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Avian Influenza Minicircle DNA-Vaccine Candidate

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Abbreviations

'	minutes	LBv	vegetable LB medium
"	seconds		(animal product free)
µg	micrograms	LPS	lipopolysaccharide
µm	micrometers	M.....	marker
AB	antibiotic	mg	milligrams
Amp	Ampicillin	MHC	major histocompatibility complex
APC	antigen presenting cell	min	minutes
BG(s)	Bacterial Ghost(s)	miniprep.....	plasmid preparation
bp.....	basepairs	nm	nanometer
BPL	beta-propiolactone	OD _{xxx}	optical density at indicated wavelength
cfu.....	colony forming unit	ori.....	origin of replication
dH ₂ O.....	deionized water	PamP	pathogen associated molecular pattern
<i>E.coli</i>	escherichia coli	parA	partitioning Resolvase
E-protein.....	Lysis Protein E from ΦX174		(Kreiss, Cameron et al. 1998)
	(Blasi, Young et al. 1988; Witte, Blasi et al. 1990)	arabinose inducible Promoter	
Gent	Gentamycin		(Bigger, Tolmachov et al. 2001)
h.....	hours	PBS	phosphate buffered saline
IS	immense system	PCR.....	polymerase chainreaction
Kan	Kanamycin	s.....	seconds
kb.....	kilobasepairs	Strep.....	Streptomycin
kDa	kilo Dalton	Tet.....	Tetracycline
l.....	liter	tp	timepoint
LacI-L'anchor.	L' anchor membrane anchored LacI	WB.....	Western blot
	repressor (Mayrhofer, Tabrizi et al. 2005)		

Summary

Objective

Goal of this work was the production of Bacterial Ghosts (BGs) loaded with pSIP-H5 minicircle, a small DNA molecule harboring a Eukaryotic Expression Cassette for the protein Hemagglutinin type 5 of the H5N1 avian influenza virus. This composes a DNA vaccine for poultry enclosed in BGs, which act as biological carrier vehicles, directly targeting the cellular as well as activating the adaptive immune system.

In combination with the Bacterial Ghost (BG) technology, the SIP (self-immobilizing-plasmid)-technique provides an easy, cheap and safe way of mass production of the vaccine.

The characteristic of the SIP-technique is the division of the original (SIP-) motherplasmid into two sub-parts, the minicircle, including, next to the H5 Expression Cassette, the ability to fix itself within the bacterial membrane and the miniplasmid, containing the undesirable sequences, which is lost with the cytoplasm upon E-lysis of the bacterial cells.

The aim was the design of a SIP plasmid containing the H5 gene, the pSIP-H5, which recombines upon arabinose induction, losing all sequences necessary for fermentation and possibly harmful when introduced into a target organism, like the antibiotic resistance cassette or the origin of replication.

pSIP-H5 would then be co-transformed with pGLysivb into *E.coli*, which induces E-lysis of the bacterial cell upon temperature shift. In one fermentation procedure recombination of the pSIP-H5 is induced, after that E-lysis is performed, H5-minicircle loaded BGs are obtained.

Next to cloning this plasmid the objective is to decide on the plasmid-carrying bacterial strain. The important properties of the strain are efficient lysis upon temperature shift and prompt and complete recombination as well as tight suppression of untimely recombination. Another beneficial property is the tropism of the bacterial strain to the vaccine recipient, such as *salmonella*, as a natural pathogen of poultry.

Results

In the presented diploma thesis the Influenza Gene H5 was cloned into the Self Immobilizing SIP Vector and the amount of bound DNA-minicircle in *E.coli* BGs was measured. pSIP-H5 minicircle-DNA binds with help of its Lac-Operator region to the membrane-anchored Lac-Repressor and thus is retained within the cell envelope and not expelled into the medium after E-Lysis of the bacteria.

The results of the sequencing of the amplified H5 Sequence of the plasmid pEPH5 showed 62 additional basepairs, which are located in the non-coding region of the fragment. No influence of that

sequence on recombination or fixation of the minicircle, carrying the into the SIP vector cloned H5 gene, was observed.

Transformation of different *E.coli* strains with pSIP-H5 showed problems with premature recombination. Within the *E.coli* strains DH10 β , ER2655 and TOP10 uninduced recombination of pSIP-H5 into miniplasmid and minicircle was confirmed.

In the *E.coli* strain TB1 without induction of expression of the parA recombinase no premature recombination was observed, however, recombination upon induction was poor and inefficient. These are the reasons why the *E.coli* strain TB1 was used for amplification of pSIP-H5. Within the *Salmonella* strain ATCC14028, which was also used for parA recombination experiments, premature recombination was shown as well. Based on these data the *E.coli* strains DH10 β , ER2655, TOP10 and TB1 as well as the *Salmonella* strain ATCC14028 were abandoned from further investigations.

Recombination of pSIP-H5 and Lysis of bacteria, the production of BGs was carried out with the *E.coli* strain NM522. Successful recombination and expression of the LacI-L'anchor was documented by plasmid preparation and Western Blot.

The H5-minicircle was detected by realtime PCR, which was applied on washed and lyophilized BG samples, amplifying the LacOs sequence, located on the minicircle.

With this technique up to 12 thousand H5-minicircles per BG (saline washed) and more than 2000 H5-minicircles per BG (washed with dH₂O) were detected.

Plasmid preparation of washed, lyophilized BGs showed a minicircle- as well as a miniplasmid band. Despite additional and adapted washing with saline later plasmid preparation still showed minicircle and miniplasmid band.

Zusammenfassung

Zielsetzung

Ziel dieser Arbeit war die Produktion von Bacterial Ghosts (BGs) beladen mit pSIP-H5 minicircle, ein kleines DNA Molekül, das eine eukaryotische Expressionkassette des Haemagglutinin Proteins Typ 5 des aviären H5N1 Influenza Virus trägt. Diese Komposition resultiert in einem DNA Impfstoff für Geflügel eingeschlossen in BGs, die als natürliches Trägermedium fungieren, direkt vom zellulären Immunsystem erkannt werden und das adaptive Immunsystem aktivieren.

In Kombination mit der Bacterial Ghost (BG) Technologie ist das SIP-system eine einfache, günstige und sichere Methode diesen Impfstoff in großen Mengen herzustellen.

Die essentielle Eigenschaft des SIP-Systems ist die Teilung des originären (SIP-) ‚Mutterplasmides‘ in zwei Untereinheiten, den Minicircle, der die H5 Expressionskassette enthält und die Fähigkeit sich an der Zellmembran zu fixieren hat, und das Miniplasmid, das alle unerwünschten Sequenzen trägt und bei der Lyse der bakteriellen Zellen mit dem Cytoplasma ausgeschwemmt wird.

Das Ziel war das Klonieren eines SIP-Vektors, pSIP-H5, der das H5 Gen enthält. Dieses Plasmid rekombiniert durch Arabinose-Induktion und verliert dadurch alle für die Fermentation notwendigen und für einen Endorganismus potentiell gefährlichen Sequenzen, wie antibiotische Resistenzkassetten oder den Origin of Replication.

pSIP-H5 sollte mit dem Lyseplasmid pGLysivb in verschiedene *E.coli* Stämme co-transformiert werden, das nach Temperaturshift die E-Lyse der Bakterienzelle induziert. In einem Fermentationsschritt rekombiniert das pSIP-H5, danach wird die E-Lyse induziert; man erhält mit H5-Minicircle beladene BGs.

Die zweite Aufgabe der Arbeit ist es, einen geeigneten Bakterienstamm für das pSIP-H5 zu finden. Die wichtigen Eigenschaften des Stammes sind effiziente Lyse nach Temperaturshift und schnelle vollständige Rekombination des pSIP-H5 nach Arabinose Induktion sowie strikte Unterdrückung von vorzeitiger Rekombination. Eine weitere günstige Eigenschaft des Stammes besteht in einem BG-Wirt Tropismus, wie es bei *Salmonellen* als natürliches Pathogen von Geflügel der Fall ist.

Resultate

In der vorliegenden Diplomarbeit wurde das Influenza Gen H5 in den Selbst-Immobilisierungs-Vektor SIP kloniert und die Menge der gebundenen Minicircle-DNA in *E.coli* BGs bestimmt. pSIP-H5 Minicircle-DNA bindet mit Hilfe seiner Lac-Operator Region an den Membran-verankerten Lac Repressor und wird dadurch in bei der E-Lyse der Bakterien nicht in das Medium ausgeschieden sondern im Zellenvelope gebunden.

Die Ergebnisse der Sequenzierung des amplifizierten H5 Stücks des Plasmids pEPH5 zeigten 62 zusätzliche Basenpaare, die im nicht-kodierenden Bereich des Fragments liegen. Ein Einfluss auf die Rekombination oder die Fixierung des Minicircles, welcher den in den SIP Vektor klonierten H5 Gens enthält, durch diese Sequenz wurde auf jeden Fall nicht festgestellt.

Die Transformation verschiedener *E.coli* Stämme mit pSIP-H5 zeigte Probleme mit frühzeitiger Rekombination auf. In den *E.coli* Stämmen DH10 β , ER2655 und TOP10 zeigte sich uninduzierte Rekombination von pSIP-H5 in die Miniplasmid- und Minicircleform. Im *E.coli* Stamm TB1 zeigte sich ohne Induktion der Expression der parA Rekombinase keine vorzeitige Rekombination, allerdings war die Rekombination nach Induktion nicht effizient. Aus diesen Gründen wurde der *E.coli* Stamm TB1 für die Amplifikation von pSIP-H5 verwendet. Im *Salmonella* Stamm ATCC14028, der für die parA Rekombinationsversuche herangezogen wurde, zeigte sich ebenfalls vorzeitige Rekombination. Aus diesem Grund wurden die *E.coli* Stämme DH10 β , ER2655, TOP10 und TB1 wie auch der *Salmonella* Stamm ATCC14028 von weiteren Untersuchungen ausgeschlossen.

Rekombination von pSIP-H5 und Lyse der Bakterien, die Produktion der BGs wurde mit dem *E.coli* Stamm NM522 durchgeführt. Die erfolgreiche Rekombination und die Expression des LacI-L'Anker Fusionsproteins wurde mit Plasmid Präparation und Western Blot überprüft.

Der H5-Minicircle wurde mit Realtime-PCR, die die LacOs Sequenz amplifizieren, welche auf dem Minicircle liegt, in Proben von gewaschenen und gefriergetrockneten BGs nachgewiesen.

Durch die Realtime-PCR wurden bis zu 12 Tausend H5-minicircles pro BG (mit Saline gewaschen) und mehr als 2000 H5-Minicircles pro BG (mit dH₂O gewaschen) nachgewiesen.

Plasmid Präparation von gewaschenen, gefriergetrockneten BGs zeigte Minicircle- wie auch Miniplasmid Banden. Trotz zusätzlichem und adaptiertem Waschen mit Saline zeigte sich in der Plasmid Präparation eine Minicircle- und eine Miniplasmid-Bande in den BGs.

Introduction

Avian Influenza type H5N1

Since 2003 when the avian influenza type A/H5N1 circulating in Southeast-Asia since 1996, became enzootic, the risk of a genetic reassortment of the highly pathogenic and contagious strain poses a threat for public health. Reported cases of felines, cats and even dogs further illustrates the unusual cross species transmission potential of the virus (Thiry, Zicola et al. 2007). It has been estimated that hundreds of millions of birds have been culled so far in an attempt to control the spreading disease. An influenza outbreak in the Netherlands in 2003 caused the culling of 30 million birds (Stegeman, Bouma et al. 2004), mounting to about half of the nations stock and pushing the EU to permitting the vaccination of poultry in this country. Vaccinating poultry not only protects birds from avian influenza, but also is an important step to control and minimize the public health risk posed by genetic reassortment of the virus.

The goal of this thesis was to create a DNA vaccine against Avian Influenza Type H5N1 by inducing immunity against the Vietnam H5-gene. The H5 gene was incorporated in a eukaryotic expression cassette, which was embedded in the plasmid pSIP-H5.

A large part of the immune response against influenza is directed against the protein HA, which is also responsible for the tissue-direction and severity of the infection (Kobasa, Takada et al. 2004; Tumpey, Garcia-Sastre et al. 2004). Another DNA vaccine using the Vietnam H5 antigen showed protective immunity against lethal H5N1 challenge in mice, 2007 (Sharpe, Lynch et al. 2007).

Bacterial Ghosts as carriers of a minicircle DNA vaccine candidate for avian influenza

Avian Influenza type H5N1 continues to be a threat for European poultry economy, sustaining the need for a vaccine. Several requirements are demanded of a possible vaccine candidate, like economical usefulness, high immunogenicity or the fulfillment of food-control standards. Important properties are low price, easy mass-production and -administration as well as efficiency and safety. In contrast to conventional vaccines, DNA vaccines can be precisely tailored, cheap and easily produced by fermentation of the bacteria carrying the DNA and pose no risk of reversion.

The specialty of the SIP-technique (self-immobilizing-plasmid) adds to the safety demand by avoiding any possibly harmful DNA sequences. Antibiotic resistance cassettes or origins of replication, which are necessary for transformation and fermentation of a plasmid, might propagate antibiotic resistance or facilitate horizontal gene transfer and therefore pose a potential risk in DNA vaccines; these sequences are lost during the SIP process.

This way of vaccine production combined with the Bacterial Ghost platform technology results in non living carrier vehicles loaded with the vaccine. The BG-DNA complex shows high structural stability even at room temperature as well as efficient immunologic availability after administration.

DNA vaccination

The first Steps in DNA Vaccine Development

The research on DNA based vaccines started its boom in the early 1990s with the report on gene expression in mouse muscle cells after naked DNA injection (Wolff, Malone et al. 1990). Little were these findings anticipated, since the use of naked DNA for injection was intended to be a control for experiments designed to assess the ability of cationic lipids to mediate expression in vivo. This discovery opened a whole new field of approaches to immunization, immunotherapy and vaccine design and production. DNA vaccines are widely investigated to address pathogen and autoimmune derived diseases as well as contribute to cancer and allergy treatment. Advanced methods and discoveries in immunology, microbiology and molecular biology facilitate engineering of specifically tailored gene vaccines in order to direct immune response (humoral, cell-based or both). In this way the immunogenicity can either be precisely limited to a desired protein or also target a larger variety of antigens; immunogenic molecular factors and pathways can be activated or influenced very selectively.

The Difference of DNA Vaccines to Conventional Vaccines

Conventional vaccines, which consist of inactivated pathogens or subunits thereof, primarily elicit humoral immune response. A certain antibody titer induced by immunization can be very important for the efficacy of later immunogenic response to that antigen. Antibodies are responsible for inactivation and opsonization of pathogens circulating in the blood, in the intestines as well as in other tissues like the mucosa or the cornea. This is done in order to either actually block and inactivate the pathogen directly or to draw the attention of the complement system or of cells of the immune system to take counter-measures. However, many infectious bacteria and any kind of virus propagate within

cells and are thus shielded from antibody attack. Proteins expressed by the cell are randomly cut to small peptides of about 10-20 amino acids by proteases and displayed at the cell surface connected to the MHC-complex (fig 1.1). This complex is recognized by the cytolytic T-cell, which eventually will induce apoptosis of the displaying cell and recruit other immune cells, if the peptide shown in the complex is foreign or abnormal as in cancer cells. This is a very important part of the immune system covering threats from internalized pathogens or cancer, killing infected or cancerous cells and with that hinder pathogen or tumor proliferation. The integral mechanism of DNA vaccines is the intracellular transcription and translation of introduced genetic information within the host cell and is therefore especially suitable to establish T-cell based immune response (Fu, Guan et al. 1998; Ulmer, Fu et al. 1998).

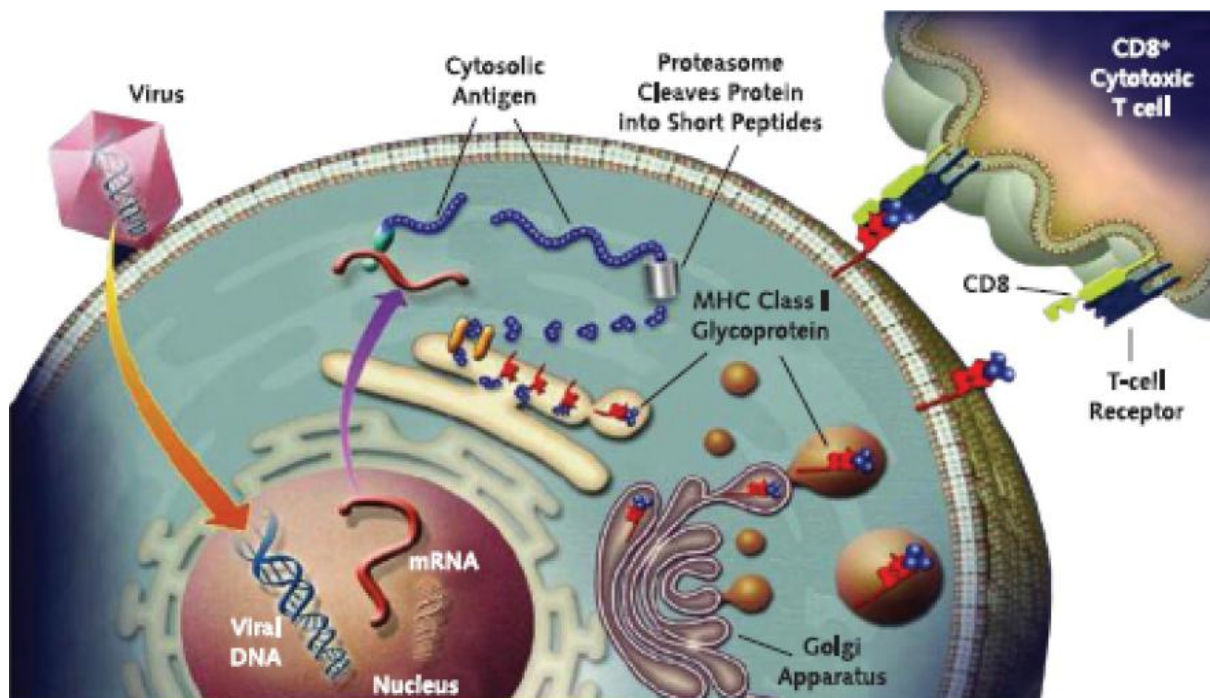


Fig 1.1: **Antigen Presentation by MHC-I.** After infection, expressed viral proteins are cleaved into oligopeptides by proteasomes and taken up by the ER, where MHC-I molecules are newly synthesized. The antigen peptide is taken up by the MHC-I molecule and via the Golgi-apparatus transported to the cell membrane and presented to the outside. This MHC-I bound antigen complex is recognized by the corresponding T-cell receptor in combination with the CD8 molecule present on CD8⁺ cytotoxic T-cells. The combined binding of the T-cell receptor and the CD8 molecule results in activation of the T-cell. In immediate consequence NF κ B, INF- γ and Interleukins are expressed, followed by Granulocyte/Macrophage-Colony Stimulating Factor. (Ulmer, Wahren et al. 2006)

Plasmid DNA vaccines induce immunogenicity in several different ways (fig 1.2). Innate immunity is activated through non methylated CpG motifs via the TLR9, which leads to activation of the adaptive immune system via inflammatory and immune response genes and is discussed below in detail (Klinman, Yamshchikov et al. 1997; Krieg, Yi et al. 1998; McCluskie, Weeratna et al. 2000; Bauer, Kirschning et al. 2001; Kovarik, Bozzotti et al. 2001). After DNA uptake the encoded antigen is

produced in situ by host-cells, which leads to the same modifications, folding and posttranslational processing of the antigen compared to the normal infection, so, very likely the antigen shows similar properties (Babiuk, Babiuk et al. 2000) for eliciting a corresponding immune response. Although it is not fully understood how DNA-uptake, transfer to nucleus and transcription mechanism come about, it has abundantly been shown, that DNA expression upon DNA introduction occurs plentifully (Wolff, Malone et al. 1990; Ulmer, Donnelly et al. 1993; Montgomery, Donnelly et al. 1994; Davis 1997; Ulmer, Deck et al. 1997). B-cell response has been shown by different groups (Kovarik, Bozzotti et al. 2001; Hobson, Barnfield et al. 2003; Rush, Mitchell et al. 2006) indicating means to initiate an antibody response after DNA vaccination. The main immune stimulatory mechanism however, is believed to be encoded gene expression followed by ubiquitination, further degradation by proteasomes and subsequent antigen peptide presentation in complex with the MHC complex, which is displayed on the outer membrane of cells (Ulmer, Deck et al. 1997; Fu, Guan et al. 1998; Uchijima, Yoshida et al. 1998). T-cell activation is restricted to antigen presenting cells, like dendritic cells or macrophages, which express the MHC-II complex. Thus, in order to activate ('prime') naïve T-cells either the APCs have to be transfected with the plasmid vaccine themselves or they take up the peptide by cross-priming (fig 1.3) (Fu, Guan et al. 1998; Donnelly, Liu et al. 2000; Heit, Maurer et al. 2003; Srivastava and Liu 2003). T-cell priming by DCs or other professional antigen presenting cells is widely considered to be the important factor of inducing cellular based immune response (Cusi, Terrosi et al. 2004). Since naked DNA uptake takes place primarily in MHC-II⁺, MHC-I⁺ cells, which is the predominant majority of cells in the body (e.g. myocytes, when vaccination route is injection into the muscle) many different groups work on vaccine delivery techniques targeting especially DCs (Denis-Mize, Dupuis et al. 2000; Donnelly, Liu et al. 2000; Kutzler and Weiner 2004; Lu, Kawakami et al. 2007).

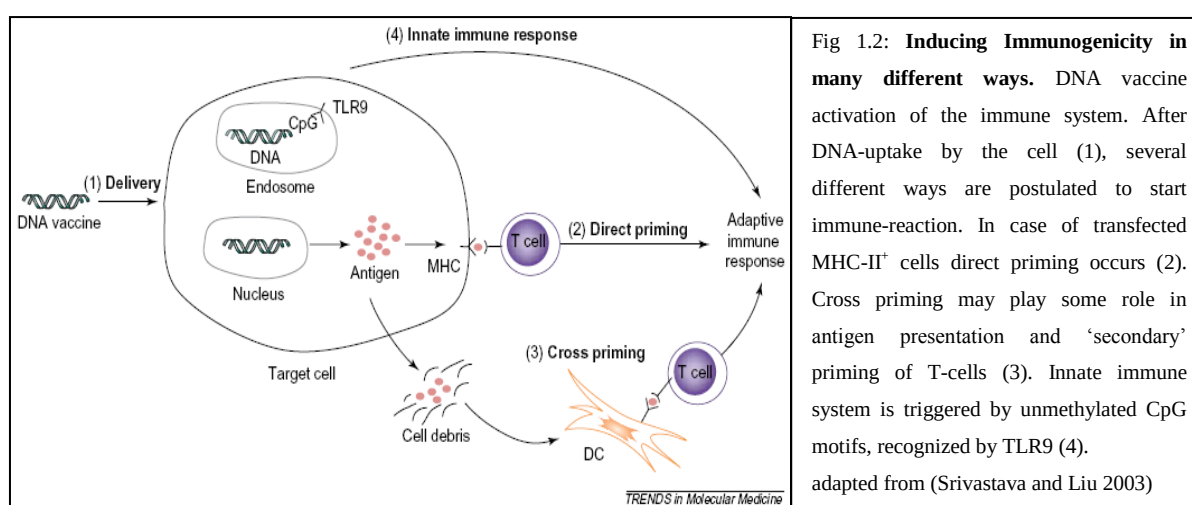
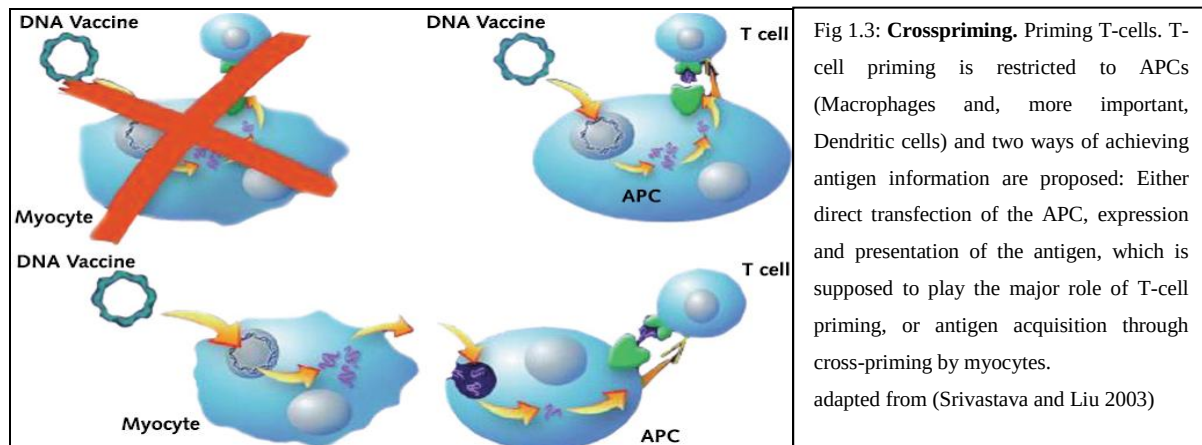


Fig 1.2: Inducing Immunogenicity in many different ways. DNA vaccine activation of the immune system. After DNA-uptake by the cell (1), several different ways are postulated to start immune-reaction. In case of transfected MHC-II⁺ cells direct priming occurs (2). Cross priming may play some role in antigen presentation and 'secondary' priming of T-cells (3). Innate immune system is triggered by unmethylated CpG motifs, recognized by TLR9 (4). adapted from (Srivastava and Liu 2003)



Drawbacks and Approaches to Problems of limited Bioavailability of introduced genes and gene products respectively

However, despite the new range of possibilities and promising results in small model organisms, DNA vaccines do not provide sufficient immune response in clinical trials so far (Cohen, Boyer et al. 1998; Boyer, Kim et al. 1999; Roy, Wu et al. 2000; Swain, Heydenburg Fuller et al. 2000; Wahren, Ljungberg et al. 2002; Hejdeman, Bostrom et al. 2004; Levine, Humeau et al. 2006). The main reason for the differences in immunogenic outcome has not yet been elucidated in detail, but is believed to be found in limited bioavailability of the immunizing agent (Ulmer, Wahren et al. 2006). Starting with low transfection rates, low expression levels, restriction of the antigen encoding plasmid DNA to injection site and, as a consequence of these shortcomings, insufficient stimulation of the innate immune system, which is responsible for a thorough activation of the adaptive immune system (reviewed by (Ulmer, Wahren et al. 2006)).

