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„Bacterial Ghosts Displaying
Chlamydia trachomatis Antigens“

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1 ZUSAMMENFASSUNG

1.1 ZIELSETZUNG

Infektionen mit dem obligat intrazellulären Gram-negativem Bakterium *Chlamydia trachomatis* gehören zu den häufigsten bakteriellen Geschlechtskrankheiten überhaupt; darüber hinaus sind sie für meisten *verhinderbaren* Fälle von Erblindungen, vor allem in Entwicklungsländern, verantwortlich. Während die Infektion oft und lange asymptomatisch verläuft, kann ein Ausbruch zu entzündlichen Beckenerkrankungen, ektopischen Schwangerschaften oder auch Unfruchtbarkeit führen.

Das Bacterial Ghost-System ist eine Technologieplattform, bei der Gram-negative Bakterien mittels Lyse durch das Protein E des Bakteriophagen ϕX174 'entleert' und getötet werden. Die leeren, bakteriellen Hüllen (sogenannte Bacterial Ghosts, abgekürzt BGs) behalten die strukturellen, morphologischen und antigenen Eigenschaften ihrer Vorgängerzellen bei. Sie können entweder direkt für Vakzinierungen verwendet werden, oder mit aktiven Substanzen beladen werden.

Eine weitere Möglichkeit ist die Verankerung rekombinanter Antigene, die im Rahmen der immunstimulierenden Umgebung der BGs präsentiert werden. Dabei kommt den BGs die Rolle als natürliches Adjuvans bei. Dies ist besonders ausgeprägt bei Bakterien des *Escherichia coli* Stamms Nissle 1917, der für seine probiotische und immunstimulierende Wirkung seit Jahrzehnten verwendet wird.

Ziel dieser Studie war es daher, BGs auf Basis von *E. coli*, bevorzugt *E. coli* Nissle zu produzieren, deren Periplasma vor der Lyse mit chlamydialen Antigenen, den Vakzinkandidaten PorB und MOMP, befüllt wurde.

Zu diesem Zweck sollten Plasmide für die Expression und den co-translationalen Export dieser beiden Antigene konstruiert werden. Desweiteren sollten unterschiedliche Methoden der Induktion der Expression, des Exports in das Periplasma sowie der Markierung und Quantifizierung von Antigenen mittels Tags ausgetestet und verglichen werden. Nach Experimenten im kleinen Maßstab, in denen zuerst Antigenexpression und anschließend Antigenexpression und Lyse getestet wurden, sollten durch Fermentationen in einem

Volumen von 20l große Mengen BGs hergestellt werden, deren Periplasma mit chlamydialen Antigenen beladen wurde.

Abschließend soll die Menge chlamydialer Antigene in BGs bestimmt werden.

1.2 ERGEBNISSE

Plasmide für die Expression, den Transport ins Periplasma, und die einfache Detektion mittels Antikörpern für zwei *Chlamydia trachomatis*-Antigene, MOMP und PorB, konnten erfolgreich kloniert werden.

Die Expression gefolgt von periplasmatischer Lokalisation von MOMP und PorB wurde mittels Western Blot gezeigt. Sie interferiert nicht mit der gleichzeitigen Protein E-vermittelten Lyse der Bakterien, und Lyse führt zu keinem merklichen Verlust an exprimierten chlamydialen Antigenen.

Bei abschließenden 20l Fermentationen wurden BGs des Stamms *E. coli* NM522 mit periplasmatischem PorB und BGs des Stamms *E. coli* NM522 mit periplasmatischem MOMP, sowie BGs des Stamms *E. coli* Nissle mit periplasmatischem PorB erzeugt.

PorB machte dabei etwa ein Fünftel des BG-Trockengewichts aus. In BGs mit MOMP betrug dessen Anteil am Trockengewicht etwa ein Drittel; die berechnete Anzahl von PorB-Molekülen pro BG betrug $2.63 \cdot 10^6$ im Fall von *E. coli* NM522 BGs und $5.08 \cdot 10^6$ im Fall von *E. coli* Nissle BGs. Für BGs des Stamms *E. coli* NM522 wurden $2.19 \cdot 10^7$ MOMP Moleküle pro BG berechnet.

Sterile BGs mit den *Chlamydia trachomatis*-Antigenen PorB und MOMP stehen für weitere Versuche bereit, um ihre Immunogenität zu prüfen.

2 SUMMARY

2.1 OBJECTIVES

Infections with the obligate intracellular Gram-negative bacterium *Chlamydia trachomatis* are a world-wide major healthcare concern. While trachoma is the world's leading cause of *preventable* blindness (primarily found in developing countries), *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen in Western countries. Infection often remains asymptomatic, but when they become acute, complications such as Pelvic Inflammatory Disease, ectoptic pregnancies and infertility can arise.

The Bacterial Ghost system is a technology platform with many recognized uses. It utilizes protein E of bacteriophage ϕX174 to lyse and thereby empty and kill Gram-negative bacteria, with the remaining empty bacterial envelopes, the so-called 'Ghosts', retaining their morphological, structural and antigenic characteristics. Bacterial Ghosts (hereafter referred to as 'BGs') of pathogenic strains can directly be used for vaccination. BGs from non-pathogenic strains can be loaded with active substances or DNA for DNA vaccinations. Another option is the anchoring of recombinant antigens from other species somewhere in the envelope of the BG, taking advantage of its immunostimulatory nature and its capability to act as a natural adjuvant. This is especially promising in the case of *Escherichia coli* Nissle 1917, a probiotic bacterial strain that has long been recognized as both safe and beneficiary.

The aim of this project was the production of BGs from *E. coli* NM522, or (preferentially) *E. coli* Nissle, with their periplasms loaded with the chlamydial vaccine candidate proteins MOMP and PorB before the initiation of lysis.

To achieve these goals, chlamydial antigens were to be cloned into inducible expression plasmids with targeting sequences for the export to the periplasm. Furthermore, different methods of expression induction, transport to the periplasm and quantification of antigen using different tag systems were to be tested and compared.

After first establishing antigen expression and combined antigen expression and E-mediated lysis in small scale experiments, large quantities of BGs with chlamydial antigen-loaded periplasms were to be produced by fermentation in a volume of 20l.

Subsequently, the amount of chlamydial antigens in BGs was to be quantified.

2.2 RESULTS

Plasmids for the expression and the periplasmatic localization of the chlamydial antigens PorB and MOMP were successfully cloned. Their correct sequence was first assessed by restriction digest pattern analysis, and later shown by the Western Blot detection of tagged proteins expressed upon induction in small scale experiments. Protein E-mediated lysis did not interfere with protein expression, and vice versa. Lysis did not lead to significant loss of protein, indicating the correct localization to the periplasm and the tight sealing of the periplasm throughout lysis.

Large scale fermentation in 20l media produced BGs of the strain *E. coli* NM522, loaded with periplasmatic MOMP and BGs of the strain *E. coli* NM522, loaded periplasmatic PorB, as well as BGs of the strain *E. coli* Nissle, loaded with periplasmatic PorB.

Quantification of chlamydial antigens showed that PorB accounted for one fifth of BG mass, whereas MOMP constituted one third of its BGs' mass. The calculated number of PorB molecules per individual BG is $2.63 \cdot 10^6$ in the case of *E. coli* NM522 BGs and $5.08 \cdot 10^6$ in the case of *E. coli* Nissle BGs. For BGs of *E. coli* NM522 a total of $2.19 \cdot 10^7$ MOMP molecules per BG were calculated.

Sterile BGs, loaded with the chlamydial antigens PorB and MOMP, are available for further experiments to assess their immunogenicity.

3 INTRODUCTION

3.1 THE BACTERIAL GHOST PLATFORM TECHNOLOGY

3.1.1 E-MEDIATED LYSIS OF GRAM-NEGATIVE BACTERIA: AN OVERVIEW

Bacteriophage ϕX174 of the Microvirus genus preys on Enterobacteriae such as *E. coli* and *Salmonella*. Virions consist of non-enveloped capsids with icosahedral symmetry, with a diameter of 25 – 27nm. The non-segmented, circular ssDNA genome has a length of 5,386bp and constitutes 26% of the virion's weight (Büchen-Osmond, 2006).

ϕX174 has been studied extensively; its genome was the first to be sequenced, (Sanger, et al., 1977) and the overlapping nature of its genome, resulting in high conservation over time, lead to speculations about it being a means of communication by an extraterrestrial society (Yokoo, et al., 1979).

Infection of bacteria with ϕX174 results in lysis and the release of progeny phage particles; lysis requires bacterial growth and is impaired when bacteria are grown on a nutrient-depleted minimal medium. The lysis protein E is the necessary and sufficient viral factor for bacterial lysis (Lubitz, et al., 1984). However, several cellular factors, notably the cell division factors FtsZ and FtsA are required for E-lysis, too (Witte, et al., 1998).

Lysis protein E is a highly hydrophobic, 91aa long structural protein with a mass of 10.5kDa and no known enzymatic function. Membrane-bound oligomerization leads to the fusion of the inner and the outer membrane, resulting in lysis of the bacteria (Bläsi, et al., 1989).

The transmembrane tunnel formed by oligomerization of protein E is preferentially located at the septum or at polar regions of bacteria; the driving force in cytoplasmic expulsion and hence lysis is the osmotic pressure difference between the cytoplasm and the environment of the bacteria (Witte, et al., 1992). A transmission electron micrograph of *E. coli* immediately after E-induced lysis, with the transmembrane tunnel indicated, is shown in Figure 1.

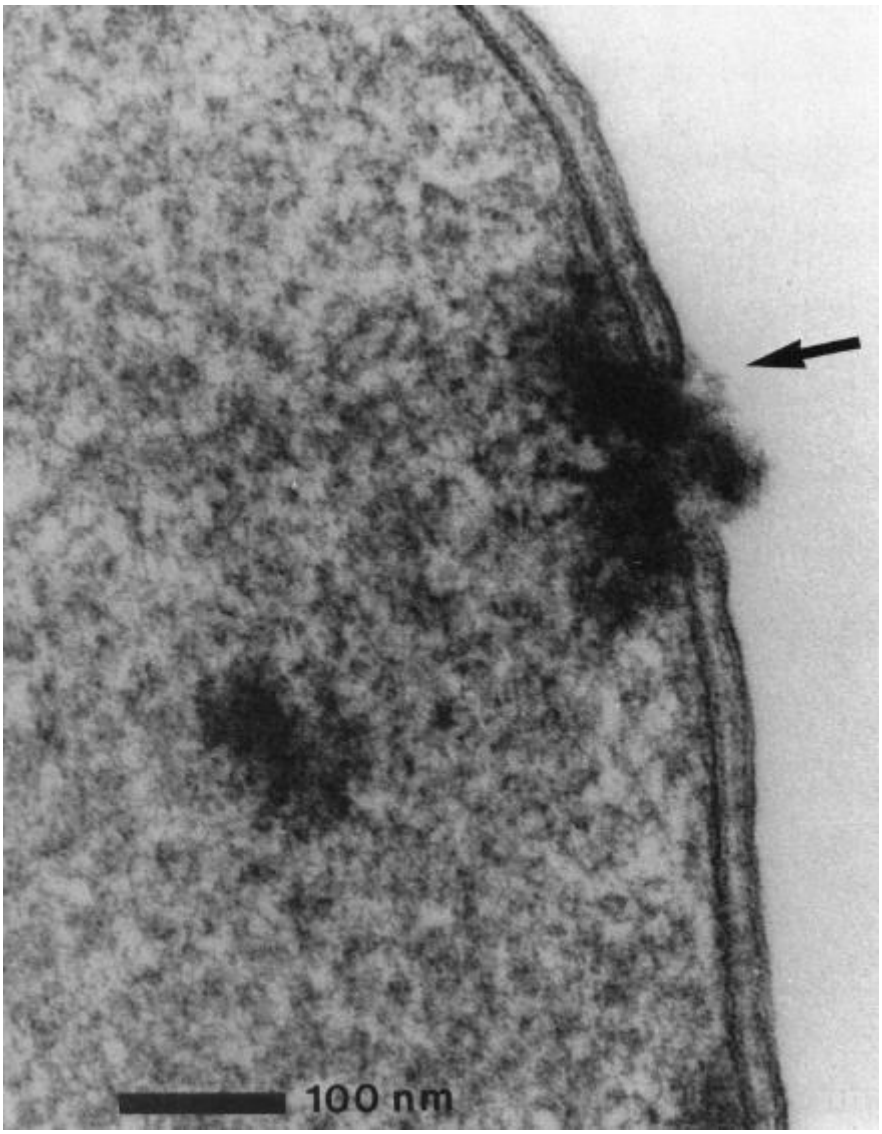


Figure 1: Transmission electron micrograph of *E. coli* immediately after E-induced lysis. Transmembrane tunnel formed by protein E oligomerization is indicated by an arrow. Taken from **(Witte, et al., 1990)**.

Lysis leads to the expulsion of cytoplasmic content (including plasmid DNA), to the fragmentation of chromosomal DNA and to the breakdown of membrane potential; while roughly 90% of the cytoplasmic content is expelled, only 5-10% of the periplasmic content is released. The overall structure and morphology of the cell envelope is not affected (Witte, et al., 1989). A scanning electron micrograph of the expulsion of cytoplasmic content is shown in Figure 2.

The empty bacterial envelopes with sealed periplasms are called ‘Bacterial Ghosts’, and are hereafter referred to as BG (singular) or BGs (plural).

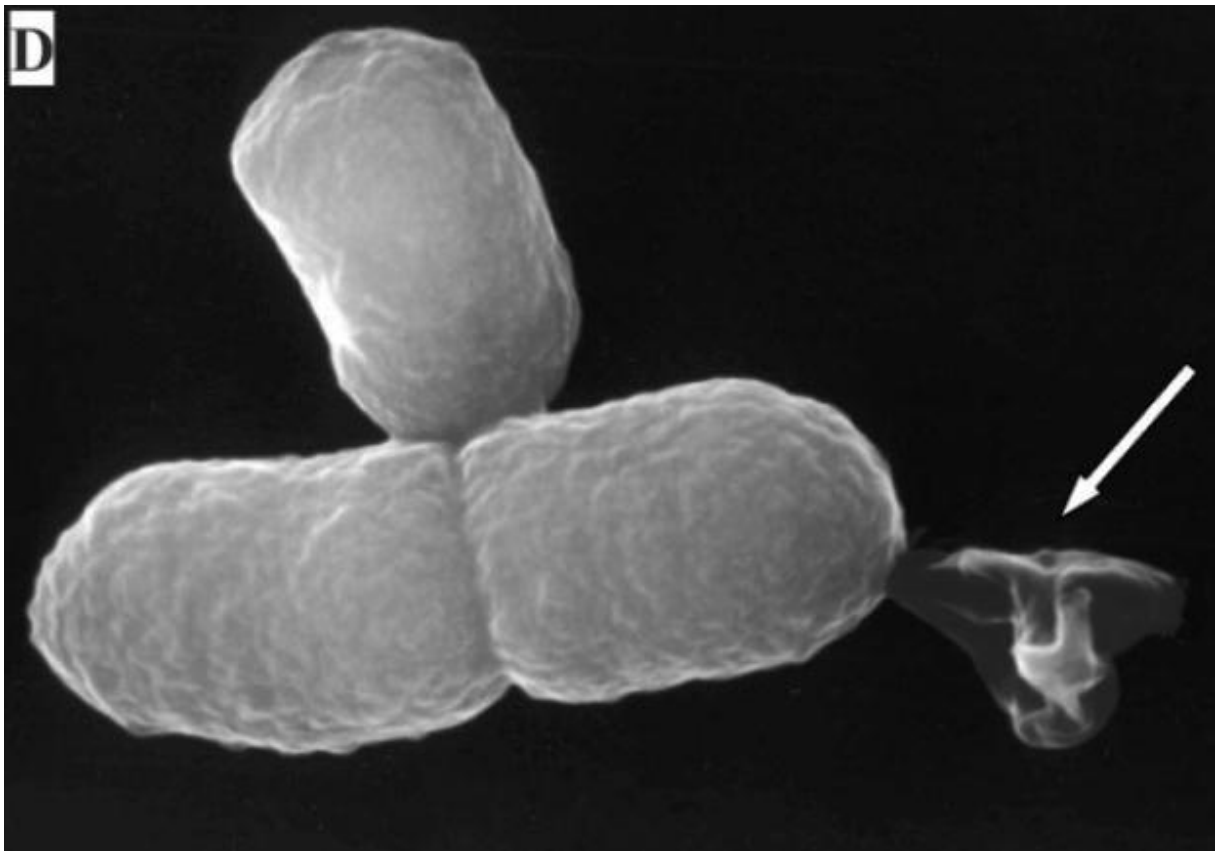


Figure 2: High resolution field emission scanning electron micrograph of a Gram-negative bacterium; expulsion of cytoplasmic content by E-induced lysis is indicated by an arrow. Taken from **(Ebensen, et al., 2004)**

A single lysis tunnel formed by protein E oligomerization is sufficient to lyse bacteria; indeed, since lysis takes place in a short time frame (≈1sec), the formation of a second lysis tunnel has to take place concomitantly (Witte, et al., 1990). The diameter of the tunnel lies typically between 40 – 80nm (Witte, et al., 1990), allowing for the easy passage of virion particles, and the expulsion of cytoplasmic components as large as ribosomes (Witte, et al., 1989).

Since as little as 100 copies of protein E are sufficient for loss of host viability (Maratea, et al., 1985) and lysis tunnel formation, tight control and suppression of gene E transcription before lysis induction is required (Bläsi, et al., 1985).

3.1.2 LYSIS PLASMID pGLYSIVB

For the regulated, inducible formation of BGs the lysis plasmid pGLysivb was developed (Haidinger, 2001); a schematic overview of the plasmid is given in Figure 3.

It is derived from the pBBR1MCS5 variant of plasmid pBBR1 (Szpirer, et al., 2001) and carries the bacteriophage ϕX174 lysis protein E under the regulation of the mutated

thermosensitive λpL/pR-cI857 promoter-repressor system. The repressor is active at temperatures below 37°C, and full transcriptional activity is reached at 42°C (Jechlinger, et al., 1999).

The plasmid carries a gentamicin resistance cassette. The MOB gene sequence has been mutated, with a decreased mobilization activity of $3 \cdot 10^{-2}$, compared to non-mutated pBBR1 (Szpirer, et al., 2001). Protein E carries an *in vivo* biotinylation sequence for easy detection of protein E with streptavidin. This increases the molecular weight of Eivb (protein E *in vivo* biotinylated) to 12.7kDa without interfering with lysis ability (Haidinger, 2001).

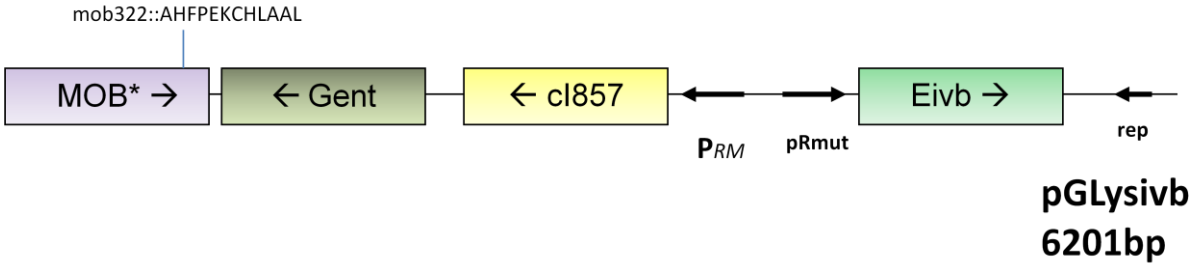


Figure 3: Schematic representation of plasmid pGLysivb. Indicated are the gentamicin resistance cassette (Gent), the mutated mobilization sequence (MOB*), the thermosensitive phage λ repressor system (cI857) and the *in vivo* biotinylated E protein (Eivb).

3.1.3 POSSIBLE APPLICATIONS

A wide range of applications for BGs has been proposed and tested, as reviewed in e.g. (Eko, et al., 1999) or (Langemann, et al., 2010). These applications include, but are not limited to:

- direct application of BGs derived from pathogenic bacteria as vaccines, possible for a wide range of Gram-negative bacteria
- usage of recombinant BGs to deliver antigens, either attached to the inner or outer membrane, transported to the periplasm or fused to S-layer monomers
- loading of BGs with active substances, leading to a targeted release in specific cell types
- loading of BGs with DNA, targeted to specific cell types for DNA vaccination

The retention of the morphological, structural and antigenic features of their living precursors, as well as the generation of sealed compartments by the fusion of outer and inner membrane, make the BG system an intriguing technology platform, as shown in Figure 4 (Langemann, et al., 2010).

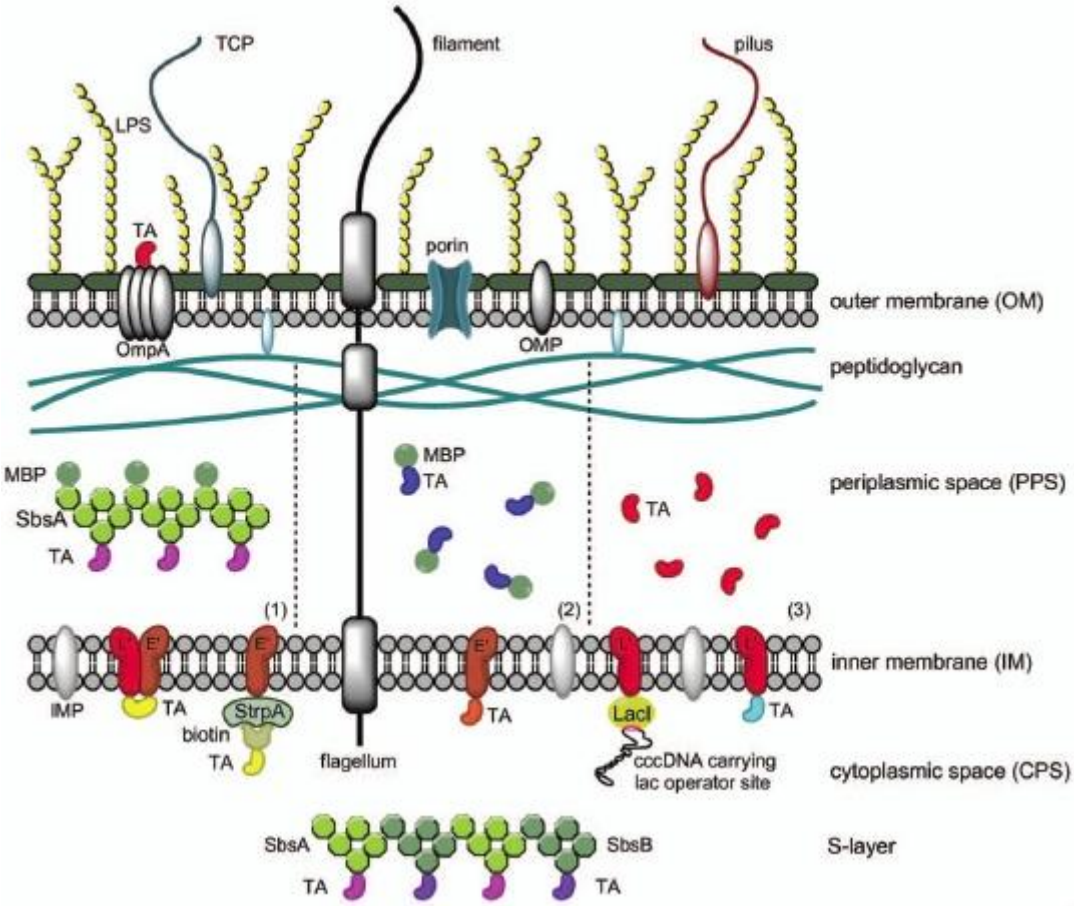


Figure 4: Presentation of antigens in the bacterial envelope/BG. Besides naturally occurring antigens (like LPS, flagellae, pili, OMPs and IMPs) acting as adjuvants, a transgenic antigen (TA) can be (1) fused to SbsA or MBP-SbsA to form a cytoplasmic/periplasmic S-layer; (2) exported to the periplasm fused to MBP; (3) exported to the periplasm using a small GIII tag; presented on the Outer Membrane as an OmpA-fusion protein; fused to inner membrane proteins like L', E', or both; attached via a biotinylation sequence to membrane-bound streptavidin; or finally cccDNA carrying a lac operator can be immobilized to the inner membrane, where LacI is bound. Taken from **(Langemann, et al., 2010)**

BGs have several advantages compared to chemically- or heat-inactivated bacteria: they retain their morphological shape and their antigenic features, which are often compromised using traditional methods. Furthermore, the loss of cytoplasmic content reduces the risk of horizontal gene transfer (Szostak, et al., 1996).

BGs are taken up efficiently by macrophages and dendritic cells, and actively stimulate macrophages and monocytes to induce a T_H1-directed immune response. The natural adjuvants found on the surface of BGs are recognized and targeted by the innate immune system (Haslberger, et al., 2000).

Furthermore, the production process for BGs is comparatively quick and cheap; lyophilized BGs are stable for years at room temperature, which makes the administration as a vaccine cold-chain independent (Szostak, et al., 1996).

3.2 *CHLAMYDIA TRACHOMATIS*

3.2.1 PROPERTIES OF *CHLAMYDIA TRACHOMATIS*

In the animal kingdom in general as well as in humans in particular, bacteria of the genus *Chlamydia* are among the most common bacterial pathogens. As non-motile, Gram-negative, obligate intracellular bacteria they are distinguished from all other microorganisms by their unique developmental cycle. *Chlamydia* are energy parasites, taking up ATP from their host-cells without generating it themselves by metabolic reactions (Schachter, 1990).

Chlamydia replicate in the host cell's cytoplasm, forming intracellular inclusions observable via light microscopy. They exist in two distinct organisational forms, the extracellular Elementary Body (EB) and the Reticulate Body (RB) which is formed inside an infected cell (Schachter, 1990).

Chlamydia lack peptidoglycan, both in their EB and RB form (Fox, et al., 1990). The structural rigidity of EBs is provided by the cross-linking of three cysteine-rich proteins found in the outer membrane, of which the Major Outer Membrane Protein (MOMP) is the most important, constituting about 60% of the outer membranes mass (Hatch, et al., 1986).

EBs are taken up by susceptible host cells via an endocytosis-related process, which is enhanced and stimulated by the bacteria. Recent studies have shown that the uptake process is likely dependent on clathrin, whereas phagocytosis, caveolae and macropinocytosis do not appear to play an important role (Hybiske, et al., 2007).

Once inside the cell, the fusion of the endosome with phagolysosomes is actively prevented by *Chlamydia* by expressing SNARE-like proteins that block SNARE-mediated membrane fusion, protecting the inclusions and the bacteria within them (Paumet, et al., 2009).

Inside the host cell, the EB reorganizes in the larger, more RNA-rich RB, which is also less rigid. This is due to the reduction of the formerly cross-linked outer membrane proteins, which also allows MOMP to act as a porin (Bavoil, et al., 1984). Chlamydial cells begin to multiply by binary fission as soon as eight hours after the infection, and approximately 18 to 24 hours after the primary infection, some of the RB reorganize into EB, are released into the environment and begin to infect further cells (Schachter, 1990).

An overview of the chlamydial lifecycle is given in Figure 5:

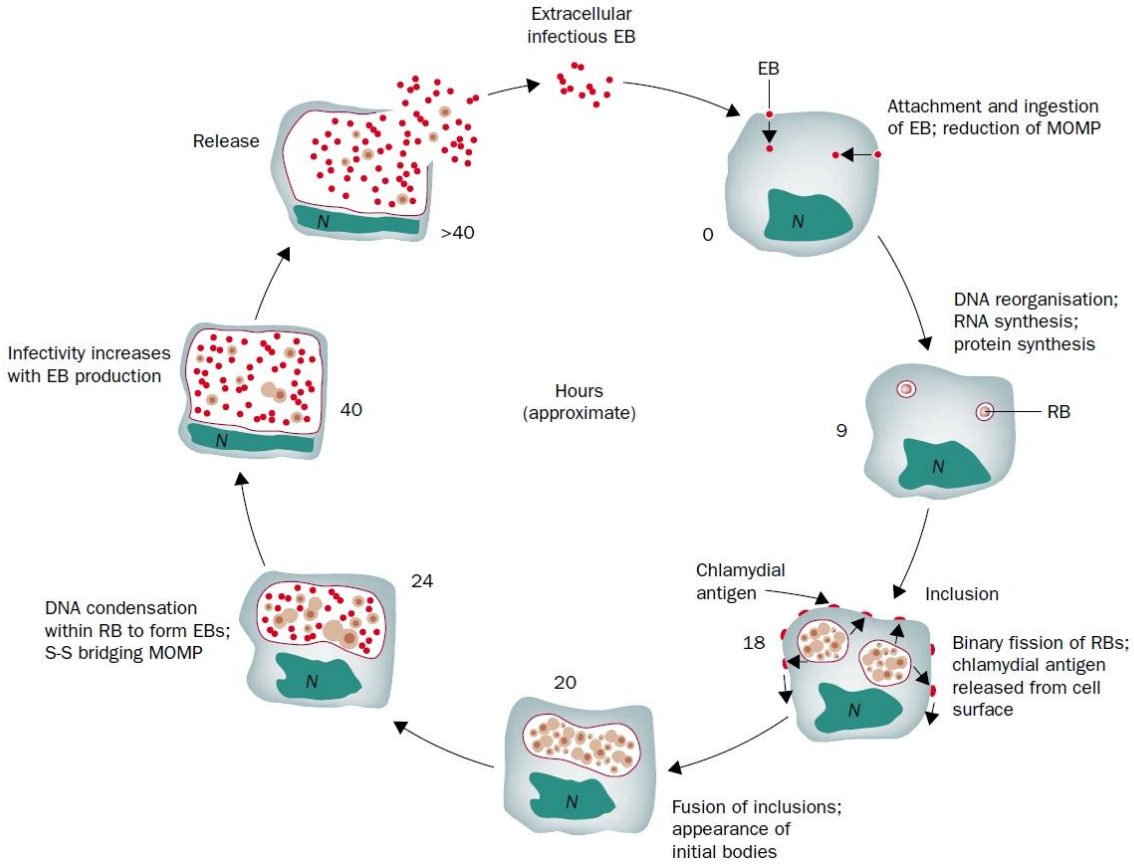


Figure 5: Lifecycle of *Chlamydia trachomatis*. EB – Elementary Bodies. RB – Reticulate Bodies. N – Nucleus. Taken from **(Mabey, et al., 2003)**

3.2.2 CLINICAL SIGNIFICANCE

Of the three known species of the *Chlamydia* genus, *C. suis*, *C. muridarum* and *C. trachomatis*, only the latter infects humans. Two other human-pathogenic species of the Chlamydiaceae family that were once considered *Chlamydia,* were recently moved to a new genus and are now called *Chlamydophila pneumonia* and *Chlamydophila psittaci* (Everett, et al., 1999).

For *C. trachomatis*, 18 serovars, clustered into two biovars, are known. The Lymphogranuloma Venereum biovar consists of serovars L1, L2, L2a and L3, whereas the Trachoma biovar consists of serovars A-K plus Ba, Da and Ia and infects superficial columnar epithelial cells (Schachter, 1978).

Of the 14 Trachoma serovars, A-C are associated with endemic trachoma, the world's leading cause of preventable blindness (Mabey, et al., 2003), whereas serovars D-K are primarily associated with sexual infections (Everett, et al., 1999).

C. trachomatis is the most common sexually-transmitted bacterial pathogen. Even though it is treatable with antibiotics, in most cases the genitourinary infection remains asymptomatic and hence undetected until major complications arise. These include ectopic pregnancies, pelvic inflammatory disease and infertility (Schachter, et al., 1998).

The need for an effective vaccination against chlamydial infections has lead to a multitude of approaches, one of which was the vaccination with inactivated bacteria, which lead to severe side-effects due to immunopathogenic components (Brunham, et al., 1994).

While patients infected with *C. trachomatis* often develop immunity against re-infection, this effect is typically temporarily only; re-infections occurring after waning immunity often lead to increased incidences of sequelae, especially infertility (Hillis, et al., 1997). This has at least partially been attributed to immunopathogenic complications arising from actions of both $CD8^+$ and $CD4^+$ T-lymphocytes, with the former dominating. In contrast, vaccine-induced immunity in a murine model is not accompanied by these sequelae upon infection challenge, while providing significant levels of protective immunity (Igietseme, et al., 2009).

The usage of BGs as efficacious vehicles for different chlamydial antigens has been proposed using recombinant *Vibrio cholera* Ghosts (rVCG) (Eko, et al., 2003). These rVCGs induced protection from infertility and lead to a significant reduction of infection clearance time in a mouse model (Ifere, et al., 2007).

3.2.3 CHLAMYDIAL ANTIGENS: PORB

PorB is a 38kDa porin that was first bioinformatically predicted after the *C. trachomatis* genome was sequenced (Stephens, et al., 1998). It is encoded by the *porB* gene and notably conserved among different serovars of *C. trachomatis*, with only minor or no sequence variations, in stark contrast to e.g. MOMP.

Like MOMP, PorB localizes to the outer membrane, but in much smaller numbers. Its transcription and translation coincides with *ompA* transcription, and PorB is present in both

RBs and EBs, throughout the whole developmental cycle. Its surface accessibility to antibodies, as well as its function as a porin have been shown experimentally (Kubo, et al., 2000).

Human sera from female *Chlamydia* patients show little or no Anti-PorB-Antibodies, with Anti-MOMP-Antibodies dominating. In mice, immunisation with PorB elicits a strong antibody response against PorB, especially if mice are subsequently infected with EBs (Kawa, et al., 2004). The possibility of neutralizing antibodies against PorB protecting against different serovars of *C. trachomatis* justifies its consideration for vaccine development.

3.2.4 CHLAMYDIAL ANTIGENS: MOMP

The Major Outer Membrane Protein, MOMP, of *C. trachomatis* is encoded by the *ompA* gene, has a mass of 40kDa and constitutes about 60% of the chlamydial outer membrane. In its oxidized, cross-linked form the protein is responsible for maintaining the structural integrity of the EB, while the reduced form found in RBs is able to act as a porin with an upper-size limit of 700Da (Caldwell, et al., 1981).

MOMP is the key immunogenic antigen of *C. trachomatis* infections, and antibodies against it dominate the humoral response of infected individuals. This evolutionary pressure has lead to a plethora of mutations, culminating in MOMP polymorphisms responsible for 18 different serovariants, which are used to classify *C. trachomatis.*

While the five transmembrane β-strands are fundamentally conserved and known as the Conserved Domains I-V (CDI – CDV), the four surface-exposed regions are highly variable and mutations are clustered here (Variable Domains, VDI – VDIV) (Nunes, et al., 2009).

The high immunogenicity of MOMP has lead to a focus on this protein for vaccine development, but its high variability would necessitate the use of multi-subunit vaccines to target the various serovars of *C. trachomatis*.

3.3 *ESCHERICHIA COLI* NISSLE 1917

Escherichia coli Nissle 1917 is an *E. coli* strain first isolated in 1917 by a German physician named Nissle, from a soldier who alone among his comrades did not develop gastrointestinal diseases (Nissle, 1918).

E. coli Nissle possesses an H1 flagellum, a K5 capsule and an unusual O6 LPS, with only one O6 antigen repeating unit attached to the R1-type core, which contributes to the semi-rough morphology of Nissle colonies and is responsible for its serum-sensitivity and hence its safety (Grozdanov, et al., 2002).

E. coli Nissle has been used as a probiotic for over 90 years, meaning that its administration in living form in adequate amounts confers health benefits to its host (International Scientific Association for Probiotics and Prebiotics, 2009).

Beside its prophylactic use that has been shown to protect against, amongst others, acute and chronic intestine inflammation (Schultz, et al., 2004) and acute secretory diarrhoea (Schroeder, et al., 2006), *E. coli* Nissle has been used therapeutically in Crohn's Disease and Ulcerative Colitis (Schultz, 2008).

E. coli Nissle has been shown to interact with Peripheral Blood T cells (PBT) via their Toll Like Receptor-2 (TLR2), resulting in the downregulation of Interleukin-2 (IL2), Tumor Necrosis Factor Alpha (TNF-α) and Interferon Gamma (INFγ) as well as the upregulation of IL-10, explaining the beneficial effects on (chronic) intestinal inflammation (Sturm, et al., 2005). Furthermore, *E. coli* Nissle contributes to intestinal immunity by stimulating the production of human β–Defensin 2 (Wehkamp, et al., 2004).

The preferential accumulation of *E. coli* Nissle in tumour tissue (Stritzker, et al., 2007) has lead to speculations about the possibility of using *E. coli* Nissle expressing apoptin as a targeted vehicle for colorectal cancer treatment (Zhou, et al., 2011).

Additionally, the use of commensal *E. coli* Nissle in prevention of HIV infection, by having *E. coli* Nissle secrete peptides inhibiting HIV fusion has been discussed (Rao, et al., 2005).

The recognized safety and the established beneficiary effects of *E. coli* Nissle make it an attractive target for the production of a recombinant BG vaccine.

3.4 ADDITIONAL DIAGNOSTIC TOOLS

3.4.1 LYSIS OBSERVATION VIA FLOW CYTOMETRY

Once E-lysis is induced, a sharp drop in culture OD_{600nm} is usually observed, which can be used as a direct, albeit crude indicator of successful lysis. More reliable data are gathered after overnight incubation of count agar plates, which allow the cfu determination before, during and after lysis. However, this method is neither able to distinguish between killed (dead, intact) and lysed (dead, translucent) cells, nor can it assess killing that takes place after the lysis process on the agar plates, during overnight incubation.

Flow cytometry was used before to assess E lysis and to sort lysed from non-lysed cells (displaying Green Fluorescent Protein, GFP) via FACS (Haidinger, et al., 2001). Recently a new method was developed to observe E-mediated lysis with a very short time delay (less than 10min) without the need of GFP expression.

Two fluorescent dyes are used to distinguish between live intact, dead intact and dead lysed cells via flow cytometry:

- RH414 stains phospholipids indiscriminately, providing a tool with which non-cellular background ('junk') can be distinguished from biogenic particles. All particles not stained by this dye are excluded from the observation.
- $DiBAC_4(3)$ stains only those cells that have lost their membrane potential, i.e. dead intact and dead lysed cells.

By excluding all non-biogenic particles via RH414 and by combining the Forward Scatter Signal (FSC) of a particle with its fluorescence signal 1 (FL1) given by $DiBAC_4(3)$, three regions can be drawn:

- Region 1 (R1), live intact cells: low FL1 signal (intact membrane potential), high FSC (opaque cells)
- Region 2 (R2), dead intact cells: high FL1 signal (no membrane potential), high FSC (intact cells maintaining their opacity)
- Region 3 (R3), dead lysed cells: medium to high FL1 signal (no membrane potential), low FSC (BGs are more translucent than intact cells)

Except for a time frame of ≈30min immediately after lysis induction, the live cell counts from flow cytometry and the cfu data obtained the next day (after plating) match very closely, showing that flow cytometry is an adequate tool for the quasi-live observation of growing, possible killing and E-mediated lysis of bacterial cultures (Langemann, et al., 2010).

3.4.2 PROTEIN QUANTIFICATION USING S-TAG

The standard method to quantify recombinant proteins in BGs relies on the comparison of signal strength from recombinant proteins and a commercially available positope, containing several epitopes for commonly used tags (Invitrogen, 2011), in a standardized concentration, using Western Blot (Schlacher, 2009).

Because of the high cost of positope, the time consuming Western Blot procedure, and the limited comparability of different-sized proteins due to different blotting efficiencies, a new quantification system for recombinant BGs was to be tested.

The S-Tag system provided by Novagen fuses a 15aa long peptide to recombinant proteins. This peptide, known either as S-Peptide or S-Tag, interacts with the S-Protein to form a functionally intact RNAse S.

The native form of RNAse S is called bovine pancreatic RNAse A; the N-terminal α–helix of the protein is connected to the globular rest of the structure via a flexible linker; cleaving the linker gives rise to the S-Peptide (the N-terminal α–helix) and the S-Protein (the residual protein), neither of which is enzymatically active. However, the S-Peptide binds with a very high affinity to the S-Protein ($K_D=10^9$M), and functionality is restored.

This can be used to purify recombinant, S-tagged proteins (with immobilized S-Protein), or for quantification: in its intact form, RNAse S cleaves poly(C). A standardized amount of S-Tag as well as a sample of bacterial culture with recombinant S-tagged proteins is incubated with substrate and S-Protein. After the reaction is stopped, the concentration of free nucleotides in the supernatant can be measured at A_{280nm}; given an excess of S-Protein and substrate, the concentration of free nucleotides is directly proportional to the amount of S-Tag, and hence of recombinant, S-tagged protein (Novagen, 2009).

4 GOALS

The aims of this work are:

- to clone the chlamydial antigens MOMP and PorB into expression vectors for export into the periplasm
- to express MOMP and PorB in *E. coli* NM522 and *E. coli* Nissle
- to express MOMP and PorB, followed by E-mediated lysis, generating BGs carrying the chlamydial antigens MOMP and PorB, in small scale and large scale experiments
- to quantify the amount of MOMP and PorB in BGs
- to test and compare different methods of expression induction (L-arabinose/IPTG), transport to the periplasm (fusion to DsbC/GIII) and antigen detection (using Myc/S-Tags) using the example of PorB

In order to achieve these goals, a number of plasmids have been designed, as detailed below.

4.1 CLONING STRATEGY pBGKB-PORB

The pBGKB vector (as seen in Figure 6) is based on Invitrogen's pBAD-GIII-B vector (Invitrogen, 2001), with the ampicillin resistance cassette having been exchanged to a kanamycin resistance cassette (Schlacher, 2009).

The plasmid incorporates a GIII export signal derived from bacteriophage fd, which directs proteins to the periplasm using the bacterial Sec transport system; the 18aa signal sequence is usually removed after crossing the inner membrane. The export signal is followed by a Multiple Cloning Site (MCS), a Myc tag and a C-terminal PolyHis tag. The fusion protein which is generated by cloning into the MCS is tightly regulated by the pBAD promoter system. Protein expression is turned on by adding L-arabinose and the expression level can simply be varied by varying the concentration of L-arabinose (Guzman, et al., 1995). The expression of the recombinant protein can be detected (and quantified) using antibodies against either the PolyHis or Myc tag.

Plasmid pMAL-PorB that served as a template for PCR amplification of *porB*, was a gift from F. Eko (Morehouse School of Medicine, Atlanta, GA, USA) and was constructed by PCR amplifying gene *porB* from *Chlamydia trachomatis* serovar D and cloning it into vector pMAL-p2x (F. Eko, personal communication). Vector pMAL-p2x is commercially available (NEB, 2011).

The cloning strategy for pBGKB-PorB construction is detailed in Figure 6.

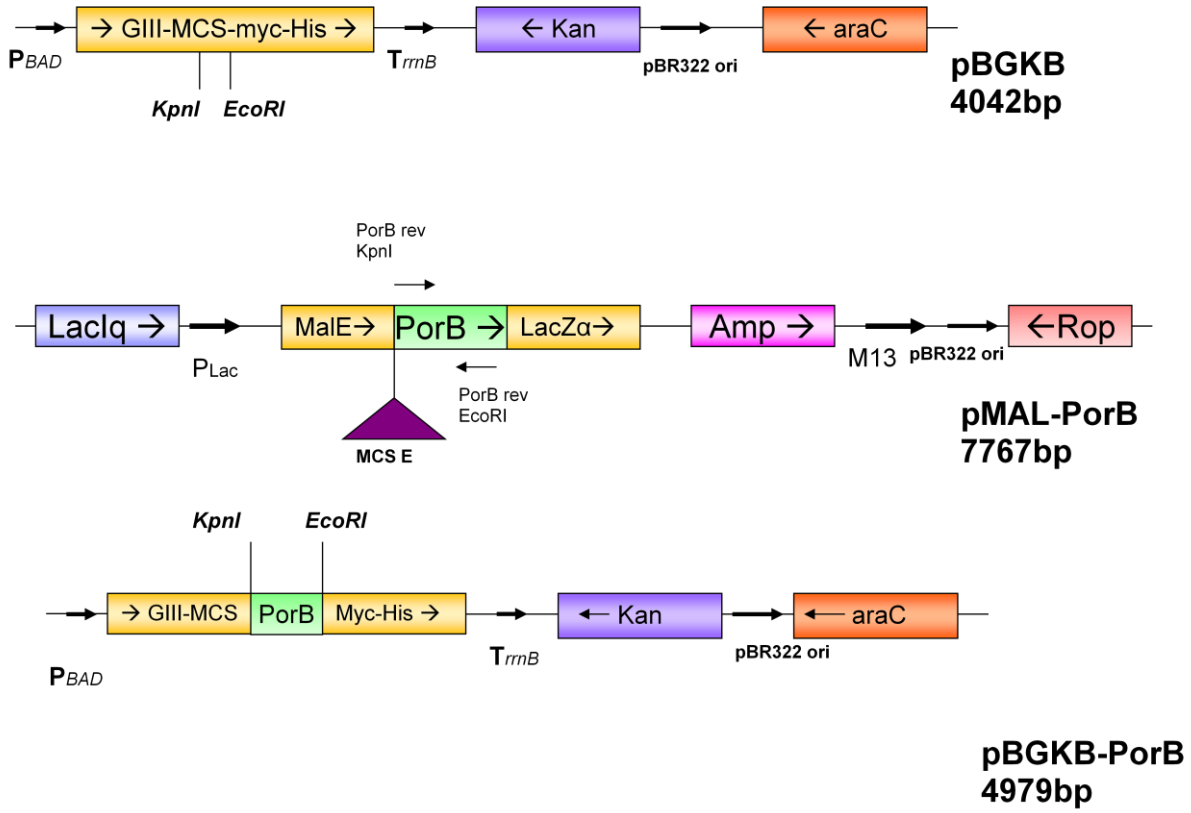


Figure 6: Cloning strategy to incorporate *PorB* into pBGBK, giving rise to pBGKB-PorB

To effect this cloning, *porB* is PCR amplified, introducing restriction sites for *Kpn*I and *Eco*RI, giving a PCR fragment of 955bp. Both the PCR product and pBGKB are then digested with those restriction enzymes and the fragments are ligated to each other afterwards.

The primer pair used for PCR amplification is given in Table 1:

Name	Enzyme	Direction	Sequence (5' – 3')	T_m
pBGKB-PorB	*Kpn*I	Fwd	ATATAGGTAC'CATGCCTGCGGGGAATCCG	60°C
pBGKB-PorB	*Eco*RI	Rev	ATATAG'AATTCCGAATTGGAATCCTCCGGAGA	60°C

Table 1: Primers used to clone *PorB* into pBGKB

The resultant plasmid pBGKB-PorB has a size of 4979bp; upon induction with L-arabinose, a 40.8kDa protein in the form of GIII-PorB-Myc-PolyHis is translated and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 38.7kDa.

4.2 CLONING STRATEGY pET40B-PORB

The pET40b vector is available from Novagen (Novagen, 2011) and designed to export proteins to the periplasm, fused to the 236aa long DsbC-tag, using the SRP transport system. It carries a kanamycin resistance cassette, and the MCS lies between a C-terminal PolyHis tag and a 15aa S-Tag. Proteins fused to the S-Tag can interact with the S-Protein to form a functionally intact RNAse S. This can be used to quantify proteins in a so called S-Tag assay (Novagen, 2011).

