Entwicklung einer neuen funktionellen Proteintechnologie in *E. coli*

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Abbreviations

A	Adenin
aa	amino acid
amp	ampicillin
Ap^R	ampicillin resistence
BHI	Brain-Heart-Infusion medium
bla	beta lactamase
BSA	bovine serum albumine
bp	base pair
C	Celsius, Cytosin
cam	chloramphenicol
cat	chloramphenicol-acetyl-transferase
D	Dalton
DNA	desoxyribonucleic acid
dNTPs	desoxynucleotriphosphates
E. coli	*Escherichia coli*
EDTA	ethylen-diamine-tetra-aceteacid
EHEC	Enterohemorrhagic *E. coli*
FunProTec	Functional Protein Technology
G	Guanin
g	gram
GOI	gene of interest
h	hour
hly	hemolysin

IPTG	isopropyl-ß-D-thiogalactoside
Kan^R	kanamycin resistence
kb	kilo base
kD	kilo dalton
l	litre
LB	Luria Broth medium
M	molar
mA	milli ampere
min	minute
mg	milli gram
ml	milli litre
mM	milli molar
mRNA	messenger RNA
mV	milli volt
MW	molecular weight
ng	nano gram
nl	nano litre
nM	nano molar
µg	micro gram
µl	micro litre
µM	micro molar
OD	optical density
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamid gel electrophoresis
PAI	pathogenicity island

PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
RT	room temperature
Rtx	repeats in toxin
s	second
SDS	sodiumdodecylsulfate
T	Thymidin
TBE	tris-borat-EDTA
Tm	melting temperature
Tricin	N-Tris-(hydroxymethyl)-methylglycin
Tris	tris-(hydroxymethyl)-aminomethan
U	unit, Uracil
UPEC	uropathogenic *E. coli*
V	volt
wt	wild-type
Xgal	5-bromo-4-chloro-3-indoyl-ß-galactoside

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1. Summary

The aim of this Ph.D thesis was the establishment of a novel Functional Protein Technology (FunProTec). It is based upon the combined application of a pro- or eukaryotic expression and secretion system, that allows a quick and easy high yield synthesis of native protein solutions or protein arrays consisting of functionally active proteins, and a compartment system, which preserves the respective proteins from the producing cells and separates them from background proteins. FunProTec is an invention of ALTANA Pharma and was patented with the international publication number WO 02/50260 A2, international publication date 27.06.2002.

In this study, FunProTec was established by utilization of a modified version of the *E. coli* alpha-hemolysin secretion system. The hemolysin secretion apparatus consists of the proteins HlyB, HlyD and TolC and can be utilized for the secretion of heterologous proteins by generating gene fusions between heterologous genes and the C-terminal signal sequence $HlyA_S$ of hemolysin (Gentschev *et al*., 1994).

In this thesis, a system for synthesis and secretion of functional active proteins was validated by generating different *E. coli* strains suitable for the secretion of heterologous proteins by the hemolysin secretion system. The genes encoding the transport proteins HlyB and HlyD and the genes encoding hemolysin (HlyA) and its activator protein (HlyC) were cloned either onto the same (case A) or onto two different plasmids (case B) in these *E. coli* strains, while the *tol*C gene was located on the bacterial chromosome. These *E. coli* strains were compared in regard to their hemolysin secretion efficiencies.

It was demonstrated, that in both cases (A and B) a DNA sequence located upstream of the *hly* genes is essential for the hemolytic activity of the respective *E. coli* strain and can not be displaced by a heterologous promoter. This sequence is also located upstream of the wild-type hemolysin gene cluster in the chromosome of uropathogenic *E. coli*. It contains the hemolysin promoter and a JUMPstart activator sequence (Hobbs and Reeves, 1994).

It was also demonstrated in this study that a defined stoichiometry between the transcripts of the genes encoding hemolysin (*hly*A) and the transcripts encoding the

transport channel *hly*B and *hly*D is responsible for efficient secretion of hemolysin and is regulated by the above mentioned activator sequence. This indicates that the hemolysin operon is regulated in a complex manner and that every genetic manipulation may influence the transcription of the *hly* genes and possibly the secretion efficiency of the respective *E. coli* strain.

In the above described *E. coli* strains A and B, the secretion efficacies of alkaline phosphatase by *E. coli* K-12 strains, the *E. coli* outer membrane protein A (OmpA), the human phosphodiesterase 1B1 and the human cytokine receptor ligand Ccl-21 were examined. None of these proteins was secreted. The secretion of heterologous proteins by the hemolysin secretion system has been described, however, with the exception of protein toxins or very small proteins or peptides, only two full-length proteins were secreted. It remains to be investigated that the loss of protein folding may facilitate the secretion. It has to be further investigated whether the application of chaperons that change the protein folding will improve protein secretion.

The present study suggest, that the hemolysin secretion system is not a universal secretion system, but it might be suitable for secretion of small, unfolded proteins as shown by the secretion of protein subunits and antibody chains (Tzatschel *et al*., 1996, Fernandez *et al*., 2000).

Moreover, the compartment systems were validated in this thesis by showing that the hemolysin protein was separated from the secreting *E. coli* strains by suitable filter systems without loosing its activity.

In conclusion, FunProTec may be used for different pro- or eukaryotic secretion systems. Its applicability depends on the properties of the protein to be secreted.

Thus, in combination with the corresponding compartment systems, FunProTec might be a suitable technology for production of recombinant proteins.

2. Zusammenfassung

Ziel dieser Arbeit war die Etablierung einer neuen „Funktionellen Proteintechnologie“ (FunProTec). FunProTec basiert auf der Kombination eines pro- oder eukaryontischen Expressions- und Sekretionssystems, welches die Sezernierung heterologer Proteine mit hoher Ausbeute in ihrer nativen Form ermöglicht, mit einem Kompartimentierungssystem, das eine einfache Abtrennung der sezernierten Proteine von der produzierenden Zelle erlaubt. FunProTec ist eine Erfindung von ALTANA Pharma und wurde mit der internationalen Publikationsnummer WO 02/50260 A2 und dem Publikationsdatum 27.06. 2002 patentiert.

FunProTec wurde in dieser Arbeit auf Basis des *E. coli*-Hämolysin-Sekretionssystems entwickelt. Dieses Typ I-Sekretionssystem uropathogener *E. coli* besteht aus den Proteinen HlyB, HlyD und TolC und ist zum Transport heterologer Proteine befähigt, insofern diese die C-terminale Signalsequenz des Hämolysin-Strukturproteins ($HlyA_s$) tragen (Gentschev *et al*., 1994).

In dieser Arbeit wurde ein System zur Synthese und Sekretion funktionell aktiver Proteine evaluiert. Zu diesem Zweck wurden *E. coli*-Stämme hergestellt, die für die Sekretion heterologer Proteine durch das Hämolysin-Sekretionssystem geeignet sind. In diesen *E. coli*-Stämmen waren die Gene für die Transportproteine HlyB und HlyD und die Gene für Hämolysin (HlyA) einschließlich seines Aktivatorproteins HlyC entweder auf demselben (A) oder auf zwei unterschiedlichen Plasmiden (B) lokalisiert. Das *tol* C-Gen war jeweils integraler Bestandteil des bakteriellen Chromosoms. Diese Stämme wurden hinsichtlich ihrer Effizienz der Hämolysin-Sekretion verglichen.

Es konnte gezeigt werden, dass in beiden Fällen A und B eine bestimmte Gensequenz, die sich stromaufwärts der *hly* Gene befindet, essentiell für die hämolytische Aktivität des entsprechenden *E. coli*-Stammes ist und nicht durch einen heterologen Promotor ersetzt werden kann. Diese Gensequenz liegt im wild-typischen Hämolysin-Gencluster ebenfalls stromaufwärts des Hämolysinoperons auf dem Chromosom uropathogener *E. coli* und enthält den Hämolysinpromotor und eine JUMPstart Aktivatorsequenz (Hobbs und Reeves, 1994).

Weiterhin wurde in dieser Arbeit erstmals gezeigt, dass eine definierte Stöchiometrie zwischen den Transkripten des Hämolysingens (*hly*A) und den Transkripten der Transportkanalgene *hly*B und *hly*D für die effiziente Sekretion von Hämolysin erforderlich ist, die vermutlich von der oben genannten Aktivatorsequenz reguliert wird. Dies weist darauf hin, dass das Hämolysin-Operon komplex reguliert ist und jede genetische Manipulation Einfluss auf die Transkription der *hly* Gene und im weiteren Sinne auf die Sekretionseffizienz des entsprechenden *E. coli*-Stammes haben kann.

In den oben beschriebenen *E. coli*-Stämmen A und B wurde die Sekretion der alkalischen Phosphatase aus *E. coli*, des Außenmembran-Proteins A (OmpA) aus *E. coli*, der humanen Phosphodiesterase 1B1 und des humanen Cytokinrezeporliganden Ccl-21 untersucht. Jedoch wurde keines dieser Proteine sezerniert. Es gibt eine Reihe an Publikationen, in welchen die Sekretion heterologer Proteine durch das Hämolysin-Sekretionssystem beschrieben ist. Mit der Ausnahme von Toxinen oder sehr kleinen Proteinen bzw. Peptiden, besitzen allerdings nur zwei Proteine ihre intakte Aminosäuresequenz. Dies deutet darauf hin, dass die Aufhebung der Proteinfaltung die Sekretion erleichtern würde. Es ist zu prüfen, ob der Einsatz von Chaperonen, welche die Faltungseigenschaften der Proteine verändern, die Proteinsekretion verbessern wird.

Die Sekretionsstudien, die in dieser Arbeit durchgeführt wurden, haben gezeigt, dass das Hämolysin-Sekretionssystem kein universelles Sekretionssystem ist. Das Hämolysin-Sekretionssystem könnte jedoch für die Sekretion kleinerer Proteine, bei denen die Faltung weitgehend aufgehoben ist, geeignet sein, wie bereits bei der Sekretion von Proteinuntereinheiten oder Antikörperketten gezeigt wurde (Tzatschel *et al*.,1996, Fernandez *et al*., 2000).

Desweiteren wurden die Kompartimentierungssysteme am Beispiel des Hämolysin Proteins validiert. Durch den Einsatz geeigneter Filtersysteme wurde die Trennung des Hämolysin-Proteins von den sezernierenden *E. coli* Stämmen erreicht, wobei die Aktivität des Proteins erhalten blieb.

FunProTec könnte sich zur Sekretion heterologer Proteine neben dem Hämolysin-Sekretionssystem unterschiedlicher pro- oder eukaryontischer Sekretionssystemen

bedienen, deren Wahl von den Eigenschaften des Proteins, welches sezerniert werden soll, abhängt. Auf diese Weise könnte es in Kombination mit den entsprechenden Kompartimentierungssystemen, eine geeignete Technologie zur Herstellung rekombinanter Proteine sein.

3. Introduction

The aim of my Ph.D thesis was the establishment of a novel „Functional Protein Technology" (FunProTec) which allows a quick and easy high yield synthesis of native protein solutions or protein arrays consisting of functionally active proteins. FunProTec is an invention of ALTANA Pharma and was patented with the international publication number WO 02/50260 A2, international publication date 27.06.2002. "FunProTec is based upon the application of a pro- or eukaryotic expression and secretion system which allows the synthesis and secretion of heterologous proteins of interest and a compartment system which preserves the respective proteins and concomitantly separates them from the background proteins of the producing cell" (ALTANA Pharma patent, 2002).