Several approaches try to overcome those problems on various levels of the immunization procedure

Vector alterations

In general vectors for gene based immunization consist of 5 essential sites, divided into two subsets. First there is the bacterial origin and a selective marker (e.g. an antibiotic resistance cassette) responsible for plasmid propagation and selection in the bacteria, which is the main production site for plasmid DNA. Second, there is the eukaryotic expression cassette, containing a eukaryotic promoter sequence, the encoded (anti-)gene as well as a polyA tail. (Xu, Mizuguchi et al. 2001) tested several different promoters and polyA sequences concerning their strength of gene expression within the vectors. Among others tested, the CMV IE promoter (cytomegalo-virus immediate early), the β -actin promoter of chicken with the β -actin intron sequence and the improved CMV-promoter containing the largest CMV intron sequence (intron A) produced the highest levels of expression. The polyA

sequences with the most stable gene expression found, were the SV40 (simian vacuolating virus 40) and the BGH (bovine growth hormone) polyAs (Xu, Mizuguchi et al. 2001).

Also codon optimization proved to be a powerful tool to increase gene expression. (Wang, Farfan-Arribas et al. 2006) showed that codon optimization enhanced the efficiency of gene expression. Surprisingly codon optimization also showed increased levels of mRNA suggesting that tRNA availability is not the only reason for improved gene expression (Uchijima, Yoshida et al. 1998; Nagata, Uchijima et al. 1999; Deml, Bojak et al. 2001; Narum, Kumar et al. 2001; Bojak, Wild et al. 2002; Wang, Farfan-Arribas et al. 2006).

Adjuvant addition

Another given reason for poor immunogenicity of DNA vaccines is the insufficient stimulation of the innate immune system. This stimulation is crucial for providing thorough immune response to any kind of vaccine. The adjuvant helps by mocking actual disease and with that, induce inflammatory response genes. Conventional vaccines, which mostly also need an adjuvant boost, are often co-administered with aluminum salts or other substances known to stimulate the innate immune response. Plasmid DNA itself has a built-in adjuvant with unmethylated CpG motifs, which are recognized by TLR9 (toll-like-receptor), which is present in lymphocytes, NK-cells and on the membrane of DC endosomes (fig 1.2) (Klinman, Yamshchikov et al. 1997; Bauer, Kirschning et al. 2001). Activation of TLR9 results in proliferation of the cell and/or secretion of immune modulatory cytokines (IL-1, IL-6, IL-12, IL-18, TNF α and INF γ), chemokines and polyreactive IgM (Kovarik, Bozzotti et al. 2001). In addition CpG motifs enhance the expression of MHC II co-stimulatory molecules like B7-2, CD40 and ICAM-1. Thus, lots of research elucidated power and modality of the immune activating properties of CpG motifs (Wagner and Bauer 2006). A recent finding of the use of control DNA (i.e. without an encoded gene) showed increased levels of INF-regulated genes in mice indicating the possibility of a TLR9 independent immunological mechanism affected by DNA (Cohen, Boyer et al. 1998). Another possibility to enhance innate immune system response is the additional cloning of genes of interleukins, chemokines and co-stimulatory molecules into the vaccine plasmid and in this way boosting inflammatory pathways (Kutzler, Robinson et al. 2005; Leitner, Bergmann-Leitner et al. 2006). It also has been tried to simply add these immune stimulants to the vaccine as adjuvant (Kutzler, Robinson et al. 2005). The composition of adjuvants also enables manipulation of immune response and directs the outcome either to cell based or humoral immunogenicity. As pars pro toto the findings of the use of IL-15 expression are mentioned. IL-15 is a very important cytokine to sustain proliferation of CD8⁺ memory T-cells. IL-15 use did not show high effect due to low expression levels in situ. However, (Kutzler, Robinson et al. 2005) showed increased efficacy in mice for HIV and influenza DNA vaccines with an optimized IL-15 expression plasmid. The study showed enhanced CD8⁺ T-cell proliferation, longevity and effector function, which lead to increased immunogenicity and protective efficacy (Zhou, Zhang et al. 2006). This way of manipulation is very helpful if

distinguished CD8⁺ T-cells are wanted as answer to intracellular pathogens. On the other hand different adjuvant composition may also elicit CD4⁺ T-cell based immune response as answer to an allergic disease.

Addition of non-directly-immunogenic sequences

(Zhou, Zhang et al. 2006) showed the possibility of increasing immunologic response by down-regulation protein expression of SOCS (suppressor of cytokine signaling) family genes in lymphocytes. This was achieved by co-administration of siRNA transcription vector with a CML-28 TAA (tumor associated antigen) expression vector. Immunogenic response and lymphocyte proliferation was significantly higher in SOCS silenced lymphocytes compared to cells immunized only with the CML-28 TAA (Kim, Hung et al. 2004; Zheng, Babiuk et al. 2005).

Immunologic availability

The limited number of transfected cells is thought to be a reason for poor immune reaction. Efforts have been made to fuse antigen-proteins with the VP22 protein of HHV type1 (Perkins, Hartley et al. 2005). This enables the spreading of the fusion protein from cell to cell to increase the number of responding cells to vaccination. Although various groups could show an increased immune response in mice and cattle, still the theory behind the rise is controversial (Ulmer, Donnelly et al. 1993; Bennett, Phillpotts et al. 1999; Dubensky, Liu et al. 2000; Singh, Briones et al. 2000; Briones, Singh et al. 2001; O'Hagan, Singh et al. 2002; Otten, Schaefer et al. 2005). It is thought that VP22-fusion proteins result in intercellular spreading.

Variation of delivery methods

Many different ways of applying foreign DNA to cells have been tried, reaching from naked DNA injection, in vivo electroporation and 'tattooing' (a method similar to former Variolation) over particle based delivery using the tendency of APCs (such as macrophages and dendritic cells) to phagocytose particles in the range of μm , to vector (viral and bacterial) associated DNA-delivery and gene gun application (Sasaki, Takeshita et al. 2003). It was shown in numerable publications that different delivery methods of DNA vaccines on the one hand show different efficiency of transfection, antigen expression and bioavailability of the antigen, and on the other hand influence the outcome of immunogenic response base, i.e. again a means to direct immune response either to cellular or humoral activation. It has been shown, that delivery systems which introduce the vaccine to the extracellular lumen (i.e. by injection) elicit humoral inclined immune response, whereas plasmid DNA directly introduced into the cytoplasm (i.e. via gene gun or electroporation) rather activates cell-based immunity. (Bagarazzi, Boyer et al. 1999; McCluskie and Davis 1999; Hobson, Barnfield et al. 2003) proposed that the reason for the difference is due to the recognition of the CpG elements by TLR9 when the plasmid is located outside the cell. This discrepancy however, remains conjectural.

Until now (2008) there are four DNA based vaccines licensed, which are for the use in animals. One is APEX-IHN® against the infectious hematopoietic necrosis Virus in rainbow trout (August 2005, CFIA), the second is West-Nile Innovator® against West-Nile Virus in horses (July 2005, CDC). Both vaccines are delivered intramuscularly.

Two more have been licensed more recently. In 2007, a therapeutical DNA vaccine against melanoma in dogs has conditionally been licensed in the US, called *Canine Melanoma Vaccine*. Companies involved are Merial, Memorial Sloan-Kettering Cancer Center and The Animal Medical Center of New York.

The last licensed DNA vaccine increases the number of piglets weaned in breeding sows; significantly decreases perinatal mortality and morbidity. It is called *LifeTide-SW5*, and used in swine. Licensed in Australia in 2007 from VGX Animal Health.

Alternative delivery: Mucosal Application

Besides those invasive, parenteral ways of introducing DNA into the body, application of suitable delivery systems onto the mucosa poses another way of immunization and the possibility to specially induce mucosal IgA-antibodies as well as a systemic response (Babiuk, Pontarollo et al. 2003). Additionally the density of DCs is very high in the mucosa, which increases the possibility of uptake. Protective mucosal immunity is crucial for inactivation of many pathogens at the entry site, thus preventing infection at the very first stage, on the other side mucosal immunization is a desirable way of applying large-scale vaccination. Its administration is easy and fast, needles as well as highly trained personnel are not needed. It was also shown, that mucosal vaccination (e.g. nasal) induces immunity in other mucosal tissues as well (e.g. vaginal) (Haslberger, Kohl et al. 2000).

Bacterial Ghosts (BGs) provide a very effective carrier vehicle for mucosally delivered DNA vaccine. Due to natural membrane proteins and further so-called Pathogen associated Molecular Patterns (PaMPs) directly targeting DCs and macrophages (Witte, Blasi et al. 1990), in this way adding to the efficient transfection of immune competent cells as well as providing intrinsic adjuvant properties like peptidoglycan, lipopolysaccharides and lipid A.

Bacterial Ghosts

The Process of Producing Bacterial Ghosts: E-Lysis

BGs are non-living, gram-negative bacterial envelopes devoid of cytoplasm but with an intact shell (fig 1.4, 1.5). This is achieved by the in vivo generation of a lysis hole through the inner and outer membrane by the phage-derived lysis-protein E from phage PhiX174 (Blasi, Young et al. 1988). This protein forms a lysis tunnel by fusing the inner and outer membranes, leaving the integrity of the envelope structure otherwise unharmed and expelling the cytoplasm by osmotic pressure difference with the surrounding medium (Szostak, Hensel et al. 1996). The so produced BGs are then subjected to lyophilisation, which renders them stable at room temperature (Eko, Hensel et al. 1994; Eko, Szostak et al. 1994; Szostak, Hensel et al. 1996; Szostak, Mader et al. 1997; Eko, Witte et al. 1999; Jalava, Hensel et al. 2002; Marchart, Dropmann et al. 2003; Panthel, Jechlinger et al. 2003; Ebensen, Paukner et al. 2004; Eko, He et al. 2004; Mayr, Haller et al. 2005; Mayr, Walcher et al. 2005; DelVecchio, Alefantis et al. 2006; Eko, Barisani-Asenbauer et al. 2007).



Fig 1.4: **Bacterial Ghosts – E-Lysis.** Lysing gram negative bacteria, showing the expulsion of cytoplasm through the lysis hole. adapted from (Tabrizi, Walcher et al. 2004)

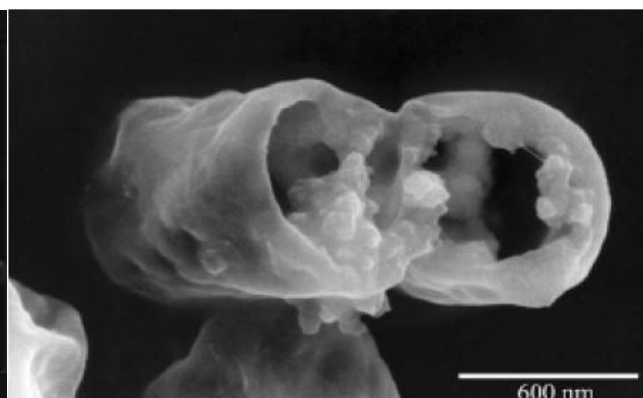


Fig 1.5: **Bacterial Ghosts – Mg-Lysis.** The lysis of the bacteria leaves the cell devoid of cytoplasm. adapted from (Ebensen, Paukner et al. 2004)

Findings suggest the building of the lysis hole near the cell division zone.

BGs are manufactured by transforming *Escherichia coli* cells with the lysis plasmid pGLysivb. This plasmid harbors the E-lysis gene under the control of the temperature dependent λ -Promoter, which is active at 42°C and silent at 36°C. Increasing the temperature from 36°C to 42°C leads to E-lysis of the bacterial cell and yields the empty BGs.

Applications of BGs

BGs are used as vaccines themselves, presenting unaltered membrane morphology, membrane bound proteins as antigens and furthermore possessing PaMPs (Pathogen associated Molecular Patterns) recognized by TLRs and other receptors of the innate immune system and in this way presenting a built in adjuvant (Paukner, Kohl et al. 2004; Tabrizi, Walcher et al. 2004; Paukner, Stiedl et al. 2006). Antigen proteins of different pathogens may also be expressed *in vivo* within the membrane complex, which makes BG a possible carrier and presenter of multiple native and foreign proteins (fig 1.6).

Furthermore BGs can be used as carrier vehicles for any kind of water soluble molecular structure such as proteins, drugs or DNA. BGs are incubated in the drug-, protein- or DNA containing solution and in this way loaded with the desired material (Walcher, Mayr et al. 2004).

In an *in situ* expression of the desired protein, it can be expressed to integrate into the inner or the outer membrane as well as be transported to the periplasmatic space (Paukner, Kudela et al. 2005; Kudela, Paukner et al. 2007). This provides a one-step loaded BG production.

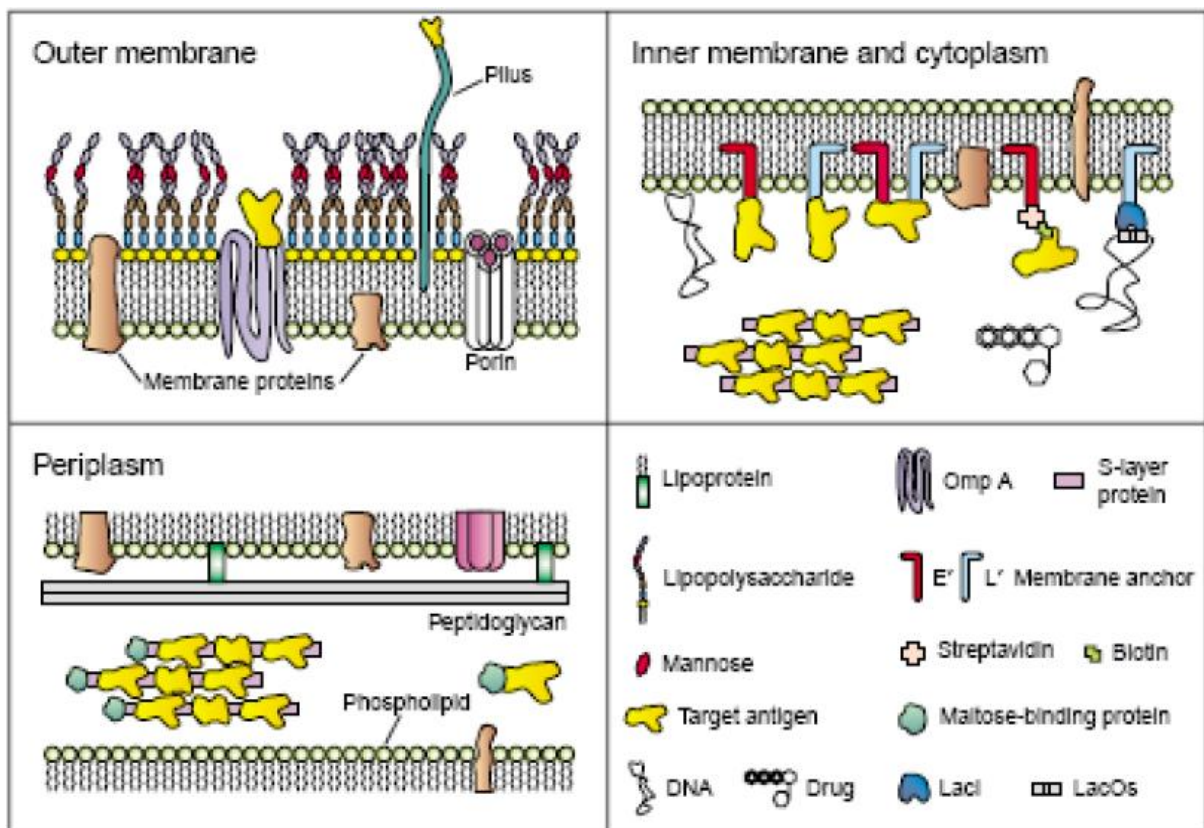


Fig 1.6: Schematic of the localization of different molecules in bacterial ghosts. adapted from (Jechlinger 2005)

It has been shown that BGs efficiently target Antigen Presenting Cells (APC) (Paukner, Kudela et al. 2005). Due to morphological and structural properties such as former mentioned PaMPs BGs are readily phagocytosed by Macrophages or Dendritic cells. The uptake of BGs loaded with DNA leads to protein expression of the encoded genes (Jechlinger, Azimpour Tabrizi et al. 2004; Paukner, Stiedl et al. 2006).

The SIP (self-immobilizing-plasmid) technique has been established to produce and retain plasmid DNA within the bacterial shell after E-lysis in one step (Mayrhofer, Tabrizi et al. 2005).

The SIP-technique

The SIP technique depicts a specially tailored system of plasmid fixation within BGs. SIP stands for Self-Immobilizing-Plasmid and describes a method of molecular interaction by using repressor-operator interaction.

The SIP plasmid contains, aside other sequences, the ParA recombinase gene with the LacI-L' gene under the shared control of the P_{BAD} -promoter (Szostak, Wanner et al. 1990), which is induced by arabinose. The induction of this promoter triggers two reactions:

- The expression of the ParA recombinase, which efficiently catalysis the recombination of the motherplasmid, in order to produce minicircle and miniplasmid.
- and LacI-L' anchor protein expression, which is a fusion protein of the L' anchor (Mayrhofer, Tabrizi et al. 2005) and the LacI repressor molecule. The LacI-L' anchor integrates into the inner cell membrane and attaches tightly on the inside of BG. On the other end the protein presents the LacI repressor molecule, which 'finds' the LacOs operator sequence on the minicircle and binds to it (Jechlinger, Azimpour Tabrizi et al. 2004).

The plasmid consists of two parts, secluded by the two recombination sites (described below). The antibiotic resistance cassette as well as the origin of replication are, together with the P_{BAD} -controlled recombinase and LacI-L' anchor, joined on the part of the motherplasmid, which will form the miniplasmid after recombination. Located on the other, the minicircle side, next to the eukaryotic expression cassette, which consists of the CMV-promoter, the gene of interest and the BGH-polyA, is only the LacOs sequence. By means of molecular interaction the LacI-L' anchor binds to the Lac-Os site on the minicircle and fixates it to the inner cell membrane (Jechlinger, Glocker et al. 2005).

This technique combined with E-lysis allows the culturing of bacteria, multiplying the antigen-encoding plasmid DNA and its recombination into minicircle and miniplasmid as well as the generating of BGs by temperature shift (Jechlinger, Azimpour Tabrizi et al. 2004) in one fermentation step. The miniplasmid as well as the lysis plasmid, which both lack the LacOs sequence, are lost after lysis. As a result the BGs are loaded with minicircle-DNA only (Darquet, Cameron et al. 1997).

The Minicircle Technique:

Definition of the Minicircle

The term minicircle refers to a small, circular DNA-molecule devoid of any sequences related to bacterial origin as first developed by (Darquet, Cameron et al. 1997; Kreiss, Cameron et al. 1998; Darquet, Rangara et al. 1999; Bigger, Tolmachov et al. 2001; Chen, He et al. 2003). Minicircles are small supercoiled minimal expression cassettes for non-viral gene transfer.

Disadvantages of using Plasmid DNA as vaccines

An easy and the most common way to produce high amounts of DNA for DNA-vaccines is plasmid propagation and preparation in bacteria. Beside the eukaryotic expression cassette, this method implies two distinguished sequences on the desired plasmid, a bacterial origin of replication and a selective marker, mostly an antibiotic resistance cassette (fig 1.7). These sequences also reduce efficiency of the plasmid in its later use in the host. After selection and propagation they are not needed any further and pose a biological safety risk, potentially causing horizontal gene transfer and antibiotic resistance distribution within the vaccine recipient. Regulatory agencies therefore recommend, e.g., totally avoiding the use of antibiotic resistance genes [FDA, 1996; EMEA, 2001]. Furthermore classical plasmid-DNA vectors contain more than 50% bacterial DNA backbone. Therefore the minicircle technique is a very efficient way to render slim, efficient and safe DNA agents. It has been shown that minicircles, besides their excellent safety profile show improved gene transfer and bioavailability properties (Laham 2004).

Producing Minicircles by Recombination

Minicircles are produced by site-specific, highly efficient recombination of the motherplasmid (fig 1.8). The two subsequent circular DNA molecules are the miniplasmid with all undesirable sequences as well as the minicircle, which carries beside the expression cassette only half of the recombination sites. After recombination the minicircle is kept within the shell of the bacteria by molecular interaction, which is described below in detail. The miniplasmid is washed out of the BG.

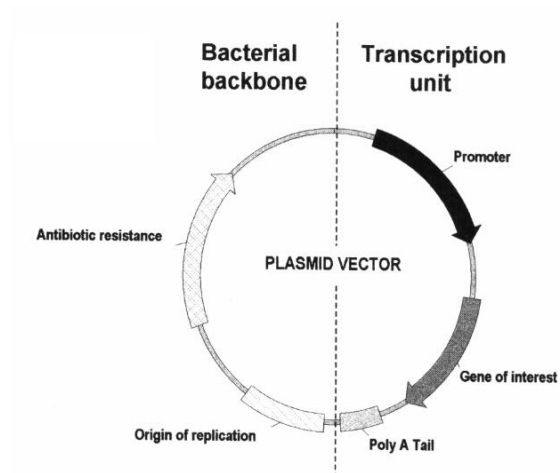


Fig 1.7: **Plasmid DNA propagated in bacteria and used for eukaryotic gene transfer.** It contains sequences related to bacterial multiplication as well as eukaryotic expression. The minicircle technique is a way to separate the potentially harmful and unwanted bacterial sequences rendering efficient and safe minicircles. adapted from (Mayrhofer, Tabrizi et al. 2005)

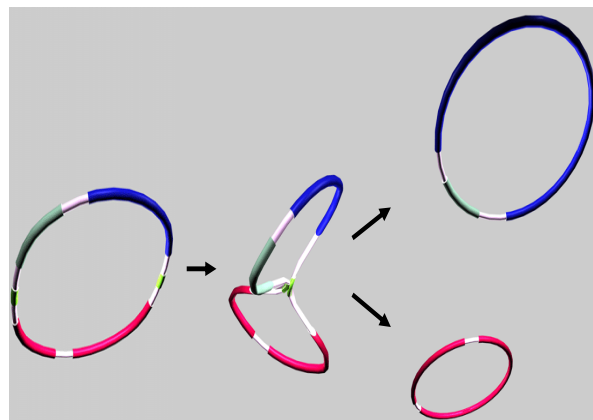


Fig 1.8: **Plasmid Recombination.** Minicircle and miniplasmid (right) are produced (middle) by recombination of motherplasmid (left). Due to sequence specific, molecular interaction, the minicircle can be separated from the miniplasmid. adapted from (Jechlinger 2005)

RESULTS

Cloning of pSIP-H5

Essential for the successful accumulation of the minicircle in the bacterial cell is stringent repression of recombination prior to induction. If the plasmid recombines early, a buildup of the self-replicating miniplasmid against the non-replicating minicircle in relative numbers would be the consequence. Before inserting the H5 gene, the performance of the origin plasmid pSIP-Ova was ensured.

Plasmid pSIP-Ova: SIP Backbone for Insertion of the H5 Gene

A -80°C glycerol stock of Firas Al Laham of *E.coli* K12 strain NM522 (pSIP-Ova, fig 2.1), was used for propagation of the plasmid, which serves as the backbone of the pSIP-H5. This plasmid was formerly tested and used by Firas Al Laham (Jechlinger, Azimpour Tabrizi et al. 2004; Tabrizi 2005). Cloning of pSIP-Ova was performed by Chakameh Azimpour and Wolfgang Jechlinger (Champeimont 2007).

pSIP-Ova includes two resolution sites (*RES 1* and *RES 2*), dividing the motherplasmid into two parts, the miniplasmid and the minicircle. On the miniplasmid the Ampicillin resistance cassette and the *LacI-L'anchor* fusion protein as well as the *ParA resolvase* gene are located. The latter two genes lie downstream of the arabinose inducible *P_{BAD}*-Promoter. Also on this side of the motherplasmid is the *pUC*-origin of replication and the *AraC* sequence, expressing the *P_{BAD}*-associated repressor protein. On the other side of the resolution sites lies the minicircle. It consists of the eukaryotic expression cassette with the *IE-CMV* promoter and the *SV40* polyA sequence; located in between is the *Ova*-gene, which, in former recombination experiments served as control gene for expression. To retain the minicircle within the BG after E-lysis, upstream of the *IE-CMV* promoter is the *LacOs* sequence. It attaches to the membrane bound *LacI-L'anchor* fusion protein, thus avoiding being expelled with the cytoplasm upon lysis.

