp4 fragment purified. The agarose gel picture shows the purified Cp4 fragment (953bp) which was obtained after PCR and restriction digest with *BglIII* and *XbaI*. Marker: 50bp DNA ladder (Fermentas)

The vector pBGKB was double digested with *BglIII* and *XbaI* restriction enzymes which cut in the multiple cloning sites. Restriction digest was carried out according to the manufacturer's instructions using Fermentas Fast Digest enzymes, resulting in a 4005bp fragment which was purified using the GeneXpress purification kit (Fig. 4.18) as described in materials and methods.

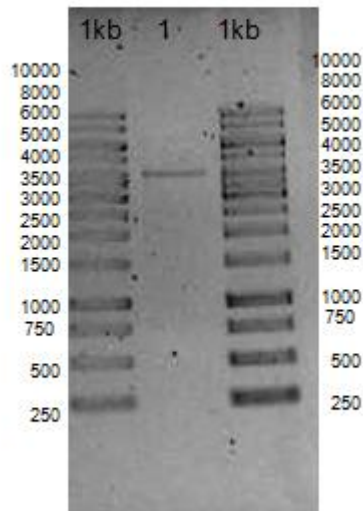


Fig. 4.18: pBGKB digests. The agarose gel picture shows the pBGKB fragment (4005bp) which was digested with restriction enzymes *BglIII* and *XbaI*. Marker: 1kb DNA ladder (Fermentas).

Ligation was done over night at 16°C using T4 DNA ligase (New England Biolabs). After transformation into MOPS-competent *E. coli* NM522 cells, clones were picked and digested with different enzymes to identify the correct pBGKBCp4 plasmid (Fig. 4.19).

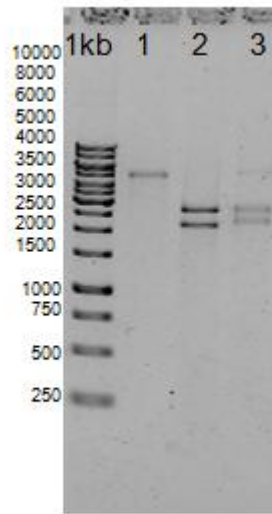


Fig. 4.19: pBGKBCp4 restriction digests. pBGKBCp4 plasmid check using different restriction enzymes which cut in the Cp4 insert of the pBGKBCp4 plasmid. Lane 1: PstI 4937bp correct, lane 2: DraI 2723/2214bp correct, lane 3: XhoI 2658/2279bp correct. Marker: 1kb DNA ladder (Fermentas).

Due to the band patterns obtained from the restriction digests (Fig. 4.19) the correctness of the pBGKBCp4 clone could be proofed.

4.2.4 Cloning pBGKBVap1

The pBGKBVap1 plasmid contains the vesicle-associated protein 1(Vap1) gene sequence of brushtail possums. The Vap1 sequence was cloned according to the cloning strategy shown in figure 4.20. The first cloning strategy involved the amplification of the Vap1 sequence from plasmid pGEX2TVap1 [52] via PCR with appropriate primers introducing *Bgl*III and *Xba*I sites.

FWD [*Bgl*III]: 5' pBGKBVap1 : ACA **AGATCT** TCCACAGAGCAAGTTCGA 3'
REV [*Xba*I] : 5' pBGKBVap1 : TAA **TCTAGA** TTTTTCCTCCTCCTGCCA 3'

All PCR attempts resulted in incorrect bands (Fig. 4.20, 4.21) not showing the expected 551bp band of the amplified Vap1 sequence.

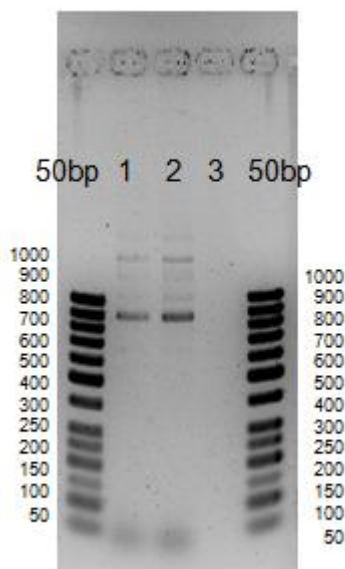


Fig. 4.20: Vap1 amplification via PCR. Agarose gel picture shows Vap1 fragment in lane 1 and 2 using Dream-taq polymerase, 52°C annealing temperature.

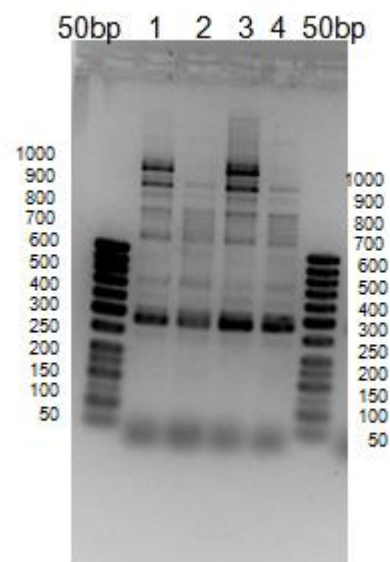


Fig. 4.21: Vap1 amplification via PCR. Agarose gel picture shows Vap1 fragment in lane 1 and 2 at 56°C annealing temperature and in lane 3 and 4 at 58°C annealing temperature, using Dream-Taq polymerase

An alternative cloning strategy of pBGKBVap1 (Fig. 4.22) involves the restriction digest of pBGKB with *Bgl*III cutting in the multiple cloning sites, resulting in linearization of the plasmid.

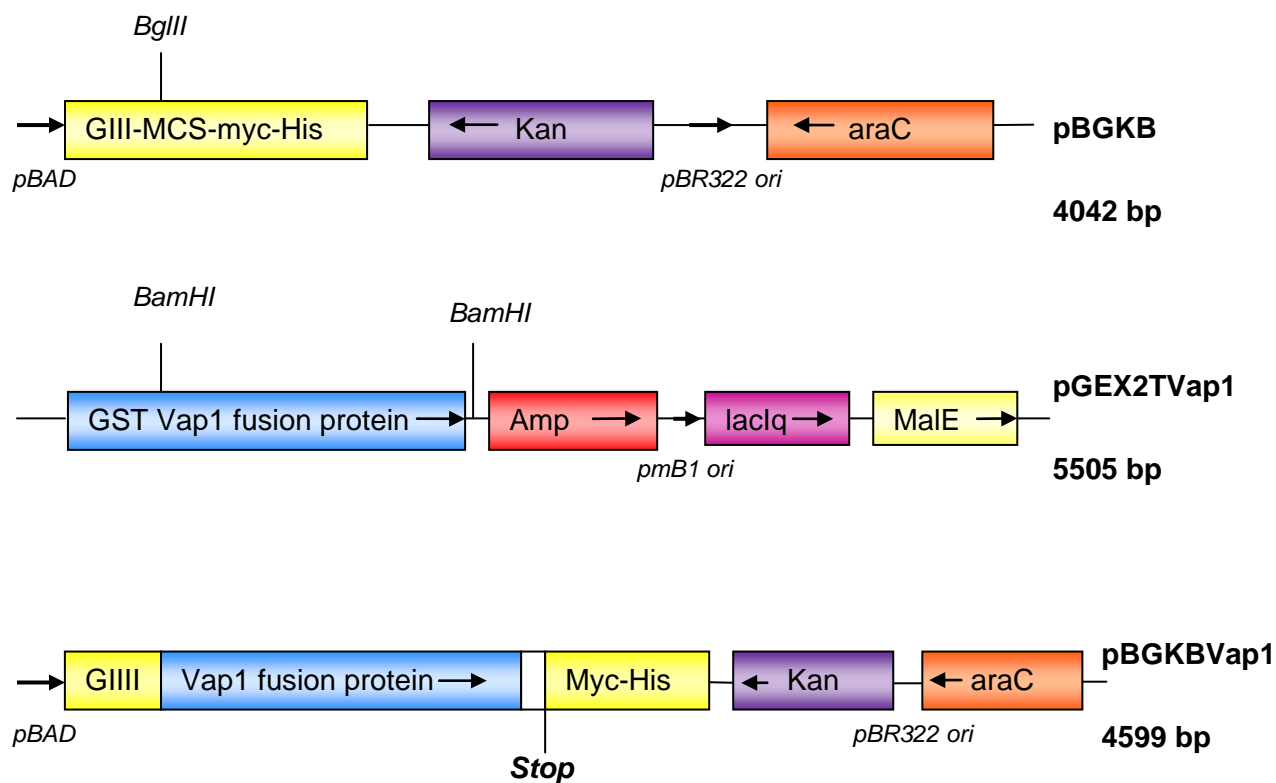


Fig. 4.22: pBGKBVap1 cloning strategy. Linearization of the vector plasmid pBGKB with BglII and restriction digest of pGEX2TVap1 with BamHI to obtain the Vap1 sequence gene. Ligation of the Vap1 fragment into the linearized vector results in the pBGKBVap1 plasmid (4599bp) where Vap1 is expressed as fusion protein together with the gIII-signal sequence but without the expression of the myc- and his-epitope.

The linearized vector plasmid was eluted from a 1% agarose gel and purified using the Zymo Clean- and Concentrator Kit. The linearized plasmid was checked on a 1% agarose gel (Fig. 23).

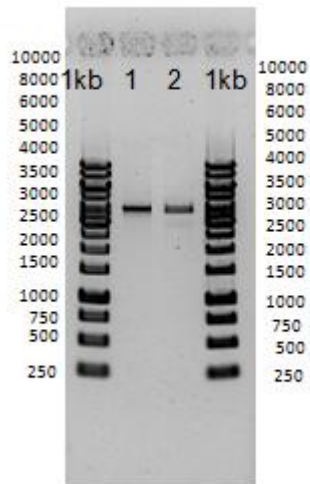


Fig. 4.23: pBGKB digest. The pBGKB plasmid was digested with *Bgl*III resulting in linearization of the plasmid (4042bp). Lane 1 and 2 purified vector fragment (4042bp). Marker: 1kb DNA ladder (Fermentas).

Due to the restriction digest problem which occurred in case of cloning pBGKBVap1, the plasmid pGEX2TVap1 was digested with *Bam*HI (Fig. 4.24) to be sure whether Vap1 has the correct length in the pGEX2TVap1 plasmid.

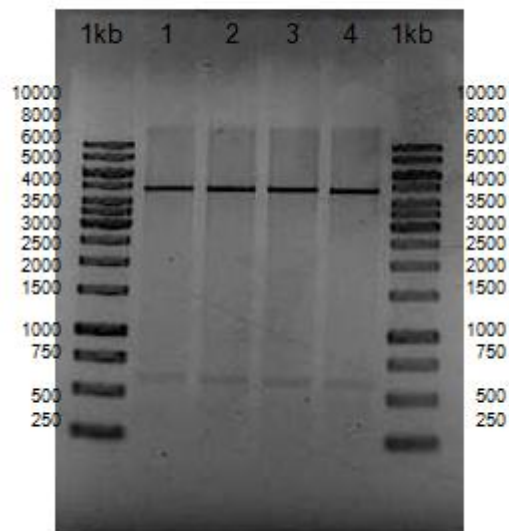


Fig. 4.24: pGEX2TVap1 restriction digests. The mother plasmid pGEX2TVap1 restriction digest with *Bam*HI resulting in 4948/551bp, where the 551bp fragment represents the Vap1 fragment.

Plasmid pGEX2TVap1 was digested with *Bam*HI which cuts twice resulting in a 551bp fragment carrying Vap1. The Vap1 fragment was eluted from a 1% agarose gel and purified using the Zymo Clean and Concentrator Kit (Fig. 4.25).

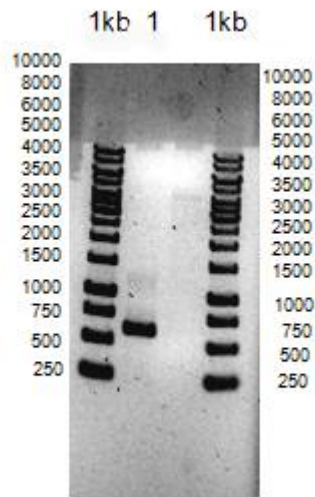


Fig. 4.25: Purified Vap1 fragment. The agarose gel picture shows the purified Vap1 fragment (551bp) which was obtained from pGEX2TVap1 using BamHI. Marker: 1kb DNA ladder (Fermentas)

Ligation of the Vap1 fragment into the linearized pBGKB vector was done over night at 16°C according to the manufacturer's instructions. After transformation into MOPS-competent E. coli NM522 cells, clones were picked and restriction digest (Fig. 26, 27) was done to identify pBGKBVap1 plasmid.

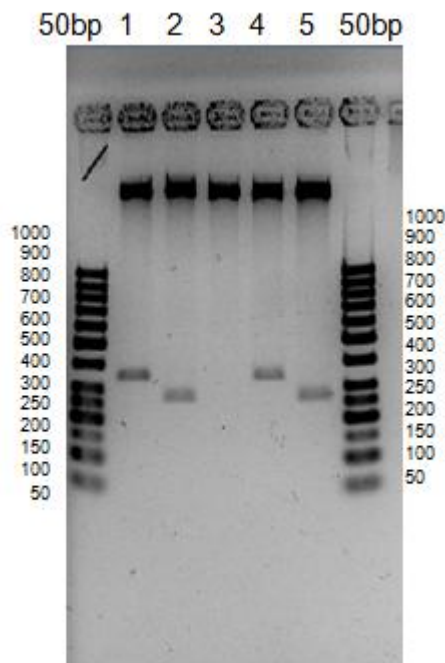


Fig. 4.26: pBGKBVap1 restriction digests. The plasmid was digested with PstI, an enzyme which cuts in the Vap1 insert region, resulting in 4429/170bp fragments when Vap1 is inserted correctly into the vector plasmid. If Vap1 inserted the other way around restriction digest with PstI would result in bands of 4149/450bp. Lane 1 and 4: restriction digests with PstI not correct, second band at around 350bp. Lane 2 and 5: restriction digest with PstI not correct, second band at around 250bp. Marker: 50bp DNA ladder (Fermentas).

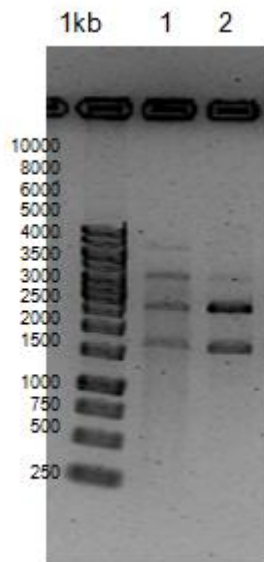


Fig. 4.27: pBGKBVap1 restriction digests. The plasmid was digested with *BspHI*, an enzyme which cuts in the *Vap1* insert. Lane 1 and 2: restriction digests with *BspHI*: 2830/1769bp correct.

Due to the band patterns obtained from the restriction digests (Fig. 4.27) the correctness of the pBGKBVap1 clone could be proofed.

4.3 Protein expression study

As protein expression of the obtained plasmids is under the control of the pBAD promoter, AraC functions as an activator and maintains protein expression when media containing high levels of arabinose is added. In order to check protein expression of the cloned plasmids an expression study was performed. Expression of the recombinant proteins was conducted according to materials and methods.

4.3.1 Protein expression study pBGKBZP2C

The expression study was performed in *E. coli* NM522 and expression was induced with L-arabinose at a final concentration of 0,2% after the OD₆₀₀ has reached 0,5. Protein expression went on for 120 minutes. The optical density OD (Fig. 4.28) as well as the colony forming units (cfu) were measured, additionally protein samples were taken.

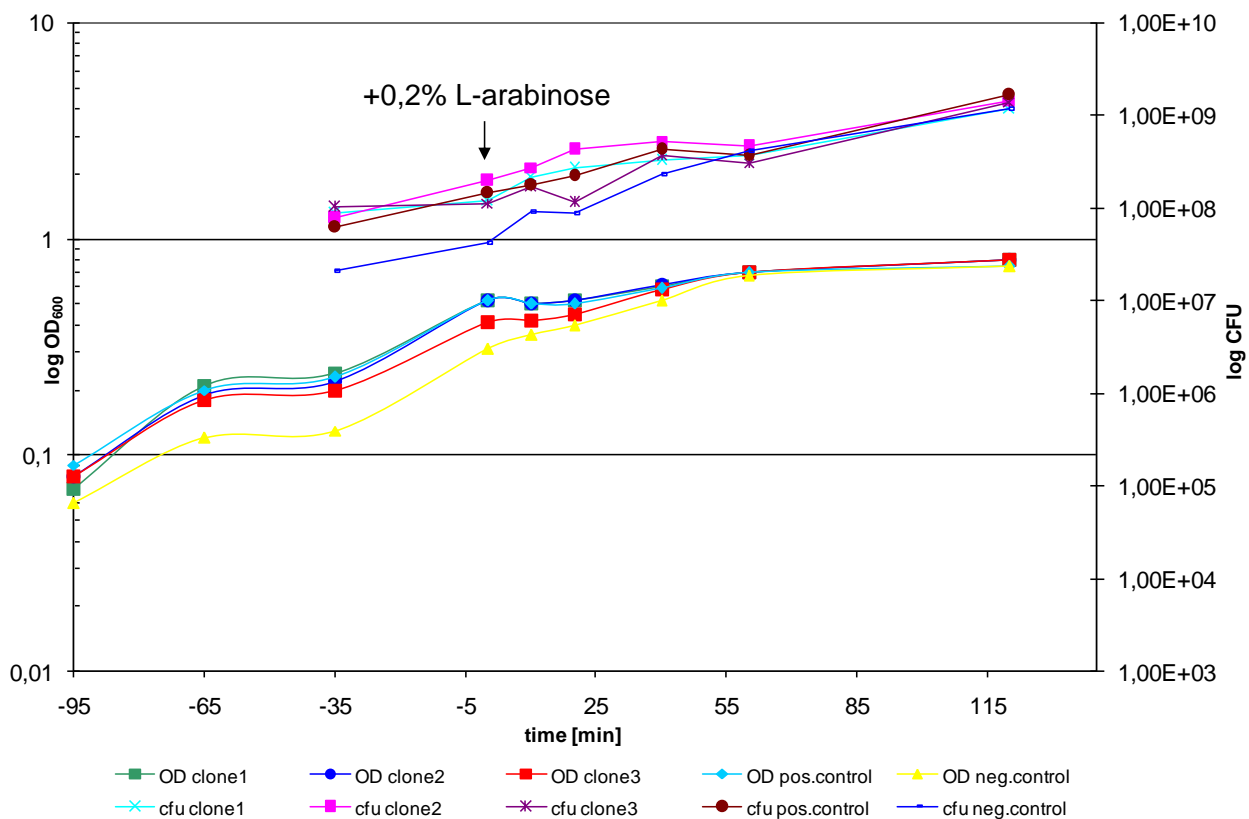


Fig. 4.28: pBGKBZP2C expression curve. ZP2C protein expression induced with 0,2% L-arabinose. The expression curve shows the OD values as well as the cfu values of pBGKBZP2C clone 1-3 and the positive control pBADGIIIcalmodulin and the negative control pBGKB. Protein expression did not impair with the growth of the pBGKBZP2C plasmids.

The samples taken from the protein expression experiment were analyzed by western blotting (see materials and methods). The ZP2C protein was detected using the anti-myc-

HRP antibody (Fig. 4.29-4.30) showing band patterns at around 37,04 kDa which appear to be quite light on this picture, whereas on the original western blot picture the bands are more intense.

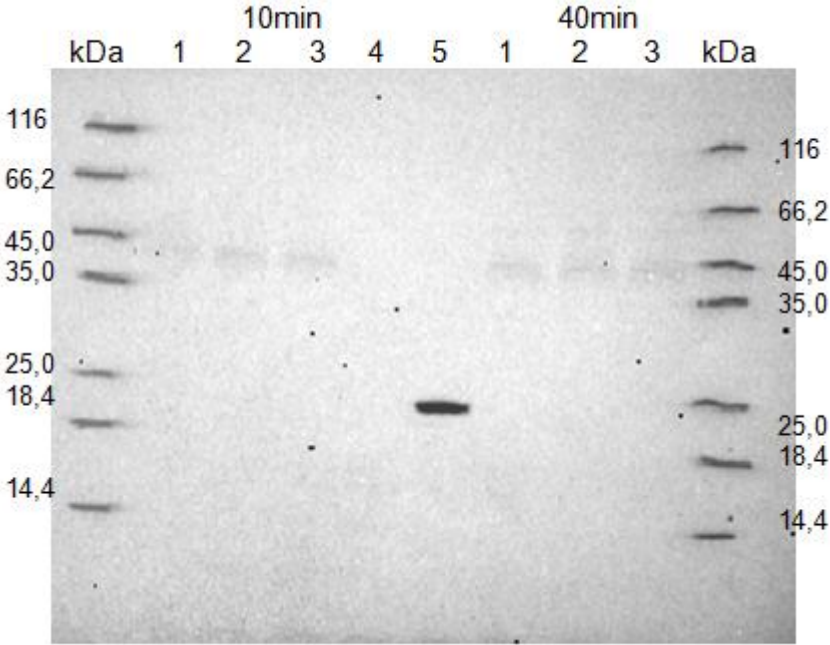


Fig. 4.29: ZP2C western blot analysis. ZP2C protein detection with anti-myc-HRP antibody. Clone 1-3: 10 minutes after L-arabinose induction and 40 minutes after L-arabinose induction bands are visible at 37,04 kDa. 4: negative control pBGKB, 5: positive control pBADGIIIcalmodulin, which shows a strong signal at about 25 kDa.

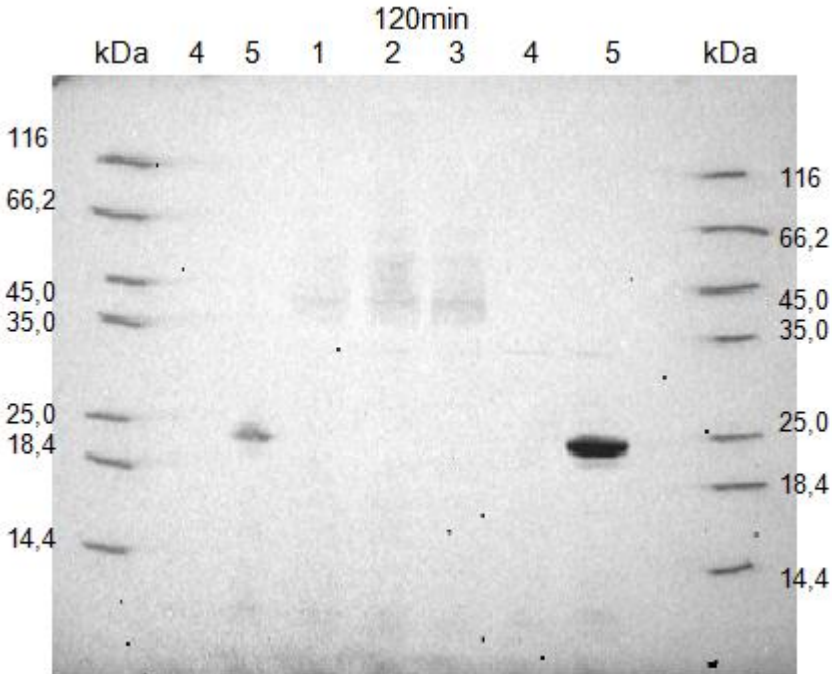


Fig. 4.30: ZP2C western blot analysis. ZP2C protein detection with anti-myc-HRP antibody. Clone1-3 are shown, 120 minutes after L-arabinose induction, bands are visible at around 37,04 kDa. 4: negative control pBGKB, 5: positive control pBADGIIIcalmodulin, which shows a strong signal at around 25 kDa.