The frequently used strategy for the production of recombinant proteins is their intracellular over-expression. To date, there are many pro- and eukaryotic expression systems, e.g. bacterial, yeast, insect and mammalian expression systems. Protein expression in bacteria, e.g. in *E. coli* has the following advantages over eukaryotic expression systems: the cultivation is very cheap, the genetics and molecular biology is known, there are many defined strains and expression vectors and the cloning and expression procedures are easy. However, it is not possible to predict whether low-usage codons in a gene may affect the efficiency of its expression in *E. coli* (Savvas *et al*., 1996). Furthermore *E. coli* is not able to form many of the posttranslational modifications, but there was some progress in disulfide bond formation during extracellular secretion (Fernandez *et al*., 2001). Moreover, proteins are sometimes degraded by intracellular proteases (Savvas *et al*., 1996).

After over-expression of recombinant proteins in suitable expression systems, the cells are usually lysed and the proteins are purified by suitable chromatographic purification methods (Vincent *et al*., 2004). However, the purification efforts are high. Moreover, there is a simultaneous loss of protein yield during the several purification steps and a frequent failure of protein refolding.

The advantage of FunProTec over this method is the secretion of the respective proteins by living cells to separate them from the bacterial background proteins. They

are expected to remain in a native state, because they are not lysed during the technical process.

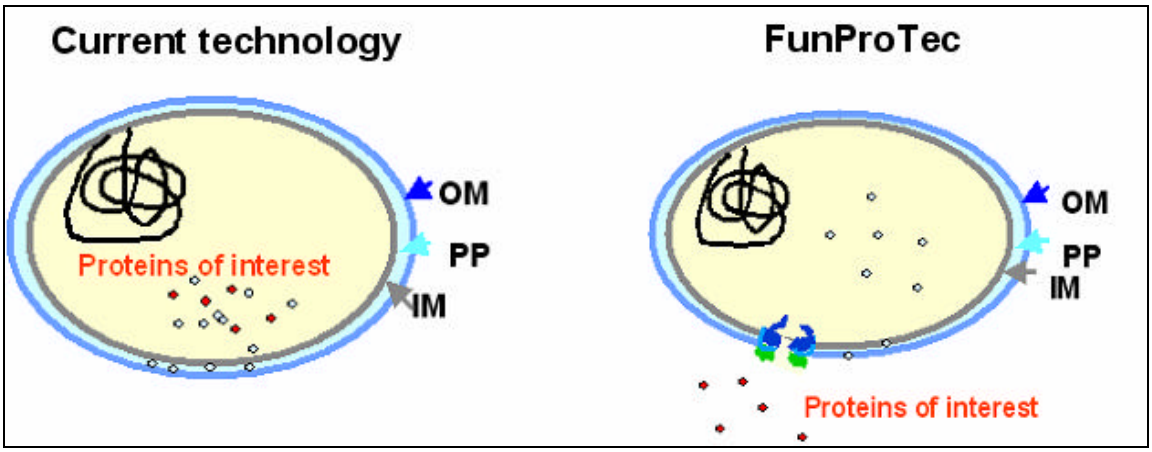


Figure 3.1: In contrast to the current technologies, FunProTec is based upon a secretion system, which secretes the proteins of interest (in the red colour) out of the cell (OM= outer membrane; IM= inner membrane; PP=periplasm).

The steady growth of DNA sequence information led to the development of protein arrays for studies of protein function, expression and protein-protein interaction (Zhu *et al*., 2003, Cutler *et al*., 2003). Many protein arrays are being developed, e.g. arrays containing synthetic peptides or display systems of different organism (Li, 2000). Further technologies used for the production of protein arrays are often based on the cell lysis of the bacterial cells. The bacterial debris is washed off and the proteins of interest are bound onto a carrier, e.g. by using of appropriate tags for immobilization and purification (Büssow *et al*., 1998; Walter *et al*., 2000; Lueking *et al*., 2001). However, the high background of bacterial proteins can interfere with specific binding studies and the lysis may denature the protein of interest.

The problem of the high background of bacterial proteins can be solved by purification of over-expressed proteins, however, this procedure is very labour intensive. FunProTec circumvents the above described problems and allows the production of arrays harbouring various proteins of an organism “as single spots, each of which is saturated with a single protein in an undenatured form” (ALTANA Pharma patent, 2002).

Moreover, FunProTec allows not only the production of protein arrays, but also the production of protein solutions “consisting of specific secreted, filtered proteins” in a small or medium scale. The proteins can be used for a lot of studies, e.g.

characterization for their structure and function. Especially in the case of protein arrays, but also in the case of protein solutions, FunProTec may serve for the identification of protein binding partners of different chemical nature, including other proteins, nucleic acids and lipids. Binding studies with array-bound proteins and soluble binding partners can be performed directly on the respective array (e.g. filter). Binding studies of protein solutions can be performed either with array-bound binding partners or with soluble binding partners. Thus, the proteins produced with FunProTec may be suitable for utilization in all conceivable binding studies, including antibody binding studies and studies on the binding of pharmaceutical compounds with array-bound target proteins. In this case, FunProTec allows studying the impact of pharmaceutical compounds on the binding behaviour and enzymatic capacities of other molecules and it is therefore a suitable system for the elucidation of mechanism of action of the respective pharmaceutical compounds (ALTANA Pharma patent, 2002).

3.1 Secretion systems

As mentioned above, FunProTec is based on the usage of pro- or eukaryotic expression and secretion systems for the expression and secretion of heterologous proteins into the extracellular space.

The requirements for protein secretion in eukaryotic cells are still poorly understood. There is often a signal peptide-mediated transport across the endoplasmic reticulum membrane and the Golgi apparatus. Conceivable eukaryotic protein secretion systems that can be applied for secretion of heterologous proteins include yeast secretion systems, e.g. the secretion systems of *Pichia pastoris* and *Saccharomyces cerevisiae* (Cereghino *et al*., 2002; Cereghino and Cregg, 2000; Cregg *et al.,* 2000). Moreover, higher eukaryotic cells are not so often used for secretion of heterologous proteins. Examples for heterologous protein secretion by higher eukaryotic cells are the secretion of human cytokines by Chinese hamster ovarian cells (Davis *et al*., 2000) and the secretion of human serum albumin to potato tubers (Farran *et al*., 2002).

There are secretion systems of Gram-positive bacteria (e.g. *Bacillus* spp., *Staphlyococcus* spp.) and Gram-negative bacteria (e.g. *E. coli*). Gram-positive

bacteria, e.g. *Bacillus* species, are able to secrete a variety of heterologous proteins into the extracellular medium (Westers *et al*., 2004).

In Gram-negative bacteria, proteins are translocated into the periplasma by the Sec or Tat pathway (Filloux *et al*., 2002; Ochsner *et al*., 2002). The periplasmic intermediate is translocated across the outer membrane by autotransporters placed in the outer membrane, so-called two-partner systems or type II secretion systems (Filloux *et al*., 2002). The transport of proteins via the type I and type III secretion system is *sec*-independent and the proteins are secreted directly from the cytoplasm (type I) into the extracellular medium or the host target cell (type III) (Cheng *et al*., 2000; Hueck *et al*., 2000; Müller *et al*., 2001; Filloux *et al*., 2002; Slepenkin *et al*., 2003). The type IV secretion system transports proteins or DNA in one or two steps through the bacterial cell membranes (Filloux *et al*., 2002). Wai *et al.* 2003 found a vesicle-mediated transport mechanism in bacteria for delivery of pathogenic effector proteins into mammalian cells.

The type I secretion system is the most frequently used secretion system of Gram-negative bacteria for secretion of heterologous proteins into the extracellular medium and the most frequently used type I secretion pathway is the hemolysin secretion system of uropathogenic *E. coli*. Conceivable advantages of the hemolysin secretion system over the other above mentioned secretion systems are that manipulations may be easy, because only three proteins are involved in the secretion process and that this secretion system may have a low degree of specifity of secreted proteins both within and between different bacteria species: It has previously been demonstrated that heterologous proteins of different origin (e.g. bacterial, viral, protozoan) were secreted by the hemolysin secretion apparatus in many Gram-negative bacteria (Spreng *et al*., 1998; Dietrich *et al*., 1998; Gentschev *et al*., 1994, 1996 a+b 1997, 1998; Mollenkopf *et al*., 1996; Spreng and Gentschev, 1998; Spreng *et al*., 1999).

In this study, FunProTec was established by using the hemolysin secretion system.

3.1.1 The hemolysin secretion system

E. coli a-hemolysin (HlyA) is a membrane damaging, pore-forming extracellular cytotoxin often produced by extraintestinal *E .coli* pathogens, predominantly by

uropathogenic *E. coli* (UPEC) (Ludwig and Goebel, 1991). The HlyA protein lyses eukaryotic cells, including erythrocytes, because of which it was termed “hemolysin” (Ludwig and Goebel, 1991). It belongs to the Rtx (repeats in toxin) family of protein toxins produced by a variety of Gram-negative bacteria (Ludwig and Goebel, 1991). Rtx cytotoxins are characterized by a C-terminal calcium binding region with a variable number of glycine-rich repeat units consisting of nine amino acids. Calcium-binding of the released Rtx toxins in the extracellular space is essential for their cytotoxic activity. Rtx toxins are transported directly from the cytoplasma into the extracellular medium by a type I secretion pathway (Ludwig and Goebel, 1991). The synthesis and secretion of the *E. coli* a-hemolysin is encoded by an operon (see Figure 3.2) consisting of four genes, designated *hly*C, *hly*A, *hly*B and *hly*D (Ludwig and Goebel, 1991).

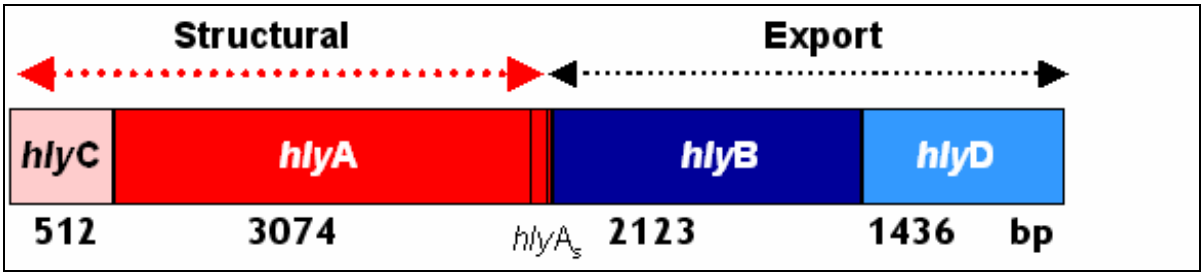


Figure 3.2: Operon for the secretion and synthesis of a-hemolysin

In the most hemolytic *E. coli* pathogens the *hly* determinants are chromosomally encoded. In about 5% of hemolytic *E. coli* the *hly* genes are localized on a plasmid (Ludwig and Goebel, 1991; Mühldorfer and Hacker, 1994).

The *hly*A gene encodes the structural protein a-hemolysin (HlyA). The co-synthesized HlyC is an acyltransferase and activates HlyA within the bacterial cytosol: It converts the nontoxic prohemolysin (proHlyA) into the toxic a-hemolysin (HlyA) by fatty acylation of two internal lysine residues (Ludwig and Goebel; Stanley *et al*., 1998, 1999; Trent *et al.*, 1998, 1999). HlyB and HlyD form together with TolC the secretion apparatus. The TolC protein is chromosomally encoded in all Gram-negative bacteria and is not part of the *hly* operon (Ludwig and Goebel, 1991; Schlör *et al*., 1997). The secretion of *E. coli* a-hemolysin by the secretion apparatus was initialized by interaction of HlyB with the C-terminal signal sequence of HlyA designated HlyA$_s$. The 60 C-terminal amino acids of HlyA, containing a helix(a1)-

linker-helix(a2) motif, are sufficient for being recognized by the secretion apparatus (Koronakis *et al*., 1989; Jarchau *et al*., 1994 Hui *et al*., 2000). The last 20 residues of the signal sequence are critical for HlyA transport (Hui *et al*., 2002). The C-terminal peptide can also be secreted by itself (Jarchau *et al*., 1994; Hui *et al*., 2000). Hui and Ling, 2002, presented a functional model of the hemolysin signal sequence consisting of two domains. The first 22 amino acids domain comprises the (a1)-helix and the linker region. The second domain covers the last eight residues of the signal sequence.