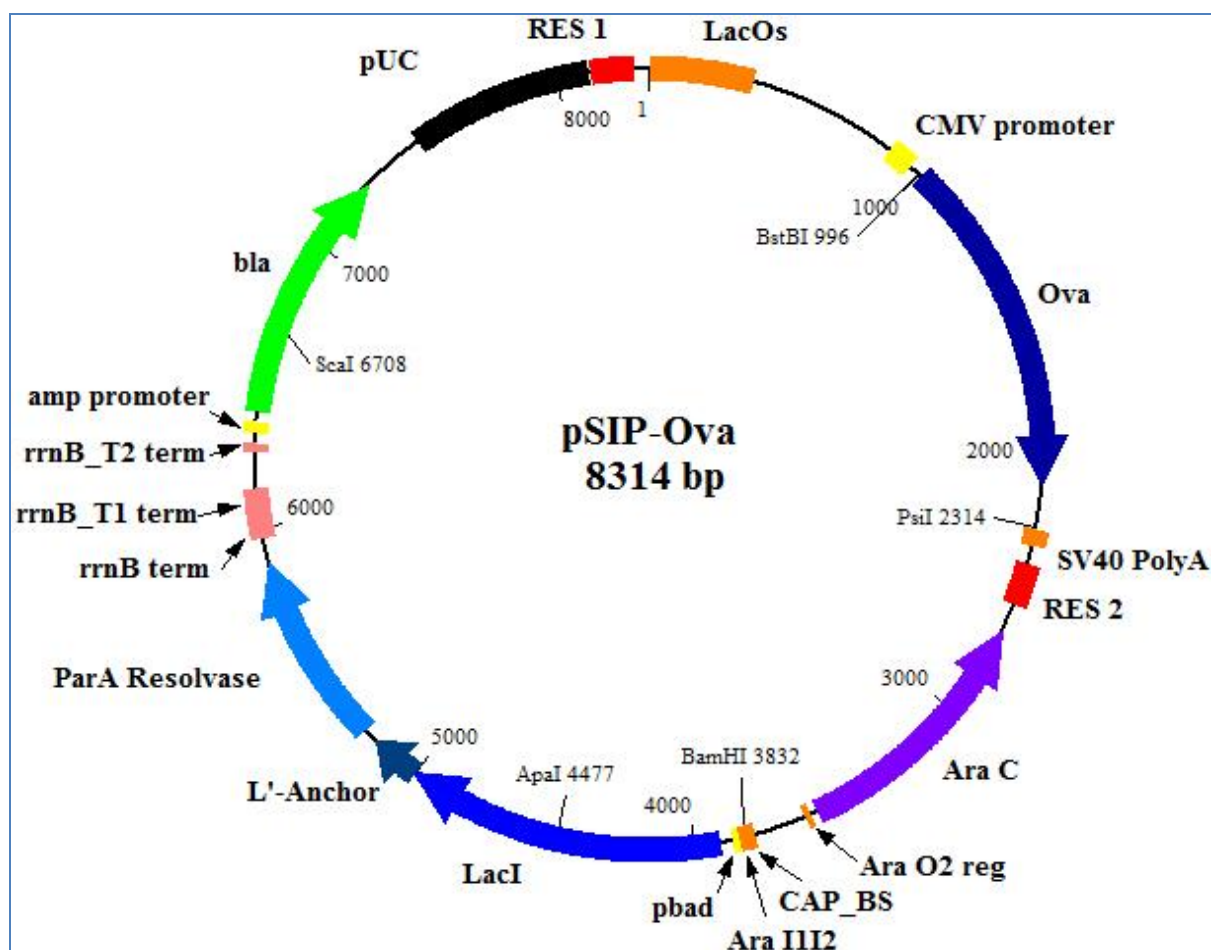
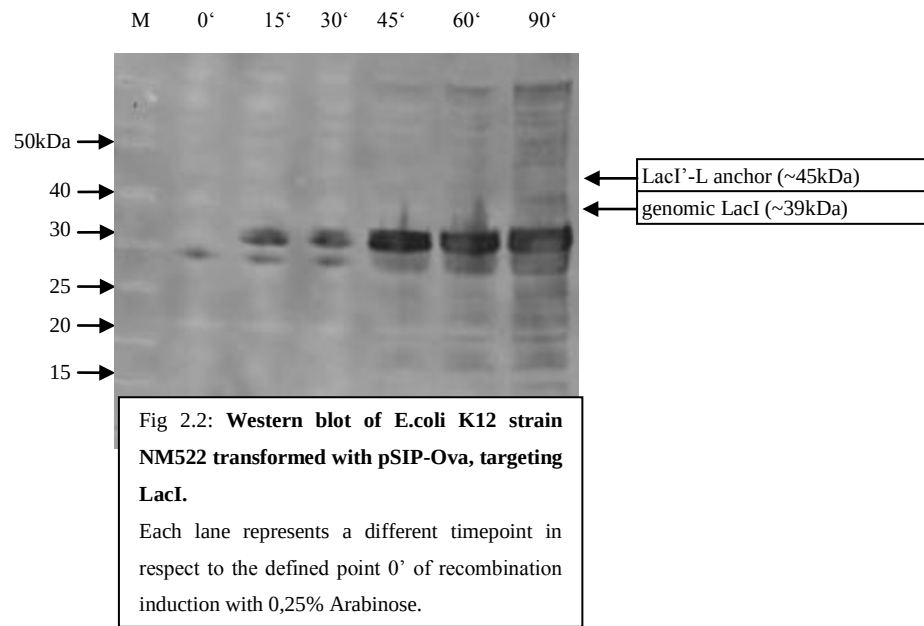


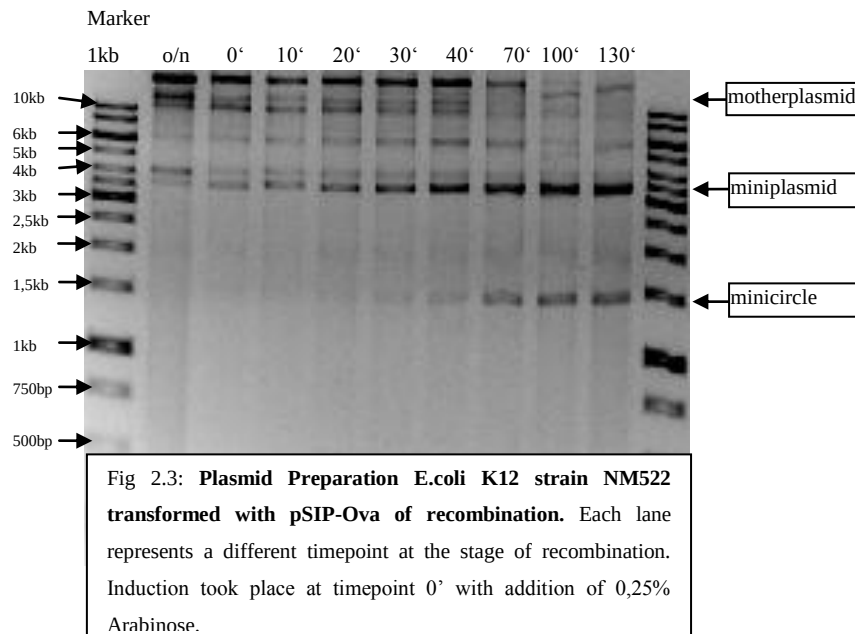
Fig 2.1: pSIP-Ova plasmid map.

amp promoter	Ampicillin Promoter	LacOs	Lac Operator Sequence
Ara C	Ara C Repressor	Ova	<i>Ovalbumin</i>
Ara I112, Ara O2	AraC regulatory sequences	ParA Resolvase	<i>ParA Resolvase</i>
amp promoter	ampicillin promoter	pbad	p _{bad} promoter
bla	ampicillin resistance cassette	pUC	pUC ori
CAP_BS	catabolite activator protein binding sequence	RES 1, RES 2	Recombination sites
CMV promoter	CytoMegaloViral eukaryotic Promoter	rrnB_T1, T2, term	terminators of 5S ribosomal RNA
L'-Anchor+LacI	Fusion Protein	SV40 PolyA	PolyA sequence of simian Virus 40

Western blots targeting *LacI* and plasmid preparations were used to show the results of *LacI-L'anchor* protein expression as well as *ParA* recombination. The experiment was designed to show tight repression and fast induction of the P_{BAD} promoter.



Western blot (fig 2.2) as well as plasmid preparation (fig 2.3) of samples taken at different timepoints, confirm expected results. LacI-L' expression can be seen 15min after arabinose induction. The single small band at about 40kDa (lane 0') is due to genomic LacI expression, which is carried on the F' episome. The anti LacI-antibody detects both LacI forms, F'-episomic LacI (39kDa) and plasmid-derived LacI-L'-anchor fusion protein (45kDa). The different molecular weight of the proteins though, allows a clear distinction.



The plasmid preparation shows uncut plasmids. The weak miniplasmid band in the o/n sample indicates slight premature recombination; the non proliferating minicircle however is lost during

overnight growth. 30min after arabinose induction the miniplasmid as well as the minicircle band appear and grow, whereas the motherplasmid band decreases.

Plasmid pEPH5: Template for the Amplification of the H5-gene

The Vietnam Hemagglutinin type 5 gene was located on the pEPH5 plasmid (fig 2.4). This plasmid, which provides the antigen in a eukaryotic expression cassette, was provided by LAH, Cuxhaven.

The *H5* gene is located between the *T7* promoter and the *bovine growth hormone* polyA sequence, *BGH*. Also between these regulatory sequences, downstream of the *H5* gene lies a Kanamycin resistance cassette. Furthermore the plasmid harbors an Ampicillin resistance cassette as well as a *lacZ_α* reporter sequence. Downstream to the Ampicillin resistance cassette lies the *pBR322* origin of replication.

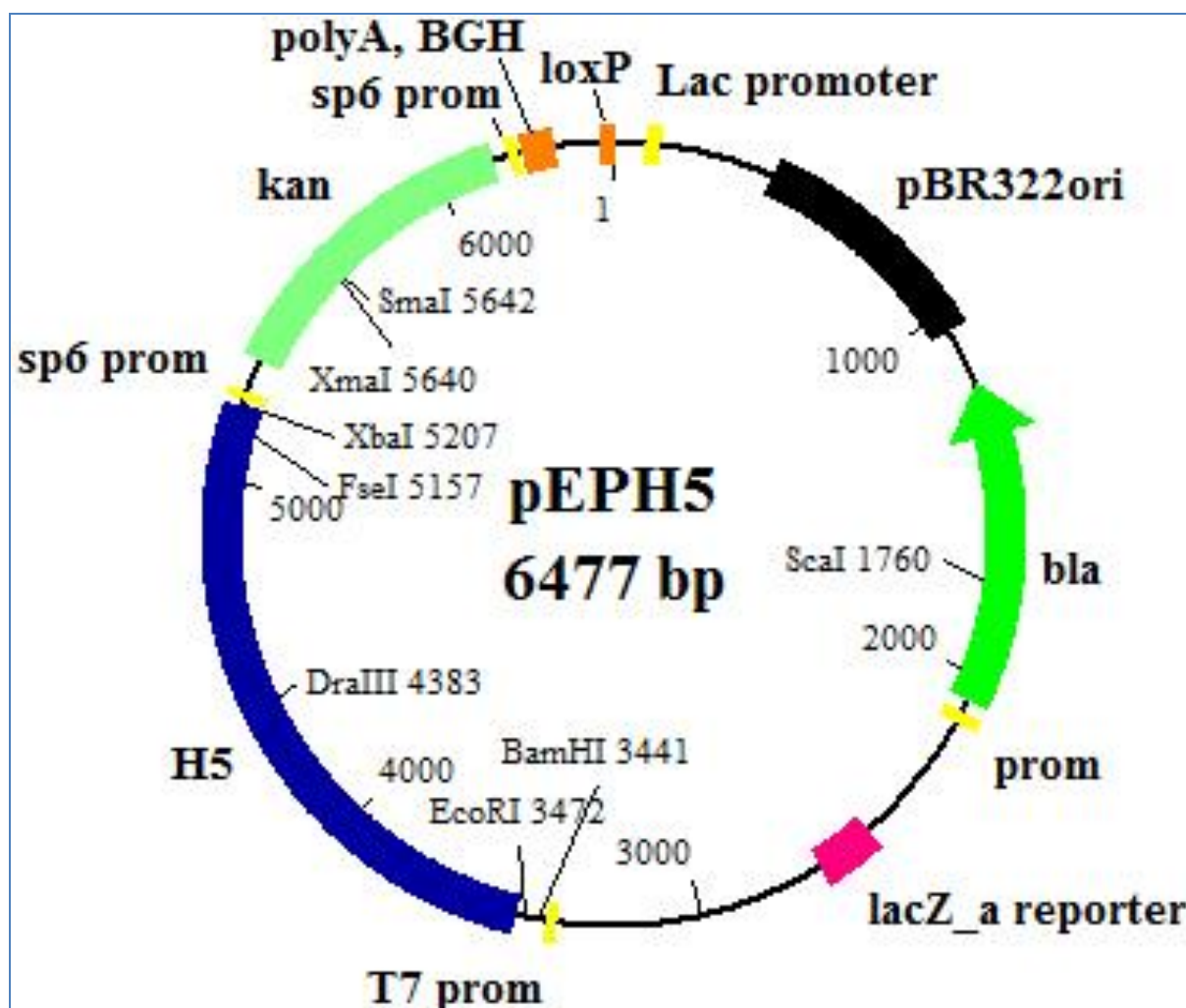
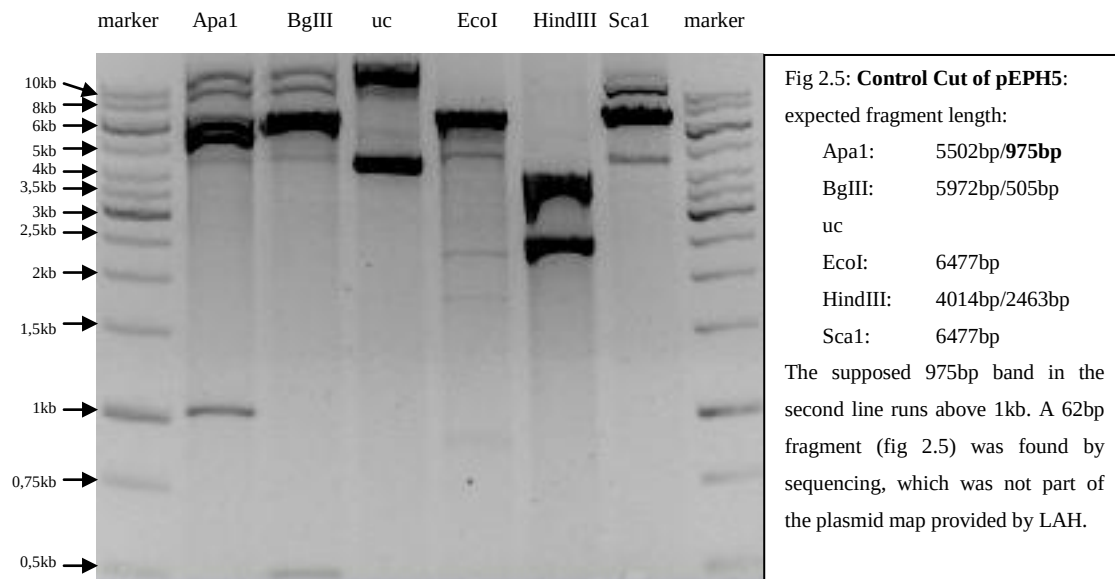


Fig 2.4: pEPH5 plasmid map.

bla	ampicillin resistance cassette	pBR322 ori	Origin of Replication
H5	type 5 Hemagglutinin	polyA, BGH	bovine growth hormone polyA sequence
kan	Kanamycin resistance cassette	prom	Ampicillin Promoter sequence
Lac Promoter	Lac Promoter sequence	SP6 prom	SP6 polymerase promoter
lacZ_α reporter	lacZ Reporter sequence	T7 prom	T7 promoter sequence
loxP	loxP site		

The plasmid was transformed into the *E.coli* K12 strain NM522. Restriction enzyme analysis of a plasmid preparation showed slightly larger bands than anticipated due to plasmid sequence provided by LAH. The anticipated 975bp band of the Apa1 restriction (lane1) exceeds the 1000bp band of the marker (fig 2.5).



Sequencing of the H5 gene followed cloning of the pSIP-H5 plasmid and showed 62 additional unknown base pairs within the eukaryotic expression cassette (fig 2.6).

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TCGAAATGGAAGATCGTGCTGCTTTCGCCATCGTAGCCTGGTGAAGAGCGACCAGATCTGCATCGGCTACCACGCCA
ACAACAGCACCGAGCAGGTGGACACCATCATGGAAAAAACGTGACCGTGACCCACGCCAGGACATCCTGGAAAAAGACCC
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CCCCATGTGCGACGAGTTCATCAACGTGCCCCGAGTGGAGCTACATCGTGGAGAAGGCCAACCCCGTGAACGACCTGTGCTAC
CCCGGCGACTTCAACGACTACGAGGAAGTGAAGCACTGCTGTCCCGCATCAACCACTTCGAGAAGATCCAGATCATCCCCA
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AGGGAAGAGATCAGCGGAGTGAAGCTGGAAAGCATCGGCATCTACCAGATCCTGAGCATCTACAGCACCGTGGCCAGCAGC
 CTGGCCCTGGCCATCATGGTGGCCGGCCTGTCCCTGTGGATGTGCAGCAACGGCAGCCTGCAGTGCAGGTGATAATCTAGAG
 GGCCCTATTC **TATAGTGTACCTAAATG**CTAGAGCTCGCTGATCATAGGGATAACAGGGTAATCGATT **ATTCAACAAAGCCA**
CGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAA
 ACAGTAATACAAGGGGTGTT **ATGAGCCATATTCAACGGGAAACGTCCTGCTCGAGGCCGCGATTAAATTCCAACATGGATGC**
TGATTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATG
CGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGAC
GGAATTATGCCTCTTCCGACCATCAAGCATTATCCGTACTCCTGATGATGCATGGTTACTCACCCTGCGATCCCCGGGAA
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CTAATCAGAATTGGTTAATTGGTTGTAACACTGGCTAGAGGGCCCTATTC **TATAGTGTACCTAAATG**CTAGAGCTCGCTGAT
 CAGCCT **CGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCCCCCGTGCCTTCCTTGACCTGGAAGGTGCCACTG**
CCACTGTCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGTGGGGTGGGG
CAGGACAGCAAGGGGGAGGATTGGGAAGACAAATGGCCGCATAAATTCTGATAGCATACATTATACGAAGTTATCTAGCAT **TT**
ATAA

Fig 2.6: **pEPH5 sequence amplified by PCR.** 3049bp of pEPH5 were amplified by PCR, introducing the restriction enzyme sites for BstBI and PsiI.

key: Vietnam type 5 Hemagglutinin *H5* (yellow), SP6 promoter (green), unknown sequence (red), Kanamycin Resistance Cassette (light blue), BGH-polyA sequence (purple), introduced sites for restriction enzymes (red letters).

The H5 sequence together with the Kanamycin resistance cassette as well as the BGH polyA were amplified by PCR, introducing the restriction sites for BstBI (**TT|C GAA**) and PsiI (**TTA| TAA**) on either end respectively. The PCR product was cut with restriction enzymes BstBI and PsiI and purified, using the PCR Purification Kit.

Cloning of plasmid: pSIP-H5

The backbone fragment of 6998bp (fig 2.7) of the pSIP-Ova plasmid originates through enzyme restriction by BstBI (**TT|C GAA**) and PsiI (**TTA| TAA**), cutting out the *Ova* gene. This vector fragment was ligated with the PCR product of pEPH5 (fig 2.7), resulting in the plasmid pSIP-H5 (fig 2.8).

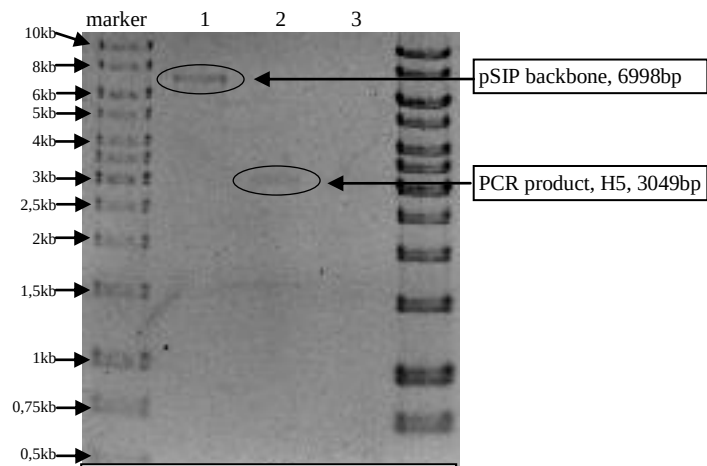
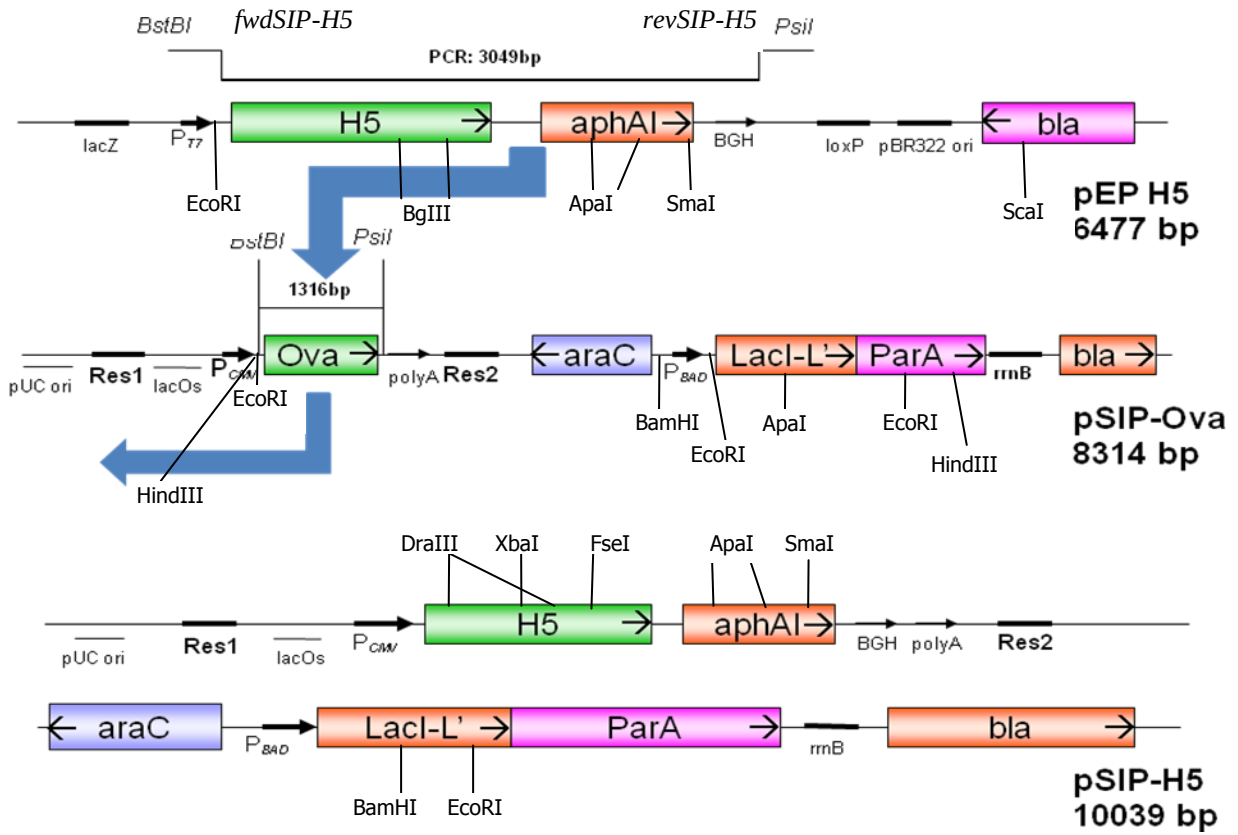


Fig 2.7: **Ligation Fragments for pSIP-H5.** The vector fragment (6998bp) was produced by double digestion (BstBI, PsiI) of pSIP-Ova. The pEPH5 originated sequence (H5, 3049bp) was produced by PCR.

Cloning strategy of pSIP-H5



Explication of Abbreviations

Abbrev.	Function	Abbrev.	Function
aphAI	Kanamycin Resistance Cassette	P_{BAD}	arabinose inducible Promoter
araC	positive and negative Regulator of P_{BAD} Promoter	P_{cmv}	Cytomegalovirus Immediate Early Promoter
BGH	Bovine Growth Hormone PolyA	P_{T7}	T7 Promoter
bla	Ampicilline Resistance Cassette	ParA	ParA Resolvase
LacI-L'	Membrane anchored (L') LacI repressor molecule	pBR322-ori	Origin of replication
lacOs	Lac Operator Sequence; LacI Binding Sequence	pUC ori	Origin of Replication
lacZ	lacZ reporter gene	Res1	Recombination Site 1
loxP	Bacteriophage P1 derived recombination site	Res2	Recombination Site 2
polyA	SV40 PolyA	rrnB	ribosomal RNA operon
Ova	Ovalbumin gene		

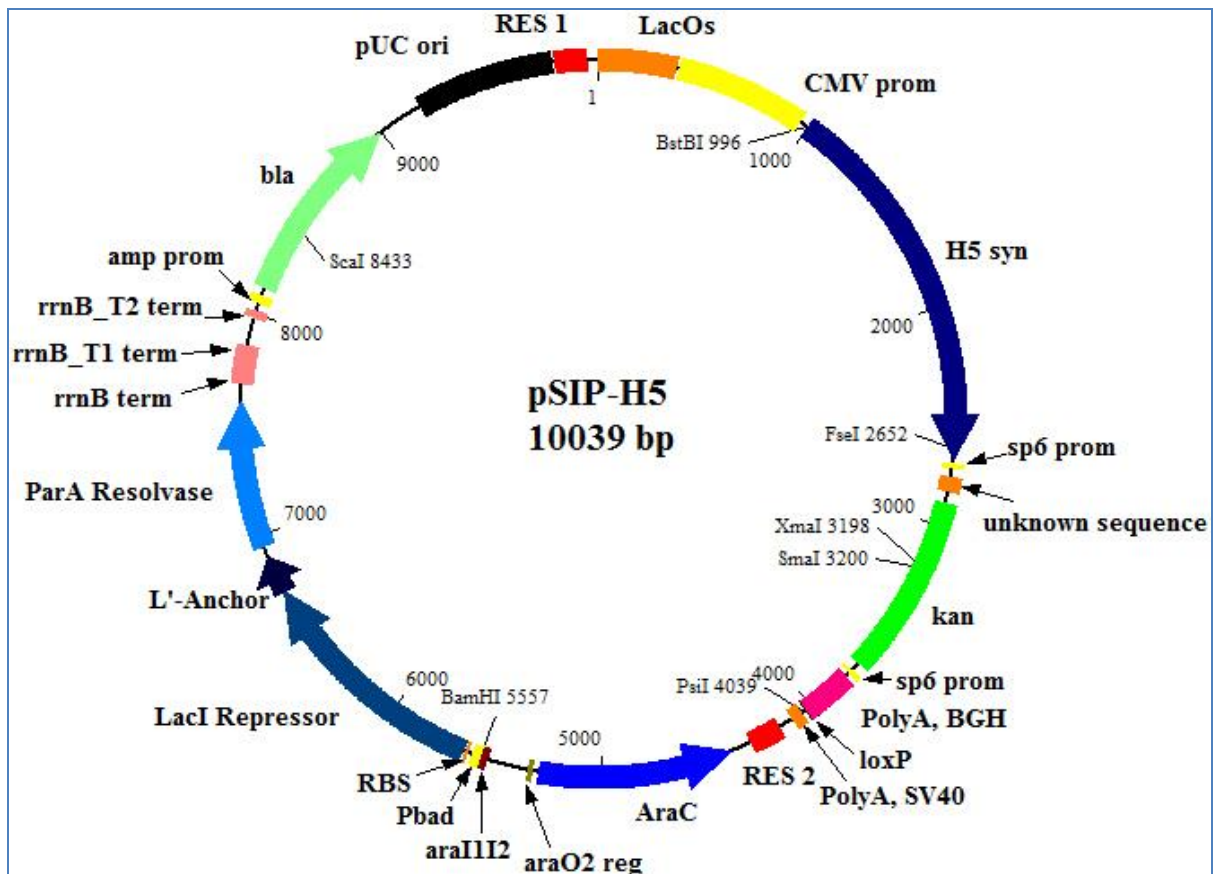


Fig 2.8: **pSIP-H5 plasmid map.** The pSIP-H5 was constructed by exchanging the *Ova* gene, located on the minicircle side of the plasmid pSIP-Ova, with the pEPH5 derived *H5* gene.