Expression of the fusion protein is induced by activation of T7 RNA Polymerase, which is not present on the plasmid, but has to be supplied from the bacterial host strain. The strain *E. coli* C41 which carries an IPTG-inducible T7 RNA polymerase on its chromosome can be used for expression experiments with pET40b vector derivates.

The cloning strategy for pET40b-PorB construction is detailed in Figure 7:

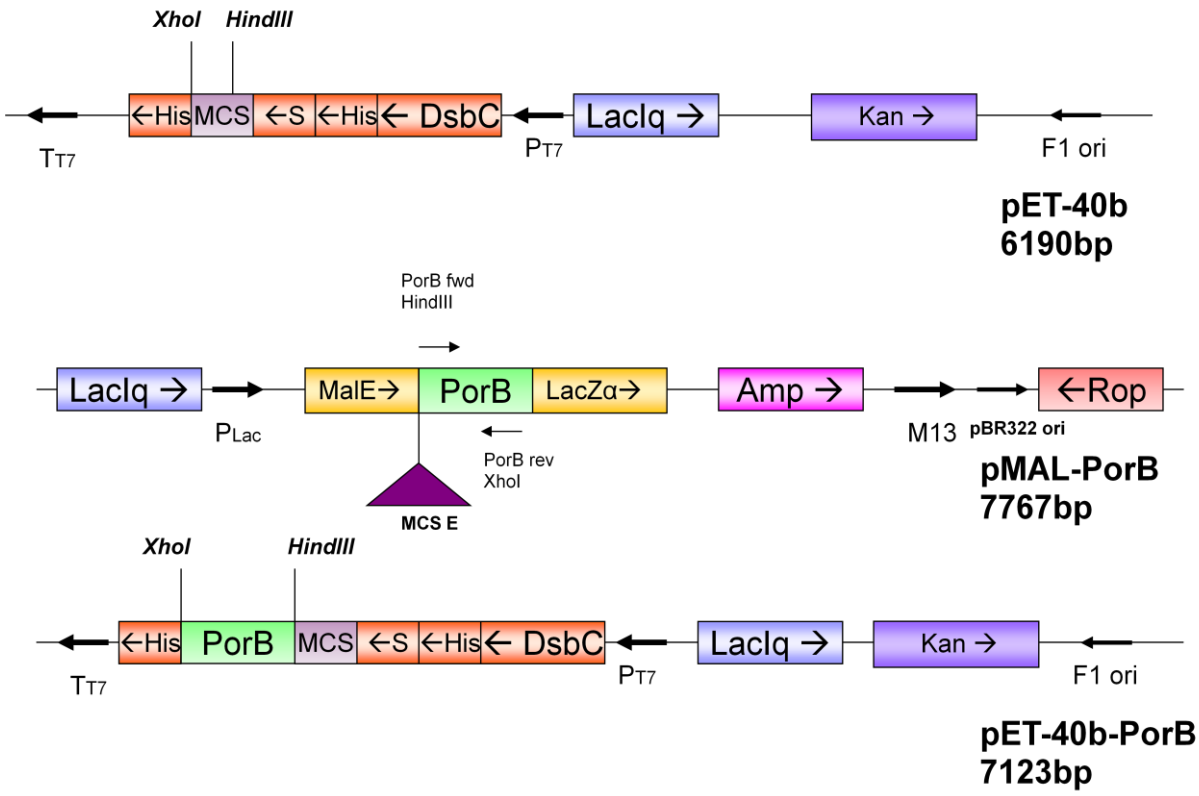


Figure 7: Cloning strategy to incorporate *PorB* into pET40b, giving rise to pET40b-PorB

To effect this cloning, *porB* is PCR amplified using pMAL-PorB as a template, introducing restriction sites for *Hind*III and *Xho*I, giving a PCR fragment of 954bp. These enzymes are used to digest the PCR product and pET40b, and the resulting fragments are ligated to each other.

The primer pair used for PCR amplification is given in Table 2:

Name	Enzyme	Direction	Sequence (5' – 3')	T_m
pET40b-PorB	*Hind*III	Fwd	TATATA'AGCTTATGCCTGCGGGGAATCCG	60°C
pET40b-PorB	*Xho*I	Rev	TATATC'TCGAGGAATTGGAATCCTCCGGAGA	60°C

Table 2: Primers used to clone *PorB* into pET40b

The resultant plasmid pET40b-PorB has a size of 7123bp; upon induction with IPTG, a 69.1kDa protein in the form of DsBC-PolyHis-S-Tag-PorB-PolyHis is translated and exported to the periplasm. While the first 20aa of DsbC, corresponding to the signal sequence for periplasmic translocation, are cleaved off by a signal peptidase, the remaining part of DsbC stays fused to PorB, in form of a 67.0kDa protein.

4.3 CLONING STRATEGY pASK

To combine the ability of S-Tag quantification with a host-strain independent inducing system where the expression level can also be varied by L-arabinose addition, a new expression plasmid is constructed and dubbed pASK (**p**lasmid **A**rabinose-inducible, **S**-Tagged, **K**anamycin resistant). Plasmid pASK was designed to export recombinant proteins to the periplasm using the GIII export signal; recombinant proteins are tagged with PolyHis and S-Tags, and their expression can be detected and quantified using Anti-His-Antibodies or S-Proteins.

The cloning strategy for pASK construction is outlined in Figure 8:

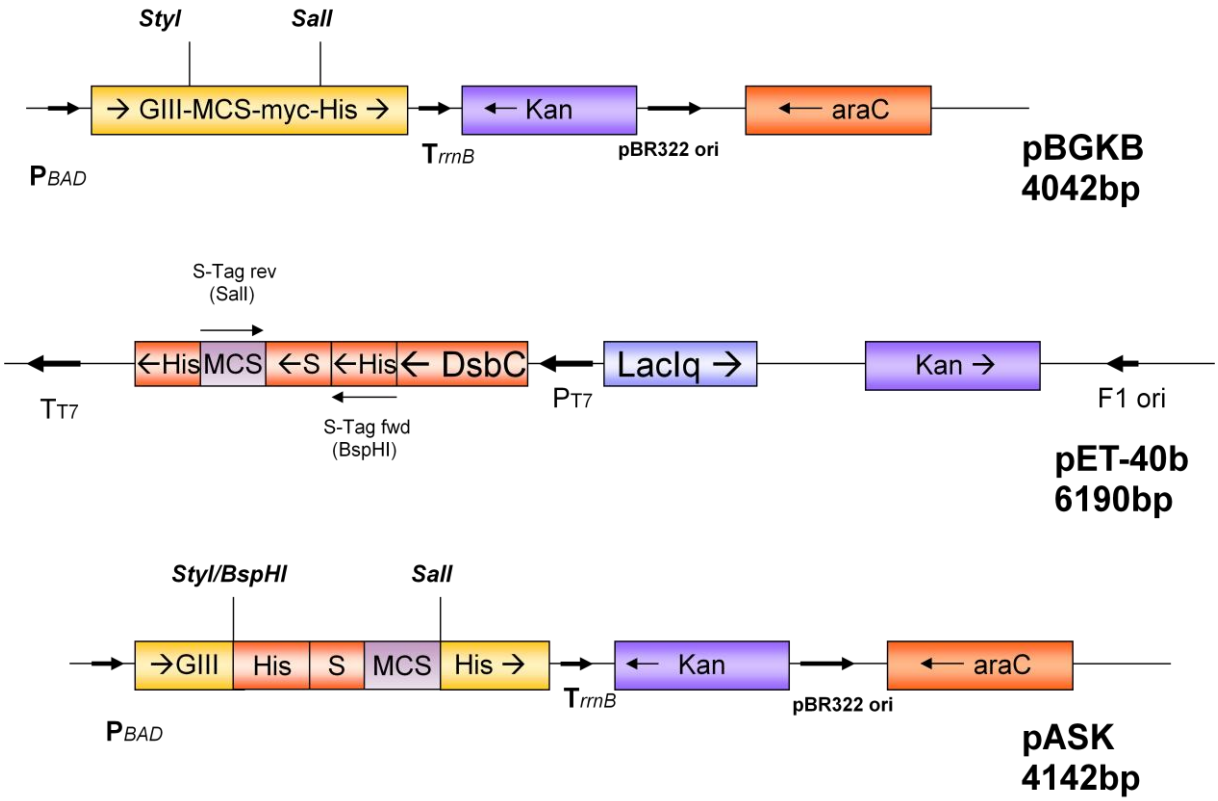


Figure 8: Cloning strategy for pASK, combining pBGKB's backbone with pET40b's MCS and S-Tag

To effect this cloning, the MCS, S-Tag and one of the His-Tags of pET40b are PCR amplified, carrying over the existing *Sal*I restriction site found in the MCS and introducing a *Bsp*HI restriction site behind the His-Tag, giving a PCR fragment of 200bp. Vector pBGKB is cleaved with *Sal*I and *Sty*I (which gives compatible ends to *Bsp*HI), the PCR product is cleaved with *Sal*I and *BspHI*, and the fragments are ligated to each other.

The primer pair given in Table 3 is used for PCR amplification:

Name	Enzyme	Direction	Sequence (5' – 3')	T_m
pASK	*Bsp*HI	Fwd	TATATT'CATGAGTCATCACCATCACCATCACTC	60°C
pASK	*Sal*I	Rev	TATATG'TCGACGGAGCTCGAATTC	60°C

Table 3: Primers used to clone pET40b's S-Tag, MCS and one of its PolyHis-Tags into pBGKB

The resultant plasmid pASK has a size of 4142bp; upon induction with L-arabinose, a 10.0kDa protein in the form of GIII-PolyHis-S-Tag-PolyHis is translated and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 8.0kDa.

4.4 CLONING STRATEGY pASK-PORB

Like pBGKB-PorB, pASK-PorB exports PorB to the periplasm upon arabinose induction, but in this case the PorB protein is also S-tagged, in addition to its Myc and PolyHis tag.

The cloning strategy for pASK-PorB construction is detailed in Figure 9:

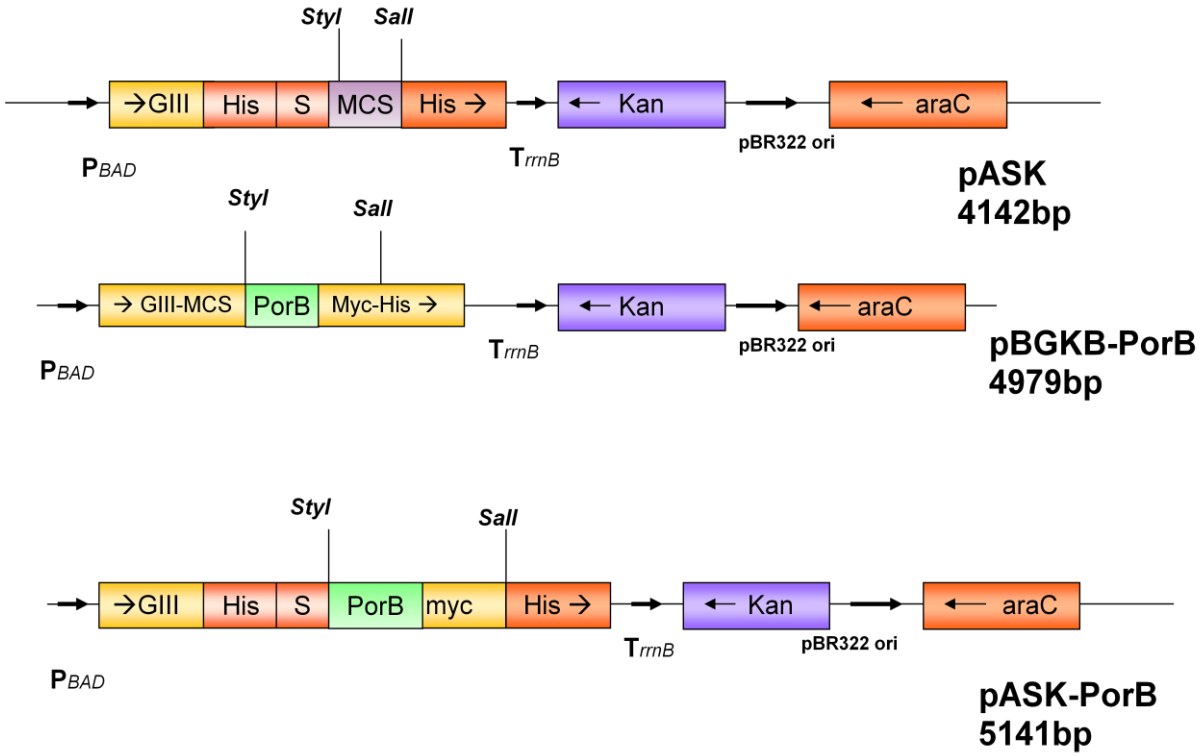


Figure 9: Cloning of pASK-PorB by cleaving PorB-Myc out of pBGBK-PorB and ligating it into pASK

In this case, no PCR amplification is necessary and *porB* including its Myc tag is cleaved from pBGBK-PorB by double digestion with *Sty*I and *Sal*I. The same restriction enzymes are used to

digest pASK, effectively removing the entire MCS, and the fragments are ligated to each other.

The resultant plasmid pASK-PorB has a size of 5141bp; upon induction with L-arabinose, a 46.6kDa protein in the form of GIII-PolyHis-S-Tag-PorB-Myc-PolyHis is translated and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 44.6kDa.

4.5 CLONING STRATEGY pBGKB-MOMP

The second chlamydial antigen used in this work is the Major Outer Membran Protein, MOMP from *Chlamydia trachomatis* serovar D. It was also a gift from F. Eko, in the form of the plasmid pKS-MOMP, which itself is a derivate of plasmid pKSEL5-2, and which has been published (Eko, et al., 2003).

The cloning strategy of pBGKB-MOMP construction is detailed in Figure 10 below:

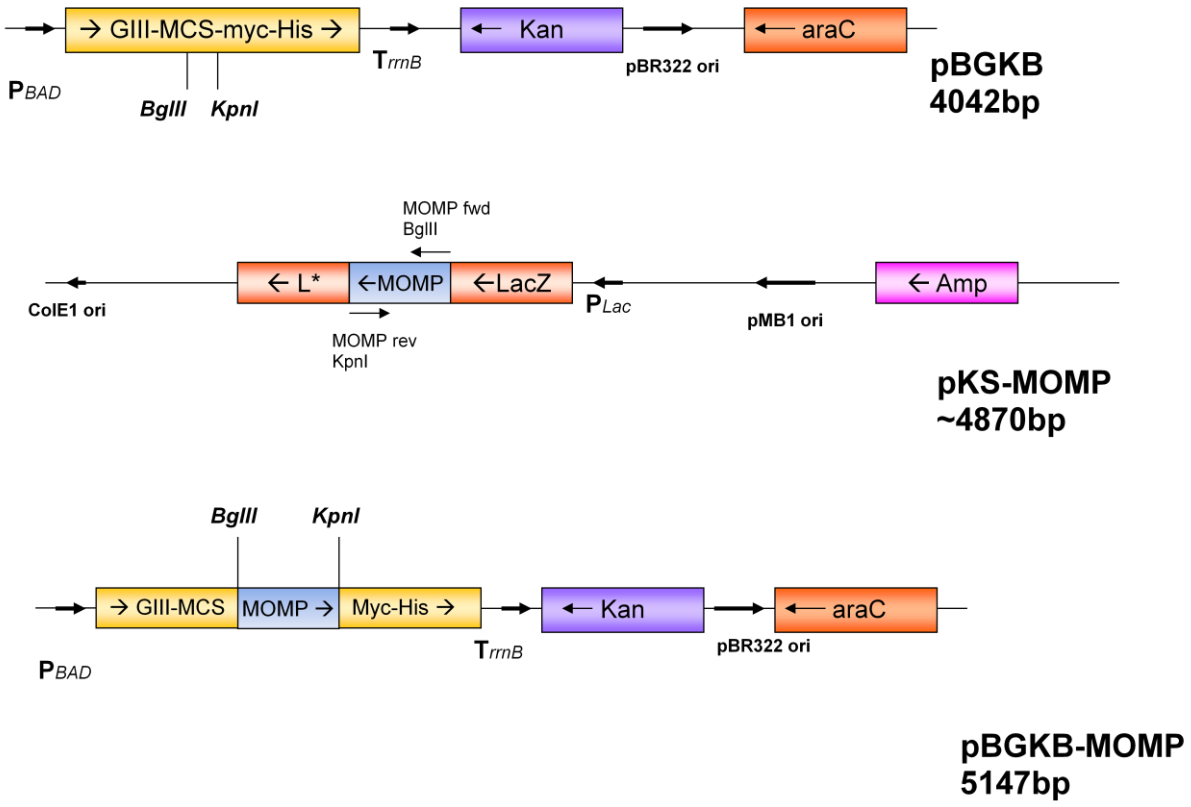


Figure 10: Cloning strategy to incorporate *MOMP* into pBGKB, giving rise to pBGKB-MOMP

To effect the cloning, *ompA* is PCR amplified introducing restriction sites for *Bgl*II and *Kpn*I, giving a PCR fragment of 1133bp. Both the PCR product and pBGKB are then digested with those two restriction enzymes, and the fragments are ligated to each other afterwards.

The primer pair given in Table 4 is used for PCR amplification:

Name	Enzyme	Direction	Sequence (5' – 3')	T_m
pBGKB-MOMP	*Kpn*I	Fwd	TATATGGTAC'CCGAAGCGGAATTGTGCATTTAC	58°C
pBGKB-MOMP	*Bgl*II	Rev	TATATA'GATCTCCTGTGGGGAATCCTGCT	60°C

Table 4: Primers used to clone *MOMP* into pBGKB

The resultant plasmid pBGKB-MOMP has a size of 5147bp; upon induction with L-arabinose, a 46.5kDa protein in the form of GIII-MOMP-Myc-PolyHis is translated and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 44.4kDa.

5 RESULTS

5.1 CLONING A NEW EXPRESSION PLASMID – pASK

Midipreps were performed of *E. coli* C2988J (pET40b) and of *E. coli* C2988J (pBGKB) to be used as source material for this cloning. The MCS, S-Tag and internal His-Tag of pET40b was PCR amplified at an annealing temperature of 60°C and an elongation time of 30sec, introducing a restriction site for *Bsp*HI while carrying over the restriction site of *Sal*I. The expected size of the PCR product was 200bp; the PCR product, run on a 2% agarose gel, can be seen in Figure 11.

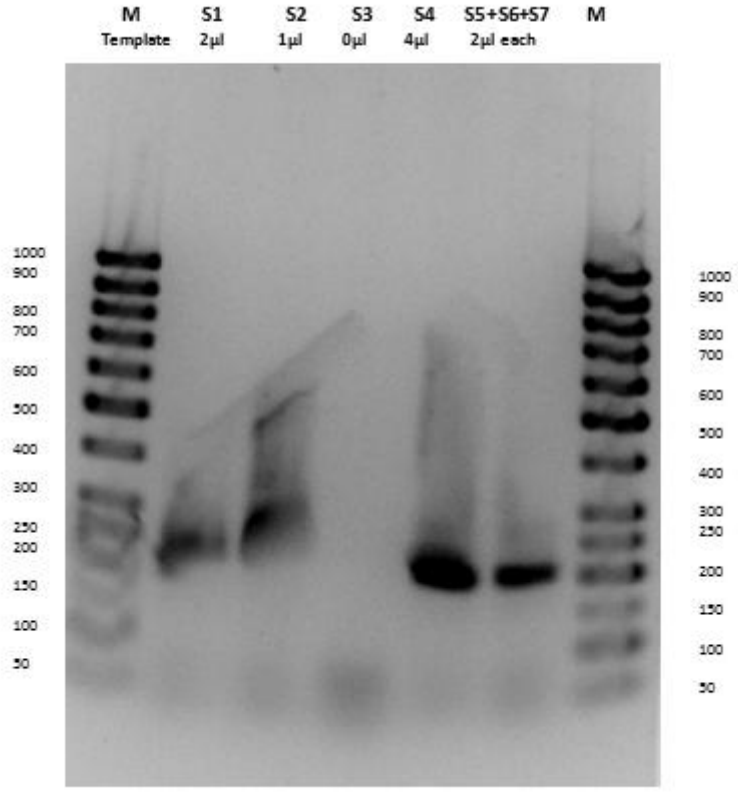


Figure 11: Pfu production PCR, using different amounts of template DNA, of the MCS, S-Tag and internal PolyHis tag of pET40b, run on a 2% agarose gel. The correct-sized band can be seen at 200bp. Marker: O'GeneRuler™ 50bp DNA Ladder (Fermentas)

After cleaning up the PCR product with the PCR Purification Kit, two large scale double digests were performed for three hours: the PCR product was cut with *Bsp*HI and *Sal*I, whereas an aliquot of pBGKB was digested with *Sty*I and *Sal*I.

Digested DNA was put on an agarose gel, with expected sizes of 190bp for the PCR fragment (see Figure 12), and 3940bp for the large fragment of pBGKB (see Figure 13).

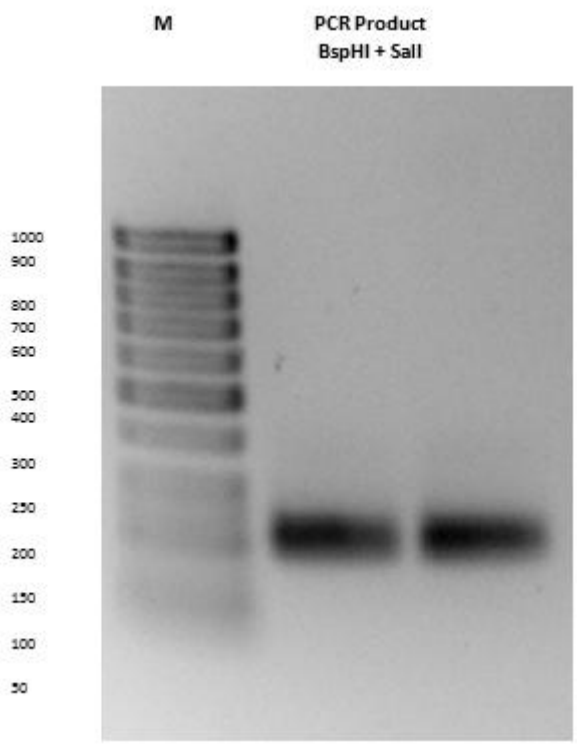


Figure 12: PCR product after double-digestion with *Bsp*HI and *Sal*I, on a 1% agarose gel. The correct sized band can be seen at 190bp. Marker: O'GeneRuler™ 50bp DNA Ladder (Fermentas)

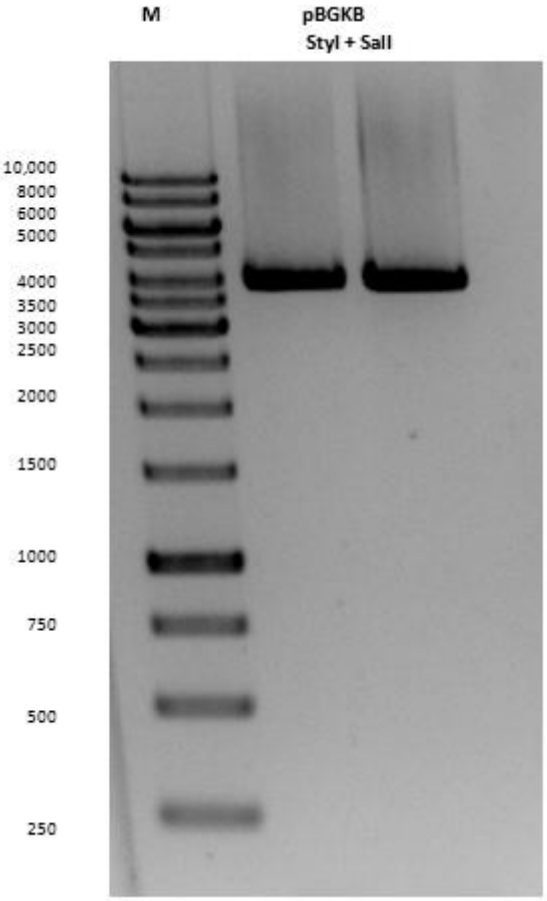


Figure 13: Vector pBGKB after double-digestion with *Sty*I and *Sal*I on a 1% agarose gel. The correct-sized band is visible at 3940bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

DNA fragments were excised from the gel and eluted in 50µl dH_2O using the Gel Extraction Kit. An aliquot of 5µl was put on a 1% agarose gel for concentration determination (not shown) and ligation was performed overnight.

The next day, the ligation mixture was transformed into MOPS-competent *E. coli* C2988J, which were then plated on LB+Kan plates. After overnight incubation at 36°C, plates showed good efficiency of transformation. Four clones were picked, inoculated overnight in LBv+Kan and miniprepped on the next day.

Miniprep DNA of different clones was first digested with a single enzyme (not shown), then a correct-sized clone was further digested with a total of four different restriction enzymes to check for the characteristic, correct pattern of pASK, as seen in Figure 14.

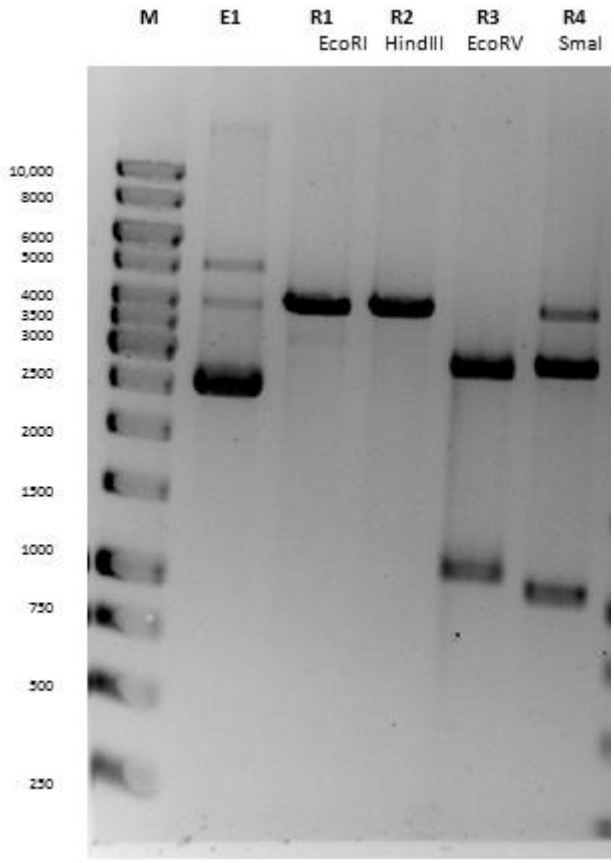


Figure 14: Digest of pASK clone A4 on a 1% agarose gel. Lane E1: undigested DNA; Lane R1: pASK digested with *Eco*RI, expected size: 4142bp; Lane R2: pASK digested with *Hind*III, expected size: 4142bp; Lane R3: pASK digested with *Eco*RV, expected sizes: 1159/2983bp; Lane R4: pASK digested with *Sma*I, expected sizes: 1075/3067bp (digest incomplete; repeated digests have found correct pattern). Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

The correctly-identified clone A4 of *E. coli* C2988J (pASK) was stored as a glycerol culture and later midiprepped for further cloning procedures; since the plasmid was not sequenced, confirmation of the correct cloning relies on the restriction digest pattern.

5.2 CLONING CHLAMYDIAL ANTIGENS

5.2.1 CLONING pBGKB-PORB

Midipreps of *E. coli* C2988J (pBGKB) and *E. coli* C2988J (pMAL-PorB) were used as source material for this cloning. Gene *porB* was PCR amplified with primers introducing restriction sites for *Kpn*I and *Eco*RI, with an annealing temperature of 60°C and an elongation time of 60sec. The size of the expected PCR product was 955bp. After the PCR run, the product was put on an agarose gel, shown in Figure 15:

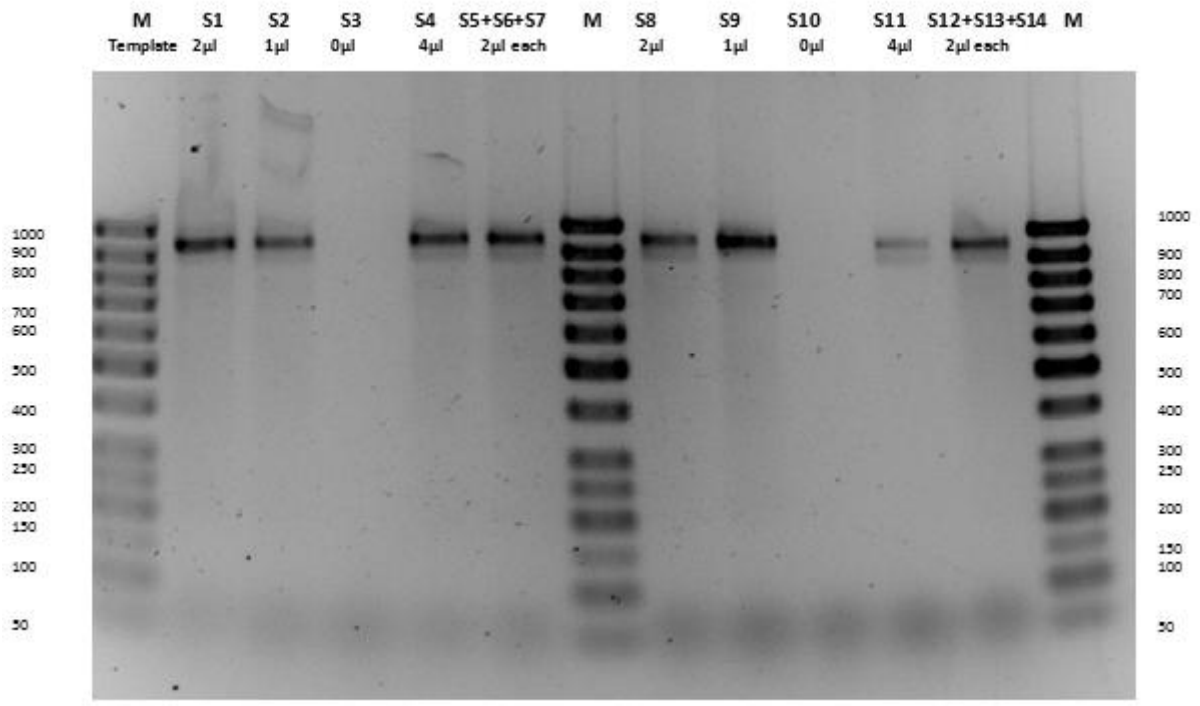


Figure 15: Pfu production PCR of PorB, using primers introducing *Xho*I and *Hind*III restriction sites in lanes S1-S7, and using primers introducing *Kpn*I and *Eco*RI restriction sites in lanes S8-14. The amount of template DNA (pMAL-PorB) is indicated under the respective lane. Correct-sized PCR product can be seen at 954bp (lanes S1-7) and 955bp (lanes S8-14). Samples were run on a 2% gel. Marker: O'GeneRuler™ 50bp DNA Ladder (Fermentas)

After cleaning up the PCR product with the PCR Purification Kit, the PCR product and the vector pBGKB were doubly-digested in a large scale restriction digest, using enzymes *Kpn*I and *Eco*RI. After three hours of digestion, fragments were put on an agarose gel, with expected sizes of 940bp for the PCR product and 4034bp for the larger vector fragment of pBGKB, as seen in Figure 16:

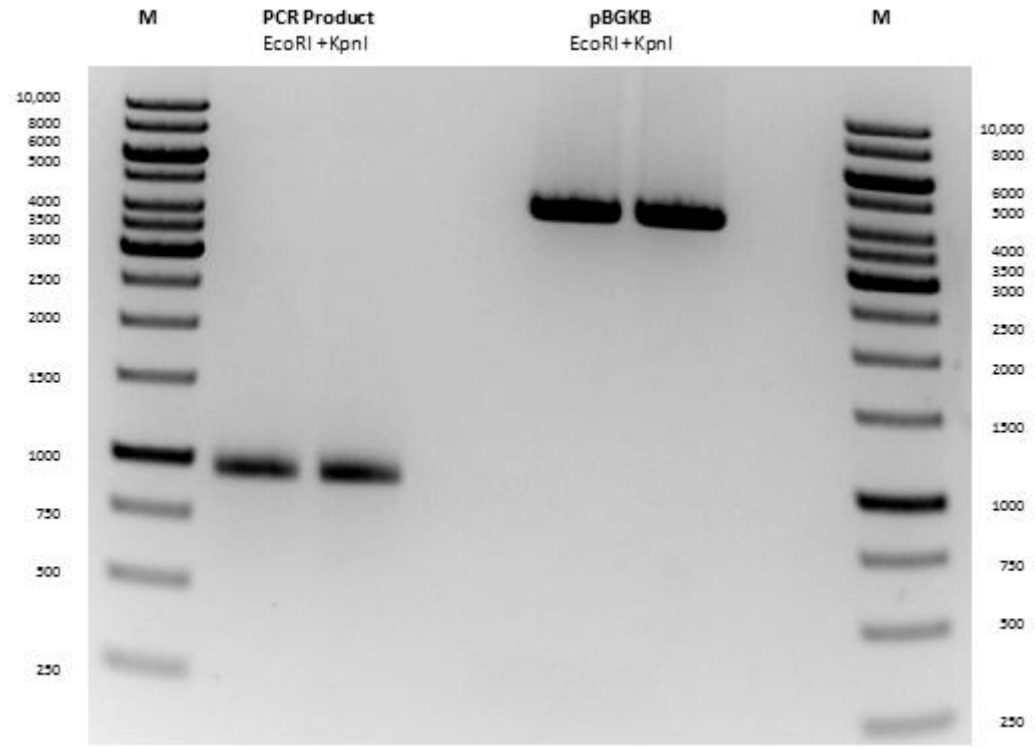


Figure 16: PorB PCR product and vector pBGKB after double digestion with *Kpn*I and *Eco*RI, on a 1% agarose gel. Correct-sized bands can be seen at 940bp (PCR product) and 4034bp (pBGKB fragment). Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Both fragments were excised from the gel and eluted in 50µl dH_2O using the Gel Extraction Kit. An aliquot of 5µl was put on a 1% agarose gel for concentration determination (not shown), the fragments were ligated to each other overnight and transformed in MOPS-competent *E. coli* C2988J cells that were then plated on LB+Kan plates.

The plates showed good growth after overnight incubation at 36°C; four colonies were picked, inoculated in 5ml LBv+Kan overnight and miniprepped on the next day. The four clones were first digested with one restriction enzyme to look for the correct size (not shown), and a conforming clone was then digested with four enzymes to look for the correct digestion pattern of pBGKB-PorB, shown in Figure 17:

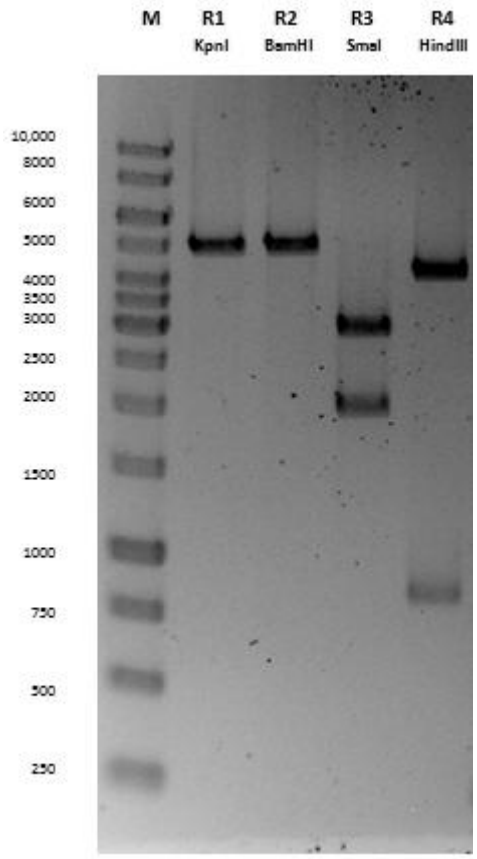


Figure 17: Digest of pBGKB-PorB clone C1 on a 1% agarose gel. Lane R1: pBGKB-PorB digested with *Kpn*I, expected size: 4979bp; Lane R2: pBGKB-PorB digested with *Bam*HI, expected size: 4979bp; Lane R3: pBGKB-PorB digested with *Sma*I, expected sizes: 1993/2986bp; Lane R4: pBGKB-PorB digested with *Hind*III, expected sizes: 829/4150bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Restriction digest patterns (not sequencing) were used to identify the correct clone C1 of *E. coli* C2988J (pBGKB-PorB); an aliquote was stored as a glycerol culture and its midiprep DNA was used to transform other strains for all further experiments with pBGKB-PorB.

5.2.2 CLONING pET40B-PORB

Midipreps of *E. coli* C2988J (pET40b) and *E. coli* C2988J (pMAL-PorB) were used as source material for this cloning. Gene *PorB* was PCR amplified with primers introducing restriction sites for *Xho*I and *Hind*III, with an annealing temperature of 60°C and an elongation time of 60sec. The size of the expected PCR product was 954bp. After the PCR run, the product was put on an agarose gel (see lanes S1-7 of Figure 15).

After the PCR was cleaned up using the PCR Purification Kit, both the PCR product (see Figure 18) and vector pET40b (see Figure 19) were subjected to a large scale double-digest, using *Xho*I and *Hind*III. After three hours of digestion, the fragments were put on an agarose

gel. The expected sizes are 942bp for the PCR fragment and 6175bp for the larger fragment of pET40b.

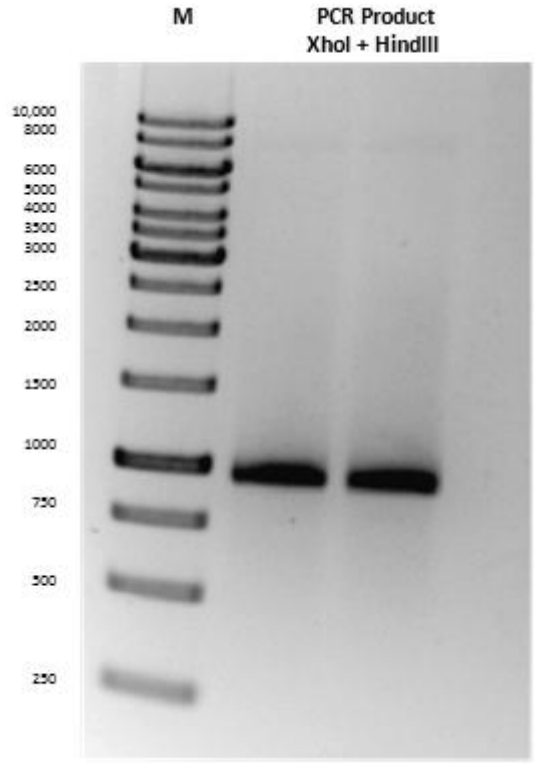


Figure 18: PCR product after double-digestion with *Xho*I and *Hind*III, on a 1% agarose gel. The correct sized band can be seen at 942bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

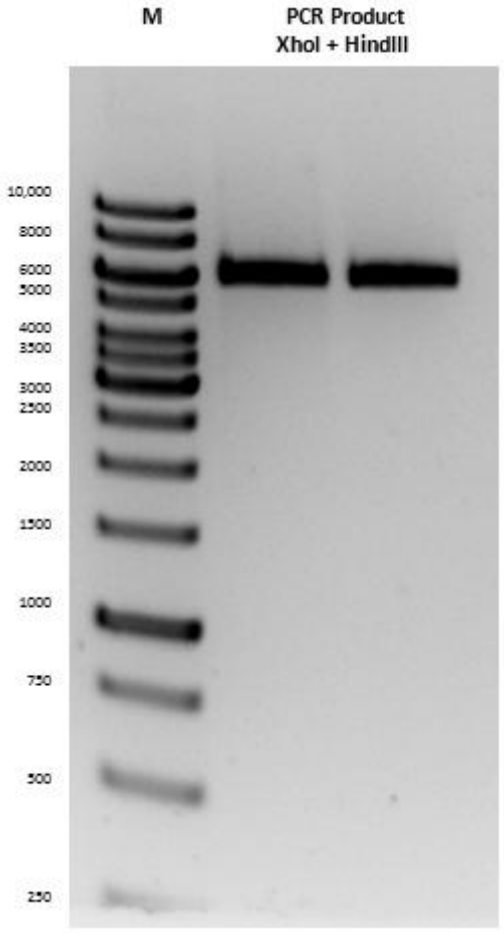


Figure 19: Vector pET40b after double-digestion with *Xho*I and *Hind*III, on a 1% agarose gel. The correct sized band can be seen at 6175bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Correct-sized fragments were excised from the gel and eluted in 50µl dH_2O using the Gel Extraction Kit; an aliquot of 5µl was put on a 1% agarose gel for concentration determination (not shown). Overnight, the fragments were ligated to each other and the ligation product was transformed into MOPS-competent *E. coli* C2988J cells and plated onto LB+Kan plates. After overnight incubation at 36°C, plates showed good growth and four colonies were picked and inoculated in 5ml LBv+Kan. After overnight inoculation, the four clones were miniprepped, and miniprep DNA was digested with one restriction enzyme to find correct-sized clones (not shown). A correct sized clone was then digested with four different restriction enzymes to look for the correct restriction digest pattern of pET40b-PorB, as seen in Figure 20:

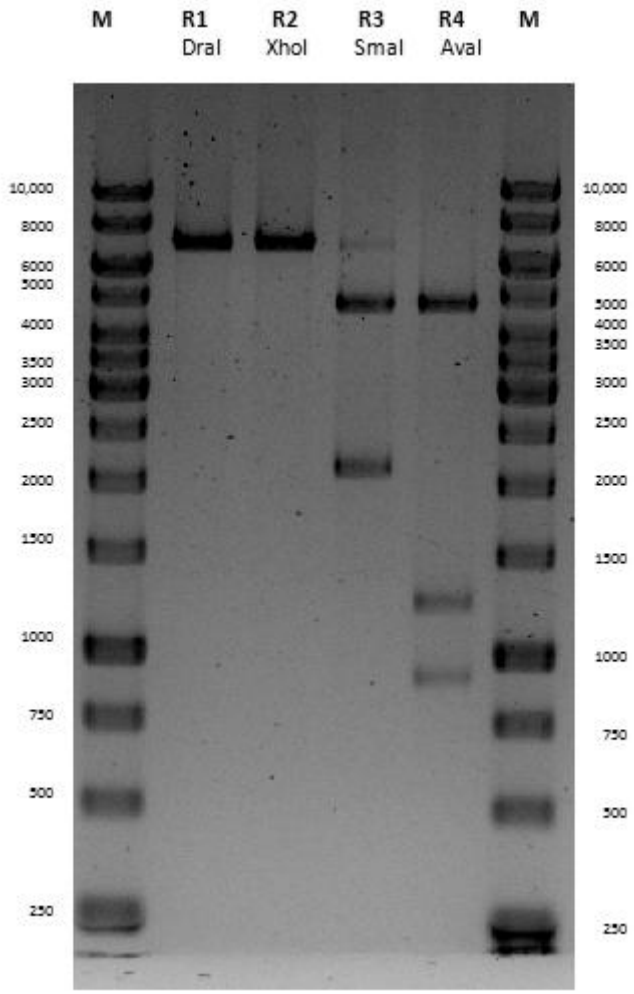


Figure 20: Digest of pET40b-PorB clone B4 on a 1% agarose gel. Lane R1: pET40b-PorB digested with *Dra*I, expected size: 7123bp; Lane R2: pET40b-PorB digested with *Xho*I, expected size: 7123bp; Lane R3: pET40b-PorB digested with *Sma*I, expected sizes: 93/2155/4875bp (smallest band too small to see); Lane R4: pET40b-PorB digested with *Ava*I, expected sizes: 93/910/1245/4875bp (smallest band too small to see). Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

The correctly-identified clone B4 of *E. coli* C2988J (pET40b-PorB) was stored as a cryoculture and its midiprep DNA was used to transform strain *E. coli* C41 for further experiments. Correctness was assessed by restriction-digest patterns as well as sequencing of regions flanking the restriction sides, performed by Microsynth (Microsynth, Balgach, Switzerland). Sequencing confirmed the correct insertion (no frameshift) of *porB* into the pET40b MCS (data not shown).

5.2.3 CLONING pASK-PORB

Midipreps of *E. coli* C2988J (pASK) clone A4 and of *E. coli* C2988J (pBGKB-PorB) clone C1 were used to perform this cloning; no PCR amplification was necessary. Instead, both vectors

were used for a large scale digest with *Sty*I and *Sal*I that linearized pASK and excised PorB (including the Myc-tag) from pBGKB-PorB. The expected sizes of the fragments were 4108bp for the pASK fragment and 1039/3948bp for the pBGKB-PorB fragments (of which the 1039bp fragment includes PorB). After three hours of digestion, the fragments were put on an agarose gel, shown in Figure 21:

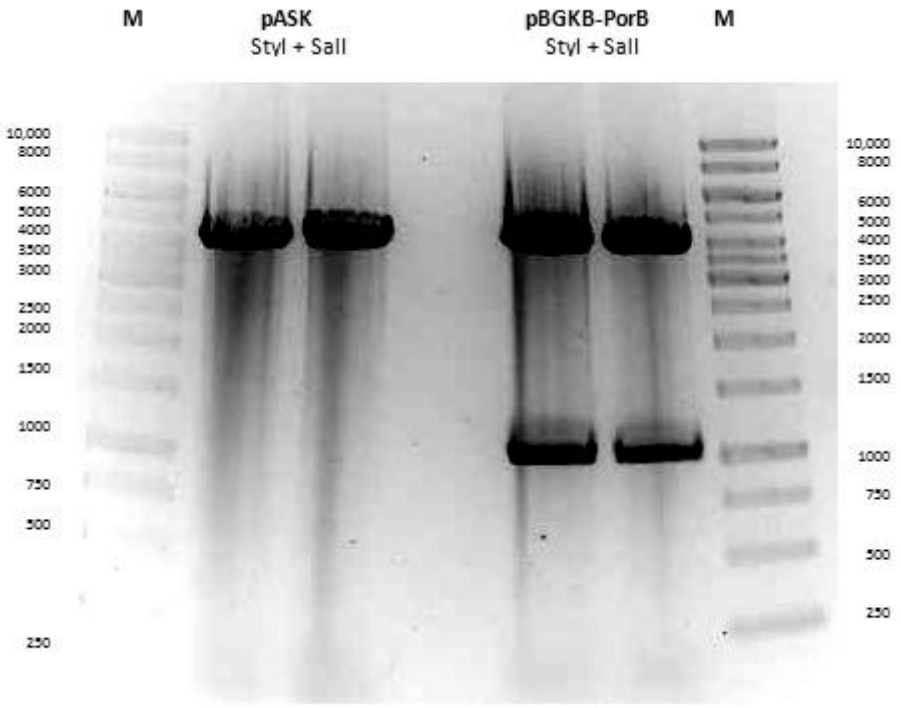


Figure 21: Vectors pASK and pBGKB-PorB after double digestion with *Sty*I and *Sal*I, on a 1% agarose gel. Correct-sized bands can be seen at 4108bp (pASK) and 1039/3948bp (pBGKB-PorB). Linearized pASK and the PorB fragment at 1039bp were excised and purified for further use. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Correct-sized bands were excised from the gel and eluted in 50µl dH_2O using the Gel Extraction Kit. An aliquot of 5µl was put on a 1% agarose gel for concentration determination (not shown). Overnight, the fragments were ligated to each other and the ligation product was transformed into MOPS-competent *E. coli* C2988J cells and plated onto LB+Kan plates. After overnight incubation at 36°C, plates showed good growth and four colonies were picked and inoculated in 5ml LBv+Kan. After overnight inoculation, the four clones were miniprepped, and miniprep DNA was digested with one restriction enzyme to find correct-sized clones (not shown). A correct sized clone was then digested with three different restriction enzymes to check the correct restriction digest pattern of pASK-PorB. This can be seen in Figure 22:

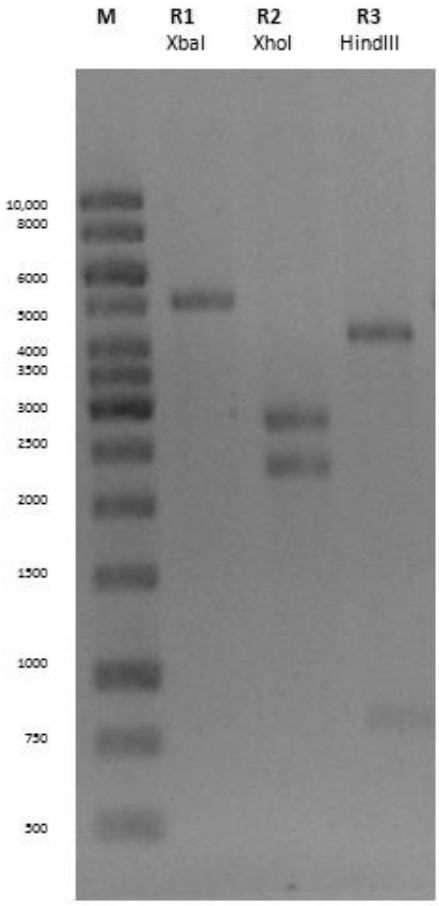


Figure 22: Digest of pASK-PorB clone A3 on a 1% agarose gel. Lane R1: pASK-PorB digested with *Xba*I, expected size: 5141bp; Lane R2: pASK-PorB digested with *Xho*I, expected sizes: 2321/2820bp; Lane R3: pASK-PorB digested with *Hind*III, expected sizes: 829/4312bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

The correctly-identified clone A3 of *E. coli* C2988J (pASK-PorB) was stored as a glycerol culture; its midiprep DNA was used to transform other strains for all further experiments with pASK-PorB. The identification relied on restriction-digest pattern analysis, and not sequencing.