Additionally protein detection was performed with a ZP2C specific serum B11 [49] as described in materials and methods resulting in a strong protein signal (Fig. 4.31, 4.32)

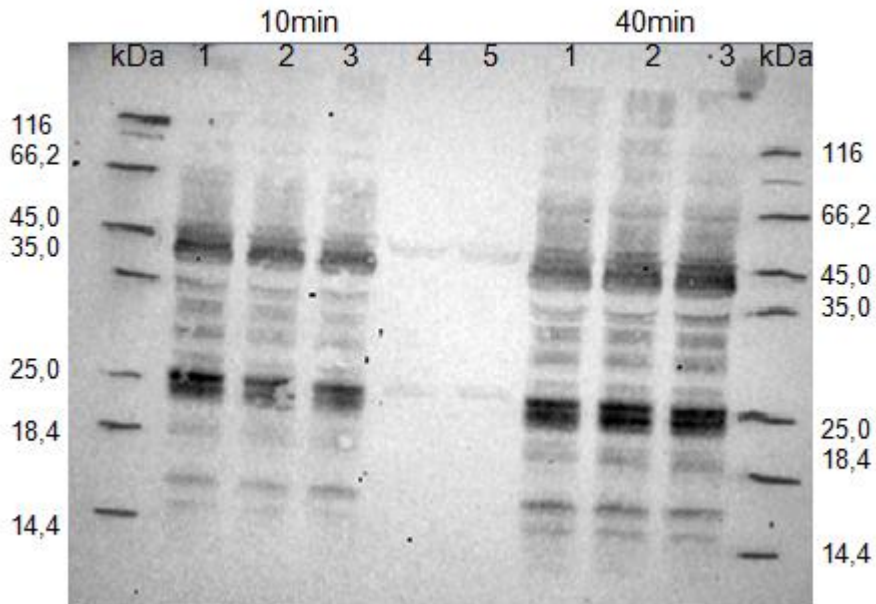


Fig. 4.31: ZP2C western blot analysis. ZP2C protein detection using the specific ZP2C serum B11. Clone 1-3 10 minutes and 40 minutes after L-arabinose induction, bands are visible at around 37,04 kDa. 4: negative control pBGKB, 5: positive control pBADGIIICalmodulin, additionally a strong signal is visible at around 25 kDa

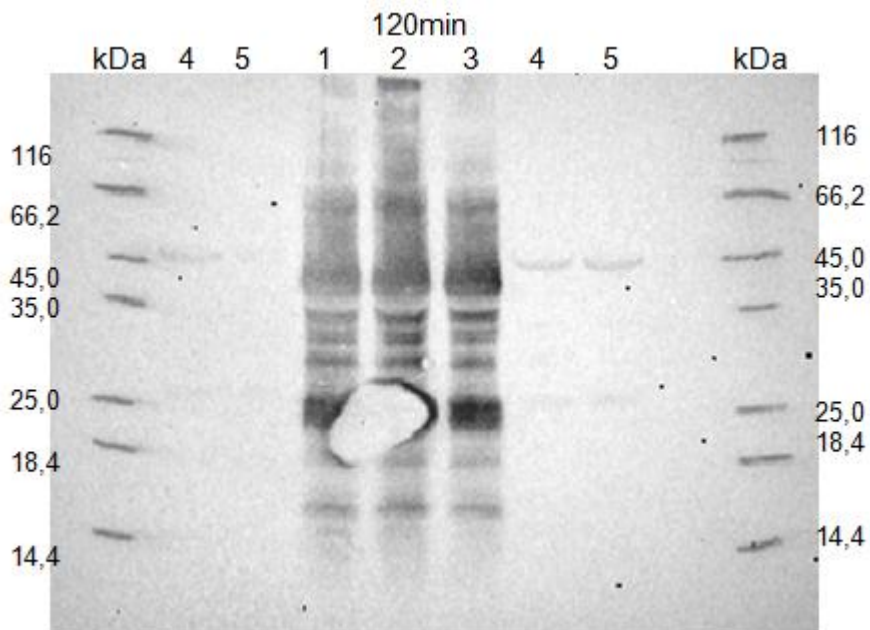


Fig. 4.32: ZP2C western blot analysis. ZP2C protein detection using the specific ZP2C serum B11. Clone 1-3 at 120 minutes after L-arabinose induction bands are visible at around 37,04 kDa. 4: negative control pBGKB, 5: positive control pBADGIIICalmodulin, additionally a strong signal is visible at around 25 kDa.

Due to the results of the western blot analysis the protein expression of the pBGKBZP2C clone was proofed to be correct (Fig. 4.32).

4.3.2 Protein expression study pBGKBZP2Copt

Expression study of ZP2Copt protein was performed in *E. coli* NM522, induced with L-arabinose at a final concentration of 0,2% after the OD₆₀₀ has reached 0,5. Protein expression went on for 120 minutes and protein samples were taken for protein detection. OD₆₀₀ values as well as cfu were measured (Fig. 4.33).

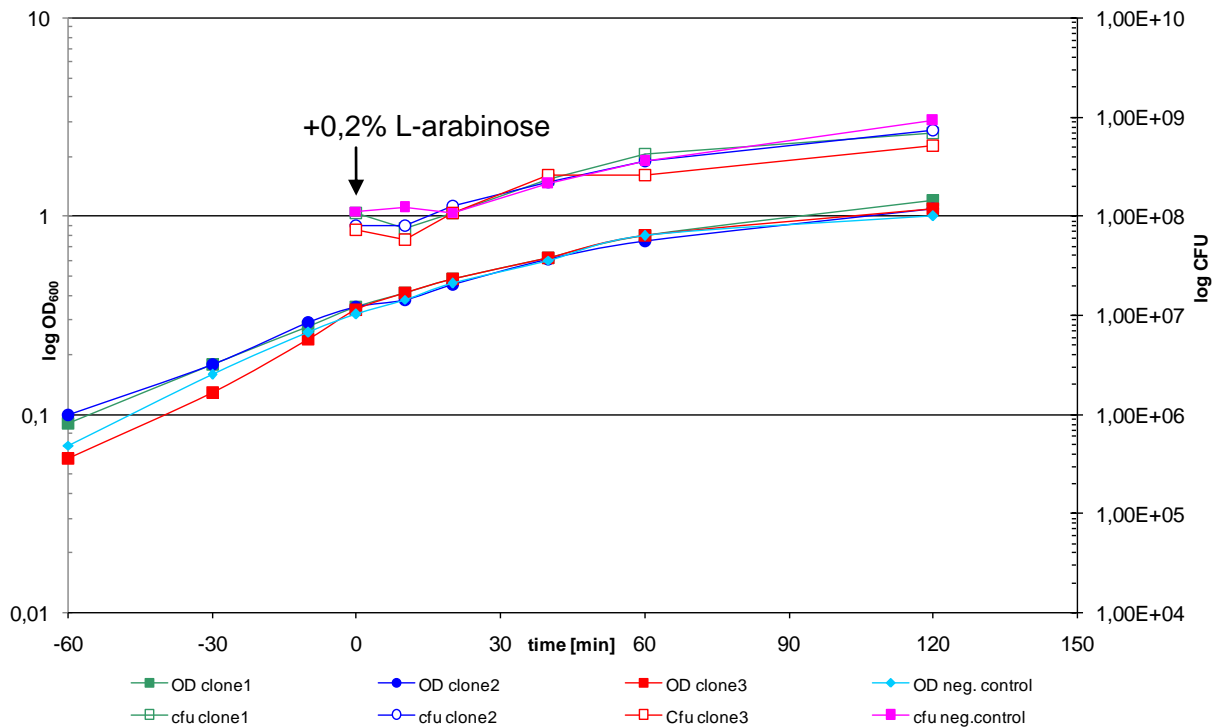


Fig. 4.33: pBGKBZP2Copt expression curve. ZP2Copt protein expression induced with 0,2% L-arabinose. The expression curve shows the OD values as well as the cfu values of pBGKBZP2Copt clone1-3 and the positive control pBADGIIIcalmodulin and the negative control pBGKB. Protein expression did not impair with the growth of the pBGKBZP2Copt plasmids.

The samples taken from the protein expression experiment were analyzed by western blotting. The ZP2Copt protein was detected using the possum specific B11 serum. Protein bands at around 39,7 kDa were detected (Fig. 4.34, 4.35).

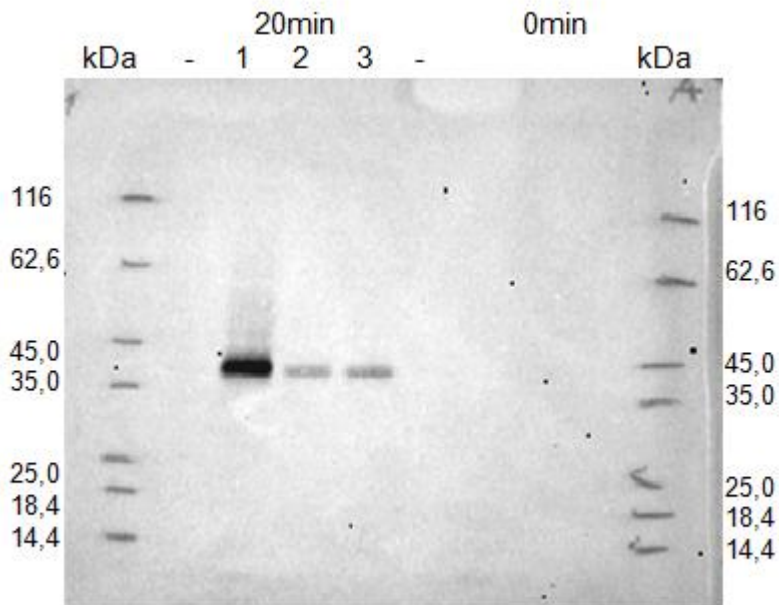


Fig. 4.34: pBGKBZP2Copt Western blot. ZP2Copt protein detection using specific B11 serum. Clone 1-3: 20 minutes after L-arabinose induction and 0 minutes after L-arabinose induction, bands are visible at 39,7 kDa. pBGKB (-) as negative control.

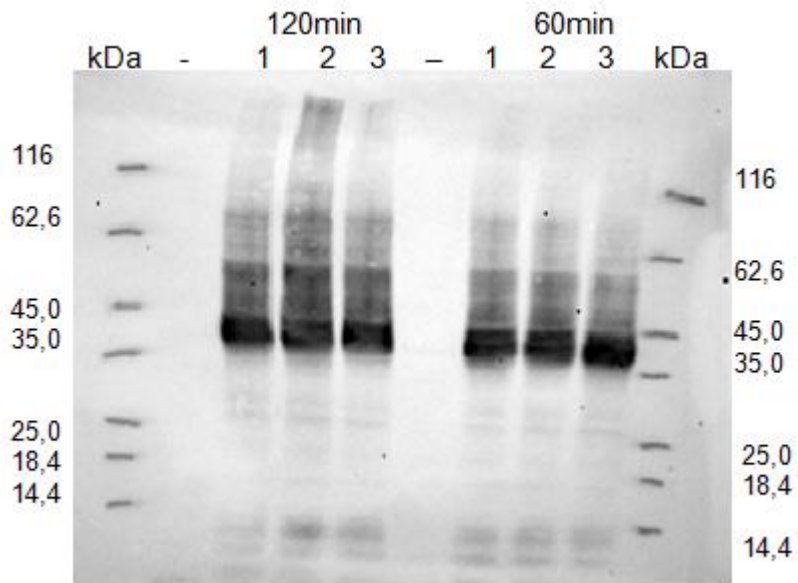


Fig. 4.35: pBGKBZP2Copt Western blot. ZP2Copt protein detection with using specific B11 serum. Clone 1-3: 120 minutes after L-arabinose induction and 60 minutes after L-arabinose induction, bands are visible at 39,7 kDa. pBGKB (-) as negative control.

Furthermore the ZP2Copt protein expression was detected using anti-myc-HRP antibody (Fig. 4.36, 4.37).

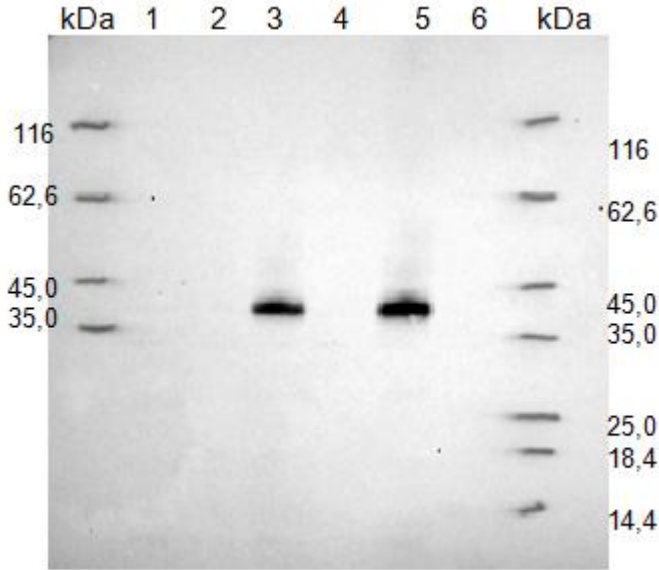


Fig. 4.36: pBGKBZP2Copt Western Blot. ZP2Copt protein detection using anti-myc-HRP. Clone 6 on lanes 1, 3 and 5 at time points 0, 10 and 20 minutes after L-arabinose induction, bands are invisible at around 39,7 kDa. pBGKB as negative control on lanes 2,4 and 6.

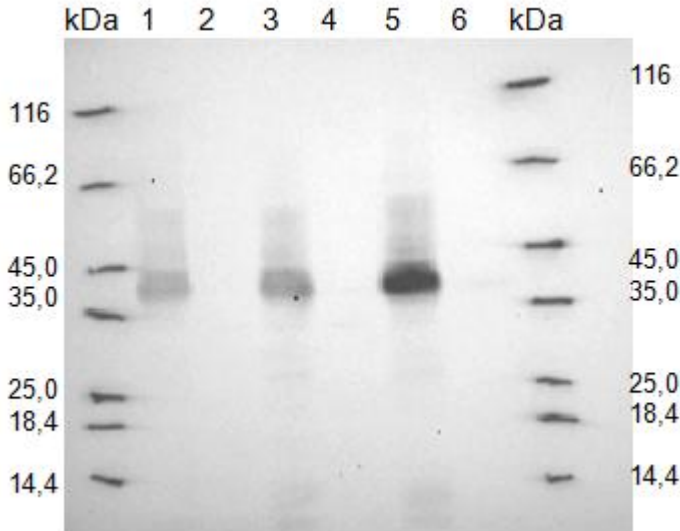


Fig. 4.37: pBGKBZP2Copt Western Blot. ZP2Copt protein detection using anti-myc-HRP. Clone 6 on lanes 1, 3 and 5 at time points 40, 60 and 120 minutes after L-arabinose induction, bands are invisible at around 39,7 kDa. pBGKB as negative control on lanes 2,4 and 6.

The western blot analysis proofed the protein expression of the pBGKBZP2Copt clone.

4.3.3 Protein expression study pBGKBCp4

The Cp4 expression study was performed in *E. coli* NM522. At an OD_{600} 0,5 L-arabinose at a final concentration of 0,2% was added to start protein expression. Protein expression went on for 120 minutes and additionally the OD_{600} values as well as the cfu were measured (Fig. 4.38).

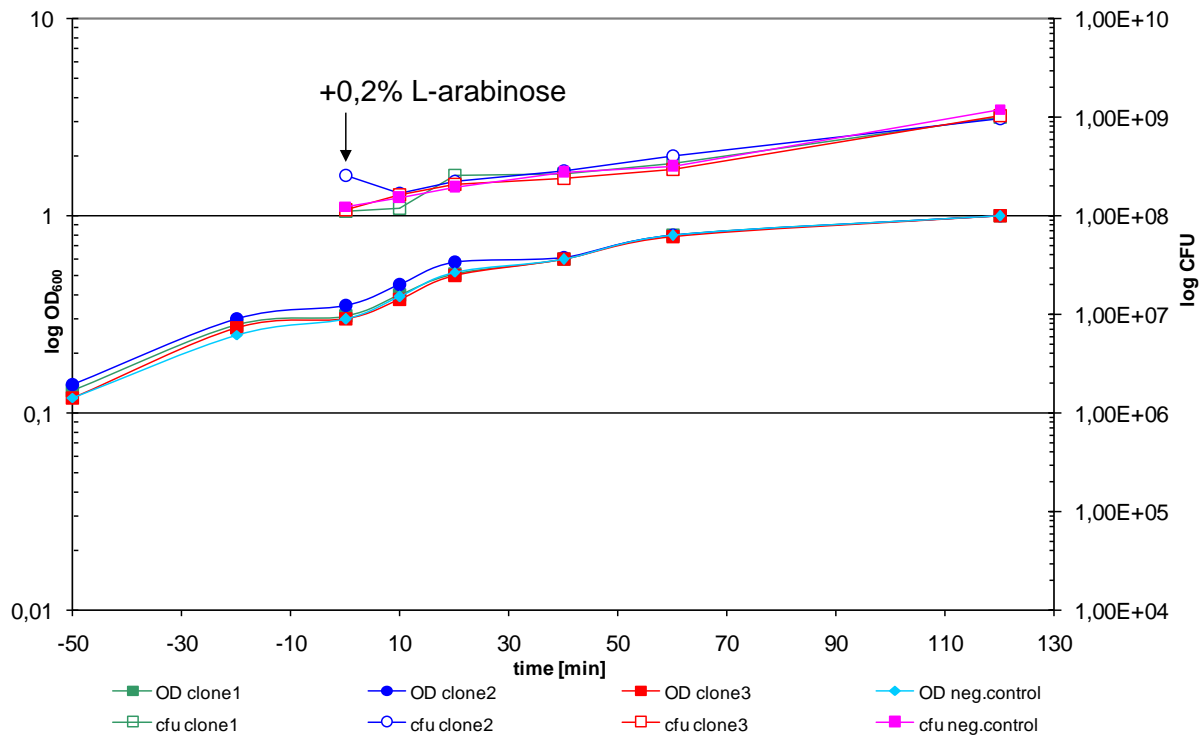


Fig. 4.38: pBGKBCp4 expression curve. The OD_{600} values as well as the cfu values of the pBGKBCp4 clones 1, 2, 3 and the negative control pBGKB can be seen on this curve. L-arabinose at a final concentration of 0,2 % was added to induce protein expression which went on for 120 minutes.

The Cp4 expression first was analyzed using the anti-myc-HRP antibody but no protein band of the correct size (39,8 kDa) could be detected. Therefore Cp4 was additionally analyzed using the specific serum Cp4 ♀TV24 (Fig. 4.39).

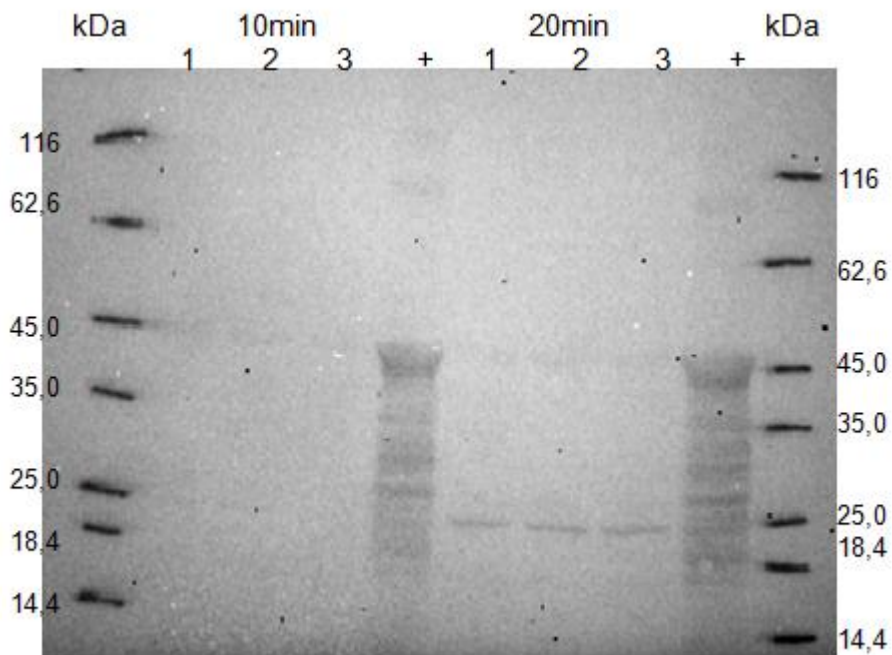


Fig. 4.39: pBGKBCp4 Western blot. The westernblot shows the clones 1, 2 and 3, 10 and 20 minutes after L-arabinose induction. As a positive control the pGEX2TCp4 protein with an expected size around 33 kDa was expressed with IPTG (0,2mM) for 60 minutes. Visible protein expression can be seen at around 25 kDa, although the Cp4 protein has a weight of 39,8 kDa.

The western blot analysis proofed the protein expression of the pBGKBCp4 clone, resulting in proteins at around 25kDa, whereas the Cp4 protein has a weight of 39,8kDa.

4.3.4 Protein expression study pBGKBVap1

The Vap1 protein expression of the plasmid pBGKBVap1 was performed in *E. coli* NM522. At an OD_{600} 0,5 L-arabinose at a final concentration of 0,2% was added to start protein expression. Protein expression went on for 120 minutes and additionally the OD_{600} values as well as the cfu were measured.

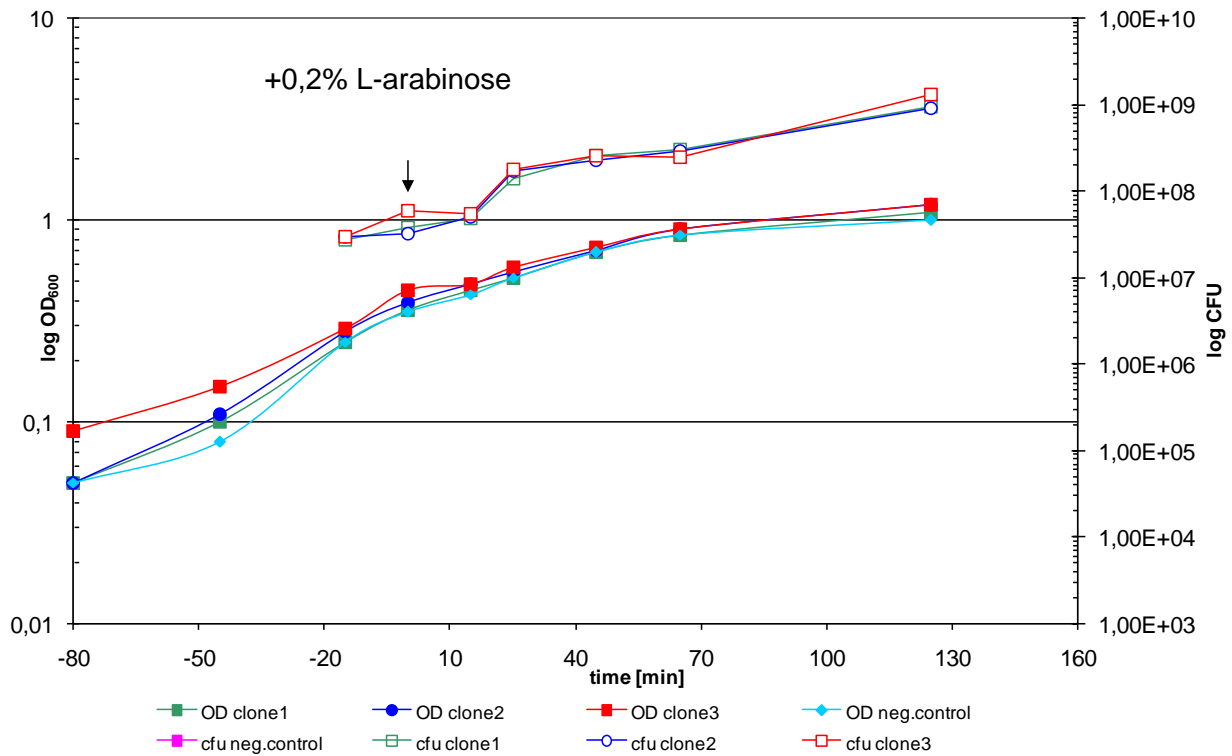


Fig. 4.40: pBGKBVap1 expression curve. The curve shows the OD_{600} values as well as the cfu values of the pBGKBVap1 clones 1, 2, 3 and the negative control pBGKB. Protein expression was induced with L-arabinose at a final concentration of 0,2%.