After transformation into *E.coli* K12 strain NM522, the plasmid was control cut and subjected to Agarose gel electrophoresis (fig 2.9).

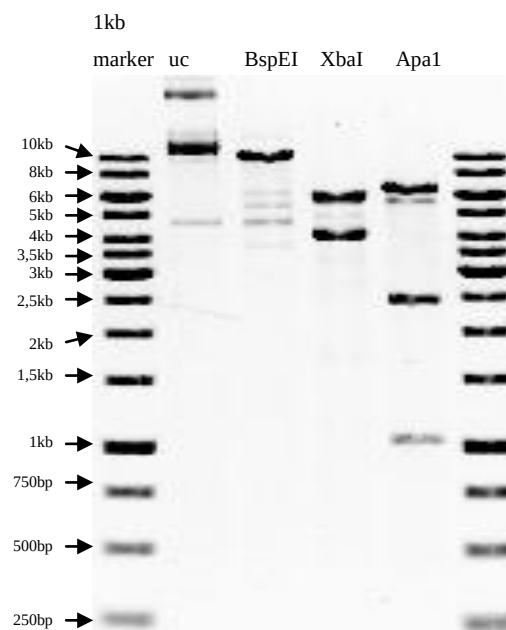


Fig 2.9: Restriction enzyme analysis of pSIP-H5. Expected fragment length:

BspEI: 10039bp

XbaI: 6020bp/4029bp

Apa1: 6549bp/2453bp/1037bp

The additional band in the Apa1 lane derives from incomplete digestion.

Pre-testings of pSIP-H5

Transformation of *E.coli* and *Salmonella* strains with pSIP-H5 and Storing of pSIP-H5

On Arabinose induction the LacI-L'anchor fusion protein will be expressed as well as the ParA Recombinase. Whereas the Recombinase will divide the plasmid into a miniplasmid and a minicircle, the membrane anchored LacI-L'anchor fusion protein will, by molecular interaction bind the LacOs sequence located on the minicircle, fixing it inside the BG.

The transformation of plasmid pSIP-H5 into different bacterial strains and its maintenance posed unexpected difficulties. The strains used were *E.coli* K12 strains NM522, DH10beta, Top10, ER2655 and TB1 as well as the *Salmonella* strain ATCC 14028. In the *Salmonella* strain as well as in the *E.coli* K12 strains DH10beta, Top10 and ER2655 the plasmid showed premature recombination (fig 2.10a&b).

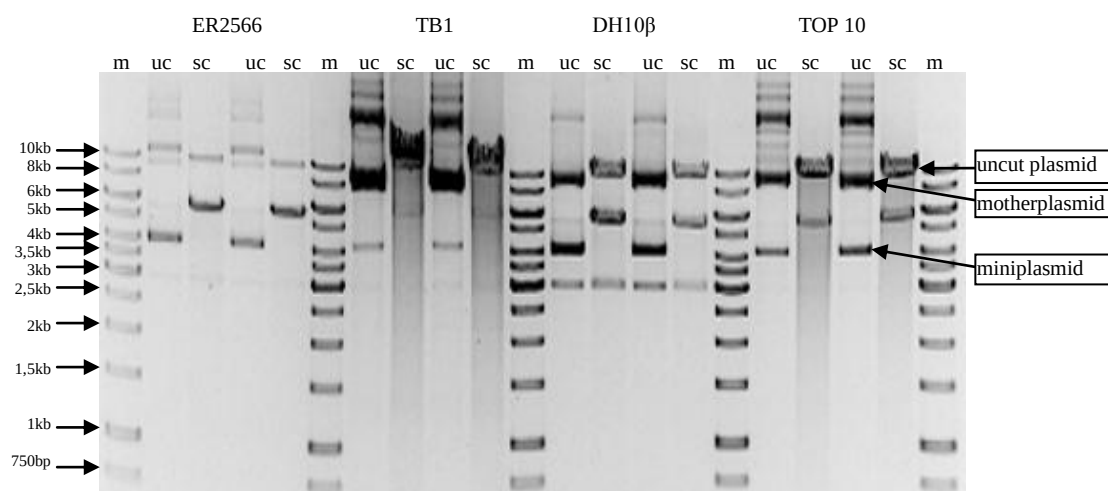


Fig 2.10a: **Plasmid Preparation of the uninduced (no Arabinose added) overnight cultures of *E.coli* strains ER2566, TB1, DH10β and TOP10 transformed with pSIP-H5.** From each strain two samples are loaded, which are either uncut or cut with the single cutter BamHI, the restriction site is located on the miniplasmid part. Miniplasmid band is visible at 5676bp in uninduced overnight cultures of ER2566, DH10β and TOP10. Linearized motherplasmid length: 10039bp

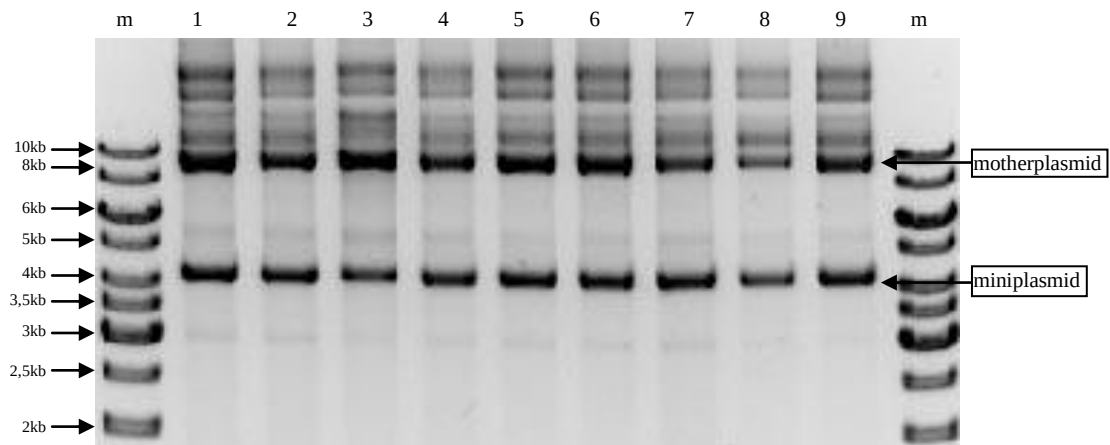


Fig 2.10b: **Plasmid Preparation of the uninduced (no Arabinose added) overnight cultures of Salmonella strain ATCC 14028 transformed with pSIP-H5.** Lanes 1-9 are different clones of *Salmonella* strain ATCC14028 from the same transformation. The lane marked with m signifies the marker.

Different variations of glucose concentration, growth temperature and growth time combined with the purification of the motherplasmid and repeated transformation with the cleaned, unrecombined motherplasmid were not successful (fig 2.11).

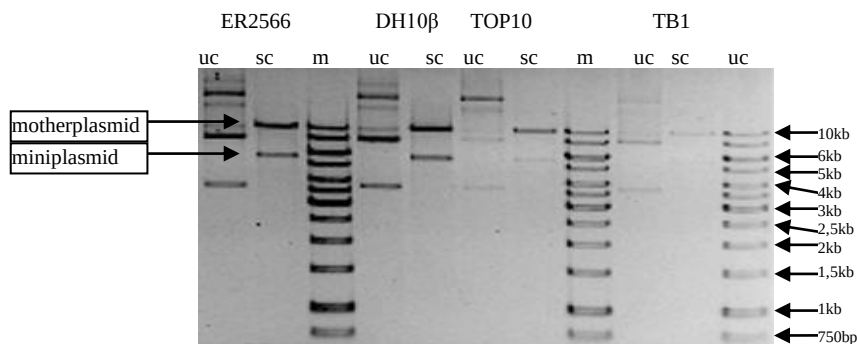
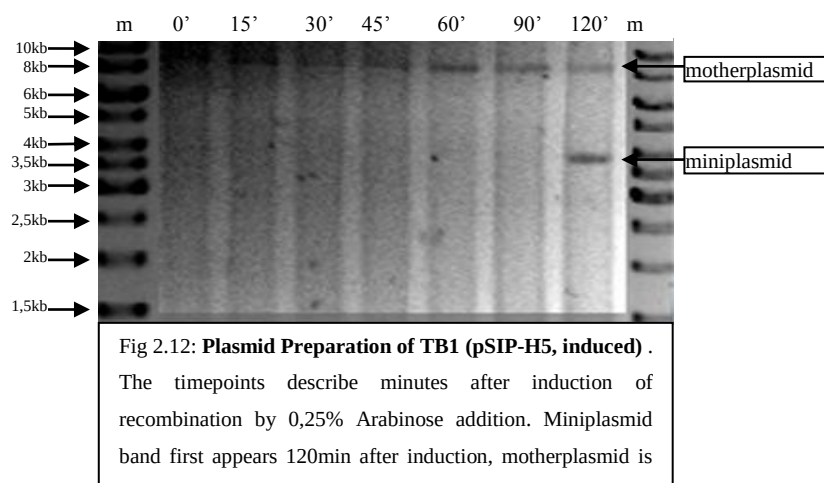


Fig 2.11: **Plasmid Preparation of uninduced overnight cultures of transformed E.coli strains ER2566, DH10β, Top10 and TB1.** Strains have been transformed with a purified motherplasmid of pSIP-H5 and recombination was not induced. For each strain two lanes are loaded with either uncut or single cut (BamHI, restriction site located on miniplasmid part of motherplasmid) plasmid.
 Miniplasmid fragment length: 5676bp
 Motherplasmid fragment length: 10039bp

pSIP-H5 transformed TB1 strain on the other hand does not show the miniplasmid band in the overnight cultures. The plasmid preparation of the recombination experiment with TB1 showed late and incomplete recombination upon arabinose addition (fig 2.12).



E.coli K12 strain TB1, for this fact, was used as safe storage for the plasmid, apparently tightly suppressing recombination of pSIP-H5. The DNA material later sent to mycosynth® for sequencing was extracted from the TB1 strain, in order to have the most unrecombined motherplasmid possible. Only strain NM522 showed a high degree of recombination after arabinose addition together with an acceptable repression of premature recombination in the presence of additional 5% glucose. The other strains were abandoned from further investigation.

Small Scale Recombination Experiments

The strain used for pSIP-H5 minicircle loaded BG production was *E.coli* K12 strain NM522. Further recombination (fig 2.13) experiments were carried out with this strain.

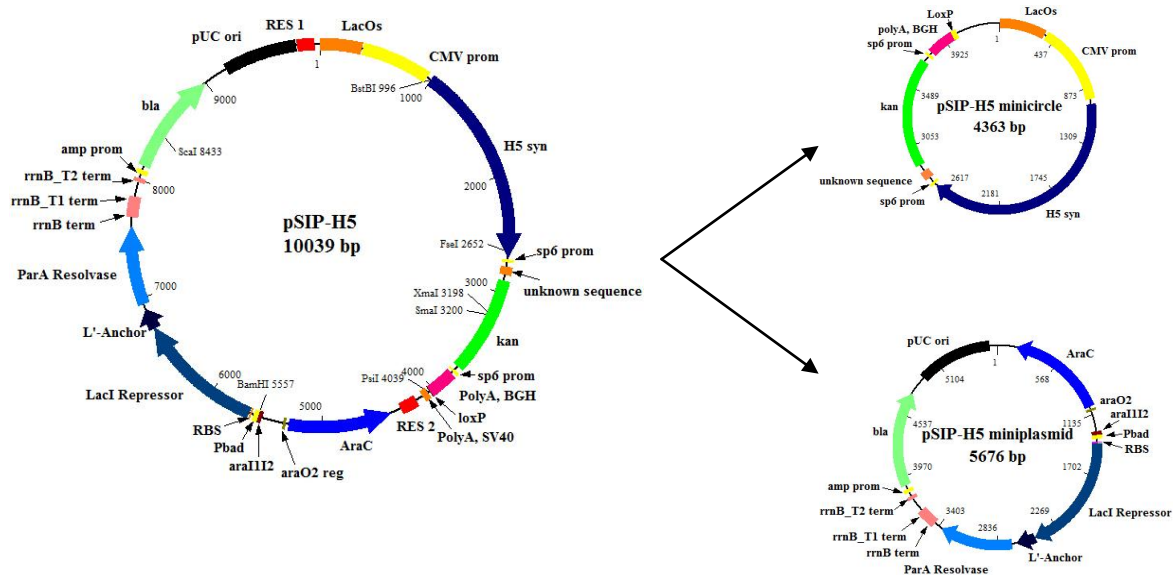


Fig 2.13: Schema of pSIP-H5 recombination. Upon P_{BAD} induction by 0,25% Arabinose the expressed ParA recombinase will recombine the resolution sites (**RES1, RES2**) present on pSIP-H5; thus producing the pSIP-H5-minicircle and the pSIP-H5 miniplasmid. Induction of the P_{BAD} promoter also leads to the expression of the LacI-L'anchor fusion protein. The H5-minicircle is fixed to the bacterial membrane due to molecular interaction with the membrane bound LacI-L'anchor fusion protein and the LacOs sequence, present on the minicircle.

Cfu counts and OD₆₀₀ measurements as well as microscopic observations of the fermentation can be seen in (fig 2.14 and fig 2.15). Plasmid preparation of certain timepoints as well as western blot analysis show appropriate recombination (2.16) and LacI-L'anchor fusion protein expression (fig 2.17). The LacI-L'anchor fusion protein expression is driven by the same promoter (P_{BAD}) as is the expression of ParA resolvase and therefore the successful western blot detection of LacI-L'anchor fusion protein also indicates the expression of parA resolvase.

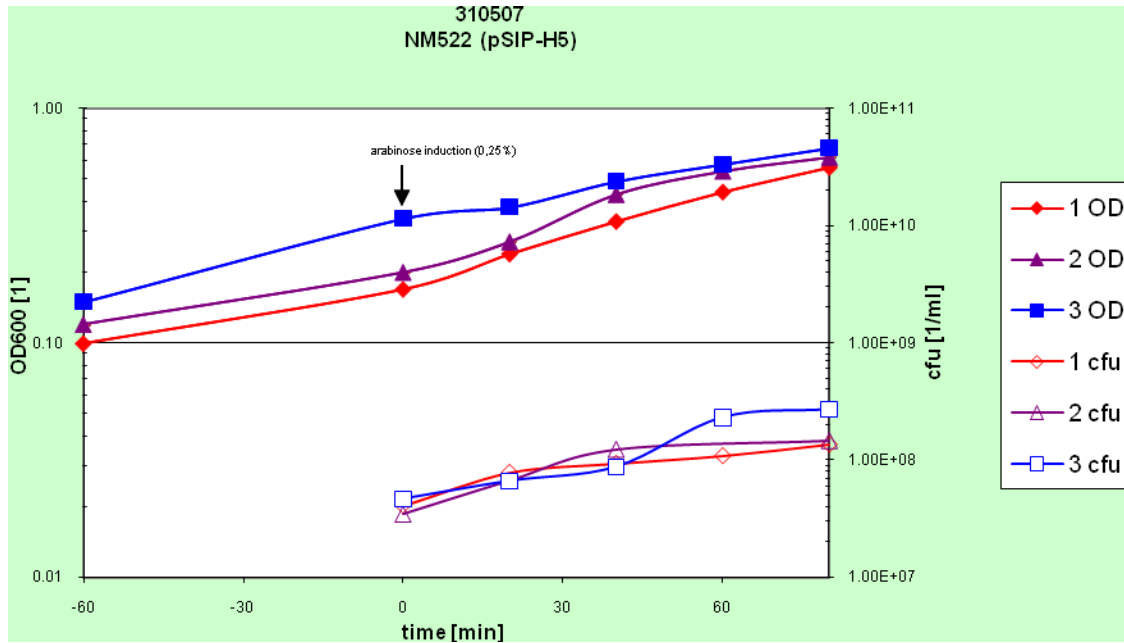


Fig 2.14: OD_{600} measurements and cfu-count of fermentation of 3 *E. coli* NM522 (pSIP-H5) clones. The arrow indicates P_{BAD} promoter induction by addition of 0,25% Arabinose.

To reach an OD_{600} of 0,2-0,3, when recombination is induced by addition of 0,25% arabinose, the medium inoculated with an amount of an overnight culture to reach an OD_{600} of 0,07-0,1 took about 60min. This time window varies slightly.

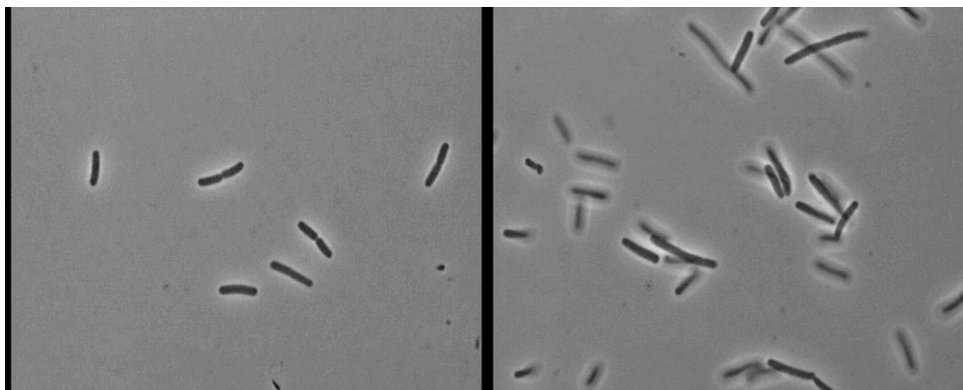
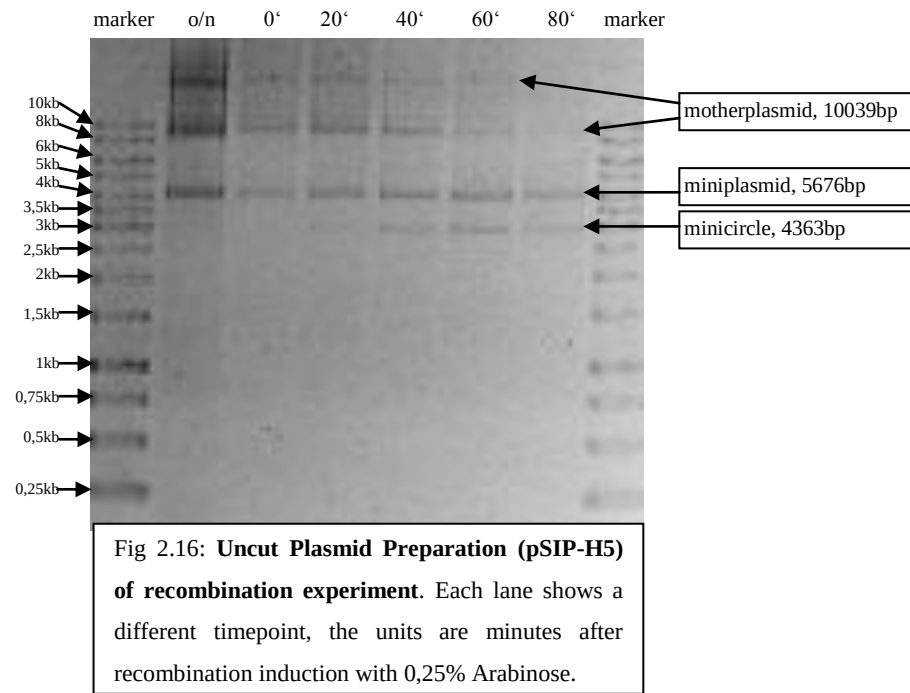
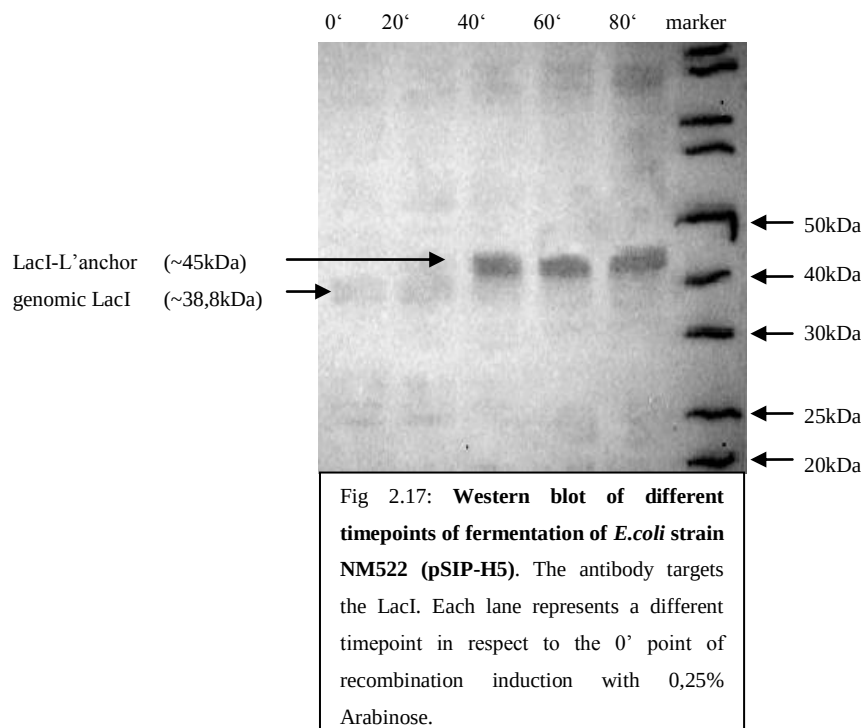


Fig 2.15: *E. coli* K12 strain NM522 (pSIP-H5) cells directly before (left) and 100min after (right) induction of recombination of pSIP-H5. After induction cells are slightly elongated.



The time between addition of 0,25% arabinose and the first appearance of the minicircle lies around 10-20min. After about 1-1,5h of recombination time the motherplasmid band has almost disappeared. After 20min the LacI-L'anchor fusion protein band is already clearly visible, which also indicates ParA-recombinase expression.



Pretestings of pSIP-H5 and pGLysivb double Transformants of *E.coli* K12 strain NM522

Lysis plasmid pGLysivb

For the induction of E-lysis to produce *E.coli* K12 strain NM522 BGs plasmid pGLysivb was used (fig 2.18).

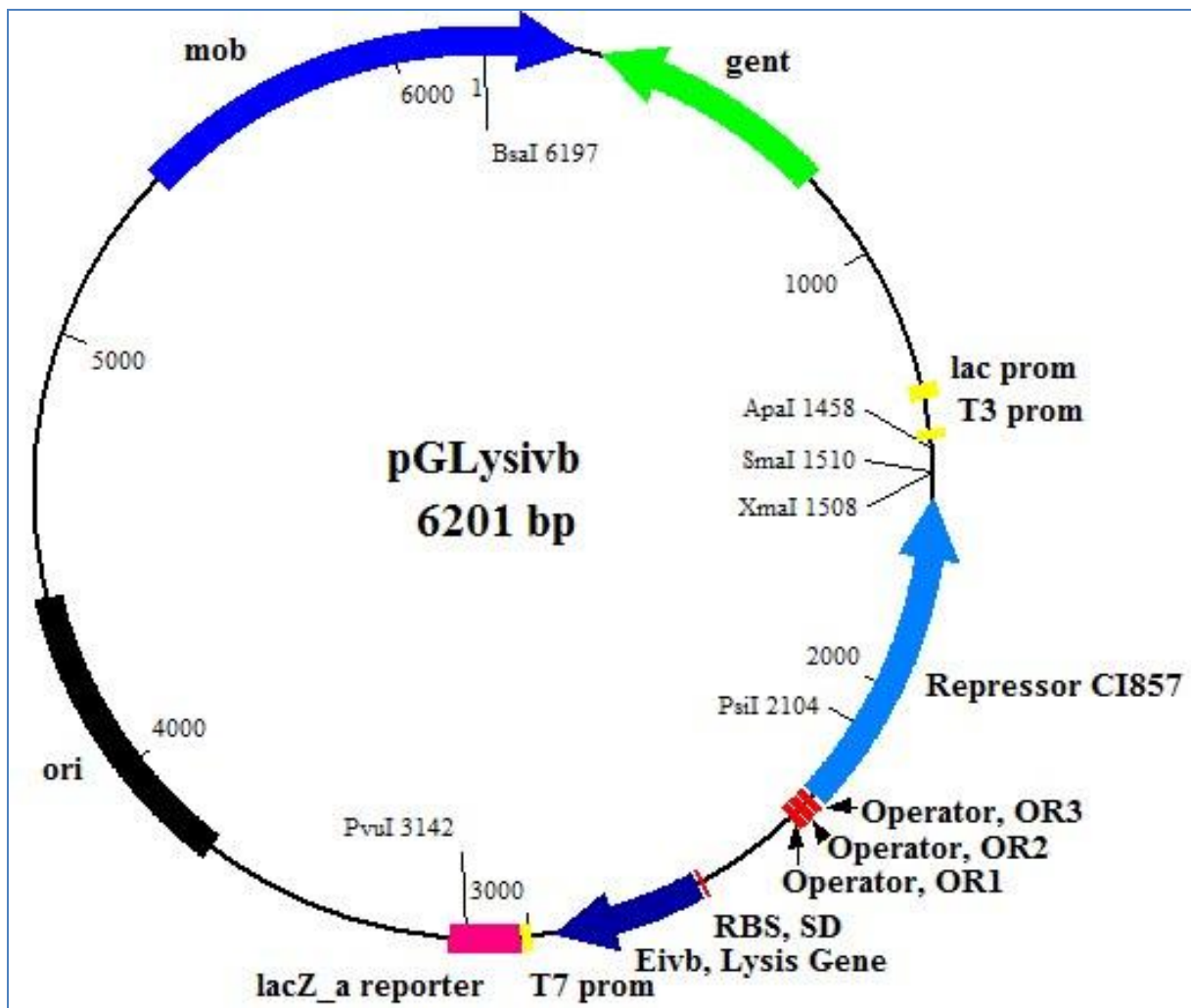


Fig 2.18: pGLysivb plasmid map.