HlyB, an inner membrane traffic ATPase, energizes the transport of HlyA. Its cytoplasmic domains recognize the HlyA C-terminal signal sequence. Consecutively, HlyB initiates the HlyA secretion and forms a transmembrane channel in the inner membrane through which HlyA is translocated. HlyD was suggested to serve as a linker between the inner and outer bacterial membrane. HlyD is inserted into the inner membrane, but its main part is localized in the periplasm where it interacts with the inner membrane protein HlyB and the outer membrane protein TolC (Ludwig und Goebel, 1991). There are studies suggesting that there is a substrate interaction with at least two segments of the HlyD cytosolic domain (Balakrishnan *et al*., 2001). It is proposed that the HlyD cytosolic domain mediates transduction of the substrate to the HlyD periplasmic domain to trigger recruitment of TolC and to assemble the type I secretion apparatus (Balakrishnan *et al*., 2001). TolC consists of three monomers. The smaller part of TolC is located in the lipid bilayer of the outer membrane and forms a ß-barrel structure. The larger part of TolC is located in the periplasm and consists of ?-helices (Lewis, 2000). By interacting with HlyB loops in the periplasm, HlyD and HlyB form a channel through the periplasm. As HlyD also has a TolC-homologous part, it is assumed to participate with TolC in the formation of a transmembrane channel in the outer membrane through which HlyA is secreted. Moreover, HlyD forms a trimer (Thanalabu, 1998) which suggests a seamless link between the TolC and HlyD (Ludwig und Goebel, 1991). It is proposed that this channel has a temporary structure (Lewis, 2000; Federici *et al*., 2004). The transport process seems to proceed by the recruitment of HlyA by HlyB which may pre-exist with HlyD (Federici *et al*., 2004). On binding ATP, TolC is engaged, and HlyA is translocated (Federici *et al*., 2004). In the presence of ?–hemolysin, TolC can associate with other translocases (Lewis, 2000; Federici *et al*., 2004).

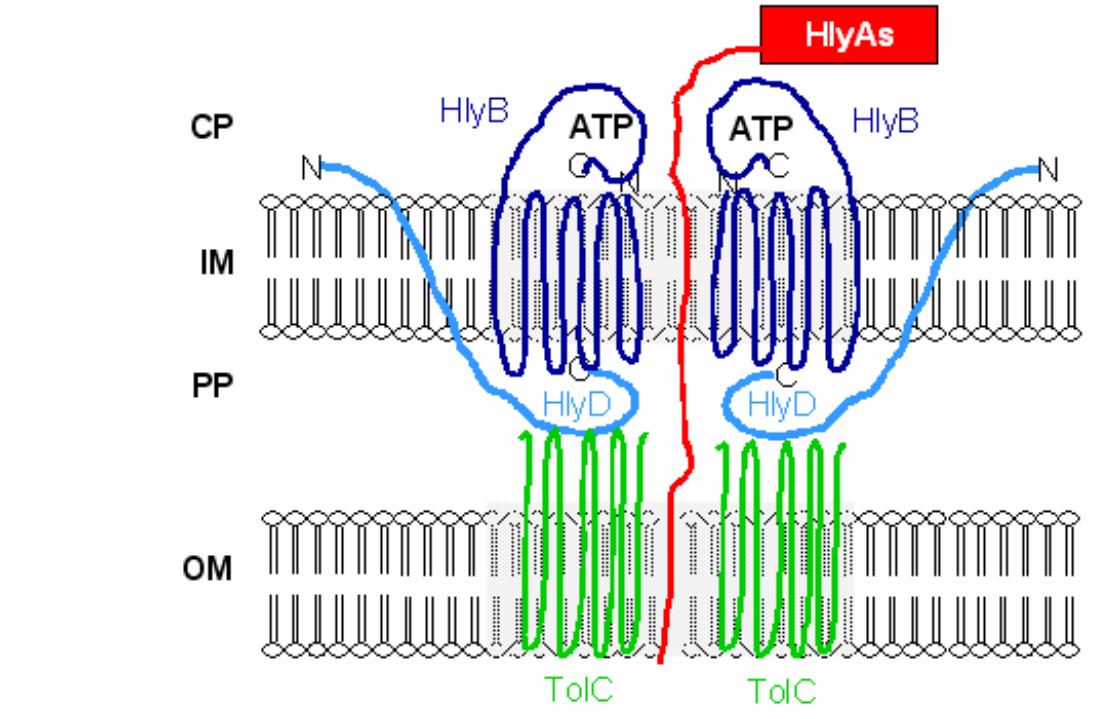


Figure 3.3: Transport channel of the hemolysin secretion system, consisting of the three proteins *Hly*B, *Hly*D, TolC (OM= outer membrane; IM= inner membrane).

Transcription of the *hly*CABD operon is strongly polar, accentuated by a stem-loop structure in the *hly*A-*hly*B intergenic sequence. Transcription has been shown to be positively controlled by cis-acting regions in the *hly* upstream regions (Nieto *et al*., 1996, Leeds *et al*., 1997). In case of the plasmid-encoded a-hemolysin, its synthesis and secretion in *E. coli* is enhanced by the *hly*R gene located at some distance upstream of the *hly*C gene (Vogel *et al*., 1988). In contrast, a *hly*R homologous activator gene has not been found in chromosomal *hly* determinants (Ludwig and Goebel, 1991), but the transcription was enhanced by a 39 bp so-called JUMPStart sequence (Hobbs and Reeves, 1994) which was also present in *hly*R (Nieto *et al*., 1996; Leeds *et al*., 1997). A part of the JUMPStart sequence is an eight bp GGCGGTAG element that is conserved by disparate *hly*CABD 5´regions and located downstream of the major promoters (Nieto *et al*., 1996) and plays a role in the RfaH enhanced transcript elongation (Leeds *et al*., 1997).

As mentioned above, the hemolysin secretion apparatus works in many Gram-negative bacteria (Spreng *et al*., 1998) and can be utilized for the secretion of heterologous proteins (Dietrich *et al*., 1998; Gentschev *et al*., 1994, 1996 a+b 1997, 1998; Mollenkopf *et al*., 1996; Spreng and Gentschev, 1998; Spreng *et al*., 1999). Heterologous proteins will be secreted by the secretion apparatus, if gene fusions are

generated between the genes of these proteins and *hly*A_S encoding the C-terminal signal sequence of HlyA. These secretion-competent fusion proteins can be secreted when HyB, HlyD and TolC are synthesized by the respective bacteria (Gentschev *et al*., 1994). The secretion of heterologous protein by the *hly* secretion system was evaluated by the group of Gentschev that developed an antigen delivery system for the presentation of secreted antigens by Gram-negative bacterial vaccine carriers (Gentschev *et al*., 1994). However, the yields of the secreted fusion proteins are very different (Gentschev *et al*., 1996). Thus, the nature of the heterologous protein seems to influence the efficiency of heterologous protein secretion via the hemolysin secretion system. E.g. proteins, which are usually secreted by the *sec*-dependent secretion pathway, are inefficiently transported by the hemolysin secretion system (Gentschev *et al*., 1997). However, this problem can be circumvented by optionally using a host cell harbouring a *sec*A mutation or cleavage of the N-terminal signal sequence (Gentschev *et al*., 1997). Furthermore, it has been demonstrated that the efficiency of heterologous protein secretion via the hemolysin secretion system can correlate with the size of the heterologous gene upstream of the $HlyA_S$ signal (Spreng and Gentschev, 1998). Unequal amounts of different proteins deriving from different efficient protein secretions can be circumvented by fusion of the proteins with an affinity tag. Thus, the proteins of interest can be bound via the affinity tag onto a carrier with a suitable binding partner until saturation will be achieved.

3.2 Compartment systems

As mentioned above, FunProTec is based on the easy separation of secreted recombinant proteins from the producing host cell. A double filter system (Figure 3.4) and a stacked microwell system (Figure 3.5) are conceivable systems as compartment systems. Due to the ALTANA Pharma patent, the compartment systems used for FunProTec should consist of at least two compartments. The upper compartment contains the host cells. It is separated from the lower compartment by a membrane filter being permeable for proteins. The heterologous proteins secreted by the host cells are collected in the lower compartment.

The principle of the double filter system was described for the detection of Shiga-like toxin producing enterohemorrhagic *E. coli* (EHEC) in fecal samples by Hull *et al*., 1993: In this study, two filters are placed on an agar plate. The bacteria grow on the

upper filter by receiving their nutrients from the agar plate. The secreted proteins bound onto the lower filter. This system was modified and adjusted to FunProTec (see Figure 3.4).

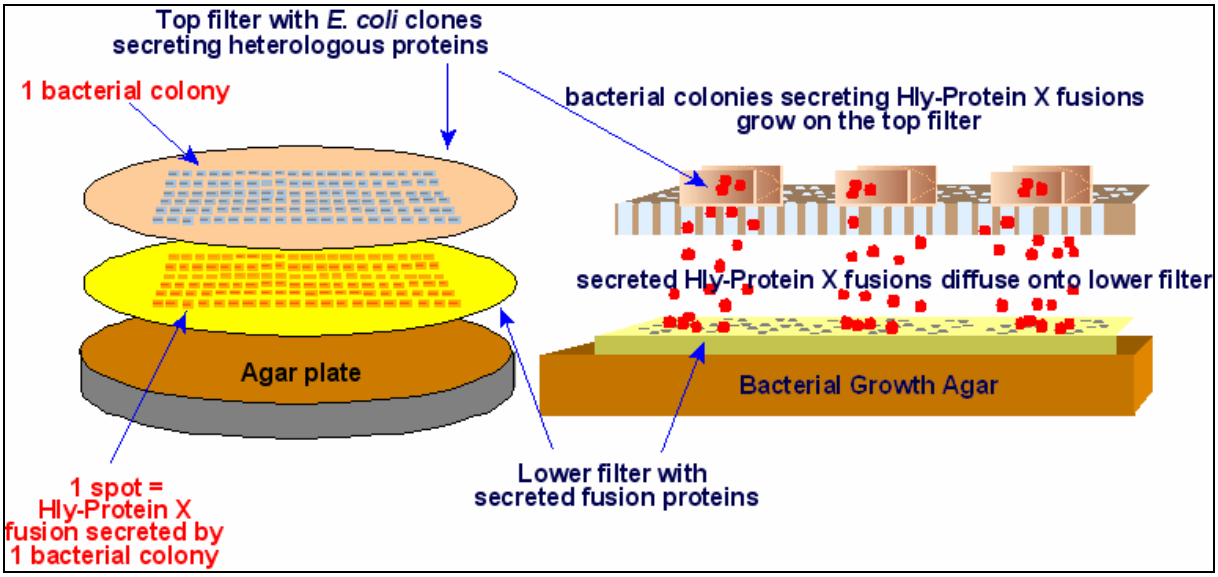


Figure 3.4: Double filter system

The double filter system can be used for the production of protein arrays, while the stacked microwell system can be applied for the production of protein solutions in micro-scale. The stacked microwell system consists of two microwell plates that are separated by a membrane filter (Figure 3.5). Liquid cultures of bacterial colonies grow in the upper microtiter plate. The lower microtiter plate contains solutions of proteins secreted by the bacterial strains grown in the upper plate. The membrane filter has a pore diameter that prevents bacterial migration, but allows the flow of the proteins secreted by the bacteria into the wells of the lower plate.