Western blot analysis of Vap1 expression was performed using the specific Vap1 serum Vap1 ♀TV17 as the protein expression of Vap1 stops before the myc-epitope (Fig. 4.41)

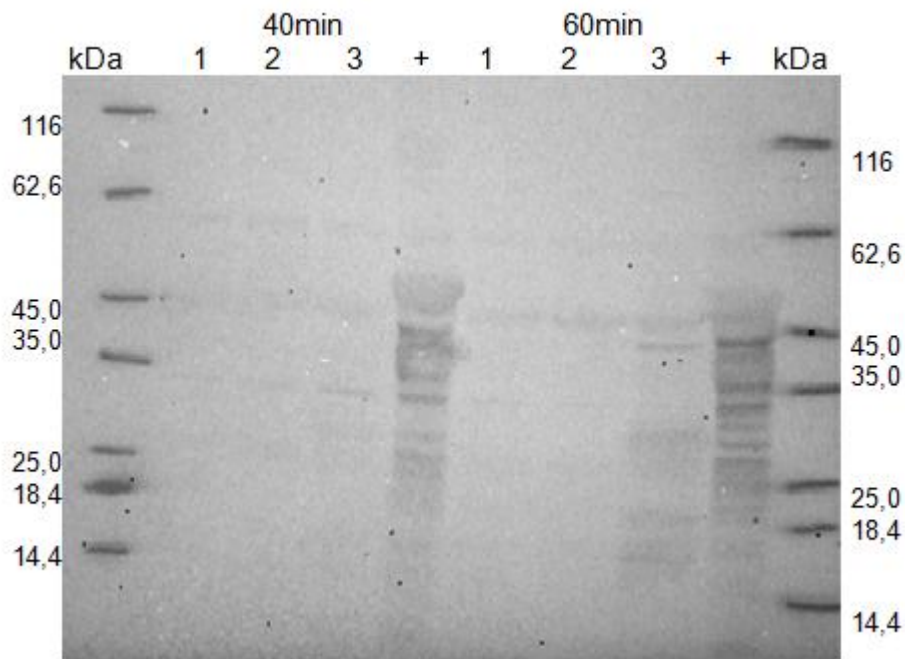


Fig. 4.41: pBGKBVap1 western blot. The picture shows the positive control pGEX2TVap1 IPTG (0,2mM) induced for 60 minutes and the pBGKBVap1 clones 1, 2, 3 40 minutes and 60 minutes after L-arabinose induction. The Vap1 protein should have a molecular weight of 20,3 kDa, but in this picture there are no clear protein bands visible.

The western blot analysis of the pBGKBVap1 clone did not show any significant bands; just background can be seen on this picture.

4.4 Transformation of expression vectors with pGLysivb

The lysis plasmid pGLysivb contains the phage lysis gene E which is under the control of the heat inducible λ P_{Rmut} promoter, as well as a gentamycin resistance cassette and the cI857 repressor. The gene E expression is under the control of the λ P_L/pR-cI857 promoter repressor system.

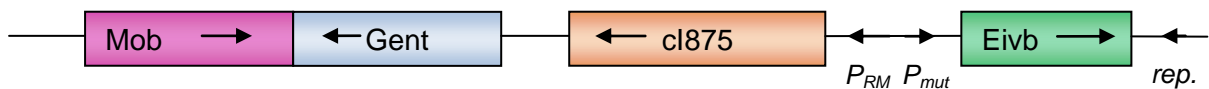


Fig. 4.42: pGLysivb plasmid. *Mob*: mobilization gene; *Gent*: gentamycin resistance cassette; *cI857*: thermo-sensitive phage λ -repressor gene; *Eivb*: in-vivo biotinylation sequence

Lysis and expression studies of the pGLysivb co-transformed clones were performed to check for lysis and protein expression.

The expression and lysis studies were done in nose flasks with 20ml autoclaved LBv media. The bacteria were grown at 36°C until OD₆₀₀ 0,2 when L-arabinose at a final concentration of 0,2% was added to induce protein expression. Lysis was induced at 42°C when OD₆₀₀ reached 0,5 and lysis was observed for 120 minutes.

4.4.1 Expression- and lysis study of *E. coli* NM522 (pBGKBZP2C) (pGLysivb)

The co-transformation of the pBGKZP2C expression plasmid with the pGLysivb lysis plasmid was performed in Mops-competent *E. coli* NM522 as described in materials and methods. Samples for cfu determination were taken as well as for western blot analysis. OD₆₀₀ and cfu curves of three different clones as well as the negative control pBGKB are shown in figure 4.43.

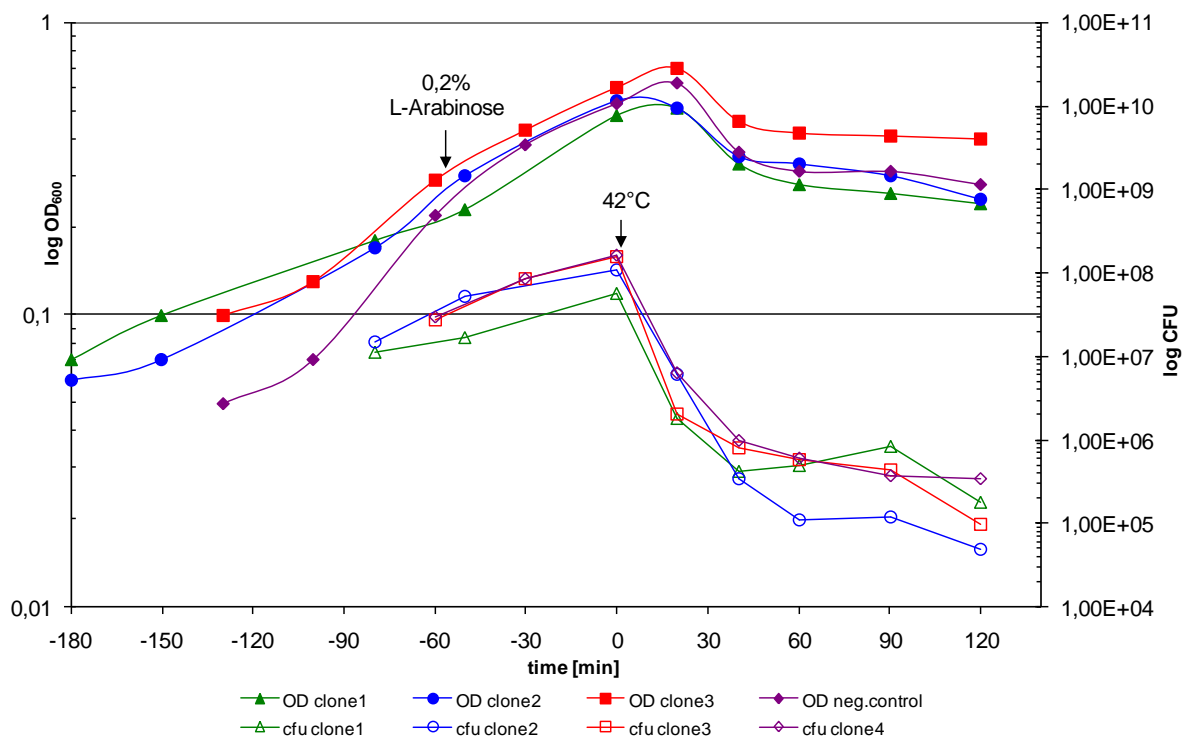


Fig. 4.43: Expression and lysis curve pBGKBZP2C/pGLysivb. Four different pBGKBZP2C/pGLysivb clones were checked for growth and lysis behavior in combination with recombinant protein expression of periplasmic ZP2C.

The ZP2C protein expression was detected using the specific B11 serum from possum in combination with anti-possum IgG as second antibody and anti-IgG HRP as third antibody (Fig. 4.44 and 4.45)

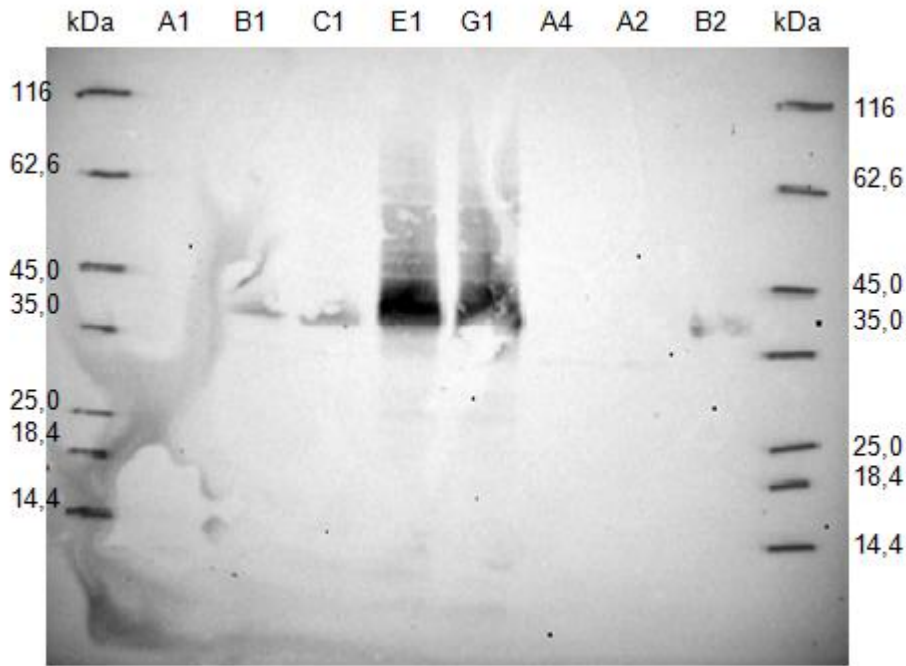


Fig. 4.44: Western blot pBGKBZP2C/pGLysivb. Time points A=0min, B=15min, C=45min, E= 60min and G=120min after L-arabinose induction. Clone 1 at time points A, B, C, E and G, visible protein detection starting with time point B, as well as clone 2 at time points A and B with a slight protein detection at time point B are shown. As a negative control (A4) pBGKBpGLysivb 30 minutes after L-arabinose induction was used.

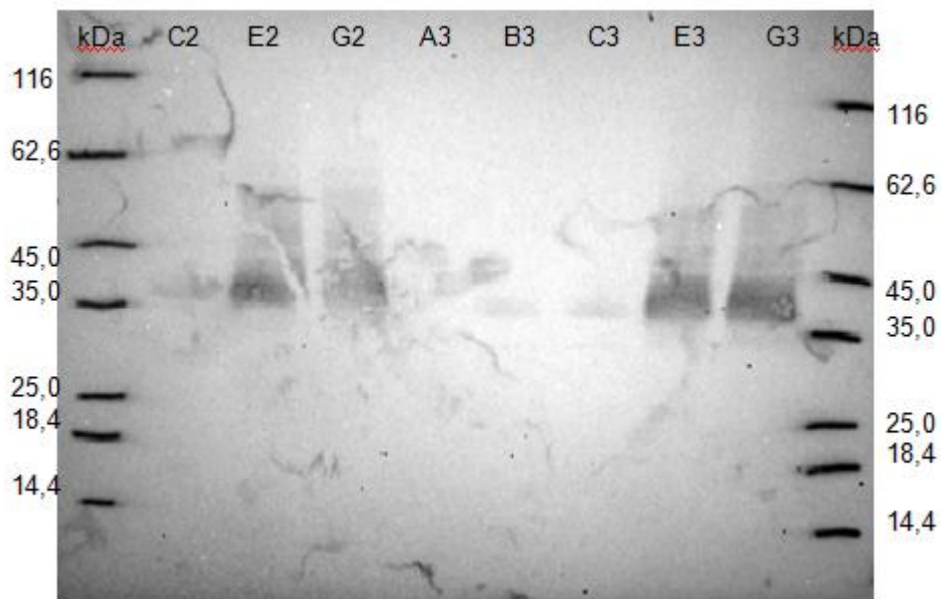


Fig. 4.45: Western blot pBGKBZP2C/pGLysivb. Time points A=0min, B=15min, C=45min, E= 60min and G=120min after L-arabinose induction. Clone 2 at time points C, E, G and clone 3 at time points A, B, C, E, G are shown. Visible protein detection starts with time point B.

Clone 1 of the plasmid pBGKBZP2C/pGLysivb showed correct ZP2C protein expression and was further used for working stock production and fermentation.

4.4.2 Expression- and lysis study of *E. coli* NM522 (pBGKBZP2Copt) (pGLysivb)

The pBGKZP2Copt expression plasmid was co-transformed with the pGLysivb lysis plasmid into Mops-competent *E. coli* NM522 strain, as described in materials and methods (Fig. 4.46).

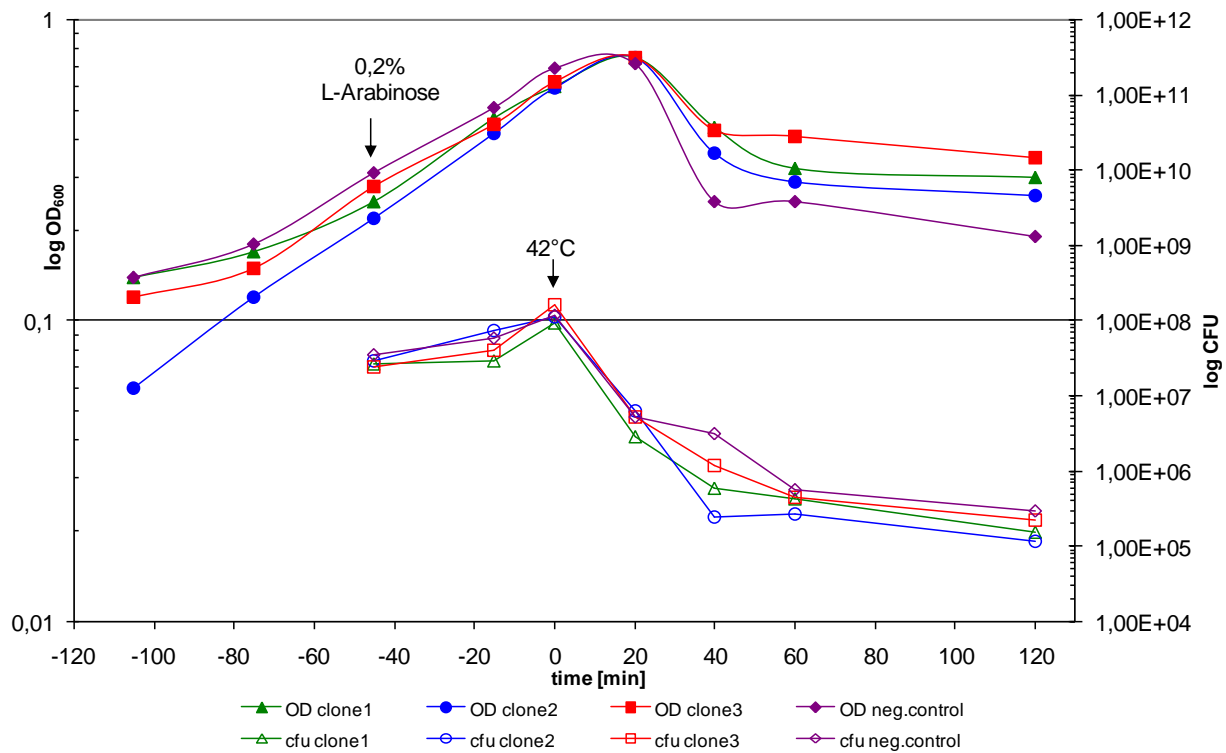


Fig. 4.46: Expression and lysis curve pBGKBZP2Copt/pGLysivb. Three different pBGKBZP2Copt/pGLysivb clones were checked for growth and lysis behavior in combination with recombinant protein expression of periplasmic ZP2Copt.

Western blot analysis was done to check the expression of the protein ZP2Copt. The anti-myc-HRP antibody was used for ZP2Copt protein detection (Fig. 4.47 and 4.48).

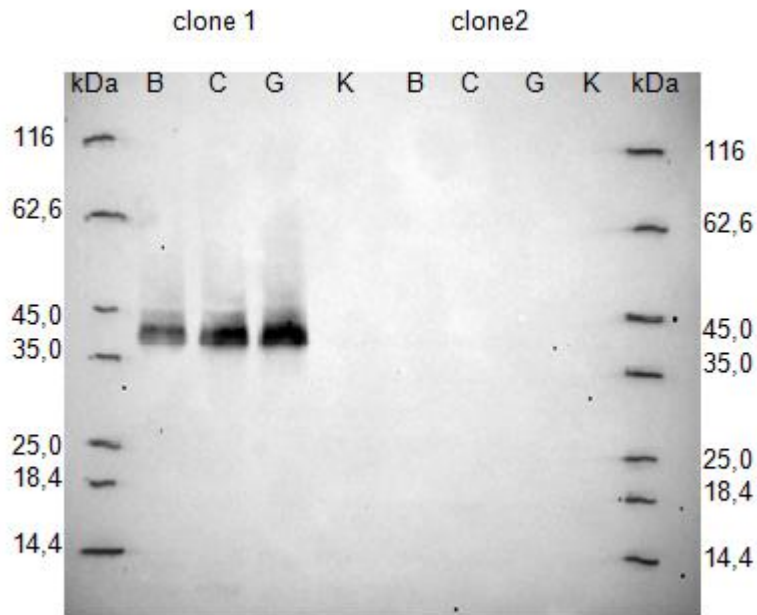


Fig. 4.47: pBGKBZP2Copt/pGLysivb western blot. Time points: B=20min, C=40min and G=120min after L-arabinose induction. The picture shows clone1 and clone2 at time points B, C and G, with visible protein detection of clone1 starting at time point B and increasing until time point G; no protein could be detected in clone 2. As a negative control (K) pBGKBpGLysivb 30 minutes after L-arabinose induction was used.

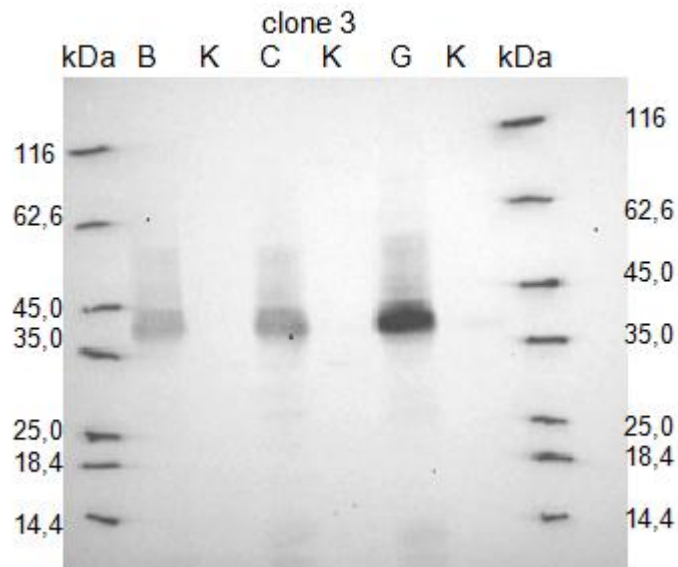


Fig. 4.48: pBGKBZP2Copt/pGLysivb western blot. Time points: B=20min, C=40min and G=120min after L-arabinose induction. The picture shows clone3 at time points B, C and G, with visible protein detection starting at time point B and increasing until time point G. As a negative control (K) pBGKBpGLysivb 30 minutes after L-arabinose induction was used.

Clone 2 of the plasmid pBGKBZP2Copt/pGLysivb showed correct ZP2Copt protein expression and was further used for working stock production and fermentation.

4.4.3 Expression- and lysis study of *E. coli* NM522 (pBGKBCp4) (pGLysivb)

The pBGKBCp4 expression plasmid was co-transformed with the pGLysivb lysis plasmid into Mops-competent *E. coli* NM522 strain as described in materials and methods (Fig. 4.49)

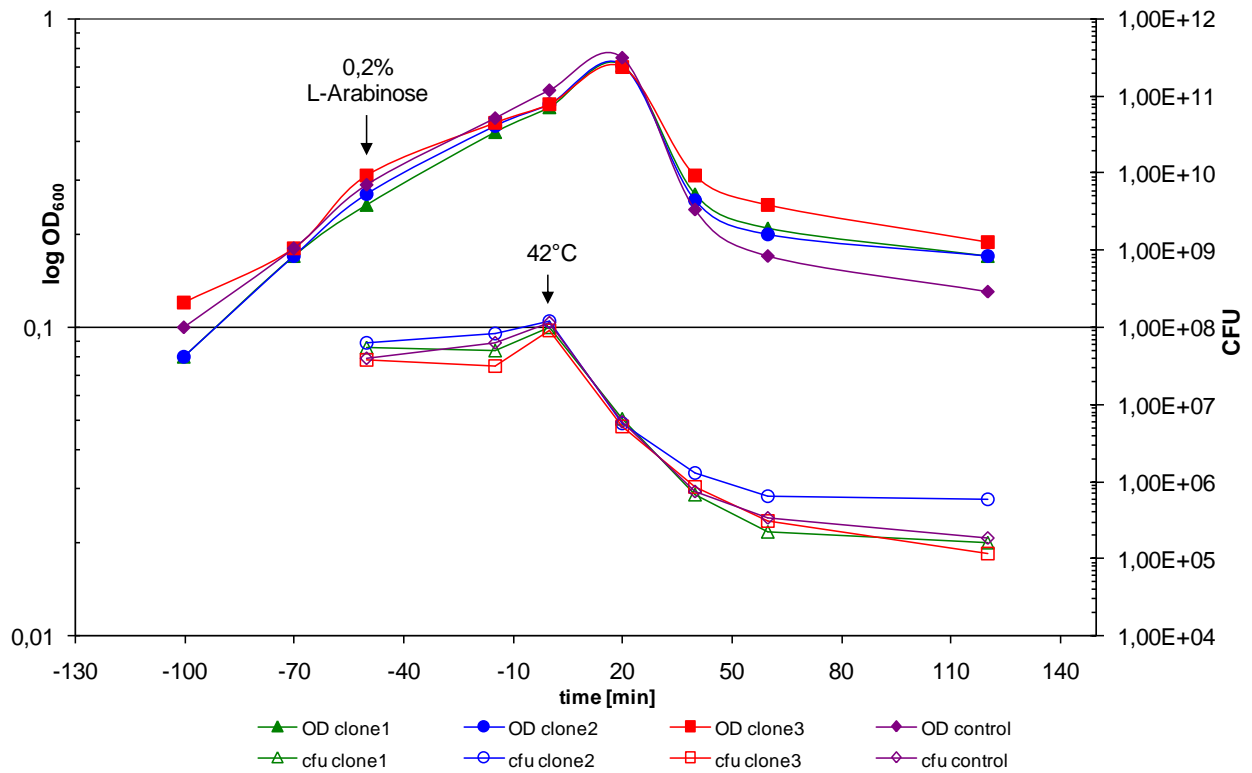


Fig. 4.49: pBGKBCp4/pGLysivb expression and lysis curve. Four different pBGKBCp4/pGLysivb clones were checked for growth and lysis behavior in combination with recombinant protein expression of periplasmic ZP2Copt.

Western blot analysis of the taken samples was performed to check for protein expression of the Cp4 protein using the anti-myc-HRP antibody as well as the Cp4 specific serum Cp4 female TV24 in combination with Rabbit anti-possum as secondary antibody and anti-Rabbit HRP as third antibody for protein detection (Fig. 4.50, 4.51 and 4.52).