5.2.4 CLONING pBGKB-MOMP

Midipreps of *E. coli* C2988J (pBGKB) and *E. coli* C2988J (pKS-MOMP) were used as source material for this cloning. Gene *MOMP* was PCR amplified with primers introducing restriction sites for *Kpn*I and *Bgl*II, with an annealing temperature of 58°C and an elongation time of 60sec. The size of the expected PCR product was 1133bp. After the PCR run, the product was put on an agarose gel, shown in Figure 23:

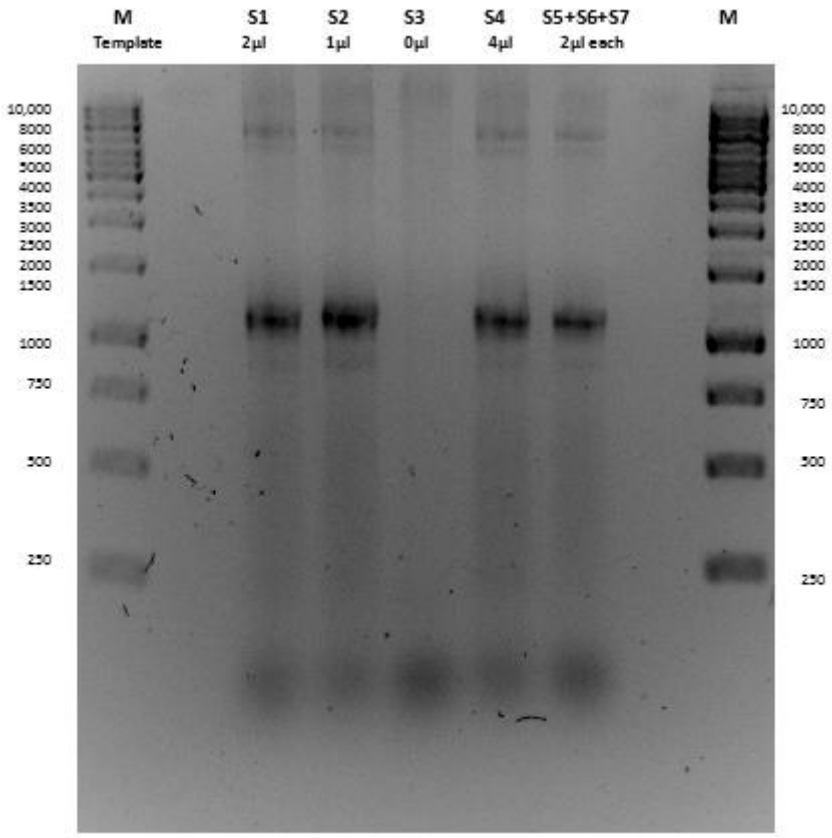


Figure 23: Pfu production PCR of MOMP, using primers introducing *Kpn*I and *Bgl*II restriction sites. The amount of template DNA (pKS-MOMP) is indicated under the respective lane. Correct-sized PCR product can be seen at 1133bp. Samples were run on a 1% agarose gel. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

After cleaning up the PCR product with the PCR Purification Kit, the PCR product and the vector pBGKB were doubly-digested in a large scale restriction digest, using enzymes *Kpn*I and *Bgl*II. After three hours of digestion, fragments were put on an agarose gel, with expected sizes of 1117bp for the PCR product and 4026bp for the larger fragment of pBGKB; the gel picture can be seen in Figure 24:

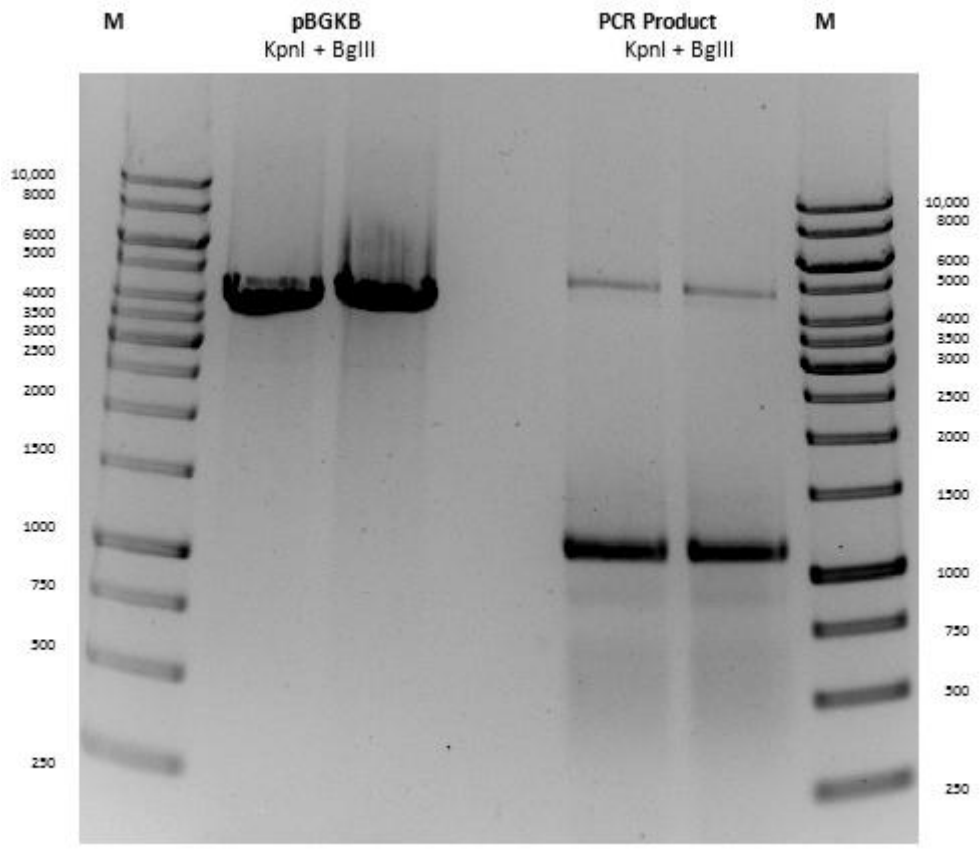


Figure 24: Vector pBGKB and PCR product of MOMP after double digestion with *Kpn*I and *Bgl*II, on a 1% agarose gel. Correct-sized bands can be seen at 4026bp (pBGKB) and 1117bp (PCR Product MOMP). Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Correct-sized bands were excised from the gel and eluted in 50µl dH_2O using the Gel Extraction Kit. An aliquot of 5µl was put on a 1% agarose gel for concentration determination (not shown). Fragments were ligated to each other overnight and the ligation product was transformed into MOPS-competent *E. coli* C2988J cells and plated onto LB+Kan plates. After overnight incubation at 36°C, plates showed good growth and twenty colonies were picked and inoculated in 5ml LBv+Kan. After overnight inoculation, the twenty clones were miniprepped, and miniprep DNA was digested with one restriction enzyme to find correct-sized clones (not shown). A correct sized clone was then digested with four different restriction enzymes to look for the correct restriction digest pattern of pBGKB-MOMP, shown in Figure 25:

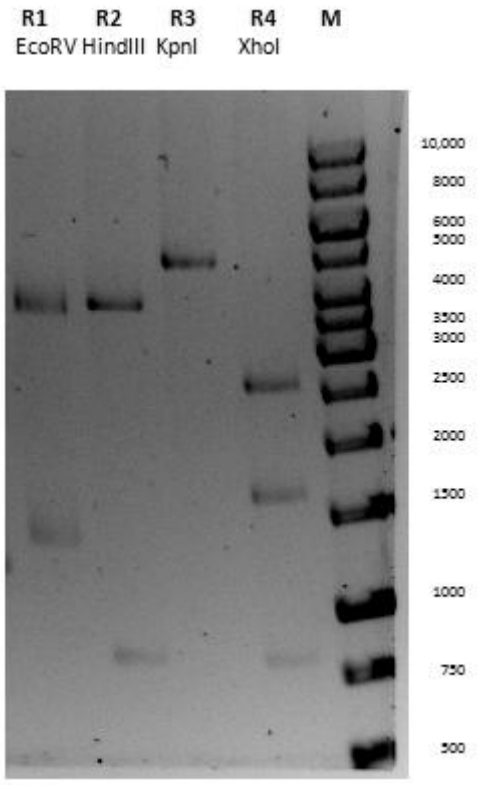


Figure 25: Digest of pBGKB-MOMP clone A11 on a 1% agarose gel. Lane R1: pBGKB-MOMP digested with *Eco*RV, expected sizes: 1371/3776bp; Lane R2: pBGKB-MOMP digested with *Hind*III, expected sizes: 315/829/4003bp (the smallest band is too small to be seen); Lane R3: pBGKB-MOMP digested with *Kpn*I, expected size: 5147bp; Lane R4: pBGKB-MOMP digested with *Xho*I, expected sizes: 818/1671/2658bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

The correctly-identified clone A11 of *E. coli* C2988J (pBGKB-MOMP) was stored as a glycerol culture; its midiprep DNA was used to transform other strains for all further experiments with pBGKB-MOMP; identification relied on restriction-digest pattern analysis, and not sequencing.

5.3 EXPRESSING CHLAMYDIAL ANTIGENS

All of the following small scale experiments were carried out in *E. coli* C2988J, except for the expression experiment of pET40b-PorB, which requires the chromosomal T7 RNA Polymerase of *E. coli* C41. While the growth rate of *E. coli* C2988J is lower than of *E. coli* NM522 (the preferred strain for antigen expression for BG production), this is not relevant for the simple determination whether antigen is expressed using the cloned plasmids.

5.3.1 EXPRESSION STUDY pBGKB-PORB

In this small scale expression experiment, two noseflasks (1 and 2) were inoculated with overnight culture of the backbone plasmid carrying *E. coli* C2988J (pBGKB) and two noseflasks (3 and 4) where inoculated with overnight culture of clone C1 of *E. coli* C2988J (pBGKB-PorB).

All four noseflasks were grown at 36°C until an OD_{600nm}≈0.3, when expression was induced in noseflasks 2 and 4 by adding 0.2% L-arabinose (time point 0min). OD values were measured and samples were collected for WB and cfu determination throughout the experiment; plates for cfu counting were incubated at 36°C overnight. A graph showing OD_{600nm} and cfu values for all four noseflasks is given in Figure 26.

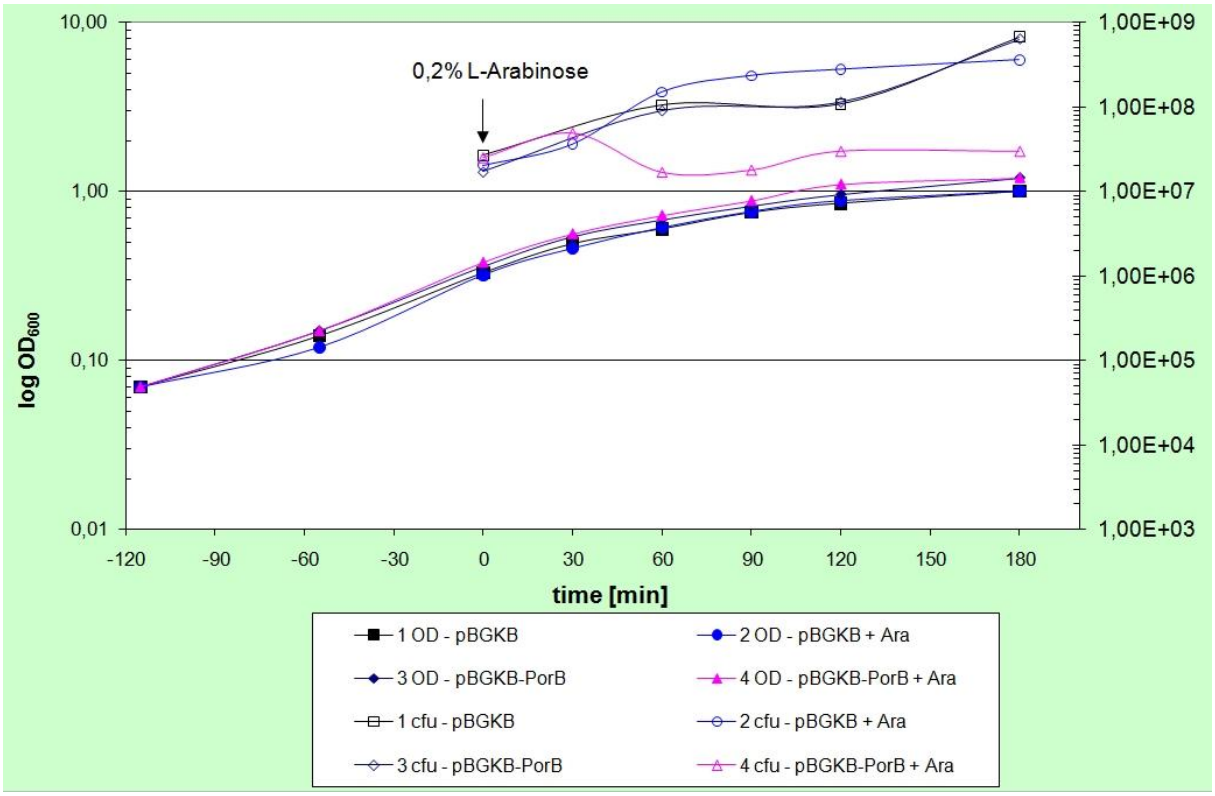


Figure 26: OD and cfu values over time during an expression experiment of *E. coli* C2988J (pBGKB) (flasks 1 and 2) and *E. coli* C2988J (pBGKB-PorB) (flasks 3 and 4). L-Arabinose was added at time point 0min to flasks 2 and 4.

While the OD values of all four flasks behave similarly, the induction of pBGKB-PorB (but not pBGKB) leads to a decrease in cfu of roughly 40% 60min after expression induction, which is not seen after 30min of expression, suggesting the mild toxicity of PorB when exported to the periplasm.

Western Blot samples just prior to L-arabinose induction and after 60min of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the nitrocellulose incubated with HRP-coupled-Anti-Myc-antibodies and developed by chemiluminiscence; the protein GIII-PorB-Myc-PolyHis was expected at a size of 40.8kDa, protein PorB-Myc-PolyHis, lacking the GIII export sequence, at a size of 38.7kDa. The developed Western Blot is shown in Figure 27.

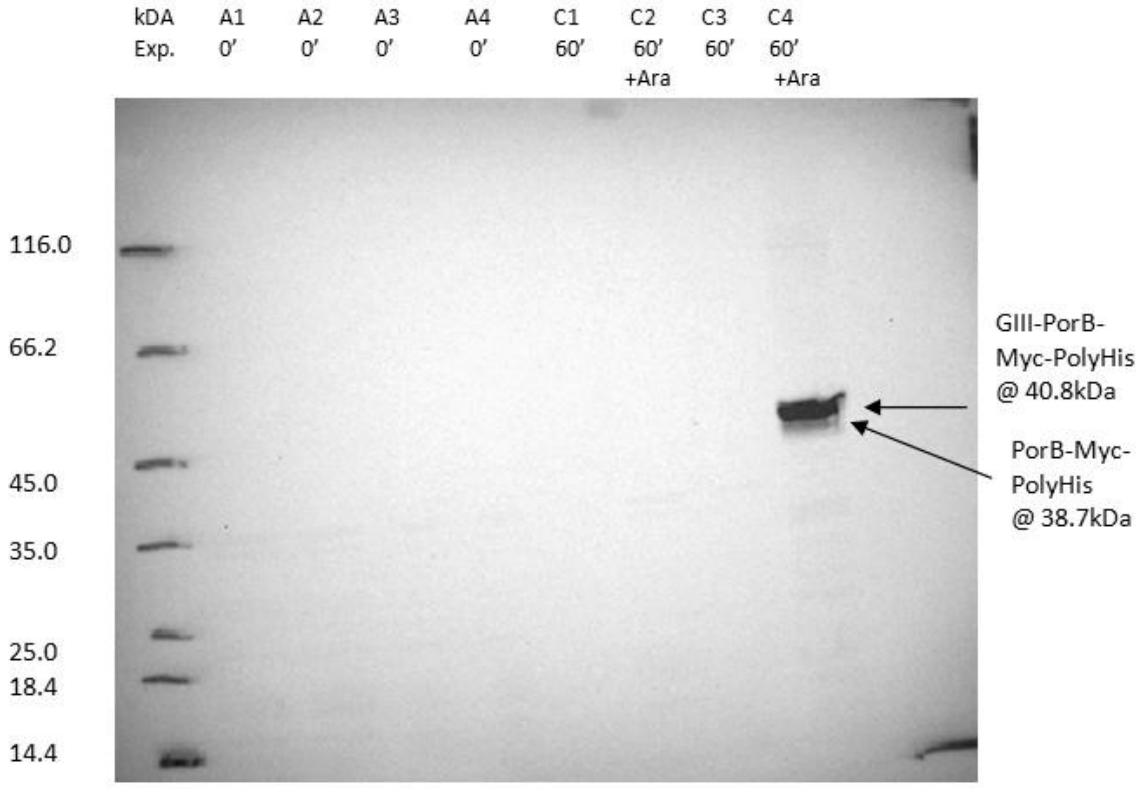


Figure 27: Western Blot of *E. coli* C2988J (pBGKB-PorB) Expression Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-antibodies and developed. Samples A are at the time point of expression induction, samples C are 60min later. Expression was induced in flasks 2 and 4.

The Western Blot shows the correct-sized signal only upon induction and only in samples where plasmid pBGKB-PorB was present (C4); hence it can be concluded that PorB can successfully be expressed from plasmid pBGKB-PorB upon induction.

5.3.2 EXPRESSION STUDY pET40B-PORB

Since the expression system of the pET40b vector system relies on T7 RNA polymerase, plasmid pET40b-PorB was transformed into MOPS-competent *E. coli* C41 cells. For the small

scale expression experiment, two noseflasks (1 and 2) were inoculated with the empty backbone plasmid carrying *E. coli* C41 (pET40b) whereas two noseflasks (3 and 4) were inoculated with clone A1 of *E. coli* C41 (pET40b-PorB). After growing at 36°C to an OD_{600nm}≈0.35, expression was induced in noseflasks 2 and 4 by adding 1mM IPTG (at time point 0min). OD_{600nm} values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values of this experiment, plotted over time are shown in Figure 28.

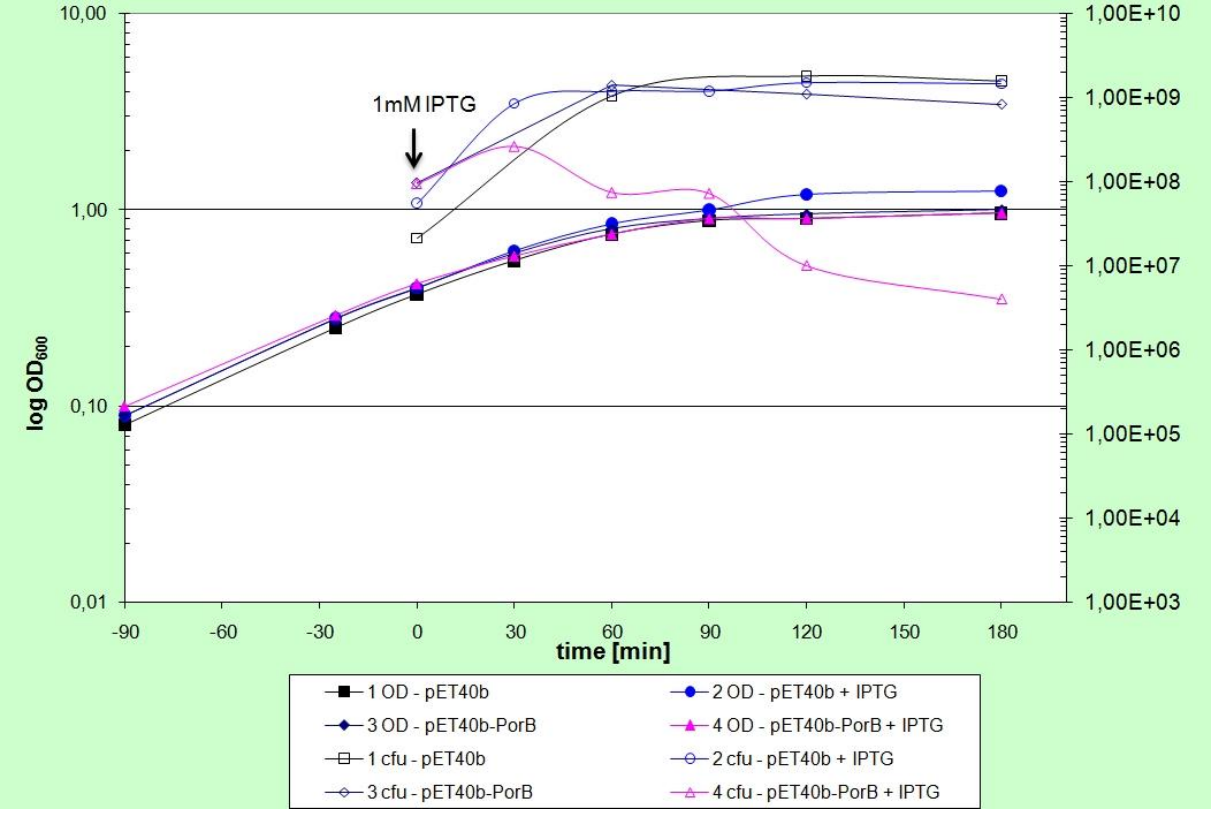


Figure 28: OD and cfu values over time during an expression experiment of *E. coli* C41 (pET40b) (flasks 1 and 2) and *E. coli* C41 (pET40b-PorB) (flasks 3 and 4). IPTG was added at time point 0min to flasks 2 and 4.

The overall picture is quite similar to the expression experiment of pBGKB-PorB: while the OD values of all four flasks behave similarly, the induction of pET40b-PorB (but not pET40b) leads to a strong decrease in cfu (almost 98%) 180min after expression induction, compared to the value before induction. This cfu decrease is apparent after 60min of expression, becoming more dramatic over time, but is not detected 30min after expression induction.

Western Blot samples just prior to IPTG induction and after 60min of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto

nitrocellulose, the nitrocellulose incubated with S-Protein-HRP and developed by chemiluminiscence; the protein DsbC-PolyHis-S-Tag-PorB-PolyHis was expected at a size of 69.1kDa (67kDa for DsbC-PolyHis-S-Tag-PorB-PolyHis with the signal sequence of DsbC cleaved off). The developed WB is shown in Figure 29.

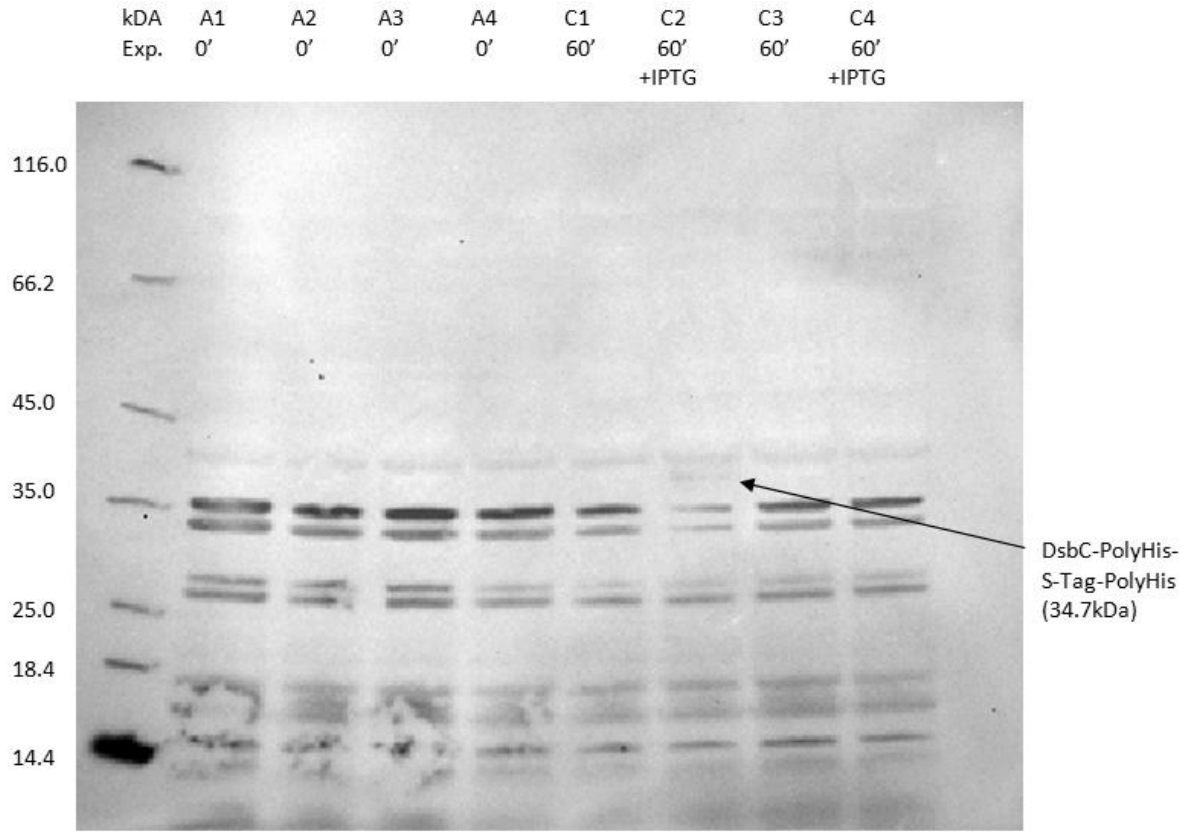


Figure 29: Western Blot of *E. coli* C41 (pET40b-PorB) Expression Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:10,000 diluted S-Protein-HRP and developed. Samples A are at the time point of expression induction, samples C are 60min later. Expression was induced in flasks 2 and 4.

Unfortunately, the Western Blot fails to detect a specific signal associated with PorB, even though the cfu values of flask 4 show a similar trend as in the experiment with pBGKB-PorB, where PorB is also expressed.

While the background signal is fairly high, a distinct and unique band can be seen in lane C2, corresponding to the expressed product of the empty backbone vector, DsbC-PolyHis-S-Tag-PolyHis at 34.7kDa. This shows the ability of the HRP-coupled S-Protein to bind to the S-Tag, which makes the absence of signal in lane C4 even more conspicuous. Anyhow, since the backbone expression is very weak (compared to the background signal) a problem with the WB development can be assumed.

Therefore, a second Western Blot (not shown) with a similar loading scheme was performed and incubated with HRP-coupled-Anti-His-antibodies, showing the same result – very weak recognition of DsbC-PolyHis-S-Tag-PolyHis, but no detection of a PorB protein and a high background signal.

No further work with this plasmid was performed.

5.3.3 EXPRESSION STUDY pASK

In order to test whether the expression of GIII-PolyHis-S-Tag-PolyHis (the gene product encoded by the newly cloned backbone vector pASK) has any influence on the viability of bacteria, a small scale expression experiment was carried out.

Two noseflasks (1 and 2) were inoculated with overnight culture of clone A4 of *E. coli* C2988J (pASK). Both noseflasks were grown at 36°C until an OD_{600nm}≈0.35, when expression was induced in noseflask 1 by adding 0.2% L-arabinose (at time point 0min). OD values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values over time are shown in Figure 30.

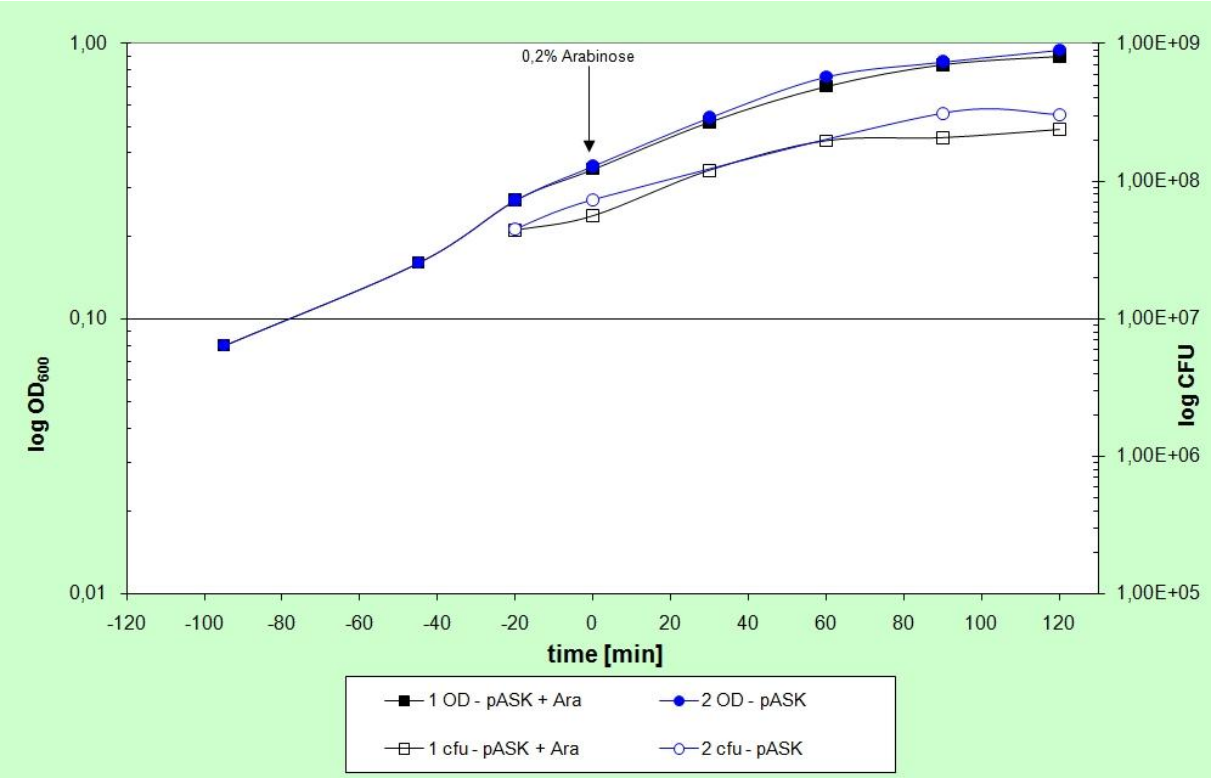


Figure 30: OD and cfu values over time during an expression experiment of *E. coli* C2988J (pASK). L-Arabinose was added at time point 0min to flask 1.

No significant difference in OD_{600nm} or cfu values could be observed, indicating that the newly cloned expression plasmid pASK *per se* is not harmful to bacterial growth when expression is induced.

Since the of GIII-PolyHis-S-Tag-PolyHis and PolyHis-S-Tag-PolyHis are only 10.0kDa and 8.0kDa, respectively, no Western Blot was performed to detect the expression product of pASK. However, a quantification using the S-Tag system was performed, as described in Chapter 5.6.4 Quantification of Recombinant Proteins Using the S-Tag System and in detail in Figure 64.

5.3.4 EXPRESSION STUDY pASK-PORB

In this small scale expression experiment, two noseflasks (1 and 2) were inoculated with overnight culture of clone A3 of *E. coli* C2988J (pASK-PorB). Both noseflasks were grown at 36°C until an OD_{600nm}≈0.35, when expression was induced in noseflask 1 by adding 0.2% L-arabinose (at time point 0min). OD values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values over time are shown in Figure 31.

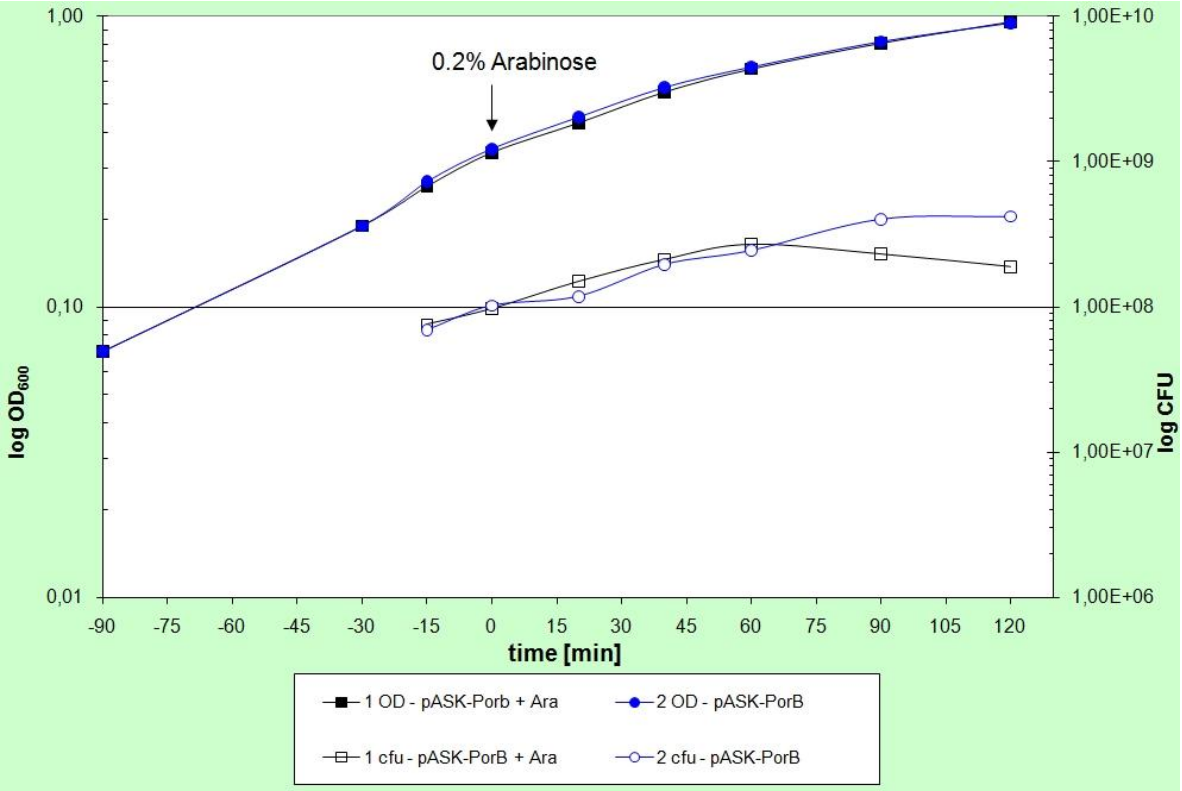


Figure 31: OD and cfu values over time during an expression experiment of *E. coli* C2988J (pASK-PorB). L-Arabinose was added at time point 0min to flask 1.

Similar to previous expression experiments of PorB (in different vector systems), induction of PorB expression leads to a decrease of viability in comparison to a non-induced sample. Interestingly the effect seen in pASK-PorB is smaller than in either pBGKB-PorB or pET40b-PorB, and after 120min of expression, the cfu is still higher than it was at the induction point (in contrast to previous experiments). As before, OD values behave nearly identical on both samples, regardless to induction.

Western Blot samples just prior to L-arabinose induction, after 20min, 60min and 120min of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the nitrocellulose incubated with S-Protein-HRP and developed by chemilumiscence; the protein GIII-PolyHis-S-Tag-PorB-Myc-PolyHis was expected at a size of 46.6kDa, and the protein PolyHis-S-Tag-PorB-Myc-PolyHis (lacking the GIII export sequence) at a size of 44.6kDa. The developed blot is shown in Figure 32.

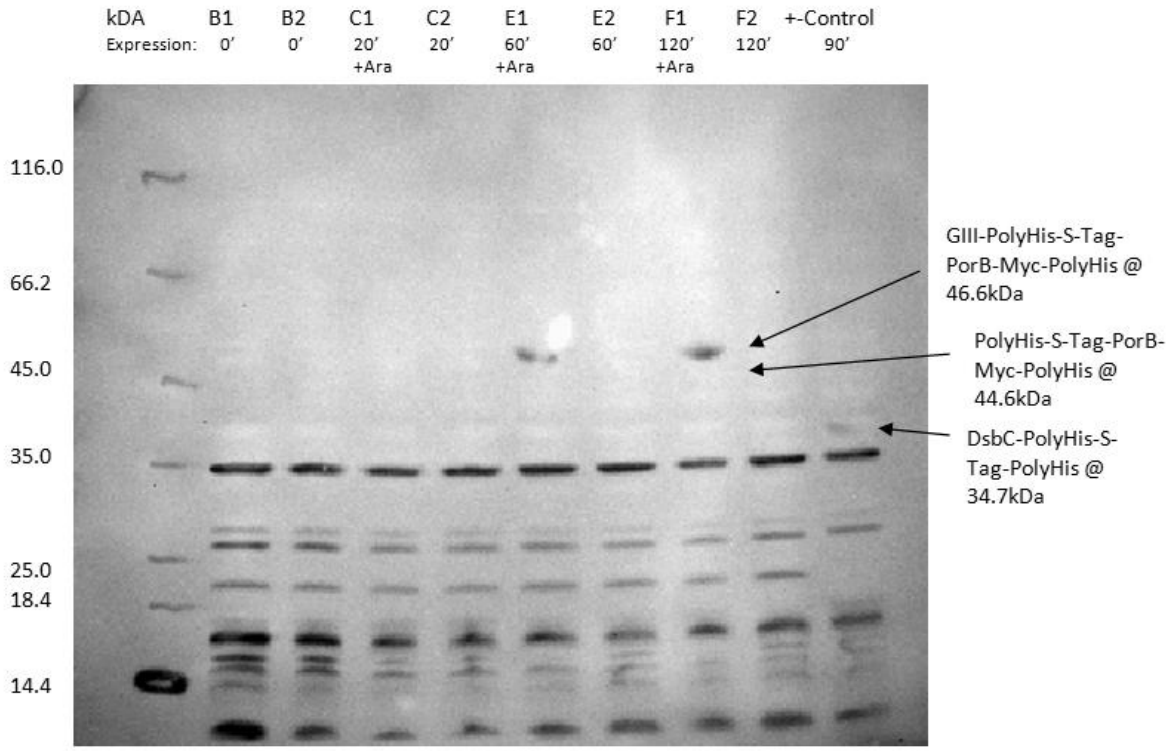


Figure 32: Western Blot of C2988J + pASK-PorB Expression Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted S-Protein-HRP and developed. Samples are at 0min (B), 20min (C), 60min (E) and 120min (F) of protein expression after induction. As a positive control, a sample from a C41 + pET40b expression experiment was used. Expression was induced in flask 1.

Again, the background signal is fairly high. The specific signals detected by the HRP-coupled-S-Protein are the positive control of DsbC-PolyHis-S-Tag-PolyHis, and the S-Tagged PorB protein of induced pASK-PorB. Signal can first be detected after 60min (but not 20min) of expression in sample E1, and again after 120min (F1).

5.3.5 EXPRESSION STUDY pBGKB-MOMP

In this small scale expression experiment, two noseflasks (1 and 2) were inoculated with overnight culture of clone A11 of *E. coli* C2988J (pBGKB-MOMP). Both noseflasks were grown at 36°C until an OD_{600nm}≈0.35, when expression was induced in noseflask 1 by adding 0.2% L-arabinose (at time point 0min). OD values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values are plotted against time in Figure 33.

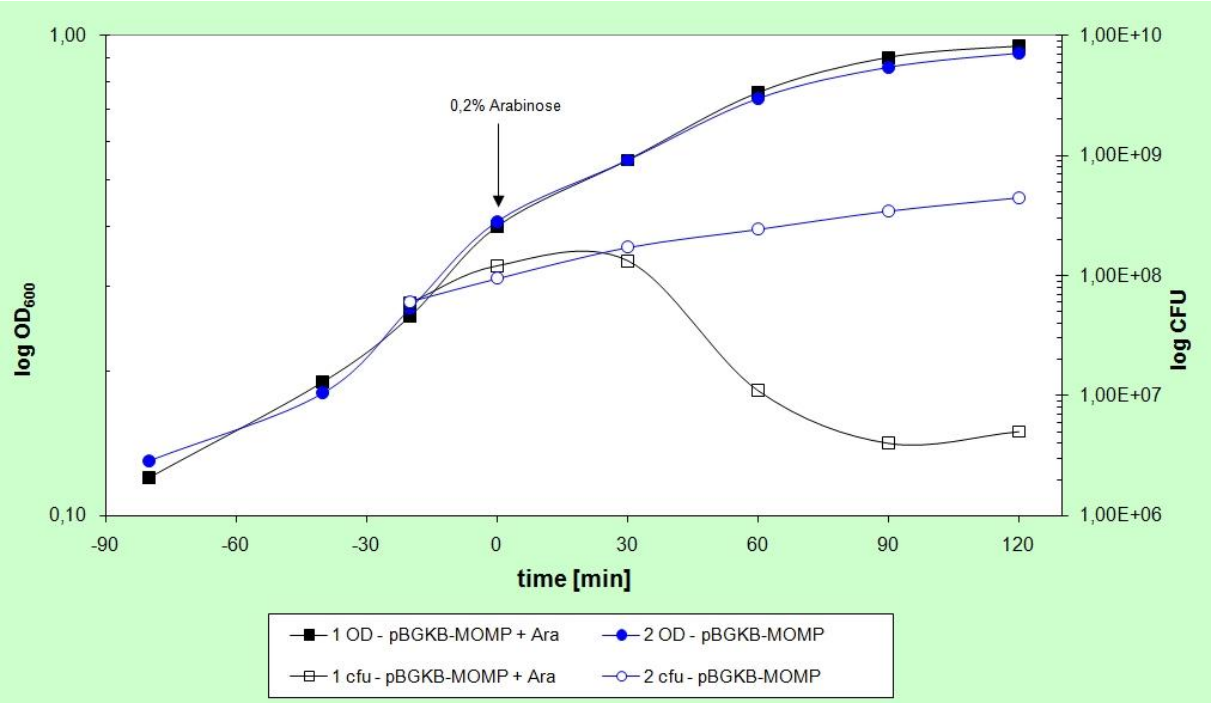


Figure 33: OD and cfu values over time during an expression experiment of *E. coli* C2988J (pBGKB-MOMP). L-Arabinose was added at time point 0min to flask 1.

As seen with PorB, the expression and export of MOMP to the periplasm seems to have a toxic effect on bacteria, with a cfu decline of 97% 90min after expression induction, in comparison to the cfu at the start of the expression. Just like in other experiments, OD values behave nearly identical regardless to induction. The cfu decrease is first apparent 60min after expression induction, becoming more dramatic over time, but is not detected 30min after expression induction.

Western Blot samples just prior to L-arabinose induction, after 30min, 60min and 90min of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the nitrocellulose incubated with HRP-coupled-Anti-Myc-Antibody and developed by chemiluminiscence; the protein GIII-MOMP-Myc-PolyHis was expected at a size of 46.5kDa, while protein MOMP-Myc-PolyHis (with the GIII signal sequence removed) was expected at a size of 44.4kDa. The developed blot is shown in Figure 34.

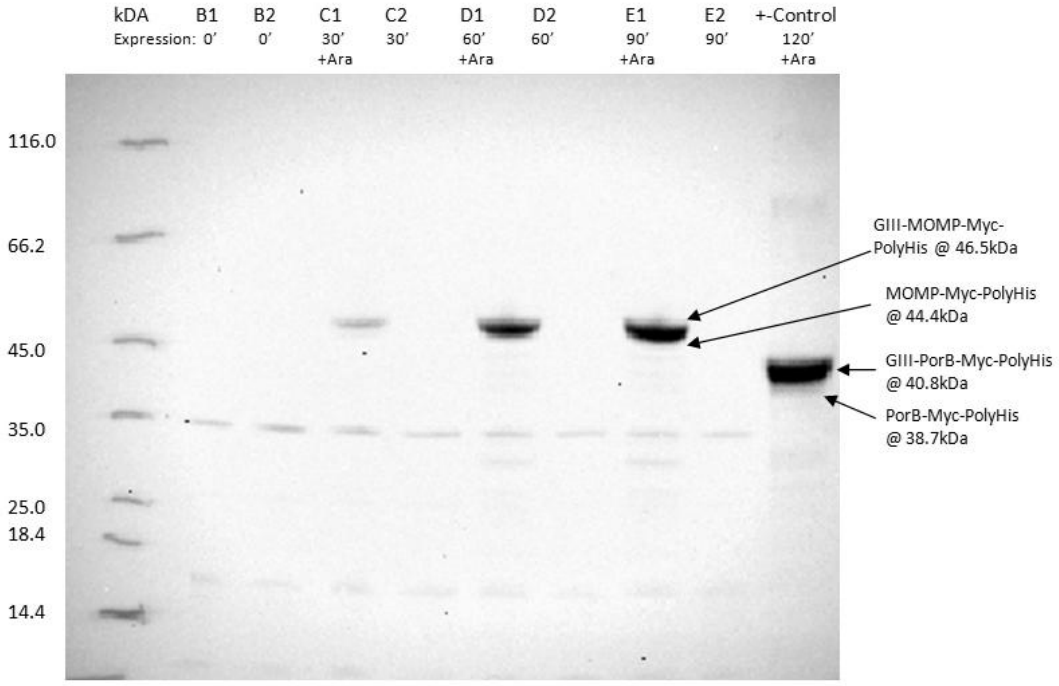


Figure 34: Western Blot of *E. coli* C2988J (pBGKB-MOMP) Expression Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-antibodies and developed. Samples were taken 0min (B), 30min (C), 60min (D) and 90min (E) after expression induction. Expression was induced in flask1. As a positive control, an induced sample of pBGKB-PorB has been used.