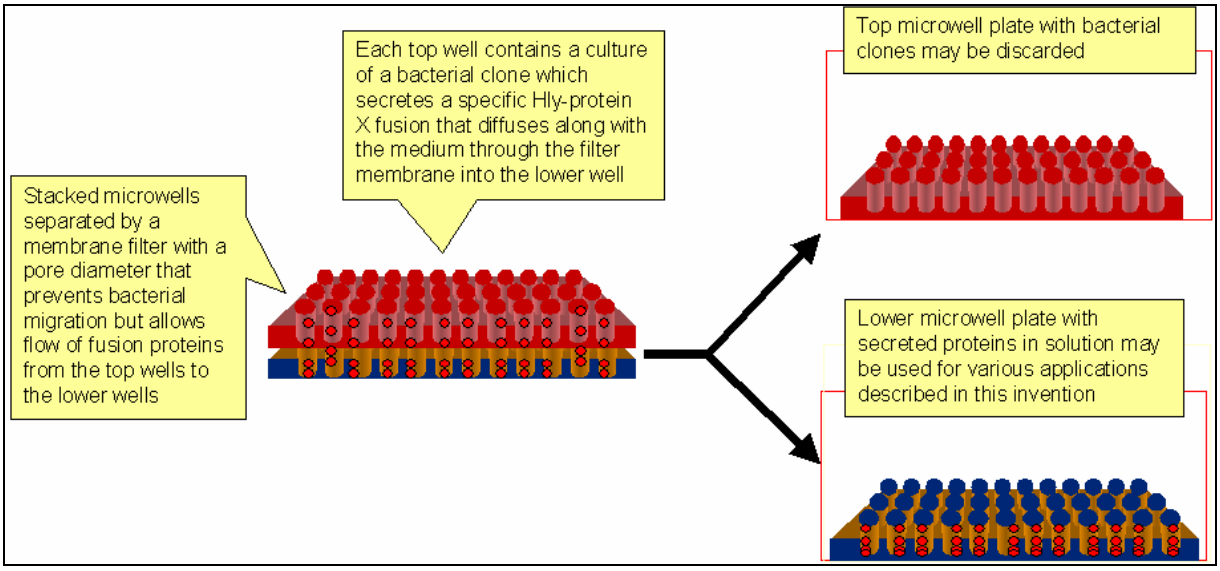


Figure 3.5: Stacked microwell system

3.3 Aim

The aim of this Ph.D thesis was to establish FunProTec by utilization of a modified version of the *E. coli* alpha-hemolysin system. The above described compartment system should be validated to preserve the native proteins of interest following their secretion by *E. coli* strains harbouring different secretion vectors. The advantages and critical parameters of the hemolysin secretion system for secretion of heterologous proteins should be investigated.

4. Material and methods

4.1 Material

4.1.1 Bacterial strains

Table 4.1: Used *E. coli* strains

***E. coli* strain**	**Characteristics or genotype**	**Reference/origin**
536	Uropathogenic *E. coli* (UPEC) wild-type (O6:K15:H31), Sm^R	Berger *et al*., 1982
536-21	Uropathogenic *E. coli* (UPEC 536) mutant, ? PaiI, PaiII	Hacker *et al*., 1983
J96	Uropathogenic *E. coli* wild-type (O4:H5:K6)	Hull *et al*., 1981
Top 10	F^-*mcr*A ?(*mrr-hsd*RMS-*mcr*BC) F 80*lac*Z?M15 ?*lac*X74 *rec*A1 *deo*R *ara*D139 ?(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Top 10F´	F {*lacI*^{q} Tn*10* (Tet^R)} *mcr*A ?(*mrr-hsd*RMS-*mcr*BC) F 80*lac*Z?M15 ?*lac*X74 *rec*A1 *deo*R *ara*D139 ?(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
BL21	BL21:DE3 strain: E F– *dcm omp*T *hs*dS(rB– mB–) *gal* (DE3) BL21 (DE3 (pLysS) strain: F– *dcm omp*T *hsd*S(rB– mB–) *gal* (DE3) [pLysS Cam^R]a	Invitrogen
C600	K-12 derivative, *sup*E44, *hsd*R, *thi*1, *thr*1, *leu*B6, *lac*Y1, *ton*A21	Appleyard, 1954
J53	K-12 derivative, F+, *met*$^-$F63, *pro*$^-$B22	Clowes and Rowley, 1954
DH5?	K-12 derivative, *sup*E44, *lac*U169, ?80*lac*Z, ? M15, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	Bethesda Research Laboratories, 1986
CC118	K-12 derivative, F- ?(*ara-leu*)7697, *ara*D139 ?(lac)X74, *pho*Ad20, *gal*E, *gal*K, *thi*, *rps*E, *rpo*B, *arg*E(Am), *rec*A1	Manoil and Beckwith, 1985
MC1000	*ara*D139, DE(*araA-leu*)7697, DE (*cod*B-*lacI*)3, *gal*K16, *gal*E15, LAM-, e14-, *rel*A1, *rps*L150(strR), *spo*T1, *mcr*B1	Casadaban, 1980
HB101	K-12 derivative, *sup*E44, *hsd*20, r_Bm_B, *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1	Boyer and Roulland-Dussoix, 1969
536-192	UPEC 536 mutant *hly*$^-$	University of Würzburg, Inge Mühldorfer
K-12 wt	K-12 wild-type, F^+, ?$^+$	Bachmann, 1987

E. coli strain	Characteristics or genotype	Reference/origin
JM109	K-12 derivative, *rec*A1, *sup*E44, *end*A1, *hsd*R17, *gyr*A96, *rel*A1, *thi* (? *lac-pro*AB)	Yanisch-Perron *et al*., 1985
LE392	K-12 derivative, *sup*E44, *sup*F58, *hsd*R514, *gal*K2, *gal*T22, *met*B1, *trp*R55, *lac*Y1	Borck *et al*., 1976
35	K-12 derivative, F^-, *trp*$^-$, *phe*$^-$, *pro*$^-$, *his*$^-$, *lac*-, Nal^R	Smith and Linggood, 1971
EN99	K-12 derivative, *ara*D, ? *lac*, *aro*B, *rps*L, *thi*	Blum, 1994
WK6	K-12 derivative, ? (*lac-pro*AB), *gal*E, *str*A, F^+, *lac*I^qZ ? M15, *pro*A^+B^+	Zell and Fritz, 1987
5K	K-12 derivative, Sm^R, *lac*Y1, *ton*A21, ?$^-$, *thr*-1, *sup*E44, *thi*, r, m^+	Blum, 1994
MC1029	K-12 derivative, araD139, ? (*ara*ABC-*leu*)7697, ? *(lac)*X74, *gal*U, *gal*K, *trp*B9601(Am), *str*A	Casadaban *et al*., 1980
DH1	K-12 derivative, *sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *relA1*	Low, 1968
Sy 327	K-12 derivative, F^-, *ara*D, ? (*lac-pro*), *arg*E (Am), Rif^r, *nal*A, *rec*A56, ? pir	Miller and Mekalanos, 1988
Sm10?pir	K-12 derivative, *thi*1, *thr*1, *leu*B6, *sup*E44, *ton*A21, *lac*Y1, *rec*A$^-$::RP4-2-Tc::Mu Km^r ? pir	Miller and Mekalanos, 1988

4.1.2 Plasmids

Table 4.2: Used vectors and plasmids

Plasmid	Genotype	Reference
pUC18	*ori*ColE1, Ap^R, *lac*Za	Yanisch-Perron *et al*., 1985
pETBlue-1	T7 promoter, f1 *ori*, pUC *ori,* *lac*Z, Ap^R	Novagen
pCRT7/CT-TOPO	T7 promoter, pUC *ori*, zeo^R, Ap^R	Invitrogen
pCR 2.1-TOPO	*lac* promoter, pUC *ori,* Ap^R, Kan^R	Invitrogen

Plasmid	Genotype	Reference
pCR-XL-TOPO	*lac* promoter, pUC *ori,* zeo^R, Kan^R	Invitrogen
pACYC Duet-1	T7 promoter, p15A *ori*, *lac*I, cam^R	Novagen
pACYC 184	p15A *ori*, *lac*I, cam^R	Novagen
pGP 704	*ori*R6K, mobRP4, Ap^R	Miller and Mekalanos, 1988
pCVD442	*sac*B in vector pGP704, Ap^R	Donnenberg and Kaper, 1991
pSPD (N/CRD)	*h-spd* (neck, C-terminus with collagen part) in pPic9K, Kan^R, *aox-1* promoter	Ken Read, Oxford
pZ132	*pde* 1B1	G. Quintini, ALTANA Pharma
pALMI-12	*yga*D-*rec*A-*ora*A in pCR 2.1-TOPO	This work, see Table 4.13 and Figure 7.1
pALMI-13	*hly*BD in pETBlue-1	This work, see Table 4.13 and Figure 7.2
pALMI-14	*yga*D-*rec*A´-Stop-*lacO/T7-hly*BD-*rec*A´´-*ora*A in pCVD442	This work, see Table 4.13 and Figure 7.3
pALMI-15	*hly*CA in pETBlue-1	This work, see Table 4.12 and Figure 7.4
pALMI-16	*hsp-d-hly*A$_s$ in pET Blue-1	This work, see Table 4.11
pALMI-17	*omp*A-*hly*A$_s$ in pETBlue-1	This work, see Table 4.11
pALMI-19	*pho*A-*hly*A$_s$ (truncated *pho*A without 63 bp endoding N-terminal signal sequence) in pETBlue-1	This work, see Table 4.11
pALMI-23	*hly*CA in pUC18	Anja Buttkewitz, Diploma thesis 2001
pALMI-26	*yga*D-*rec*A´-Stop-*lacO/T7-hly*BD-*rec*A´´-*ora*A in pCR 2.1-TOPO	This work, see Table 4.13 and Figure 7.5
pALMI-59	*hly*CABD with *hly*C upstream sequence (J96) in pACYCDuet-1	This work, see Table 4.11

Plasmid	Genotype	Reference
pALMI-60	*ccl-21* in vector pQE-30 (Qiagen), T5 promoter, Ap^R	Karsten Keldermann, ALTANA Pharma, 2003
pALMI-63	*hly*A_s*hly*BD with *Eco 47* III cleavage site in pCR-XL-TOPO	This work, see Table 4.11
pALMI-64	*hsp-d* in pALMI-63	This work, see Table 4.11
pALMI-76	*ccl-21* in pALMI-63	This work, see Table 4.11
pALMI-83	*hly*CABD with *hly*C upstream sequence (J96) in pCR-XL-TOPO	This work, see Table 4.11
pALMI-84	*hly*BD in pACYC Duet-1	This work, see Table 4.12 and Figure 7.6
pALMI-89	*yga*D-*rec*A´-Stop-*lacO/T7-hly*BD-*cat-rec*A´´-*ora*A in pCVD442	This work, see Table 4.13
pALMI-121	*hly*C upstream sequence, 3´part *hly*A, *hly*BD (J96) in pACYC-Duet-1	This work, see Table 4.11
pALMI-122	*hly*BD with *hly*C upstream sequences in pACYC Duet-1	This work, see Table 4.12 and Figure 7.7
pALMI-130	*hly*A_s*hly*BD with *hly*C upstream sequences in pACYC Duet-1	This work, see Table 4.12 and Figure 7.9
pALMI-131	*hly*CABD with *hly*C upstream sequence (J96) in pCR-XL-TOPO (mutated *Nsi* I site)	This work, see Table 4.11
pALMI-133	0,8 kb *hly*C upstream sequence with 3´-*Nsi* I site in pCR-XL-TOPO	This work, see Table 4.11
pALMI-135	*pho*A-*hly*A_s (truncated *pho*A without 63 bp encoding N-terminal signal sequence) in pCRT7 /CT-TOPO	This work, see Table 4.12 and Figure 7.10
pALMI-137	*h-spd-hly*A_s-*hly*BD in pETBlue-1	This work, see Table 4.11
pALMI-138	*hly*CABD in pCRT7/CT-TOPO	This work, see Table 4.12 and Figure 7.11