Eivb	Lysis Gene E from Bacteriophage ΦX174, with additional <i>in vivo</i> biotinylation sequence.	ori	Origin of Replication, rep
gent	Gentamycin Resistance Cassette	RBS, SD	Ribosome binding site, Shine-Dalgarno Sequence
lac prom	lac promoter	Repressor ci857	temperature sensitive Repressor for λp_R Promoter
lacZ_a	lacZ_α reporter sequence	T3 promoter	bacteriophage T3 promoter sequence
mob	mobilization sequence (Barlett P 1950)	T7 promoter	bacteriophage T7 promoter sequence
OR1, 2, 3	partitioned λp_R Promoter		

The plasmid pGLysivb was used for its well-investigated properties and its overall lysis efficiency of more than 99,7% (Jechlinger, Szostak et al. 1999). The *Eivb* lysis gene, derived from Phage Φ X174 is under the control of the temperature sensitive λ -P_{mut}-Promoter. This promoter is tightly repressed by the thermally instable interaction with the gene product of the *repressor* *cl857* at the bacterial growth temperature of 36°C and active at 42°C (Standardization 2007), thus enabling propagation of the lytic protein. The selective marker is a Gentamycin resistance cassette.

Successful transformation of *E.coli* K12 strain NM522 with the plasmid pGLysivb is shown on one hand by growth on selective antibiotic agar plates and as well by plasmid preparation (fig. 2.19 a and b).

Transformation of *E.coli* K12 strain NM522 with pSIP-H5 and pGLysivb

MOPS competent *E.coli* K12 strain NM522 was transformed simultaneously with pSIP-H5 and pGLysivb, growing cultures on selective plates and clones were picked. Plasmid preparation showed corresponding bands (fig 2.19 a and b), further investigations combining recombination and lysis were carried out.

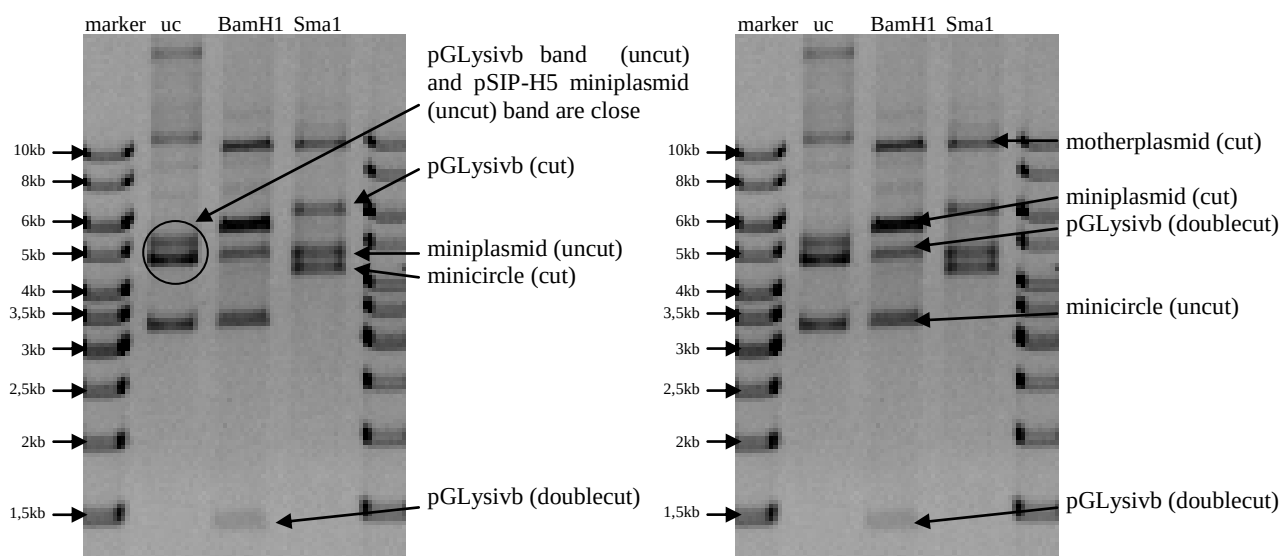


Fig 2.19a: Restriction Enzyme Analysis of *E.coli* NM522 transformed with pSIP-H5 and pGLysivb.

pSIP-H5	motherpl.	miniopl.	minicircle
BamHI	10039bp	5675bp	uncut
SmaI	10039bp	uncut	4364bp

pGlysiVB	
BamHI	4768bp/1433bp
SmaI	6201bp

Fig 2.19b: Restriction Enzyme Analysis of *E.coli* NM522 transformed with pSIP-H5 and pGLysivb:

pSIP-H5	motherpl.	miniopl.	minicircle
BamHI	10039bp	5675bp	uncut
SmaI	10039bp	uncut	4364bp

pGlysiVB	
BamHI	4768bp/1433bp
SmaI	6201bp

For the restriction enzyme analysis the plasmids were prepared from a recombination experiment, which consisted of motherplasmid as well as recombined plasmid. This way the unrecombined as well as the recombined state of pSIP-H5 can be seen.

Recombination of pSIP-H5 and Lysis Experiment

Fermentation of *E.coli* K12 strain NM522 transformed with pSIP-H5 and pGLysivb was carried out. OD₆₀₀ measurements and cfu diagram can be seen in fig 2.16.

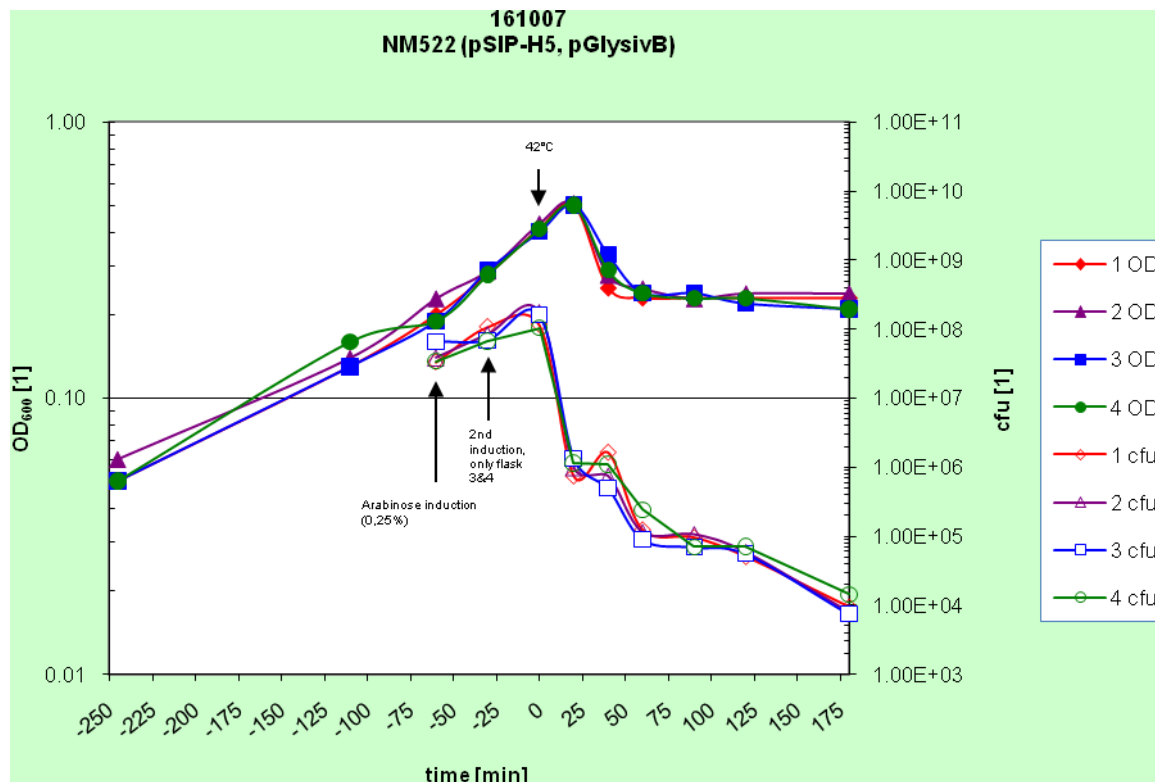


Fig 2.20: **OD₆₀₀ measurements and cfu-count of four different *E.coli* K12 strain NM522 (pSIP-H5) clones.** The arrows indicate addition of 0,25% Arabinose (*induction*, *2nd induction*) to induce recombination of pSIP-H5. A second addition of Arabinose was applied only to clone 3 and 4. Temperature shift from 36°C to 42°C activating E-lysis by induction of the temperature sensitive λ -P_{mut}-promoter controlling the expression of the *Eivb* on pGLysivb is also indicated by an arrow.

The time window from inducing recombination of pSIP-H5 to activating E-lysis was 1h. To ensure a stable concentration of arabinose another addition of the same amount (375 μ l of 20% arabinose for 30ml medium) was applied 30min after the first to clone 3 and 4.

About 20 minutes after the time of activation of the pGLysivb by a temperature shift from 36°C to 42°C, corresponding to 1h after induction of recombination of pSIP-H5, cfu-count drops, followed by

a drop of OD₆₀₀ measurements about 40min later. The cfu curve shows an average lysis efficiency of 99,992%.

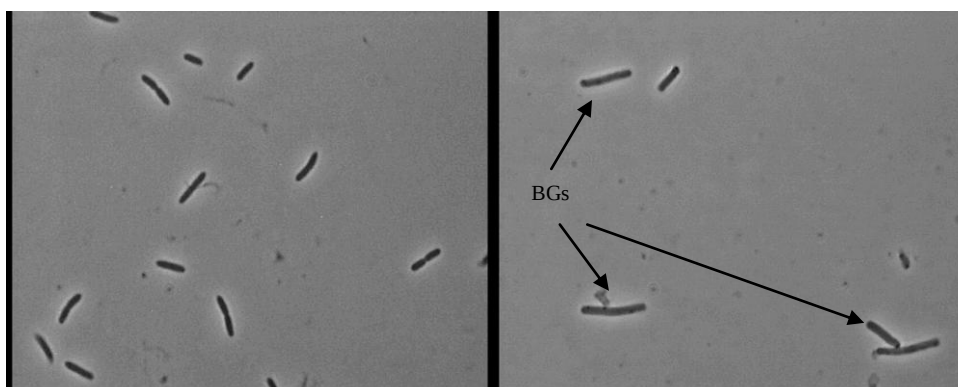


Fig 2.21: E.coli K12 strain NM522 cells harboring pSIP-H5 and pGLysivb before and after E-Lysis induction. The left picture shows the cells 60min after recombination induction of pSIP-H5 by an addition of 0,25% Arabinose and immediately before lysis induction. The right picture shows BGs 180min after E-Lysis induction by temperature shift from 36°C to 42°C. Cells after lysis induction are transparent.

In the left picture *E.coli* cells can be seen before activation of pGLysivb by temperature shift from 36°C to 42°C. In the right picture the microscopy sample was taken 180 minutes after temperature shift displaying BGs. Plasmid pSIP-H5 recombination and expression of LacI-L' anchor were observed by plasmid preparation and western blotting (figs 2.22 and 2.23).

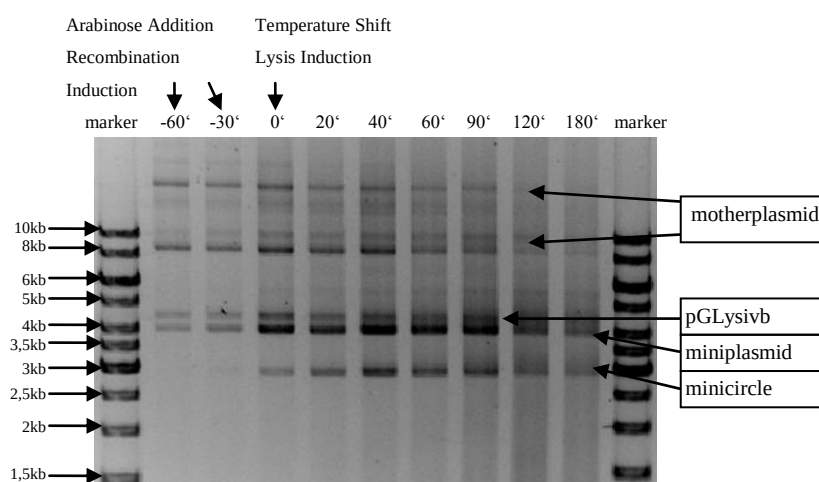
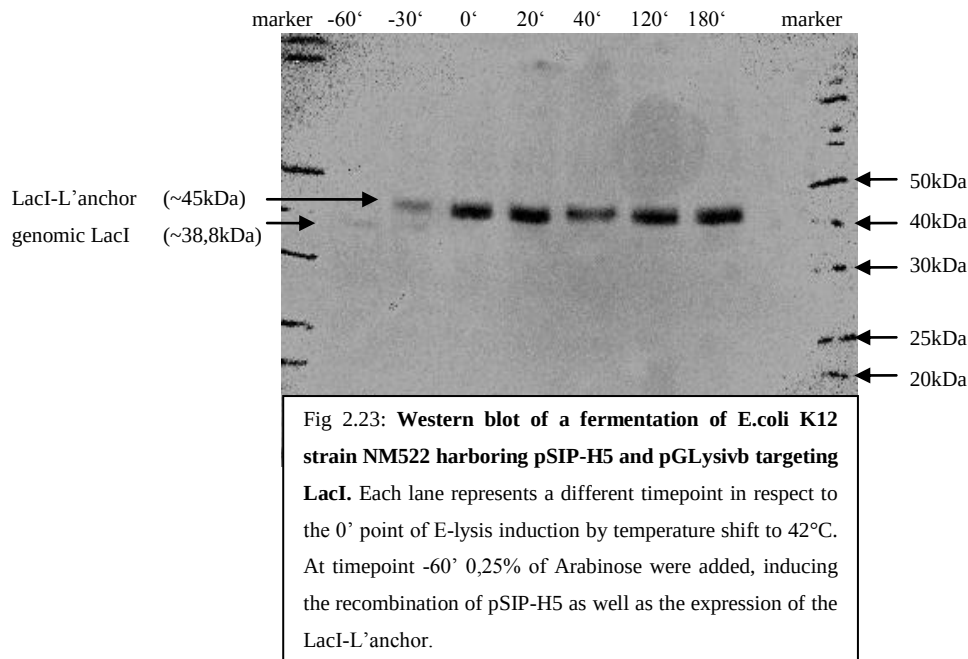


Fig 2.22.: Uncut Plasmid Preparation of *E.coli* K12 strain NM522 (pSIP-H5, pGLysivb). The arrows indicate the addition of 0,25% Arabinose inducing the recombination of pSIP-H5 as well as the expression of the LacI-L' anchor fusion protein. Also the Temperature shift from 36°C to 42°C is indicated by an arrow. The lanes show different timepoints of the fermentation, [min in respect of temperature shift (at 0 minutes)].

The first slight minicircle band can be seen 30min after induction of recombination of pSIP-H5. Also the sample of -60min (lane 2) shows a faint miniplasmid band, which points to recombination of pSIP-H5 already in the o/n culture.



In the western blot the first LacI-L'anchor fusion protein band (~45kDa) is visible 30min after addition of 0,25% arabinose (lane 3, tp -30'). After the second addition of 0,25% arabinose (lane 4, 0') the band appears stronger. Also the genomic LacI is visible at the time of arabinose addition and at -30' but vanishes later on.

Fermentation of *E.coli* strain NM522 (pSIP-H5, pGLysivb)

Fermentation Process

Two clones of *E.coli* K12 strain NM522 harboring pSIP-H5 and pGLysivb were disponible for fermentation in the 30l bioreactor. The state of recombination of plasmid pSIP-H5 of different glycerin stocks was tested by restriction enzyme analysis, to decide with which clone to carry on (fig 2.24).

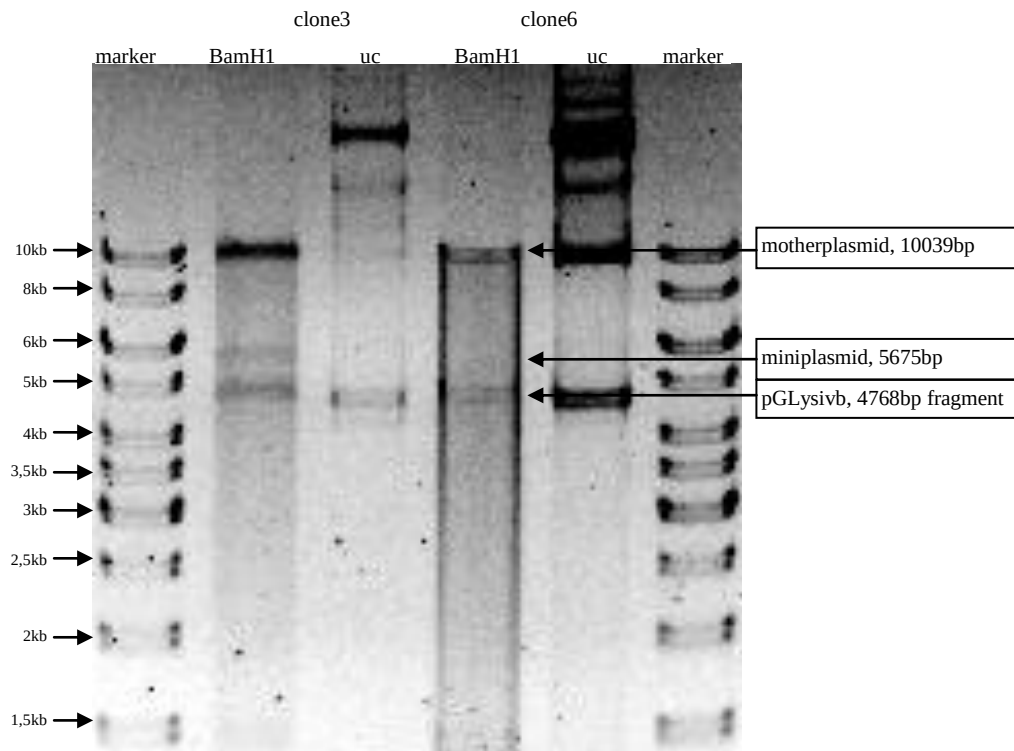


Fig 2.24: Restriction Enzyme Analysis of two clones of E.coli K12 strain NM522 harboring the plasmids pSIP-H5 and pGLysivb.
Clone 3 shows a slight miniplasmid band. No miniplasmid band is visible in the lane of clone 6.

Expected fragment length:

pSIP-H5	motherplasmid	miniplasmid	minicircle
BamHI	10039bp	5675bp	uncut
pGLysivB			
BamHI	4768bp/1433bp		

Clone 6 shows only the 10039bp band of the unrecombined motherplasmid of the pSIP-H5, clone 3 also shows a slight miniplasmid band at 5675bp, which indicates uninduced recombination of pSIP-H5 in the o/n culture. For these reasons clone 6 was chosen for the large scale fermentation.

Inoculation culture showed an OD_{600} of 0,981, the 1:10 dilution showed an OD_{600} of 0,122. After inoculation of the fermenter, the medium within the fermenter showed an OD_{600} of 0,05. The fermentation process was closely observed by the IRIS program (fig 2.25).

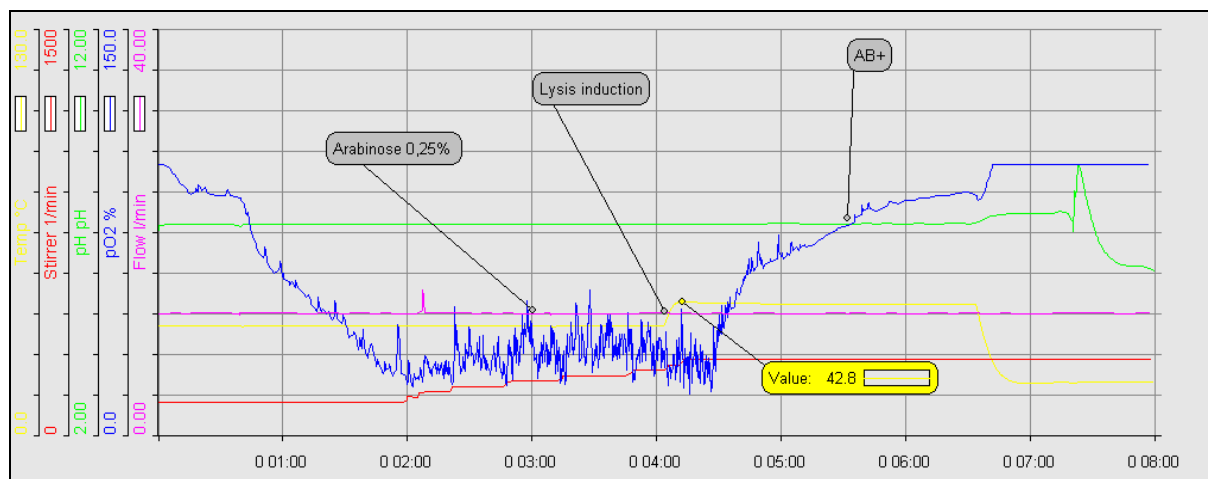


Fig 2.25: **Observation of the fermentation of E.coli K12 strain NM522 harboring pSIP-H5 and pGLysivb.** Recombination of pSIP-H5 was induced by addition of 0,25% Arabinose after three hours of fermentation. E-Lysis induction by temperature shift followed one hour later after 4h of fermentation. The final killing of all bacteria was carried out by addition of antibiotics. Curves show temperature [°C](yellow), stirring frequency [1/min](red), pH value (green), air flow [l/min](purple) and partial Oxygen pressure [%](blue).

The IRIS diagram shows pH-value, Oxygen concentration, airflow-rate, stirring per minute and temperature. After three hours of fermentation, at an OD_{600} of 0,6 0,25% arabinose was added, inducing the P_{BAD} promoter and thus the expression of ParA recombinase as well as the LacI-L'anchor fusion protein. pH is stable as is the temperature and the air flow. The stirring frequency increases steadily to keep pO_2 above 5%. The value of the partial Oxygen pressure indirectly corresponds to the growth of the culture metabolizing Oxygen. About 30 minutes after lysis induction the pO_2 value starts to rise, pointing to the dying of the bacteria. 90 minutes after induction of lysis surviving cells are killed with antibiotics.

Further observation of fermentation can be found in fig 2.26.

NM522pSIP-H5-Ghosts

Date: 29.11.2007	Clone: 6 (21.09.07)
Starting time: 9:00	Strain: NM522
End time: 19:00	Plasmids: pGLysivb, pSIP-H5
ON culture OD: 0,981(pure)/0,122(diluted 1:10)	Recombinant Protein Expression: LacI-L'anchor / ParA Recombinase
Inoc. Volume: 2 L	Expression Induction: 0,25% arabinose
Medium: LBv	Expression Induction Timepoint: G
Antibiotics: Gent + Amp	Lysis Induction: 42°C
Temperature: 35°C	Lysis Induction Timepoint: I
Total Volume: 22 L	Killing: Strep / Tet (L)
Acid: F.A.: 0ml	Volume harvested: ~20l
Base: A.W.: 0ml	Harvested by: Separator, washing H₂O and 0,5mM saline
Antifoam A: 0ml	OD separator flowthrough: 0,11 / 0,063/0,053/0,047
E-Blot: not performed	Yield: 1070mg
R-Blot: OK (by MRA)	Particles / mg: 7,57E08 (ww); 2,41E08 (sw)

RT:	Sterility: OK by AFA
Microscopy: ghosts	Efficiency: 99,92%

timetable

Time	Minutes	Sample	Microscopy	OD	OD 1/10	pH	Flow/pO2	rpm	Temp
		ON		0,981	0,122				
11:00	-240'	A		0,05		7,2	12	120	35°C
11:30	-210'	B	alive; elongated	0,165		7,2	12	120	35°C
12:00	-180'	C		0,21		7,2	12	120	35°C
12:30	-150'	D	alive; elongated	0,32		7,2	12	120	35°C
13:00	-120'	E		0,382		7,2	12	140	35°C
13:30	-90'	F		0,451		7,2	12	160	35°C
14:00	-60'	G	nice, short cells; elongated cells in minority	0,58		7,2	12	180	35°C
14:40	-30'	H	OK	0,65		7,2	12	220	35°C
15:00	0'	I	single dead cells OK	0,853	0,109	7,2	12	240	42°C
15:30	30'	J	ghosts	0,766	0,089	7,2	12	280	42°C
16:00	60'	K	ghosts	0,578	0,07	7,2	12	280	42°C
16:30	90'	L	OK	0,533	0,068	7,2	12	280	42°C
16:45	105'	M		0,562	0,06	7,2	12	280	42°C
17:00	130	N		0,563	0,064	7,2	12	280	42°C
17:15	145	O		0,494	0,06	7,2	12	280	42°C
17:30	160	P		0,514	0,059	7,2	12	280	42°C

Notes:

arabinose (0,25%) was added to the vessel at the time point G.

Lysis was induced by temperature upshift to 42°C at timepoint I.

RT samples (2x) were taken every time point (starting from B) and stored at -80 °C.

20l were treated with Streptomycin and Tetracycline (double dose) for 60 min (samples were taken at the point 0' and then every 15 min).

WB samples were taken every time point starting from B to the end of process except M and O.

fig 2.26: Fermentation-report

The overall growth of *E.coli* K12 strain NM522 was observed by OD₆₀₀ measurements and cfu count (fig 2.27).