Expressed MOMP can successfully be detected first 30min after expression induction, and strongly after 60min and 90min, at the expected size. No signal at the correct size can be seen without induction.

5.4 SMALL SCALE EXPRESSION/LYSIS EXPERIMENTS

After the successful expression of the chlamydial antigens from plasmids pBGKB-PorB, pASK-PorB and pBGKB-MOMP had been shown, those plasmids were to be co-transformed with the E-lysis plasmid pGLysivb into *E. coli* NM522 and into *E. coli* Nissle, since those two strains yield higher growth rates and are the preferable strains for *in vivo* immunization trials.

Small scale expression/lysis experiments attempted to show that it was possible to:

- first express and export chlamydial antigens to the periplasm,
- to induce lysis afterwards, and
- to retain the antigens in the BGs.

Representative expression/lysis curves are shown in the following chapters.

5.4.1 EXPRESSION/LYSIS OF pBGKB-PORB IN *E. COLI* NM522

The goal of this first small scale expression/lysis experiment was to determine the optimal time of expression before lysis induction in regards to lysis efficiency and expression yield. Two noseflasks (1 and 2) were inoculated with a clone of *E. coli* NM522 (pBGKB-PorB, pGLysivb).

After reaching an OD_{600nm}≈0.3, 0.2% L-arabinose was added to all three flasks. In flask 1, lysis was induced by temperature shifting to 42°C after 30min of expression, whereas in flask 2 this was done after 60min (time point 0min for both cases).

OD_{600nm} values were measured and samples were collected for WB and cfu determination throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values are plotted against time in Figure 35.

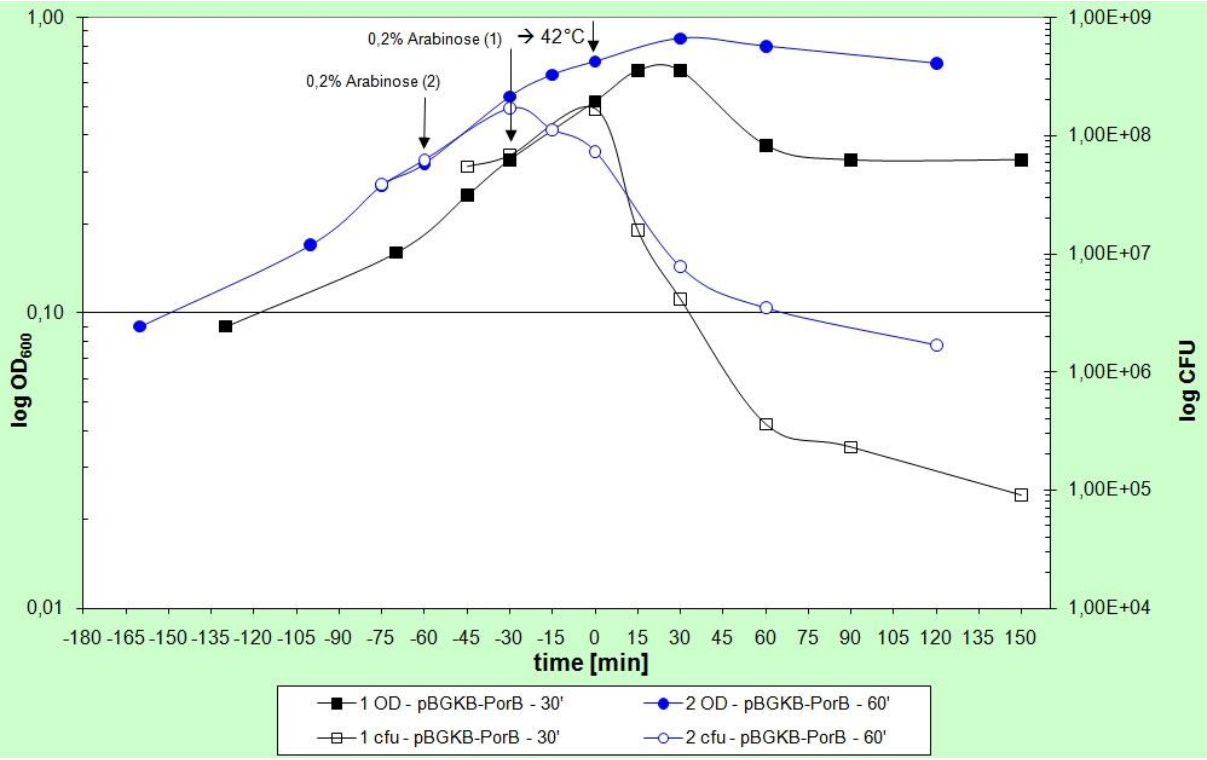


Figure 35: OD and cfu values over time during an expression/lysis experiment of *E. coli* NM522 (pBGKB-PorB, pGLysivb). L-Arabinose was added at time point -30min to flask 1, and time point -60min to flask 2. Lysis was induced by shifting to 42°C at time point 0min.

After expression induction with L-Arabinose, OD_{600nm} increases in both flasks until lysis induction at time point 0min, whereas cfu values increase only in flask 1 up until lysis

induction, while in flask 2 cfu values start to drop after 45min of expression, even before lysis induction at time point 0min. While the cfu decreases in both flasks, it does less so in flask 2, where lysis was preceded by 60min of expression. While flask 1 has a lysis efficiency of 99.86% from time point 0min to time point 120min (as determined by cfu counting), lysis efficiency of flask 2 is only at 97.70% percent in the same time frame, indicating interference of prolonged PorB expression with successful lysis. Additionally, killing is also apparent before lysis induction in flask 2, whereas cfu still increases during the expression time of PorB in flask 1. Indeed, PorB expression seems to become toxic between 30min and 45min of expression, just about the time lysis becomes effective as well in flask 1, where lysis is induced after 30min of expression.

Western Blot samples just prior to L-arabinose induction, after 60min and after 120’ of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence, as seen in Figure 36. Protein GIII-PorB-Myc-PolyHis was expected at a size of 40.8kDa, protein PorB-Myc-PolyHis at a size of 38.7kDa.

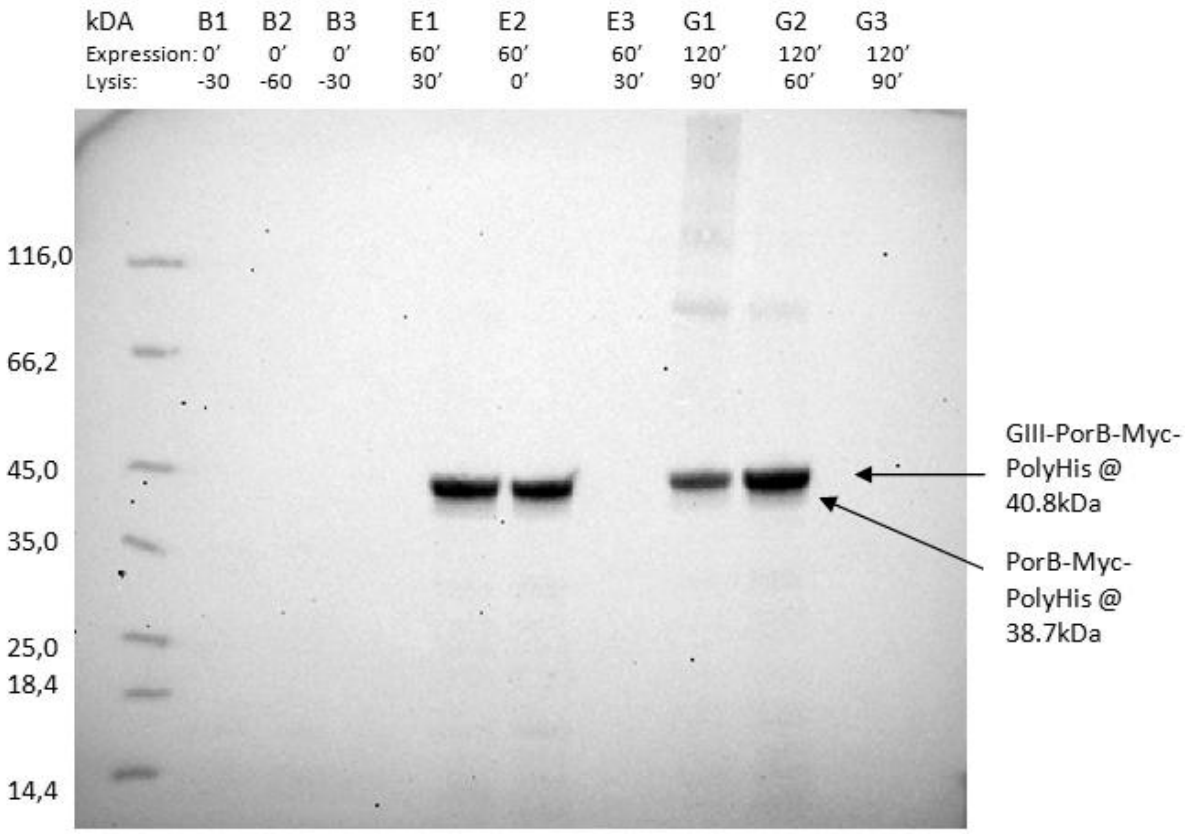


Figure 36: Western Blot of *E. coli* NM522 (pBGKB-PorB, pGLysivb) Expression/Lysis Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. Samples B are at the time point of expression induction, samples E are 60min later – after 30min of lysis for samples 1 and 3, and just before lysis induction sample 2. Samples G are 120min after expression induction, after 90min of lysis for 1 and 3, and 60min of lysis for 2. Samples 3 are *E. coli* NM522 (pBGKB, pGLysivb), and are used as an expression-negative control.

The expression of PorB can be seen in induced samples with no visible background expression. Only a slight decrease over time was detected (compare sample E1 with sample G1) which could be due to protein degradation or due to loss of expressed protein that was not exported to the periplasm before E-lysis. Because of higher lysis efficiency for shorter expression time and less killing by recombinant protein, future experiments will use an expression time of 30min before lysis induction.

5.4.2 EXPRESSION/LYSIS OF pBGKB-PORB IN *E. COLI* NISSLE

E. coli Nissle was co-transformed with plasmids pBGKB-PorB and the E-lysis plasmid pGLysivb, at very low transformation efficiencies. In a small scale expression/lysis experiment, the successful expression and export to the periplasm of PorB followed by E-

lysis was to be shown. As a lysis negative control, *E. coli* Nissle was also co-transformed with pBGKB-PorB and pBBR1MCS5 (the backbone plasmid of pGLysivb).

One noseflask (sample 1) was inoculated with a clone of *E. coli* Nissle (pBGKB-PorB, pGLysivb), while a second noseflask (sample 2) was inoculated with *E. coli* Nissle (pBGKB-PorB, pBBR1MCS5).

After reaching an OD_{600nm}≈0.3, 0.2% L-arabinose was added to both flasks. After 30min of expression, both flasks were shifted to 42°C, activating the thermosensitive promoter of pGLysivb (time point 0min).

OD_{600nm} values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values are plotted against time in Figure 37.

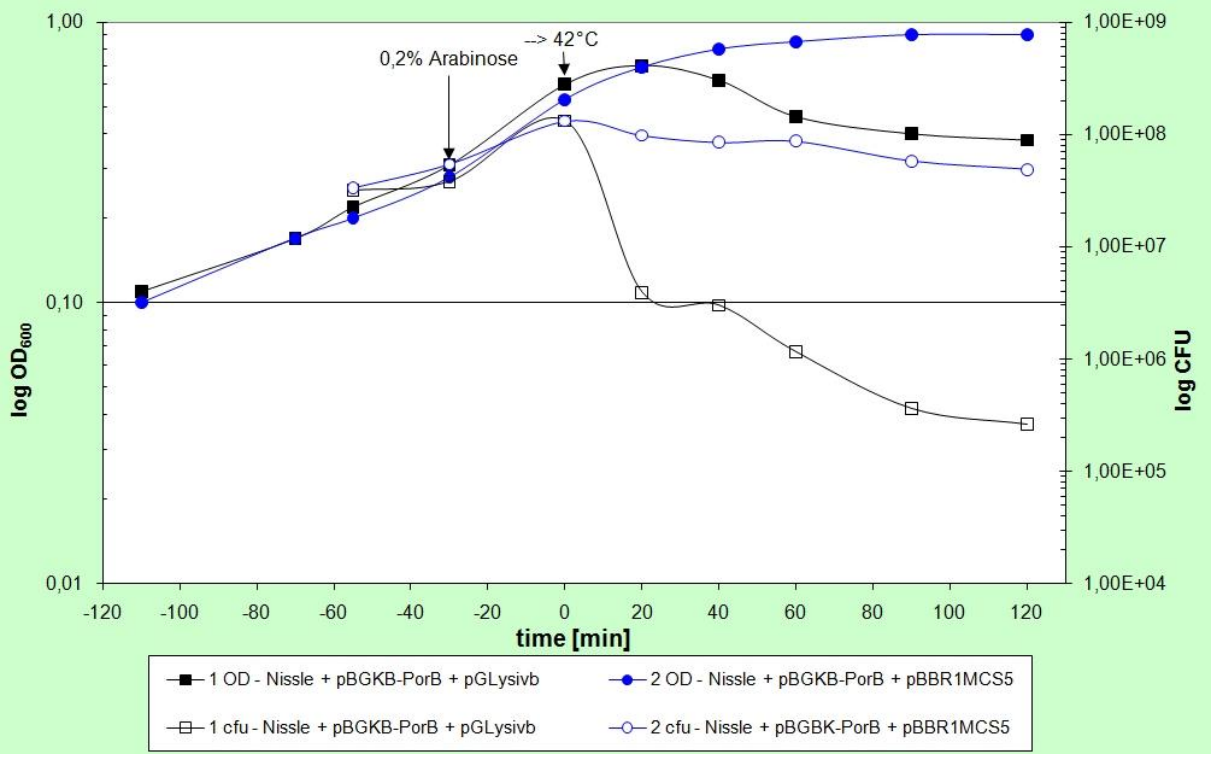


Figure 37: OD and cfu values over time during an expression/lysis experiment of *E. coli* Nissle (pBGKB-PorB, pGLysivb), with *E. coli* Nissle (pBGKB-PorB, pBBR1MCS5) used as a lysis negative control. L-Arabinose was added at time point -30min to flasks 1 and 2; lysis was induced by shifting to 42°C at time point 0min.

In both samples cfu values rise until the temperature shift to 42°C, after which they drop rapidly for sample 1, indicating successful E-lysis, and stagnate for sample 2, indicating the toxic effects of prolonged PorB expression and export to the periplasm. OD_{600nm} values

continue to rise for sample 2, and drop for sample 1 after the temperature shift. The lysis efficiency for *E. coli* Nissle (pBGKB-PorB, pGLysivb) is 99.80%, as determined by cfu counting.

Western Blot samples just prior to L-arabinose induction, just prior to lysis induction and after 40min and 90min of lysis were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence, as seen in Figure 38. Protein GIII-PorB-Myc-PolyHis was expected at a size of 40.8kDa, and protein PorB-Myc-PolyHis at a size of 38.7kDa.

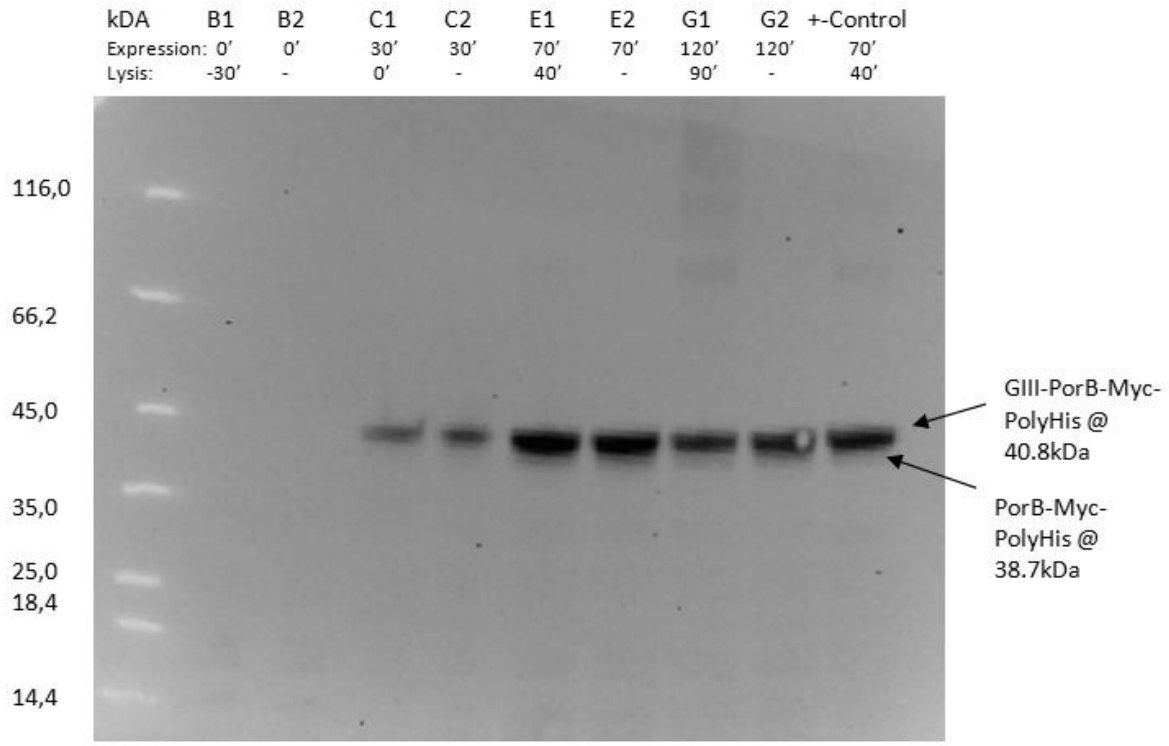


Figure 38: Western Blot of *E. coli* Nissle (pBGKB-PorB, pGLysivb) Expression/Lysis Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). As a positive control, a PorB sample from an *E. coli* NM522 (pBGBK-PorB, pGLysivb) Expression/Lysis Experiment was used. Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. Samples B are at the time point of expression induction, samples C at the time point of lysis induction.

The signal of Myc-tagged PorB can first be seen 30min after expression induction at the correct-size. Signal is strongest after 70min of expression/40min of lysis in flask 1 and while the signal somewhat diminishes with time, it does so in both the samples where lysis was induced (samples 1) and where no lysis took place (samples 2). Therefore, this slight signal decrease is most likely due to protein degradation, and not due to loss during the E-lysis process.

5.4.3 EXPRESSION/LYSIS OF pASK-PORB IN *E. COLI* NM522

After the successful co-transformation of pASK-PorB (as well as pASK as an expression-negative backbone plasmid) and pGLysivb into *E. coli* NM522, two noseflasks were inoculated with *E. coli* NM522 (pASK, pGLysivb) overnight culture (samples 1 and 2) and two other noseflasks (samples 3 and 4) were inoculated with *E. coli* NM522 (pASK-PorB, pGLysivb); all four noseflasks were incubated in a water bath at 36°C

After reaching an OD_{600nm}≈0.3, 0.2% L-arabinose was added to all four flasks. After 30min of expression, flasks 2 and 4 were shifted to 42°C, inducing lysis at time point 0min.

OD_{600nm} values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values are plotted against time in Figure 39.

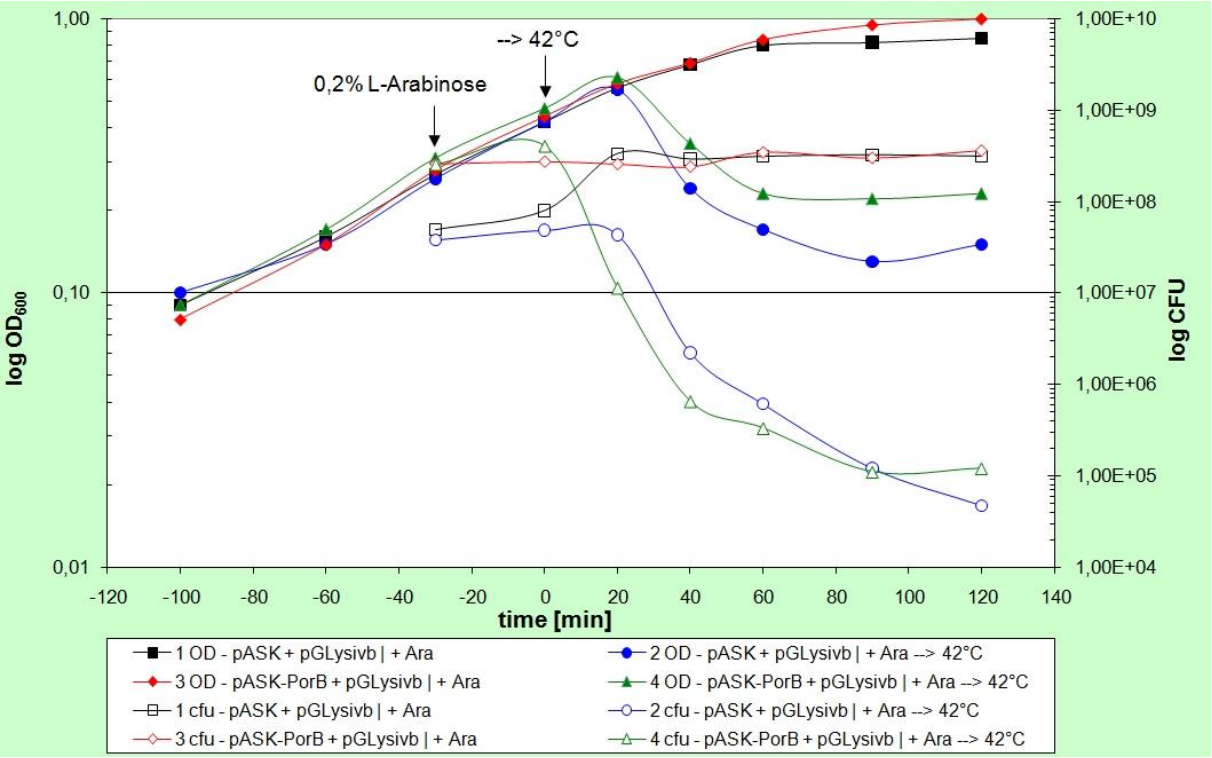


Figure 39: OD and cfu values over time during an expression/lysis experiment of *E. coli* NM522 (pASK-PorB, pGLysivb), with *E. coli* NM522 (pASK, pGLysivb) used as an expression negative control. L-Arabinose was added at time point -30min to all four flasks; lysis was induced by shifting flasks 2 and 4 to 42°C at time point 0min.

Whereas the induction of pASK and pASK-PorB without lysis induction leads to cfu stagnation (samples 1 and 3), combination of expression induction and lysis induction (samples 2 and 4) leads to strong decrease of cfu after temperature shift to 42°C, with

calculated lysis efficiencies (from cfu counting) of 99.97% for *E. coli* NM522 (pASK-PorB, pGLysivb) and of 99.90% for *E. coli* NM522 (pASK, pGlysivb).

Western Blot samples just prior to lysis induction and after 60min of lysis (in flasks 2 and 4) were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled S-Protein and developed by chemiluminiscence, as seen in Figure 40. Protein GIII-PolyHis-S-Tag-PorB-Myc-PolyHis was expected at a size of 46.6kDa, and protein PolyHis-S-Tag-PorB-Myc-PolyHis at a size of 44.6kDa.

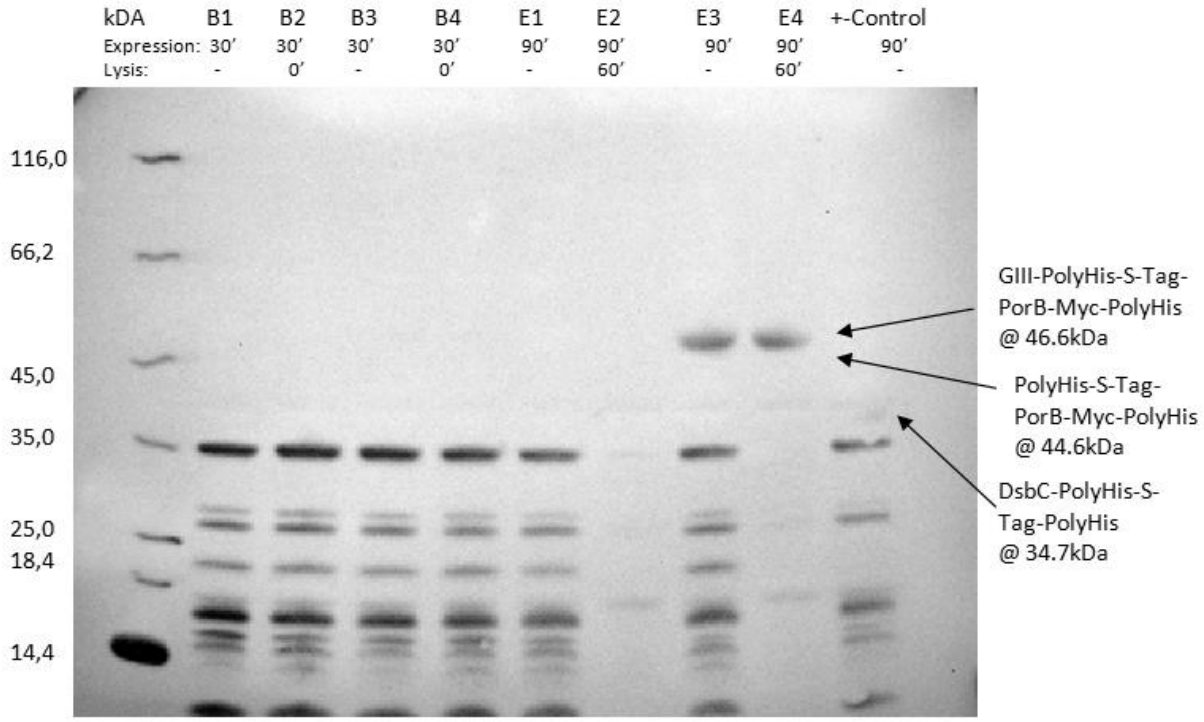


Figure 40: Western Blot of *E. coli* NM522 (pASK-PorB, pGLysivb) Expression/Lysis Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5,000 diluted S-Protein-HRP and developed. Samples are at 30min (B) and 90min (E) of protein expression. As a positive control, a sample from an *E. coli* C41 (pET40b) Expression Experiment was used. Expression was induced in all flasks, whereas lysis was induced only in flasks 2 and 4.

While the background signal is again very high, S-tagged PorB is recognized 90min after induction in both samples carrying pASK-PorB, regardless whether they underwent lysis or not. No signal is visible after 30min of expression in either sample. Expulsion of cytoplasmic content during E-lysis is evident by the strongly diminished background signal in samples E2 and E4 (where lysis was induced).

5.4.4 EXPRESSION/LYSIS OF pBGKB-MOMP IN *E. COLI* NM522

After the successful co-transformation of pBGKB-MOMP and pGLysivb into *E. coli* NM522, four noseflasks were inoculated with overnight cultures of four different clones and grown at 36°C.

After reaching an OD_{600nm}≈0.3, 0.2% L-arabinose was added to all four flasks. After 30min of expression, all flasks were shifted to 42°C, inducing lysis at time point 0min.

OD_{600nm} values were measured and samples for WB and cfu determination were collected throughout the experiment; plates for cfu counting were incubated at 36°C overnight. OD_{600nm} and cfu values are plotted against time in Figure 41.

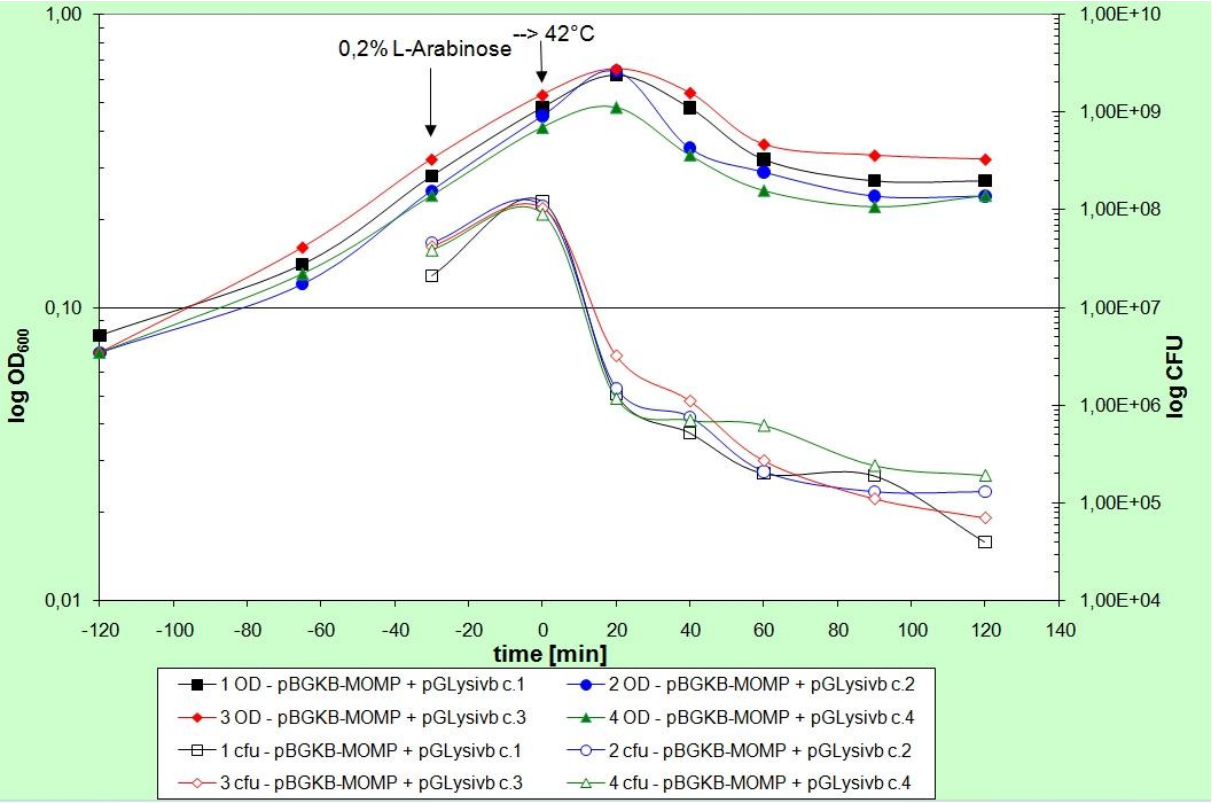


Figure 41: OD and cfu values over time during an Expression/Lysis Experiment of four different clones of *E. coli* NM522 (pBGKB-MOMP, pGLysivb). L-Arabinose was added at time point -30min; lysis was induced by shifting all four flasks to 42°C at time point 0min.

All four clones show similar OD_{600nm} and cfu behaviour; while cfu increases after induction of MOMP expression at time point -30min and until lysis induction at time point 0min, it drops

sharply afterwards, with lysis efficiencies between 99.79% (clone 4) and 99.97% (clone 1), as calculated by cfu counting.

Western Blot samples just prior to lysis induction and after 60min of lysis were separated on two identical 4-12%-Bis-Tris-Gels with MES buffer. The gels were blotted onto nitrocellulose; one of the membranes incubated with HRP-coupled-Anti-Myc-Antibodies (see Figure 42), the second first with Goat-Anti-MOMP-Antibody and then with HRP-coupled-Anti-Goat-Antibodies (see Figure 43), and developed by chemilumiscence. Protein GIII-MOMP-Myc-PolyHis was expected at a size of 46.5kDa, protein MOMP-Myc-PolyHis at a size of 44.4kDa.

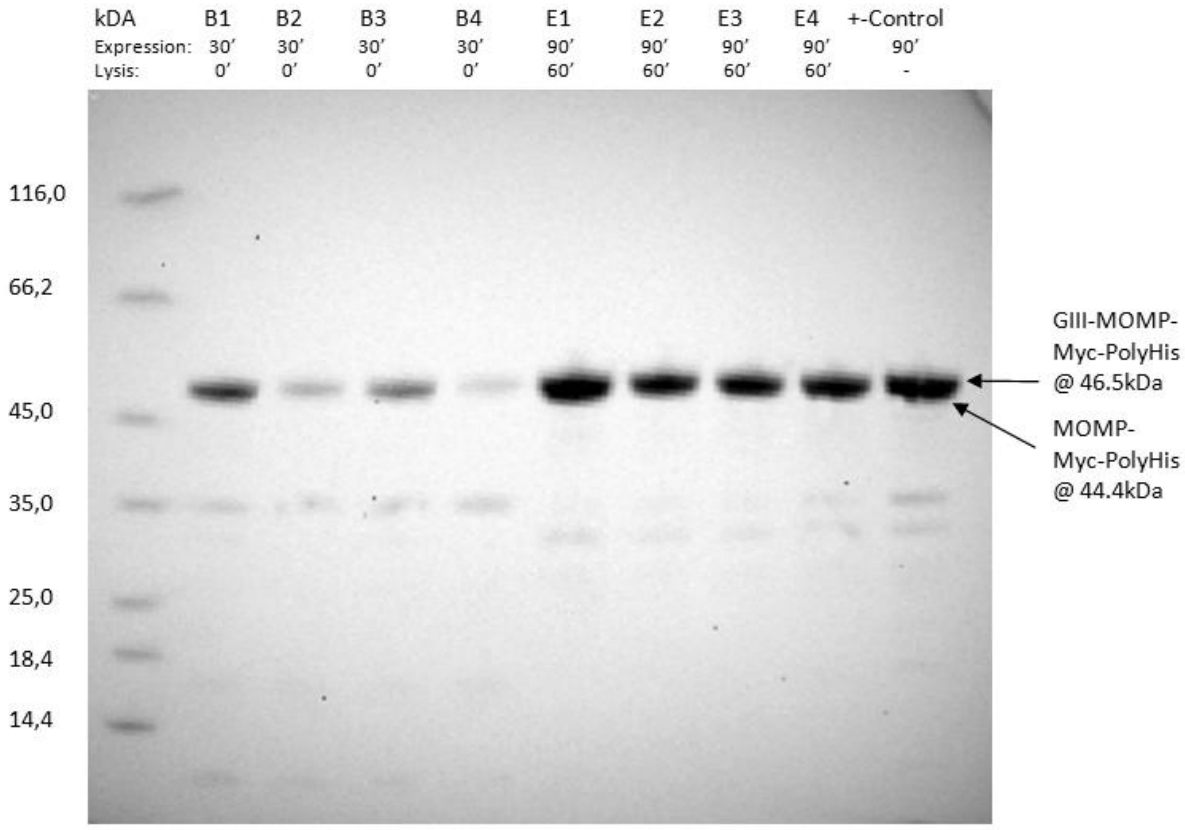


Figure 42: Western Blot of *E. coli* NM522 (pBGKB-MOMP, pGLysivb) Expression/Lysis experiment with four different clones, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. Samples were taken 0min (B) and 60min (E) after lysis induction. As a positive control, an induced sample of an *E. coli* C2988J (pBGKB-MOMP) Expression Experiment was used.

Myc-tagged MOMP can be detected in all samples at the correct size; while the concentration varies considerably 30min after expression induction, after 60min of lysis the amount of MOMP is roughly equal in all clones, with a slightly stronger signal for flask 1.

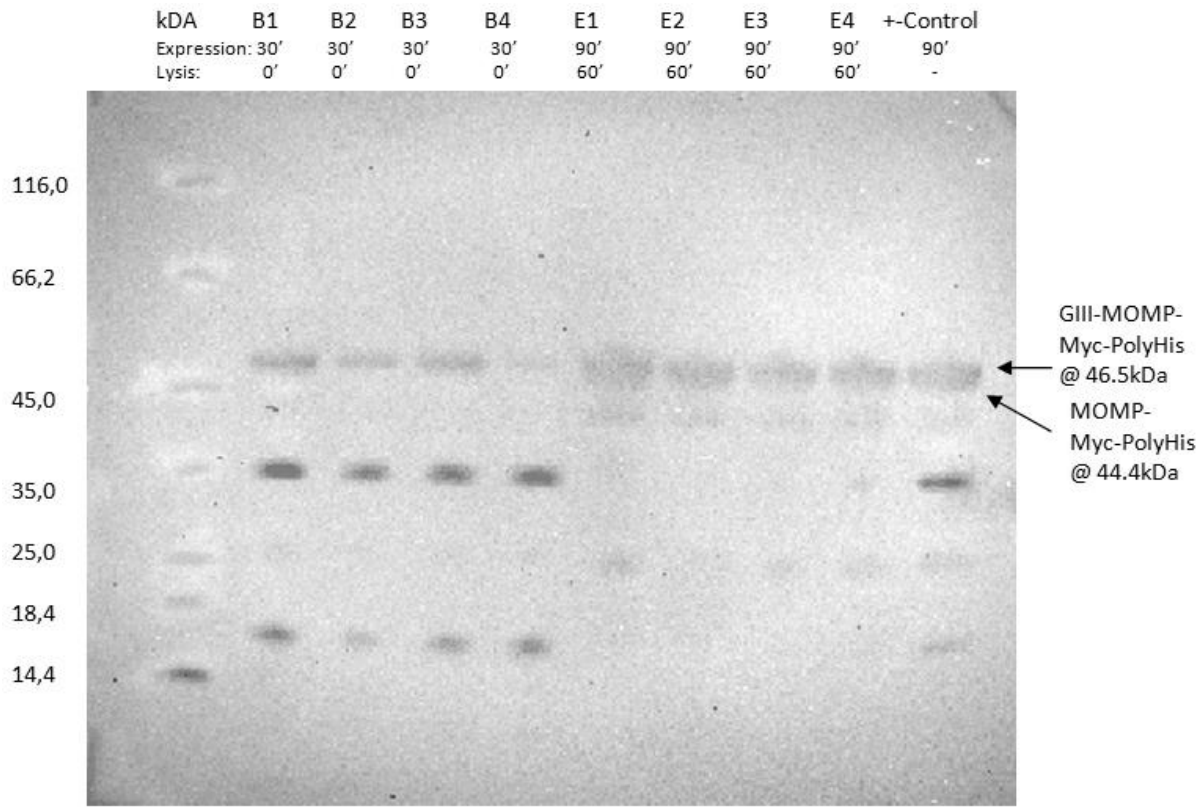


Figure 43: Western Blot of *E. coli* NM522 (pBGKB-MOMP, pGLysivb) Expression/Lysis experiment with four different clones, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:500 diluted Goat-Anti-MOMP-Antibodies and with 1:5000 diluted HRP-coupled-Anti-Goat-Antibodies, and developed. Samples were taken 0min (B) and 60min (E) after lysis induction. As a positive control, an induced sample of an *E. coli* C2988J (pBGKB-MOMP) Expression Experiment was used.

MOMP can also be detected with polyclonal Anti-MOMP-Antibody preparation, even though the signal:noise ratio is worse compared to detection via the Myc-tag (see Figure 42). In addition to the correct-sized product, two smaller fragments at roughly 16kDa and 35kDa are detected. They disappear after lysis induction, suggesting a previous cytoplasmic location.

5.4.5 EXPRESSION/LYSIS OF pBGKB-MOMP IN *E. COLI* NISSLE

Even though numerous attempts were made, using different midipreps and freshly prepared MOPS-competent cells, in my hands it was not possible to co-transform pBGKB-MOMP and pGLysivb into *E. coli* Nissle.

5.5 LARGE SCALE EXPRESSION/LYSIS EXPERIMENTS

For the large scale production of BGs ,fermentation was performed in a volume of 22l LBv pH 7.2, with antibiotics kanamycin and gentamicin added. In 30min intervals, samples were withdrawn to measure OD_{600nm}, check bacterial viability via the microscope, prepare Western Blot samples and plate dilutions for cfu determination on count agar plates; flow cytometry was performed for live-monitoring of the growth and lysis process.

Important fermentation parameters such as flow, stirring rate, temperature or pO_2 were documented using the IRIS software.

After an initial growth phase at 35°C, protein expression was induced by adding 0.2% L-arabinose; after 30min of expression for PorB, and after 20min of expression for MOMP, lysis was induced by temperature upshift of the culture to 42°C.

After 120min of lysis, harvesting by Tangential Flow Filtration and killing of viable bacteria using β-Propiolactone took place. After washing, the concentrate was aliquoted and lyophilized. Plates for cfu determination were incubated at 36°C overnight.

5.5.1 *E. COLI* NM522 (pBGKB-PORB, pGLYSIVB) FERMENTATION

After testing several clones of *E. coli* NM522 (pBGKB-PorB, pGLysivb) for differences in lysis efficiency and PorB expression, working stocks of clone A3 of *E. coli* NM522 (pBGKB-PorB, pGLysivb) were prepared. These were used to inoculate an overnight culture for fermentation.

OD_{600nm} values and cfu counts (taken the next day) plotted against time are shown in Figure 44.

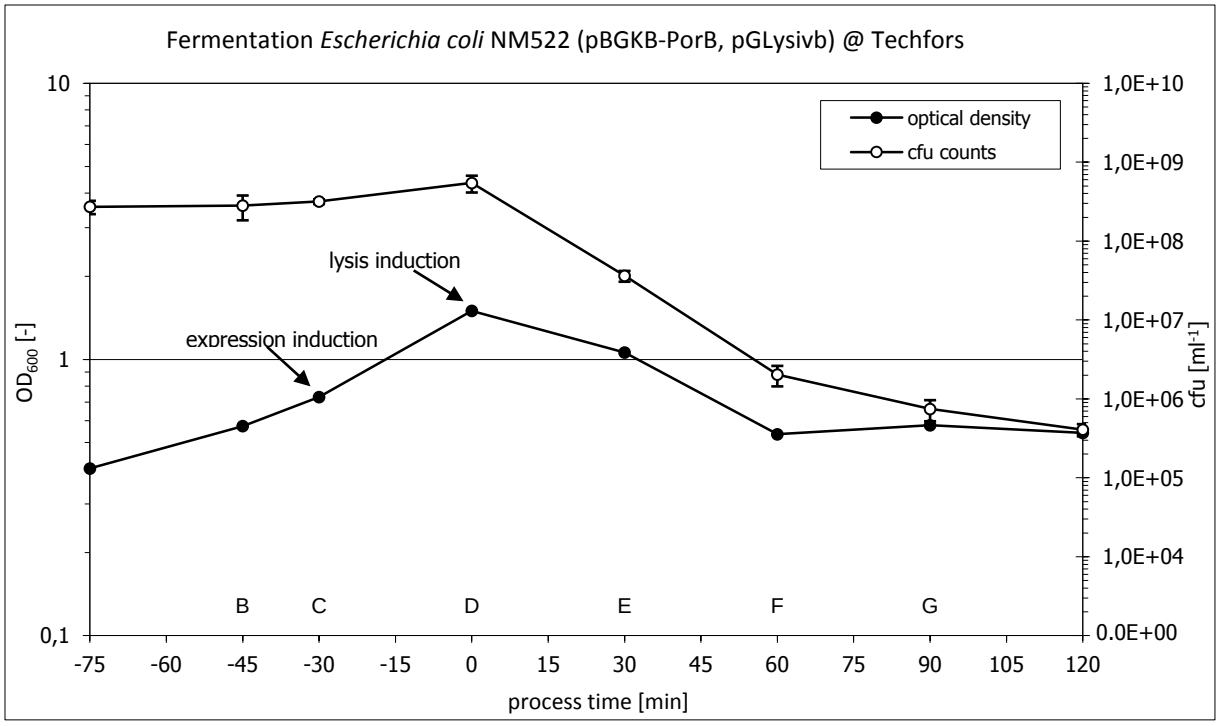


Figure 44: OD and cfu values over time during fermentation in 22l of *E. coli* NM522 (pBGKB-PorB, pGLysivb). L-Arabinose was added at time point C (-30min); lysis was induced by increasing the temperature to 42°C at time point D (0min).

While both OD_{600nm} and cfu values increase after expression induction, they drop sharply after lysis induction ($5.45*10^8$cfu/ml at D to $4.09*10^5$cfu/ml at H – a lysis efficiency of 99.93%).

No flow cytometry was performed for this fermentation because of malfunctioning equipment. Microscopic analysis (see Figure 45) using the membrane potential staining dye $DiBAC_4(3)$ also showed no killing before lysis induction. The development of BGs is clearly seen in Figure 45C after lysis induction by temperature shifting to 42°C.

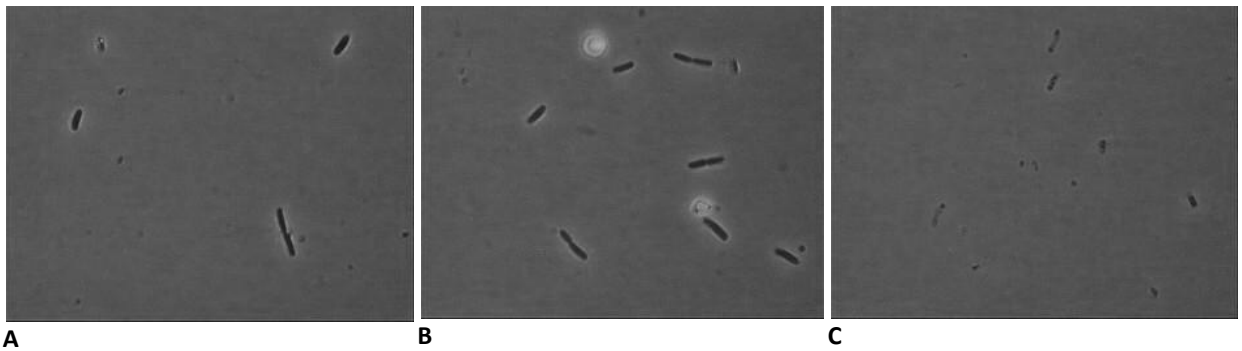


Figure 45: Light microscopy of samples taken during the fermentation process. Intact cells at time point C after 45min of growth (A), intact cells at time point D after 30min of protein expression (B) and translucent lysed cells at time point F after 60min of lysis (C).