Plasmid	Genotype	Reference
pALMI-139	*pho*A (truncated *pho*A without 63 bp endoding N-terminal signal sequence) in pALMI-130	This work, see Table 4.12 and Figure 7.12
pALMI-140	*hly*CA (without *hly*A_s) in pALMI-130	This work, see Table 4.12 and Figure 7.13
pALMI-141	*hly*CA in pCRT7/CT-TOPO	This work, see Table 4.11 and Figure 7.14
pALMI-168	*hly*CA with *hly*C upstream sequences in pUC18	This work, see Table 4.12 and Figure 7.15
pALMI-176	*ccl-21* including factor Xa cleavage site in pALMI-130	This work, see Table 4.12 and Figure 7.16
pALMI-177	*omp*A-*hly*A_s (truncated *omp*A with 63 bp encoding N-terminal signal sequence) in pCRT7/CT-TOPO	This work, see Table 4.12 and Figure 7.17
pALMI-178	*pho*A-*hly*A_s (truncated *pho*A without 63 bp encoding N-terminal signal sequence and 72 bp encoding C-terminal residue) in pCRT7/CT-TOPO	This work, see Table 4.12 and Figure 7.18
pALMI-186	*pde* 1B1 in pALMI-130	This work, see Table 4.12 and Figure 7.19
pALMI-198	*pho*A (without 63 bp encoding N-terminal signal sequence and 72 bp encoding C-terminal residue) in pALMI-130	This work, see Table 4.12 and Figure 7.20

4.1.3 Cosmids

Table 4.3: Used cosmid

Cosmid	Genotype	Reference
pCOS10	Pai I of uropathogenic *E. coli* (UPEC) 536 in vector pHC79, Ap^R	Knapp *et al*., 1986

4.1.4 Oligonucleotids

Table 4.4: Sequencing primers

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 67	3´-1341-*hly*A start	-	GTGCAGTGGTTATTGTTGTCA
Mibi 68	5´-1320-*hly*A start	+	ATGACAACAATAACCACTGCAC
Mibi 69	3´-4394-*hly*A end	-	TTATGCTGATGTGGTCAGGGTTATGAG
Mibi 71	5´-2841-*hly*A	+	GAAGAAGGAAAACGTCTGGAG
Mibi 72	3´-2861-*hly*A	-	CTCCAGACGTTTTCCTTCTTC
Mibi 73	5´-*hly*A_s	+	TCAACTTATGCAGACCTGGATAATCTG
Mibi 74	3´-*hly*A_s	-	TTATGCTGATGCGGTCAAAGTTATTGA
Mibi 75	5´-4467-*hly*B start	+	ATGGATTCTTGTCATAAAATTGATTATGGG
Mibi 76	3´-4494-*hly*B start	-	CCCATAATCAATTTTATGACAAGAATCCAT
Mibi 77	3´-6589-*hly*B end	-	TTAGTCTGACTGTAACTGATATAAG
Mibi 78	5´- 6565-*hly*B end	+	CTTATATCAGTTACAGTCAGACTAA
Mibi 79	5´-5521-*hly*B	+	GACGAACATATGGGACAAACA
Mibi 80	3´-5541-*hly*B	-	TGTTTGTCCCATATGTTCGTC
Mibi 81	3´-819-*hly*C start	-	CTCTAATGGTTTGTTTATATTCAT
Mibi 84	5´-796-*hly*C start	+	ATGAATATAAACAAACCATTAGAG
Mibi 85	5´-6608-*hly*D start	+	ATGAAAACATGGTTAATGGGGTTC
Mibi 86	3´-8044-*hly*D end	-	TTAACGCTCACGTAAACTTTCTGT
Mibi 88	5´-7311-*hly*D	+	GCCGTCTGGATGATTTCAGTAG

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 89	3´-7332-*hly*D	-	CTACTGAAATCATCCAGACGGC
Mibi 142	5´-1-*hly*C	+	ATGAACAGAAACAATCCATTAGAGGTTCTT
Mibi 226	3`-1413-*pho*A	-	TTTCAGCCCCAGAGCGGCTTTCAT
Mibi 253	3`-790-*pho*A	-	AGGCAGCATCGCTCACCAACTGATAACCAC
Mibi 259	5´-1-*pde*1B1	+	ATGGAGCTGTCCCCCCGCAGTCCTCC
Mibi 267	5´-814-*pde*1B1	+	CAGTCCATTGCTTCTTGCTCCGCACAGGGA
Mibi 268	3´-843-*pde*1B1	-	TCCCTGTGCGGAGCAAGAAGCAATGGACTG
Mibi 269	5`-347-*pho*A	+	CTATAACGGCGCGCTGGGCGTCG
Mibi 270	3`-1020-*pho*A	-	TCGAGATCGACCGTCTCGCC
Mibi 277	5´-4911-*hly*B	+	AATACAGAAAAATATTTATT GAAACCCTTG
Mibi 278	3´-4940-*hly*B	-	CAAGGGTTTCAATAAATATTTTTCTGTATT
Mibi 279	5´-6011-*hly*B	+	CAACGTTTTTATATTCCTGA AAATGGCCAG
Mibi 280	3´-6040-*hly*B	-	CTGGCCATTTTCAGGAATATAAAAACGTTG
Mibi 281	5´-7061-*hly*D	+	CGGTATCAAATTCTGAGCCG GTCAATTGAA
Mibi 282	3´-7090-*hly*D	-	TTCAATTGACCGGCTCAGAATTTGATACCG
Mibi 283	5´-7661-*hly*D	+	ACAGCGGAAACACTGATGGT TATCGTTCCG
Mibi 284	3´-7690-*hly*D	-	CGGAACGATAACCATCAGTGTTTCCGCTGT
Mibi 287	5´-4821-*hly*B	+	AGGGGCATATTATTCTTATT GCTTCCCGTT
Mibi 288	5´-5761-*hly*B	+	GGATTTCCAGCAGGTTGGTA TATCAGTTAC
Mibi 289	3´-5791-*hly*B	-	GTAACTGATATACCAACCTGCTGGAAATCC
Mibi 290	5´-6311-*hly*B	+	ATTGCAAGGGCGCTGGTGAA CAACCCTAAA
Mibi 292	3´-1721-*pde*1B1	-	ATCCAGATTCCCATTCTGGTTGTGTTCA
Mibi 345	5´-351-*omp*A	+	TCTGGGTGGCATGGTATGGCGTGCAGACAC

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 302	5´-1061-*hly*C	+	GGTTCATTGACTGGATTGCT CCTTTCGGGG
Mibi 303	5´-1451-*hly*A	+	TGCGGGAAACAGACTCATTT TACTTATCCC
Mibi 304	5´-1751-*hly*A	+	TGTACTGTCAACGTTTCAAA ATTTTCTGGG
Mibi 305	5´-2101-*hly*A	+	CAGATGCAGATACCGGAACT AAAGCTGCAG
Mibi 306	5´-2451-*hly*A	+	GTATCCTCAGGTATTAGTGC TGCTGCAACG
Mibi 307	5´-3001-*hly*A	+	AGTCCGGAAAATATGAATAT ATTACCGAGT
Mibi 308	5´-3351-*hly*A	+	CAGGAAGTTGTGAAGGAGCA GGAGGTTTCA
Mibi 309	5´-3701-*hly*A	+	TAATGGCGGAGACGGGGATG ATGAGCTTCA
Mibi 310	5´-4001-*hly*A	+	TAAAGCTGAA GGTAATGTTC TTTCCATTGG
Mibi 351	5´-337-*omp*A	+	GACATCTACACTCGTCTGG
Mibi 352	5`-668-*omp*A	+	TCAACTTCAACAAAGCAAC
Mibi 430	3´-1038-*omp*A	-	AGCCTGCGGCTGAGTTACAACGTCTTTG
Mibi 515	3´-424-*pde*1B1 (U86078)	-	GGCCTTTGGCCCGGGCCTGCTGGGTGAAGG
Mibi 516	5´-624-*pde*1B1 (U86078)	+	TTGAACCAGGCAGCAGATGACCATGCCCTG
Mibi 517	5´-1224-*pde*1B1 (U86078)	+	ATCAGCCACCCAACCAAGCAGTGGTTGGTC
Mibi 518	5´-1364-*pde*1B1 (U86078)	+	GGCACAGTCTCAGATAGGGTTCATCGACTT
Mibi 546	5´-408-*pde*1B1 (U86078)	+	GACTGGCTGGCCTCCACCTTCAC
Mibi 1015	5´-*hly*C (J96) upstream	+	GCTGCAGTAAAACACGAGTATACGTC

***If no other accession number is mentioned, the positions of the *hly* genes refer to the 8215 bp nucleotide sequence of the *E. coli* plasmid pHLHYX (Hess *et al*., 1986; accession number M14107). The positions of the *E. coli* genes refer to *E. coli K*-12 (NC000913).**

Table 4.5: Primers used for cloning procedures

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 86	3´-8044-*hly*D end	-	TTAACGCTCACGTAAACTTTCTGT
Mibi 142	5´-1-*hly*C	+	ATGAA AGAAACAATCCATTAGAGGTTCTT
Mibi 145	3´-4394-*hly*A	-	TTATGCTGATGCGGTCAAAGTTATTGAGTT
Mibi 148	5´-ATG-64-*pho*A	+	ATGCGGACACCAGAAATGCCTGTTCTGGAA
Mibi 149	**5´-1393-1416-*pho*A**+5´-1-21 *hly*A_s	+	**AAAGCCGCTCTGGGGCTGAAA**TCAACTTATGCAGACCTGGAT
Mibi 150	3´-21-1-*hly*A_s+**3´-1416-1393-*pho*A**	-	ATCCAGGTCTGCATAAGTTGA**TTTCAGCCCCAGAGCGGCTTT**
Mibi 151	5´-4466-*hly*B	+	ATGGATTCTTGTCATAAAATTGATTATGGG
Mibi 167	5´-1-*yga*D	+	ATGACTGACAGTGAACTGATGCAG
Mibi 168	*3´-501-ora*A	-	TCAGTCGGCAAAATTTCGCCAAATCTCC
Mibi 245	5´-***Cla* I site**-7008-pALMI-13	+	**CCATCGATGG**CTAACCTGACCTAAAATTGTGAGCGCTCACAA
Mibi 246	3´-***Cla* I site**-8044-*hly*D	-	**CCATCGATGG**TTAACGCTCACGTAAACTTTCTGT
Mibi 261	5´-ATG-1-*h-spd*(N/CRD)	+	ATGCCGGGATTGAAGGGGGACAAAGGCATT
Mibi 262	**5´-506-525-*h-spd***+5´-1-20 *hly*A_s	+	**GTCTTGTGGTCTGCGAGTTC**TCAACTTATGCAGACCTGGA
Mibi 263	3´-20-1-*hly*A_s+**3´-525-506 *h-spd***	-	TCCAGGTCTGCATAAGTTGA**GAACTCGCAGACCACAAGAC**
Mibi 292	3´-1721-*pde*1B1 (U86078)	-	ATCCAGATTCCCATTCTGGTTGTGTTCA
Mibi 297	5´-1-*omp*A	+	ATGAAAAAGACAGCTATCGCGATTGCAGTG
Mibi 298	**5´-1019-1038-*ompA***+5´-1-20-*hly*A_s	+	**TTGTAACTCAGCCGCAGGCT**TCAACTTATGCAGACCTGGA