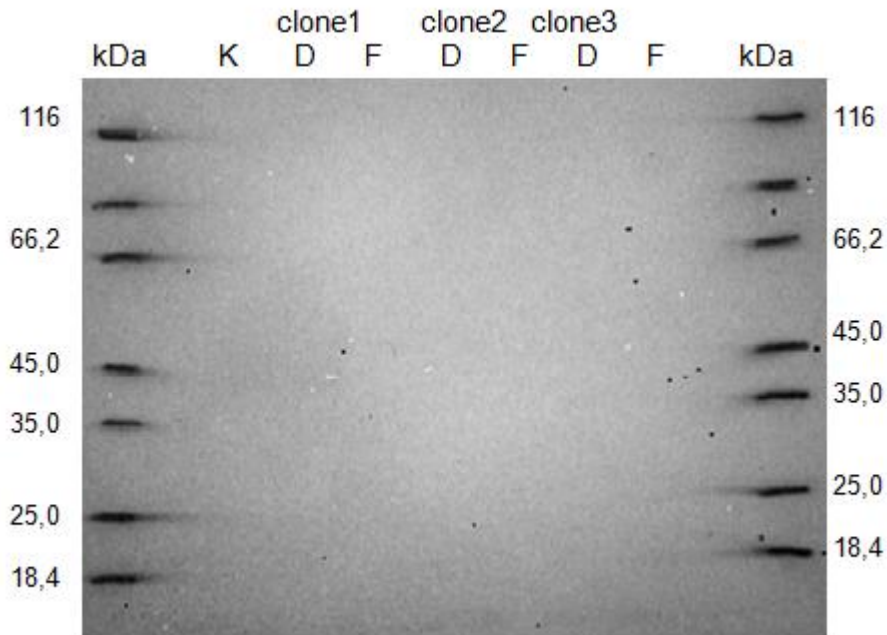


Fig. 4.50: pBGKBCp4/pGLysivb Western blot. Detection of Cp4 protein with anti-myc-HRP antibody. The picture shows the control plasmid (K) pBGKB/pGLysivb 30 minutes after L-arabinose induction and three different pBGKBCp4/pGLysivb clones at time points: D: 20min, F: 60min after L-arabinose induction. No protein could be detected.

As there was no protein expression detected using anti-myc-HRP antibody the western blot analysis was repeated using the possum Cp4 female TV24 specific serum for development.

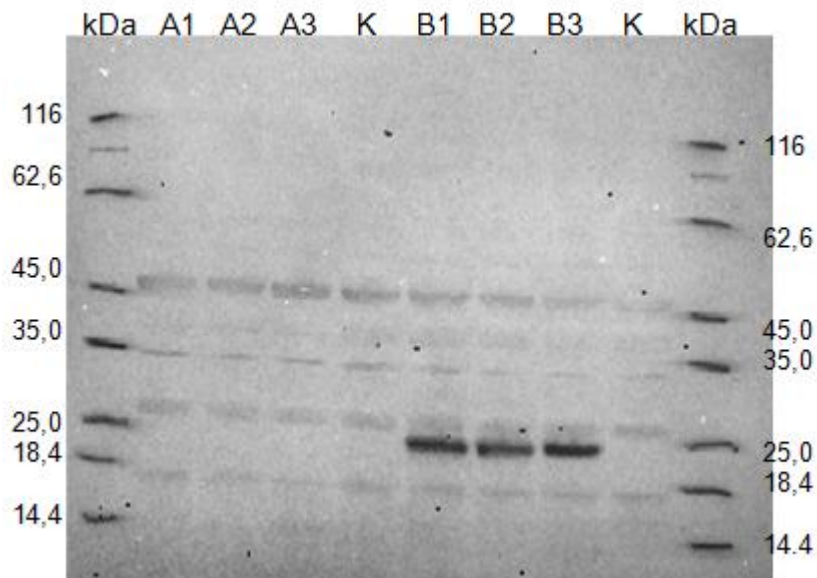


Fig. 4.51: pBGKBCp4/pGLysivb Western blot. Detection of Cp4 protein with possum Cp4 female TV24 specific serum. The picture shows the protein expression of the clone: C1, C2 and C3 directly at L-arabinose induction, time point A and 20 minutes after L-arabinose induction, time point B. Protein bands with a weight of around 25kDa were detected. The expected size of the expressed protein from plasmid gIII-Cp4-myc-polyH protein has a molecular weight of 39,8 kDa. As a negative control (K) pBGKB/pGLysivb 30 minutes after L-arabinose induction was used.

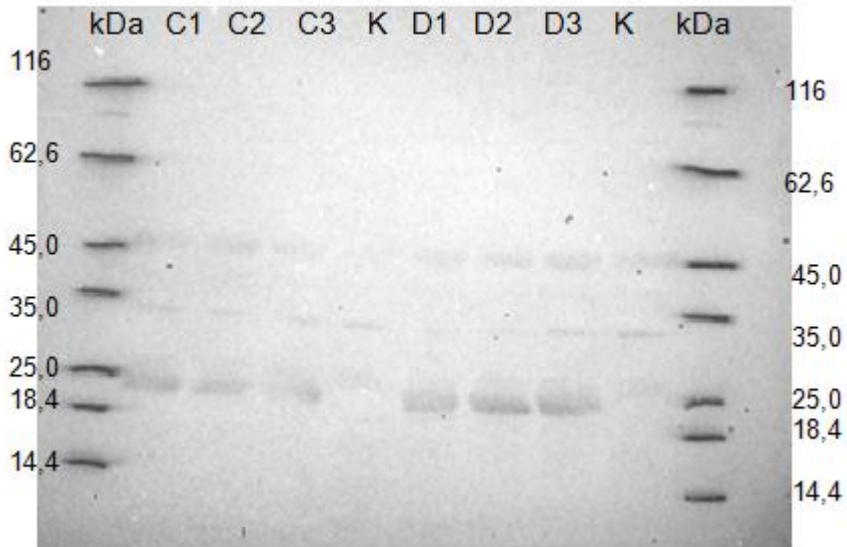


Fig. 4.52: pBGKBCp4/pGLysivb Western blot. Detection of Cp4 with possum Cp4 female TV24 specific serum. The picture shows the protein expression of the clones 1, 2 and 3, 40 (C) and 60 (D) minutes after L-arabinose induction. Protein bands with a weight of around 25kDa were detected. The expected size of the expressed protein from plasmid gIII-Cp4-myc-polyH protein has a molecular weight of 39,8 kDa. As a negative control (K) pBGKB/pGLysivb 30 minutes after L-arabinose induction was used.

As the Cp4 protein expression showed bands only at around 25 kDa and not the correct size of 39,8 kDa, pBGKBCp4 was not further used for fermentation.

4.5 Fermentation

Fermentation was carried out as described in materials and methods with the strain *E. coli* NM522 including the expression plasmid pBGKBZP2C and the lysis plasmid pGLysivb and with *E. coli* NM522, containing the expression plasmid pBGKBZP2Copt and the lysis plasmid pGLysivb. The process of fermentation was performed in a 30L fermenter (20L working volume) with the appropriate antibiotics kanamycin (for the expression plasmid) and gentamycin (for the lysis plasmid).

4.5.1 Fermentation of *E. coli* NM522 (pBGKBZP2C) (pGLysivb)

E. coli NM522 (pBGKBZP2C) (pGLysivb) clone 1, tested in chapter 4.4.1 was used for fermentation in 22L volume. Bacterial growth went on until the OD₆₀₀ reached a value of 0,8 when L-arabinose was added up to a final concentration of 0,2% to induce protein expression (Fig. 4.53). The addition of L-arabinose did not impair growth and after 110 minutes of bacterial growth the temperature was shifted up to 42°C to induce lysis (Fig. 4.53) which went on for another 90 minutes causing a drop in cfu from 10⁹ to 10⁵ (per ml). The surviving bacteria were finally killed by the addition of β-propiolactone (0,075% end-concentration) for another 60 minutes (Fig. 4.53).

During fermentation process samples were taken for OD measurement and cfu determination as well as samples for protein analysis and microscopy. OD and cfu curves can be seen in figure 4.53. Microscopic observations showed intact bacterial cell shape in living cells and ghosts.

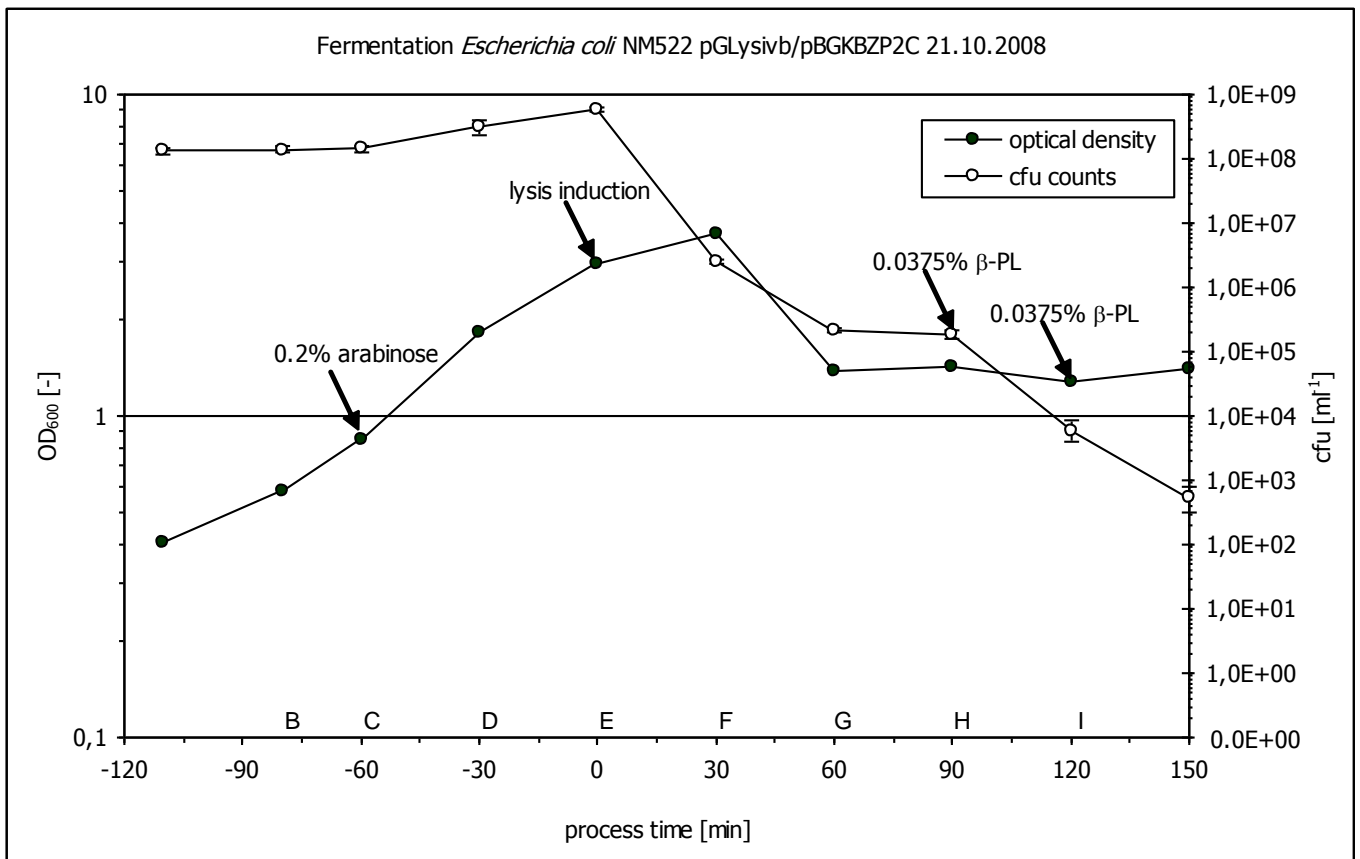


Fig. 4.53: Fermentation curve of NM522 (pBGKBZP2C) (pGLysivb). The figure shows the OD curve as well as the cfu curve of the fermentation. L-arabinose was added up to an end-concentration of 0,2% when OD has reached 0,8 and lysis was induced 110 minutes after bacterial growth. As the arrows in the figure show the cfu drop down from 10^8 to 10^5 (per ml) resulting in a lysis efficiency of 99,97%

The lysis efficiency of the fermentation was calculated to be 99,97% according to the cfu values.

All important fermentation parameters such as flow, stirrer, pH, temperature and oxygen are documented during the process by the IRIS software. A diagram showing all the documented parameters can be seen in figure 4.54.

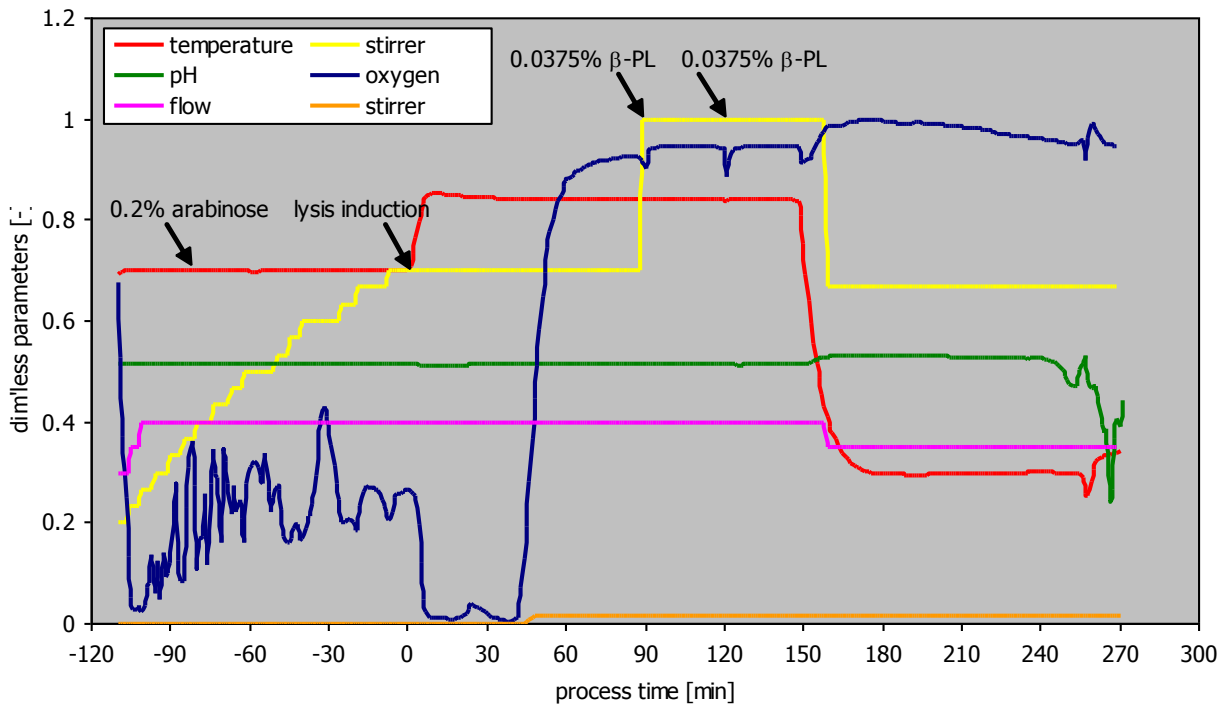


Fig. 4.54: The IRIS diagram. This diagram shows pH-value regulated to a constant pH of 7,2 green colour curve, oxygen concentration regulated to keep the oxygen above 5% blue colour curve, airflow-rate yellow colour curve, stirring per minute brown colour curve and temperature regulated manually red colour curve.

Further data concerning the fermentation process can be found in the following data sheet (Fig. 4.55).

Pre-culture	
Volume: 4*500 ml	Additives: Gentamycin, Kanamycin
Medium type: LBv	Other: -
Date: 2008/10/21	Clone: 1 (2008/09/18 by SSC)
Starting time: 09:45	Strain: Escherichia coli NM522
End time: 14:05	Plasmids: pGLysivb, pBGKBZP2C
ON culture OD: 2.207 / 0.639	Recombinant Protein Expression: ZP2C-myc-polyH
Inoc. Volume: 1.40 l	Expression Induction: 0.2% L-arabinose
Medium: LBv	Expression Induction Time point: C
Antibiotics: Gentamycin, Kanamycin	Lysis Induction: 42°C
Temperature: 35°C	Lysis Induction Time point: E
Total Volume: ~ 22 L	Killing: 2 * 0.0375% β-PL (H/I)
Acid: F.A.: 38.6 ml	Volume harvested: ~ 20 l
Base: A.W.: 116.8 ml	Harvested by: separator
Antifoam A: 17.0 ml	OD separator flow: 0.035 / 0.194 / 0.105 / 0.101
E-Blot: OK (by SSC)	Yield: 7128mg
R-Blot: OK (by SSC)	Particles / mg: 1,65 x 10⁹
RT: -	Sterility: OK (by AFA)
Microscopy: okay	Efficiency: 99.97 %

Fig. 4.55: Fermentation data sheet. NM522 (pBGKBZP2C) (pGLysivb) fermentation

After harvesting of the cells by separation the bacterial ghosts were washed (starting with a volume of 20L down to 1L) with autoclaved, sterile water and aliquoted into flasks for lyophilization. The total yield of the fermentation was 7128 mg with calculated $1,65 \times 10^9$

particles per mg (Fig. 4.55). The sterility of the lyophilisate was checked by sterility testing with 3 x 10mg. No survivors were detected in the preparation by this test (Fig. 4.55).

Western blot analysis of the ZP2C antigen expression using the possum specific B11 serum for protein detection resulted in clear bands at the weight of the ZP2C protein of 37,04 kDa after induction with L-arabinose at time point C. This did not decrease after lysis induction assuming the periplasmatic transport of the ZP2C protein (Fig. 4.56).

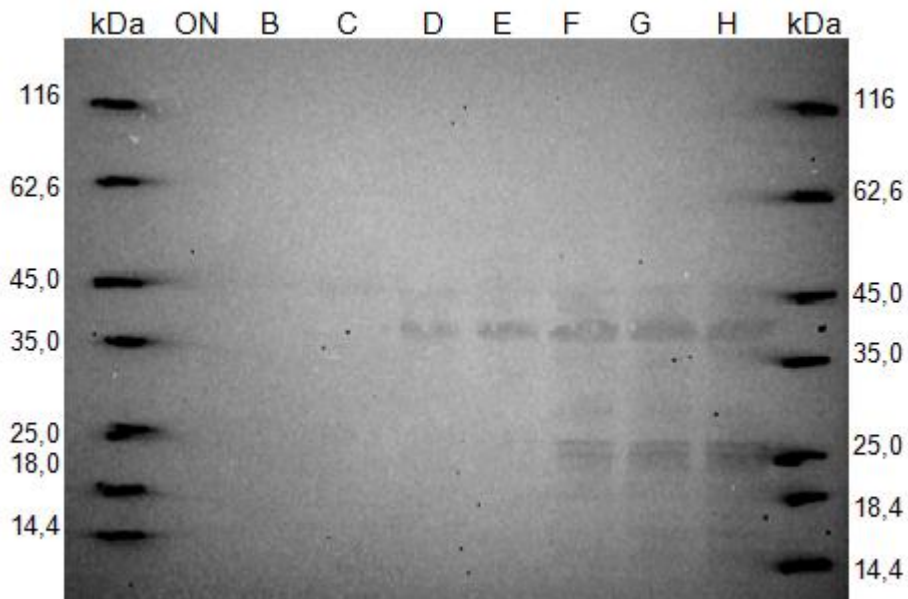


Fig. 4.56: NM522 pBGKBZP2C/pGLysivb fermentation western blot. Fermentation samples shown: overnight culture sample (ON), B: 30 min of bacterial growth, C: time of L-arabinose induction, D: 30 min after L-arabinose induction, E: time of temperature shift up to 42°C, F: 30 min after lysis induction, G: 60 min after lysis induction, H: 90 min after lysis induction. The molecular weight ZP2C is 37,04 kDa.

Western blot analysis of the ZP2C antigen expression using anti-myc-HRP antibody showed comparable results (Fig. 4.57).

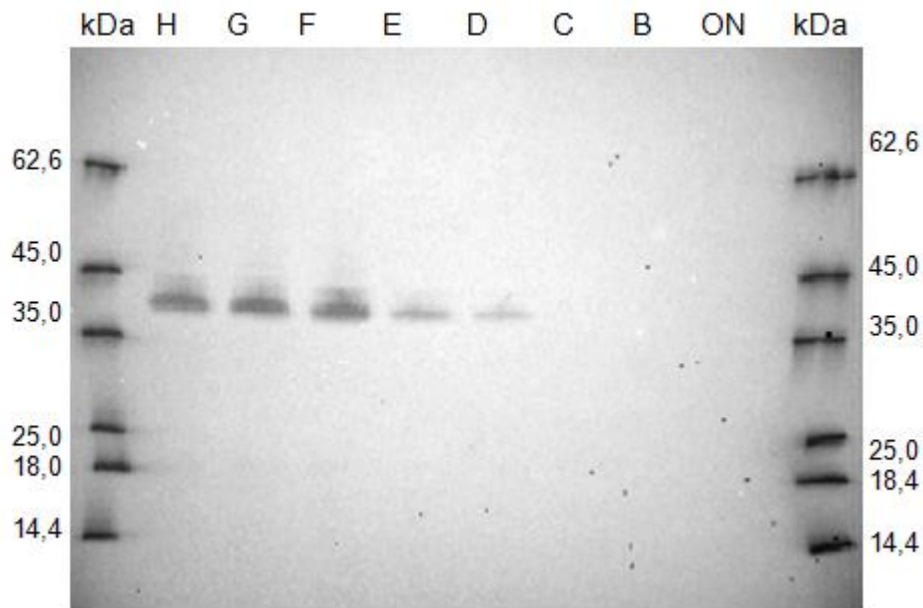


Fig. 4.57: NM522 pBGKBZP2C/pGLysivb fermentation western blot. The picture shows fermentation samples of different time points. H: 90 min after lysis induction, G: 60 min after lysis induction, F: 30 min after lysis induction, E: time point of temperature shift up to 42°C, D: 30 min after L-arabinose induction, B: 30 min after bacterial growth and ON: overnight culture. The molecular weight ZP2C is 37,04 kDa.

Furthermore the expression of the ZP2C protein was quantified using western blot analysis. The Positope (Invitrogen) [5µg/µl] was used as standard with a 1:2 dilution series. The amounts loaded per lane were the following:

- 500ng standard 1
- 250ng standard 2
- 125ng standard 3
- 62,5ng standard 4
- 31,25ng standard 5

The lyophilized ZP2C bacterial ghosts were diluted in loading buffer as described in materials and methods and the following amounts were loaded per lane:

- U1 = 4µg
- U2 = 10µg
- U3 = 20µg
- U4 = 40µg

The membrane was developed using anti-myc-HRP antibody (Fig. 4.58)

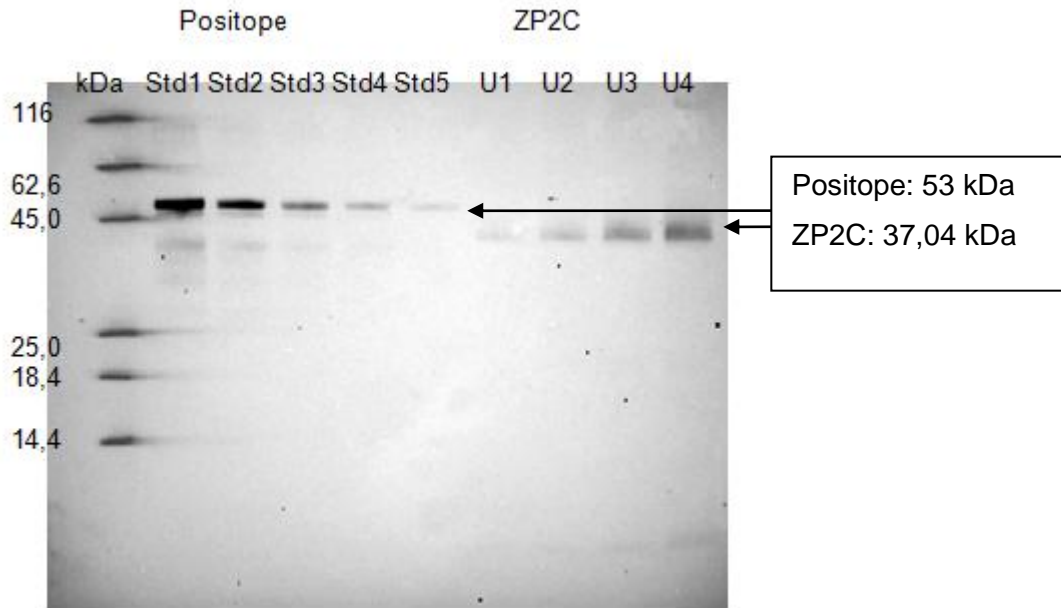


Fig. 4.58: Quantification western blot analysis of lyophilized ZP2C ghosts. Positope functions as standard with a known concentration. Different amounts of ZP2C ghosts were loaded as samples for quantification.