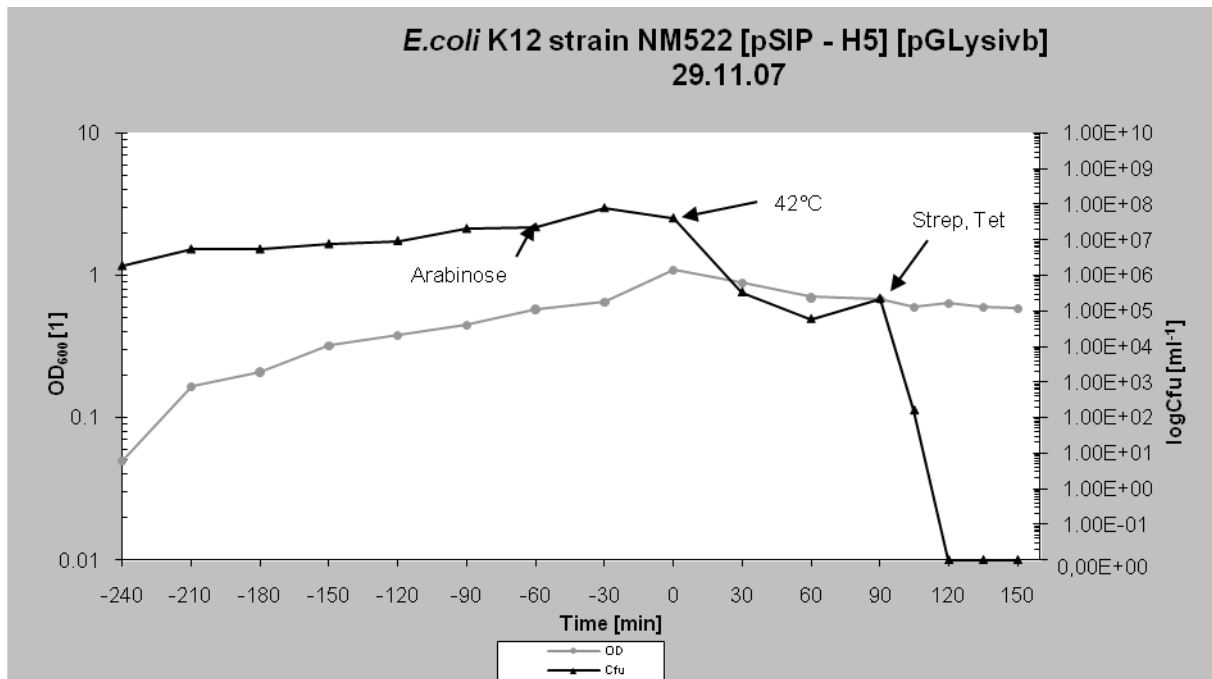
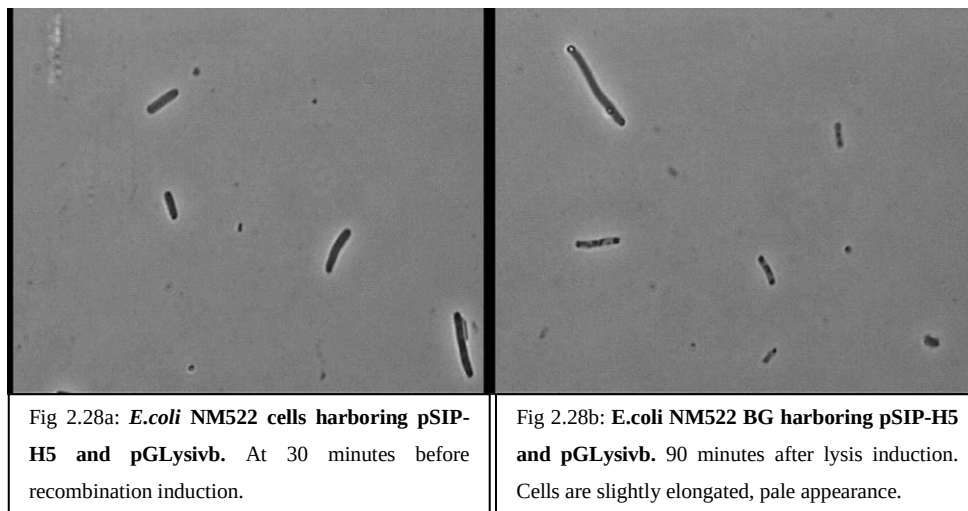


Fig 2.27: OD₆₀₀ measurements and cfu count of fermentation of *E.coli* K12 strain harboring pSIP-H5 and pGLysivb. After 3 hours of fermentation 0,25% Arabinose were added (arrow) inducing recombination of pSIP-H5. After 4 hours of fermentation E-Lysis was induced (arrow). After 90 minutes of lysis, surviving cells were killed by antibiotic addition.

Quality Control of large scale Fermentation

To confirm recombination as well as expression of the LacI-L' anchor, lysis and bacterial appearance, the results of microscopic observation (fig 2.28a&b), plasmid preparation (fig 2.29) and western blot targeting LacI (fig 2.30).



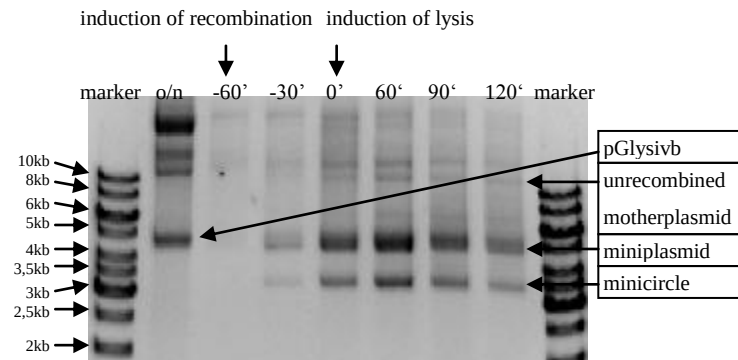


Fig 2.29: **Plasmid preparation at different timepoints of fermentation of E.coli K12 strain NM522 harboring pSIP-H5 and pGLysivb.** The o/n bands appear stronger due to dilution differences. After 4 hours of fermentation (0') E-lysis is induced by temperature shift of 36°C to 42°C. Miniplasmid and minicircle bands appear about 30 minutes (-30') after addition of 0,25 Arabinose, inducing recombination of pSIP-H5

The miniplasmid and minicircle band appear at the timepoint -30 minutes, 30 minutes after P_{BAD} activation by arabinose induction. Due to the very similar molecular weight of miniplasmid and pGLysivb the two bands cannot be kept apart without restriction enzyme digest. After 220min the motherplasmid band has almost vanished, thus leaving only miniplasmid and minicircle, a sign of efficient recombination. However, after lysis the miniplasmid is still present and apparently not fully washed out of the ghost. A former fermentation product washed with ddH₂O did not show sufficient extraction of the remaining miniplasmid, therefore the harvested material was divided and either washed with water or with 500mM saline, to extract the remaining miniplasmid from the shell (fig 2.27).

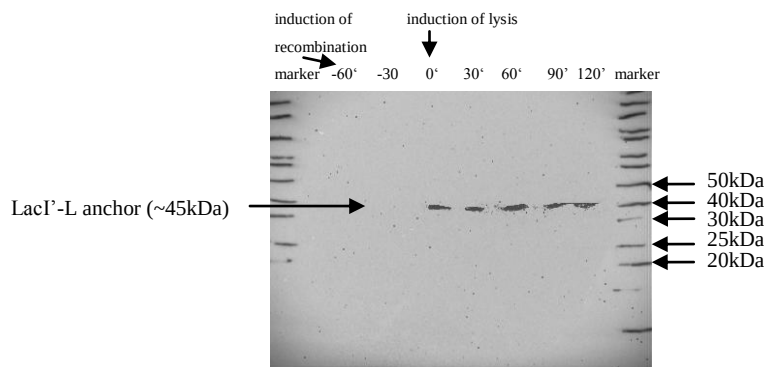


Fig 2.30: Westernblot of fermentation of E.coli K12 strain NM522 harboring pSIP-H5 and pGLysivb. The antibody targets LacI. Each sample is taken at a different timepoint in respect to the 0min point of lysis induction. Recombination of pSIP-H5 is induced 60 minutes before lysis.

The western blot shows LacI-L'anchor expression 60min after arabinose addition, which corresponds to 4 h of fermentation. With the expression of the LacI-L'anchor the pSIP-H5 minicircle is fixated within the bacterial shell and kept from expulsion.

Washing of the BGs

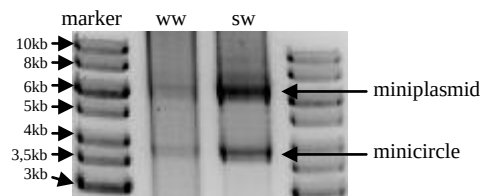


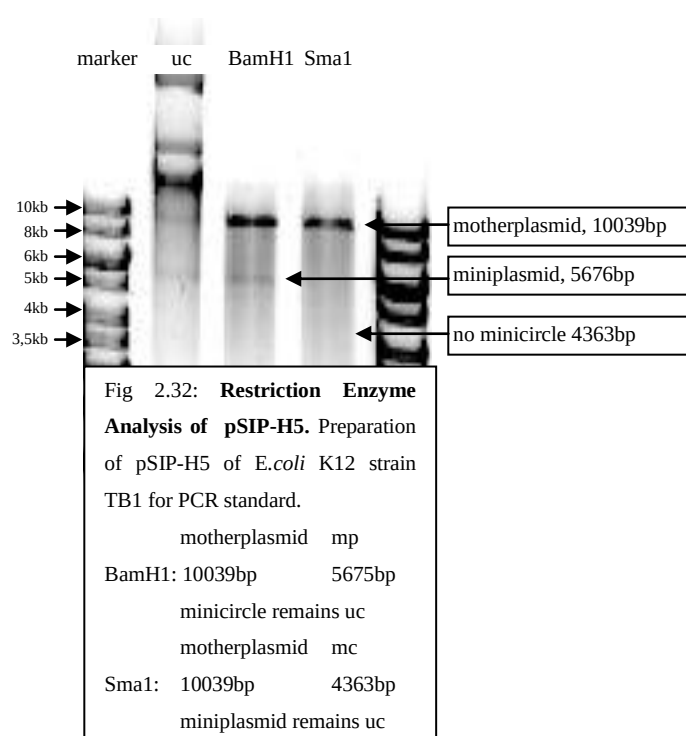
Fig 2.31: Plasmid Preparation of BGs. The harvested BGs were either water-washed (ww) or saline-washed (500mM) (sw).

Harvested ghosts were washed either with water or with saline (500mM). Solubility of the BGs in saline was far less than in water. Plasmid Preparation shows bands of the minicircle as well as the miniplasmid (fig 2.31). The expected result was for the saline washed BGs to contain less miniplasmid due to favoured extraction of randomly bound miniplasmid to LacI-L'anchor fusion protein bound minicircle.

Quantification of minicircle DNA using realtime-PCR

Realtime-PCR Standard targeting pSIP-H5 minicircle

After unsuccessful tries to extract clean minicircle from purifying agarose gel as a standard for the rt-PCR, purified, unrecombined motherplasmid was used instead, taking into account a molecular weight relation of 0,4284. Plasmid preparation of the *E.coli* K12 strain TB1, which was formerly transformed with pSIP-H5 and had shown very tight repression of recombination, was control cut (fig 2.32) to confirm plasmid status. The DNA concentration in the preparation was evaluated by OD_{260/280} measurement and used as rt-PCR standard. Primers used were *fwdLacOs* [5'-gac ttc gaa atg gaa aag atc gtg ctg-3'] and *revLacOs* [5'-ata aca tta taa tgc tag ata act tcg tat aa-3'].



Although this plasmid preparation shows a slight miniplasmid band in the BamH1 lane, it was used as rt-PCR standard. A control rt-PCR was carried out with the standard preparation alone in different dilutions (fig 2.33).

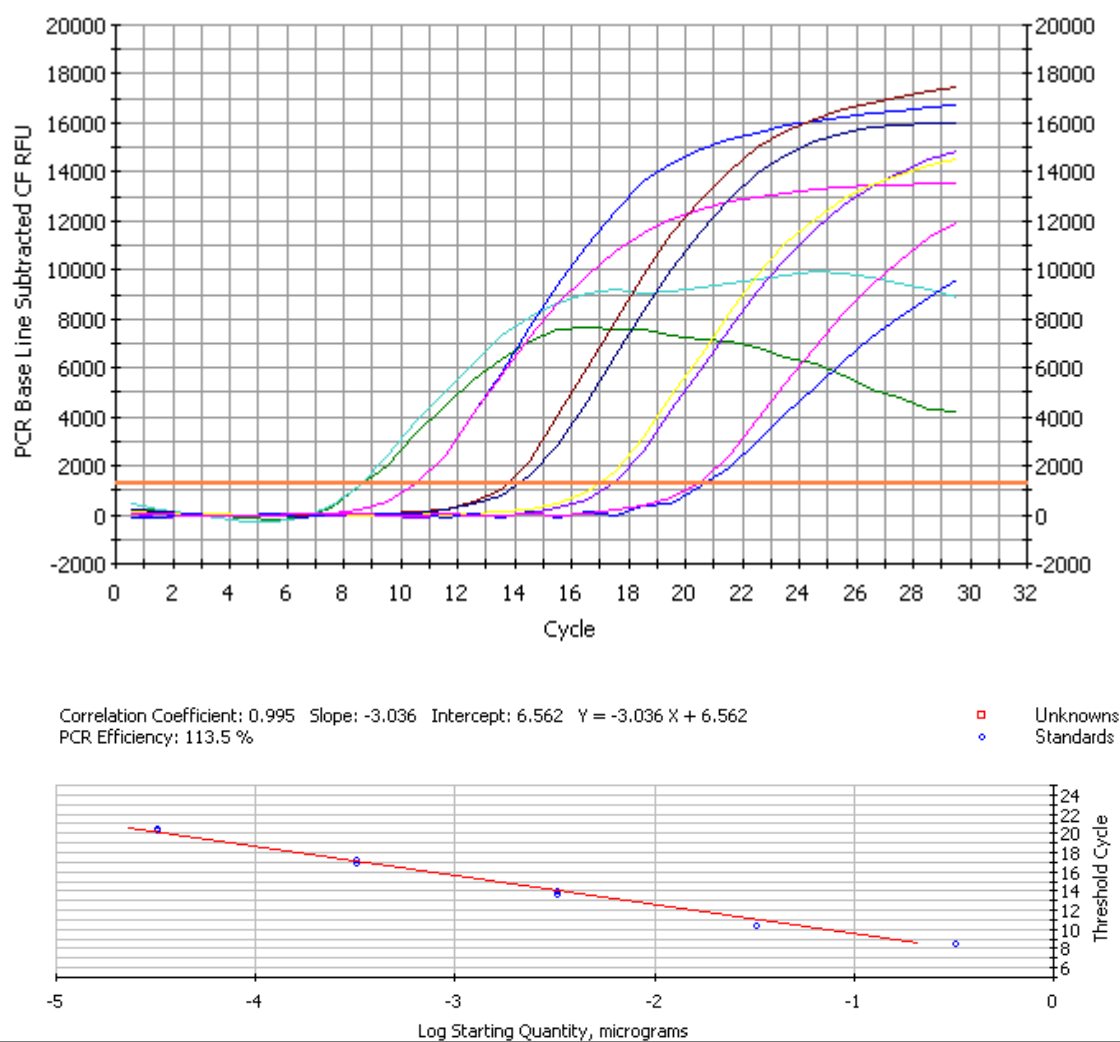
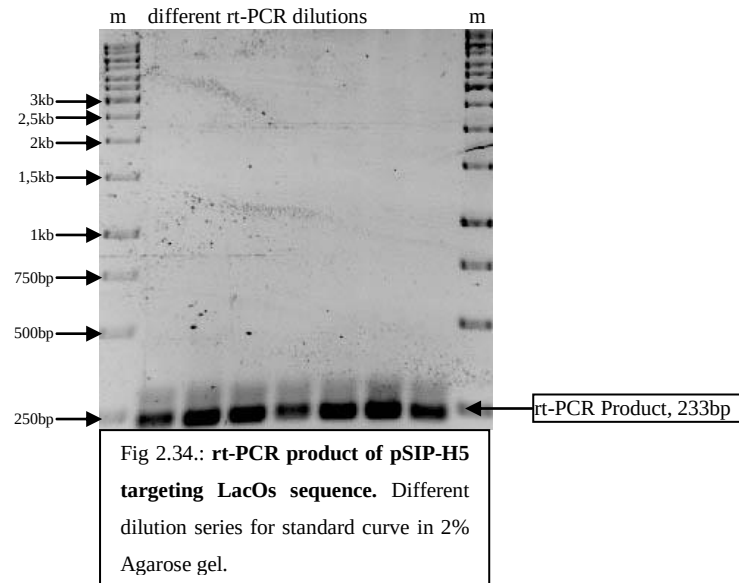


Fig 2.33: **BioRad® iCycler rt-PCR process observation.** Realtime PCR-run of standard dilutions. Targeted sequence was LacOs, situated on the minicircle part of the motherplasmid pSIP-H5 (DNA used: motherplasmid, pSIP-H5).
 Above: Graph: Amplification per cycle. For each concentration two wells were used.
 Below: Standard curve with a correlation coefficient 0,995.

The outcome of the control rt-PCR of the pSIP-H5 standard dilution series shows a correlation coefficient of 0.995 and a PCR efficiency of 113.5%. The length of the PCR product was further checked by agarose gel electrophoresis (fig 2.34).



The gel electrophoresis shows the band at 233bp in all samples. The preparation was used to create a standard curve based on dilution series, to be able to quantify the amount of minicircle loaded in the BGs.

rt-PCR using BGs as sample

To quantify the amount of propagated and fixated minicircle within the BGs, a realtime PCR was carried out, targeting the LacOs sequence, which is present on the minicircle (and the motherplasmid). Subjected to the rt-PCR were whole BGs loaded with pSIP-H5 minicircle, from both water washed as well as saline washed harvested material (2.31).

Following can be found the documentation of the iCycler rt-PCR observation (2.35).

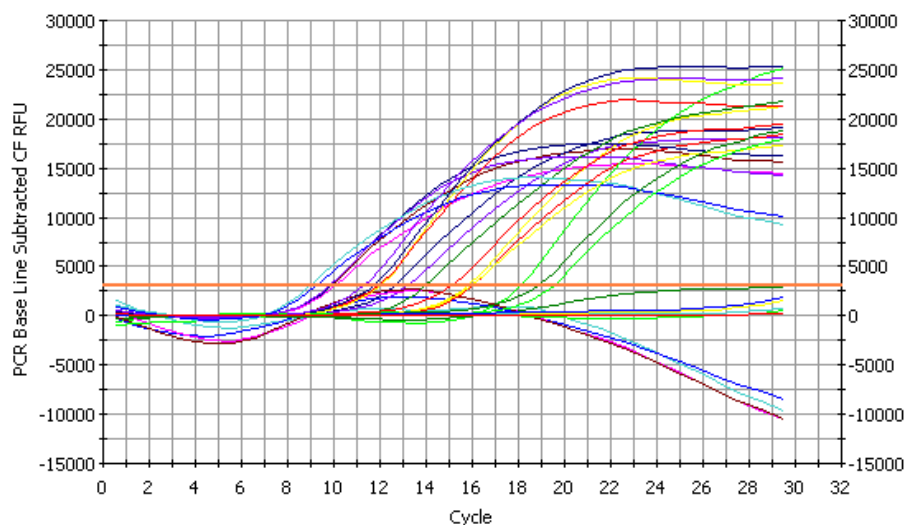


Fig 2.35: **rt-PCR of pSIP-H5 minicircle loaded BGs, targeting the LacOs sequence.** Different lines show standard curve samples as well as BG samples of unknown minicircle DNA concentration.

The threshold is set by the program itself, calculating the intersection which produces the highest correlation coefficient of the functions in order to get the most accurate standard curve (fig 2.36), rendering samples below the threshold invalid.

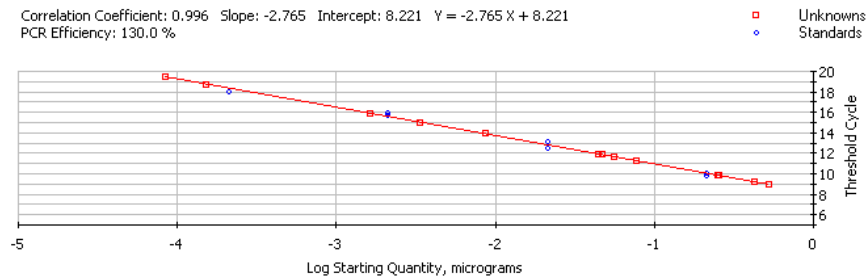


Fig 2.36: **Standard curve of rt-PCR targeting LacOs sequence.** Whereas the blue circles show the standard dilutions, the red squares depict the Unknowns, which are also applied in dilutions.

The Correlation Coefficient is 0,996, the PCR efficiency is 130%. The melting curve can be found below (fig 2.37).

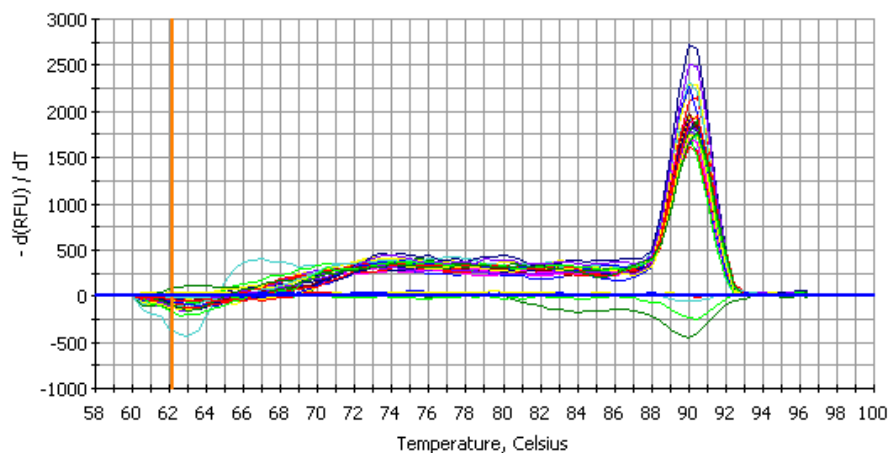
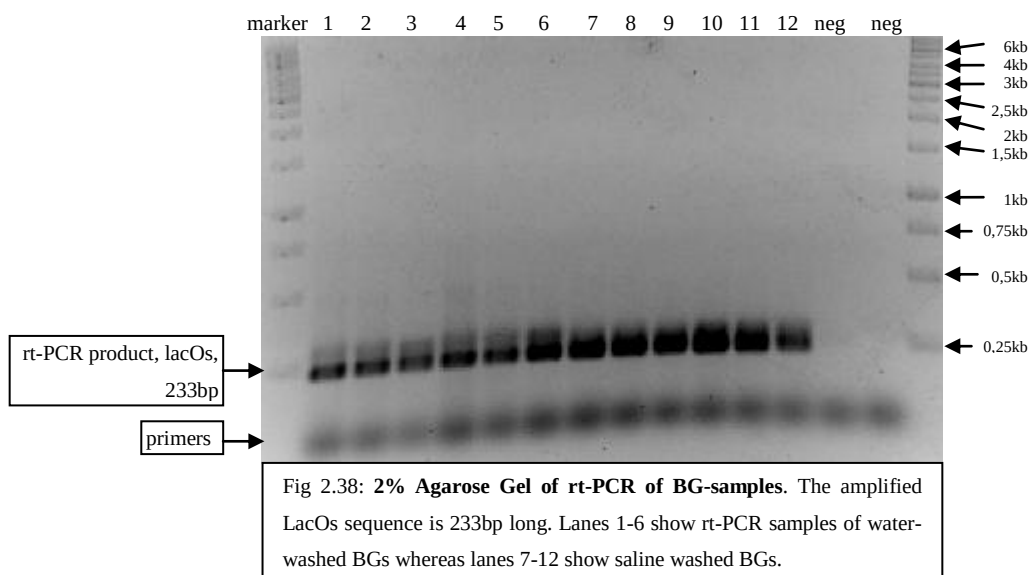


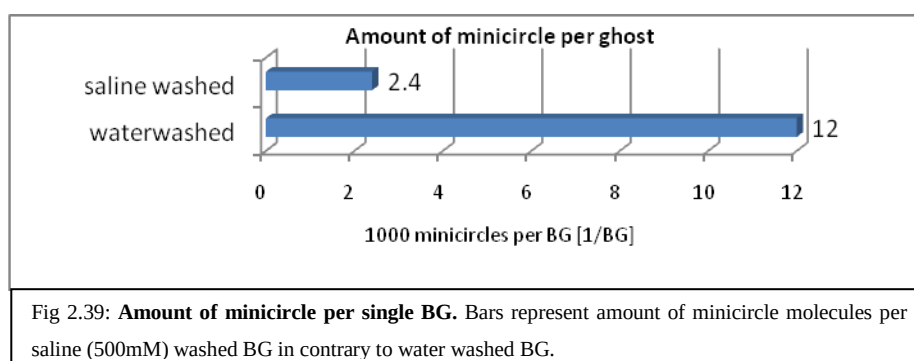
Fig 2.37: **Melting curve of rt-PCR targeting LacOs sequence of BGs loaded with pSIP-H5 minicircle.** Samples are the standard dilutions as well as BGs loaded with an unknown minicircle concentration.

Agarose gel electrophoresis of the standard curve dilution samples as well as of the BG-samples loaded with pSIP-H5 minicircle shows bands at 233bp (fig 2.38).



PCR was positive for all tested samples, water washed (lanes 1-6) and saline washed (lanes 7-12) alike.

The calculated amount of minicircle fixated in the BG based on the results of the rt-PCR lies between 44,7 μ gDNA/mg lyophilised material for water washed BG and 3,2 μ gDNA/mg lyophilised material for saline washed BG (2.39).