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 299	3´-20-1-*hly*A_s+**3´-1038-1019-*omp*A**	-	TCCAGGTCTGCATAAGTTGA**AGCCTGCGGCTGAGTTACAA**
Mibi 432	3´-526-*hsp-d*	-	GAACTCGCAGACCACAAGACGCTTTTCTCCAC
Mibi 447	5´-***Eco 47*III Site**-1-*hly*A_s	+	**AGCGCT**TCAACTTATGCAGACCTGGATAAT
Mibi 472	5´-***Xba* I**-*hsp-d*	+	**CGAGATCTCG**ATGCCGGGATTGAAGGGGGA
Mibi 493	3´-CCC-**factor Xa**-489 pALMI-60	-	CCC**ACGACCTTCGAT**TGGCCCTTTAGGGGT
Mibi 502	5´-***Xba* I Site**-*ccl-21*	+	**GCTCTAGA**ATGAGGATGGAGGGGCTCAGG
Mibi 631	5´-***Bam* HI**-4466-*hly*B	+	**CGGATCCG**ATGGATTCTTGTCATAAAATT
Mibi 644	5´-***Bam* HI**-786 bp upstream *hly*C	+	**CGGGATCCCG**ATTGTGCGAAGGCATGGCATATTT
Mibi 702	3´-4540-*hly*B	-	AACAGAGACGTTATGGTATTGGGCTAA
Mibi 739	3´-***Nsi* I**-*hly*C upstream (J96)	-	**AACTGCAGAACCAATGCAT**TATAAATGATACTATCTCCATTGATAA
Mibi 742	5´-799-*hly*C	+	TGAACAGAAACAATCCATTAGAGGTTCTTGGGCATGTATCC
Mibi 743	3´-4211-*hly*A	-	TGCATCATTCCCATACACATAACTTGCCTT
Mibi 744	5´-2-*pho*A	+	TGCGGACACCAGAAATGCCTGTTCTGGAA
Mibi 745	3´-1413-*pho*A	-	TTTCAGCCCCAGAGCGGCTTTCATGGTGTA
Mibi 746	5´-*hly*C upstream sequence (J96) mutagenesis primer	+	GGCAGCGTCGTATGCTTATGTTTTTATTTC
Mibi 747	3´-*hly*C upstream sequence (J96) mutagnesis primer	-	GAAATAAAAACATAAGCATACGACGCTGCC

Oligonucleotide designation	Gene specifity and position	Forward (+) or reverse (-)	Sequence (5`-3`)
Mibi 799	5´-**TG**-157-pALMI-60	+	**TG**AGTGATGGAGGGGCTCAGGACTGTT
Mibi 808	3´-*pho*A-1341	-	ATGCGGGCCATACGCCGCAATACGCAA
Mibi 977	5´-117-*pde*1B1	+	TGGAGCTGTCCCCCCGCAGTCCTCC
Mibi 1028	5´-ATG-64-*omp*A	+	ATGGCTCCGAAAGATAACACCTGGTACACT

4.1.5 Antibodies

Table 4.6: Antibodies used for Western blotting

Antibody designation	Conjugation	monoclonal/polyclonal	Source
Goat anti-rabbit	Horse radish peroxidase	monoclonal	Diagen
Rabbit anti-$HlyA_S$	-	polyclonal	Trenzyme
Rabbit anti-Ccl-21 (Anti-human 6Ckine) (rabbit)	-	polyclonal	R&D Systems
Rabbit anti-PhoA (Anti-alkaline phosphatase)	-	polyclonal	Chemicon

4.1.6 Bacterial growth media

All Solutions and media were autoclaved.

LB medium: 0,5% NaCl (Merck)
1% Bacto tryptone (Difco)
0,5% Yeast Extract (Difco)
H_2O_{bidest}

LB agar with 100 µg/ml IPTG, 100 µg/ml Xgal and 50 µg/ml ampicillin:

Product of MBI Fermentas

BHI-medium: 185 g brain heart infusion bouillon (Difco)
12,5 g Yeast Extract (Difco)
fill up with H_2O_{bidest} to 5 l

MacConkey agar (GIBCO BRL): 8,5 g/l peptone 190
8 g/l peptone 100
5 g/l peptone 140
10 g/l lactose
12 g/l agar
1,5 g/l bile salts No.3
5 g/l NaCl
0,03 g/l Neutral red
0,001 g/l Crystal violett
H_2O_{bidest}

Soc medium (Invitrogen^{Tm}): 2% tryptone
0,5% Yeast Extract
2,5 mM KCl
10 mM $MgCl_2$
20 mM glucose
H_2O_{bidest}

Blood agar plates (Oxoid): Columbia Agar with sheep blood Plus

M9 Medium: M9 Salts: 6 g/l Na_2HPO_4
3 g/l KH_2PO_4
1 g/l NH_4Cl
H_2O_{bidest}

Subsequent addition of a sterile filtered solution, consisting of:

2 ml 1 M $MgSO_4$
0,4% glucose
100 µl 1 M $CaCl_2$
0,004% thiamine

Medium for freezing of bacteria strains:

750 µl of an overnight culture were frozen in 250 µl 87% Glycerol (sterile) at –70°C.

4.1.7 Supplements

Table 4.7: Antibiotic supplements for selective growth media of *E. coli*.

	Ampicillin (Sigma)	Kanamycin (Sigma)	Chloramphenicol (Sigma)
Stock solution	50 mg/ml	50 mg/ml	20 mg/ml
Final concentration	50 µg/ml	25 µg/ml	20 µg/ml
Diluted in	H_2O_{bidest}	H_2O_{bidest}	Ethanol

The antibiotics that were diluted in H_2O_{bidest} were sterile filtered using a 0.45 µM-micron filter. The antibiotic was added to the culture medium at 45°C.

Xgal (5-Bromo-4-Chloro-3-Indolyl-ß-galactoside)

(MBI Fermentas)

Stock solution: 2% Xgal solution in dimethylformamide

Ten ml per litre of stock solution was added to the culture medium (45°C).

IPTG (1-Isopropyl-ß-D-1-thiogalactopyranoside) (Merck)

Stock solution: 100 mM

Ten ml per litre of stock solution was added to the culture medium (45°C).

4.1.8 Reagents

Sterile $H_2O_{bidest.}$ was used for all buffers and solutions.

Reagents:

Agarose (Seakem, FMC Bio Products)
Ammonium chloride (Merck)
Boric acid (Merck)
Bromphenol blue (Merck)
BSA (Sigma)
Calcium chloride (Merck)
Disodium hydrogen phosphate (Merck)
Dimethyl formamide (Merck)
EDTA (Titriplex III, Merck)
Ethanol (Merck)
Isopropanol (Merck)
Ethidium bromide (Sigma)
Glucose (Sigma)
Glycerol (Merck)
87% Glycerol (Merck)
Kalium dihydrogen phosphate (Merck)
Magnesium sulfate (Merck)
Sodium acetate (Merck)
Sodium chloride (Merck)
Sieving Agarose (Seakem, Biozym)
Thiamine (Sigma)
Trisbase (Tris(hydroxymethyl-)aminomethan) (Merck)
Xylene cyanol (Sigma)

4.1.9 Equipment and computer software

Analysis balance: *Mettler AE 163, Mettler PM 4600 Delta Range*

Centrifuges: *Hermle Z 360 K, Sorvall RC 5 C, Eppendorf 5417 R*

Computer programs: *Microsoft Office 2000,Vector NTI Suite 6, Paint Shop Pro 6*

DNA- und RNA gele chambers: *Bio-Rad*

Elektroporator: *Bio-Rad Gene Pulser II*

Freezer –20°C: *Liebherr Premium*

Freezer –80°C: *Heraeus Hera freeze*

Fridge: *Liebherr Premium*

Incubator: *Heraeus Typ B 5060 E*

Magnetic stirrer: *Klett Summerson*

Micropipets: *Heidolph MR 2000*

Microwave: *Siemens Typ HF 0616*

Mixer: *Vortex Genie 2*

Power Supply for gel electrophoresis: *Phero-stab 500 Biotech Fischer, Bio- Rad Power Pac 300, Apelex PS 304*

PCR cycler: *Eppendorf Mastercycler gradient, Biometra UNO II*

pH meter: *Knick Digital pH-Meter*

Photometer: *Perkin Elmer UV/VIS-Spectrometer Lambda 25, Unicam UV500 (Thermo Spectronic), Gene Quant II (Pharmacia Biosciences)*

Pipets: *Gilson pipetman P2, P10, P20, P100, P1000 Eppendorf Reference, Eppendorf Research pro*

Pipet boy: *Hirschmann Laborgeräte pipetus akku*

Printer: *HP Laserjet 4000*

Protein mini gel chamber: *Bio-Rad Mini-Protean II*

Shaker: *B. Braun Biotech International Certomat R, GFL 3017*

Lamina flow: *Heraeus Typ HS 12 Herasafe*

Table top centrifuge: *Eppendorf Centrifuge 5415C*

Table incubator*: Eppendorf Thermomixer 5436*

UV transilluminator: *MWG Biotech, Bachofer 312 nm*

Vacuum manifold: *Promega Vac-Man Laboratory Vacuum Manifold, KNF Neuberger Laboport*

Western blot apparatus*: Hoefer scientific instruments*

4.2 Methods

4.2.1 Isolation of genomic bacterial DNA

The Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) was used for the isolation of genomic bacterial DNA. Its preparation was carried out according to the protocol provided by Promega.

4.2.2 Isolation of bacterial plasmid DNA

Bacterial plasmids were isolated using the NucleoSpin-Kit (Macherey/Nagel, Düren) or the Fast Plasmid Mini Kit (Eppendorf, Hamburg). The HiSpeed Plasmid Midi Kit (Qiagen, Hilden) was used for the plasmid midi preparations. The plasmid preparations were carried out according to the protocol provided by the manufacturers.

4.2.3 Isolation of total bacterial RNA

Isolation of total RNA from *E. coli* was carried out using the MasterPure^{TM} Complete DNA and RNA Purification Kit (Epicentre, Madison, USA) according to the protocol provided by the manufacturer.

4.2.4 Precipitation of DNA

For DNA precipitation, the DNA containing solution was mixed with two volumes of ethanol and 1/10 volume of 3 M sodium acetate pH 4,8 , incubated for several hours or over night at –70°C, consecutively centrifuged for 15 min at 4°C. The resulting pellet was washed with 70% ethanol for 3 min at 4°C to remove residual salts. The supernatant was carefully discarded. The DNA containing pellet, was lyophilized in a vacuum centrifuge for 10 min and consecutively resuspended in an appropriate volume of buffer or H_2O.

4.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation, identification and purification of DNA. An appropriate concentration of agarose was used for separation of fragments with different lengths as described in the Table 4.8.

Table 4.8: Agarose gel concentration used for the separation of DNA fragments of different length

Agarose [%]	Fragment length [kb]
0,5	1-30
0,7	0,8-12
1,0	0,5-10
1,2	0,4-3
1,5	0,2-3
2 (Sieving Agarose)	0,01-1

For production of agarose gels, agarose was added to 1x TBE buffer and heated in a microwave until it was solved. After cooling of the agarose solution to about 50°C, ethidium bromide was added at a final concentration of 5 µl/100 ml. The agarose gel was poured at room temperature. Before loading the gel with DNA samples, 1x sample buffer was added to the samples. Electrophoresis was carried out in horizontal gel chambers in 1x TBE buffer at 100-140 V for a minimum of 30 min. The DNA was visualized by using UV light. For documentation, photos were taken with a video camera (Gene genius, Bio imaging system).