With the use of the QuantityOne Software in the ChemiDocXRS program quantification of the unknown ZP2C antigen was performed. The results of the quantification are shown on the following quantification curve (Fig. 4.59).

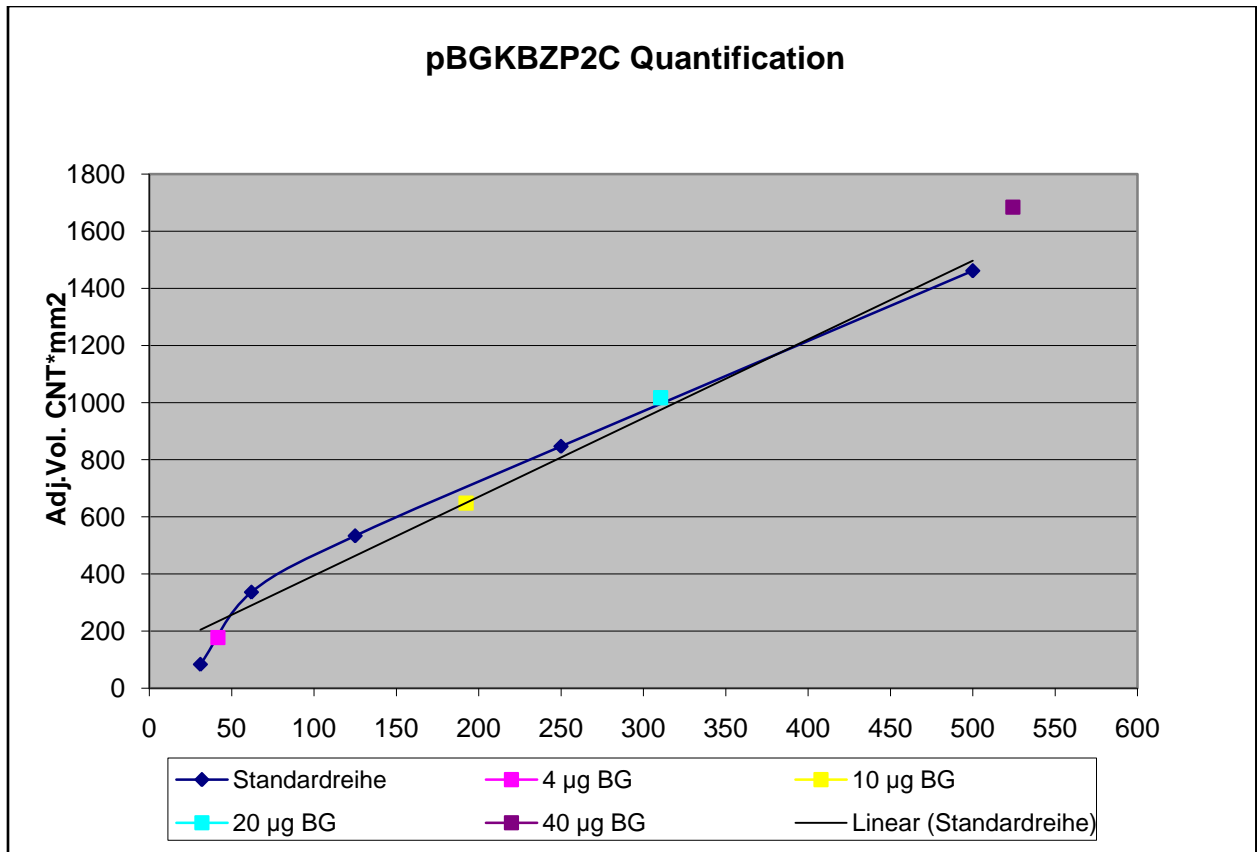


Fig. 4.59: Quantification curve ZP2C ghost. The linear Positope standard curve is shown in black; the colorful dots mark the different ZP2C concentrations.

Due to the standard curve the concentration of the ZP2C antigen had been calculated as the following:

U1: 4µg BG: 42 ng/20µl = 10.39ng ZP2C / µg BG

U2: 10µg BG: 192 ng/20µl = 19.23ng ZP2C / µg BG

U3: 20µg BG: 310 ng/20µl = 15.52ng ZP2C / µg BG

U3: 40µg BG: 524 ng/20µl = 13.11ng ZP2C / µg BG

An average of 14,6 ng ZP2C/µg bacterial ghost was determined. The ghost preparation was sent for animal trials to New Zealand to the Landscape Research Group.

4.5.2 Fermentation of *E. coli* NM522 (pBGKBZP2Copt) (pGLysivb)

NM522 (pBGKBZP2Copt) (pGLysivb) clone 2 tested in chapter 4.4.2 was used for fermentation in 22L volume. Bacterial growth went on until the OD_{600} reached a value of 0,8 when L-arabinose was added up to a final concentration of 0,2% to induce protein expression (Fig.4.60). The addition of L-arabinose did not impair growth and after 120 minutes of bacterial growth at 36°C the temperature was shifted up to 42°C to induce lysis (Fig.4.60), which went on for another 60 minutes causing a drop in cfu from 10^8 to 10^5 (per ml). The surviving bacteria were finally killed by β -propiolactone (0,075% end-concentration) for another 60 minutes (Fig. 4.60).

During fermentation process samples were taken for OD measurement and cfu determination as well as samples for protein analysis and microscopy. OD and cfu curves can be seen in figure 4.60. Microscopic observations showed intact bacterial cell shape in living cells and ghosts.

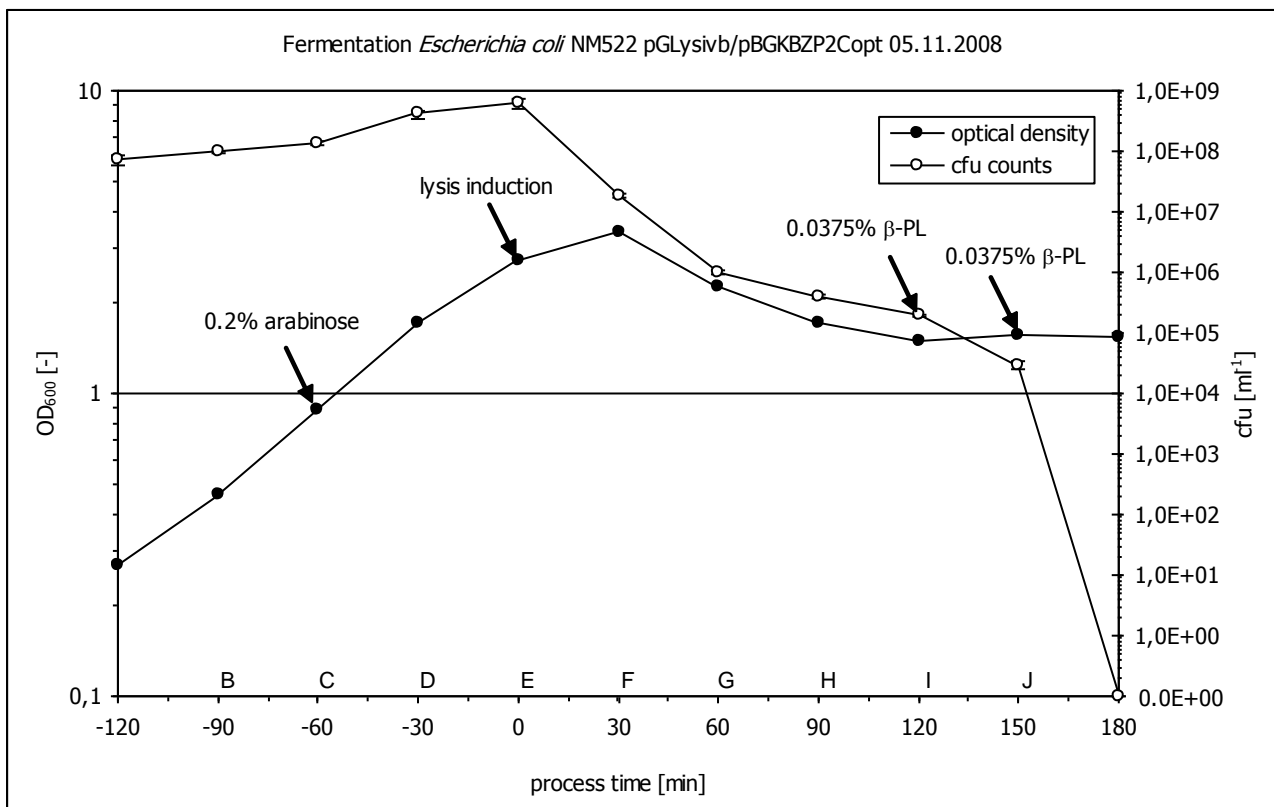


Fig. 4.60: Fermentation curve of NM522 (pBGKBZP2Copt) (pGLysivb) clone2. OD values as well as cfu values are shown. L-arabinose was added up to an end-concentration of 0,2% after 60 minutes of bacterial growth, when the OD_{600} was at 0,8 to induce protein expression. After 120 minutes of bacterial growth temperature was shifted up to 42°C to induce lysis (see arrows). Due to bacterial lysis the cfu dropped from 10^8 up to 10^5 (per ml) resulting in a lysis efficiency of 99,97%.

The lysis efficiency of the fermentation was calculated 99,97%.

All important fermentation parameters such as flow, stirrer, pH, temperature and oxygen are documented during the process by the IRIS software. A diagram showing all the documented parameters can be seen in figure 4.61.

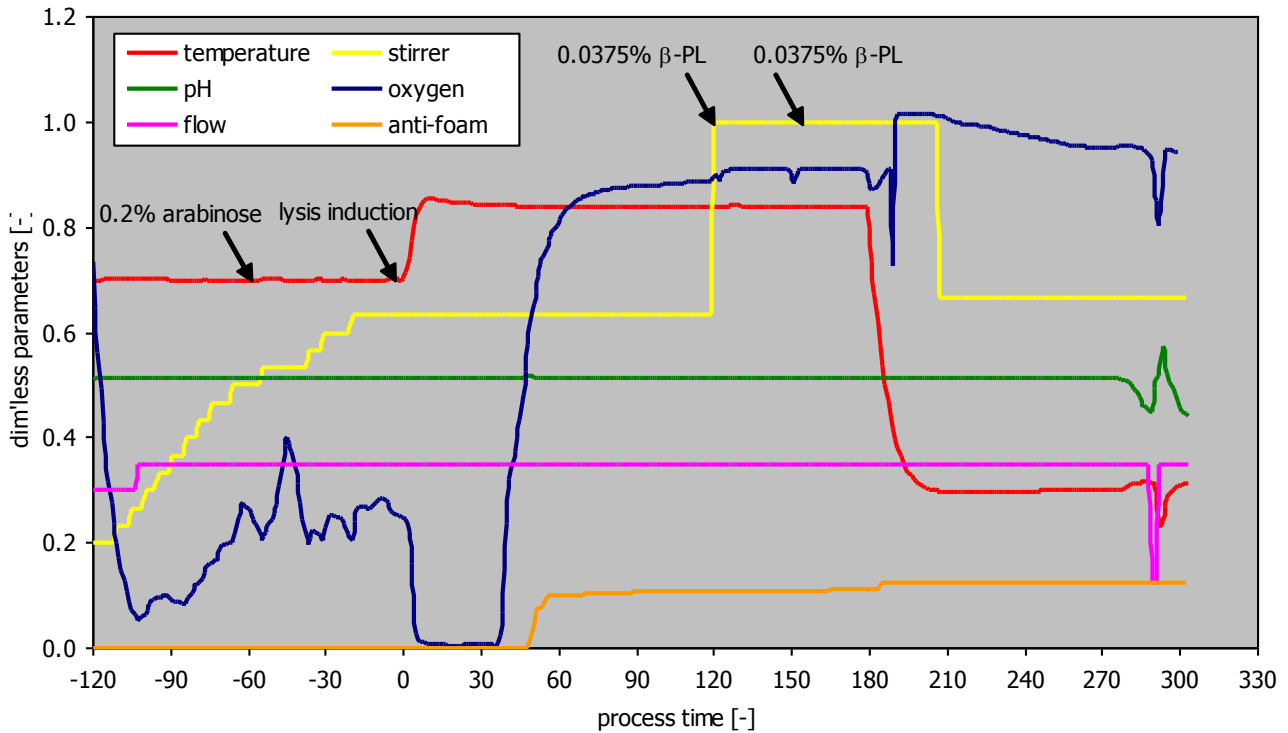


Fig. 4.61: IRIS diagram. This diagram shows pH-value regulated to a constant pH of 7,2 green colour curve, oxygen concentration regulated to keep the oxygen above 5% blue colour curve, airflow-rate yellow colour curve, stirring per minute brown colour curve and temperature regulated manually red colour curve.

Further fermentation data are documented in the fermentation sheet (Fig. 4.62).

Pre-culture	
Volume: 4*500 ml	Additives: Gentamycin, Kanamycin
Medium type: LBv	Other: -
Date: 2008/11/05	Clone: 2 (2008/09/27 by SSC)
Starting time: 08:45	Strain: <i>Escherichia coli</i> NM522
End time: 13:45	Plasmids: pGLysivb, pBGKBZP2Copt
ON culture OD: 1.807 / 0.320	Recombinant Protein Expression: ZP2Copt-myc-polyH
Inoc. Volume: 1.80 l	Expression Induction: 0.2% arabinose
Medium: LBv	Expression Induction Time point: C
Antibiotics: Gentamycin, Kanamycin	Lysis Induction: 42°C
Temperature: 35°C	Lysis Induction Time point: E
Total Volume: ~ 22 L	Killing: 2 * 0.0375% β-PL (I/J)
Acid: F.A.: 43.2 ml	Volume harvested: ~ 20 l
Base: A.W.: 129.7 ml	Harvested by: separator
Antifoam A: 123.4 ml (?)	OD separator flow: 0.016 / 0.055 / 0.060 / 0.042
E-Blot: -	Yield: 6847mg
R-Blot: OK (by SSC)	Particles / mg: 1,85x10⁹
RT: -	Sterility: OK (by AFA)
Microscopy: okay, elongation	Efficiency: 99.97 %

Fig. 4.62: Fermentation data sheet. NM522 (pBKGBZP2Copt) (pGLysivb) fermentation.

After harvesting of the cells by separation the bacterial ghosts were washed (starting with a volume of 20L down to 1L) with autoclaved, sterile water and aliquoted into flasks for lyophilization. The total yield of the fermentation was 6847 mg with calculated $1,85 \times 10^9$ particles per mg (Fig. 4.62). The sterility of the lyophilisate was checked by sterility testing with 3 x 10mg. No survivors were detected in the preparation by this test (Fig. 4.62).

Western blot analysis of the ZP2Copt antigen expression using the possum specific B11 serum for protein detection resulted in clear bands at the weight of the ZP2Copt protein of 39,07 kDa after induction with L-arabinose at time point C. This did not decrease after lysis induction assuming the periplasmatic transport of the ZP2Copt protein (Fig. 4.63).

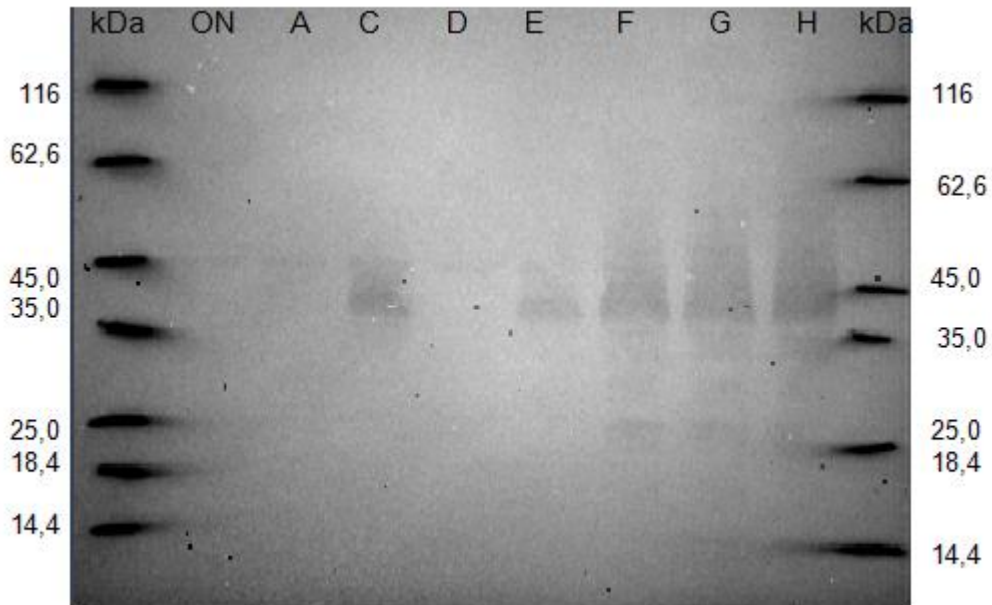


Fig. 4.63: NM522 (pBGKBZP2Copt) (pGLysivb) western blot. Development with B11 serum to detect protein expression of ZP2Copt. Loaded time points: ON: overnight, A: start of bacterial growth, C: time point of L-arabinose induction, D: 30 min after L-arabinose induction, E: time point of lysis induction, F: 30 min after lysis induction, G: 60 min after lysis induction, H: 90 min after L-arabinose induction. Expression of the ZP2Copt protein can be seen at time points C, E, F, G, H with a molecular weight of 39,07 kDa.

Additionally western blot analysis was performed using the anti-myc-HRP antibody for the detection of the antigen.

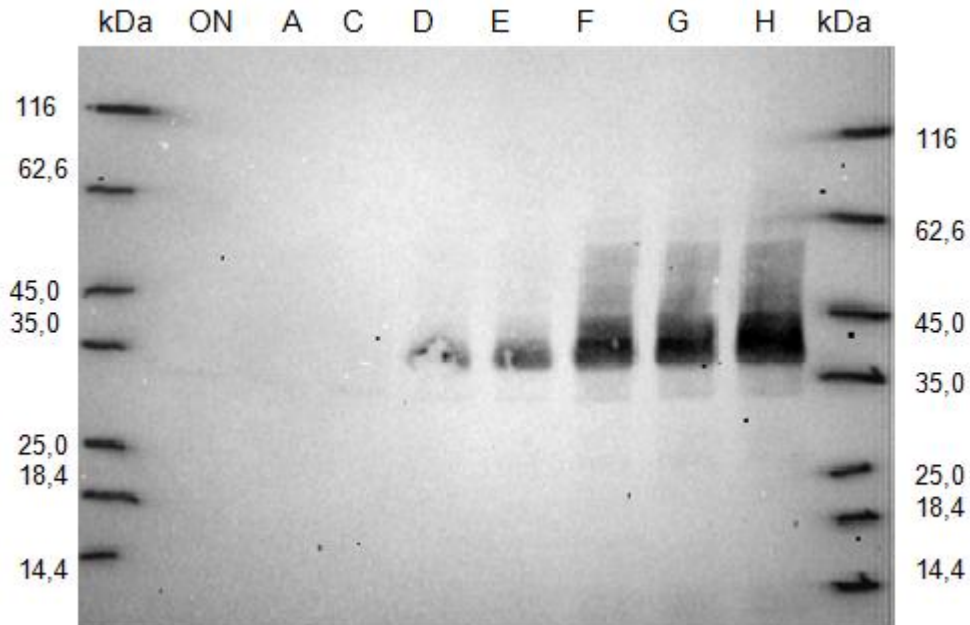


Fig. 4.64: NM522 (pBGKBZP2Copt) (pGLysivb) clone2 western blot. Development with anti-myc-HRP antibody. Loaded time points: ON: overnight, A: start of bacterial growth, C: time point of L-arabinose induction, D: 30 min after L-arabinose induction, E: time point of lysis induction, F: 30 min after lysis induction, G: 60 min after lysis induction, H: 90 min after L-arabinose induction. Visible expression of ZP2Copt protein at time points: D, E, F, G, H with a molecular weight of 39,07 kDa.

Furthermore the expression of ZP2Copt protein was quantified using western blot analysis and the Positope (Invitrogen) [5µg/µl] was used as standard with a 1:2 dilution series. The amounts loaded per lane were the following:

- 500ng standard 1
- 375ng standard 2
- 300ng standard 3
- 250ng standard 4

The lyophilized ZP2C bacterial ghosts were diluted in loading buffer as described in materials and methods and the following amounts were loaded per lane:

- U1 = 0,4µg
- U2 = 0,8µg
- U3 = 1,2µg
- U4 = 1,6µg
- U5 = 2,0µg

The membrane was developed using anti-myc-HRP antibody (Fig. 4.65)

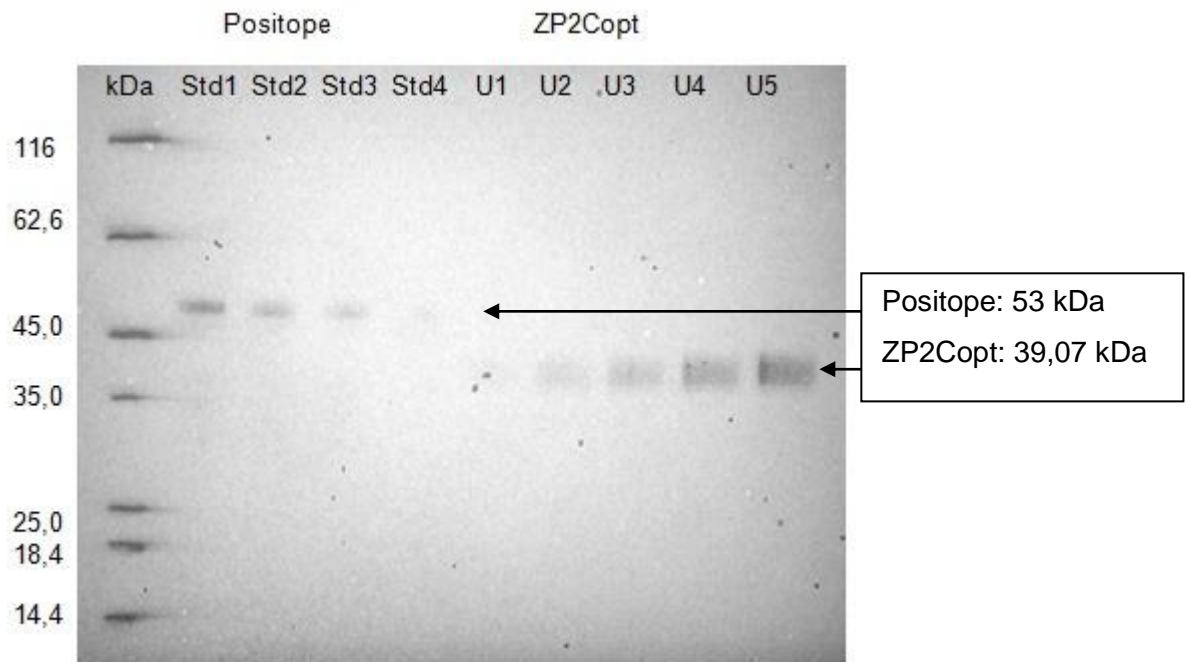


Fig. 4.65: Quantification of pBGKBZP2Copt/pGLysivb clone2. Positope functions as standard with a known concentration. Different amounts of ZP2Copt ghosts were loaded as samples for quantification.