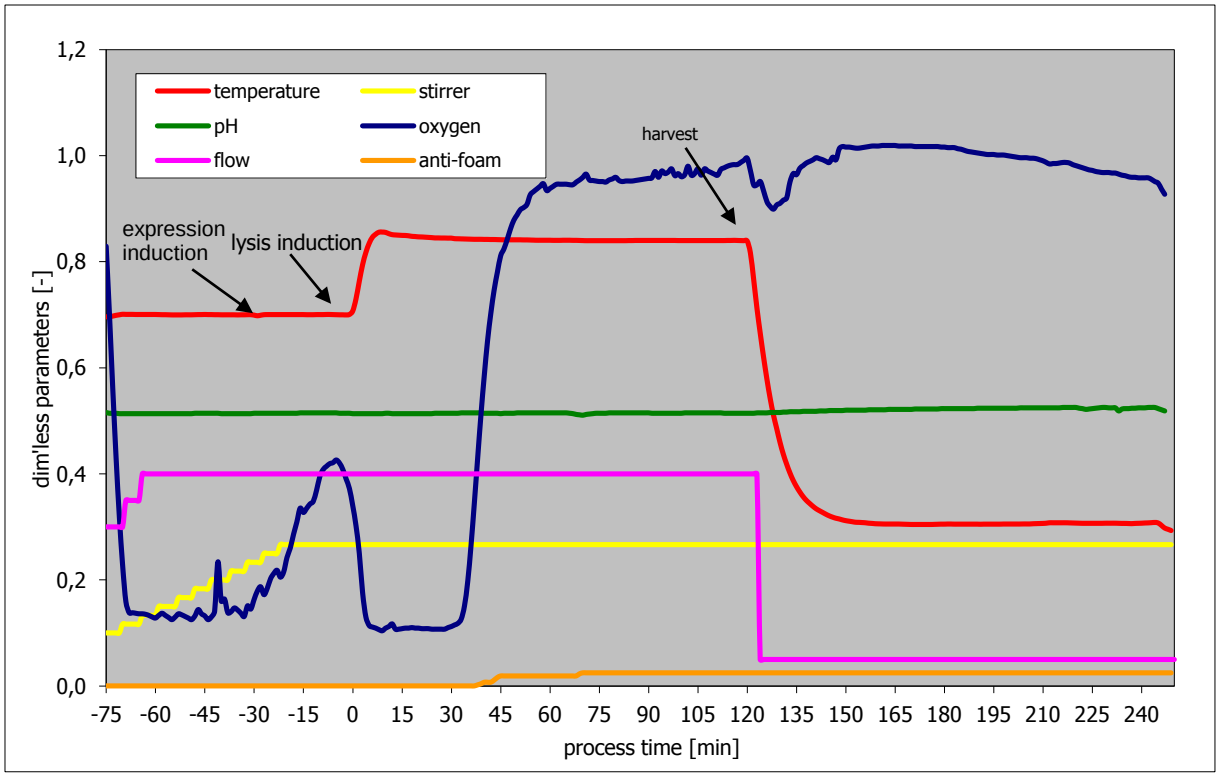


Figure 46: IRIS diagram documenting fermentation parameters like pH (regulated at 7.2), temperature (35°C during growth and expression, 42°C during lysis), flow rate, stirring rate, oxygen concentration and addition of anti-foam.

In the IRIS diagram of this fermentation (see Figure 46), the oxygen curve is of particular interest, which typically registers a sharp drop within minutes of lysis induction, followed by a strong increase in oxygen concentration after 30min of lysis. Additionally, anti-foam is added after roughly 40min of lysis to counteract the foaming initiated by expulsion of cytoplasmic content during lysis.

Several other specifications as well as follow-up experiments of this particular fermentation are detailed in Table 5 below:

***E. coli* NM522 (pBGKB-PorB, pGLysivb) Bacterial Ghosts**	
Pre-Culture	
Volume: 4*500ml	**Additives**: Gentamicin + Kanamycin
Medium type: LBv	**Other**: -
Date: 2009-10-15	**Clone**: c. A3 (2009-09-30/ WS 2009-10-15 FHO)
Starting time: 8:19	**Strain**: NM522
End time:	**Plasmids**: pGLysivb, pBGKB-PorB
ON culture OD: 5.39	**Recombinant Protein Expression**: GIII-PorB-Myc-PolyHis
Inoculation Volume: 1.6l	**Expression Induction**: L-Arabinose, 0.2%
Medium: LBv	**Expression Induction Time Point**: C
Antibiotics: Gentamicin + Kanamycin	**Lysis Induction**: 42°C
Temperature: 35°C	**Lysis Induction Time Point**: D
Total Volume: ≈22l	**Killing**: after harvest, 0.075% ß-PL (2*0.0375%)
Acid: F. A.: 31 units	**Volume harvested**: 20l
Base: A. W.: 30 units	**Harvested by**: TFF
Antifoam A: 25 units	**OD separator flow**: -
Eivb blot: -	**Yield**: 6447mg
Recombinant blot: OK (by FHO)	**Particles/mg**: 1.69 x 10^9
BPL Test: Survivors (by AME)	**Sterility**: OK (by EDZ)
Microscopy: ok	**Efficiency**: 99.925%

Table 5: Data sheet of *E. coli* NM522 (pBGKB-PorB, pGLysivb) Fermentation

While some colonies after ß-PL killing could be detected, PCR analysis could not detect gentamicin or kanamycin resistance genes in these colonies, suggesting a later contamination. Sterility tests of the freeze-dried lyophilisate indicate a sterile product. From a total of 20l fermentation volume, harvesting and lyophilisation yielded **6447mg of BGs** dry weight. Calculating from a cfu count of 5.45*10^8/ml at the time point of lysis induction, and a harvested volume of 20l, the lyophilisate contains **1.69*10^9particles/mg**.

Western Blot samples taken throughout the fermentation process were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence, as seen in Figure 47. Protein GIII-PorB-Myc-PolyHis was expected at a size of 40.8kDa, protein PorB-Myc-PolyHis at a size of 38.7kDa.

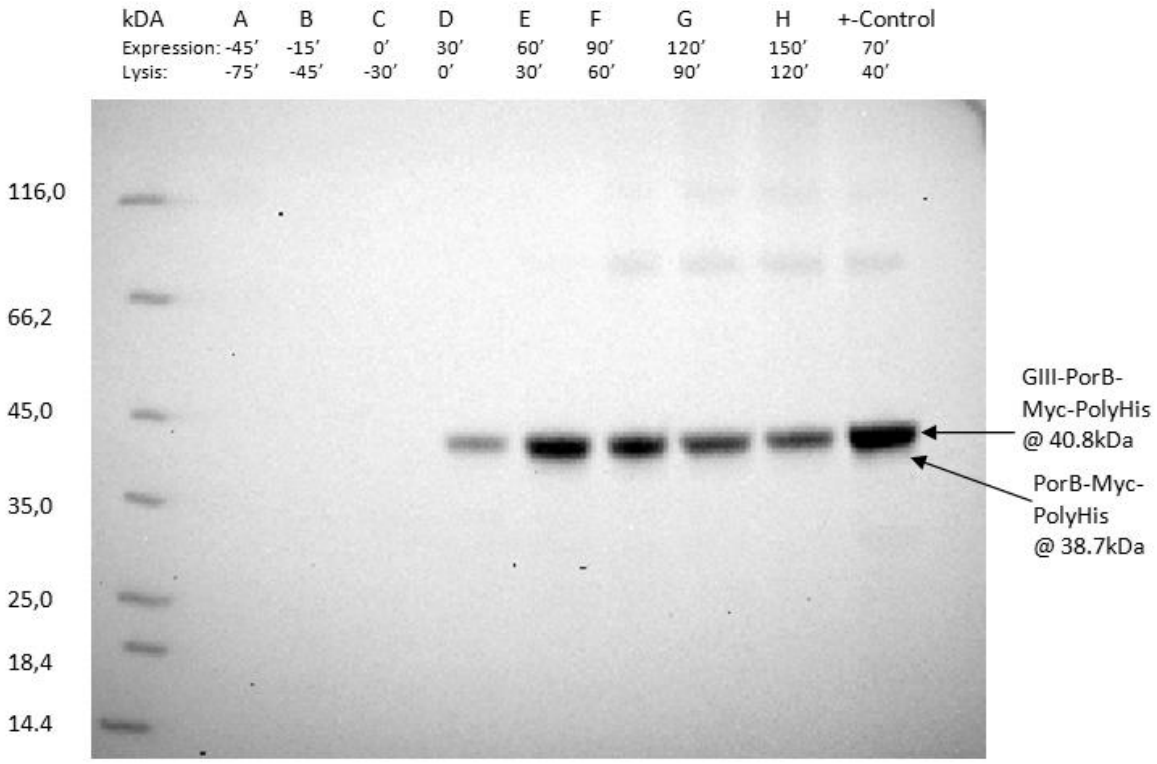


Figure 47: Western Blot of *E. coli* NM522 (pBGKB-PorB, pGLysivb) Fermentation, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. As a positive control, a sample from a previous small scale experiment was used.

Myc-tagged PorB is detected after induction and reaches its highest concentration after 60min of expression/30min of lysis. While the concentration slightly decreases afterwards, the majority of protein is retained during lysis.

5.5.2 *E. COLI* NISSLE (pBGKB-PORB, pGLYSIVB) FERMENTATION

Clone A1 of *E. coli* Nissle (pBGKB-PorB, pGLysivb) already tested in small scale experiments (see 5.4.2 Expression/Lysis of pBGKB-PorB in *E. coli* Nissle) was used to produce working stocks. These were used to inoculate an overnight culture for fermentation.

OD_{600nm} values and cfu counts plotted against time are shown in Figure 48.

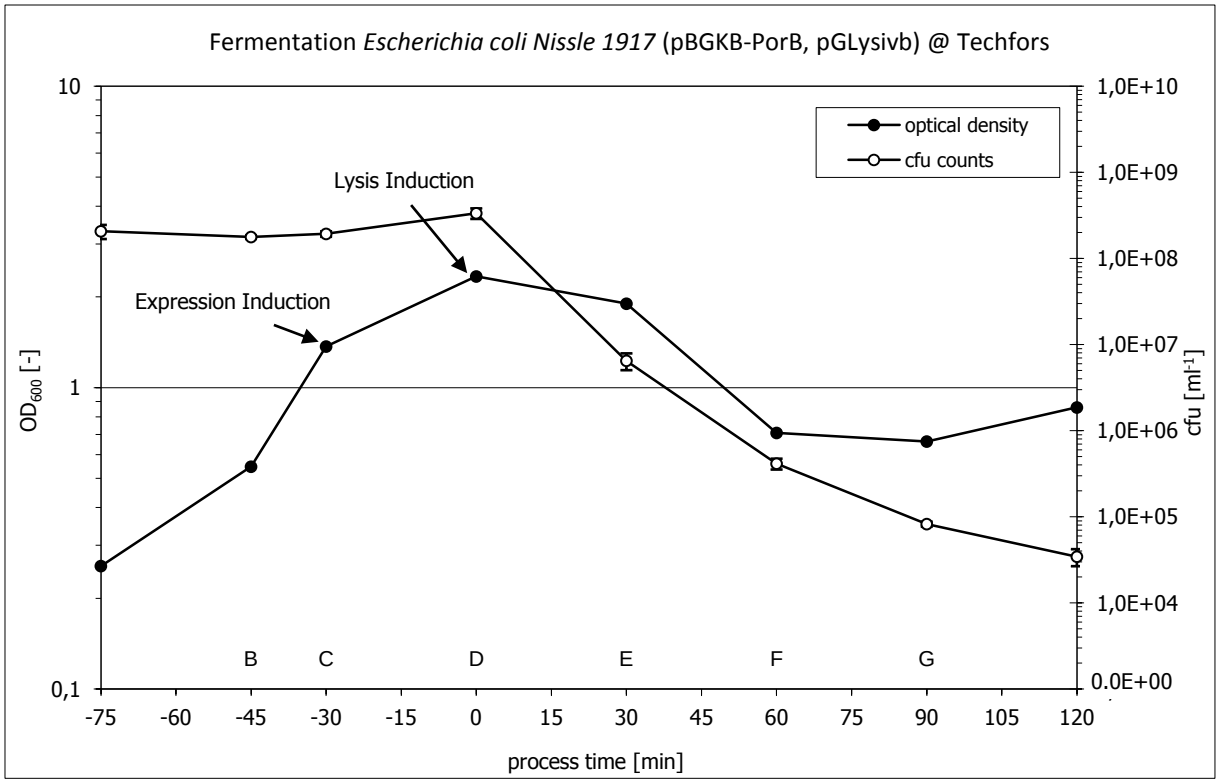


Figure 48: OD and cfu values over time during fermentation in 22l of *E. coli* Nissle (pBGKB-PorB, pGLysivb). L-Arabinose was added at time point C (-30min); lysis was induced by increasing the temperature to 42°C at time point D (0min).

While both OD_{600nm} and cfu values increase after expression induction, they drop sharply after lysis induction ($3.36*10^8$cfu/ml at D to $3.45*10^4$cfu/ml at H – a lysis efficiency of 99.99%).

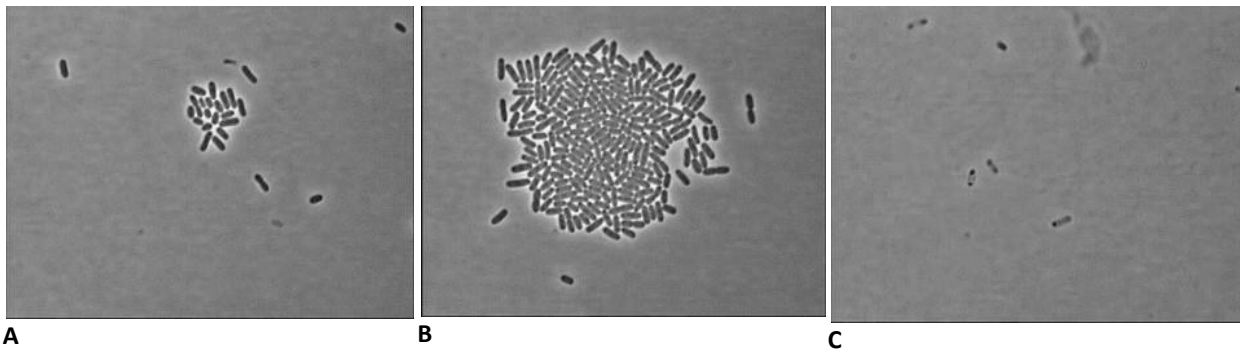
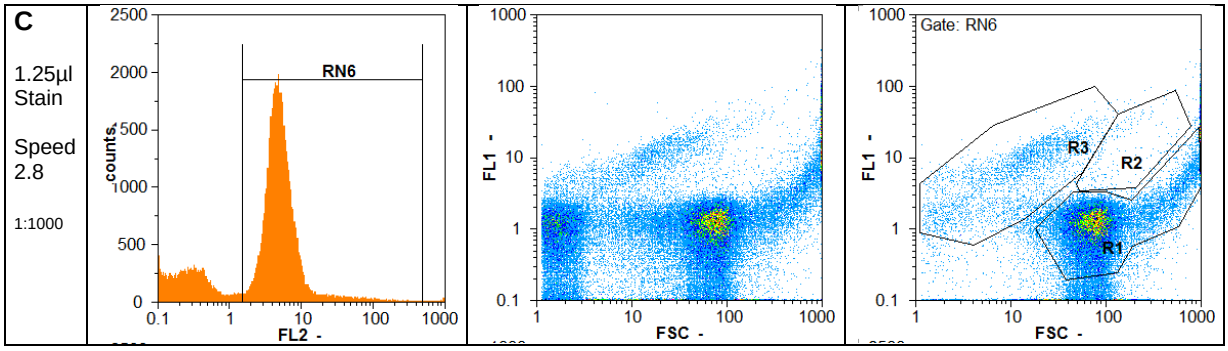


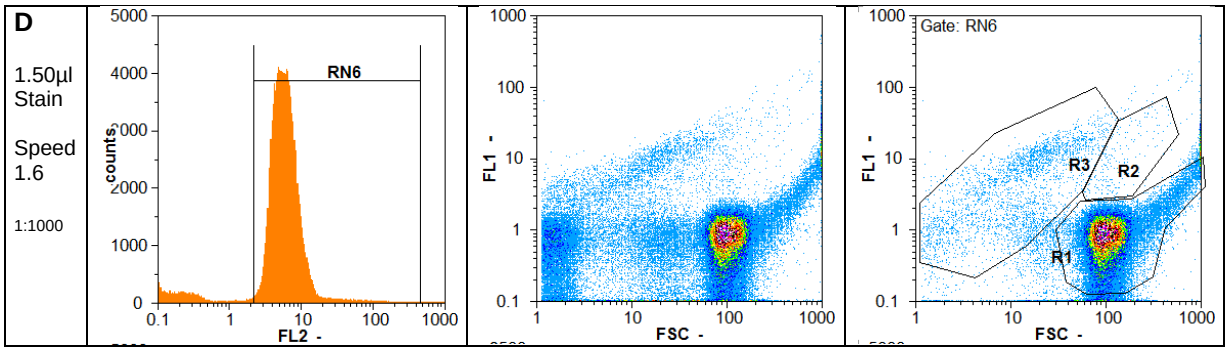
Figure 49: Light microscopy of samples taken during the fermentation process. Intact cells at time point C after 45min of growth (A), intact cells at time point D after 30min of protein expression (B) and translucent lysed cells at time point F after 60min of lysis (C).

Microscopic analysis (see Figure 49) using the membrane potential staining dye $DiBAC_4(3)$ also showed no killing before lysis induction. The development of BGs is clearly seen in Figure 49C after lysis induction by temperature shifting to 42°C. Note the typical clustering of *E. coli* Nissle seen for growth in LBv medium before lysis is induced.

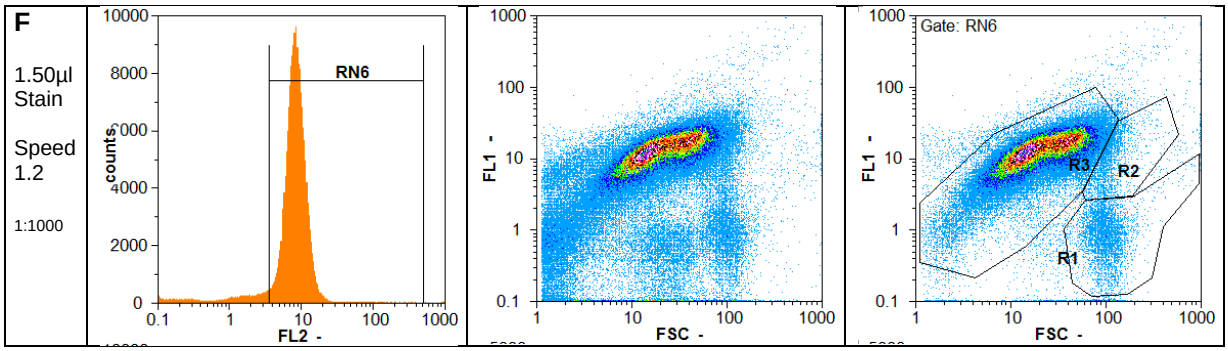
Flow Cytometry was used to observe the influence of expression of foreign antigen (PorB) and induction of lysis on cells. Three exemplary results are given below in Figure 50:



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	1.83 e 8	0.97 e 6	1.47 e 7	1.99 e 8
% Gated	70.0%	0.4%	5.6%	76.0%



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	4.85 e 8	0.93 e 6	1.47 e 7	5.01 e 8
% Gated	81.5%	0.2%	2.5%	84.2%



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	2.32 e 7	1.15 e 7	8.90 e 8	9.25 e 8
% Gated	2.5%	1.2%	95.1%	98.8%

Figure 50: Flow cytometric analysis of fermentation samples, prior to expression induction (C), after 30min of expression, prior to lysis induction (D) and after 60min of lysis (F). Samples were stained with the indicated volume/ml of $DiBAC_4(3)$ and RH414. Gate R1 denotes live, intact cells; R2 dead, intact cells and R3 dead, lysed cells.

While a small amount of particles is gated in the R3 region even before expression or lysis induction, no toxic effect of PorB expression during the first 30min can be seen when samples C and D are compared. After 60min of lysis (time point F), 95% of particles are gated in the R3 (BG) region.

As seen before in the IRIS diagram, the oxygen concentration drops within minutes of lysis induction, only to sharply rise again after roughly 30min of lysis, as seen in Figure 51.

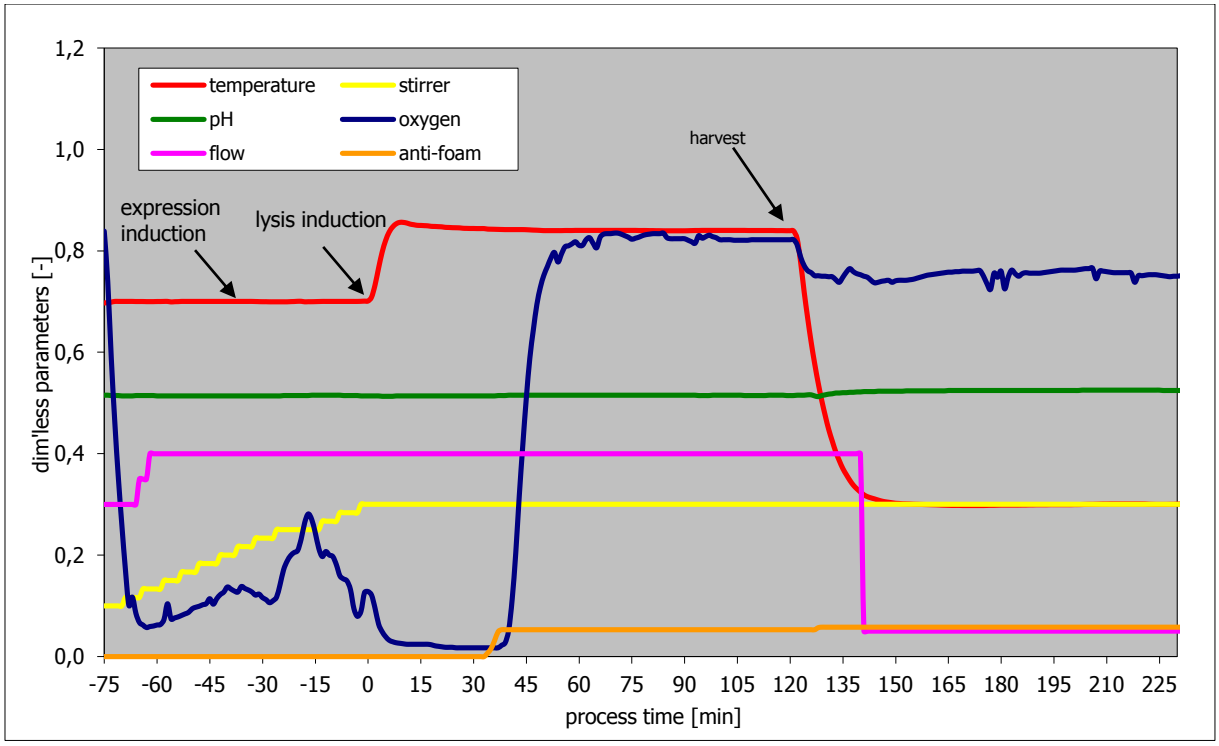


Figure 51: IRIS diagram documenting fermentation parameters like pH (regulated at 7.2), temperature (35°C during growth and expression, 42°C during lysis), flow rate, stirring rate, oxygen concentration and addition of anti-foam (used to counteract the foaming initiated by expulsion of cytoplasmic content during lysis).

Several other specifications as well as follow-up experiments of this particular fermentation are detailed Table 6 below:

***E. coli* Nissle 1917 (pBGKB-PorB, pGLysivb) Bacterial Ghosts**	
Pre-Culture	
Volume: 4*500ml	**Additives**: Gentamicin + Kanamycin
Medium type: LBv	**Other**: pH 7.6
Date: 2009-11-19	**Clone**: c.1 (2009-10-22/ WS 2009-11-05 FHO)
Starting time: 9:18	**Strain**: **E. coli** Nissle 1917
End time:	**Plasmids**: pGLysivb, pBGKB-PorB
ON culture OD: 6.18	**Recombinant Protein Expression**: GIII-PorB-Myc-PolyHis
Inoc. Volume: 1.4l	**Expression Induction**: Arabinose, 0.2%
Medium: LBv	**Expression Induction Time Point**: C
Antibiotics: Gentamicin + Kanamycin	**Lysis Induction**: 42°C
Temperature: 35°C	**Lysis Induction Time Point**: D
Total Volume: ≈22 L	**Killing**: after harvest, 0.075% ß-PL (2*0.0375%)
Acid: F. A.: 34 units	**Volume harvested**: 20l
Base: A. W.: 49 units	**Harvested by**: TFF
Antifoam A: 15 units	**OD Separator flow**: -
Eivb blot: -	**Yield**: 10376 mg
Recombinant blot: OK (by FHO)	**Particles/mg**: 7.12 x 10^8
BPL Test: OK (by AME), no survivors	**Sterility**: OK (by EDZ)
Microscopy: ok, cluster	**Efficiency**: 99.990%

Table 6: Datasheet of *E. coli* Nissle (pBGKB-PorB, pGLysivb) Fermentation

No survivors after ß-PL killing could be detected, and sterility tests of the freeze-dried lyophilisate indicate a sterile product. From a total of 20l fermentation volume, harvesting and lyophilisation yielded **10376mg of BGs** dry weight. Calculating from a cfu count of 3.36*10^8/ml at the time point of lysis induction, and a harvested volume of 20l, the lyophilisate contains **7.12*10^8particles/mg**.

Western Blot samples taken throughout the fermentation process were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence as seen in Figure 52. Protein GIII-PorB-Myc-PolyHis was expected at a size of 40.8kDa, and protein PorB-Myc-PolyHis at a size of 38.7kDa.

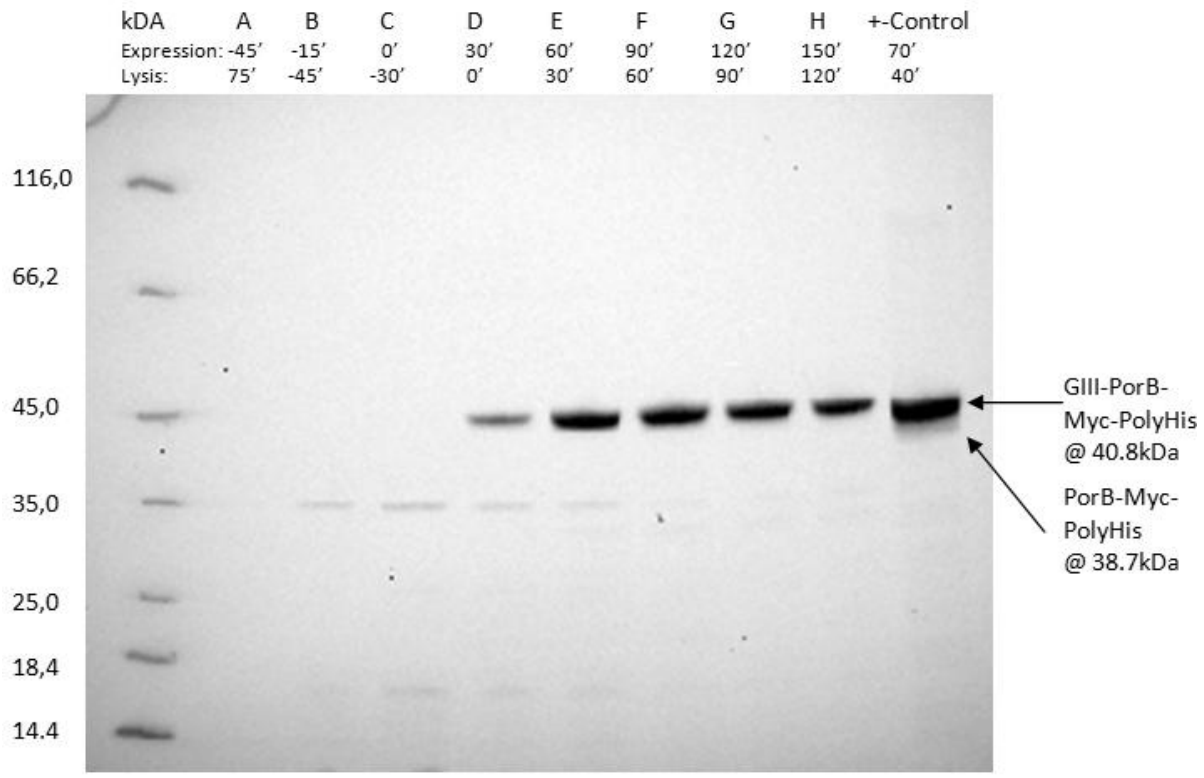


Figure 52: Western Blot of *E. coli* Nissle (pBGKB-PorB, pGLysivb) Fermentation, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. As a positive control, a sample from a previous small scale experiment was used.

A faint signal of Myc-tagged PorB can first be seen 30min after expression induction (D); after 60min of expression/30min of lysis (E), the signal reaches its maximal intensity; in contrast to the fermentation in *E. coli* NM522, where lysis lead to a small but noticeable loss of PorB, (almost) no loss of product upon lysis induction is visible in this *E. coli* Nissle fermentation.

5.5.3 *E. COLI* NM522 (pBGKB-MOMP, pGLYSIVB) FERMENTATION

Working stocks were prepared from clone A3 of *E. coli* NM522 (pBGKB-MOMP, pGLysivb). These were used to inoculate an overnight culture for fermentation. In contrast to PorB fermentations, due to the toxicity of MOMP expression, E-lysis was induced by temperature upshift to 42°C already 20min after induction of MOMP expression.

OD_{600nm} values and cfu counts plotted against time are shown in Figure 53.

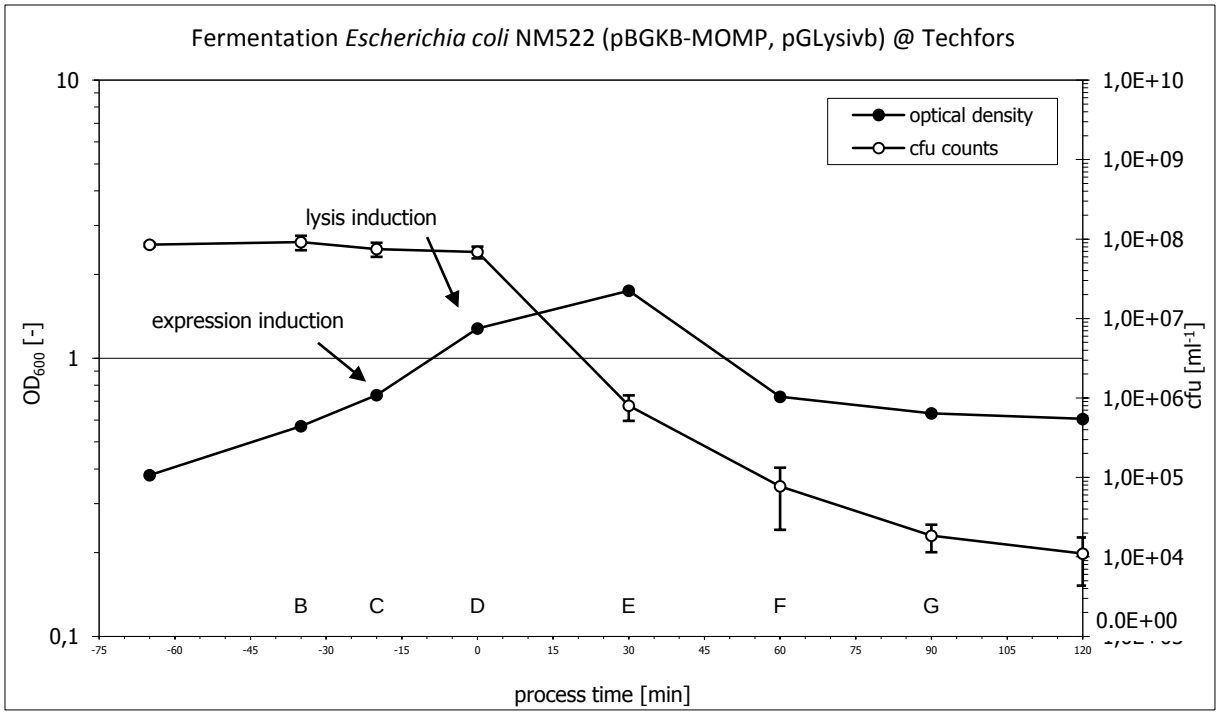


Figure 53: OD and cfu values over time during fermentation in 22l of *E. coli* NM522 (pBGKB-MOMP, pGLysivb). L-Arabinose was added at time point C (-20min); lysis was induced by increasing the temperature to 42°C at time point D (0min).

While OD_{600nm} increases from the time point of induction until lysis induction, the cfu more or less stagnates during the whole fermentation, until lysis is induced. After that, both values drop sharply (from $6.90*10^7$cfu/ml at time point D to $1.10*10^4$cfu/ml at time point H – a lysis efficiency of 99.98%). In contrast to cfu determination, FACS live cell counts do increase by 50% from the start of the fermentation until lysis is induced (compare Figure 55).

Microscopic analysis (see Figure 54) using the membrane potential staining dye $DiBAC_4(3)$ also showed no killing before lysis induction. The development of BGs is clearly seen in Figure 54C after lysis induction by temperature shifting to 42°C.

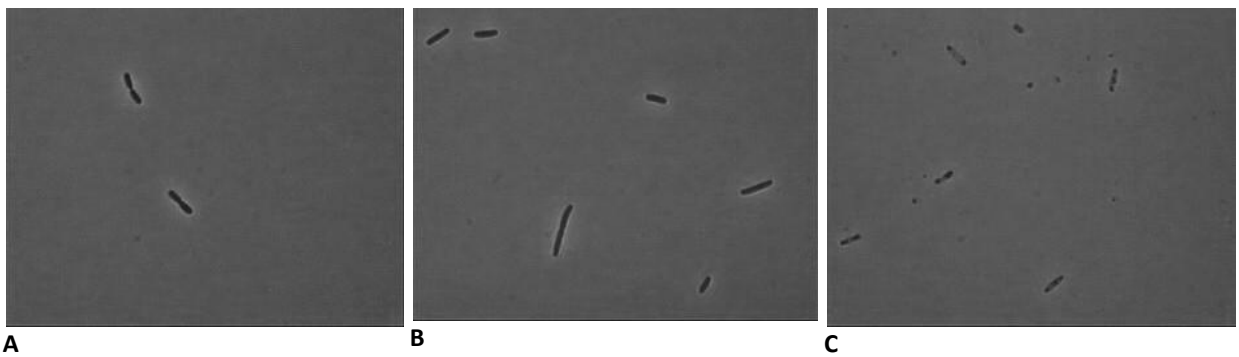
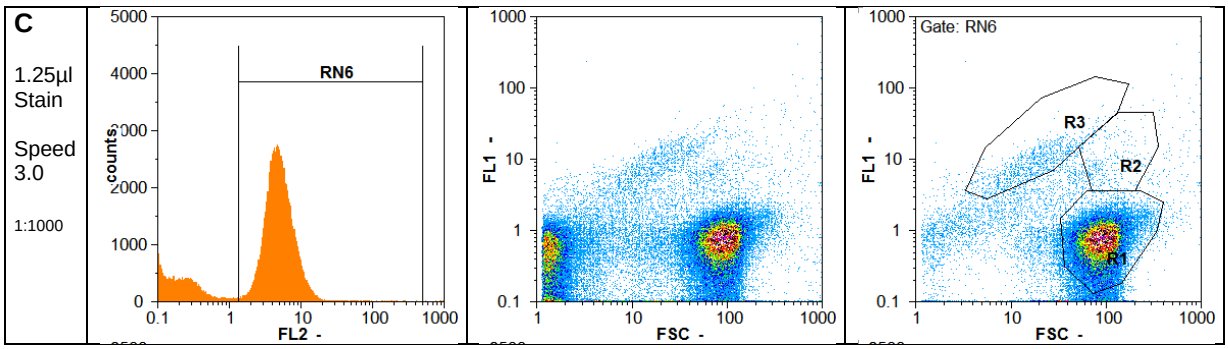
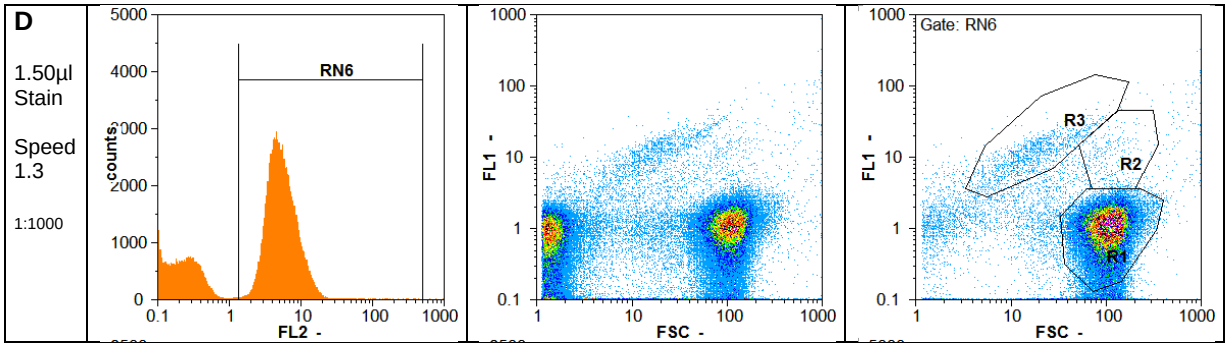


Figure 54: Light microscopy of samples taken during the fermentation process. Intact cells at time point C after 45min of growth (A), intact cells at time point D after 20min of protein expression (B) and translucent lysed cells at time point F after 60min of lysis (C).

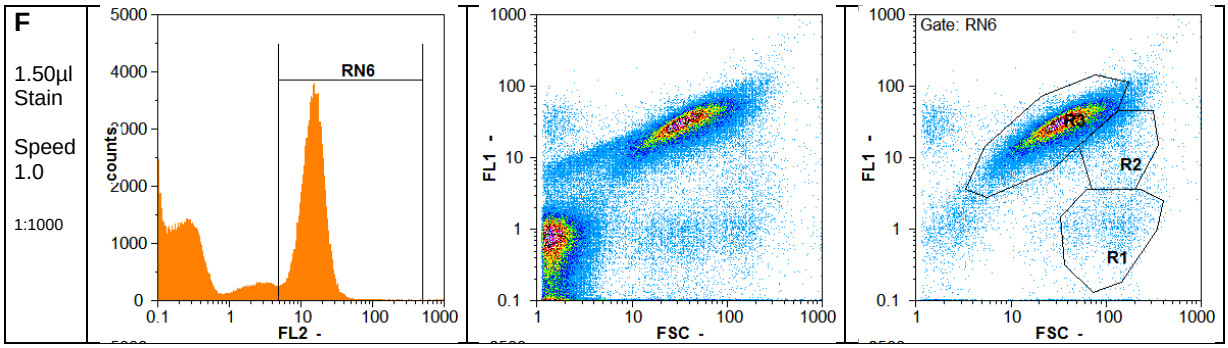
Flow Cytometry was used to observe the influence of foreign antigen expression (MOMP) and induction of lysis on cells. Three exemplary results are given below in Figure 55:



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	3.21 e 8	1.56 e 6	4.71 e 6	3.28 e 8
% Gated	80.6%	0.4%	1.2%	82.1%



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	3.62 e 8	1.65 e 6	6.53 e 6	3.70 e 8
% Gated	81.5%	0.4%	1.5%	83.3%



	R1 - Living	R2 - Dead / Intact	R3 - Ghost	Total Particles
Count / ml	6.19 e 6	1.50 e 7	4.15 e 8	4.36 e 8
% Gated	1.4%	3.3%	90.2%	94.8%

Figure 55: Flow cytometric analysis of fermentation samples, prior to expression induction (C), after 20min of expression, prior to lysis induction (D) and after 60min of lysis (F). Samples were stained with the indicated volume/ml of $DiBAC_4(3)$ and RH414. Gate R1 denotes live, intact cells; R2 dead, intact cells and R3 dead, lysed cells.

Only a small amount of particles is gated in the R3 region before expression or lysis induction, and no toxic effect of MOMP expression during the first 20min can be seen when samples C and D are compared. After 60min of lysis (time point F), 90% of particles are gated in the R3 (BG) region.

Important fermentation parameters such as flow, stirring rate, temperature or pO_2 are documented using the IRIS software and detailed below in Figure 56:

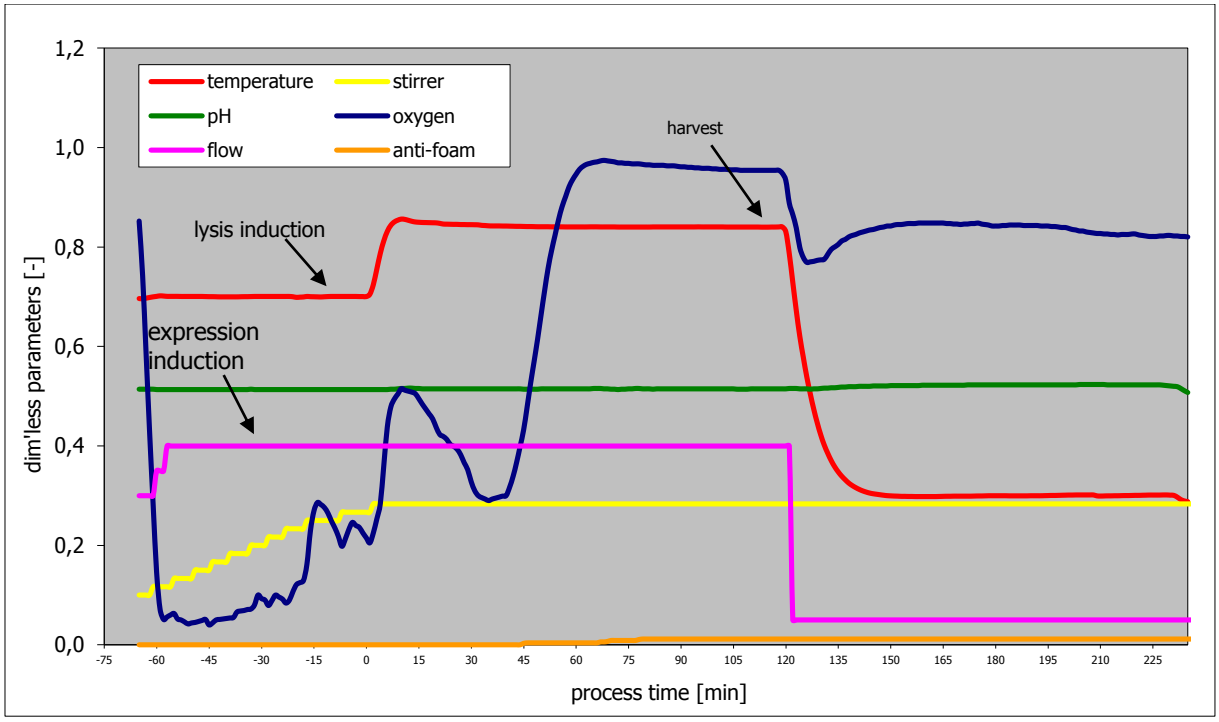


Figure 56: IRIS diagram documenting fermentation parameters like pH (regulated at 7.2), temperature (35°C during growth and expression, 42°C during lysis), flow rate, stirring rate, oxygen concentration and addition of anti-foam (used to counteract the foaming initiated by expulsion of cytoplasmic content during lysis).

Paralleling previous fermentations, the oxygen concentration drops rapidly upon lysis induction, only to sharply rise again after roughly 30min of lysis, as seen in the IRIS diagram.

Several other specifications as well as follow-up experiments of this particular fermentation are detailed in Table 7 below:

E. coli NM522 (pBGKB-MOMP, pGLysivb) Bacterial Ghosts	
Pre-Culture	
Volume: 4x500ml	**Additives**: Gentamicin + Kanamycin
Medium type: LBv	**Other**: pH 7.64
Date: 2009-12-10	**Clone**: c. A3 (2009-12-03/ WS 2009-12-07 FHO)
Starting time: 8:55	**Strain**: *E. coli* NM522
End time:	**Plasmids**: pGLysivb, pBGKB-MOMP
ON culture OD: 5.69	**Recombinant Protein Expression**: GIII-MOMP-Myc-PolyHis
Inoc. Volume: 1.47l	**Expression Induction**: Arabinose, 0.2%
Medium: LBv	**Expression Induction Time Point**: C
Antibiotics: Gentamicin + Kanamycin	**Lysis Induction**: 42°C
Temperature: 35°C	**Lysis Induction Time Point**: D
Total Volume: ≈22l	**Killing**: after harvest, 0.075% ß-PL (2*0.0375%)
Acid: F. A.: 38 units	**Volume harvested**: 20l
Base: A. W.: 22 units	**Harvested by**: TFF
Antifoam A: 12 units	**OD separator flow**: -
Eivb blot: -	**Yield**: 6209mg
Protein Gel: OK (by FHO)	**Particles/mg**: 2.1 x 10^8
BPL Test: OK (by AME), no survivors	**Sterility**: OK (by EDZ)
Microscopy: ok	**Efficiency**: 99,984%

Table 7: Datasheet of *E. coli* NM522 (pBGKB-MOMP, pGLysivb) Fermentation

No survivors after ß-PL killing could be detected, and sterility tests of the freeze-dried lyophilisate indicate a sterile product. From a total of 20l fermentation volume, harvesting and lyophilisation yielded **6209mg of BGs** dry weight. Calculating from a cfu count of 6.90*10^7/ml at the time point of lysis induction, and a harvested volume of 20l, the lyophilisate contains **2.10*10^8particles/mg**.

Western Blot samples taken throughout the fermentation process were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence, as seen in Figure 57. Protein GIII-MOMP-Myc-PolyHis was expected at a size of 46.5kDa, and protein MOMP-Myc-PolyHis at 44.4kDa.

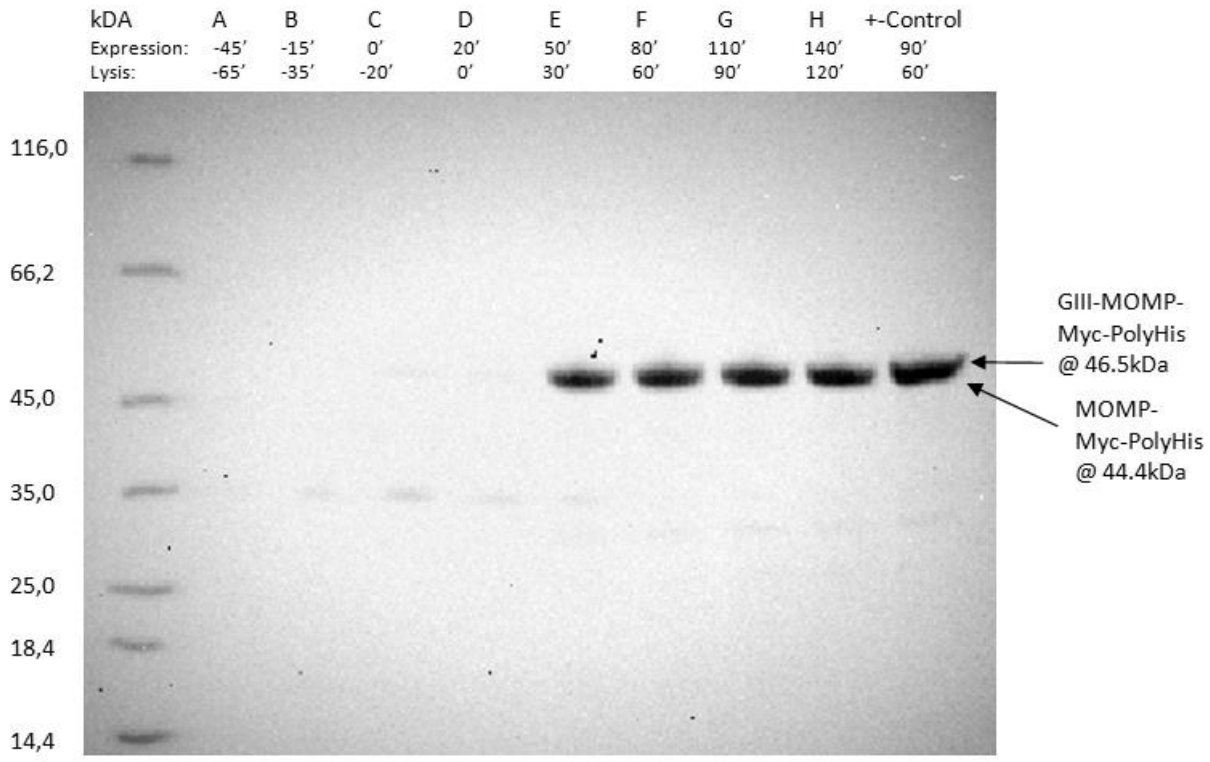


Figure 57: Western Blot of *E. coli* NM522 (pBGKB-MOMP, pGLysivb) Fermentation, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. As a positive control, a sample from a previous small scale experiment was used.