Sterilizing Process after Fermentation

Standard Sterilizing Procedure

Safety guidelines for biotechnology products proposed by the WHO demand a reliable prevention of any bacterial growth, infectivity (in case of conventional vaccine production) or other form or possibility of DNA propagation in biotechnology products (Lubitz 2008). The killing-efficiency of E-lysis in *E.coli* ranges up to about 5 logs. Several different methods are in use of achieving total inactivation. Besides the snuc-technique (recombinant *in situ* expression of staphylococcal nuclease) (Morgeaux 1995) and different antibiotics, β -Propiolactone (BPL) (fig 2.40) poses a consistent possibility of deactivating any further growth or propagation. Easily hydrolyzed in aqueous environment, it is a strongly alkylating agent, which interacts with nucleic acids, proteins and any other nucleophilic reagent (Savel'ev 1966; Pedreira and Tauraso 1969; Daly, Orszulak et al. 1991; Brault, Bouilly et al. 1996; Scheidler, Rokos et al. 1998; Brault, Renault et al. 1999; Stittelaar, Boes et al. 2000). BPL is widely used as disinfecting agent from sterilizing cultivation apparatus for microorganisms over plasma sterilization of blood and tissue transplants to vaccine production (Mayrhofer, Tabrizi et al. 2005).

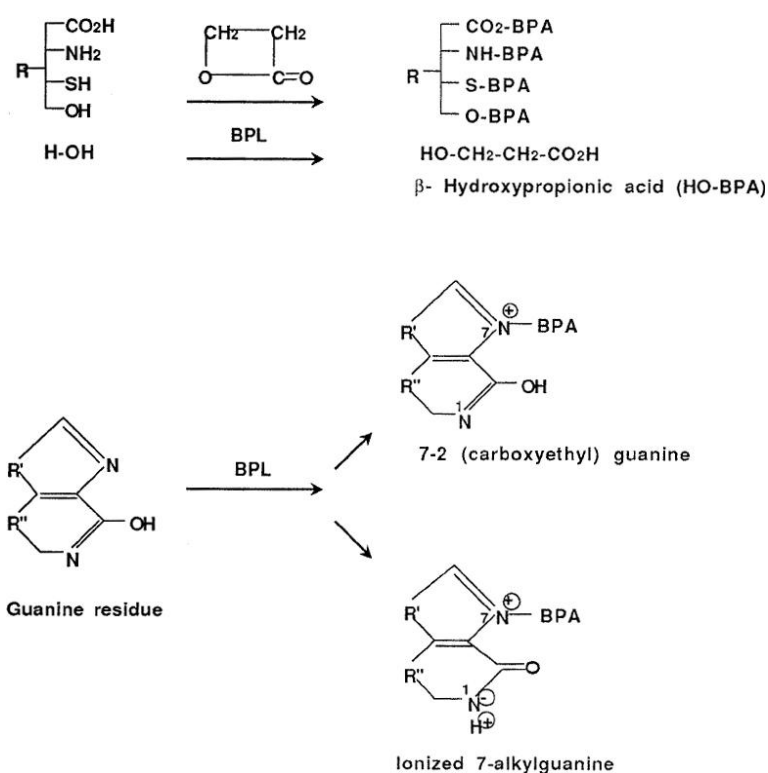


Fig 2.40: **Mode of action of β -Propiolactone**

Above: general mode of β -Propiolactone reaction on nucleophilic reagents.

Below: Alkylation of Guanine residue.

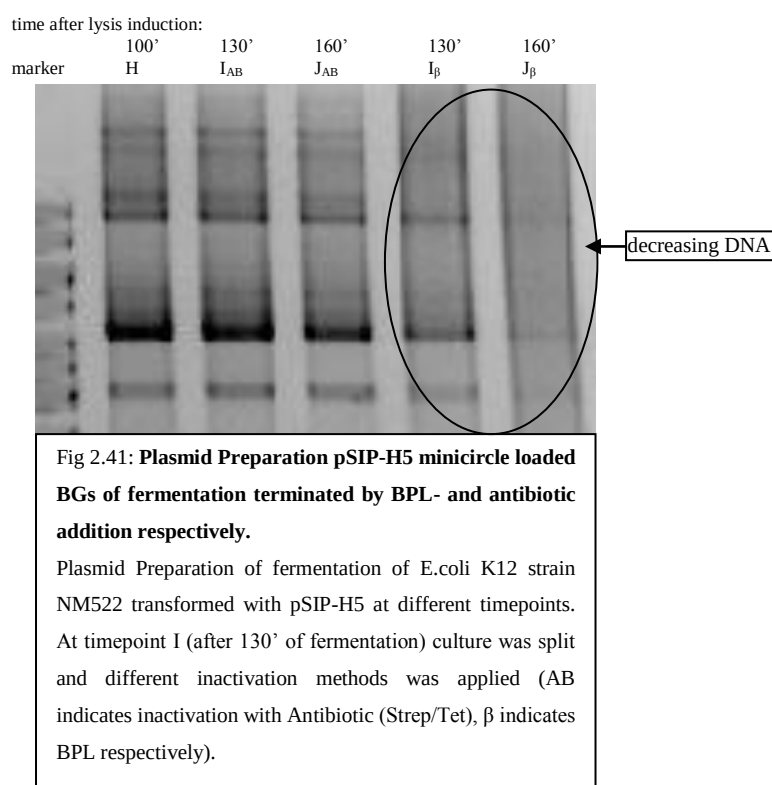
adapted from [Barlett, P., 1950]

The mode of action and the impact of this agent on DNA highly depend on applied concentration and temperature and reaches from weak alkylation of mostly Guanine residues to complete destruction of nucleosides leaving only the phosphate-backbone unharmed. Due to its relative instability the effect of BPL itself is very transitory, and after hydrolization it completely loses its toxicity, rendering beta-hydroxy-propionic acid (fig 2.40).

The concentration used for final bacterial inactivation of the fermentation broth is 0,0375%; this dose of 7,5ml BPL (20l culture) is added twice with a 30 minutes time gap at the end of fermentation.

Decrease in DNA Quality through the Use of β -Propiolactone

Only a few applications of BGs involve the later on-use of DNA within the BG. It was found that for the *in vivo* loading of BGs with pSIP-H5 minicircle, the use of BPL as killing agent is not suitable due to DNA decrease (fig 2.41).



BPL-DNA Interaction Experiment

Since these findings were not anticipated, questions were raised on whether the decrease is a direct effect of the BPL or rather a result of other factors on BPL-treated DNA. The conjecture is that bacterial enzymes are responsible for the degradation of DNA treated with BPL in this distinct concentration. To prove this hypothesis a DNA preparation was incubated with sterile-filtered medium

of different fermentation timepoints (before and after lysis) and BPL was added. The DNA again was retracted and checked by agarose gel electrophoresis (fig 2.42).

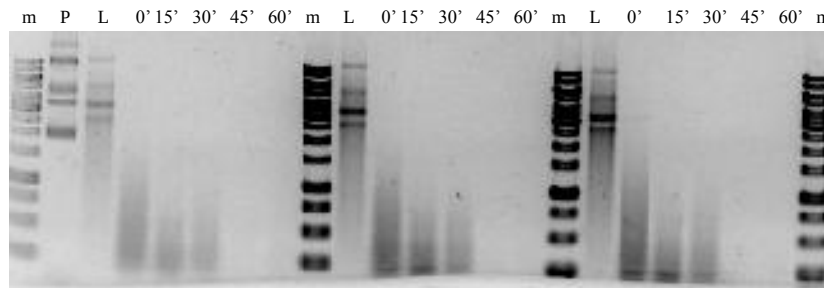


Fig 2.42: **DNA Preparation (pUC18) after being incubated with β -Propiolactone in sterile filtered cultivation medium.** The different lanes represent different fermentation timepoints at which the medium for incubation is taken from the fermenter (fermentation of *E.coli* NM522 (pSIP-H5 and pGLysivb)) and sterile-filtered; β -Propiolactone was added and this medium then was used to incubate with the DNA preparation. L corresponds with the timepoint of Lysis induction (immediately before), whereas the minute indications correspond to time after Sterilization with Antibiotic.

It was found that before Lysis, where the medium is mainly free of any cytoplasmic content (i.e. enzymes), the BPL alone does not have any influence on the length of the DNA (lanes L). Although there is a difference in band pattern to the original DNA (lane P), the molecular weight of the DNA is not greatly influenced. On the contrary, 2h after induction of Lysis (at the time when antibiotic was added), i.e. the medium now carries much cytoplasm expelled from bacteria, the degradation of DNA is obvious. After 45' no more DNA can be found on the gel.

The conclusion is, that BPL alone in the concentration used (0,04%) does not harm DNA directly (lane L), but rather renders it subject to enzymatic degradation by bacterial enzymes (lanes 0' to 60'). In any case, for our purposes of generating pSIP-H5 minicircle harboring *E.coli* BGs, BPL is no useful inactivation agent due to uncertain interference with DNA ranging from alkylation of Guanin residues to breakdown of the Pphosphate-ribose backbone.

Discussion

Overview

The aim of this work was the construction of the pSIP-H5 plasmid out of the pSIP-Ova and the pEPH5 plasmid, transform it into a suitable host bacterium, perform E-lysis and thus obtain BGs loaded with pSIP-H5 minicircle in a one-step fermentation. The BGs loaded with the minicircle, which harbors a eukaryotic H5-expression-cassette, consequently function as carrier for a DNA vaccine against Avian Influenza Virus type H5N1 for poultry.

Several steps led to this object and are here discussed separately.

- Construction of the plasmid pSIP-H5
- Transformation of the plasmid pSIP-H5 into a suitable host bacterium
- Transformation of the plasmid pGLysivb into the pSIP-H5 carrying strain
- Fermentation of *E.coli* K12 strain NM522 (pSIP-H5, pGLysivb) in the 30l bioreactor to produce pSIP-H5 minicircle loaded BGs for cell culture or animal testing
- Quantification of pSIP-H5 minicircle molecule per BG

Construction of the plasmid pSIP-H5

The amplification product of the H5 gene, derived from the pEPH5 plasmid, by PCR was sent to Mycosynth®, Switzerland, for sequencing. The result showed 62bp not documented in the sequence provided by LAH, Germany ([fig 2.6](#)). The additional 62bp are found in a non-coding region and did not have any measurable effect on constructing the plasmid pSIP-H5 ([fig 2.8](#)). What effect this might have on the expression of the H5 gene in targeted eukaryotes can only be investigated by cell-culture- or animal tests. Furthermore two single-cut restriction enzyme sites were introduced with the amplification, BstBI and PsiI ([fig 2.8](#)). The properties of pSIP-Ova ([fig 2.1](#)), from which the SIP backbone (Laham 2004; Mayrhofer, Tabrizi et al. 2005) derives, were tested in growth, expression of the ParA as well as the LacI-L' anchor fusion protein and recombination ([figs 2.2, 2.3](#)) before the OVA gene was replaced by the H5 gene. Expression of the LacI-L' anchor fusion protein, controlled by the P_{BAD} promoter occurs about 30 minutes to one hour after induction by arabinose ([fig 2.2](#)). Also the efficiency of recombination of pSIP-H5 by parA resolvase is confirmed by plasmid preparation. No motherplasmid could be detected 70 minutes after induction of recombination ([fig 2.3](#)). These findings are comparable to former publications (Laham 2004).

After the insertion of the H5 gene into the SIP backbone and transformation of the plasmid into *E.coli* K12 strain NM522 a restriction enzyme analysis showed slightly larger bands than anticipated due to the sequence provided by LAH ([fig 2.5](#)). The reason was the 62bp fragment found in sequencing.

Transformation of the plasmid pSIP-H5 into a suitable host bacterium

After transformation of pSIP-H5 into several different *E.coli* and *Salmonella* strains plasmid preparation showed premature recombination in the *E.coli* strains ER2566, DH10 β and Top10 ([fig 2.10a](#)) as well as in the *Salmonella* strain ATCC 14028 ([fig 2.10b](#)). Several different setups of glucose concentration and growth time could not repress the P_{BAD} promoter tightly in these strains ([fig 2.11](#)). As a consequence the strains ER2566, DH10 β and Top10 as well as the *Salmonella* strain ATCC 14028 were abandoned from further investigations. No previously generated data on those strains in relation to SIP-plasmid performance was accessible. *E.coli* K12 strain TB1 showed no premature recombination but very long response time to P_{BAD} activation. Recombined miniplasmid or minicircle could first be detected 120 minutes after induction by arabinose ([fig 2.12](#)). TB1 was also abandoned from fermentation and BG production but used as a storage strain for pSIP-H5. The sequence sent to Mycosynth®, Switzerland was prepared from this strain.

After successful transformation of pSIP-H5 into the *E.coli* K12 strain NM522 recombination ([fig 2.13](#)) and LacI-L'anchor fusion protein expression were tested again. No difference to the pSIP-Ova results was found. Plasmid preparation over time shows recombination 20 minutes after induction with arabinose, motherplasmid could not be detected after 80 minutes of recombination time, pointing to complete recombination ([fig 2.16](#)). Formerly generated data indicates recombination completion already 15, 20 or between 20 and 40 minutes after arabinose induction (compare (Jechlinger 2005) [pSIP-Ova, pSIP-CafI, pSIP-PA and pHCNparA respectively]).

Western blot analysis shows LacI-L'anchor fusion protein expression 40 minutes after induction by arabinose ([fig 2.17](#)). The time difference after induction of recombination onset (20 minutes on the plasmid preparation) and LacI-L'anchor fusion protein expression (40 minutes on western blot) has to be found in the different techniques of detection, since recombination can only take place after parA recombinase expression, which is tightly connected with LacI-L'anchor expression ([fig 2.8](#)). Comparison of these findings with former data could not be made due to different time resolution in sample taking (Champeimont 2007).

Data on growth of the culture observed by OD₆₀₀ as well as cfu count can be found in ([fig 2.14](#)). Activation of the P_{BAD} promoter does not seem to influence growth or appearance of bacteria ([fig 2.15](#)).

Transformation of pGLysivb into the *E.coli* K12 strain NM522 (pSIP-H5)

E.coli K12 strain NM522 (pSIP-H5) subsequently was transformed with pGLysivb in order to generate BGs by E-lysis. Successful transformation was confirmed by plasmid preparation and subsequent restriction enzyme analysis ([fig 2.19a&b](#)). All different states of the plasmid can be seen, the unrecombined motherplasmid, miniplasmid and minicircle, uncut as well as single cut. It can be seen that the uncut bands of pGLysivb and the pSIP-H5 minicircle are situated close together at about 5kb, leading to possible confusion of the bands at lower resolution.

Growth time of the double transformed strain (pSIP-H5, pGLysivb) is slower than of the strain only harboring pSIP-H5, mounting up to 250 minutes of growth time to reach an OD₆₀₀ of 0,2-0,3 from an initial (inoculation-) OD₆₀₀ of 0,05-0,1 ([fig 2.20](#)). In order to provide a constant concentration of arabinose in an estimated longer recombination time, a second addition of 0,25% arabinose was carried out 30 minutes after the first.

Plasmid preparation of the fermentation shows a slight miniplasmid band already in the second lane ([fig 2.22](#), -60min), which depicts the culture immediately before induction of recombination. This band points to slight premature recombination. The lane of -30 minutes (3rd lane) already shows a slight minicircle band indicating onset of recombination upon arabinose induction. In the lane of 0 minutes (4th lane, 60 minutes after first dose of 0,25% arabinose) minicircle is clearly visible, miniplasmid band strength increases strongly. 60 minutes after lysis induction by temperature shift, the pSIP-H5 motherplasmid band starts diminishing (lanes 7-10), which in this case next to recombination of the plasmid, can also be attributed to expulsion of the plasmid with the cytoplasm following E-lysis. Strong miniplasmid and minicircle bands however, suggest abundant recombination, although there are no data available to confirm completeness of the recombination event.

Already 30 minutes after the first induction of the P_{BAD} promoter by arabinose addition, western blot shows a LacI band at 45kDa, signifying LacI-L'anchor fusion protein expression ([fig 2.23](#)), correlating with former results of protein expression ([fig 2.17](#)). Visible bands in the second and third lane (-60 and -30 minutes) just below 40kDa show genomic LacI expression of *E.coli* host strain NM522 ([fig 2.23](#)). Genomic LacI conversely, does not attach to the membrane since it is not fused to the L'anchor and therefore is expelled with the cytoplasm upon lysis.

There is no direct indication, that second addition of arabinose has any lasting effect on recombination. The overall duration of recombinant protein expression (LacI-L'anchor fusion protein and parA resolvase) and recombination itself does not alter significantly from results of single-dose addition of arabinose.

Averaged lysis efficiency over all 4 clones adds up to 99,992% following cfu count ([fig 2.20](#)) topping results of 99,7% of lysis efficiency for the plasmid found in earlier investigations (Champeimont

2007). Microscopic pictures of the culture one hour after addition of arabinose (immediately before lysis induction) and three hours after lysis induction ([fig 2.21](#)) shows *E.coli* K12 strain NM522 bacteria and BGs respectively.

Lysis and recombination profile as well as confirmed P_{BAD} controlled protein expression led to the progression to fermentation of the *E.coli* K12 NM522 (pSIP-H5, pGLysivb) in the 30l bioreactor; thus producing enough BGs loaded with the pSIP-H5 minicircle.

Fermentation of *E.coli* K12 strain NM522 (pSIP-H5, pGLysivb) in the 30l bioreactor to produce pSIP-H5 minicircle loaded BGs for cell culture or animal testing

In order to determine the clone fermented in the bioreactor, plasmid preparations were done, examining the recombination state of the clone ([fig 2.24](#)). To produce BGs effectively loaded with the pSIP-H5 minicircle, it is a prerequisite to keep the motherplasmid in a non-recombined state until fermentation, when recombination is deliberately induced. Any untimely generation of miniplasmid by recombination means a growing disadvantage for motherplasmid carrying bacteria, propagating slower and continuously reducing the motherplasmid/miniplasmid ratio within the culture.

Due to the restriction enzyme analysis it was observed, that clone 3 already showed a slight miniplasmid band, leaving clone 6 (without miniplasmid band) the favored candidate for large scale fermentation.

The growth time of the culture to reach an OD_{600} of 0,6 in the bioreactor (which corresponds to a culture OD_{600} of 0,2-0,3 in the 30ml flask) was 3 hours after inoculation, which is about 3 to 4 times longer than other growth times of (in relation to plasmids and strains) comparable fermentations (Jechlinger, Azimpour Tabrizi et al. 2004).

Recombination was induced by addition of 250ml of 20% arabinose resulting in the induction concentration of 0,25% arabinose within the fermentation vessel of 20l. After 1 hour of recombination, corresponding with former results (this work), E-lysis was induced with a temperature shift from 36°C to 42°C and maintained for 90 minutes before killing surviving cells with addition of antibiotics.

Plasmid Preparation of the fermentation shows visible and increasing minicircle band 30 minutes after P_{BAD} promoter induction (starting in lane 4, -30' indicating 30 minutes before lysis induction) ([fig 2.29](#)).

Western blot shows recombinant protein expression 1 hour after induction ([fig 2.30](#)) in accordance to previously established data (Laham 2004; Tabrizi 2005).

Due to influence of BPL on DNA another killing agent, a mixture of antibiotics was used instead. The interaction of BPL with the DNA attached to the BGs is dealt with above in detail.

cfu count shows a lysis efficiency of 99,92% ([fig 2.27](#)) which is in accordance with formerly raised data. Also in conformity with publicized data is the amount of cfu in the culture, rising to 8,1E07/ml (Laham 2004).

BGs were harvested by centrifugation, washed with dH₂O or 500mM saline and lyophilized. Plasmid preparations of similar amounts of lyophilized BGs show higher amount of DNA if saline washed than in those washed with dH₂O ([fig 2.31](#)). A reason for this counter intuitive result can be found in the solubility of BGs in saline, which is by far smaller than in dH₂O. A change in the minicircle (molecularly bound to the membrane with the LacI-L'anchor)/miniplasmid (arbitrarily bound to the BG) ratio could not be observed.

rt-PCR targeting the lacOs sequence present on the pSIP-H5 minicircle as well as on the motherplasmid, naturally, was carried out to quantify the minicircle load of the BGs. The standard used was pSIP-H5 motherplasmid ([fig 2.32](#)), since no pSIP-H5 minicircle could be found after purification steps of plasmid preparations. Taking into account a weight factor between pSIP-H5 motherplasmid (standard DNA) to the minicircle (DNA to detect in the samples) of 0,4284.

PCR showed an average DNA load of 2,4E03 minicircles per saline washed BG and 12,0E03 minicircles per water washed BG respectively ([fig 2.39](#)). The difference in the amount of minicircle per BG between water washed and saline washed BGs may be found in the chaotropic properties of the saline, more eagerly disrupting weak molecular bonds (hydrogen bonds, van der Waals-interactions or hydrophobic effects), arbitrary (miniplasmid) and defined (minicircle) interactions alike.

Compared to the pSIP-Ova minicircle load per ghost of 575 of the former work of Firas Allaham the amount reached in this work lies about 4 to 20 times higher (Laham 2004). There are no data concerning the amount of pSIP-H5 miniplasmid still persisting in the BGs.

Cell-culture and animal experiments are still to be waited upon to draw precise conclusions concerning cellular transfection, bioavailability and immunologic efficacy.

Material and Methods

Bacterial Strains

E.coli K12 strains

NM522: sup E thi-1Δ(Lac-proAB)Δ(mcrB-hsdSM) 5(rK-mK-) (F'proABlacIqZΔM15) (MB69)
(Source: Stratagene, Heidelberg, Germany)

ER2655: FλfhuA2[lon] ompT lacZ::T7geneI gal sulA11 Δ(mcrC-mrr)114::IS10 R (mcr-73::miniTn10 TetS) 2 R(zgb-210::(TetS) endA1 [d cm] (Source: kindly provided by Wolfgang Jechlinger, vet. Uni. Wien)

DH10β: F⁻ mcr AΔ(mrr-hsdRMS-mcrBC)Φ80dlacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU galK nupG rpsL λ⁻; (Source: kindly provided by Wolfgang Jechlinger, vet. Uni. Wien)

TOP10: F-mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG

TB1: F⁻ ara Δ(lac-proAB) [Φ80dlac Δ(lacZ)M15] rpsL(Str^R) thi hsdR (Source: kindly provided by Wolfgang Jechlinger, vet. Uni. Wien)

Salmonella Species

Salmonella enterica subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium deposited as *Salmonella typhimurium* (Loeffler) Castellani and Chalmers Designations: CDC 6516-60 [4016, CIP 104115, NCTC 12023] Isolation: tissue, animal([172299] pools of heart and liver from 4-week-old chickens) Depositor: CDC History: CDC University of Missouri.

(inserted from the ATCC homepage: ATCC number: 14028; wildtype

<http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=14028&Template=bacteria>)

SALMONELLA VAC E; ATC vet QI01AE01; vaccine strand for active immunization against Salmonella Enteritidis, Phage type 4 (LAH, Cuxhaven, Germany)

Plasmids

Plasmid	Length [bp]	Origin	Induction	Expression	Resistance	Source
pGLysivb	6201	Rep	42°C	Gene E	Gentamycin	Lab
pEPH5	6477	pUC 19	T7 promoter	Vietnam-H5	Ampicillin, Kanamycin	LAH, Cuxhaven
pSIP-Ova	8314	pUC 19	arabinose	L'-anchor, ParA, Ovalbumin	Ampicillin	Lab, Firas al Laham
pSIP-H5	10039	pUC 19	arabinose	L'-anchor, ParA, Vietnam-H5	Ampicillin, Kanamycin	Lab, this work
miniplasmid	5676	pUC 19	arabinose	L'-anchor, ParA, Vietnam-H5	Ampicillin	Lab, this work
minicircle	4363	-	eukaryotic, through CMV- IE Promoter	Vietnam-H5	Kanamycin	Lab, this work

Microbiologic Techniques

E.coli and *Salmonella* o/n cultures

When taken from the -80°C stock, *E.coli* as well as the *Salmonella* strains are incubated in 5ml LBv medium; depending on carried plasmid, antibiotics or other supplements are added. The eprouvettes containing the medium are kept in a rotor or shaker overnight at 37°C or 28°C respectively; incubating Agar plates are also kept 37°C or 28°C respectively using a temperature regulated incubator (*Heraeus BK5060E*) or *Controlled Environment Incubator Shaker, New Brunswick Scientific*.

Storage of clones

All stocks are stored at -80°C in 25% Glycerol.

Double Transformation of *E.coli* NM522 with pSIP-H5 and pGLysivb

To finally have both plasmids in the *E.coli* strain, two options were considered. At first, it was tried to generate MOPS competent cells out of the strain already transformed with pSIP-H5. This, however, did not work and transformants were not obtained.

In the end it was decided to double transform naked MOPS competent *E.coli* cells. Transformants were picked and incubated o/n in 5ml LBv with Ampicillin, Kanamycin and 5% glucose. From these cultures glycerol stocks were made and kept at -80°C.

Growth, Protein Expression and Lysis of *E.coli* K12 strain NM522 in small scale experiments

All small scale experiments are carried out in 100ml noseflasks (30ml culture volume). LBv Medium is autoclaved within the noseflasks and inoculated with the o/n culture to an OD₆₀₀ between 0,07 and 0,1. The noseflasks are incubated in a stirring waterbath at 36°C and constant OD₆₀₀ measurements are carried out with the *Spectronic 20 Milton Roy Spectrophotometer*, defining the growth period of the culture and timing of sample taking. At an OD₆₀₀ of 0,3 expression of the *P_{BAD}* promoter is induced by adding 375µl of 20% arabinose, adding up to a total arabinose concentration within the flask of 0,25%. This expression-OD₆₀₀ was chosen, because an expression time of about one hour was anticipated, leaving the culture enough time to reach an OD₆₀₀ of between 0,4 and 0,5 when lysis will be induced by temperature shift from 36°C to 42°C.

If the experiment does not include lysis, expression is carried on for 1-2 hours, taking samples due to protocol.

Temperature at 42°C for lysis induction is maintained for 3-4 hours for sampling. The cultures are discarded by autoclaving.

Sample taking for Plasmid Preparation, Western Blot, Microscopic Observation

To observe culture growth and lysis efficiency the OD₆₀₀ is measured constantly and noted. Furthermore samples are taken at distinct timepoints, which themselves are chosen to match requirements established by former observation and are therefore due to change. Following scheme gives an idea of the sample-taking timetable (table 1).