With the use of the QuantityOne Software in the ChemiDocXRS program quantification of the unknown ZP2Copt antigen was performed. The results of the quantification are shown on the following quantification curve (Fig. 4.66)

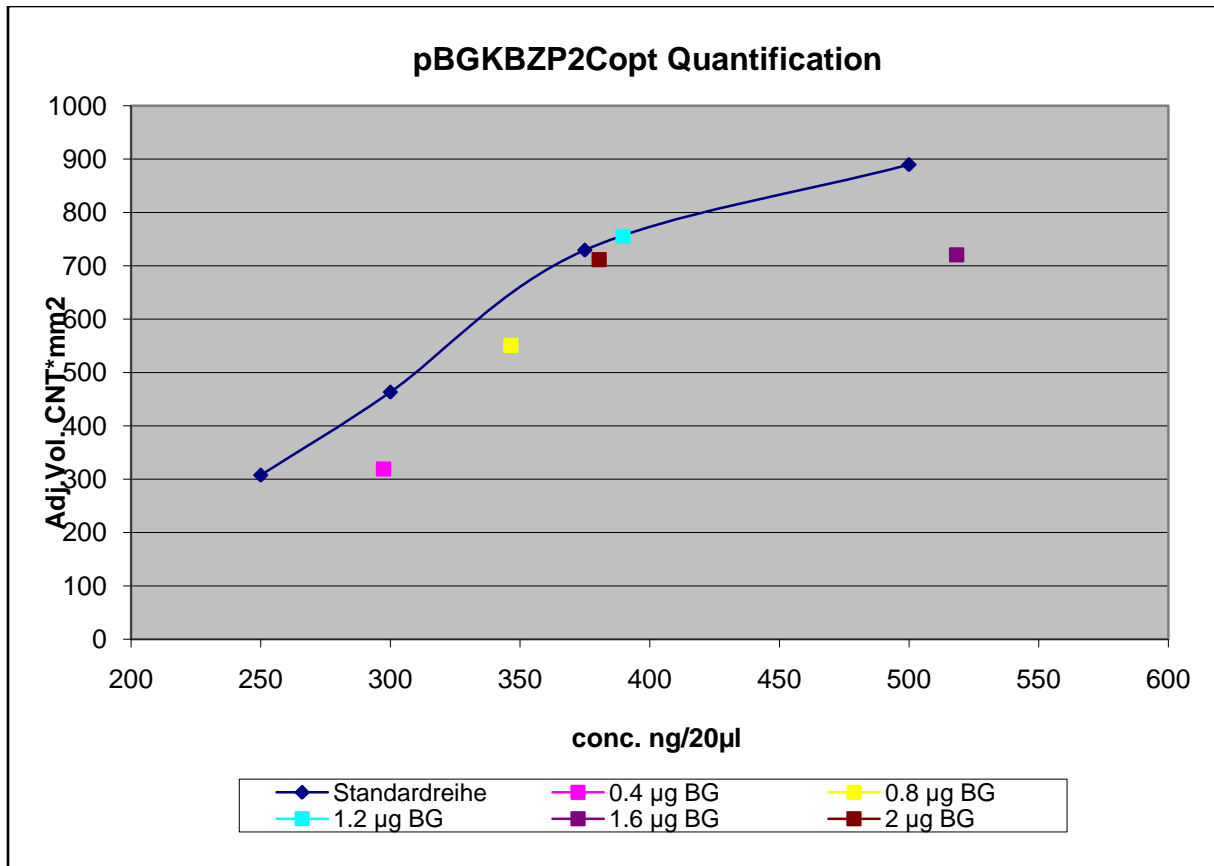


Fig. 4.66: ZP2Copt ghosts quantification curve. The linear Positope standard curve is shown in black; the colorful dots mark the different ZP2Copt concentrations.

Due to the standard curve the concentration of the ZP2Copt antigen had been calculated as the following:

U1: 0,4µg BG: 297,35 ng/20µl = 743,37 ng ZP2C / µg BG

U2: 0,8µg BG: 346,39 ng/20µl = 432,98 ng ZP2C / µg BG

U3: 1,2µg BG: 389,68 ng/20µl = 324,73 ng ZP2C / µg BG

U4: 1,6µg BG: 518,39 ng/20µl = 323,99 ng ZP2C / µg BG

U5: 2,0µg BG: 380,45 ng/20µl = 190ng ZP2C / µg BG

An average of 403 ng ZP2Copt / µg bacterial ghost was determined. The ghost preparation was sent for animal trials to New Zealand to the Landscare Research Group.

4.6 Trouble shooting pBGKBCp4

Due to the fact that protein expression of the pBGKBCp4 plasmid always resulted in products around 25 kDa, instead of 37,7 kDa there was no fermentation made with the pBGKBCp4 clone. Furthermore the pBGKBCp4 clones were digested again using different restriction enzymes to be sure to have the Cp4 sequence cloned into the pBGKB vector. Additionally to this restriction digest another western blot analysis was done to verify the protein expression of Cp4 protein.

4.6.1 Restriction digest and western blot analysis of pBGKBCp4

The restriction analysis of pBGKBCp4 was performed with different enzymes, where some enzymes cut in cloned Cp4 insert sequence (Fig. 4.67)

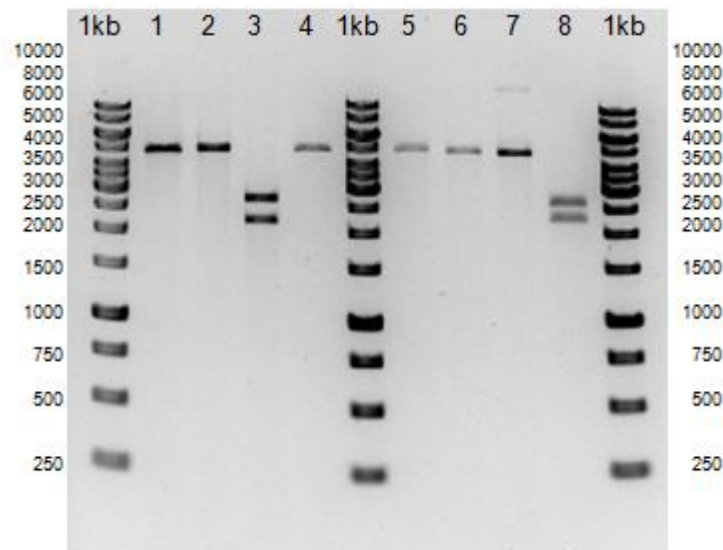


Fig. 4.67. pBGKBCp4 restriction digest.

1: BamHI	4937bp	correct	5: PstI	4937bp (insert cut)	correct
2: BglII	4937bp	correct	6: SacI	4937bp (insert cut)	correct
3: DraI	2212/2723bp	correct	7: XbaI	4937bp	correct
4: EcoRI	4937bp (insert cut)	correct	8: XhoI	2279/2658bp	correct

Marker: 1kb DNA ladder (Fermentas).

All tested restriction digests showed correct band patterns, even the enzymes which cut in the Cp4 insert gave correct bands.

Additionally the NM522 (pBGKBCp4) expression study was repeated and fresh protein samples were collected to perform western blot analysis to check for correct protein expression of Cp4 at around 37,7 kDa (Fig. 4.68, 4.69).

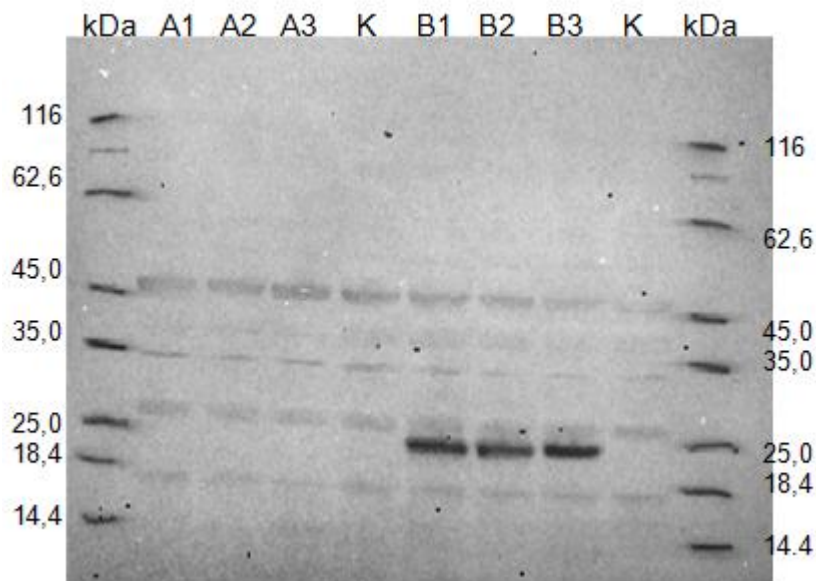


Fig. 4.68: Cp4 western blot analysis. Detection with possum specific Cp4 female TV24 serum. Clone 4, 5, 6 can be seen at 0 min: time point of L-arabinose induction and at 20 min: 20 minutes after L-arabinose induction, showing protein bands at around 25 kDa, instead of 37,7 kDa. As a negative control (K) pBGKB, 30 minutes after L-arabinose induction was used.

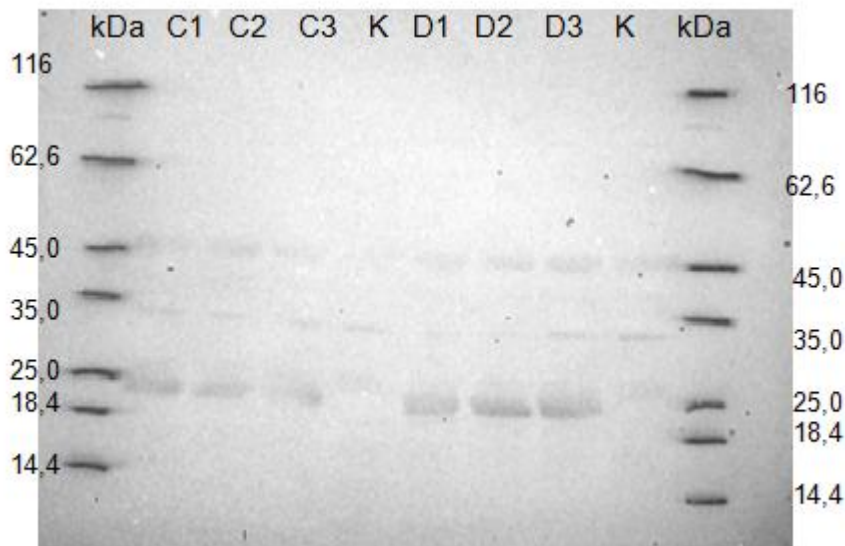


Fig. 4.69: Cp4 western blot analysis. Detection with possum specific Cp4 female TV24 serum. Clone 4, 5, 6 can be seen at 40 min: 40 minutes after L-arabinose induction and at 60 min: 60 minutes after L-arabinose induction, showing protein bands at around 25 kDa, instead of 37,7 kDa. As a negative control (K) pBGKB, 30 minutes after L-arabinose induction was used.

Although the restriction digests showed expected results, the protein expression of Cp4 could not clearly be shown. Bands at a lower weight could only be detected via specific possum serum, but detection with anti-myc-HRP showed no results.

This might be due to the fact that there is no correct expression of Cp4 from start to end, and therefore expression is stopped within the insert, just before the myc-epitope.

To detect possible mutations in the Cp4 sequence which might have led to the stop of the protein expression, sequencing of the Cp4 insert was done.

4.6.2 Sequencing of Cp4

For sequencing of Cp4, the pBGKBCp4 plasmid was transformed into E. coli C2988J and a midiprep was made as described in materials and methods; 200µl of the midiprep (elution 1) were sent for sequencing to Microsynth, CH.

Following primers were designed to start sequencing 100bp before the Cp4 sequence and to end sequencing 100bp behind the Cp4 sequence:

FWD: 5' TTT GGG CTA ACA GGA GGA ATT 3'
REV: 5' ATC TGT ATC AGG CTG AAA ATC 3'

The sequencing results were blasted: confirmed sequence against theoretical sequence, using the EBLOSUM62 blast matrix:

Aligned Sequences	2
EMBOSS_001	1
EMBOSS_001	2
Gap penalty	10.0
Extend penalty	0.5
Length	983
Identiy	963/983 (98.0%)
Similarity	963/983 (98.0%)
Gaps	3/983 (0.3%)
Score	5836.0

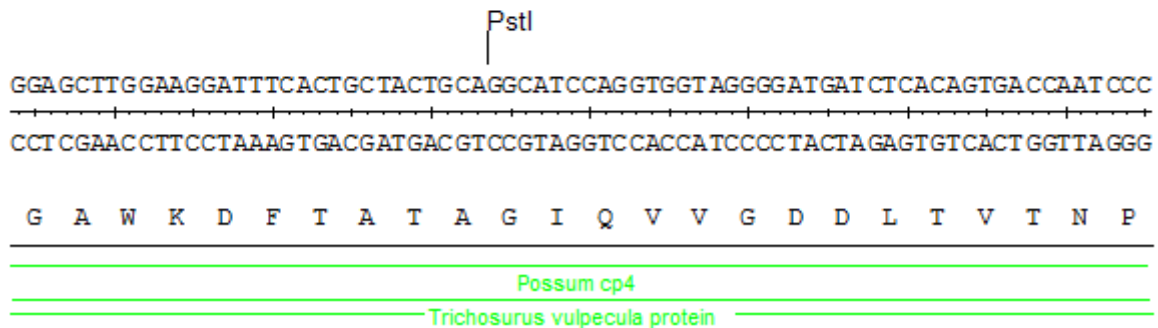
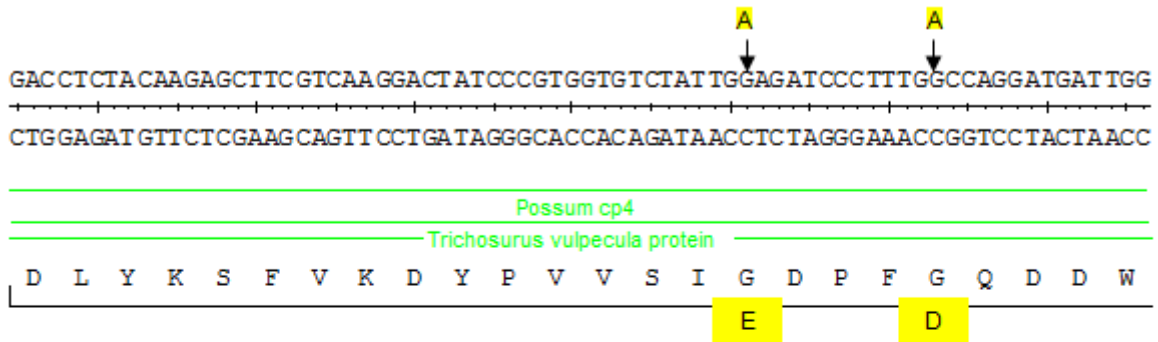
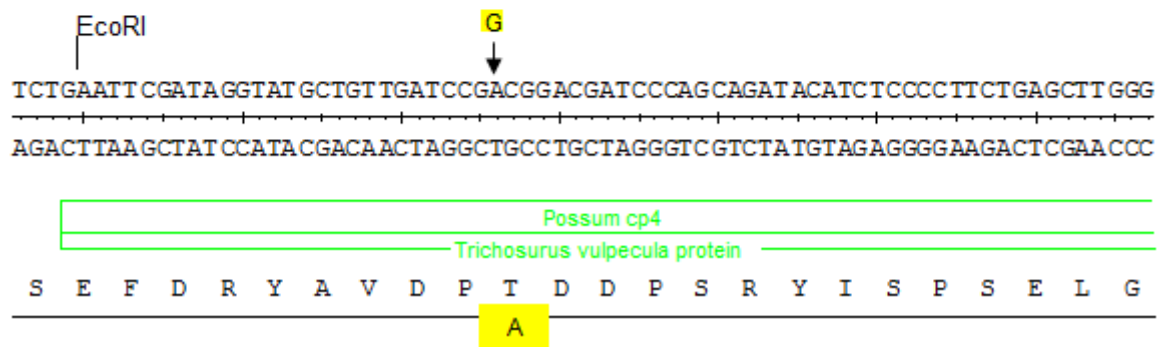
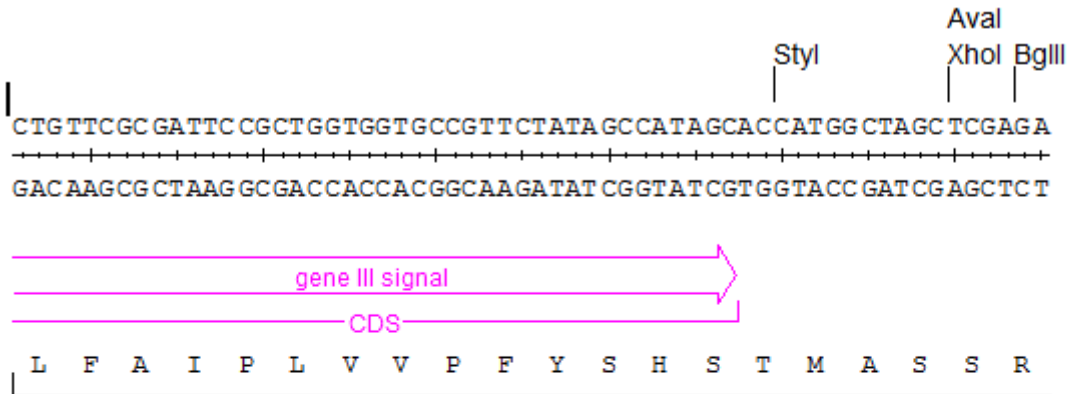
confirmed
sequence

theoretical
sequence

EMBOSS_001 1 CTGTTTCGCGATTCCGCTGGTGGTGCCGTTCTATAGCCATAGCACCATGGC
EMBOSS_001 1 CTGTTTCGCGATTCCGCTGGTGGTGCCGTTCTATAGCCATAGCACCATGGC
EMBOSS_001 51 TAGCTCGAGATCTGAATTCGATAGGTATGCTGTTGATCCGACGGACGATC
EMBOSS_001 51 TAGCTCGAGATCTGAATTCGATAGGTATGCTGTTGATCCGGCGGACGATC
EMBOSS_001 101 CCAGCAGATACATCTCCCCTTCTGAGCTTGGGGACCTCTACAAGAGCTTC
EMBOSS_001 101 CCAGCAGATACATCTCCCCTTCTGAGCTTGGGGACCTCTACAAGAGCTTC
EMBOSS_001 151 GTCAAGGACTATCCCGTGGTGTCTATTGGAGATCCCTTTGGCCAGGATGA
EMBOSS_001 151 GTCAAGGACTATCCCGTGGTGTCTATTGAAGATCCCTTTGACCAGGATGA
EMBOSS_001 201 TTGGGGAGCTTGGAAAGGATTTCACTGCTACTGCAGGCATCCAGGTGGTAG
EMBOSS_001 201 TTGGGGAGCTTGGAAAGGATTTCACTGCTACTGCAGGCATCCAGGTGGTAG
EMBOSS_001 251 GGGATGATCTCACAGTGACCAATCCCAAGCGCATTGAAAAGGCTGTGAAC
EMBOSS_001 251 GGGATGATCTCACAGTGACCAATCCCGAGCGCATTGAAAAGGCTGTGAAC
EMBOSS_001 301 GAGAAAGCCTGCAACTGCCTCCTCCTCAAAGTGAACCAGATTGGCTCCGT
EMBOSS_001 301 GAGAAAGCCTGCAACTGCCTCCTCCTCAAAGTGAACCAGATTGGCTCCGT
EMBOSS_001 351 GACTGAATCTCTCCAGGCGTGCAAGCTGGCCCAGTCCAATGGATGGGGAG
EMBOSS_001 351 GACTGAATCTCTCCAGGCGTGCAAGCTGGCCCAGTCCAATGGCTGGGGAG
EMBOSS_001 401 TGATGGTTTCTCATCGTTCTGGGGAGACTGAAGATACCTTTATTGCAGAC
EMBOSS_001 401 TGATGGTTTCTCATCGTTCTGGGGAGACTGAAGATACCTTTATTGCAGAC
EMBOSS_001 451 CTGGTGGTGGGCCTCTGCACTGGGCAGATCAAGACCGGTGCCCCCTTCCG
EMBOSS_001 451 CTGGTGGTGGGCCTCTGCACTGGGCAGATCAAGACCGGTGCCCCCTGCCG
EMBOSS_001 501 ATCTGAGCGTCTGGCACAAGTATATCACAGCTTCTCAGAATTGAGCAAGA
EMBOSS_001 501 ATCTGAGCGTCTGGC-CAAGTATAAC-CAGCTTCTCAGAATTGAGGAAGA
EMBOSS_001 551 GATGGGCAGCAAGGTTAAATTTGCTGGAAGGAACTTCAGAAACCCTCAGG
EMBOSS_001 549 GCTGGGCAGCAAGGTTAAATTTGCTGGAAGGAACTTCAGAAACCCTCAGG
EMBOSS_001 601 CCAAGTAAGCTCTGTGAGCAGGAGGCCTATGAGCTCGCAGGTGCCAGTCA
EMBOSS_001 599 CCAAGTAAGCTCTGTGACCAGGAGGCCTATGAGCTCGCAGGTGCCAGTCA
EMBOSS_001 651 CCTCTGTAGAAGTCTGCTCCTGCCCTACGAGTGGGCAGCACCAAGTACTA
EMBOSS_001 649 CCTCTGTAGAAGTCTGCTCCTGCCCTAAGAGTGGGCAGCACCAAGTACTA

```
EMBOSS_001      701 GACCAGTTTTGTTTTGGGCGCAGGGCCCTCCGCTGCTCAACTTCCCTCTC
      |
      |
EMBOSS_001      699 GACCAGTTTTGTTTTGGGCGCAGGGCCCTCCGCTGCTCAGCTTCCCTCTC
      |
      |
EMBOSS_001      751 CCTTCCACCTTTCTGTTATGTTCTCACTGCTTCGTTAGAAGTGTCTTACA
      |
      |
EMBOSS_001      749 CCTTCCACCTTTCTGTTATGTTCTCACTGCTTCGTTAGAAGTGTCTTACA
      |
      |
EMBOSS_001      801 CCAGAGAAGACCTGATGTTTGTTCTCTGTCGGAAAACCCCTTATCATCTT
      |
      |
EMBOSS_001      799 CCAGAGATGACCTGATGTTTGTTCTCTGTCGGAAAACCCCTTATCATCTT
      |
      |
EMBOSS_001      851 GTGACTGGGATAGAACTCACTTGGTCTCACCCCAACCCTGGTGTGTTTATGT
      |
      |
EMBOSS_001      849 GTGACTGGGATAGAACTCACTTGGTCTCACCCCAACCCTGGTGTGTTTATGT
      |
      |
EMBOSS_001      901 GTGGGAGCCCTGTCCCTTGTGATGCAGTCCCGATGAGCCACAGGCCAGCT
      |
      |
EMBOSS_001      899 GT-GGAGCCCTGTCCCTTGTGATGCAGTCCCGATGAGCCACAGGCCAGCT
      |
      |
EMBOSS_001      951 CCTTAGTGGCTTCTATGTGCAGAATAAAAAGTAT      983
      |
      |
EMBOSS_001      948 CCTTAGTGGCTTCTATGTGCAGAATAAAAAGTAT      980
```

Obtained results from the blast program of the theoretical and the confirmed sequence were displayed using the Map Draw of DNASTAR program for a more detailed information. In the following data sheet, the yellow letters mark the differences in the nucleotide sequence as well as in the amino acids.