Myc-tagged MOMP is detected first 50min after induction (not after 20min) and remains at a stable level throughout the whole experiment, with no loss occurring during lysis.

5.6 QUANTIFYING CHLAMYDIAL ANTIGENS IN BGs

Chlamydial antigens presented in BGs after fermentation were quantified using two different techniques: (1) the established protocol of comparing signal strength of a positope of known size and concentration and signal strength of known concentrations of lyophilised BGs on a Western Blot and (2) the S-Tag system that is based on measuring an enzymatic reaction that is proportional to the amount of S-tagged protein in a sample.

5.6.1 QUANTIFICATION OF PORB IN *E. COLI* NM522 BGs

In order to quantify the amount of recombinant PorB per µg *E. coli* NM522 BGs, the signals obtained from a serial dilution of BGs (Techfors Fermentation of *E. coli* NM522 (pBGKB-PorB, pGLysivb), see 5.5.1) with known concentration and a serial dilution of positope (containing

the Myc epitope) with known concentration were compared on a Western Blot, as seen in Figure 58.

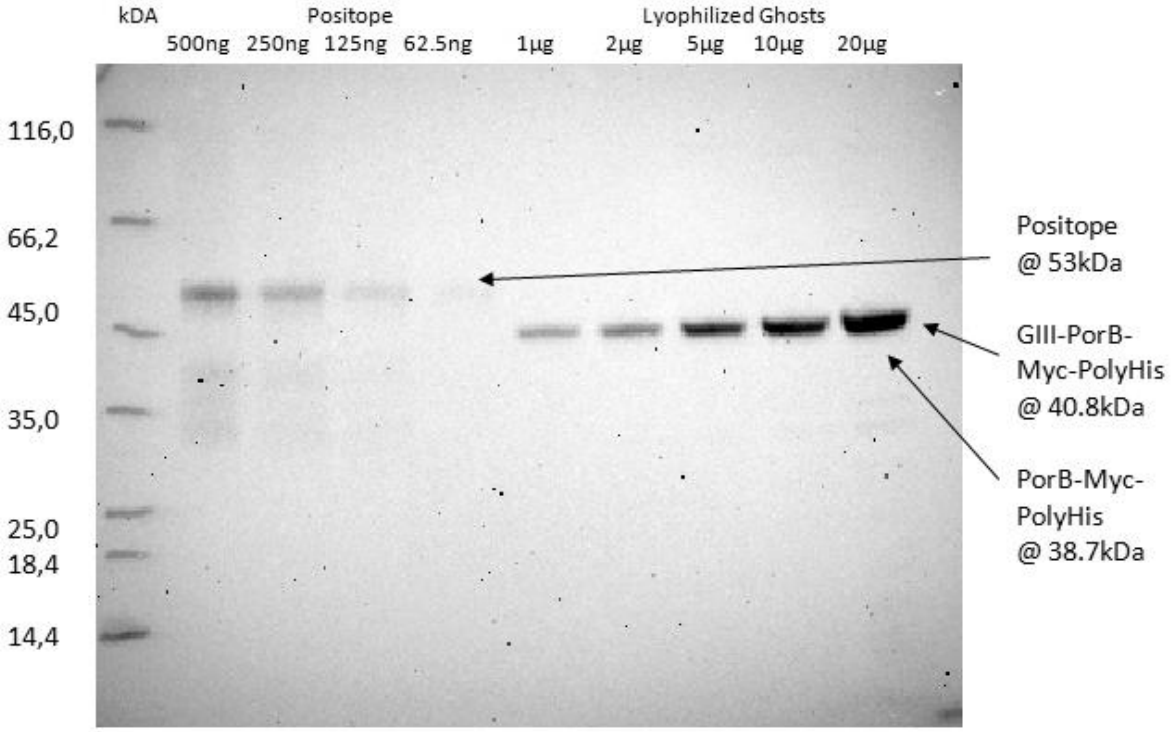


Figure 58: Western Blot of *E. coli* NM522 (pBGKB-PorB, pGLysivb) BGs and positope, using Unstained Protein Molecular Weight Marker (Fermentas). Samples were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. The amount of lyophilized BGs, as well as of positope per lane is indicated.

The chemiluminiscence signals generated by HRP-coupled-Anti-Myc-Antibodies at the site of positope and PorB bands were measured using the ChemidocXRS machine; with the standard curve provided by the positope, the amount of PorB per µg BG could be calculated, as outlined in Figure 59:

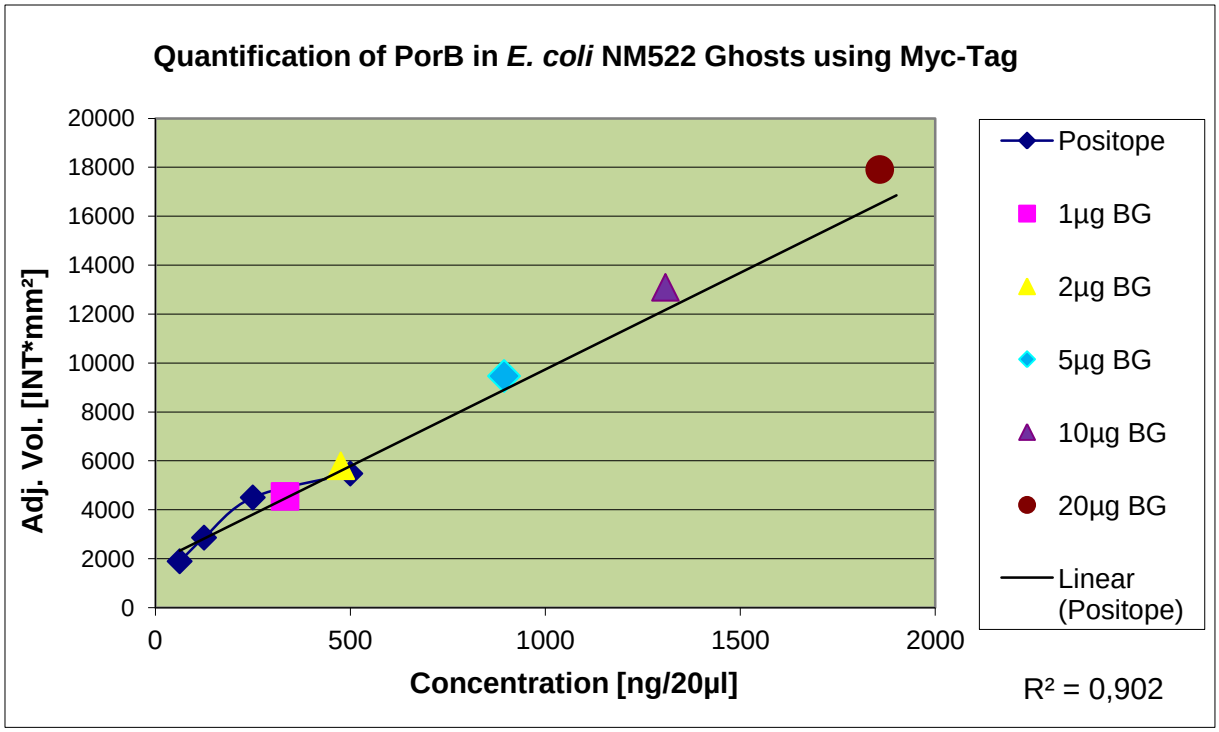


Figure 59: Quantification curve for *E. coli* NM522 (pBGKB-PorB, pGLysivb) BGs. Signals generated by positope with known concentration are indicated by blue diamonds, the linear regression curve is shown in black. Signals generated by BG samples are indicated as detailed in the legend.

The square of the correlation coefficient is R^2=0.902, indicating a good fit of the standard curve provided by the positope. Calculated on that basis, Table 8 shows the amounts of PorB that are found in *E. coli* NM522 BGs.

Lane	Amount of BGs	Calc. Total Amount of PorB	Calc. Amount PorB/µg BG
U1	1µg	332.72ng	332.72ng
U2	2µg	475.56ng	237.78ng
U3	5µg	894.21ng	178.84ng
U4	10µg	1308.02ng	130.80ng
U5	20µg	1857.50ng	92.88ng

Table 8: Calculating the average amount of PorB per µg of *E. coli* NM522 BGs

On average, one microgram of *E. coli* NM522 BGs contains **194.60 ± 94.35ng PorB.** Therefore, recombinant PorB constitutes roughly one fifth of the lyophilized BGs. Taking in account that only 1µg and 2µg BG samples fall on the standard curve given by the positope, a different amount of PorB/µg BG is calculated; if only these two values are included, one microgram of *E. coli* NM522 BGs contains **285.25 ± 67.13ng PorB.**

The molecular weight of PorB of 38.7kDa translates into a mass of $6.42 \cdot 10^{-20}$g, therefore, one microgram of *E. coli* NM522 BGs contains $4.44 \cdot 10^{12}$ PorB molecules (using the second

calculated value). Assuming a particle count of $1.69 \cdot 10^6$ BGs/µg, **a single *E. coli* NM522 BG contains $2.63 \cdot 10^6$ PorB molecules**.

5.6.2 QUANTIFICATION OF PORB IN *E. COLI* NISSLE BGS

For the quantification of recombinant PorB per µg *E. coli* Nissle BG (Techfors Fermentation of *E. coli* Nissle (pBGKB-PorB, pGLysivb), see 5.5.2), the signals obtained from a serial dilution of BGs with known concentration and a serial dilution of positope (containing the Myc epitope) with known concentration were compared on a Western Blot, as seen in Figure 60.

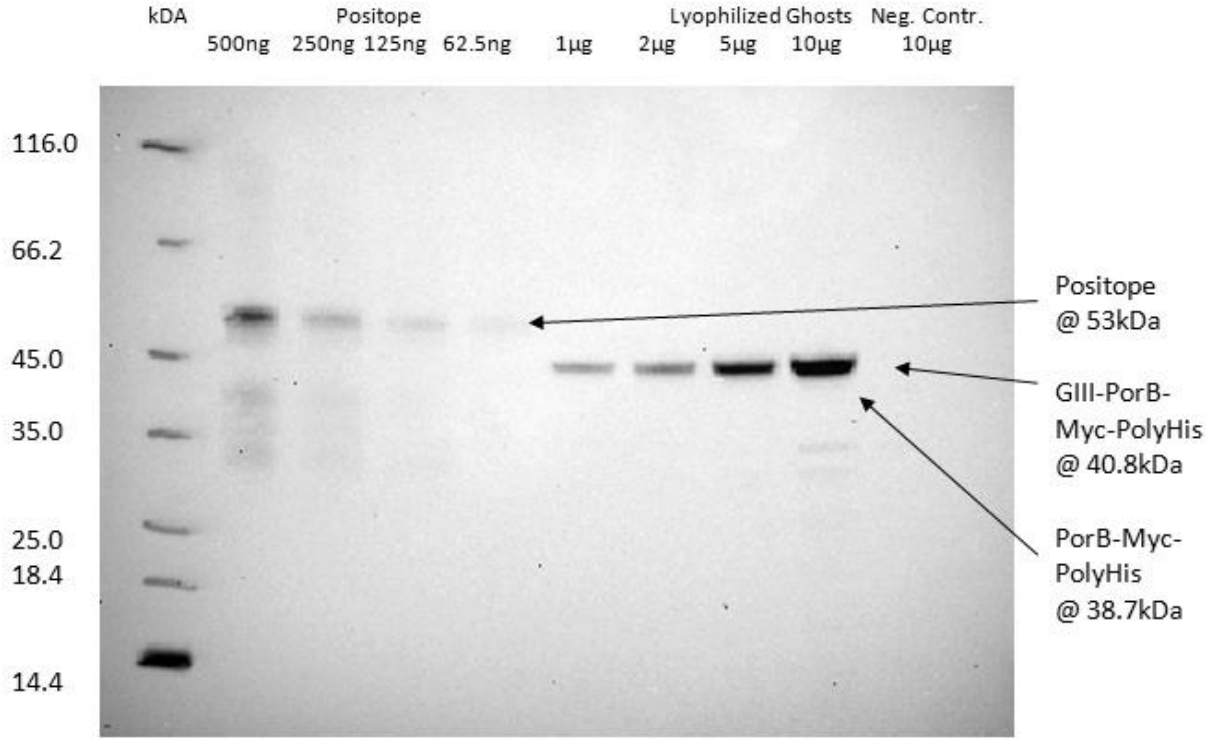


Figure 60: Western Blot of *E. coli* Nissle (pBGKB-PorB, pGLysiv) BGs and positope, using Unstained Protein Molecular Weight Marker (Fermentas). Samples were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. The amount of lyophilized BGs, as well as of positope per lane is indicated. As a negative control, BGs from an *E. coli* Nissle (pGLysivb) fermentation were used.

The chemiluminiscence signals generated by HRP-coupled-Anti-Myc-Antibodies at the site of positope and PorB bands were measured using the ChemidocXRS machine; with the standard curve provided by the positope, the amount of PorB per µg BG could be calculated, as seen in Figure 61:

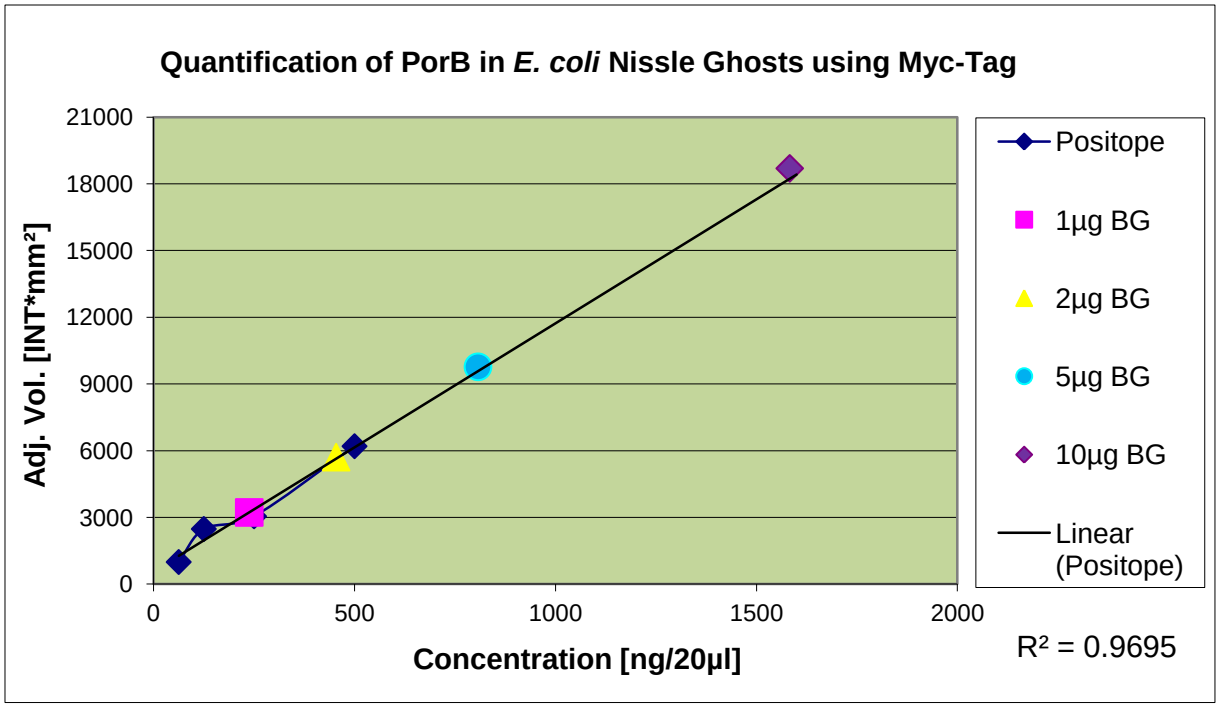


Figure 61: Quantification curve for *E. coli* Nissle (pBGKB-PorB, pGLysivb) BGs. Signals generated by positope with known concentration are indicated by blue diamonds, the linear regression curve is shown in black. Signals generated by BG samples are indicated as detailed in the legend.

The square of the correlation coefficient is R^2=0.9695, indicating a very good fit of the standard curve provided by the positope. Calculated on that basis, Table 9 details the following amounts of PorB are found in BGs.

Lane	Amount of BGs	Calc. Total Amount of PorB	Calc. Amount PorB/µg BG
U1	1µg	238.04ng	238.04ng
U2	2µg	454.18ng	227.09ng
U3	5µg	807.14ng	161.43ng
U4	10µg	1582.81ng	158.28ng

Table 9: Calculating the average amount of PorB per µg of *E. coli* Nissle BGs

On average, one microgram of *E. coli* Nissle BGs contains **196.21 ± 42.24ng PorB.** Therefore, recombinant PorB constitutes roughly one fifth of the lyophilized BGs. Taking in account that only 1µg and 2µg BG samples fall on the standard curve given by the positope, a different amount of PorB/µg BG is calculated; if only these two values are included, one microgram of Nissle BGs contains **232.57 ± 7.74ng PorB.**

These values are remarkably similar to the amount of PorB found in *E. coli* NM522 BGs, indicating a good reproducibility using the pBGKB-PorB vector.

The molecular weight of PorB of 38.7kDa translates into a mass of $6.42 \cdot 10^{-20}$g, therefore, one microgram of *E. coli* Nissle BGs contains $3.62 \cdot 10^{12}$ PorB molecules (using the second calculated value). Assuming a particle count of $7.12 \cdot 10^{5}$ BGs/µg, **a single *E. coli* Nissle BG contains $5.08 \cdot 10^{6}$ PorB molecules**.

5.6.3 QUANTIFICATION OF MOMP IN *E. COLI* NM522 BGS

To quantify the amount of recombinant MOMP per µg *E. coli* NM522 BG (from Techfors Fermentation of *E. coli* NM522 (pBGKB-MOMP, pGLysivb), see 5.5.3), the signals obtained from a serial dilution of BGs with known concentration and a serial dilution of positope (containing the Myc epitope) with known concentration were compared on a Western Blot, as seen in Figure 62.

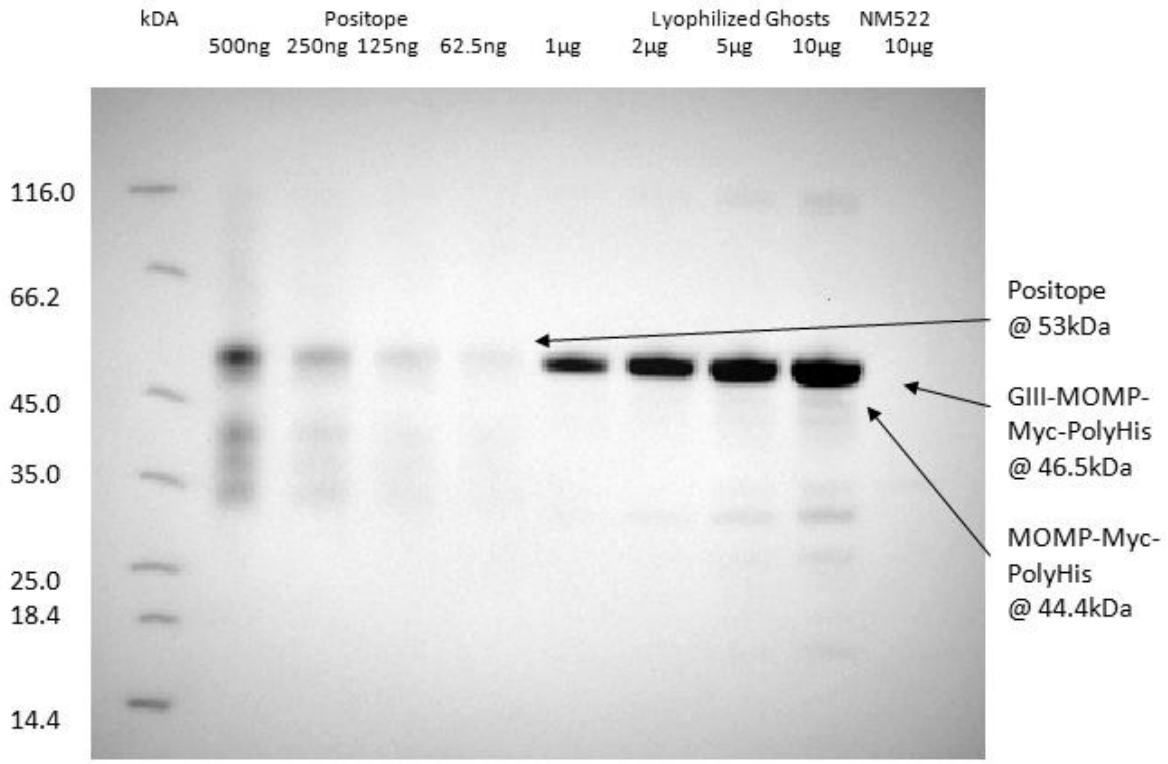


Figure 62: Western Blot of *E. coli* NM522 (pBGKB-MOMP, pGLysivb) BGs and positope, using Unstained Protein Molecular Weight Marker (Fermentas). Samples were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. The amount of lyophilized BGs, as well as of positope per lane is indicated. BGs from a fermentation of *E. coli* NM522 (pGLysivb) were used as a negative control.

The chemiluminiscence signals generated by HRP-coupled-Anti-Myc-Antibodies at the site of positope and MOMP bands were measured using the ChemidocXRS machine; with the

standard curve provided by the positope, the amount of MOMP per µg BG could be calculated, as seen in Figure 63:

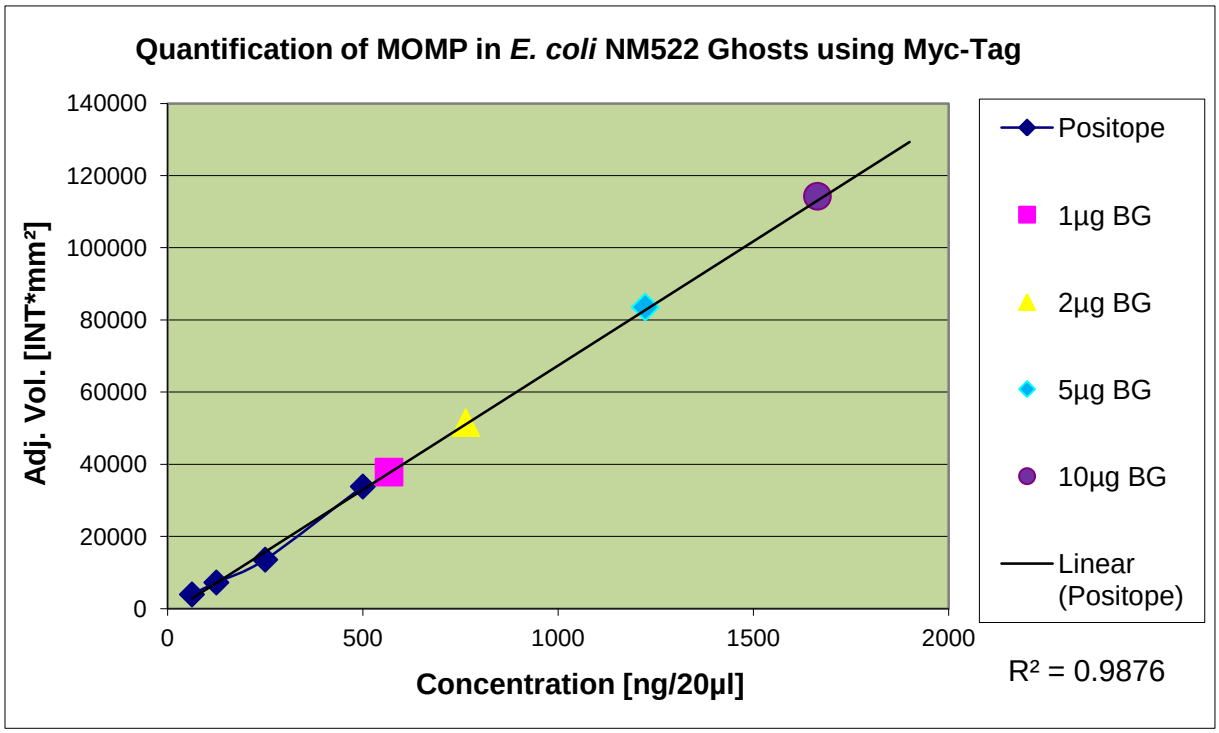


Figure 63: Quantification curve for *E. coli* NM522 (pBGKB-MOMP, pGLysivb) BGs. Signals generated by positope with known concentration are indicated by blue diamonds, the linear regression curve is shown in black. Signals generated by BG samples are indicated as detailed in the legend.

The square of the correlation coefficient is R^2=0.9876, indicating a very good fit of the standard curve provided by the positope. Calculated on that basis, Table 10 gives the following amounts of MOMP for *E. coli* NM522 BGs.

Lane	Amount of BGs	Calc. Total Amount of MOMP	Calc. Amount MOMP/µg BG
U1	1µg	567.15ng	567.15ng
U2	2µg	763.64ng	381.82ng
U3	5µg	1223.16ng	244.63ng
U4	10µg	1663.95ng	166.40ng

Table 10: Calculating the average amount of MOMP per µg of *E. coli* NM522 BG

On average, one microgram of NM522 BGs contains **340.00 ± 175.67ng MOMP.** Therefore, recombinant MOMP constitutes an impressive one third of the lyophilized BGs.

All samples are out of the range of the standard curve; the sample using 1µg BG comes closest to the upper limit of the standard curve, and gives a value of 567.15ng MOMP per microgram BG – more than half of the BGs' dry mass.

The molecular weight of MOMP of 44.44kDa translates into a mass of $7.38 \cdot 10^{-20}$g, therefore, one microgram of *E. coli* NM522 BGs contains $4.61 \cdot 10^{12}$ MOMP molecules (using the first calculated value). Assuming a particle count of $2.10 \cdot 10^{5}$ BGs/µg, **a single *E. coli* NM522 BG contains $2.19 \cdot 10^{7}$ MOMP molecules**.

5.6.4 QUANTIFICATION OF RECOMBINANT PROTEINS USING THE S-TAG SYSTEM

Samples were collected during an Expression Experiment of *E. coli* C2988J (pASK) and an Expression/Lysis Experiment of *E. coli* NM522 (pASK-PorB, pGLysivb) (already described above) and treated according to protocol, as detailed in 7.10.3 Quantification Using the S-Tag System.

In the case of *E. coli* C2988J (pASK), the quantification result is shown in Figure 64:

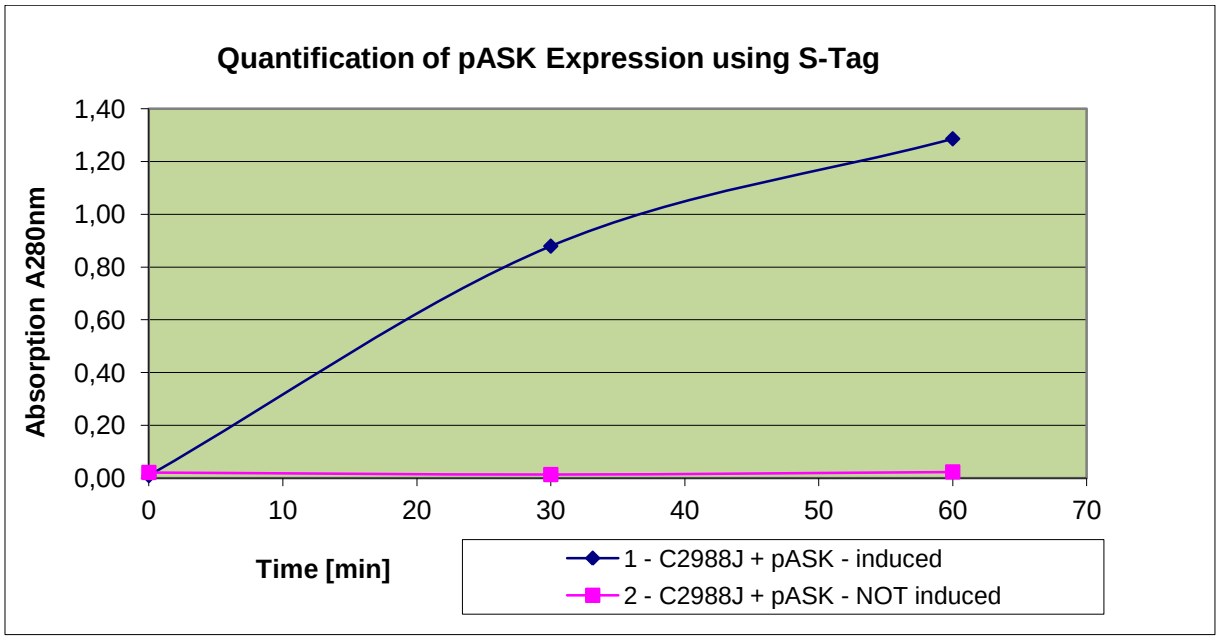


Figure 64: S-Tag Detection Assay, using induced (1) and non-induced (2) samples of *E. coli* C2988J (pASK). Time point 0min is the moment of L-Arabinose addition to (1).

While the RNAse S activity as measured by A_{280nm} absorption remains at baseline levels in non-induced samples (2), and is at baseline levels at the induction point for sample 1 (0min), absorption increases rapidly when pASK is induced by addition of 0.2% L-Arabinose. The calculated amount of GIII-PolyHis-S-Tag-PolyHis (the gene product of pASK) is 0.002pmol/µl at time point 0min, 0.161pmol/µl after 30min and reaches 0.236pmol/µl 60min after expression induction, while it stays at 0.004pmol/µl for the non-induced sample.

An S-Tag standard sample was used for calculating unknown samples.

Using samples from a *E. coli* NM522 (pASK-PorB, pGLysivb) and an *E. coli* NM522 (pASK, pGLysivb) Expression/Lysis Experiment, the following data were collected, as shown in Figure 65:

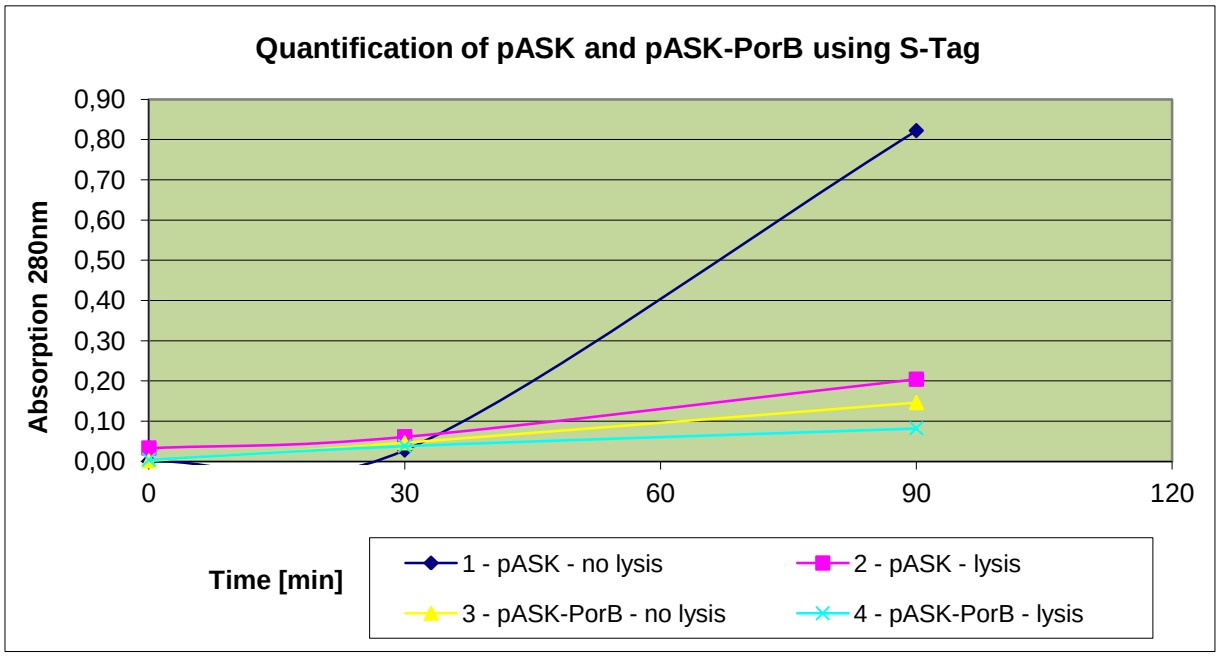


Figure 65: S-Tag Detection Assay, using samples of *E. coli* NM522 (pASK, pGLysivb) (1-2) as well as samples of *E. coli* NM522 (pASK-PorB, pGLysivb) (3-4). Time point 0min is the moment of L-Arabinose addition to all samples. Lysis was induced at time point 30min for samples (2) and (4).

In general, the yield was much lower than in the previous experiment, even after 30min of expression, before loss of protein due to lysis might have occurred. In sample 1, where pASK expression was induced but no lysis occurred, 0.153pmol/µl are reached after 90min of expression, whereas lysis (in sample 2) reduces this number to 0.038pmol/µl. Anyhow, at time point 30min (just before lysis induction), no real difference was observed between samples 1 and 2.

Yields for pASK-PorB translation products were even lower, possibly reflecting the larger mass of the recombinant protein. After 90min of expression, 0.027pmol/µl PorB are detected in samples 3, but when lysis is induced (sample 4) this number is reduced to 0.015pmol/µl. This is somewhat conflicting with the Western Blot seen in Figure 40, where sample E3 does not appear to be twice as intensive as sample E4.

6 DISCUSSION

6.1 TESTING THE S-TAG SYSTEM

While the S-Tag Assay, as described in the Materials & Methods chapter, is less time consuming than a Western Blot (90min compared to roughly 8 hours), it also gives less information. While in theory S-tagged proteins can be quantified, the enzymatic reaction measured in this assay only gives information about the total S-Tag present in samples. Whether the S-Tag is still attached to the recombinant protein, or the protein has undergone degradation, is not immediately visible.

By coupling the signal strength (indicating concentration) with information about protein size, Western Blot quantification techniques are intrinsically more informative, and signal given by degraded protein can be excluded from the quantification calculation. On the other hand, quantification via WB is strongly dependent on the blotting behaviour of the protein and needs a standard protein of similar size compared to the protein that has to be quantified. The S-Tag system is independent of protein size and a unique standard can be used for proteins of any size. Also multiple samples can be analyzed in parallel, making it a suitable for tool for the pretesting several clones. To allow quantification using the S-tag for BGs, further studies are necessary to implement this technique successfully.

Given the need of multiple assays for statistically significant data and the necessity of many pipetting steps, the established WB quantification system might at the moment be the more accurate and more economic mode to quantify recombinant proteins in BGs. For the future, a combination of both techniques, using the S-Tag also for detection on WB, is desired.

6.2 CLONING EXPRESSION VECTORS

To analyse expression of foreign proteins transported to the periplasm, three different vectors were used, differing in the promoter/repressor system, the transport signal to the periplasm and the tags used.

A new expression system for the export of S-tagged proteins to the periplasm upon L-arabinose addition was successfully cloned in the form of pASK. Its correct size was proven by the correct pattern after restriction digests (see Figure 14).

The pASK vector was used as an expression backbone vector for PorB, a chlamydial outer membrane protein, giving rise to pASK-PorB (see Figure 22). PorB was also successfully cloned into an IPTG-inducible, T7 RNA polymerase dependent vector system for the DsbC-dependent periplasmic export of S-tagged proteins, pET40b. The correct size and restriction digest pattern of the resulting plasmid pET40b-PorB was confirmed (see Figure 20).

Another expression vector used for foreign protein expression and periplasmic export of proteins upon L-Arabinose induction is pBGKB. Restriction digests again showed the correct size and pattern of pBGKB-PorB (see Figure 17) and pBGKB-MOMP (see Figure 25), the latter encoding the chlamydial Major Outer Membrane Protein, MOMP.

All expression vectors used conferred kanamycin resistance to their host and signalled protein transport to the periplasm. No difference in cloning efficiency was observed for the different backbone vectors; expected restriction digest patterns were seen after control digests, but no sequence analysis was performed.

6.3 EXPRESSING CHLAMYDIAL ANTIGENS

After expression vectors were cloned successfully, their function was tested in small scale experiments. The effect of recombinant protein expression on viability and on cell shape was observed using cfu counting and light microscopy, while protein expression was detected via Western Blotting using antibodies against tags fused to the expressed proteins.

Neither PorB nor MOMP expression and periplasmic transport had notable effect on cell shape, but both influenced the viability of bacteria negatively. PorB lead to a decrease in growth rate once expression was induced, resulting in cfu stagnation or mild killing (see Figure 26 and Figure 31). This effect was even more pronounced for MOMP, where killing reached almost 99% after 120min of expression (see Figure 33). For both PorB and MOMP, these effects were only seen in the cfu behaviour after more than 30min of protein expression.

Western Blots were able to detect Myc-tagged PorB (see Figure 27) and MOMP (see Figure 34) as well as S-tagged PorB from pASK-PorB (see Figure 32), usually faintly after 20min of expression, and at saturated levels after 60min. Little or no degradation over time is detectable, and no protein is present before the induction of protein expression.

While the same stagnation/killing effect is seen upon induction of pET40b-PorB (see Figure 28), no protein could be detected in Western Blots using HRP-coupled-S-Protein, even though the gene product of the empty vector pET40b can be detected (see Figure 29), but only faintly. No ready explanation can be given for that. From the cfu behaviour it can be concluded that PorB is expressed, but to ensure the functionality of the S-Tag, sequencing would be necessary. Also changing to another antibody could solve this problem, as low efficiency of the HRP-coupled-S-Protein can be seen when trying to detect the gene product of pET40b.

In all cases where a stagnation of killing was observed in the cfu, no such effect was seen in OD_{600nm} measurements. Together with the microscopy analysis an increase in cell size can be excluded. A possible explanation for this behaviour could be a delayed killing effect by the protein, coming into effect only during ON incubation on the plate. This is supported by observations via flow cytometry that failed to detect large-scale killing in the first 30min of protein expression during fermentations.

6.4 SMALL SCALE BG PRODUCTION

Expression plasmids that were shown to be able to express their cloned proteins were co-transformed with lysis plasmid pGLysivb into *E. coli* NM522, and if possible, *E. coli* Nissle to perform small-scale Expression/Lysis Experiments in order to test whether foreign antigens interfere with lysis and whether they are lost upon lysis induction.

6.4.1 SMALL-SCALE BG PRODUCTION IN *E. COLI* NM522

Plasmids pASK-PorB, pBGKB-PorB and pBGKB-MOMP were used in Expression/Lysis experiments in *E. coli* NM522. In all three cases expression of recombinant proteins under the chosen conditions did not negatively influence lysis efficiency, even though in the case of

MOMP it is possible that killing contributes to the reduction in cfu used for the calculation of lysis efficiency.

Lysis efficiencies of clones selected for later fermentation were:

- 99.97% for *E. coli* NM522 (pASK-PorB, pGLysivb) c. B2 (fermentation not performed)
- 99.96% for *E. coli* NM522 (pBGKB-PorB, pGLysivb) c. A3 (used for fermentation) and
- 99.93% for *E. coli* NM522 (pBGKB-MOMP, pGLysivb) c. A3 (used for fermentation).

Western Blot analysis of Expression/Lysis experiments showed similar amounts of expressed protein regardless whether lysis was induced or not, implying that little or no loss due to lysis occurs. This is especially striking in contrast to other cellular proteins, that are visibly lost on lysis induction (see Figure 40). Therefore it can be concluded that the expressed proteins are located within the membrane complex. Even though the exact location was not determined, the presence of the signal sequence for the periplasm makes a periplasmatic localization most likely.

These findings suggest that neither PorB nor MOMP infer under given conditions with E-mediated lysis, and E-mediated lysis does not lead to the loss of these antigens in significant numbers.

6.4.2 SMALL-SCALE BG PRODUCTION IN *E. COLI* NISSLE

Even though numerous approaches were tried, in my hands it was not possible to transform pBGKB-MOMP into *E. coli* Nissle, neither alone nor in combination with pGLysivb. Interestingly, also the precursor plasmid that was used to clone pBGKB-MOMP, pKS-MOMP, could not be transformed into *E. coli* Nissle in my hands.

In contrast, pBGKB-PorB (as well as its precursor plasmid, pMAL-PorB) was easily (even though with reduced efficiencies compared to an *E. coli* NM522 transformation) transformed into *E. coli* Nissle.

An Expression/Lysis Experiment of *E. coli* Nissle (pBGKB-PorB, pGLysivb) showed similar results as in *E. coli* NM522. Lysis was efficient (99.80% for c. A1, that was used for fermentation), but nearly one log stage lower than seen in NM522 (pBGKB-Porb, pGLysivb)

(99.96% lysis efficiency). Again, lysis did not affect the concentration of PorB in comparison with non-lysed samples (see Figure 38).

6.5 LARGE SCALE BG PRODUCTION

For the large scale production of BGs carrying chlamydial antigens, *E. coli* NM522 (pBGKB-PorB, pGLysivb) c. A3, *E. coli* NM522 (pBGKB-MOMP, pGLysivb) c. A3, as well as *E. coli* Nissle (pBGKB-PorB, pGLysivb) c. A1 were used for fermentations in 22l volume. Sterility of the final product was shown by follow-up experiments.

Quantification using Western Blots with a known concentration of positope showed that PorB constituted 20% of *E. coli* NM522 (see Table 8) and *E. coli* Nissle (see Table 9) BGs, and that MOMP accounted for one third of the mass of *E. coli* NM522 BGs (see Table 10).

In contrast to the pretestings in Expression/Lysis experiments, where lysis efficiency of *E. coli* NM522 (pBGKB-PorB, pGlysivb) was higher than in *E. coli* Nissle, the opposite was observed for fermentations, but a repeated number of fermentations would be necessary for any statistically sound conclusions.

Additionally, in the three fermentations performed, the one using *E. coli* Nissle had a roughly 60% higher yield than fermentations using *E. coli* NM522, but once again, not enough fermentations were performed for statistically sound conclusions.

Furthermore, the calculated particle numbers per microgram of BG dry-weight varies by almost a log stage (2.1×10^5 for *E. coli* NM522 (pBGKB-MOMP, pGLysivb) and 1.69×10^6 for *E. coli* NM522 (pBGKB-PorB, pGLysivb)). In addition to more fermentations, a second, independent particle count method would be necessary to test these results.

Below in Table 11 is given an overview detailing the large scale BG production:

	***E. coli* NM522 (pBGKB-PorB, pGLysivb)**	***E. coli* Nissle (pBGKB-PorB, pGLysivb)**	***E. coli* NM522 (pBGKB-MOMP, pGLysivb)**
Lysis Efficiency:	99.925%	99.990%	99.984%
Yield:	6447mg	10376mg	6209mg
Particles/µg:	$1.69 \cdot 10^6$	$7.12 \cdot 10^5$	$2.1 \cdot 10^5$
ng Antigen/µg BG:	194.60 ± 94.35ng	196.21 ± 42.24ng	340.00 ± 175.67ng
Proteins/Particle:	$2.63 \cdot 10^6$ PorB/BG	$5.08 \cdot 10^6$ PorB/BG	$2.19 \cdot 10^7$ MOMP/BG

Table 11: Product details of BGs displaying chlamydial antigens produced during this study.

Previous studies attempted

- to display fertility proteins in the periplasm of BGs to induce immunocontraception in possums (Schlacher, 2009)
- to display human choriongonadotropin-ß fused to heat labile enterotoxin B (hCG-ß-LTB) in the periplasm of BGs for immunocontraception induction (Hodul, 2010) or
- to display luteinizing hormone releasing hormone (LHRH) in the periplasm of BGs for wildlife control by immunocontraception (Champeimont, 2008).

In these cases, quantified antigen accounted for:

- 0.35ng/µg BG to 6.35ng/µg BG in the case of LHRH (Champeimont, 2008)
- 44.51ng/µg BG to 49.75ng/µg BG in the case of hCG-ß-LTB (Hodul, 2010) and
- 14.6ng/µg BG to 403ng/µg in the case of the possum antigen ZP2C (Schlacher, 2009).

The quantified yields of roughly 200ng/µg BG for PorB-displaying BGs and 340ng/µg BG for MOMP-displaying BGs compare favourably to previous experiments.

Furthermore, the calculated amounts of proteins per individual BG are found in a range between $2.63 \cdot 10^6$ PorB/BG for *E. coli* NM522 (pBGKB-PorB, pGLysivb) and $2.19 \cdot 10^7$ MOMP/BG for *E. coli* NM522 (pBGKB-MOMP, pGLysivb), and thus are between 30 and 300times higher than in previous studies where proteins per particle were calculated (Champeimont, 2008).

In previous studies, the particle numbers for fermentations were found to be between 1.65 x 10^6/µg and 1.85 x 10^6/µg (Schlacher, 2009), between 1.89 x 10^6/µg and 2.85 x 10^6/µg (Champeimont, 2008) and between 1.19 x 10^5/µg and 1.05 x 10^6/µg (Hodul, 2010).

The calculated particle numbers for fermentations of BGs displaying chlamydial antigens were found to be well within these numbers.

6.6 CHLAMYDIAL ANTIGENS IN BGS

Regarding the goals stated before this study – to express chlamydial antigens in *E. coli*, to localize them in the periplasm and to retain them there during and after E-lysis, followed by their quantification – it can be concluded that they were completed successfully.

The efficaciousness of BGs as natural adjuvants, displaying chlamydial antigens, to induce (protective) immunity has to be addressed in future studies. With BGs derived from *E. coli* NM522 displaying PorB and MOMP, and *E. coli* Nissle displaying PorB in sufficient, an further preclinical studies can be initiated.

7 MATERIALS AND METHODS

7.1 BACTERIAL STRAINS, MEDIA, CULTIVATION

7.1.1 BACTERIAL STRAINS

The following strains of *Escherichia coli* have been used in this work.

For cloning and plasmid storage, *E. coli* C2988J is used; *E. coli* NM522 and *E. coli* Nissle are used for Expression/Lysis experiments, and *E. coli* C41 is used for experiments with plasmids of the pET40b variant, which requires a T7 RNA polymerase (which *E. coli* C41 carries on its chromosome).