The numbers on the right (1-4) indicate the noseflasks of which corresponding samples (western blot, miniprep, cfu or microscope) are taken; in this case from all of them. To take the samples, noseflasks are rapidly transferred under the lamina. 1ml is taken for miniprep as well as for western blot samples. Both are spun down 1min at 13krpm and supernatant is discarded for miniprep- or kept separately

for western blot samples respectively and kept at -20°C until used (for further information on western blot process see *Western blot* in *General SOPs* below). For plasmid preparation the *PeqLab miniprep Kit I* or *II* was used, following manufacturers manual. 100µl of the culture are used to commence dilution for cfu count (for dilution scheme and further handling see *Determination of live cell count via colony forming units* in *General SOPs* below). For microscopic observation another 8µl are taken out and put on a microscopic slide immediately.

table 1.: Timetable and sample-taking schedule

MRA

10/16/2007			NM522 psipH5, pGlysiB	NM522 psipH5, pGlysiB	NM522 psipH5, pGlysiB	NM522 psipH5, pGlysiB					
Kolben			1	2	3	4					
Time:	Min:	Nr.	30 ml LBv + Amp + Gent 36°C + 0,25% arabinose at A, 42°C at C	30 ml LBv + Amp + Gent 36°C + 0,25% arabinose at A, 42°C at C	30 ml LBv + Amp + Gent 36°C + 0,25% (375µl) arabinose at A and again at B 42°C at C	30 ml LBv + Amp + Gent 36°C + 0,25% (375µl) arabinose at A and again at B 42°C at C	OD	CFU	WB	Miniprep	microscope
		O/N					1-4			1-4	
9:45	-245		0.05	0.06	0.05	0.05					
12:00	-110		0.13	0.14	0.13	0.16					
12:50	-60	A (OD 0,2)	0.20	0.23	0.19	0.19	1-4	1-4	1-4	1-4	
13:20	-30	B	0.28	0.29	0.29	0.28	1-4	1-4	1-4	1-4	
13:50	0	C	0.41	0.43	0.40	0.41	1-4	1-4	1-4	1-4	1-4
14:10	20	D	0.50	0.51	0.50	0.50	1-4	1-4	1-4	1-4	
14:30	40	E	0.25	0.28	0.33	0.29	1-4	1-4	1-4	1-4	1-4
14:50	60	F	0.23	0.25	0.24	0.24	1-4	1-4		1-4	
15:20	90	G	0.23	0.23	0.24	0.23	1-4	1-4		1-4	
15:50	120	H	0.23	0.24	0.22	0.23	1-4	1-4	1-4	1-4	
16:50	180	I	0.23	0.24	0.21	0.21	1-4	1-4	1-4	1-4	1-4

inoculate flasks with OD: 0,07-0,1
at A: induction with 0,25% arabinose

Fermentation of *E.coli* K12 strain NM522

Machine, Sterility and Observance

For the large scale fermentation a 30L bioreactor is used (*Techfors S1820, Infors AG*) containing 22L of working LBv medium, which is sterilized *in situ* by autoclaving. To prove sterility the milieu within the bioreactor (pO₂, pH, temperature, air-flow, impeller frequency) is closely observed by the *IRIS*-program starting 18h before inoculation, furthermore immediately before inoculation medium samples are taken and checked for sterility by plating.

Inoculation

4 times 5ml LBv medium are inoculated with a scratch of the chosen clone of the -80°C glycerol stock. When medium is dense, this volume is used to inoculate 4 times 50ml of LBv medium, which then are incubated until dense and on their part used to inoculate 4 times 500ml of LBv medium. These 500ml are incubated o/n and used to inoculate the fermenter. At all times during growth of the inoculation culture the medium is kept with corresponding antibiotics and supplements. When sterility of the fermenter is shown, it is inoculated by 1-2l (depending on OD₆₀₀) of the o/n culture.

Process

Also during fermentation the whole growth process is observed by the *IRIS* program. 50ml samples are taken and handled correspondingly to the *small scale experiments*. Additionally, at the large scale fermentation 15ml samples are taken in pairs for rt-PCR.

Killing

To inactivate remaining live bacteria after the lysis process, a double dose of Streptomycin and Tetracyclin (4,4g Streptomycin [0,22g/l] and 440mg Tetracyclin respectively [0,022g/l]) were added, differing from normal killing method. Reasons for the adaption of the fashion of inactivation can be found in *Adaption of the killing process after fermentation in results*.

Harvesting

The ghosts are harvested by centrifugation using the *Westfalia Seperator, type CTC1-06-107*. The harvest process is closely observed and inflow and outflow are regulated by hand, keeping an outflow OD₆₀₀ of less than 0,1 to efficiently extract all material.

Washing and Lyophilisation

The harvested material is washed with dH₂O or 500mM saline respectively. In both cases material is divided into 3 parts and washed with a total of 7200ml in three washing steps á 2400ml. Between the washing steps the tubes are centrifuged at 8krpm for 15min at 4°C in a cooling centrifuge, *Hermle ZK401*. After the last washing step the pellet is resuspended in 200ml (either dH₂O or 500mM saline) and divided into 11 lyophilisation bottles. After pre-cooled at -20°C, the bottles are kept at -80°C. Bottles are lyophilised for 3 days in a lyophilisator, *LYOLAB B, LSL SECFROID*.

Calculation of lysis efficiency and particles/mg ratio

To obtain lysis efficacy, the highest and the lowest cfu are compared and their quotient results in efficacy:

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

To obtain the particles/mg of the lyophilized material again the highest cfu is multiplied by the volume of culture, divided by the lyophilized weight of the harvest.

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

Molecular Techniques

Cloning of pSIP-H5: Overview

Procedure:

1. Transformation of plasmid Prep of pEPH5 redissolved from filter paper sent from LAH, Cuxhaven, into *E. coli* strain; plated on Ampicillin Agar.
2. Inoculation of Transformants in 5ml LBv + Amp, o/n on 37°C, plasmid preparation of pEPH5.
3. PCR of template H5, using primers noted above, introducing the restriction sites BstBI and PstI to the fragment.
3. Control of PCR fragment on 1% Agarose gel (check size: 3049bp)
4. Inoculation of NM522 (pSIP-Ova): 5ml LBv + Amp [100µg/ml] + 5% glucose
5. Plasmid preparation of pSIP-Ova
6. Restriction of pSIP-Ova as well as the H5 PCR fragment using BstBI and PstI
7. 1% Agarose separation of fragments (check sizes: 6998bp and 1316bp). Use Gel-extraction-kit to extract 6998bp fragment out of gel as well as for purifying PCR fragment.
8. Ligation of pSIP backbone fragment and PCR H5 fragment. After incubation of 6h at 4°C NM522 cells were transformed with half of the ligation solution and plated on Ampicillin plates +5% glucose. The other half is incubated on 4°C o/n and transformed afterwards.
9. Incubate Transformants in 5ml LBv + Amp + 5% glucose
10. Plasmid preparation of culture
11. Control with restriction-digest of pSIP-H5
12. Sequence of H5 has to be controlled by sequencing
13. Construct has to be tested in noseflask

Preparing the plasmid pEPH5 for PCR

The plasmid pEPH5 was sent to us by LAH, Cuxhaven on filter paper, received on 23rd of April, 2007. Three small areas, where plasmid solution has been applied were marked with pen. Under the Lamina and with sterilized scissors one of the patches was cut out, put into a 1,5ml Eppendorf tube and soaked in 50µl of microbiological water (ddH₂O), resuspending the plasmid for 5min. After centrifugation at 13krpm for 1min 10µl of the solution were used to transform 100µl of competent *E.coli* NM522. The Transformants were plated on Agar plates with Ampicillin and incubated o/n at 37°C. Growing clones

were picked the next day and 5ml LBv tubes with Ampicillin were inoculated and incubated at 37°C in the rotor. The plasmid preparation then was used for PCR, following the procedures stated below.

Amplification of the H5 sequence from pEPH5 by PCR

PCR: Template: pEPH5
PCR fragment: 3049bp

Primer:

FwdSIP-H5:

5'-GAC TTC GAA ATG GAA AAG ATC GTG CTG3' T_m= 62°C

BstBI

RevSIP-H5:

3'-ATA ACA TTA TAA TGC TAG ATA ACT TCG TAT AA' T_m= 51°C

PsiI

PCR cycle: initial cycle:
95°C 3min
30 cycles:
95°C 30sec
50°C 30sec
72°C 100sec
1 cycle:
72°C 10min
end
4°C ∞

Recipe:	50µl	pcr MasterMix fermentas
	40µl	ddH2O
	1µl	fwd primer
	1µl	rev primer
	8µl	template DNA

Purifying the H5-insert

After checking the molecular weight of the PCR fragment, it was restricted with BstBI and PsiI to prepare the endings for ligation. After restriction process DNA was purified applying the purifying steps of the Gel-extraction-kit.

Preparing pSIP backbone from plasmid pSIP-Ova

A -80°C glycerol stock ('projects') of Firas Al Laham (Srivastava and Liu 2003) of *E.coli* K12 strain NM522 harboring the pSIP-Ova plasmid was used for inoculating 5ml LBv tubes +Ampicillin and 5% glucose, in order to repress the P_{BAD} -Promoter, and incubated at 37°C in the rotor o/n. When the cultures were dense, the plasmid pSIP-Ova was prepped and control-cut. *E.coli* K12 strain NM522 was transformed with the plasmid, cells plated on Agar plates with Ampicillin and 5% glucose and incubated o/n at 37°C. Positive clones were picked and used for inoculating 5ml LBv tubes +Ampicillin and 5% glucose. From these cultures -80°C glycerol stocks were made. This strain then was used for preparation of the pSIP backbone fragment. The plasmid preparation was cut with BstBI and PsiI. Due to different manufactures the performance of the restriction enzymes were tested. BstBI was bought from Fermentas™ as a FastDigest®-enzyme whereas or PsiI was bought from NEB™. The test showed that actually each combination of buffer and enzyme worked and the plasmid was cut correctly and efficiently, no difference in efficiency or accuracy was found (data not shown).

Purifying the pSIP backbone

Plasmid pSIP-Ova preparation was double digested with BstBI and PsiI in NEB-Buffer B for 2h at 37°C. The fragments (6998bp, the backbone and 1316bp, the Ova-gene) were separated by electrophoresis in a 1% Agarose gel. The backbone-band was cut out under mild UV-illumination and the DNA purified using the QIAGEN™ (QIAquick® PCR Purification Kit) following manufactures manual.

Ligation of the pSIP backbone with H5-gene

Both DNA preparations were applied to agarose gel electrophoresis. The amounts of DNA of each fragment within the ligation recipe were set up in accordance to the intensity of the bands in the gel. After 6h of incubation at 4°C 10µl were taken and used for transformation into NM522 MOPS competent cells. They were incubated at 37°C o/n on Ampicillin Agar plates with 5% glucose. The rest of the Ligation-solution was further incubated at 4°C o/n and used for NM522 MOPS competent cell transformation the next day. The cells again were striked out on Ampicillin Agar plates with 5% glucose and incubated at 37°C o/n. Transformants were picked from both incubations and inoculated in 5ml LBv tubes with Ampicillin and 5% glucose. The cultures were incubated on 37°C o/n in the rotor and glycerol stocks were made.

Quantification of the minicircle within the BGs

To determine the amount of minicircle DNA within the Bacterial Ghosts Realtime-PCR was used. The sequence amplified by rt-PCR was the LacOs sequence which is present on the minicircle, but not on the miniplasmid. As rt-PCR sample 5mg of whole lyophilized BGs were resuspended in 500µl ddH₂O. This solution was diluted 1:20, 1:2e02, 1:2e03, 1:2e04 and 1:2e05. It also was made sure, that any original genomic sequence, which might still be attached to the membrane shell and be amplified by the primers, would not forge the measurements. Primers are kept at -80°C.

recipe: 12,5µl SybrGreen® pcr Mastermix of BioRad®

5,5µl ddH₂O

1,0µl fwd primer (equals 1pmol)

1,0µl rev primer (equals 1pmol)

5,0µl template (different BG dilutions or Standard dilutions respectively)

primers: fwd: 5'-TGG AAC TCA ATA CGA CGG CG- 3'

rev: 5'-CGG TCG TGA AGG TGT TGC TC- 3'

amplifying a sequence of 233 bp

Calculation of minicircle per ghost:

$$\frac{Mass_{total\ minicircle\ DNA}\ [kg]}{Number\ of\ BG\ [1] \times Mass_{of\ 1\ minicircle}\ [kg]} = Number\ of\ minicircles\ per\ BG\ [1]$$

Mass of the DNA will be obtained by the rt-pcr.

Number of BG will be determined through weight of the sample and particle/mg ratio of sample (calculation see *Calculation of lysis efficiency and particles/mg ratio* above).

Mass of minicircle is calculated by the following:

$$Mass_{dsDNA-molecule}\ [kg] = number\ of\ kb\ [1] \times 6,6 \times 10^5 \times 1,66 \times 10^{-27}\ [kg]$$

The mass of 1kb of dsDNA is 6,6x10⁵Da. One Da equals 1,66x10⁻²⁷kg.

In respect to the 4363bp long minicircle, the weight of one molecule is: 4,7801E-12µg

rt-PCR Standard

The standard was purified motherplasmid. The length of the two DNA molecules (motherplasmid and minicircle) differs by a factor of 0,4284. The DNA concentration of the standard was calculated by following equation:

$$[DNA] = OD_{280} \times DF \times MF$$

The [DNA] will result in [µg/ml]

The OD₂₈₀ is optical density at 280nm

DF is the Dilutionfactor

MF is the Multiplicationfactor, which depends on the type of nucleic acid measured. In this case this is dsDNA, and the dilutionfactor is 50.

rt-PCR program

The program for amplification was transferred from the protocol of the same pcr of **Firas Al-Laham**:

initial cycle

95C° 3min

30 cycles

94°C 45sec

60°C 90sec

1 cycle

4°C ∞

Fluorescent measurements were taken each extension step. A Melting Curve was established at the end of amplification cycles by increasing temperature every 5s from 60°-95°C. At the end of the run the PCR machine cools down to 4°C in order to maintain good DNA quality, if the samples are not taken immediately for further use.

General Standard Operating Procedures:

Agarose-Gel

Corresponding to DNA-length either a 1% (10^3 - 10^4 bp) or 2% (10^2 - 10^3 bp) Agarose-Gel was used to separate DNA-fragments. Following recipe was used to produce the Agarose-Gel, perform the separation, staining DNA to take a photograph of the stained DNA:

1.0 Casting of the Gel:

- 1.1 3g of Agarose (6g respectively, for 2% Gels) were mixed with 300ml 1xTAE
- 1.2 Mixture was heated with microwave (750W) for several minutes until boiling point, shaken several times and heated again, until any solid Agarose dissolved.
- 1.3 Liquid Agarose was cooled down to hand warm and then cast into the gel casting tray, where several combs were applied.
- 1.4 When gel was solidified, it was stored in 1xTAE solution until used

2.0 Loading of the Gel:

- 2.1 A part with enough slots was cut from the gel.
- 2.2 DNA is applied into the slots after mixing with 6xloading buffer, mostly 2 μ l loading buffer with 10 μ l DNA solution, but amounts may vary, depending on DNA concentration of the solution.
- 2.3 For the marker mainly 5 μ l of the 1kb DNA ladder was used.

3.0 Running of the Gel:

- 3.1 A voltage of about 150V was applied for about 30-50min
- 3.2 Depending on the length of the gel or the DNA fragment, the applied times of running of the gel varies and the end of the run is determined by the stained lanes of the loading buffer, whenever they reach the end of the gel.

4.0 Staining of the Gel:

- 4.1 After the run the gel is transferred to a staining bath, which contains GelRed® (BIOTREND).
- 4.2 After 10 to 15min, depending on DNA-concentration, length and dye-concentration the gel was stained sufficiently to be photographed, illuminated at 250nm.

Antibiotics

All antibiotics used, were bought from (Carl Roth, Germany) in solid state, and dilution and end concentration respectively were made in the lab.

Name	[stock solution]	[final solution]	making of
Ampicillin	50mg/ml	100µg/ml	2g Ampicillin + 40ml dH ₂ O
Gentamycin	10mg/ml	20µg/ml	0,4g Gentamycin + 40ml dH ₂ O
Kanamycin	25mg/ml	50µg/ml	1g Kanamycin + 40ml dH ₂ O

All antibiotics were stored at 4°C. Ampicillin was not longer than 7 days at 4°C, otherwise stored at -20°C. Long-term storage of all antibiotics at -20°C.

Production of CaCl₂/RbCl₂ competent cells

1. Growing of the cells
 - 1.1 Noseflask with 30ml medium is inoculated with the bacteria.
 - 1.2 Culture is grown in waterbath at 36°C under sterile conditions to an OD₆₀₀ of 0,5.
2. Treatment with MOPS solutions
 - 2.1 Centrifuge culture at 4°C at 4krpm for 10min.
 - 2.2 Decant supernatant
 - 2.3 Resuspend pellet in 6ml cold MOPS I and keep on ice for 10min.
 - 2.4 Centrifuge culture at 4°C at 4krpm for 10min.
 - 2.5 Decant supernatant
 - 2.6 Resuspend pellet in 6ml cold MOPS II and keep on ice for 30min.
 - 2.7 Centrifuge culture at 4°C at 4krpm for 10min.
 - 2.8 Decant supernatant
 - 2.9 Resuspend Pellet in 480µl MOPS II and 180µl glycerol.
 - 2.10 Keep on ice for 10min
 - 2.11 Aliquot in 100µl and store at -70°C

Determination of live cell count via colony forming units

For several important timepoints during the fermentation, like inoculation, expression induction and lysis induction and termination the culture concentration is very important. The cell count is determined crudely by OD₆₀₀ measurement and precisely by cfu total. For the plating of the bacterial samples a spiral plater (WASP-system, Don Whitley Scientific Ltd, West Yorkshire, GB) was used and

colonies counted by a colony counter (*Synbiosis ProtoCOL Colony Counter 3.15, Synoptics Ltd., Cambridge, UK*).

1.0 Dilution of the culture sample:

- 1.1 OD₆₀₀ is measured and determines the dilution of the sample for plating. The accordance is given by following table:

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
1,0-2,0	10 ⁶		0,5-0,2	10 ³
			0,2-0,01	10 ²

- 1.2 The culture is diluted with 0,85% saline, using eprouvettes containing 9ml saline for 1:10¹ dilution.
 - 1.2.1 9ml saline + 1ml culture

eprouvettes containing 9,9ml saline are used for 1:10² dilutions.

 - 1.2.2 9,9ml saline + 100µl culture
- 1.3 50µl and 100µl of the end-dilution are plated with the spiral plater.
- 1.4 Plates are incubated at 36°C or 28°C depending on the period of incubation until colonies are large enough to be read by the colony counter.

Only plates between 20 and 500 colonies are counted.

MOPS-Transformation

All transformations of *E.coli* were CaCl₂/RbCl₂ transformations, carried out following the subsequent protocol.

1. 2-10µl of DNA (depending on plasmid preparation) are pipetted to 100µl of competent cells (see SOP on preparation of CaCl₂/RbCl₂-competent cells)
2. Mixture is kept on ice for 30'
3. Heat shock at 36°C or 42°C (depending on the plasmid) for 2'

4. Mixture is kept on ice for 5'
5. 700µl of LBv-medium +5% glucose is added and cells are incubated for 1h at 37°C
6. Solution is stroke on 2 agar plates with the corresponding antibiotic, 5% glucose, 100µl and the rest.
7. Plates are incubated mostly overnight at 36°C

Preparing of electrocompetent cells

1. Growing of the culture
 - 1.1 Noseflask with 30ml medium is inoculated with the bacteria
 - 1.2 Bacteria is grown in waterbath at 36°C under sterile conditions to an OD₆₀₀ of 0,4
2. Treatment of the culture
 - 2.1 Keep on ice for 30min
 - 2.2 Centrifuge at 4°C and 4krpm for 10min
 - 2.3 Decant supernatant
 - 2.4 Resuspend pellet in 30ml 10% glycerol
 - 2.5 Centrifuge at 4°C and 4krpm for 10min
 - 2.6 Decant supernatant
 - 2.7 Resuspend pellet in 15ml 10% glycerol
 - 2.8 Centrifuge at 4°C and 4krpm for 10min
 - 2.9 Decant supernatant
 - 2.10 Resuspend pellet in 600µl 10% glycerol
 - 2.11 Centrifuge at 4°C and 4krpm for 5min
 - 2.12 Decant supernatant
 - 2.13 Resuspend pellet in 300µl 10% glycerol
 - 2.14 Aliquot 50µl
 - 2.15 Store at -70°C

Recipes

Low Salt LBv-Medium (Lennox)

All growth of bacteria took place in Lennox medium or agar, which was either freshly prepared or stored at 4°C for not longer than two weeks after autoclaving.

The recipe for 1l:

1. 10g Peptone

5g Yeast extract

5g NaCl

2. The ingredients were dissolved in 900ml dH₂O
3. The pH was adjusted to 7,4 with NaOH (1N)
4. If Agar was made, 15g of Agar were added at this point
5. After completing to 1l with dH₂O the medium (agar or no) was autoclaved

Any supplements like antibiotics or glucose are to be added after autoclaving and after the solution had cooled down to hand warm temperature.

In case of Agar, when hand warm temperature was reached, it was poured into petri dishes (ca. 20ml) next to the gas flame and left covered, until solidified and used immediately or stored at 4°C not longer than 2 weeks.

MOPS I

10.47g MOPS I (100mM)

0.47g CaCl₂ x 2H₂O (10mM)

0.6g RbCl₂ (10mM)

Dissolve ingredients in 400ml dH₂O and adjust to pH=7 using KOH. Fill up to 500ml and sterilize by autoclaving.

MOPS II

10.47g MOPS I (100mM)

5.15g CaCl₂ x 2H₂O (10mM)

0.6g RbCl₂ (10mM)

Dissolve ingredients in 400ml dH₂O and adjust to pH=6,5 using KOH. Fill up to 500ml and sterilize by autoclaving.

Digest with Fermentas® FastDigest® Enzymes

All restriction enzyme cuts were done with Fermentas® FastDigest® Enzymes except otherwise stated.

1. Enzymes are stored at -20°C, when working enzymes are kept in the freeze-block

1.1 Recipe:

3µl of DNA (plasmid preparation)

1µl of FastDigest® restriction enzyme

1µl of FastDigest® Buffer

5µl of ddH₂O

- 1.2 Ingredients are mixed together in an Eppendorf under the lamina
- 1.3 Eppendorf is incubated at 37°C for about 20min

Ligation

For the Ligation the T4 ligase, NEB, was used and the whole process was performed due to manufacturers' instructions. Insert to vector relation was approximately 1:10. The plasmid pSIP-H5 was ligated in two ways, differing in the ligation time. After mixing of the ligation ingredients, it was incubated at 4°C for about 6h, when the first aliquot was taken and used for transformation, the rest of the solution was kept at 4°C overnight and again an aliquot was taken and a second transformation was carried out.

Western Blot

The presence of the LacI-L' anchor was determined by westernblotting. The LacI-L' anchor gene lies upstream of the recombinase gene and both are under control of the same promoter P_{BAD} .

At different time points samples of the growing and lysing culture were taken. Usually 1ml of culture was centrifuged and the supernatant was separated from the pellet and kept, at -20°C

1.0 Taking of the sample:

- 1.1 1ml of the culture is taken into an eppendorf tube and centrifuged at 10000rpm for 1-2min. It is important to know the OD_{600} of the culture at the corresponding timepoint.
- 1.2 The supernatant is transferred to an extra tube.
- 1.3 If the sample is not directly prepared for western blot it is kept at -20°C

2.0 Preparing the sample for western blot:

- 2.1 The measured OD_{600} is multiplied by 250, which then gives the amount of NuPage Reducing Sample-Buffer, Invitrogen, added to the pellet.
- 2.2 The pellet is thoroughly resuspended in the buffer.
- 2.3 The tube then is heated to 99°C for ten minutes, to denature all protein.

3.0 Applying the sample on the gel (4-12% gradient BisTris-Gel Novex, Invitrogen):

- 3.1 The precast gel is stored at 4°C and placed into the tank chamber just before electrophoresis. MES-running buffer, Invitrogen, is added, sufficient to cover both sides (up and down) of the gel.
- 3.2 The sample is centrifuged shortly at high-speed to sediment any solids.
- 3.3 15µl of the supernatant are applied per slot.

3.4 5µl of marker (PageRuler™ Unstained Molecular Weight Marker, Fermentas) are applied at each end of the sample-set.

3.5 The electrophoresis takes place at 120V (for two gels in one tray) for about 40min. The sample buffer shows blue bands which indicate the running-front.

4.0 Blotting the sample

4.1 The gel is placed upon a nitrocellulose membrane, which is already wetted in transfer buffer, the edges of the gel marked with a pen.

4.2 A special order of sponge-pads and filter paper (all saturated with NuPage Transfer buffer, Invitrogen) are applied to either side of the gel. This whole stack is then placed into the blotting apparatus.

4.3 The blotting takes place at 60V (for two gels) and for about 1h.

4.4 For cooling reasons water was added to the outside of the blotting apparatus.

5.0 Staining and Blocking of the membrane:

5.1 The gel and filter paper are discarded and the membrane stained with Ponceau S stain.

5.2 After the marker bands are penciled over, the membrane is washed again with water in order to remove the Ponceau S staining again.

5.3 Blocking Solution (1x Roti-Block™, Roth) is added to cover all of the membrane.

5.4 The membrane is stored in the Blocking Solution at 4°C overnight.

6.0 Antibody incubation:

6.1 After the blocking the membrane is washed 3 times for about 5-10 minutes in TBST on the shaker

6.2 About 20ml of the primary antibody, a rabbit derived anti-LacI antibody (1:5000) is used and incubated for 1h on the shaker.

6.3 The antibody is taken off the membrane and stored for further use. The membrane is washed three times for about 5-10minutes each washing step in TBST.

6.4 The membrane is incubated for 1h in the secondary antibody, a goat anti-rabbit antibody associated with HRP (1:5000).

6.5 The membrane is washed three times, 5-10min each step, in TBS.

7.0 Staining and imaging of the membrane:

7.1 The membrane is soaked in 6ml of premixed Chemiluminescent Substrate SantaCruz Biotechnology for several minutes

7.2 The membrane is documented in the Chemidoc device

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