G
↓
AAGCGCAT TGAAAAGGCT GTGAA CGAGAAAGCCT GCAACTGCCT CCTCC TCAAA GTGAA CCAGATTGGCTCC
TTCGCGTAACTTT TCCGACACTT GCTCTTT CGGACGTTGACGGAGGAGGATTT CACTT GGTCTAACCGAGG

Possum cp4
Trichosurus vulpecula protein
K R I E K A V N E K A C N C L L L K V N Q I G S

E
GTGACTGAATCTCTCCAGGCGTGCAAGCTGGCCCAGTCCAATGGATGGGGAGTGATGGTTTCTCATCGT TCT
CACTGACTTAGAGAGGTC CGCAC GTTCGACCGGGTCAGGTTACC TACCC CTCAC TACCAAAGAGTAGCAAGA

C
↓
V T E S L Q A C K L A Q S N G W G V M V S H R S
Possum cp4
Trichosurus vulpecula protein

GGGGAGACTGAAGATACTTTAT TGCAGACCTGGTGGTGGGCCT CTGCACTGGG CAGAT CAAGACCGGT GCC
CCCCTCTGACTTCTATGGAAATAACGTCTGGACCACCACCCGGAGACGT GACCC GTCTAGTTCTGGCCACGG

Possum cp4
Trichosurus vulpecula protein
G E T E D T F I A D L V V G L C T G Q I K T G A

G **Deletion** **A** **Deletion** **G** **C**
↓ ↓ ↓ ↓ ↓ ↓
CCCTTCCGATCTGAGCGT CTGGCACAAGTATATCACAGCTTCTCAGAAT TGAGCAAGAGATGGGCAGCAAGG
GGGAAGGCTAGACTCGCAGACCGTGTTCATATAGTGTGGAAGAGTCTTAACTCGTTCTCTACCCGTCGT TCC

Frameshift →
P F R S E R L A Q V Y H S F S E L S K R W A A R
E **K**
Possum cp4
Trichosurus vulpecula protein

TTAAATTT GCTGGAAGGAACTTCAGAAACCCCTCAGGCCAAGTAA GCTCT GTGAG CAGGAGGCCTATGAG CTC
AATTTAAACGACCTTCCT TGAAGTCTTTGGGAGT CCGGTT CATT CGAGACACTC GTCTCT CCGGATACTC GAG

Possum cp4

Trichosurus vulpecula protein

L N L L E G T S E T L R P S K L C E Q E A Y E L

GCAGGTGC CAGT CACCTC TGTAGAAGTC TGCTCCTGCC TACGAGTGGG CAGCA CCAAGTACTA GACCA GTT
CGT CCACG GTCAGTGGAGACATCTTCAGACGAGGACGGGATGCT CACCC GTCGT GGTTCATGAT CTGGT CAA

A G A S H L C R S L L L P Y E W A A P S T R P V

Possum cp4

Trichosurus vulpecula protein

TTGTTTTGGGCGCAGGGG CCTCCGCTGCTCAACTTCCCT CTCCC TTCCACCTTT CTGTTATGTT CTCAC TGC
AACAAAAC CCGCGTCCCC GGAGG CGACGAGTTGAAGGGA GAGGGAAGGT GGAAA GACAATACAA GAGTGACC

L F W A Q G P P L L N F P L P S T F L L C S H C

Possum cp4

Trichosurus vulpecula protein

TTCGTTAGAACTG TCTTACACCAGAGAAGACCTGATGTTTGGTT CTCTGTCGGAAAACCCTTATCATCT TGT
AAGCAATCTTGACAGAAT GTGGT CTCTTCTGGAC TACAAACCAA GAGACAGCCT TTTGG GAATAGTAGAACA

Possum cp4

Trichosurus vulpecula protein

F V R T V L H Q R R P D V W F S V G K P L S S C

The theoretical sequence would give the following amino acid sequence:

LFAIPLVVPFYSHSTMetASSRSEFDRYAVDPTDDPSRYISPSEL
GDLYKSFVKDYPVVSIGDPFGQDDWGAWKDFTATAGIQVVGDD
LTVTNPKRIEKAVNEKACNCLLLKVNQIGSVTESLQACKLAQSN
GWGVMetVSHRSGETEDTFIADLVVGLCTGQIKTGAPFRSERLA
QVYHSFSELSKRWAARLNLLGTSETLRPSKLCQEAYELAGA
SHLCRSLLLPYEWAAPSTRPVLFWAQGPPLLNFPSTFLLCSH
CFVRTVLHQRDPVWFSVGKPLSSCDWDRITWSHPQPWCLCV
GALSLLCSPDEPTGQLLSGFYVQNKs

In contrast the confirmed sequence gives the amino acid sequence shown below:

LFAIPLVVPFYSHSTMetASSRSEFDRYAVDPADDDPSRYISPSEL
GDLYKSFVKDYPVVSIEDPFDQDDWGAWKDFTATAGIQVVGDD
LTVTNPERIEKAVNEKACNCLLLKVNQIGSVTESLQACKLAQSN
GWGVMetVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLA
KYNQLLRIEEEELGSKVKFAGRNFRNPQAK Stop AL Stop P G G L Stop A
RRCQSPL Stop KSAPALRVGSTKY Stop TSFVLGAGASAAQLPSPF
HLSVMetFSLLR Stop SCLTPE Met T Stop CLVLCRKTLLIL Stop LG Stop N
HLVSPPTLVLMetCGALSLSL Stop CSPDEPTGQLLSGFYVQNKs

According to the mutations in the confirmed Cp4 sequence, the expression product of the confirmed sequence results in the following amino acid sequence:

MKLLFAIPLVVPFYSHSTMASSRSEFDRYAVDPADDPSRYISPSELGDLYKSFVKDYPVVSIEDPFDQDDWGAWKDFTATAGIQVVGDDLTVTNPERIEKAVNEKACNCLLLKVNQIGSVTESLQACKLAQSNGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIEEEELGSKVKFAGRNFRNPQAK.

The expression of the Cp4 sequence stops at the first frameshift stop and yields in a protein with a molecular weight of 22,7 kDa and an isoelectric point of 5.2.

Due to these results, it cannot be said whether the original plasmid pGEX2TCp4 already had these mutations inside. To answer this question sequencing of the Cp4 part of the pGEX2TCp4 would be necessary.

4.7 Trouble shooting pBGKBVap1

Restriction digests as well as western blot analysis never gave correct results. After several different cloning strategies for cloning the Vap1 gene sequence into the pBGKB vector plasmid, the last option was to sequence the Vap1 sequence to be sure whether the sequence is correct or not.

4.7.1 Sequencing of Vap1

For sequencing of Vap1, the pBGKBVap1 plasmid was transformed into E. coli C2988J and a midiprep was made as described in materials and methods; 200µl of the midiprep (elution 1) were sent for sequencing to Microsynth, CH.

Following primers were designed to start sequencing 100bp before the Vap1 sequence and to end sequencing 100bp behind the Vap1 sequence:

FWD: 5' TTT GGG CTA ACA GGA GGA ATT 3'
REV: 5' ATC TGT ATC AGG CTG AAA ATC 3'

The sequencing results were blasted: confirmed sequence against theoretical sequence, using the EBLOSUM62 blast matrix:

Aligned Sequences	2
EMBOSS_001	1
EMBOSS_001	2
Gap penalty	10.0
Extend penalty	0.5
Length	735
Identiy	324/735 (44.1%)
Similarity	324/735 (44.1%)
Gaps	232/735 (31.6%)
Score	1375.5

confirmed
sequence

theoretical
sequence

EMBOSS_001 1 TC-CACAGAG-CAAGTTCGACGAGTACGTACGGTGATCACCGAGAACGAG
EMBOSS_001 1 TCTCACAGTGACCAATCC--CGAG--CGCATTGAAAAGGCTGTGAACGA-
EMBOSS_001 49 GAGGGCGTGCAACAAGCGCTCAACTTCG-----CCAT
EMBOSS_001 46 GAAAGCCTGCAAC-TGC-CTCCTCCTCAAAGTGAACCAGATTGGCTCCGT
EMBOSS_001 81 CAAAGAATTTAACCGGGCCAGCAA-C--GACAAGTAC---GGCAGCCGAG
EMBOSS_001 94 GACTGAATCTCTCCAGGCGTGCAAGCTGGCCCAGTCCAATGGCTGGGGAG
EMBOSS_001 125 TGT-----TCAGGGTGCT-GCGAGTCAGGA-----AGCAG--
EMBOSS_001 144 TGATGGTTTCTCATCGTTCTGGGGAGACTGAAGATACCTTTATTGCAGAC
EMBOSS_001 154 CTTGTGGCTGG-----TGTGAAATACTATATTGATG---CTGAGG
EMBOSS_001 194 CTGGTGGTGGGCTCTGCACTGGGCAGATCAAGACCGGTGCCCCCTGCCG
EMBOSS_001 191 TT-----CGTCGAACCACGTGCACCAAG----TC-----TGTGGCTGACC
EMBOSS_001 244 ATCTGAGCGTCTGGCCAAGTATAACCAGCTTCTCAGAATTGAGGAAGAGC
EMBOSS_001 227 T----TGCAAG-----CTGC-----CCCTAT---C
EMBOSS_001 294 TGGGCAGCAAGGTTAAATTTGCTGGAAGGAACTTCAGAAAACCTCAGGCC
EMBOSS_001 245 ATGAGGAC-----CCAG-----CTCTGAAAAAGCA----CTCCGTCTG
EMBOSS_001 344 AAGTAAGCTCTGTGACCAGGAGGCTATGAGCTCGCAGGTGC-CAGTCAC
EMBOSS_001 279 CGTATTTGAGGTGTACACTATTCCTTGGT-TGGGCAAAACCACCTCTTG
EMBOSS_001 393 CTCTGTAGAAGTCTGCTCCTGCCCTAAGAGTGGGCAGCACCAAGTACT--
EMBOSS_001 328 AAGAACGAATGCAAAGATGCCG-AGGCGCCTCC-TTGCACA-----CATC
EMBOSS_001 441 -AGACCAGTTTTGTTTTGGGCGCAGGGCCTCCGCTGCTCAGCTTCCCTC
EMBOSS_001 371 TGCGACCAGCCTTAGCTGCAGCTGTTCTCTCCTTTTTGAAAAAAGAGCAA
EMBOSS_001 490 TCCCTTCCACCTTT-CTG-TGATGTTCTCACTGCTTCG---TTAGAGCTG
EMBOSS_001 421 TATTACAAC-----CC----ATTTGATTCAATTATTGCTTAAC---TG
EMBOSS_001 535 TCTTACACCAGAGATGACCTGATGTTTGGTTCTCTGTGCGAAAACCTTA
EMBOSS_001 456 TAAGTTAGTG-CTGTG-----CAAGGTA
EMBOSS_001 585 TCATCTTGTGACTGGGATAGAATCACTTGGTCTCACCCCAACCTGGTG
EMBOSS_001 478 ATGA----TGAATC---TCCT-----CCCTCAG-
EMBOSS_001 635 TTGATGTGTGGAGCCCTGTCTTGTGATGCAGTCCCGATGAGCCCACAGG
EMBOSS_001 500 -----C----TGGC----AGGAGGAGGAAATAA 519
EMBOSS_001 685 CCAGCTCCTTAGTGGCTTCTATGTGCAGAATAAAA 719

As the blast results show, the theoretical sequence differs strongly from the confirmed sequence.

Amino acids from theoretical sequence gIII-Vap1:

Met K K L L F A I P L V V P F Y S H S T **Met** A S S R S S T E Q V R R V R T V I T E N E E
 G V Q Q A L N F A I K E F N R A S N D K Y G S R V F R V L R V R K Q L V A G V K Y Y I D
 A E V R R T T C T K S V A D L A S C P Y H E D P A L K K H S V C V F E V Y T I P W L G K
 T T L L K N E C K D A E A P P C T H L R P A L A A V L S F L K K E Q Y Y N P F D S L L
 L N C K L V L C K V **Met Met** E S P P S A G R R R K **Stop**

It is very unlikely that the high degree of mutation is due to PCR mismatches and it therefore can be supposed that the confirmed sequence is already present in the original plasmid pGEX2TVap1. Proof of this would be possible by sequencing of the Vap1 sequence of pGEX2TVap1.

In summary the goal of this study was to produce an immunocontraceptive vaccine for opossums depending on the bacterial ghost system. Four different possum - immunocontraceptive target sequences were used: ZP2C, ZP2Copt, Cp4 and Vap1. Because of erroneous sequences of Vap1 and Cp4 only the sequences ZP2C and ZP2Copt could be cloned into the BG-system and were further used for protein production in the fermenter.

Plasmids	Production	Results
pBGBKZP2C	fermentation	14,6 ng ZP2C / µg BG
pBGKBZP2Copt	fermentation	403 ng ZP2Copt / µg BG
pBGKBCp4	sequencing	EBLOSUM62 blast identity: 98,0%
pBGKBVap1	sequencing	EBLOSUM62 blast identity: 44,1%

5 Discussion

The purpose of this work was to create a bacterial ghost delivery system containing a recombinant antigen to induce immune response against target contraceptive antigens in brushtail possums. Former studies showed that immunization of female possums against recombinant zona pellucida protein-2 (ZP2) reduced embryo production by 72-75% [1] and delivery of ZP2C antigen in a bacterial ghost vaccine had already been proofed to impair the fertilization process in immunized animals [16]. In the present study plasmids were constructed which carry target molecule sequences acting during embryonic possum development. Four different immunocontraceptive target gene sequences ZP2C, ZP2Copt, Cp4 and Vap1 were cloned into the pBGKB vector and bacterial ghosts were produced.

5.1 Vector plasmid

Work was started with the composition of the appropriate vector plasmid. Outgoing from three different plasmids pBADGIIIA, B, C the vector was cloned. In this case the ampicillin resistance cassette was replaced by kanamycin in order to avoid the application in wildlife or human. The pBGKB plasmid was chosen to be used as vector for further cloning.

5.2 Construction of possum protein expressing plasmids

The immunocontraceptive target sequences ZP2C, ZP2Copt, Cp4 and Vap1 were cloned into the pBGKB vector using two different cloning strategies: amplification of the desired sequence via PCR or restriction digest to cut the target gene sequence out. The obtained gene sequences were cloned into the multiple cloning site of the pBADGIII derived pBGKB vector and therefore provide all of the necessary reagents to express recombinant protein.

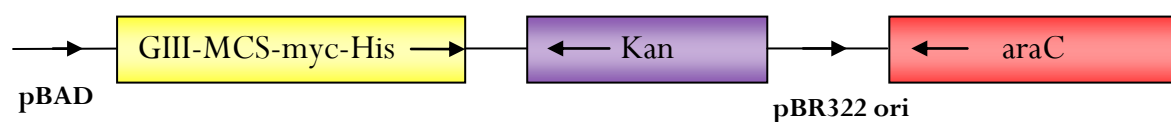


Fig. 5.1: Vector plasmid pBGKB. pBAD promoter, gIII-signal sequence, MCS – multiple cloning site, myc- and his-epitope, kan – kanamycin resistance cassette, pBR322 – origin of replication

The desired gene sequences were expressed as fusion proteins together with the gIII signal sequence. This sequence is derived from the bacteriophage fd gene III protein which

encodes for pIII peptide and is important for the transport of the protein into the periplasmic space where it is additionally cleaved off.

5.3 Expression study

Western blot analysis of the ZP2C and ZP2Copt plasmids showed diverse signals, differing between the usage of the low signal anti-myc-HRP antibody and the usage of the possum specific B11 serum which showed quite strong signals. L-arabinose protein expression of ZP2C and ZP2Copt never impaired the growth of the E. coli NM522 bacterial strain.

Western blot analysis of Cp4 plasmid only illustrated signals when developed with the possum specific Cp4 serum; anti-myc-HRP never showed any bands. Serum development resulted in a Cp4-expression at around 25 kDa, although the Cp4-protein should be at 37,7 kDa.

Due to these results the pBGKBCp4 clones were checked to proof their correctness using restriction digest; but although restriction digest approved the correctness of the clones, the protein expression could not clearly be shown.

This might be due to the fact that there is no correct expression of Cp4 from start to end, and maybe expression was stopped within the insert, just before the myc-epitope.

For pBGKBVap1 protein expression no significant bands could be detected; therefore the plasmid was checked for correctness using restriction digest which showed quite different results. Restriction digest of pBGKBVap1 clones with the enzyme *PstI* should result in bands of 4429/170bp if the Vap1 gene sequence was cloned into the vector plasmid in the correct way. Cutting the wrong inserted Vap1 plasmid restriction digest with *PstI* would show bands of 4199/400bp. In this case restriction digest of pBGKBVap1 with *PstI* resulted in bands at around 250bp/350bp and was therefore neither correct nor incorrect inserted Vap1 gene sequences.

In addition to these results the pBGKBVap1 clones were digested with *BspHI*, an enzyme which also cut in the Vap1 inserted gene sequence and gave information about the correct insertion of the sequence into the vector plasmid. Restriction digest with *BspHI* resulted in bands of 2830/1769bp and showed correct results.

However, western blot analysis never showed any bands and protein expression was only detectable using the possum specific Vap1 serum, because the Vap1 fusion protein expression stopped before the myc-epitope.

5.4 Expression and lysis study

The pBGKBZP2C as well as the pBGKBZP2Copt plasmids were co-transformed with the plasmid pGLyisvb. This lysis plasmid has a gentamycin resistance cassette and lysis is regulated by the thermosensitive λ pL/pR-cl857 system.

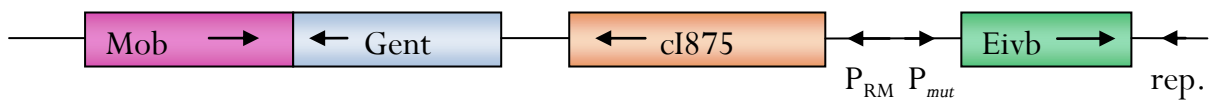


Fig. 5.2: Lysis plasmid pGLyisvb. *Mob*- mobilization sequence, *Gent*- gentamycin resistance cassette, *cl857*-repressor, *Eivb*- C-terminal fusion gene of protein E and *in vivo* biotinylation sequence.

As the proteins are under the control of the pBAD promoter, expression is tightly regulated and induced by the addition of L-arabinose at a final concentration of 0,2%. The expression of the desired proteins did not impair the growth of *E. coli* NM522. Temperature shifting from 35°C up to 42°C induced the expression of the protein E which resulted in the E-mediated transmembrane lysis tunnel structure. In this study the best clones of the plasmids were chosen to be used for fermentation.

5.5 Fermentation

Fermentation process was carried out in a 30L fermenter with the best clones of the plasmids pBGKBZP2C and pBGKBZP2Copt.

Clone 1 of the plasmid pBGKBZP2C carrying the lysis plasmid pGLyisvb was used for fermentation. After bacterial growth reached an OD₆₀₀ of 0,8 L-arabinose at a final concentration of 0,2% was added to start protein expression. After 110 minutes of bacterial growth temperature was shifted from 35°C up to 42°C to induce lysis which went on for another 90 minutes. Finally the surviving cells were killed for 60 minutes, using β -propiolactone.

Lysis of pBGKBZP2C clone1 fermentation resulted in a cfu-drop from 10^9 to 10^5 (per ml), whereas lysis efficiency of the fermentation was 99,97%.

Clone 2 of the plasmid pBGKBZP2Copt carrying the lysis plasmid pGLysvib was used for fermentation. After bacterial growth reached an OD_{600} of 0,8 L-arabinose at a final concentration of 0,2% was added to start protein expression. After 120 minutes of bacterial growth temperature was shifted from 35°C up to 42°C to induce lysis, which went on for another 60 minutes. Finally the surviving cells were killed for 60 minutes, using of β -propiolactone.

Lysis of pBGKBZP2Copt clone 2 fermentation resulted in a cfu-drop from 10^9 to 10^5 (per ml), whereas lysis efficiency of the fermentation was 99,97%.

5.6 Quantification

After harvesting of the bacterial ghosts containing ZP2C and ZP2Copt antigen, quantification of antigen protein within the bacterial ghosts was performed.

pBGKBZP2C as well as pBGKBZP2Copt bacterial ghosts were quantified using the Positope from Invitrogen [$5\mu\text{g}/\mu\text{l}$] as a standard.

In case that the pBGKBZP2Copt plasmid contains the brushtail possum zona pellucida 2 (ZP2) C-terminal sequence gene with a codon optimized (opt.) for the protein expression in *Escherichia coli*, a higher protein expression was expected. Due to the quantification results of pBGKBZP2C and pBGKBZP2Copt bacterial ghosts, a significant difference in the amount of protein expression could be obtained.

The ZP2C antigen expression in the pBGKBZP2C bacterial ghost resulted in an average of 14,6 ng ZP2C/ μg bacterial ghost.

In contrast the ZP2Copt antigen expression in the pBGKBZP2Copt bacterial ghost showed a much higher amount of expressed protein, resulting in an average of 403 ng ZP2Copt/ μg bacterial ghost.

5.7 Trouble shooting Cp4

As the protein expression of the pBGKBCp4 plasmid always showed bands at around 25 kDa when developed with the possum specific Cp4 serum and never showed any bands when developed with the anti-myc-HRP antibody, the Cp4 gene sequence was send for sequencing.

Sequencing results were quite disappointing, illustrating next to several point mutations also frame shifts which led to the formation of stop-codons, with the first stop in the middle of the Cp4 sequence. The Cp4 gene sequence part until the first frameshift stop had a molecular weight of 22,7 kDa, which had already been proofed in different western blot analysis developed with the specific possum serum against Cp4.

As a future step the Cp4 gene region should be obtained by sequencing, to avoid and exclude further mutations and to be sure to work with the correct, un-mutated Cp4 gene sequence.

5.8 Trouble shooting Vap1

Also pBGKBVap1 plasmid which never showed any bands at western blot analysis was send to be sequenced and gave an even more devastating result; where blast research of the confirmed and theoretical sequence showed an similarity of 44,1%. Too many mutations in the confirmed Vap1 gene sequence suggested that the plasmid pGEX2TVap1 from which the Vap1 gene sequence was taken from must had already been mutated.

As a future step the Vap1 gene sequence has to be cloned again and correctness of the obtained Vap1 element should be checked by sequencing.

For future projects it has to be found out whether possum responses to target proteins like pBGKBZP2C, pBGKBZP2Copt and furthermore their effects on fertility in vivo in the possum will have to be examined as well. Overall, concerning immunologically based fertility control in possums to be effective in long term, multiple fertility – inhibiting antigens or usage of different antigens are important, thereby maximizing the effectiveness of immunocontraception for possum biocontrol.

6 Materials and methods

6.1 Bacterial strains, media, cultivation

6.1.1 Bacterial Strain

- 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 C2988J (NEB 5-alpha competent E .coli) *fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44* Φ80Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17* (Source: New England Biolabs, Germany)

6.1.2 Cultivation medium for Escherichia coli

Cultivation is carried out in LB-Medium which consists of 10g Peptone, 5g Yeast extract and 5g NaCl, adjusted to a pH of 7,4.

6.1.3 Cultivation of Escherichia coli

E. coli are stored as glycerin stocks at -80°C in 25% glycerol.

For growth E. coli are incubated in 5ml LBv (eprouvettes). Depending on the carried plasmids the respective antibiotic and/or other supplements are added as well. Growth is performed at 36°C in a rotating incubator (Heraeus BK5060E).

6.1.4 Antibiotics

Name	Stock	Final conc.	µl/5ml
ampicillin	50mg/ml	100µg/ml	10
kanamycin	25mg/ml	50µg/ml	10
gentamycin	10mg/ml	20µg/ml	10

6.2 Plasmids

6.2.1 Backbone plasmids

Name	Length	Resistance	Origin	Reference	Features
pBADGIIIA	4145 bp	Amp	pBR322	Invitrogen	GIII-myc
pBADGIIIB	4147 bp	Amp	pBR322	Invitrogen	GIII-myc
pBADGIIIC	4149 bp	Amp	pBR322	Invitrogen	GIII-myc
pBGKA	4040 bp	Kan	pBR322	this work	GIII-myc
pBGKB	4042 bp	Kan	pBR322	this work	GIII-myc
pBGKC	4044 bp	Kan	pBR322	this work	GIII-myc

6.2.2 Lysis plasmid

Name	Length	Resistance	Origin	Reference	Features
pGLysivb	6201 bp	Gent	Rep	[53]	Eivb

6.2.3 Expression plasmids

Name	Length	Resistance	Origin	Reference	Features
pBGKBZP2C	5132 bp	Kan	pBR322	this work	GIII-ZP2C-myc
pBGKBZP2Copt	4994 bp	Kan	pBR322	this work	GIII-ZP2Copt-myc
pBGKBCp4	4937 bp	Kan	pBR322	this work	GIII-Cp4-myc
pBGKBVap1	4599 bp	Kan	pBR322	this work	GIII-Vap1-myc

Name	Length	Resistance	Origin	Reference	Features
pMalZP2C	7699 bp	Amp	pMB M13	[49]	MBP-ZP2C
pMalZP2Copt	7717 bp	Amp	pMB M13	[49]	MBP-ZP2Copt

Name	Length	Resistance	Origin	Reference	Features
pGEX2TCp4	5907 bp	Amp	pMB1	landcare research	GST-Cp4
pGEX2TVap1	5505 bp	Amp	pMB1	landcare research	GST-Vap1

6.3 Buffer and Solutions

50% Glycerol:

- 25ml 100% glycerol
- 25ml ddH₂O
- Mix and autoclave

0,85% Saline Medium:

- 8,5g NaCl
- 1l ddH₂O
- Filled into eprovettes 9 ml and 9,9 ml using a dispenser and autoclave

Mops I stock solution:

- 10,47g MOPS (100mM)
- 0,74g CaCl₂ x 2H₂O (10mM)
- 0,6g RbCl₂ (10mM)
- Dissolve in 400ml ddH₂O, pH adjusted to 7,0 with KOH , sterilized by autoclaving

Mops II stock solution:

- 10,47g MOPS (100mM)
- 5,15g CaCl₂ x 2H₂O (10mM)
- 0,6g RbCl₂ (10mM)
- Dissolve in 400ml ddH₂O, pH adjusted to 7,0 with KOH , sterilized by autoclaving

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

1%, 2% Agarose:

- 3g (or 6g) Agarose
- 300ml 1x TAE Buffer

Gelred:

GelRed™ Nucleic Acid Gel Stain 1000x in water (Biotium order No. 41003)

- 15µl GelRed stain
- 5ml 1M NaCl
- 45ml water

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 Methon
- 850ml deionized water

1x Blocking Solution:

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

1x TBS:

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

1xTBST:

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

PonceauS

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

Luminol Reagent:

- 1,5ml ECL-Reagent A (Santa Cruz Biotechnology)
- 1,5ml ECL-Reagent B (Santa Cruz Biotechnology)

6.4 Chemicals

Chemicals, solutions, buffer and media are purchased from Roth (ROTH Carl, Germany)

6.5 Enzymes

All enzymes are purchased from Fermentas Life Science and New England Biolabs; ligation, restriction digests as well as double restriction digests and dephosphorylation were performed according to the manufacturer's instructions.