- *Escherichia coli* K12 NM522: supE thi-1 Δ(Lac-proAB) Δ(mcrB-hsdSM)5 ($r_K^- m_K^+$) [F' proAB $lacI^q$ZΔM15] – obtained from Stratagene, Heidelberg, Germany (Stratagene, 2011)
- *Escherichia coli* K12 C2988J (NEB 5-alpha competent *E. coli*): fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 – obtained from New England Biolabs, Ipswich, MA, USA (NEB, 2011)
- *Escherichia coli* K12 C41 (OverExpress™ C41(DE3)): F^- ompT hsdSB (r_B^- m_B^-) gal dcm (DE3) – obtained from Lucigene, Middleton, WI, USA (Lucigene, 2011)
- *Escherichia coli* Nissle 1917*:* O6:K5:H1 ΔpMut1 ΔpMut2 – obtained from Ardeypharm, Herdecke, Germany (Nissle, 1918)

7.1.2 CULTIVATION MEDIUM

Bacteria are cultivated in "Lennox Lysogeny Broth" (LBv), 1l of which consists of 10g soy peptone, 5g animal product free (vegetable) yeast extract and 5g NaCl, with pH adjusted to 7.2.

Plate Count Agar (ROTH) is used for agar plates for cfu counting. 23.5g are dissolved in 1l dH_2O, autoclaved and poured at ≈45°C using a plate pouring machine.

Lennox LB-Agar (ROTH) is used for agar plates supplemented with antibiotics. 35g are dissolved in 1l dH_2O and autoclaved. Antibiotics are added when agar is hand-warm, and plates are poured next to the flame.

7.1.3 BACTERIAL CULTIVATION

Bacteria are inoculated from either frozen cryocultures or single colonies on agar plates and grown in test tubes in a volume of 5ml LBv plus antibiotic(s) and other additives as needed. Samples are incubated at 36°C in an incubating wheel.

For long time storage, bacterial cultures are kept in 25% v/v glycerol at -70°C (in a total volume of 1.8ml).

7.1.4 ANTIBIOTICS

Stock solutions are stored at +4°C for immediate use and at -20°C for long time storage. From these, necessary dilutions to achieve the final desired concentration in culture media are made, as detailed in Table 12.

Antibiotic	[Stock Solution]	[Desired Concentration]	µl stock solution/5ml
Ampicillin	50mg/ml	100µg/ml	10
Gentamicin	25mg/ml	50µg/ml	10
Kanamycin	10mg/ml	20µg/ml	10

Table 12: Antibiotics and their concentration used in this work.

7.2 CHEMICALS

Chemicals used for buffers, solutions and media, as well as some ready to use stock solutions (e.g. 10x TBS, 20x TAE) are obtained from ROTH (ROTH Carl, Karlsruhe, Germany), except noted otherwise.

For Western Blots, the NuPage System including gels, buffers and chambers is used (Invitrogen, Paisley, UK)

7.3 USED APPLIANCES

The following appliances have been used during this work:

Name	Description	Manufacturer
BK7200	Incubator	WTB Binder, Tuttlingen, Germany
Sigma 3K30	Cooling centrifuge	Sigma Aldrich, St. Louis, MO, USA
Biofuge pico	Tabletop centrifuge	Heraeus, Hanau, Germany
Minispin	Tabletop centrifuge	Eppendorf, Hamburg, Germany
Uniflow UVUB 1200 Biohazard	Laminar Flow	Kojair, Vilppula, Finland
Spectronic 20+	Nose flask photometer	Milton Roy, Ivyland, PA, USA
UV-160	Spectrophotometer	Shimadzu, Kyoto, Japan
DW spiral plater	Spiral plater	Don Whitley Scientific, Shipley, UK
ProtoCOL 92000	Colony counter	Bartelt Labor- und Datentechnik, Graz, Austria
ChemidocXRS	Gel detection system	Bio-Rad, Hercules, CA, USA
Lyolab B	Secfroid Lyophilisator	Inula, Vienna, Austria
iCycler IQ	PCR machine	Bio-Rad, Hercules, CA, USA
Leica DMRB Microscope	Microscope	Leica Microsystems, Wetzlar, Germany
CyFlow	Flow Cytometry Analyzer	Partec, Görlitz, Germany
Techfors	30l Fermenter	Infors HT, Bottmingen, Switzerland

Table 13: List of appliances and machines and their manufacturers used in this work.

7.4 BUFFERS AND SOLUTIONS

7.4.1 50% GLYCEROL

- 25ml dH_2O
- 25ml 100% Glycerol
- Mix well and autoclave before using

7.4.2 0.85% SALINE

- 8.5g NaCl
- Fill up to 1l with dH_2O
- Distribute to test tubes using a dispenser for 9.0ml and 9.9ml, autoclave before using

7.4.3 MOPS I SOLUTION

- 10.47g MOPS (100mM)
- 0.74g $CaCl_2$ x $2H_2O$ (10mM)
- 0.6g $RbCl_2$ (10mM)
- Dissolve in 400ml dH_2O
- Adjust pH to 7.0 with KOH
- Fill up to 500ml and autoclave before using

7.4.4 MOPS II SOLUTION

- 10.47g MOPS (100mM)
- 5.15g $CaCl_2$ x $2H_2O$ (70mM)
- 0.6g $RbCl_2$ (10mM)
- Dissolve in 400ml dH^2O
- Adjust pH to 6.5 with KOH
- Fill up to 500ml and autoclave before using

7.4.5 20% L-ARABINOSE STOCK SOLUTION

- 20g L-Arabinose
- Fill up to 100ml with dH_2O
- Sterilize by filtration (Steritop 0.22µm pore size, obtained from Millipore, Billerica, MA, USA)

7.4.6 ANTIBIOTIC STOCKS

- Weigh in antibiotics:
    - 2g D(-)-α-aminobenzylpenicillin sodium salt (Ampicillin) for a stock concentration of 50mg/ml
    - 0.4g Gentamicin sulfate for a stock concentration of 10mg/ml
    - 1g Kanamycin monosulfate for a stock concentration of 25mg/ml
- Dissolve and fill up to 40mg with dH_2O

- Sterilize via filtration using syringes (Millex filters, obtained from Millipore, Billerica, MA, USA)
- Stocks are aliquoted into 1ml aliquots (ampicillin) or 8ml aliquots (gentamicin, kanamycin) and stored at 4°C during use or at -20°C for long-time storage.

7.4.7 1x TRIS-ACETATE-EDTA (TAE) BUFFER

- 50ml 20x TAE stock solution
- 950ml dH_2O

7.4.8 AGAROSE GEL

- Weigh in appropriate amount of agarose (e.g. 3g for 1% gel)
- Dissolve in 300ml 1x TAE buffer
- Melt in the microwave until a clear solution is reached
- Cool down on magnetic stirrer to ≈40°C
- Pour into gel electrophoresis tray with appropriate combs

7.4.9 1x GEL RED NUCLEIC ACID GEL STAIN SOLUTION

- 15µl GelRed (obtained from Biotium, Hayward, CA, USA)
- 5ml 1M NaCl
- 45ml dH_2O
- Replace after 3-4 days of using

7.4.10 1x TRIS-BUFFERED SALINE (TBS)

- 100ml 10x TBS stock solution
- 900ml dH_2O

7.4.11 1x TRIS-BUFFERED SALINE TWEEN-20 (TBST)

- 100ml 10x TBST stock solution
- 900ml dH_2O

7.4.12 1x NUPAGE SAMPLE BUFFER

- 6.5ml 1x TBS
- 2.5ml NuPAGE® LDS Sample Buffer (4x)
- 1ml NuPAGE® Reducing Agent (10x)

7.4.13 1x NUPAGE MES RUNNING BUFFER

- 50ml 20x NuPAGE® MES Running Buffer
- 950ml dH_2O

7.4.14 1x TRANSFER BUFFER

- 50ml 20x NuPAGE® Transfer buffer
- 100ml Methanol
- 850ml dH_2O

7.4.15 1x BLOCKING SOLUTION

- 3ml 10x Blocking Solution
- 27ml dH_2O

7.4.16 PONCEAU-S MEMBRANE STAIN SOLUTION

- 0.2g Ponceau-S
- 3.0g Trichloric Acetic Acid
- Dissolve in and fill up 100ml with dH_2O
- Can be reused multiple times

7.4.17 LUMINOL CHEMILUMINISCENCE DEVELOPING AGENT

- Mix 2ml of solution A with 2ml of Solution B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) directly before use

7.5 ENZYMES

Enzymes are purchased either from Fermentas (Thermo Fischer Scientific, Waltham, MA, USA) or New England Biolabs.

Manufacturer's instructions are followed and buffers supplied with enzymes are used when performing restriction digests, ligations, PCR, and other enzymatic reactions.

7.6 MICROBIOLOGICAL TECHNIQUES

7.6.1 MOPS-COMPETENT CELLS

- Inoculate 500ml LBv medium with an overnight culture and grow in a shaking water bath at +36°C until OD_{600} of ≈0.5
- Centrifuge 10min at +4°C at 1660g
- Decant supernatant
- Resuspend pellet in 100ml MOPS I and keep on ice for 10min
- Centrifuge again for 10min at +4°C at 1660g
- Decant supernatant
- Resuspend pellet in 100ml MOPS II and keep on ice for 30min
- Centrifuge for 10min at +4°C at 1660g
- Decant supernatant
- Resuspend pellet in 8ml MOPS II and 3ml 50% glycerol
- Keep 10min on ice
- Aliquot in 100µl portions and freeze at -70°C
- Check competent cells by microscopy and single cell strike on LB agar plates.

7.6.2 TRANSFORMATION OF MOPS-COMPETENT CELLS

- Add DNA (e.g. 2µl of miniprep DNA or up to 10µl of ligation) to a 100µl aliquot of competent cells
- Keep 30min on ice

- Heat shock at +36°C or +42°C for 2min (depending whether a thermosensitive plasmid is used)
- Keep 5min on ice
- Add 700µl of medium and regenerate for 1h at proper temperature
- Strike cells on LB agar plates with the corresponding antibiotics (100µl and the remaining rest)
- Incubate agar plates overnight at +36°C

7.6.3 ISOLATION OF PLASMID DNA (MINIPREP)

Between 1.5ml and 3ml of an overnight culture are harvested via centrifugation and the plasmid DNA is isolated from the pellet using the small volume PeqGOLD Miniprep Kit I (PeqLab, Erlangen, Germany), following the manufacturer's instructions. (PeqLab, 2011)

Up to 60µl of plasmid DNA solution are isolated and used directly or stored at -20°C.

7.6.4 ISOLATION OF PLASMID DNA (MIDIPREP)

100ml LBv (with selective antibiotic(s) added) are inoculated in the evening and grown overnight in a shaking water bath at +35°C. Cells are harvested via centrifugation and plasmid DNA is isolated from the pellet using Promega's PureYield™ Plasmid Midiprep System (Promega, Madison, WI, USA), following the manufacturer's instructions. (Promega, 2011)

DNA is eluted twice, first in 1000µl (Elution 1 – E1) and secondly in 300µl (E2); DNA concentration and purity is determined by absorption measurements at A_{260nm} and A_{280nm}, using the empirical equation:

$$\left[\text{DNA}\left(\frac{ng}{\mu l}\right)\right] = A_{260nm} \times \text{dilution factor} \times 50\left(\frac{ng}{\mu l}\right)$$

7.7 ENZYMATIC REACTIONS

7.7.1 RESTRICTION DIGESTS

For site-specific restriction digests, FastDigest enzymes by Fermentas are used. Digests are carried out at +37°C for at least 15min. After digestion is complete, fragment lengths are routinely checked on agarose gels. Preparative digests are either excised and eluted from the agarose gel, or directly purified using columns (see below).

7.7.1.1 SMALL SCALE (CONTROL) DIGEST

- 8µl of miniprep plasmid DNA or 3µl of midiprep plasmid DNA
- 9µl (miniprep) or 14µl (midiprep) dH_2O
- 2µl 10x FastDigest buffer
- 1µl FastDigest enzyme

7.7.1.2 LARGE SCALE (PREPARATIVE) DOUBLE DIGEST

- 34µl midiprep DNA
- 4µl 10x FastDigest buffer
- 1µl FastDigest enzyme 1
- 1µl FastDigest enzyme 2

Note: incubate at least 60min to account for higher amount of DNA

7.7.2 ISOLATION OF DNA FRAGMENTS

Doubly-digested DNA is run on agarose gels to check the quality of the digest and to purify correct-sized fragments. Bands corresponding to the desired DNA fragments are excised from the gel under low intensity UV light. The gel is then liquefied and the DNA eluted using the PureLink™ Quick Gel Extraction Kit from Invitrogen (Invitrogen, Paisley, UK), following the manufacturer's instructions. (Invitrogen, 2011)

PCR product is cleaned up using the PureLink™ PCR Purification Kit (Invitrogen, Paisley, UK), following the manual. (Invitrogen, 2011)

7.7.3 LIGATION

- For standard ligation, prepare the following mix:
    - 3µl plasmid DNA
    - 5µl insert DNA
    - 2µl 10mM ATP
    - 2µl 10x T4 DNA Ligase Buffer
    - 7µl dH_2O
    - 1µl T4 DNA ligase (NEB)
- Incubate at +4°C overnight or at room temperature for 180min

Note: Different vector:insert ratios may be necessary; a control ligation including only the insert (or the vector) may be included to check ligation ability of the insert (or the vector) itself.

7.8 PCR AMPLIFICATION

Target DNA sequences are amplified using PCR with sequence specific binding primers that have incorporated restriction sites used for cloning in their non-binding sites.

Generally, a small scale Taq test PCR is performed first to assess whether a correct-sized product is synthesized. This is followed by a small scale Pfu test PCR, which in turn (if correctly performed) is followed by a large scale Pfu production PCR.

7.8.1 TAQ TEST PCR

Materials are thawed on ice. The 2x PCR Master Mix (Fermentas) contains dNTPs, polymerase and the needed buffer. To ensure minimum contamination, pipetting is done under the laminar flow hood.

- Prepare master mix on ice:
    - 50µl 2x PCR Master Mix
    - 1µl Primer 1 (50pmol/µl)
    - 1µl Primer 2 (50pmol/µl)
    - 40µl dH_2O

- Pipette into three labelled PCR tubes:
    - 23µl master mix + 2µl template DNA – tube 1
    - 23µl master mix + 1µl template DNA + 1µl dH_2O – tube 2
    - 23µl master mix + 2µl dH_2O (negative control) – tube 3
- Run the following PCR program:
    - Step 1: Denaturation 95°C 3min
    - Step 2 (30x):
        - Denaturation 95°C 30sec
        - Annealing T_m°C 30sec
        - Elongation 72°C 1min/1000bp
    - Step 3: Final Elongation: 72°C 10min
- Check quality of PCR product on agarose gel.

7.8.2 SMALL SCALE PFU TEST PCR

Materials are thawed on ice. To ensure minimum contamination, pipetting is done under the laminar flow hood.

- Prepare master mix on ice:
    - 10µl 10X Pfu Buffer
    - 10µl dNTPs (2mM)
    - 1µl Primer 1 (50pmol/µl)
    - 1µl Primer 2 (50pmol/µl)
    - 1µl Pfu Polymerase (Fermentas)
    - 69µl dH_2O
- Pipette into three labelled PCR tubes:
    - 23µl master mix + 2µl template DNA – tube 1
    - 23µl master mix + 1µl template DNA + 1µl dH_2O – tube 2
    - 23µl master mix + 2µl dH_2O (negative control) – tube 3
- Run the following PCR program:
    - Step 1: Denaturation 95°C 3min
    - Step 2 (30x):
        - Denaturation 95°C 30sec

- Annealing T_m°C 30sec
- Elongation 72°C 1min/1000bp

- Step 3: Final Elongation: 72°C 10min

Check quality of PCR product on agarose gel.

7.8.3 LARGE SCALE PFU PRODUCTION PCR

Materials are thawed on ice. To ensure minimum contamination, pipetting is done under the laminar flow hood.

- Prepare master mix on ice:
  - 30µl 10X Pfu Buffer
  - 30µl dNTPs (2mM)
  - 3µl Primer 1 (50pmol/µl)
  - 3µl Primer 2 (50pmol/µl)
  - 3µl Pfu Polymerase (Fermentas)
  - 207µl dH_2O
- Pipette into three labelled PCR tubes (as control):
  - 23µl master mix + 2µl template DNA – tube 1
  - 23µl master mix + 1µl template DNA + 1µl dH_2O – tube 2
  - 23µl master mix + 2µl dH_2O (negative control) – tube 3
- Pipette into four labelled PCR tubes (for production)
  - 46µl master mix + 4µl template DNA – tube 4
  - 46µl master mix + 2µl template DNA + 2µl dH_2O (3x) – tubes 5 - 7
- Run the following PCR program:
  - Step 1: Denaturation 95°C 3min
  - Step 2 (30x):
    - Denaturation 95°C 30sec
    - Annealing T_m°C 30sec
    - Elongation 72°C 1min/1000bp
  - Step 3: Final Elongation: 72°C 10min

Check quality of PCR product on agarose gel. Purify PCR product of tubes 5 – 7 and use for downstream applications.

7.9 USED PRIMERS

All primers are ordered from and synthesized by Microsynth (Microsynth, Balgach, Switzerland). Sequence specific binding regions are coloured green, introduced restriction sites are coloured red. An apostrophe (‘) denotes the position where the restriction enzyme cleaves.

Note: In the case of the pASK reverse primer, the restriction site is also a binding site.

The melting temperature T_m has been calculated using the (crude) empiric equation:

$$T_m\ (°C) = (n \cdot G + n \cdot C) \times 4°C + (n \cdot A + n \cdot T) \times 2°C$$

Name	Enzyme	Direction	Sequence (5’ – 3’)	T_m
pBGKB-PorB	*Kpn*I	Fwd	ATATAGGTAC’CATGCCTGCGGGGAATCCG	60°C
pBGKB-PorB	*Eco*RI	Rev	ATATAG’AATTCCGAATTGGAATCCTCCGGAGA	60°C
pASK	*BspHI*	Fwd	TATATT’CATGAGTCATCACCATCACCATCACTC	60°C
pASK	*Sal*I	Rev	TATATG’TCGACGGAGCTCGAATTC	60°C
pET40b-PorB	*Hind*III	Fwd	TATATA’AGCTTATGCCTGCGGGGAATCCG	60°C
pET40b-PorB	*Xho*I	Rev	TATATC’TCGAGGAATTGGAATCCTCCGGAGA	60°C
pBGKB-MOMP	*Kpn*I	Fwd	TATATGGTAC’CCGAAGCGGAATTGTGCATTTAC	58°C
pBGKB-MOMP	*Bgl*II	Rev	TATATA’GATCTCCTGTGGGGAATCCTGCT	60°C

Table 14: Primers, their sequences, T_m and the restriction sites introduced by them.

7.10 ANALYTICAL TECHNIQUES

7.10.1 AGAROSE GEL ELECTROPHORESIS

Agarose gels are poured with concentrations of 1% for fragments ranging from 10^3 – 10^4bp or 2% for fragments smaller than 10^3bp. For control digests, small-welled gels that can accommodate 10µl samples are used. For preparative gels, large wells that hold up to 20µl samples are used.

For fragments ranging from 10^3 – 10^4bp, 5µl of GeneRuler™ 1kb DNA Ladder (Fermentas) are used as a size marker. For smaller fragments, 5µl of O'GeneRuler™ 50bp DNA Ladder (Fermentas) are used.

10µl samples are mixed with 2µl of 6x Loading Dye (Fermentas) and loaded onto the gel. Gels are run at 160V until the loading dye's lower front reaches the gel's end, incubated on a shaker in 1x GelRed solution for 15min, exposed to UV radiation and photographed using the ChemiDoc.

7.10.2 WESTERN BLOT ANALYSIS

For separation of proteins according to their electrophoretic mobility followed by blotting and antibody detection, the Novex NuPAGE® Bis-Tris Electrophoresis System using the XCell SureLock mini-cells (Invitrogen) has been used according to the manufacturer's instructions. (Invitrogen, 2011)

As a size marker, the Unstained Protein Molecular Weight Marker, ranging in size from 14.4 to 116kDa has been used (Fermentas).

7.10.2.1 SCALING THE SAMPLE AMOUNT

For Western Blot analysis, samples can be gathered from expression experiments (live cells), expression and/or lysis experiments (lysed bacteria) or samples can be lyophilized BGs. Additionally, for quantification purposes it is necessary to establish a standard curve using a dilution series. Sample preparation protocols are explained in the following sections.

7.10.2.1.1 USING A BACTERIAL CULTURE

From an ongoing experiment, 1ml of culture are withdrawn under sterile conditions and centrifuged for 3min at 17,000g. Supernatant is removed and the ensuing pellet can either be stored at -20°C or prepared for Western Blots directly (see below) according to OD_{600nm} values:

Before lysis induction : OD_{600nm} x 250 = volume in µl of 1x NuPAGE sample buffer

After lysis induction : highest OD_{600nm} x 250 = volume in µl of 1x NuPAGE sample buffer

7.10.2.1.2 USING LYOPHILIZED BGS

10mg of BGs are weighed in using an analytical balance and dissolved in 1000µl dH_2O using vigorous vortexing. 200µl of the BG suspension are mixed with 200µl of 2x NuPAGE sample buffer and incubated for 10' at +99°C (1:2 dilution).

After centrifugation at 17,000g for 3min, 100µl are mixed with 400µl 1x NuPage sample buffer (1:5 dilution) to give a concentration of 1µg/µl.

To establish standard dilutions, 2µl, 5µl, 10µl and 20µl of the 1µg/µl sample of the BGs are filled up with 1x NuPAGE sample buffer to 20µl and loaded onto a gel.

7.10.2.1.3 QUANTIFICATION USING A POSITOPE

In order to quantify proteins, a detectable standard with a known concentration of 25ng/µl, called Positope (Invitrogen) is used for a serial dilution.

60µl of the positope (already delivered in sample buffer) are incubated at +99°C for 5min. 20µl of the positope are directly applied to the gel, the remainder is diluted with 40µl 1x NuPAGE sample buffer (1:2 dilution). These serial dilutions are repeated twice, and 20µl of each are loaded onto the gel, giving the following standards:

- Std1: 20µl positope are directly applied to the gel 500ng / 20µl
- Std2: 40µl positope + 40µl NuPAGE sample buffer 250ng / 20µl
- Std3: 40µl Std2 + 40µl NuPAGE sample buffer 125ng / 20µl
- Std4: 40µl Std3 + 40µl NuPAGE sample buffer 62.5ng / 20µl

7.10.2.2 SAMPLE PREPARATIONS

- Dissolve pellet in the appropriate volume 1x NuPAGE sample buffer
- Vortex vigorously
- Incubate at +99°C for 10min
- Spin down at 17,000g for 3min

7.10.2.3 SDS-PAGE

- For SDS-PAGE, pre-cast 10 well NuPAGE® Novex 4-12% Bis-Tris Gels (Invitrogen) are used with 1x MES running buffer
- Load 20µl of prepared samples and 5µl of protein marker into the pre-cast wells
- Run SDS-PAGE at 180V according to the protocol (see above)

7.10.2.4 TRANSFER

- A semi-dry blotting sandwich with Whatman paper soaked in 1x transfer buffer is assembled in an XCell II Blot module according to the manufacturer's instructions (see above)
- Proteins separated through electrophoresis are transferred onto 0.2µm nitrocellulose for 60min at 30V (one gel per apparatus) or at 60V (two gels per apparatus)
- Successful transfer of proteins onto the nitrocellulose membrane is controlled via Ponceau-S staining, allowing the labelling of marker bands. Ponceau-S is afterwards rinsed off with dH_2O.

7.10.2.5 BLOCKING

- The membrane is incubated in 30ml 1x RotiBlock solution
- Incubation is at least 90min on the shaker at room temperature or over night at +4°C

7.10.2.6 ANTIBODY INCUBATION

- The membrane is washed with 1x TBST for 3x 5min, 1x 10min
- The membrane is incubated with the primary antibody diluted in 10ml 1x TBS
- The membrane is washed again with 1x TBST (1x TBS if Streptavidin-HRP is used) for 3x 5min, 1x 10min
- If necessary, the membrane is incubated with a secondary antibody diluted in 10ml 1x TBS, 0.3% BSA, 0.05% NaN_3
- Afterwards, the membrane is washed again with 1x TBST for 3x 5min, 1x 10min

7.10.2.7 WESTERN BLOT DEVELOPMENT

- Incubate membrane in 4ml Luminol Developing Agent for 3min at the shaker
- Photograph the membrane in white light using the Chemidoc XRS
- Shut off the Chemidoc's lamps, expose the membrane for a total of 200sec to the camera
- Combine the white-light photograph and the chemiluminiscence photograph using Quanitity One software, and quantify signal strength if applicable.

7.10.3 QUANTIFICATION USING THE S-TAG SYSTEM

- Harvest 1ml of bacterial culture by centrifugation; use samples from cultures not carrying an S-Tag encoding plasmid as blank
- Remove supernatant, resuspend pellet in 100µl of 1% SDS. Vortex thoroughly and heat at +70°C for 10min
- Centrifuge for 1min at max speed and transfer supernatant to a new tube. Dilute 10-100fold in dH_2O
- Assemble the following components in a set of sterile 1.5ml microcentrifuges:

	Tube 1	**Tube 2**	**Tube 3**
dH_2O	348µl	346µl	346µl
S-Tag Standard	-	2µl	2µl
Sample Lysate	-	-	2µl
Blank Lysate	2µl	2µl	-
10x S-Tag Assay Buffer	40µl	40µl	40µl
S-Tag Grade S-Protein	10µl	10µl	10µl

- Incubate the tubes at +37°C for exactly 5min
- Stop the reaction by adding 100µl ice-cold 25% TCA, vortex and place on ice for 5min
- Centrifuge the tubes at max speed for 10min
- Read the absorbance of the supernatants at A_{280nm}. Zero the spectrophotometer with sample #1
- To calculate the amount of S-tagged protein in the sample, use the following equation:

$$\frac{A_{280nm}\text{Tube \#3}}{2\mu\text{l}} \times \frac{0.1\text{pmol S-Tag-Standard}}{\text{A}_{280\text{nm}}\text{Tube \#2}} = \frac{\text{pmol}}{\mu\text{l}}$$

7.11 USED ANTIBODIES

Antibodies were used to detect antigens on Western Blots. The biotinylated E-Protein used for lysis could be detected via HRP-conjugated streptavidin; for detection of MOMP, a polyclonal antibody was used. Genes cloned into expression vectors were fused either to a PolyHis-Tag, a Myc-Tag or both, and could be detected via the corresponding antibodies, of which an overview is given below.

7.11.1 PRIMARY ANTIBODIES

Name	Isolated from	Specificity	Dilution	Source
Anti-MOMP-AB	Goat	*C. trachomatis* MOMP	1:500 in TBS	Santa Cruz Biotechnology

Table 15: Primary, non-HRP-coupled antibodies used during this work

7.11.2 HRP-COUPLED ANTIBODIES

Name	Isolated from	Specificity	Dilution	Source
Anti-Goat-HRP	Rabbit	Goat-IgG	1:5000 in TBS	Sigma-Aldrich
Streptavidin-HRP	n/a	Biotin	1:5000 in TBS	Invitrogen
S-Protein-HRP	n/a	S-Tag	1:5000 in TBS	Novagen
Anti-His-HRP	Mouse	C-terminal $(His)_6$	1:5000 in TBS	Invitrogen
Anti-Myc-HRP	Mouse	EQKLISEEDL	1:5000 in TBS	Invitrogen

Table 16: HRP-coupled primary and secondary antibodies used during this work

7.12 GROWTH, EXPRESSION AND LYSIS STUDIES (NOSEFLASKS)

7.12.1 GROWTH AND LYSIS (SMALL SCALE)

E. coli NM522 carrying a lysis plasmid is inoculated in 5ml LBv plus antibiotic and grown overnight at +36°C.

100ml nose flasks with 25ml LBv plus antibiotic and a magnetic stirrer are inoculated with ≈1ml over night culture (starting OD_{600nm} should be at 0.1) and grown in a water bath at +36°C at a stirring rate of 300rpm. OD_{600nm} measurements are taken using a noseflask spectrophotometer until an OD_{600nm} of 0.5 is reached. Lysis is then induced, for thermosensitive lysis plasmids by transferring nose flasks into a second water bath at +42°C. Lysis is observed for 120min.

7.12.1.1 MICROSCOPIC OBSERVATION

10µl of culture are withdrawn at regular intervals and observed using a microscope, first to assess viability and growth, and later to look for BGs. These appear translucent in the microscope (as opposed to live or dead cells), with darker spots accounting for membrane engulfments due to E-lysis.

7.12.1.2 DETERMINATION OF LYSIS EFFICIENCY VIA CFU

In order to assess the efficiency of the lysis process, colony forming unit (cfu) counts are performed at regular intervals.

100µl of culture are withdrawn at regular intervals (at least twice before lysis induction, to assess the growth rate of the bacterial culture before lysis takes place) and diluted in 9.9ml 0.85% saline (10^2 dilution). Samples are further diluted to 10^3 – 10^6 in respect to the OD_{600nm} and the status of the culture (see Table 17).

Before Lysis Induction		**After Lysis Induction**	
OD_{600}	**Dilution**	**OD_{600}**	**Dilution**
<0.20	10^2	1.00 – 2.00	10^5
0.20 – 0.50	10^4	0.50 – 1.00	10^4
0.50 – 1.00	10^5	0.20 – 0.50	10^3
1.00 – 2.00	10^6	<0.20	10^2

Table 17: Dilutions used for plating of cultures before and after lysis induction

From the respective dilutions, 50µl are plated in duplicates in logarithmic manner on count agar plates using a spiral plater. After overnight incubation at +36°C, plates are counted using the Colony Counter machine and cfu counts per ml calculated.

7.12.2 GROWTH AND EXPRESSION (SMALL SCALE)

E. coli NM522 carrying an expression plasmid is inoculated in 5ml LBv plus antibiotic and grown over night at +36°C.

100ml nose flasks with 25ml LBv plus antibiotic and a magnetic stirrer are inoculated with ≈1ml over night culture (starting OD_{600nm} should be at 0.1) and grown in a water bath at +36°C at a stirring rate of 300rpm. OD_{600nm} measurements are taken using a noseflask spectrophotometer until an OD_{600nm} of ≈0.35 is reached, when expression is induced by adding a chemical inducer at an appropriate concentration (e.g. L-arabinose with a final concentration of 0.2% for plasmids using the pBAD promoter system).

Like in a lysis experiment, cfu counts and microscopy analyses are performed to assess the influence of foreign protein expression on the viability, shape and size of bacteria. Additionally, aliquots of 1ml are withdrawn for later Western Blot analysis. These samples are also taken at least once before expression induction to distinguish between non-induced baseline expression and induced expression.

Samples are centrifuged for 3min at 17,000g; after the withdrawal of supernatant, pellets can be stored at -20°C.

7.12.3 GROWTH, EXPRESSION AND LYSIS (SMALL SCALE)

E. coli NM522 carrying expression and lysis plasmids is inoculated in 5ml LBv plus antibiotics and grown over night at +36°C.

100ml nose flasks with 25ml LBv plus antibiotics and a magnetic stirrer are inoculated with ≈1ml overnight culture (starting OD_{600nm} should be at 0.1) and grown in a water bath at +36°C at a stirring rate of 300rpm. OD_{600nm} measurements are taken using a noseflask spectrophotometer until an OD_{600nm} of ≈0.35 is reached, when expression is induced by adding a chemical inducer at an appropriate concentration (e.g. L-arabinose with a final concentration of 0.2% for plasmids using the pBAD promoter system).

Depending on the toxicity of the expressed gene, interference of the expressed gene with lysis and the desired accumulation of the expressed gene, lysis can be co-induced with

expression induction, or induced at a later time point, by shifting to +42°C (for a thermosensitive lysis plasmid).

Cfu counts and microscopy analysis are performed to assess the influence of foreign protein expression on the viability, shape and size of bacteria and later, E-lysis. Additionally, aliquots of 1ml are withdrawn for later Western Blot analysis. These samples are also taken at least once before expression induction to distinguish between non-induced baseline expression and induced expression.

Samples are centrifuged for 3min at 17,000g. After the withdrawal of supernatant, pellets can be stored at -20°C.

The typical setup of an Expression/Lysis experiment can be seen in Table 18 below. The intervals between inoculation, expression induction and lysis induction at time point 0min are subject to variations and dependent on bacterial growth rate; typical approximate values are given for illustration only. Expression is induced at time point “B”, lysis at time point “C”.

Time	App. OD_{600nm}	Sample	OD_{600}	Microscopy	CFU	Western Blot
≈ -120min	0.10		√			
≈ -60min			√			
≈ -40min	0.25	**A**	√		√	
≈ -20min	0.35	**B**	√	√	√	√
0min	0.50	**C**	√	√	√	√
20min		**D**	√		√	√
40min		**E**	√		√	√
60min		**F**	√	√	√	√
90min		**G**	√		√	√
120min		**H**	√	√	√	√

Table 18: Typical setup of an Expression/Lysis experiment; expression time (time difference between points B and C) is subject to deliberations considering toxicity of the expressed protein and expected yield

7.13 GROWTH, EXPRESSION AND LYSIS (FERMENTATION)

For the large scale production of BGs, fermentation is performed in a 30l fermenter, using 22l LBv as a medium.

7.13.1 PREPARATIONS, STERILITY AND MONITORING

LBv medium is autoclaved (30min at +121°C) at least 18h prior to use, from which time on the conditions inside the fermenter are monitored using the IRIS software, which gives online information about pH, temperature, oxygen saturation and flow.

Blank samples of medium taken from the fermenter prior to inoculation are plated on plate count agar to verify sterility. A second blank sample is taken after addition of antibiotics. After setting the pO_2 value to 100% of the fully saturated medium, standard conditions are set and regulated by the IRIS software and documented in the fermentation report.

7.13.2 OVERNIGHT CULTURE

For the overnight culture, four 2l flasks containing 500ml medium plus antibiotics are inoculated from glycerol stocks, with 800µl stock per flask (necessitating two glycerol stocks with 1.8ml of cryoculture each for one fermentation).

The flasks are incubated overnight at +34°C in a shaking water bath.

7.13.3 FERMENTATION PROCESS

After inoculating the fermenter with overnight culture to an OD_{600nm} ≈0.4, growth is monitored and 50ml samples are taken every 30min via the sterilized sample valve. Like in small scale experiments described before, samples are used for OD_{600nm} measurement, microscopy, cfu analysis (in independent duplicates) and for preparation of Western Blots samples, but also for Flow Cytometry analysis (see below). Expression is induced by injecting the chemical inducer (e.g. L-arabinose) at an OD_{600nm} ≈0.75 via a septum. E-lysis is induced 15-30min later by temperature increase to +42°C. Lysis is conducted after pO_2 levels reach the plateau and flow cytometric analyses show complete lysis (≈120min).

7.13.4 ONLINE MONITORING VIA FLOW CYTOMETRY

To monitor the status of the bacterial culture and distinguish between live cells, dead intact cells and dead empty cells (BGs), flow cytometry is used.

Samples from the fermentation broth are diluted in 0.85% saline 10^3 to 10^4fold in order to keep count rates at roughly 1000sec^{-1}. To 1ml diluted sample, 1µl each of two fluorescent dyes ($DiBAC_4(3)$ and RH414, both obtained from Anaspec, Fremont, CA, USA) are added.

After vortexing, the sample is run through a CyFlow analyzer, with flow rates adjusted to keep the count rate at ≈1000sec^{-1}.

By excluding non-cellular background not stained by RH414 and combining the Forward Scatter Signal (FSC) and the Fluorescence Signal (FL1) of $DiBAC_4(3)$, three different areas can be defined:

- Region 1 (R1), live intact cells: low FL1 signal (intact membrane potential), high FSC (opaque cells)
- Region 2 (R2), dead intact cells: high FL1 signal (no membrane potential), high FSC (intact cells maintaining their opacity)
- Region 3 (R3), dead lysed cells: high FL1 signal (no membrane potential), low FSC (BGs are more translucent than intact cells)

This allows an “online” (approximately 10min after sample taking) observation of the bacterial culture, and more importantly, for the differentiation between lysis and killing because of e.g. foreign gene expression, which is not possible via cfu determination.

7.13.5 HARVESTING

After completed lysis, BGs are harvested via Tangential Flow Filtration (TFF) in a 0.2µm hollow fibre module at a temperature of 15°C, reducing the volume from 22l to 2l.

7.13.6 KILLING

To kill remaining, non-lysed cells, β-Propiolactone (BPL – obtained from Ferak, Berlin, Germany) is added to the 2l concentrate in two 0.0375% doses 30min apart. Inactivation is done while stirring at 42°C.

7.13.7 WASHING AND LYOPHILIZATION

To remove medium and residual cytoplasmic content from the product, the broth is washed with a total of 5l of sterile dH_2O by diafiltration, using a smaller 0.2µm hollow fibre module. During the non-steady state diafiltration, the volume is further reduced to 400ml, a 55fold reduction from the starting volume.

The 400ml of BG suspension are aliquoted into weighted lyophilisation bottles, stored at -20°C over night, followed by -80°C over night storage and are lyophilized in a Lyolab B machine for three days. Lyophilized BG bottles are weighted and the dry weight yield is calculated and labeled. BG bottles are stored at RT.

7.13.8 STERILITY TESTING

To test the sterility of the final product, about 10mg of lyophilisate are resuspended in 1.5ml LBv medium in triplicates.

- 1ml of the suspension are used for pour plating with 20ml of hand-warm agar.
- 100µl of the suspension are plated on a count agar plate
- 200µl of the suspension are plated on a count agar plate
- 100µl of the suspension are used to inoculate 5ml LBv and incubated overnight.
    - 100µl of this enrichment are plated on a count agar plate
    - 200µl of this enrichment are plated on a count agar plate
- All plates are incubated at +36°C for at least 24h.

7.14 CALCULATING LYSIS EFFICIENCY AND YIELD

Lysis efficiency is calculated by comparing the highest cfu value with the lowest cfu value:

$$\text{Efficiency } [\%] = \left(1 - \frac{\text{cfu}_{\text{lowest}}[ml^{-1}]}{\text{cfu}_{\text{highest}}[ml^{-1}]}\right) \times 100\%$$

Particle yield per mg is calculated by multiplying the cfu per ml prior to lysis induction with the total volume of the fermentation process, divided by the total yield after harvesting:

$$\frac{\text{Particles}}{mg}[mg^{-1}] = \frac{\text{cfu}_{\text{highest}}[ml^{-1}] \times \text{volume}_{\text{total}}[ml]}{\text{yield}_{\text{total}}[mg]}$$

To calculate the average amount of quantified antigen per BG, first the number of proteins per µg BG is calculated. To this end, the protein's mass in kDa has to be converted to gram:

$$1Da = 1.660538921 \cdot 10^{-24} g$$

Using this calculated mass per µg, the number of proteins per µg BG is calculated:

$$\frac{\text{Proteins}}{\mu g}[\mu g^{-1}] = \frac{\text{Protein Yield } \left[\frac{g}{\mu g}\right]}{\text{Protein mass } [g]}$$

Using the above calculated particle yield per mg, the amount of protein per particle (BG) can be derived:

$$\frac{\text{Proteins}}{\text{Particle}} = \frac{\frac{\text{Proteins}}{\mu g}[\mu g^{-1}]}{\frac{\text{Particles}}{\mu g}[\mu g^{-1}]}$$

8 APPENDIX

8.1 IMMUNOCONTRACEPTION IN POSSUMS

8.1.1 INTRODUCTION

The brushtail possum (*Trichosorus vulpecula*) is a recent addition to the biosphere of New Zealand; it was introduced by humans to start a fur industry, but the lack of natural predators lead to a rapid and (for other species) disastrous proliferation (Montague, 2000). Possums harm trees and other plants, destroy bird's habitats and are a vector of diseases affecting cattle and deer (National Science Strategy Committee for the Control of Possums and Bovine Tuberculosis., 2000).

As a cost-effective, long-term and humane method to reduce possum numbers and their impact on the biosphere of New Zealand, the development of an immunocontraceptive to decrease possum fertility was suggested (Magiafoglou, et al., 2001).

Several target molecules acting during early embryonic stages of the possum's development were identified to be used for a BG derived immunocontraceptive (Walcher, et al., 2008).

Among them is the Coat Protein 4 (CP4), a protein found in the shell coat of the possum's egg, unique among marsupials and therefore an ideal candidate for immunocontraception (Selwood, 2000). CP4 is secreted by the oviduct and uterus, and plays an important role in late cleavage of blastocyte development, as well as in epithelial maintenance (Frankenberg, et al., 1998).

8.1.2 GOALS

Among several other immunocontraceptive target proteins, the CP4 of the possum's egg was to be cloned into an expression vector system for the periplasmatic presentation, followed by E-mediated lysis to produce CP4-loaded BGs.

While the *Zona pellucida* protein 2 C-terminal region (ZP2C) was successfully cloned and used for production of BGs, in previous studies in this laboratory CP4 was not successfully

cloned into the expression vector system pBGKB, due to an overwhelming amount of mutations (Schlacher, 2009).

Since mutations were possibly already present in the original plasmids used to amplify the CP4 gene, it was decided to synthesize CP4 – in its *E. coli*-codon optimized form – *de novo*. The coding sequence as described at Genbank was used (Genbank accession number: EF121769.1), and synthesis was done by Microsynth (Microsynth, Balgach, Switzerland).

The CP4opt gene was cloned into a standard vector by Microsynth. The CP4opt gene was transferred from this plasmid (pSlo1.0A-CP4opt) into pBGKB without the need for PCR amplification as shown in Figure 66.

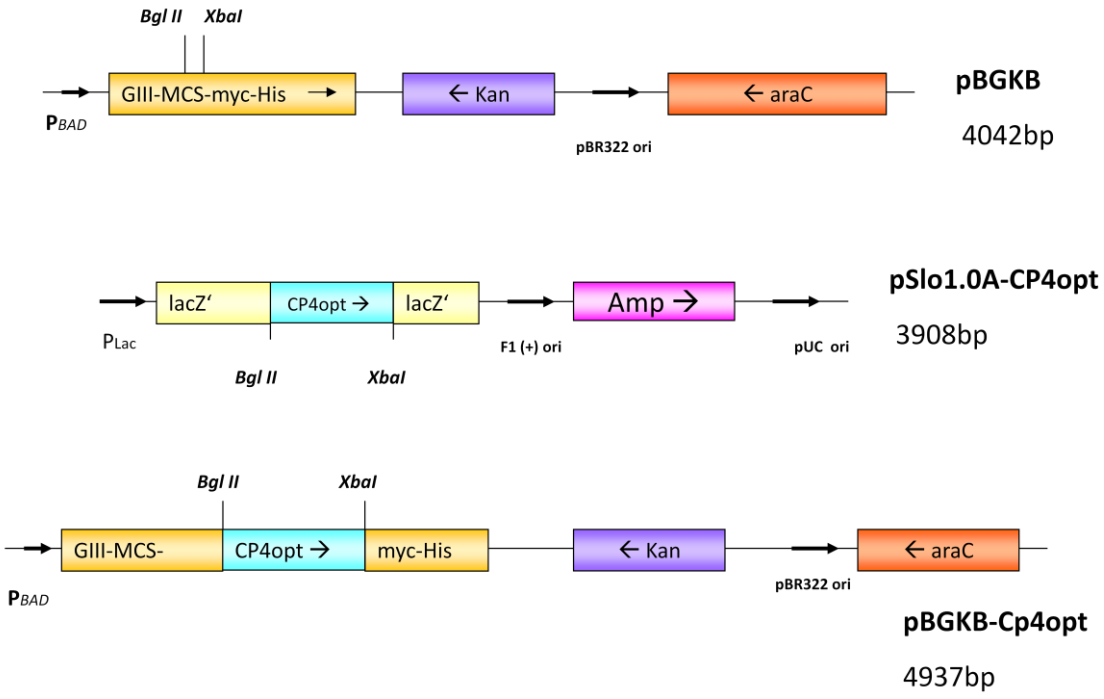


Figure 66: Cloning strategy to incorporate CP4opt into pBGBK, giving rise to pBGKB-CP4opt

The resultant plasmid pBGKB-CP4opt has a size of 4937bp; upon induction with L-arabinose, a 39.8kDa protein in the form of GIII-CP4-Myc-PolyHis is expressed and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 37.0kDa.

Additionally, as a positive control to check both induction with L-Arabinose as well as the detection with Anti-Myc-Antibodies, pBAD-GIII-Calmodulin was used in an early experiment. This plasmid is available from Invitrogen (Invitrogen, 2008) and its features are detailed in Figure 67.

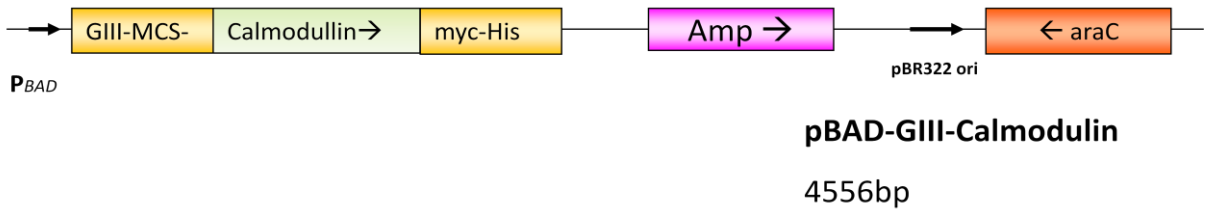


Figure 67: Schematic representation of plasmid pBAD-GIII-Calmodulin. Indicated are the ampicillin resistance cassette (Amp), the pBR322 origin of replication (pBR322 ori), the P_{BAD} promoter (P_{BAD}), the regulatory AraC protein (araC) and the GIII-Calmodulin-Myc-PolyHis fusion gene.

The pBAD-GIII-Calmodulin has a size of 4556bp; upon induction with L-arabinose, a 21.3kDa protein in the form of GIII-Calmodulin-Myc-PolyHis is expressed and exported to the periplasm. When the GIII export sequence is cleaved off, the resultant protein has a size of 19.2kDa.

8.1.3 RESULTS

8.1.3.1 CLONING pBGKB-CP4OPT

Midipreps of *E. coli* C2988J (pBGKB) and of *E. coli* C2988J (pSlo1.0A-CP4opt) were used to perform this cloning; no PCR amplification was necessary. Instead, both vectors were used for a large scale digest with *Bgl*II and *Xba*I that 'linearized' pBGKB and excised CP4opt from pSlo1.0A-CP4opt. After three hours of digestion, the fragments were put on an agarose gel; correct sized bands were excised and eluted from the gel using the Gel Extraction Kit. An aliquot of purified DNA was put on an agarose gel (see Figure 68); the expected sizes of the fragments were 4005bp for the pBGKB fragment and 948bp for the CP4opt.

Ligation was performed overnight; the ligation mixture was transformed into MOPS-competent *E. coli* C2988J, which were then plated on LB+Kan agar plates. After overnight incubation at 36°C, plates showed good efficiency of transformation. Four clones were picked, inoculated overnight in LBv+Kan and miniprepped on the next day.

Miniprep DNA of different clones was first digested with a single enzyme (not shown), then a correct-sized clone was further digested with a total of four different restriction enzymes to check for the characteristic, correct pattern of pBGKB-CP4opt, as seen in Figure 69.