Stock solution 10xTBE:
108 g/l Trisbase
55 g/l Boric acid
40 ml/l 0,5 M EDTA
$H_2O_{bidest.}$

10x sample buffer:
50% Glycerol
0,4% Bromphenol blue
0,4% Xylene cyanol
$H_2O_{bidest.}$

DNA ladder

The following DNA markers were used for estimation of the length of DNA fragments:

100 bp ladder: 100 Base-Pair ladder (Pharmacia Amersham, Freiburg)
1 kb ladder: Smart ladder (Eurogentec)

4.2.6 DNA elution from agarose gels

The Gel Extraction Kit (Qiagen, Hilden) or the S.N.A.P. UV free Gel Extraction Kit (Invitrogen, Karlsruhe) were used for DNA elution from agarose gels. If the Gel Extraction Kit was applied, the DNA was visualized by using Gel Star (BioWhittaker, Walkersville, USA). The method was carried out according to the protocol provided by manufacturers.

4.2.7 Determination of DNA concentration and purity

The concentration and purity of plasmid DNA, gel eluted DNA, chromosomal DNA or PCR products were determined either by agarose gel electrophoresis or by photometry using a Gene Quant II (Pharmacia Biosciences) to measure OD_{260} and OD_{280} of the DNA solutions. OD_{260}/OD_{280} is a value for the purity of DNA. A value of 1,7-2,0 is defined as pure.

4.2.8 DNA restriction

Enzymes and appropriate buffers of Pharmacia Amersham, Freiburg and Roche Diagnostics, Mannheim were used. The DNA restriction was carried out according to the protocol provided by manufacturers.

4.2.9 Cleavage of 3´-overhanging DNA ends or refilling of 5´recessed ends

T4 DNA polymerase (Pharmacia Amersham, Freiburg) or the Klenow enzyme (Pharmacia Amersham) were used for the cleavage of 3´-overhanging ends or the refilling of 5´-recessed ends, respectively to generate blunt ends. The reactions were carried out at 37°C according to the protocol provided by Pharmacia Amersham.

4.2.10 Dephosphorylation of DNA fragments

„Shrimp alkaline phospatase“ (Roche Diagnostics, Mannheim) or the Calf intestine alkaline phosphatase (Pharmacia Amersham, Freiburg) were used for dephosphorylation of DNA fragments. The reactions were carried out at 37°C according to the protocol provided by manufacturers.

4.2.11 Polymerase chain reaction (PCR)

PCRs were carried out either in PCR cyclers „Mastercycler gradient“ (Eppendorf) or in „Uno II“ (Biometra) using microwell plates (Eppendorf/ Whatman) or PCR-tubes (Eppendorf/Whatman) in a volume of 50 µl according to the information provided by the manufacturer. Different DNA polymerases were used for different applications: Taq polymerase (Invitrogen-Gibco, Karlsruhe), polymerases of Roche Diagnostics, Mannheim (Expand ^{TM} Long Template PCR-System), Eppendorf, Hamburg (Triple Master PCR System) or Stratagene, La Jolla, USA (Herculase). For generation of blunt ends the PfuTurbo polymerase of Stratagene was used. The cycling parameters were chosen according to the protocol provided by the manufacturers of the polymerases: The annealing temperature was two degree below the lowest melting temperature of the primers and was calculated according to: Tm [°C] = 69,4+ 0,41 x GC content [%] -650/ length of primer.

For DNA amplification used whole bacterial colonies as the template source, a bacterial colony was picked from the agar plate and resuspended in 50-100 µl H_2O. The bacterial suspension was heated to lyse the bacteria and to denature DNA for 10 min at 95°C. Consecutively, it was stored on ice to prevent renaturation. 1 - 5 µl of the bacterial suspension was used in a PCR reaction with a volume of 50 µl.

4.2.11.1 Crossover-PCR

Crossover-PCR (see Figure 4.1) was used to generate gene fusions between two genes designated genes A and B. In the first PCR reaction, a forward primer, encoding 20-30 bp of the 5´region of gene A (start), and a reverse primer, encoding 20 bp of the 3`region of gene A (end) with additional about 10 bp of the 3` part of gene B (start), as well as gene A was used.

In a second PCR a forward primer encoding about 20 bp of the 5`region of gene B (end) with an additional small 5`region of gene A (start) and a reverse primer encoding 20-30 bp of the 3`region of gene B (start), as well as gene B was used.

The purified PCR products of the first and second PCR were used as templates in the third PCR reaction using a forward primer encoding about 20-30 bp of the 5´region of gene A and a reverse primer encoding about 20-30 bp of the 3`region of gene B. This three steps procedure resulted in a fusion between the gene A and gene B.

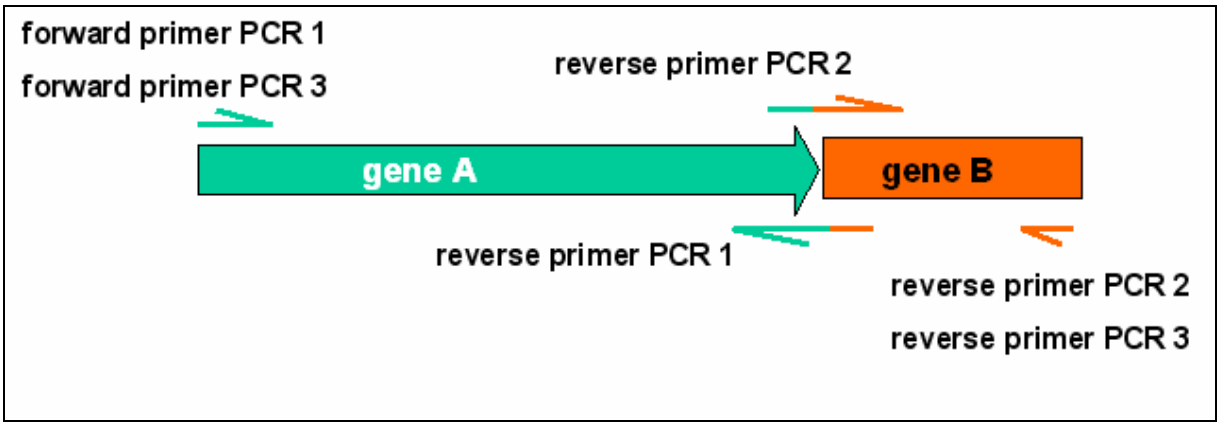


Figure 4.1: Principle of Crossover-PCR

4.2.11.2 Reverse transcriptase (RT) PCR

First-Strand cDNA Synthesis Kit (Pharmacia Amersham, Freiburg) was used for the generation of cDNA from an RNA template. The reactions were performed according to the protocol provided by Amersham. One µg of total RNA was used for the PCR reaction with 0,2 µg random hexanucleotide primers (Pharmacia Amersham, Freiburg) in a final volume of 15 µl. If the cDNA was used as template for TaqMan PCR, it was diluted 1:25 in 0,1x TE buffer.

4.2.12 Real Time TaqMan PCR

Real Time TaqMan PCR (Applied Biosystems, USA) was applied for relative quantification of the *hly* transcripts in different *E. coli* strains using a so-called Fluorescence Resonance Energy Transfer (FRET): The probes have a reporter dye (fluorophore) at their 5`end and a quencher dye (quencher) at the 3`end. The

reporter dye´s emission is suppressed by the quencher dye as a result of the close proximity of the dyes. Thus, the probe is only weakly fluorescent when excited by a light source. The TaqMan probe is highly specific and anneals between the upstream and downstream primer during the PCR. During PCR the AmpliTaq Gold DNA Polymerase (TaqMan PCR Universal Master Mix, Applied Biosystems) with 5´-3´exonuclease activity cleaves the fluorophore from the probe. As a consequence, the fluorophore removes itself from the quencher, because of which fluorescence can be detected. Its amount is in direct proportion to the amount of target DNA accumulated during the PCR. An unspecific DNA bound probe will rather be displaced by the polymerase before being cleaved.

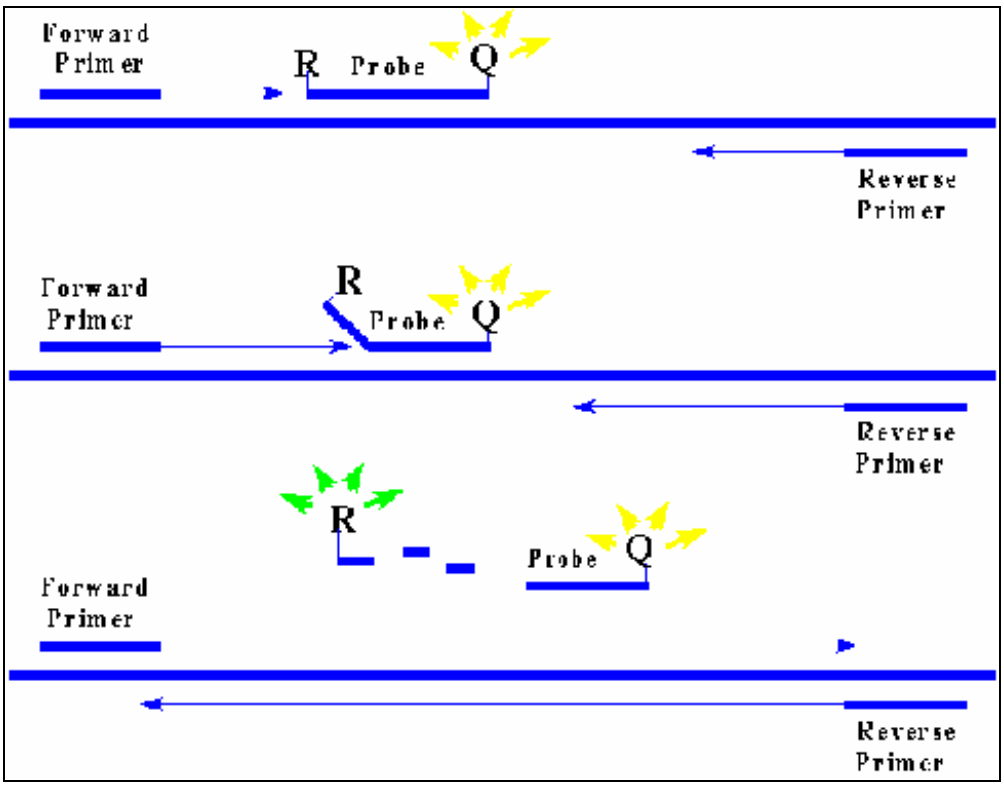


Figure 4.2: Principle of TaqMan PCR. The reporter (R) dye is illustrated in green, the quencher (Q) dye in yellow. When the target sequence is amplified, the probe is cleaved and the reporter starts to fluorescence. The fluorescence signal is detected by the Perkin Elmer Abi Prism 7700 Sequence Detector.

The PCR reaction parameters of the TaqMan PCR reaction are listed in Table 4.9. The probes and primers used in TaqMan PCR are summarized in Table 4.10. Primers and probes were diluted in 0,1xTE buffer. Probes were labelled with the

fluorescence reporter FAM at the 5´end and the fluorescence quencher TAMRA at the 3´end. The *pho*A gene was detected using a vic-labeled probe.