6.6 Microbiological techniques

6.6.1 Preparation of CaCl₂/RbCl₂ competent cells

30ml of autoclaved LB-medium are inoculated with 1ml of a fresh overnight E. coli culture and are grown in a 36°C water bath under continuous shaking until an OD₆₀₀ of 0,5 is reached. The culture is then centrifuged for 10 minutes at 4°C and 4000rpm; the supernatant is decanted and the resulting pellet is resuspended in 6ml cooled MOPS I solution and is kept on ice for further 10 minutes. The solution is centrifuged for 10 minutes at 4°C and 4000rpm and the supernatant is decanted; pellet gets resuspended in 6ml cooled MOPS II solution and is kept on ice for further 30 minutes. Centrifugation step is performed for 10 minutes at 4°C and 4000 rpm and the pellet is resuspended in 480µl MOPS II and 180µl 50% glycerol. Additionally the solution is kept on ice for 10 minutes before it's aliquoted into 100µl portions and stored at -80°C.

6.6.2 Transformation of CaCl₂/RbCl₂ competent cells

CaCl₂/RbCl₂ competent cells (100µl aliquot) are thawed on ice and 2µl of DNA miniprep (Peqlab Miniprep Kit I or Kit II) or 10µl of ligation product are added to competent cells and kept on ice for 30 minutes. Heat shock is performed for 2 minutes depending on the DNA plasmid at 42°C or lysis plasmids at 36°C. After 5 minutes on ice, 700µl of autoclaved LB-medium is added and cells are regenerated for 1 hour at 36°C on the shaker. Finally 100µl and the remaining rest are stroke on agar plates with the corresponding antibiotics and incubated over night at 36°C.

6.6.3 Preparation of plasmid DNA (miniprep)

For plasmid DNA preparation in small volume the PeqLab Kit I (Erlangen, Germany) (over night culture 1-5ml) and Kit II (over night culture 3-15ml) are used according to the manufacturer's instructions. Plasmid DNA is further used for digestion or ligation or is stored at -20°C.

6.6.4 Preparation of plasmid DNA (midiprep)

For plasmid DNA preparation in medium volume (100ml) the Promega Midiprep Kit (Mannheim, Germany) is used according to the manufacturer's instructions. Plasmid DNA is further used for digestion or ligation or is stored at -20°C.

6.7 Enzymatic reactions

6.7.1 DNA restriction digest

All restriction digests are performed with Fermentas (St. Leon-Rot, Germany) Restriction enzymes under the manufacturer's instructions:

6.7.2 DNA ligation

All DNA ligations are performed with the T4 DNA ligase from New England Biolabs (Frankfurt am Main, Germany):

- 3µl DNA vector
- 5µl DNA insert
- 1µl T4 DNA Ligase Buffer (10x)
- 1µl T4 DNA Ligase
- 10µl Ligation Mix is incubated at 16°C over night

6.7.3 Isolation and purification of DNA fragments

Isolation and purification steps were performed with the GeneXpress Clean and Concentrator Kit (GmbH, Germany), GE-Healthcare Purification Kit (Vienna, Austria), and Zymo Gel DNA Recovery Kit (California, USA). The isolation and purification was done according to the manufacturer's instructions. The DNA fragment is further used for cloning steps or is stored at -20°C.

6.8 Amplification of DNA fragments using PCR

All PCR reactions are performed with the iCycler Thermal Cycler features from Bio-Rad according to the manufacturer's instructions using different PCR enzymes from Fermentas (St. Leon-Rot, Germany):

- 2x PCR Master Mix (Fermentas)
- Pfu DNA Polymerase (Fermentas)
- Dream Taq DNA Polymerase (Fermentas)
- High Fidelity DNA Polymerase (Fermentas)

6.8.1 Test-PCR small scale

PCR materials are thawed on ice; all further steps are carried out on ice under the lamina flow:

100µl master mix:

- 50µl 2x PCR Master Mix (Fermentas)
- 1µl Primer 1 (Fwd) 50pmol/µl
- 1µl Primer 2 (Rev) 50pmol/µl
- 40µl dH₂O

The master mix is divided into three labeled PCR eppis (each 23µl):

- Eppi 1: 23µl master mix + 2µl unknown DNA (sample)
- Eppi 2: 23µl master mix + 1µl unknown DNA + 1µl dH₂O (positive control)
- Eppi 3: 23µl master mix + 2µl dH₂O (negative control)

The PCR program includes following steps:

- Step1: Denaturation: 1 cycle
95°C 3min
- Step2: Denaturation, Annealing, Elongation: 25-30cycles
 - Step2a: 95°C 30sec
 - Step2b: 48°C-60°C 30sec (dependent on Primer T_m)
 - Step2c: 72°C 1min/1000bp product
- Step3: Final Elongation: 1cycle
72°C 10min

6.8.2 PCR large scale

PCR materials are thawed on ice; all further steps are carried out on ice under the lamina flow:

300µl master mix:

- 30µl dNTPs Mix (2mM)
- 30µl 10x Buffer + MgSO₄
- 3µl Primer 1 (Fwd) 50pmol/µl
- 3µl Primer 2 (Rev) 50pmol/µl
- 3µl Polymerase (Pfu; High Fidelity; Dream-Taq)
- 207µl dH₂O

For control: the master mix is divided into three labeled PCR eppis (each 23µl):

- Eppi 1: 23µl master mix + 2µl unknown DNA (sample)
- Eppi 2: 23µl master mix + 1µl unknown DNA + 1µl dH₂O (positive control)
- Eppi 3: 23µl master mix + 2µl dH₂O (negative control)

For production: the master mix is divided into four labeled PCR eppis (each 46µl):

- Eppi 4-7: 46µl master mix + 4µl unknown DNA (sample)

The PCR program includes following steps:

- Step1: Denaturation: 1 cycle
95°C 3min
- Step2: Denaturation, Annealing, Elongation: 25-30cycles
 - Step2a: 95°C 30sec
 - Step2b: 48°C-60°C 30sec (dependent on Primer T_m)
 - Step2c: 72°C 1min/1000bp product
- Step3: Final Elongation: 1cycle
72°C 10min

6.9 Primers used for PCR

6.9.1 Cloning pBGKA, B, C

Vector pBHR1 as PCR template

PCR fragment: 896bp

Fwd Primer: [KanFWD(NcoI)]

5' pBGKA, B, C: TTA **CCATGG** TGT TAC ATT GCA CAA GAT AA 3'

T_m= 59°C

Rev Primer: [KanREV(NcoI)]

5' pBGKBA, B, C: ATT **CCATGG** TTA GAA AAA CTC ATC GAG CAT 3'

T_m=62°C

6.9.2 Cloning pBGKBZP2C

Vector pMalZP2C as PCR template

PCR fragment: 1001 nt

Fwd Primer: [KpnI]

5'pBGKBZP2C: ACA **GGTACC** AAT GGC TCA AGA CTA CAT 3'

T_m=50°C

Rev. Primer: [XbaI]

5'pBGKBZP2C: TAA **TCTAGA** TTA GAT GAC CCA GGA CAA 3'

T_m=52°C

6.9.3 Cloning pBGKBZP2Copt

Vector pMalZP2Copt as PCR template

PCR Fragment: 983 nt

Fwd Primer: [BglIII]

5'pBGKBZP2Copt: ACA **AGATCT** AAT GGC AGC CGT CTG C 3'

Tm=52°C

Rev. Primer: [XbaI]

5'pBGKBZP2Copt: TAA **TCTAGA** CTG CTA CCC GGG CAG 3'

Tm=56°C

6.9.4 Cloning pBGKBcp4

Vector pGEX2Tcp4 as PCR template

PCR fragment: 953nt

Fwd Primer: [BglIII]

5'pBGKBcp4: ACA **AGATCT** GAA TTC GAT AGG TAT GCT 3'

Tm=50°C

Rev. Primer: [XbaI]

5'pBGKBcp4: TAA **TCTAGA** TTC TGA ATA CTT TTA TTC TGC 3'

Tm=54°C

6.9.5 Cloning pBGKBVap1

Vector pGEX2Tvap1 as PCR template

PCR fragment: 551nt

Fwd Primer: [BglII]

5'pBGKBvap1: ACA **AGATCT** TCC ACA GAG CAA GTT CGA 3'

T_m=54°C

Rev. Primer: [XbaI]

5'pBGKBcp4: TAA **TCTAGA** TTT TTC CTC CTC CTG CCA 3'

T_m=54°C

6.10 Analytical techniques

6.10.1 Electrophoresis

For separation of DNA fragments gel electrophoresis is performed; according to the DNA length 1% (length 10³-10⁴bp) or 2% (10²-10³bp) agarose gels are used:

3g (1% gel) or 6g (2% gel) of agarose are weighted in and dissolved in 300ml 1x TAE. The solution is boiled for several minutes in the microwave until the solution is clear. After cooling down the gel is poured into appropriate gel-tray and the polymerized gel is stored in 1x TAE solution for further use.

For DNA separation 10µl DNA solution is mixed with 2µl of 6x loading dye (Fermentas) and is loaded on the agarose gel. For length control also 5µl of DNA marker (Fermentas, 1kb loading dye) are loaded.

For gel run 160V for 30 minutes are used until the loading dye reaches the end of the gel.

Gel staining is performed in 1x GelRed solution.

6.10.2 Western Blot Analysis

For the separation of proteins the NuPAGE® Bis-Tris Electrophoresis System from Invitrogen is used according to the manufacturer's instructions.

Protein dissolved in 1x NuPage® Sample Buffer and denaturated at 99°C for 10 minutes is centrifuged for three minutes, maximum speed. 15µl of your sample and 5µl of appropriate protein marker are loaded into sample wells and the run is performed at 180V for 60 minutes.

6.10.2.1 Transfer

A semi-dry blotting sandwich is prepared according to the manufacturer's instructions for usage in an xCell II Blot Module. The protein transfer to a nitrocellulose membrane and takes place at 60V for 30 minutes (one gel) or 60 minutes (two gels).

6.10.2.2 Ponceau S staining

After protein transfer the nitrocellulose membrane is stained with Ponceau S to identify the marker bands. Ponceau S is washed off with deionized water and membrane is blocked with 1x Blocking Solution (1x Roti-Block™, Roth) over night at 4°C.

6.10.2.3 Antibody Incubation & development

After blocking the membrane is washed 3 x 5 minutes, 1 x 10 minutes with TBST and incubated with the appropriate first antibody for one hour. After antibody incubation the membrane is washed 3 x 5 minutes and 1 x 10 minutes with TBS or TBST. If necessary incubate the membrane in the same way with the second and third antibody for an hour. The last washing step is performed with TBS 3 x 5 min and 1 x 10 min. For development the membrane is incubated with 3ml ECL-chemiluminescent reagent from Santa Cruz Biotechnology the membrane according to manufacturer's instructions. Documentation of the membrane is done in the BioRad ChemiDoc machine using the Quantity One software.

6.10.2.4 Preparation of Western blot samples

- *From bacterial culture:*

1ml of bacterial culture is taken at appropriate time points and centrifuged for 3 minutes at 10.000rpm. Supernatant is removed and the pellet is stored at -20°C for further use. Pellets are resuspended according to the OD₆₀₀ in NuPage Sample buffer following these calculations:

Before lysis: OD₆₀₀ value x 250 = volume of 1x NuPage Sample buffer.

After lysis: OD600 (highest value before lysis) x 250 = volume of 1x NuPage Sample buffer.

The mixture is incubated for 10min at 99°C and additionally stored at -20°C or used for western blot analysis.

- *From lyophilized bacterial ghosts:*

10mg of lyophilized bacterial ghosts are resuspended in 1ml ddH₂O. 200µl of resuspended ghosts are mixed with 200µl NuPage Sample Buffer and heated for 10 minutes at 99°C. The sample is centrifuged and 100µl supernatant sample are taken diluted with 400µl NuPage Sample buffer (1:5 dilution). 2µl, 5µl, 10µl and 20µl of the 1:5 diluted sample (filled up with NuPage Sample Buffer to a total volume of 20µl) are loaded for quantification.

- *For protein quantification:*

For protein quantification the Positope from Invitrogen is used. The Positope [5µg/µl] is already diluted in Sample Buffer and is heated for 5 minutes at 99°C. 40µl of Positope are diluted with 40µl of NuPage Sample Buffer (0,5µg/20µl). A serial dilution is made (1:2) by transferring 40µl of each standard in 40µl NuPage Sample Buffer.

- Std.5: 0,5µg/20µl 500ng
- Std.4: 0,25µg/20µl 250ng
- Std.3: 0,125µg/20µl 125ng
- Std.2: 0,0625µg/20µl 62,5ng
- Std.1: 0,031,25µg/20µl 31,25ng

20µl of each standard are loaded on the same gel with dilutions of the unknown sample.

6.11 List of used antibodies

Direct detection antibodies	Species type	Uses	Dilution	Recognizes	Source
Anti-myc-HRP	mouse monoclonal IgG antibody	WB	1 to 5.000 in TBS	recombinant proteins containing the c-myc epitope	Invitrogen
Anti-His(C-term)-HRP	mouse monoclonal IgG antibody	WB	1 to 5.000 in TBS	polyhistidine amino acid sequence at the C-terminus of a protein	Invitrogen

Serum detection antibodies	Species type	Uses	Dilution	Recognizes	Source
B11 ZP2C Serum	Possum	WB	1 to 1.000 in TBS	recombinant ZP2C protein	LCR
Cp4 serum tv24 female	Possum	WB	1 to 500 in TBS, BSA, NaN3	recombinant Cp4 protein	LCR
Vap1 serum tv17 female	Possum	WB	1 to 500 in TBS, BSA, NaN3	recombinant Vap1 protein	LCR

Secondary antibodies	Species type	Uses	Dilution	Recognizes	Source
Rabbit-anti-Possum HRP	Rabbit	WB	1 to 1.000 in TBS	Possum IgG	LCR
Anti-rabbit HRP	Goat	WB	1 to 20.000 in TBS	Rabbit IgG	LCR

6.12 Growth, expression and lysis of E. coli NM522

6.12.1 Growth and lysis in small scale

E. coli NM522 carrying a lysis plasmid is inoculated in 5ml LBv with the appropriate antibiotics and are grown over night at 36°C.

Growth and lysis are performed in 100ml nose-flasks containing 25ml autoclaved LB-medium and a magnet stirrer. Appropriate antibiotics are adjusted to LBv-medium with antibiotics in nose-flasks and inoculation with overnight culture is done, to reach an OD₆₀₀ of 0,1. The nose-flasks are stirred at 330rpm in a 36°C water bath and OD₆₀₀ is measured until an OD₆₀₀ of 0,5 is reached. The OD is measured by the Spectronic 20 Milton Roy spectrophotometer.

When OD₆₀₀ has reached 0,5 the nose flasks are shifted to a water bath of 42°C and lysis is followed for 120 minutes.

6.12.1.1 Microscopy

Lysis of bacteria is checked additionally by microscopy: Lysed bacteria differ from normal bacteria by their weak contrast; they appear not as dark as intact bacteria.

6.12.1.2 Cfu determination

For exact data analysis of growth and lysis viable cell counts (cfu) are performed. Therefore samples from nose flasks are diluted in 0,85% saline (NaCl). For 1:100 dilutions 9,9ml saline eprouvettes are used 0,1ml bacterial sample. For 1:10 dilutions 9,0ml saline eprouvettes are used with 1ml bacterial sample. In a logarithmic manner 50µl and 100µl of the final dilution are plated on count agar plates using a spiral plater. After incubation at 36°C over night the colonies are counted in the Synbiosis ProtoCOL Colony Counter machine (3.15, Synoptics Ltd., Cambridge, UK) using the colony counter program. Finally the cfu values are illustrated as a logarithmic curve, together with the OD₆₀₀ values in dependency of time.

Following dilutions according to the OD₆₀₀ of growing and lysing bacterial cultures are used as a reference:

Before lysis/growth		After lysis induction	
OD ₆₀₀ (pure)	Dilution	OD ₆₀₀ (pure)	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

6.12.2 Growth and expression study in small scale

E. coli NM522 containing an expression plasmid are inoculated in 5ml LBv-medium with appropriate antibiotics and are grown over night at 36°C.

100ml nose flasks containing 25ml autoclaved LBv with appropriate antibiotics and a magnet stirrer are inoculated with the overnight culture to reach an OD₆₀₀ of 0,1. The nose flasks are stirred at 330rpm in a 36°C water bath. Optical density is measured until an OD₆₀₀ of 3-3,5 is reached. For protein expression L-arabinose is added to an end concentration of 0,2% (time point 0 of protein expression). Expression is performed for further 60 minutes at 36°C. Samples are taken every 30 minutes according to the following scheme:

time [min]	OD ₆₀₀	Cfu	microscope	western blot samples
-30	0,25-0,3	X	X	X
0	0,4-0,5	X		X
10		X		X
20		X		X
40		X	X	X
60		X		X

6.12.3 Expression and lysis study in small scale

E. coli NM522 containing an expression plasmid as well as a lysis plasmid are inoculated in 5ml LBv-medium with appropriate antibiotics and are grown over night at 36°C.

100ml nose flasks containing 25ml autoclaved LBv with the appropriate antibiotics and a magnetic stirrer are inoculated with the overnight culture to reach an optical density of 0,25-0,3. The nose flasks are stirred at 330rpm in a 36°C water bath. Optical density is measured until an OD₆₀₀ of 0,25-0,3 is reached. Expression of the recombinant protein is achieved by adding L-arabinose to an end concentration of 0,2%. Expression is performed for 60 minutes at 36°C. Afterwards the temperature is shifted up to 42°C to induce lysis. Samples are taken according to the following scheme:

time [min]	sample	OD ₆₀₀	cfu	microscope	western blot samples
-60	A	X	X	X	X
-30	B	X	X		X
0	C	X	X	X	X
20	D	X	X		
40	E	X	X		
60	F	X	X		X
90	G	X	X	X	
120	H	X	X		X

6.12.4 Glycerine stocks of bacteria

To each labeled glycerin tube 900µl of 50% glycerin and 900µl of bacterial culture are added; the storage glycerin stocks are all prepared in duplets, are mixed by shaking and stored at -70°C.

6.12.5 Consistency study

For consistency study *E. coli* carrying the lysis plasmid is inoculated in 5ml LBv with the appropriate antibiotic and are grown over night at 36°C. In general nine different clones are tested and the study is performed with 3 clones at the same time. 100ml nose flasks containing 25ml autoclaved LBv with antibiotics and a magnet stirrer are inoculated with overnight culture to reach an optical density of 0,1. The nose flasks are incubated at 36°C in water baths under stirring at 330rpm.

After an OD₆₀₀ of 0,5 is reached the nose flasks are shifted to a water bath with 42°C to induce lysis and samples are taken according to the following scheme:

time [min]	OD ₆₀₀ (expected)	OD ₆₀₀	cfu	microscope
-120		X		
-60		X		
-30	0,25-0,3	X	X	X
0	0,4-0,5	X	X	
10		X	X	
20		X	X	
40		X	X	
60		X	X	X
90		X	X	
120		X	X	

6.13 Fermentation

The production of bacterial ghosts is performed in a 30L fermenter (TECHFORS S1820 Infors AG, Switzerland). For fermentation a total volume of 22 liters LBv is used.

6.13.1 Media preparation and previous settings

For fermentation 22 liters of LBv are autoclaved (30min, 121°C) 18 hours before fermentation starts. Within this time the IRIS program observes temperature, pH, oxygen and flow in the fermenter. Furthermore sterility of the medium is checked by taking blank samples and plating them on plate count agar.

6.13.2 Overnight culture

Two glycerol working stocks stored at -80°C are thawed and 800µl are inoculated into 4 x 500ml autoclaved LB each in 2L flasks. The 500ml flasks are incubated over night in a water bath at 34°C.

6.13.3 Fermentation process

The fermenter is inoculated with overnight culture to an OD₆₀₀ of about 2. During fermentation parameters like temperature, oxygen flow, pH and stirring are observed by the IRIS program. 50ml samples are taken via the sterilized sample valve.

6.13.4 Killing

After growth and lysis, none lysed bacteria are killed by using 0,075% β -propiolactone added as two equal doses of 0.0375% 30min apart.

6.13.5 Harvesting

Bacterial ghosts are harvested by separation (Westfalia Seperator, type CTC1-06-107).

6.13.6 Washing and lyophilisation

The collected bacterial ghosts are washed 3 times with 2400ml sterile ddH₂O and centrifuged at 4°C (Hermle, ZK401) for 15 minutes at 8000 rpm. After the washing steps the bacterial ghosts are resuspended in 200ml sterile ddH₂O separated into 11 lyophilisation bottles and kept at -20°C, bottles are transported to -80°C and lyophilisation is performed in a Lyolab B (LSL Secfroid) machine for three days.

6.13.7 Calculation of lysis efficiency and particles per mg

For lysis efficiency the highest and the lowest cfu value are compared, resulting in the following equation:

$$Efficacy [\%] = \left(1 - \frac{cfu_{lowest} [ml^{-1}]}{cfu_{highest} [ml^{-1}]} \right) \times 100\%$$

The particles per mg are calculated with the value of the highest CFU which is multiplied by the ml amount of the harvested bacterial ghost culture and divided by the lyophilized weight of the entire harvested material resulting in an equation as follows:

$$Particles/mg [mg^{-1}] = \frac{cfu_{highest} [ml^{-1}] \times volume_{total} [ml]}{weight_{lyophilized} [mg]}$$

6.13.8 Sterility testing of the material

10mg of lyophilisate are resuspended in 1,5ml LBv, 1ml resuspension are used for pour plating by adding 20ml hand warm LB agar to the sample in an empty agar plate. In addition 100µl and 200µl are plated and all plates are incubated over night at 36°C. Additionally 100µl are inoculated in 5ml LBv and incubated over night at 36°C. On the next day 100µl and 200µl of this enrichment are plated and incubated over night at 36°C.

6.14 Sequencing

Sequence analysis was done at Microsynth (Balgach, Switzerland) using the A1 premium run service. Therefore a midiprep of the desired plasmid was performed and 200µl of the first midiprep elution was sent to be sequenced. A DNA concentration of minimum 100ng/µl was necessary.

6.14.1 Blast results

The obtained sequences from the A1 premium run service (confirmed sequence) were aligned and blasted against the theoretical sequence (expected by DNASTar program using Genbank information) using the EBLOSUM62 blast matrix.

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