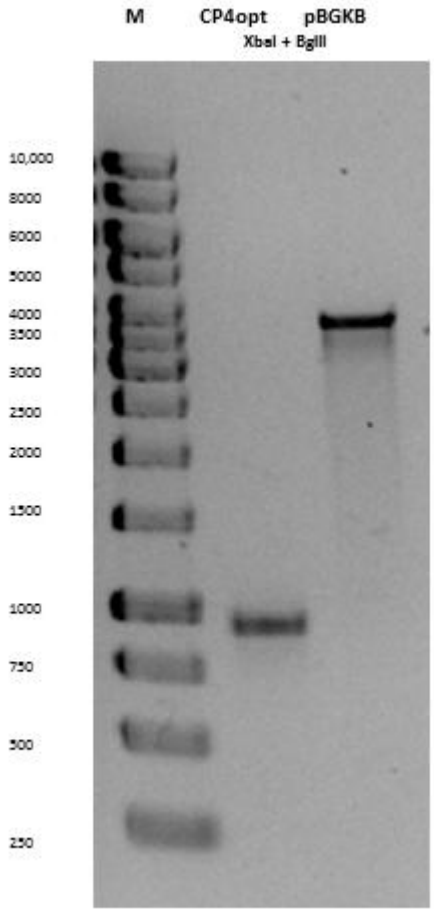


Figure 68: Vectors pBGKB and purified CP4opt after double digestion with *Bgl*II and *Xba*I, and purification, on a 1% agarose gel. Correct-sized bands can be seen at 948bp (CP4opt) and 4005bp (linearized pBGKB). Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

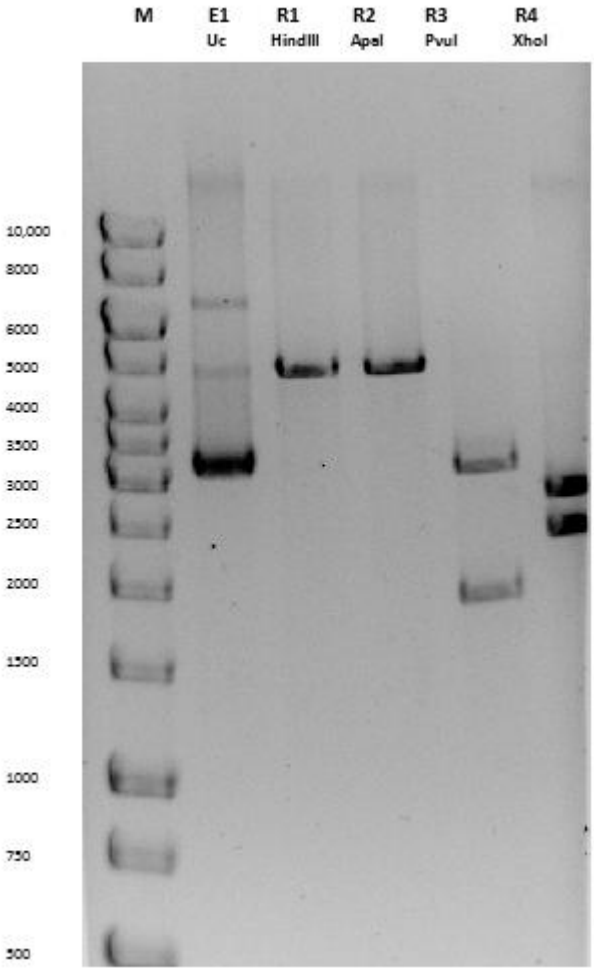


Figure 69: Digest of pBGKB-CP4opt clone A1 on a 1% agarose gel. Lane R1: pBGKB-CP4opt digested with *Hind*III, expected size: 4937bp; Lane R2: pBGKB-CP4opt digested with *Apa*I, expected size: 4937bp; Lane R3: pBGKB-CP4opt digested with *Pvu*I, expected sizes: 1865/3072bp; Lane R4: pBGKB-CP4opt digested with *Xho*I, expected sizes: 2279/2658bp. Marker: GeneRuler™ 1kb DNA Ladder (Fermentas)

Clone A1 of *E. coli* C2988J (pBGKB-CP4opt) was identified as correct by restriction digest pattern analysis and stored as a glycerol culture; no sequencing analysis was performed. The clone was used for a small scale Expression Experiment in *E. coli* C2988J and its midiprep DNA was used for transformation in *E. coli* NM522.

8.1.3.2 SMALL-SCALE EXPRESSION EXPERIMENT

In this small scale Expression Experiment, eight noseflasks were inoculated with overnight culture of six different *E. coli* C2988J (pBGKB-CP4opt) clones, one noseflask with *E. coli* C2988J (pSlo1.0A-CP4opt), and one noseflask with *E. coli* C2988J (pBAD-GIII-Calmodullin).

All noseflasks were grown at 36°C until an OD_{600nm}≈0.3, when expression was induced by adding 0.2% L-Arabinose to pBGKB vectors and 5mM IPTG to pSlo1.0A-CP4opt (time point 0min). OD_{600nm} were measured and WB samples were collected throughout the experiment, but no cfu counts were analysed; the OD_{600nm} over time are given in Figure 70.

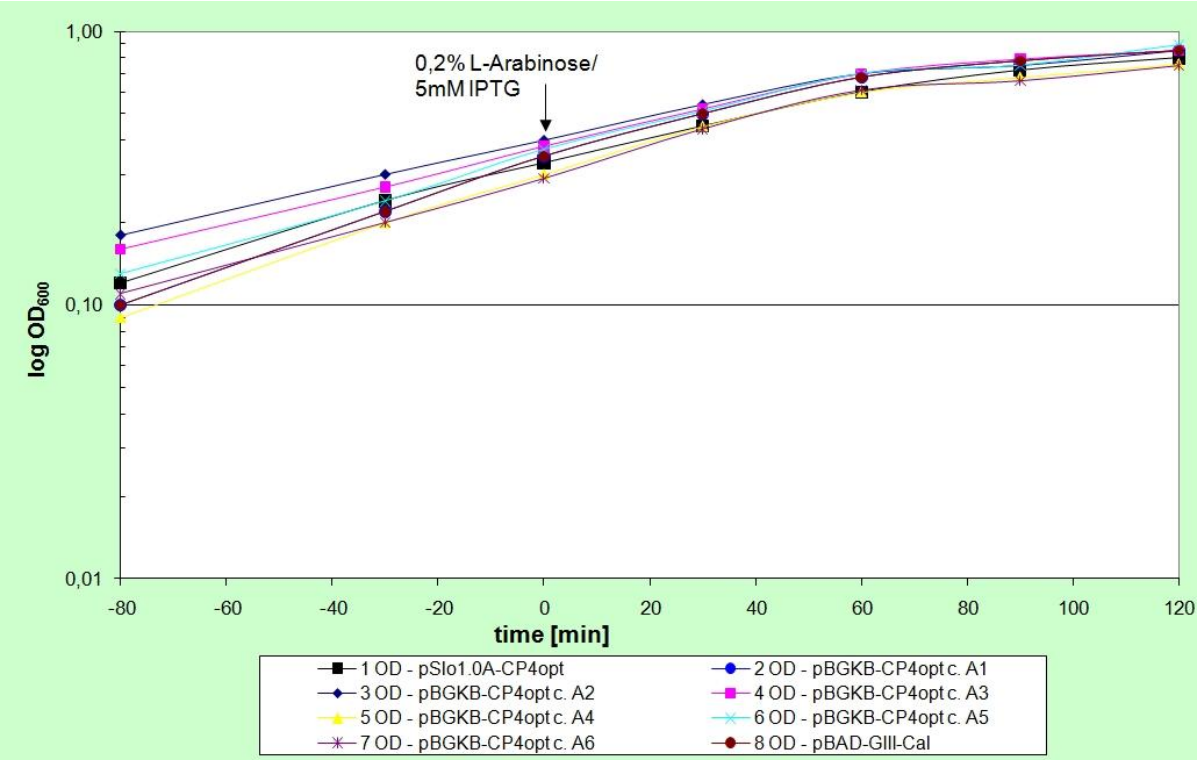


Figure 70: OD values over time during an Expression Experiment of *E. coli* C2988J (pSlo1.0A-CP4opt) (flask 1), six clones of *E. coli* C2988J (pBGKB-CP4opt) (flasks 2 – 7) and *E. coli* C2988J (pBAD-GIII-Calmodullin) (flask 8). L-Arabinose was added at time point 0min to flasks 2-8, and IPTG to flask 1.

The induction of protein expression had no noticeable influence on OD_{600nm}.

Western Blot samples after 90min of protein expression were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the nitrocellulose incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence and can be seen in Figure 71. Protein GIII-CP4opt-Myc-PolyHis was expected at a size of 39.8kDa, CP4opt-Myc-PolyHis at a size of 37.0kDa, GIII-Calmodulin-Myc-PolyHis at 21.3kDa and Calmodulin-Myc-PolyHis at a size of 19.2kDa.

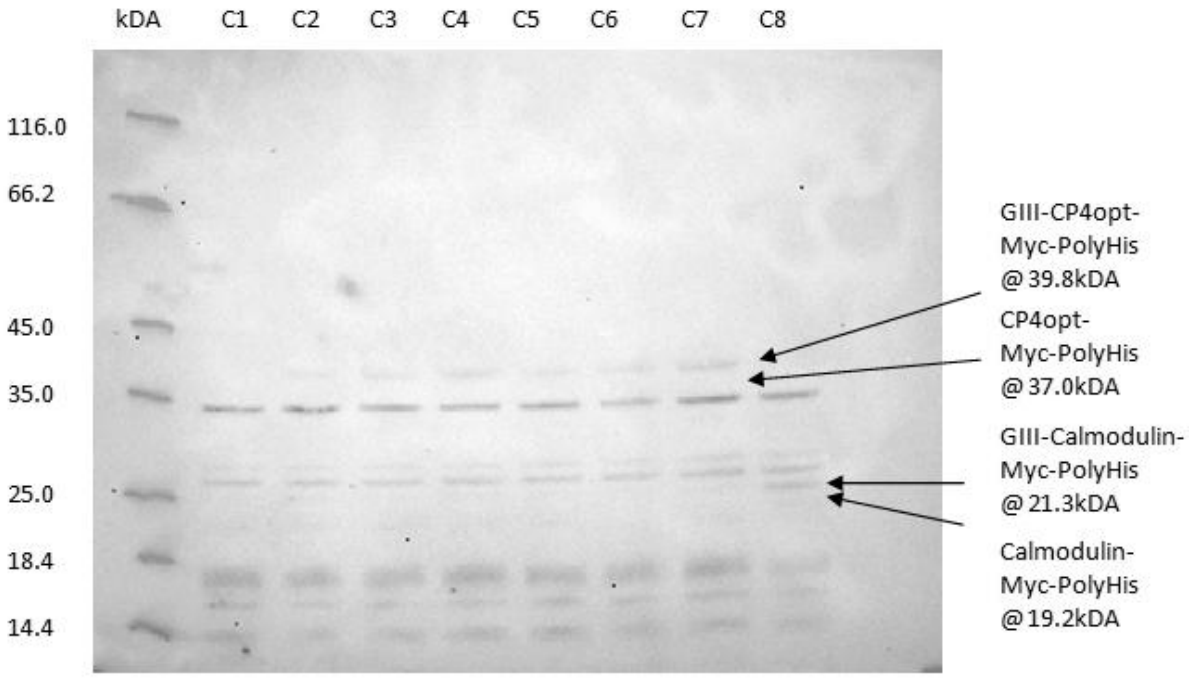


Figure 71: Western Blot of *E. coli* C2988J (pBGKB-CP4opt) Expression Experiment, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. Samples were taken 90min after expression induction.

Very faintly, and at the correct position, the putative CP4 signal can be seen in samples C2-7 after induction. Since CP4 is not Myc-tagged in sample C1, no detection was expected for pSlo1.0A-CP4opt samples. The positive control sample of pBAD-GIII-Calmodulin also shows a very faint sample at the expected size (GIII-Calmodulin-Myc-PolyHis at 21.3kDa and Calmodulin-Myc-PolyHis at a size of 19.2kDa). As also the positive control's signals are very weak, better pictures can be expected from repeated blots and/or new batches of antibodies.

8.1.3.3 SMALL-SCALE EXPRESSION/LYSIS EXPERIMENT

A total of eight different clones of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) were tested. While lysis efficiency was >99.8% in all cases, the amount of CP4 detectable by Western Blot Analysis was very low and often hardly distinguishable from non-specific background signal. The small-scale Expression/Lysis Experiment of one clone (A8) that was later used for fermentation is shown below in Figure 72:

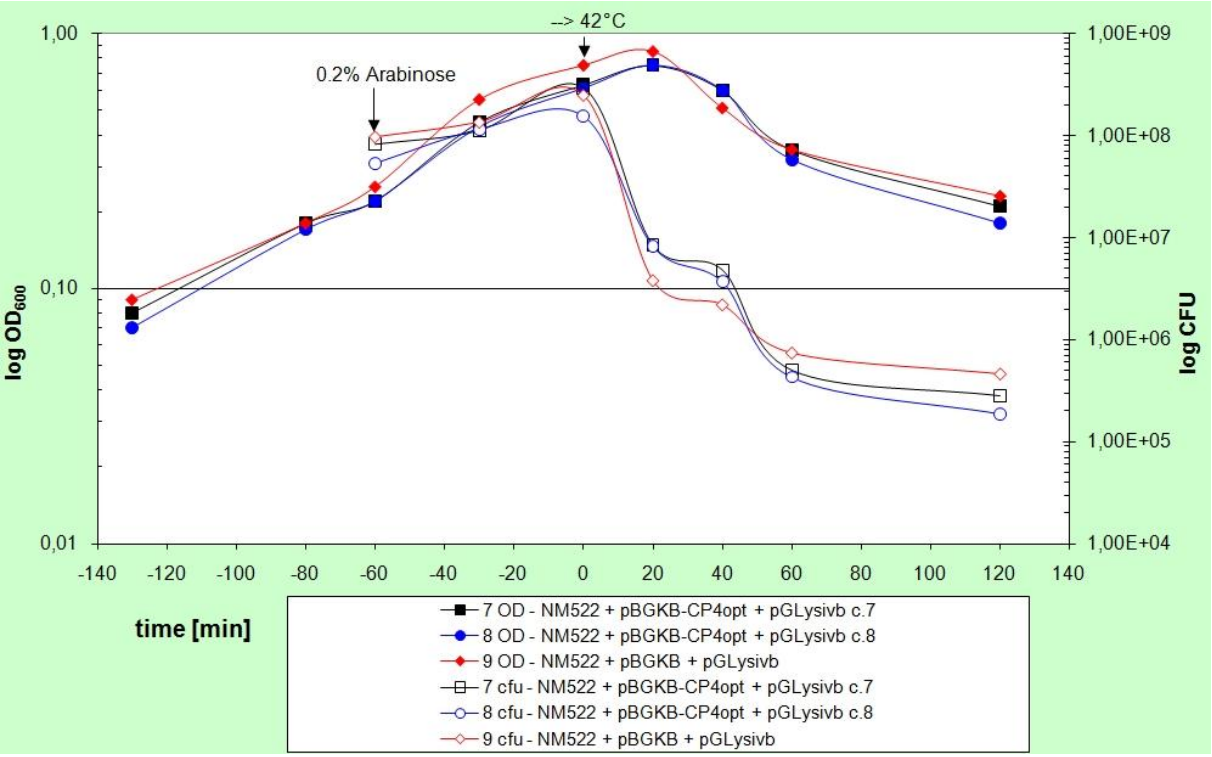


Figure 72: OD and cfu values over time during an Expression/Lysis Experiment of two different clones of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) and one clone of *E. coli* NM522 (pBGKB, pGLysivb). L-Arabinose was added at time point -60min; lysis was induced by shifting all four flasks to 42°C at time point 0min.

Lysis efficiency is at 99.88%, regardless whether pBGKB-CP4opt or the empty backbone plasmid pBGKB is induced by 0.2% L-Arabinose addition.

Western Blot samples after 60' of protein expression and after 60min of lysis were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the nitrocellulose incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemiluminiscence, as seen in Figure 73. Protein GIII-CP4opt-Myc-PolyHis was expected at a size of 39.8kDa, and protein CP4opt-Myc-PolyHis at a size of 37.0kDa.

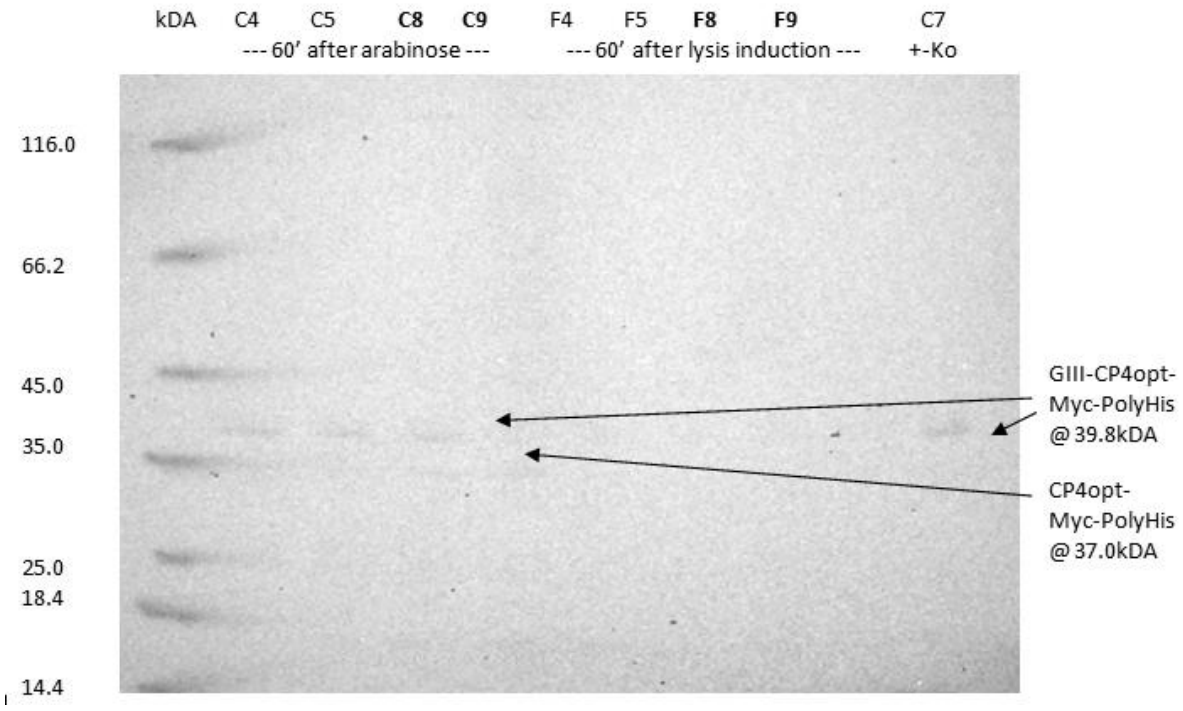


Figure 73: Western Blot of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) Expression/Lysis experiment, using a sample from *E. coli* C2988J (pBGKB-CP4opt) Expression Experiment as a positive control (C7), and using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. The nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. Samples were taken 60min after expression induction, and again 60min after lysis induction.

While CP4 can faintly be detected before lysis (C8), the signal drops further after lysis induction (F8) suggesting that the product is partially lost during E-lysis. Nevertheless, a fermentation was performed to see growth and expression behaviour in large scale.

8.1.3.4 *E. COLI* NM522 (pBGKB-CP4OPT, pGLYSIVB) FERMENTATION

After testing several clones of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) for differences in lysis efficiency and CP4 expression, working stocks of clone A8 of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) were prepared. These were used to inoculate an overnight culture for fermentation.

Fermentation was performed in a volume of 22l LBv pH 7.2, with antibiotics kanamycin and gentamicin added. In 30min intervals, samples were withdrawn to measure OD_{600nm}, check bacterial viability via the microscope, prepare Western Blot samples and plate dilutions for cfu determination on count agar plates. No flow cytometry was performed for this fermentation because of malfunctioning equipment.

Protein expression was induced after 30min of growth at 35°C by adding 0.2% L-Arabinose; after 90min of expression, lysis was induced by temperature upshift of the culture to 42°C; OD_{600nm} values and cfu counts over time are shown in Figure 74.

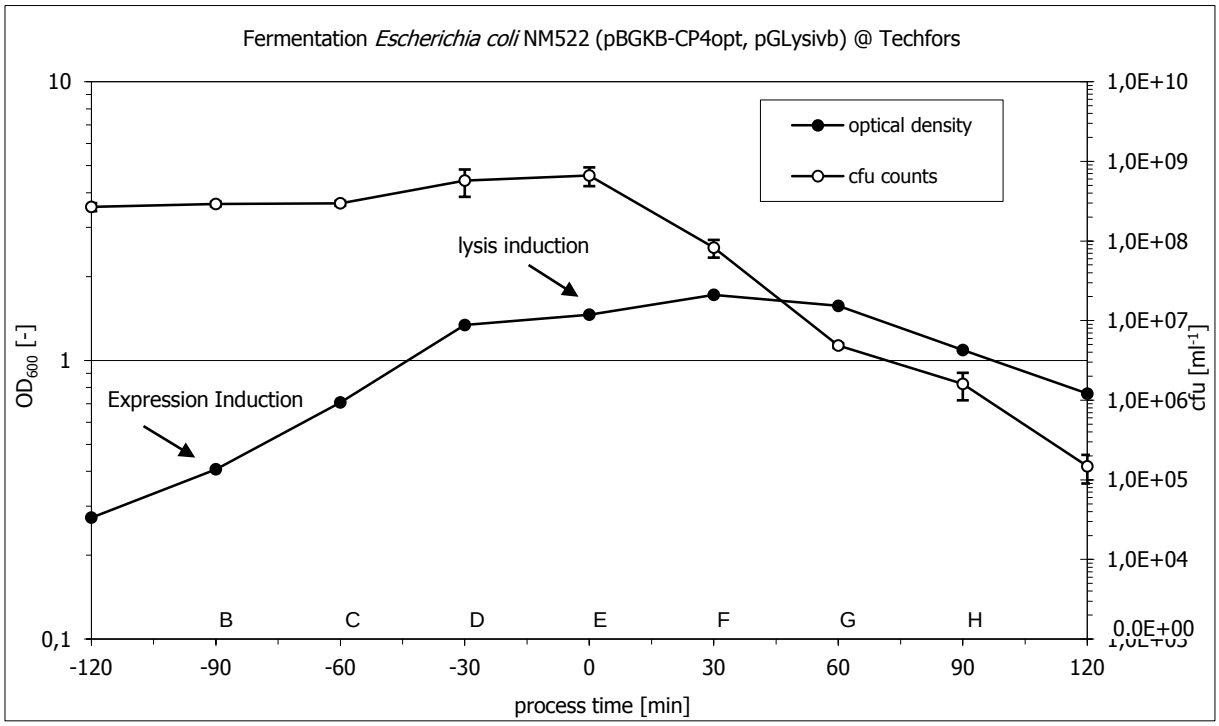


Figure 74: OD and cfu values over time during fermentation in 22l of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb). L-Arabinose was added at time point B (-90min); lysis was induced by increasing the temperature to 42°C at time point E (0min).

Both OD_{600nm} and cfu increase from the time point of expression induction until lysis induction, but the cfu increase is only a little more than twofold in the 120min until lysis induction. After that, both values drop sharply (from $6.65*10^8$cfu/ml at time point E to $1.48*10^5$cfu/ml at time point I – a lysis efficiency of 99.978%).

Important fermentation parameters such as flow, stirring rate, temperature or pO_2 are documented using the IRIS software and detailed below in Figure 75:

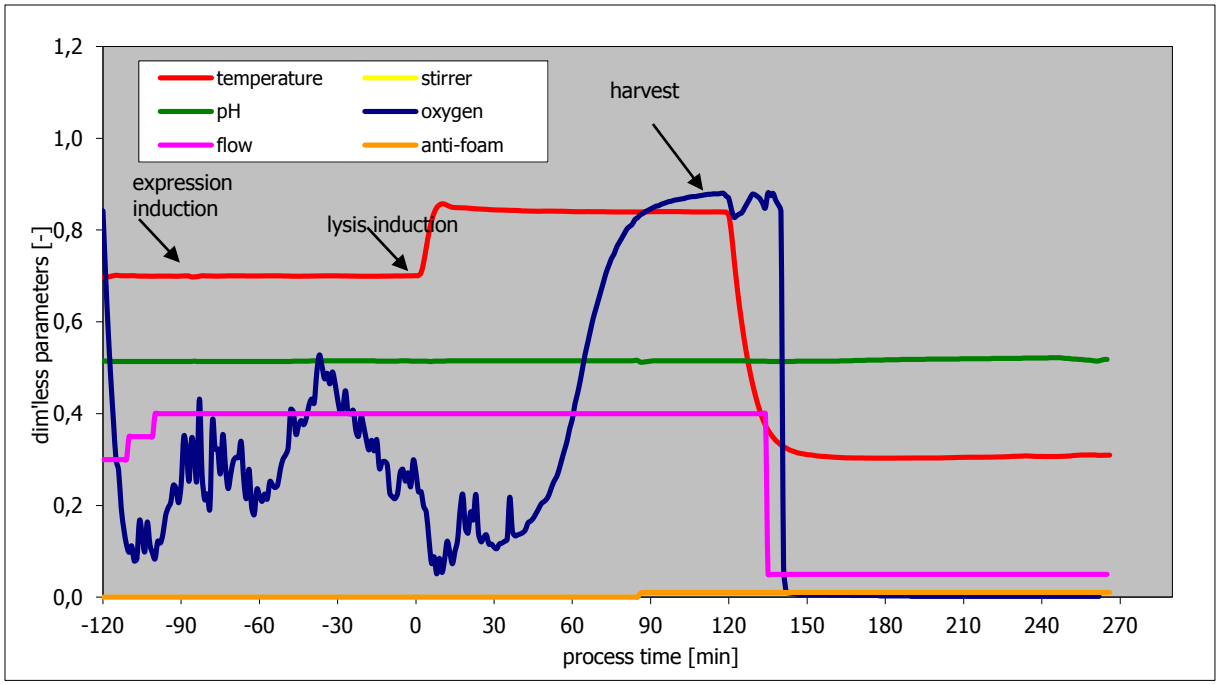


Figure 75: IRIS diagram documenting fermentation parameters like pH (regulated at 7.2), temperature (35°C during growth and expression, 42°C during lysis), flow rate, stirring rate, oxygen concentration and addition of anti-foam (used to counteract the foaming initiated by expulsion of cytoplasmic content during lysis).

In the IRIS diagram, the oxygen curve is of particular interest, as the oxygen curve does not show the characteristic sharp drop upon lysis induction, but only a smaller drop followed by a stepwise increase and finally a typical shift up to the plateau. This final increase happens at about 60min, while normally it is seen already after 30min.

Several other specifications as well as follow-up experiments of this particular fermentation are detailed below in Table 19:

***E. coli* NM522 (pBGKB-Cp4opt, pGLyisvb) Bacterial Ghosts**	
Pre-Culture	
Volume: 2l	**Additives**: Gentamicin + Kanamycin
Medium type: LBv	**Other**: GJS80, *7 drops Antifoam (1:3)*
Date: 2009-07-01	**Clone**: c.8 (2009-05-20/WS 2009-06-24 FHO)
Starting time: 09:35	**Strain**: *E. coli* NM522
End time:	**Plasmids**: pGLysivb, pBGKB-CP4opt
ON culture OD: 1.407/0.315	**Recombinant Protein Expression**: GIII-CP4opt-Myc-PolyHis
Inoc. Volume: 2l	**Expression Induction:** L-Arabinose, 0.2%
Medium: LBv	**Expression Induction Time Point**: B
Antibiotics: Gentamicin + Kanamycin	**Lysis Induction: 42°C**
Temperature: 35°C	**Lysis Induction Time Point**: E
Total Volume: ≈22l	**Killing**: after harvest, 0.075% BPL (2*0.0375%)
Acid: F.A.: 52 units	**Volume harvested**: 20l
Base: A.W.: 75 units	**Harvested by**: TFF

antifoam A: 10 units	**OD separator flow**: -
Eivb blot: -	**Yield**: 7480mg
Recombinant blot: OK (by FHO)	**Particles/mg**: 1.78 x 10^9
BPL killing: Survivors (by AME)	**Sterility**: OK (by EDZ)
Microscopy: ok / some dead / some elongated	**Efficiency**: 99.978%

Table 19: Fermentation data sheet of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) fermentation

While colonies after ß-PL killing could be detected, PCR analysis of these colonies failed to detect the gentamicin resistance gene, suggesting a later contamination. Sterility tests of the freeze-dried lyophilisate indicate a sterile product. From a total of 20l fermentation volume, harvesting and lyophilisation yielded **7480mg of BGs** dry weight. Calculating from a cfu count of 6.65*10^8/ml at the time point of lysis induction, and a harvested volume of 20l, the lyophilisate contains **1.78*10^9particles/mg**.

Western Blot samples taken throughout the fermentation process were separated on a 4-12%-Bis-Tris-Gel with MES buffer. The gel was blotted onto nitrocellulose, the membrane incubated with HRP-coupled-Anti-Myc-Antibodies and developed by chemilumiscence; the protein GIII-CP4opt-Myc-PolyHis was expected at a size of 39.8kDa, while CP4opt-Myc-PolyHis was expected at 37.0kDa. Several Western Blots of this fermentation were done because of the very low signal. Besides detection employing Anti-Myc-Antibodies, Anti-CP4-Serum (not shown) and Anti-His-Antibodies were used, but all showed the same results of very low recombinant protein expression, as seen in Figure 76.

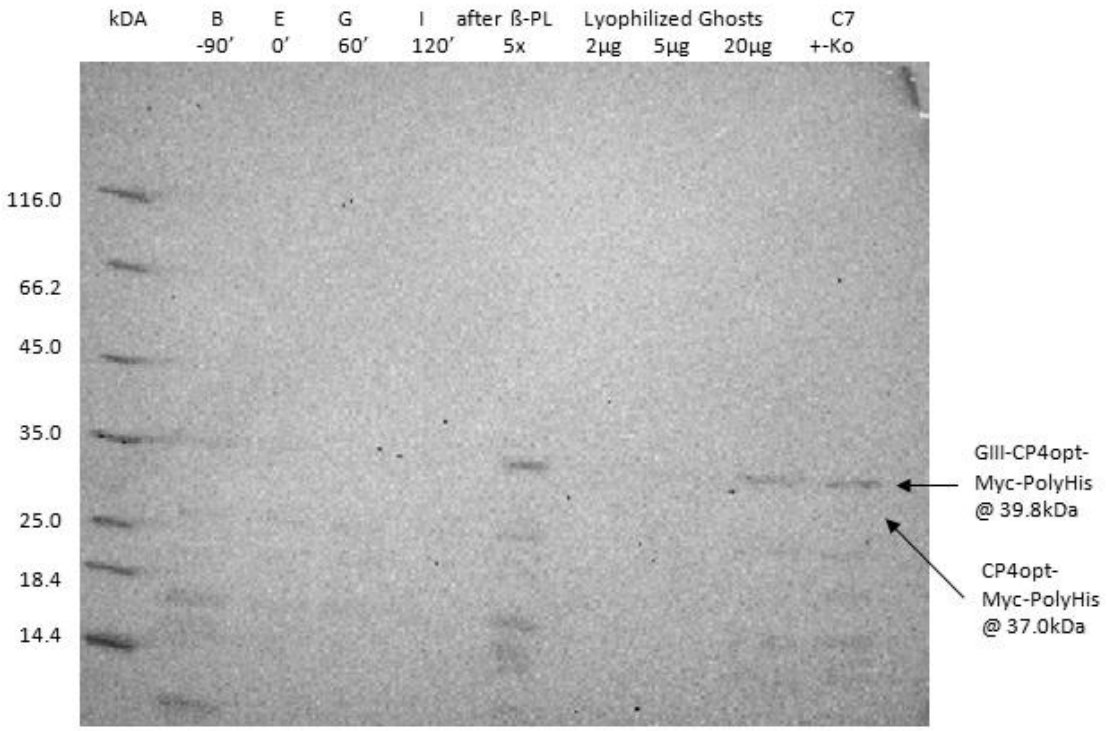


Figure 76: Western Blot of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) Fermentation and BGs, using Unstained Protein Molecular Weight Marker (Fermentas). Proteins were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-His-Antibodies and developed. Samples after β-PL treatment are five-fold concentrated compared to samples taken during the fermentation process. Different concentrations of lyophilized BGs were also loaded onto the gel (2µg, 5µg, 20µg). As a positive control, a sample from a previous small scale experiment was used.

While detection of CP4 after lysis is possible, it is only seen in highly concentrated samples (such as after ß-PL treatment), or high amounts of lyophilized BGs, indicating its low abundance.

8.1.3.5 QUANTIFICATION OF CP4 IN *E. COLI* NM522 BGS

In order to quantify the amount of recombinant CP4 per µg *E. coli* NM522 BG, the signals obtained from a serial dilution of BGs (Techfors Fermentation of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb), see 8.1.3.4) with known concentration and a serial dilution of positope (containing the Myc-epitope) with known concentration were compared on a Western Blot, as seen in Figure 77.

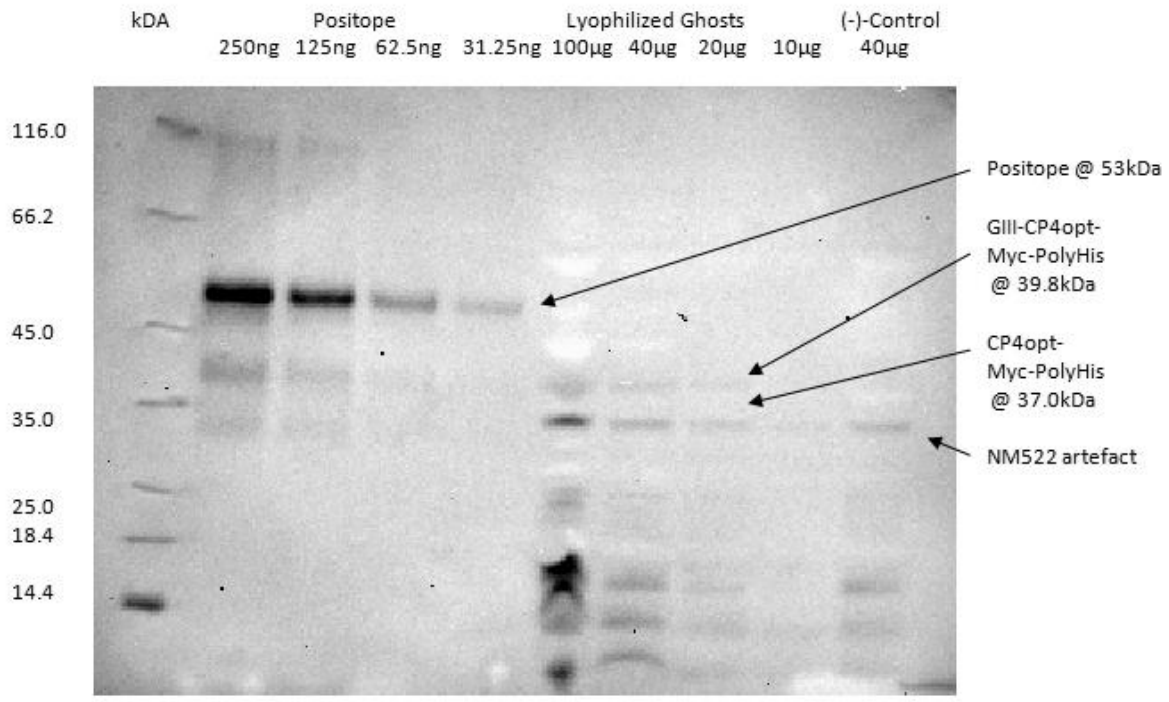


Figure 77: Western Blot of *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) BGs and positope, using Unstained Protein Molecular Weight Marker (Fermentas). Samples were separated on a 4-12% Bis-Tris-Gel with MES Running Buffer, the gel was blotted onto nitrocellulose. Nitrocellulose was incubated with 1:5000 diluted HRP-coupled Anti-Myc-Antibodies and developed. The amount of lyophilized BGs, as well as of positope per lane is indicated. As a negative control, lyophilized BGs from an *E. coli* NM522 (pGLysivb) Fermentation were used.

The chemiluminiscence signals generated by HRP-coupled-Anti-Myc-Antibodies at the site of positope and CP4 bands were measured using the ChemidocXRS machine; with the standard curve provided by the positope, the amount of CP4 per µg BG could be calculated, as outlined in Figure 78:

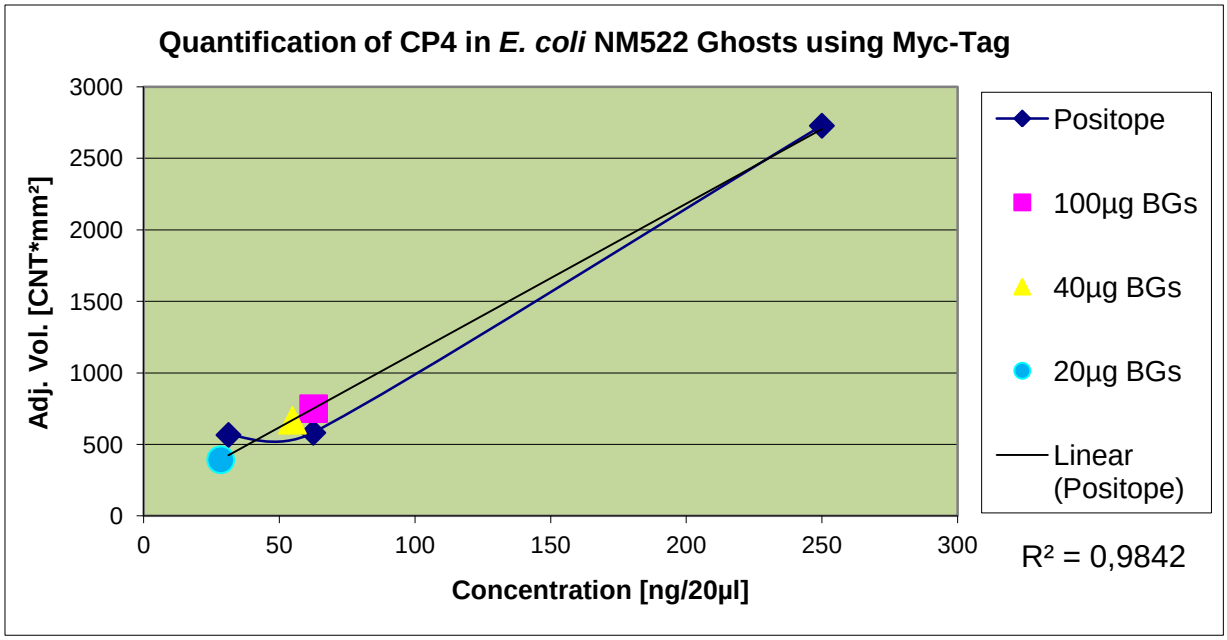


Figure 78: Quantification curve for *E. coli* NM522 (pBGKB-CP4opt, pGLysivb) BGs. Signals generated by positope with known concentration are indicated by blue diamonds, the linear regression curve is shown in black. Signals generated by BG samples are indicated as detailed in the legend.

The square of the correlation coefficient is R^2=0.9842, an acceptable fit of the standard curve provided by the positope. Calculated on that basis, the amounts shown in Table 20 of CP4 are found in BGs.

Lane	Amount of BGs	Calc. Total Amount of CP4	Calc. Amount CP4/µg BG
U1	100µg	62.64ng	0.63ng
U2	40µg	54.85ng	1.37ng
U3	20µg	28.38ng	1.42ng

Table 20: Calculating the average amount of CP4 per µg of *E. coli* NM522 BGs

On average, one microgram of *E. coli* NM522 BGs contains **1.14 ± 0.44ng CP4.**

The molecular weight of CP4 of 37.0kDa translates into a mass of $6.14 \cdot 10^{-20}$g, therefore, one microgram of *E. coli* NM522 BGs contains $1.86 \cdot 10^{10}$ CP4 molecules. Assuming a particle count of $1.78 \cdot 10^{6}$ BGs/µg, **a single *E. coli* NM522 BG contains $1.04 \cdot 10^{4}$ CP4 molecules**.

8.1.4 DISCUSSION

In contrast to previous attempts (Schlacher, 2009), it was possible to clone the CP4opt gene into the periplasm anchoring backbone vector pBGKB. The plasmid pBGKB-CP4opt was checked for correct size by restriction digests, but not sequence analysed, since the CP4opt sequence itself was synthesized and sequenced prior to shipping by Microsynth. Expression

was determined to be very low in Expression Experiments. In Expression/Lysis Experiments, a slight decrease of CP4 after lysis induction, as seen e.g. in Figure 73, might hint at incomplete transport into the periplasm. However, no CP4 expression at all was observed in samples taken during fermentation process, only concentrated samples and lyophilized BGs were shown to contain traces of CP4.

While the total amount of CP4 – calculated to be 1.14ng per µg BG – is comparatively low, this does not necessarily reflect its antigenicity or the strength of the induced immune response. On average, 10,000 CP4 molecules are present on a single BG, values that are well in the range of previous experiments (Champeimont, 2008).

More than 7g of BGs displaying CP4 are available for shipping to New Zealand to assess their immunogenicity, and thereby the concept of using BGs for immunocontraception.

8.2 USED ABBREVIATIONS

The abbreviations and symbols used during this work are given in Table 21:

Abbreviation	Meaning
µg	microgram
µl	microlitre
aa	amino acid
AB	antibody
BG(s)	Bacterial Ghost(s)
bp	base pair
C. trachomatis	*Chlamydia trachomatis*
cfu	colony forming unit
CP4	Coat Protein 4 of *Trichosorus vulpecula*
CP4opt	DNA sequence of Coat Protein 4 of *Trichosorus vulpecula,* codon-optimized for *E. coli*
Da	Dalton, atomic mass unit 1Da = $1.660538921 \cdot 10^{-30}$g
dH_2O	deionised water
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
fwd	Forward
g	Gram
g	standard acceleration due to free fall, i.e. 9.81m/s²
hCG-ß-LTB	human choriongonadotropin-ß fused to heat labile enterotoxin B
HRP	Horseradish Peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_D	dissociation constant
kb	kilo base pair
kDa	kilo Dalton
l	litre
LHRH	Luteinizing Hormone Releasing Hormone
LPS	Lipopolysaccharide
M	Molar, i.e. mol/L
MCS	Multiple Cloning Site
mg	milligram
min	minute(s)
ml	millilitre
ng	nanogram
nm	nanometre
nt	nucleotide
OD_{600nm}	Optical Density at 600nm
ORF	Open Reading Frame
ori	Origin of Replication
PCR	Polymerase Chain Reaction
PPS	Periplasmic Space

rev	reverse
rpm	rounds per minute
sec	Second
T_m	Annealing Temperature
WB	Western Blot

Table 21: List of used abbreviations and their meanings

8.3 SYMBOLS USED IN CLONING STRATEGIES

The following symbols were used to denote genes, promoters, and other genetic elements in schemes showing cloning strategies:

8.3.1 GENES

Symbol	Meaning
→ GIII-MCS-myc-His →	GIII export sequence of Gene III protein from bacteriophage fd, followed by a Multiple Cloning Site, a Myc tag and a PolyHis Tag.
←His MCS ←S ←His ← DsbC	Gene encoding the Thiol:disulfide interchange protein DsbC precursor protein for the export to the periplasm, followed by PolyHis tag, followed by an S-Tag, followed by a Multiple Cloning Site and again a PolyHis Tag
← Kan	Kanamycin resistance cassette
← Amp	Ampicillin resistance cassette
← Gent	Gentamicin resistance cassette
← araC	Gene AraC, encoding the repressor protein of the L-arabinose operon of *E. coli*
MOB* →	Gene encoding a mutated mobility protein
Eivb →	Gene encoding the lysis gene E of bacteriophage ΦX174 fused to an *in vivo*-biotinylation sequence
← cI857	Thermosensitive allele of the λ phage repressor gene
LacIq →	LacI gene, regulated by a mutated high expression lacI promoter
←Rop	Repressor of Primer gene, regulating the plasmids copy number
MalE→	Gene encoding the N-terminal part (323aa) of Maltose Binding Protein, which targets fused proteins to the PPS

LacZα→	Gene encoding the lacZα protein, allowing for Blue-White-Screening of transformants
← L*	Gene encoding the lysis protein L of the bacteriophage M2 for C-terminal anchoring in the Inner Membrane
PorB →	Gene encoding protein PorB from *Chlamydia trachomatis*
MOMP →	Gene encoding Major Outer Membran Protein from *Chlamydia trachomatis*
CP4opt →	Gene encoding codon-optimized Coat Protein 4 from *Trichosorus vulpecula*
Calmodullin→	Gene encoding Calmodulin

Table 22: Symbols used for genes in cloning strategies

8.3.2 OTHER GENETIC ELEMENTS

Symbol	Meaning
→ P_{BAD}	Promoter of the *ara* operon
→ T_{rrnB}	Terminator region of the *E. coli* rrnB gene
→ **pBR322 ori**	Origin of Replication, taken from plasmid pBR322
→ P_{Lac}	Promoter of the *lac* operon
→ M13	M13 intergenic region, Origin of Replication
← P_{T7}	Promoter recognized by the T7 RNA polymerase
← T_{T7}	Terminator region for the T7 RNA polymerase
← F1 ori	Origin of Replication of bacteriophage F1
→ **pUC ori**	Origin of Replication of the pUC plasmid

 pMB1 ori	Origin of Replication of the pMB1 plasmid
 ColE1 ori	Origin of Replication of the ColE1 plasmid

Table 23: Symbols used for genetic elements other than ORFs in cloning strategies

8.4 PLASMID OVERVIEW

An overview of plasmids used in this work is given in Table 24.

Plasmid	Expression	Marker	Ori	Size	Reference	Page
pBGKB	GIII-Myc-PolyHis	Kan	pBR322	4042bp	(Schlacher, 2009)	23
pBAD-GIII-Calmodulin	GIII-Calmodulin-Myc-PolyHis	Amp	pBR322	4556bp	(Invitrogen, 2011)	-
pSlo1.0A-Cp4opt	lacZ-CP4opt-lacZ	Amp	pUC	3908bp	This Work	122
pET40b	DsbC-PolyHis-S-Tag-PolyHis	Kan	pBR322	6190	(Novagen, 2011)	-
pKS-MOMP	LacZ-MOMP-L*	Amp	ColE1	≈4870bp	(Eko, et al., 2003)	-
pMAL-PorB	MBP-PorB	Amp	pBR322	7767	Eko, pers. communication	-
pGLysivb	Eivb	Gent	Rep	6201	(Haidinger, 2001)	175
pET40b-PorB	DsbC-PolyHis-S-Tag-PorB-PolyHis	Kan	pBR322	7123bp	This Work	28
pASK	GIII-PolyHis-S-Tag-PolyHis	Kan	pBR322	4142bp	This Work	29
pASK-PorB	GIII-PolyHis-S-Tag-PorB-Myc-PolyHis	Kan	pBR322	5141bp	This Work	30
pBGKB-PorB	GIII-PorB-Myc-PolyHis	Kan	pBR322	4979bp	This Work	26
pBGKB-MOMP	GIII-MOMP-Myc-PolyHis	Kan	pBR322	5147bp	This Work	31
pBGKB-CP4opt	GIII-CP4opt-Myc-PolyHis	Kan	pBR322	4937bp	This Work	122

Table 24: Overview of plasmids and their origin used in this work.

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