Table 4.9: TaqMan PCR reaction parameters

reagent	concentration stock	concentration final	µl per 1 rxn
2 x TaqMan Universal MasterMix	2 x	1 x	12,50
FP forward primer	10 µM	900 nM	2,25
RP reverse primer	10 µM	900 nM	2,25
P probe	10 µM	200 nM	0,50
template (cDNA)	diverse	diverse	2,50
water			5,00
total volume µl			25,00

Table 4.10: Primers and probes used for TaqMan PCR reaction

primer and probes	length (bases)	melting temperature [°C]	Position	Sequenz 5´- 3´	Label
forward primer *pho*A	21	59	766-786	GCAGTCACCTGTACGCCAAAT	no
reverse primer *pho*A	21	59	820-840	AATGGCTTTGTCGGTCATCTG	no
probe for *pho*A	29	68	790-818	CAACGTAATGACAGTGTACCAACCCTGGC	5´-Vic/Fam 3´-TMARA
forward primer *hly*A	21	58	253-273	GGCACGGCGATTACTAAACAG	no
reverse primer *hly*A	28	59	318-345	GTCTAATTGTGGTGCAAAGATAGTCACT	no
probe for *hly*A	22	68	295-316	CTCATTGGCCTCACCGAACGGG	5´-FAM 3´-TMARA
forward primer *hly*B	20	58	672-691	TGTTGAGTTGGGTGCCAAAC	no
revese primer *hly*B	22	60	727-748	CACCAACACGACGACTCTCAAA	no
probe for *hly*B	22	68	696-717	CCGGCATTTACTGGCGCTACCG	5´-FAM 3´-TMARA
forward primer *hly*C	25	59	239-263	AAGACTGGACTTCAGGTGATCGTAA	no
reverse primer *hly*C	21	58	294-314	TTGTACAGGGCACCGTTATCC	no
probe for *hly*C	27	69	265-292	TGGTTCATTGACTGGATTGCTCCTTTCG	5´-FAM 3´-TMARA
forward primer *hly*D	24	59	196-219	GGGTTTCTGGTTATTGCTTTCATT	no
reverse primer *hly*D	24	59	255-278	CTGAGTGTTAATTTCCCATTTGCA	no
probe for *hly*D	26	68	228-253	TTTAGGCCAGGTGGAAATTGTTGCCA	5´-FAM 3´-TMARA

4.2.13 Ligation

T4-DNA–Ligase (NEB, Frankfurt) was used for DNA ligation which was carried out according to the protocol provided by NEB. 20-200 ng linearized purified vector was used in the ligation reaction. For sticky-end ligations, a 3-5 fold molar excess of

purified insert over vector, for blunt-end ligations, a 10 fold molar excess of insert over vector was used. The amount of insert was calculated according to the following formula:

$$\frac{\text{insert (bp)}}{\text{vector (bp)}} \times \text{ng vector} \times \begin{cases} \text{x 3 (sticky ends)} \\ \text{x 5-10 (blunt ends)} \end{cases} = \text{ng insert}$$

4.2.14 Capillary sequencing

A PCR reaction using a single primer precedes the sequencing reaction. A mixture of all four dNTPs each present in limiting quantities and each labelled with a dye that fluoresces at different colour is used in this PCR reaction. Because all four unlabeled nucleotides are also present, chain elongation proceeds normally, until a labelled dNTP was inserted instead of a nucleotide. After the PCR reaction, there are amplified fragments of different length ending with a dye terminator. The fragments having a negative charge move through the capillaries of the sequencing machine toward the positive charged pole. Shorter fragments move faster than longer fragments. A laser excites the dyes that are analyzed by a detector.

The PCR for the sequencing reaction was carried out in GeneAmp 9700 PE (Perkin Elmer) PCR cyclar. The standard PCR reaction was performed for 10 sec at 96°C, for 5 sec at 50°C and for 2 min at 60°C with 25 cycles in a volume of 10 µl. The amount of plasmid DNA was 250-300 ng, the amount of the used primer was 5 pmol. HPLC purified water (Merck) was used in all reactions. The reaction buffer and the corresponding nucleotide mix (Applied Biosystems) were used according the protocol provided by Applied Biosystems. After the PCR, the reactions were filled up with H_2O to 20 µl and purified with DyeEx 2.0 Spin Kit (QIAGEN). The sequencing reaction was started with 10 µl purified PCR reaction and 10 µl formamide in ABI Prism 310 and ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

4.2.15 Site specific mutagenesis *in vitro*

The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) was used for site specific mutagenesis: e.g. introduction of point mutations, insertions or deletions. The carrying out was done according to the protocol provided by Stratagene.

4.2.16 Plasmid constructs

The plasmids constructed in this study are described in Table 4.11-4.13.

Table 4.11: Generation of plasmids used for further construction of secretion plasmids (see Table 4.12).

Plasmid designation	Basic vector	Restriction site/ Overhang	Cloned Insert	Source of insert	Primer for amplification of insert
pALMI-16	pETBlue-1	T/A	*hsp-d-hly*A_s	*P_SP_D(N/CRD)* pCOS10	Mibi 261 Mibi 262 Mibi 263 Mibi 145 Crossover PCR
pALMI-17	pETBlue-1	T/A	*omp*A-*hly*A_S	*E. coli* C 600 lysate pCOS10	Mibi 297 Mibi 299 Mibi 298 Mibi 145 Crossover PCR
pALMI-19	pETBlue-1	*Eco* RV	*pho*A-*hly*A_S **Truncated:** 63 bp encoding N-terminal signal sequence	*E. coli* C 600 lysat pCOS10	Mibi 148 Mibi 150 Mibi 149 Mibi 145 Crossover PCR
pALMI-59	pACYCDuet-1	*Not* I, *Kpn* I	*Kpn* I-*hly*C upstream sequence-*hly*CABD-*Not* I	pALMI-83 x *Not* I and *Kpn* I: 7.9 kb fragment	-
pALMI-63	pCR-XL-TOPO	T/A cloning	*hly*A_s*hly*BD	pCOS10	Mibi 447 Mibi 86
pALMI-64	pALMI-63	*Xba* I, *Eco* 47 III	*Xba* I-*hsp-d* (without stop)	pALMI-16	Mibi 472 Mibi 432
pALMI-76	pALMI-63	*Xba* I, *Eco* 47 III	*ccl*-21 with additional sequences encoding factor Xa	pALMI-60	Mibi 502 Mibi 493
pALMI-83	pCR-XL-TOPO	T/A	*hly*CABD with *hly*C upstream region (789 bp)	UPEC J96 lysate	Mibi 644 Mibi 86

Plasmid designation	Basic vector	Restriction site/ Overhang	Cloned Insert	Source of insert	Primer for amplification of insert
pALMI-121	pALMI-59	*Nsi* I	No insert, religation of Nsi I restricted pALMI-59 (8,5 kb fragment)	-	-
pALMI-131	pALMI-83	-	Site directed mutagenesis of *Nsi* I site	-	Mibi 746 Mibi 747
pALMI-133	pCR2.1-TOPO	T/A	*Bam* H I-*hly*C upstream sequence-*Nsi* I	pALMI-131	Mibi 644 Mibi 739
pALMI-137	pETBue-1	T/A	*h-spd-hly*A_s-*hly*BD	pALMI-64	Mibi 261 Mibi 86
pALMI-141	pCRT7/CT-TOPO	T/A	*hly*CA	pCOS10	Mibi 142 Mibi 145

Table 4.12: Plasmid constructs generated for secretion studies of $HlyA_s$-fusion protein:

Name	Basic vector	Restriction site/Overhang	Cloned Insert	Source of insert	Primer for amplification of insert
pALMI-15	pETBlue-1	*Eco* RV	*hly*CA	pCOS10	Mibi 142 Mibi 145
pALMI-84	pACYC-Duet-1	*Bam* HI, *Eco* RV	5´-*Bam* HI-*hly*BD	pCOS10	Mibi 631 Mibi 86
pALMI-122	pALMI-84	*Hpa* I	5´-795 bp of *hly*C upstream sequence -251 bp-*hly*A -74 bp-*hly*B-3´	pALMI-121	Mibi 644 Mibi 702
pALMI-130	pALMI-122	*Bam* H I, *Nsi* I	*hly*C upstream sequence	pALMI-133 restricted with *Bam* H I and *Nsi* I	-
pALMI-135	pCRT7/CT-TOPO	T/A cloning	***pho*A-*hly*A_s** **Truncated *pho*A:** 63 bp encoding N-terminal signal sequence	pALMI-19	Mibi 145 Mibi 148

Name	Basic vector	Restriction site/Overhang	Cloned Insert	Source of insert	Primer for amplification of insert
pALMI-139	pALMI-130	*Nsi* I, after blunting	***pho*A** (blunt) * **Truncated:** 63 bp encoding N-terminal signal sequence	pALMI-19	Mibi 744 Mibi 745
pALMI-140	pALMI-130	*Nsi* I, after blunting	*hly*CA (blunt) *	pALMI-15	Mibi 742 Mibi 743
pALMI-168	pALMI-23	*Bam* H I	0,8 kb *hly*C upstream sequence and part of *hly*C	pALMI-140 restricted with *Bam* H I : 1,1 kb fragment	-
pALMI-176	pALMI-130	*Nsi* I, after blunting	***ccl*-21** (blunt) * with additional sequences encoding factor Xa	pALMI-76	Mibi 799 Mibi 493
pALMI-177	pCRT7/CT-TOPO	T/A cloning	***omp*A-*hly*A$_s$** **Truncated *ompA***: 63 bp encoding N-terminal signal sequence	pALMI-17	Mibi 1028 Mibi 145
pALMI-178	pCRT7/CT-TOPO	T/A cloning	***pho*A-*hly*A$_s$** **Truncated *phoA***: 63 bp encoding N-terminal signal sequence and 72 bp encoding C-terminal residue	pALMI-198	Mibi 145 Mibi 148
pALMI-186	pALMI-130	*Nsi I* after blunting	***pde* 1B1** (blunt) *	pZ132	Mibi 977 Mibi 292
pALMI-198	pALMI-130	*Nsi* I, after blunting	***pho*A** (blunt) * **Truncated**: 63 bp encoding N-terminal signal sequence and 72 bp encoding C-terminal residue	pALMI-19	Mibi 744 Mibi 808

* The PCR product should not have a start codon, but an additional TG at the 5`terminus to retain the start codon ATG after insertion into the blunted *Nsi* I site. It also should not have a stop codon for fusion with the signal sequence.

Table 4.13: Plasmids constructs for generation of suicide vector pALMI-89

Name	Basic vector	Restriction site	Cloned Insert	Source of insert	Primer for amplification of insert
pALMI-12	pCR 2.1-TOPO	T/A	*yga*D-*rec*A-*ora*A	*E. coli* J 53 lysate	Mibi 167 Mibi 168
pALMI-13	pETBlue-1	T/A	*hly*BD	pCOS10	Mibi 151 Mibi 86
pALMI-14	pCVD442	*Sph* I/*Sac* I	*yga*D-*rec*A´-*Stop-lacO/T7-hly*BD-*rec*A´´-*ora*A	pALMI-26 restricted with *Sac* I and *Sph* I: 6,3 kb fragment	-
pALMI-26	pALMI-12	*Cla* I	*Stop-lacO/T7-hly*BD	pALMI-13	Mibi 245 Mibi 246
pALMI-89	pALMI-26	*Pme* I	*cat*	pACYC-184	Trenzyme supplied by 5'-*Sma* I-*cat* 3'-*Sma* I-*cat*

4.2.17 Integration of the T7 promoter controlled *hly*BD genes into the bacterial chromosome of the *E. coli* strain BL21:DE3

Plasmid pALMI-89 (Table 4.13) was used as vector for integration of the T7 promoter controlled *hly*BD genes into the bacterial chromosome of the *E. coli* strain BL21:DE3. It is a lamda pir-dependent suicide plasmid that can only replicate in bacteria harbouring the Pir protein, such as in the *E. coli* K-12 strains Sy327 and Sm10 lamda pir. Plasmid pALMI-89 confers ampicillin resistance and sensitiveness against growth in the presence of 5% sucrose at a growth temperature of 30°C. It also contains the T7 promoter controlled *hly*BD genes, including the gene for the chloramphenicol acetyl transferase as a second selection marker, flanked with 1 kb *rec*A gene specific sequences. The chromosomal *rec*A gene in the *E. coli* strain not possessing the Pir protein, will be destroyed following introduction of plasmid pALMI-89 and a double crossover event between the *rec*A sequences on plasmid pALMI-89 and the chromosomal *rec*A gene, in consequence of which, the *hly*B and *hly*D genes will be inserted into the chromosomal *rec*A gene. The positive selection of cointegrants and integrants were done using LB agar plates supplemented with 5, 7.5, 10, 12.5 and 20 µg/ml chloramphenicol.

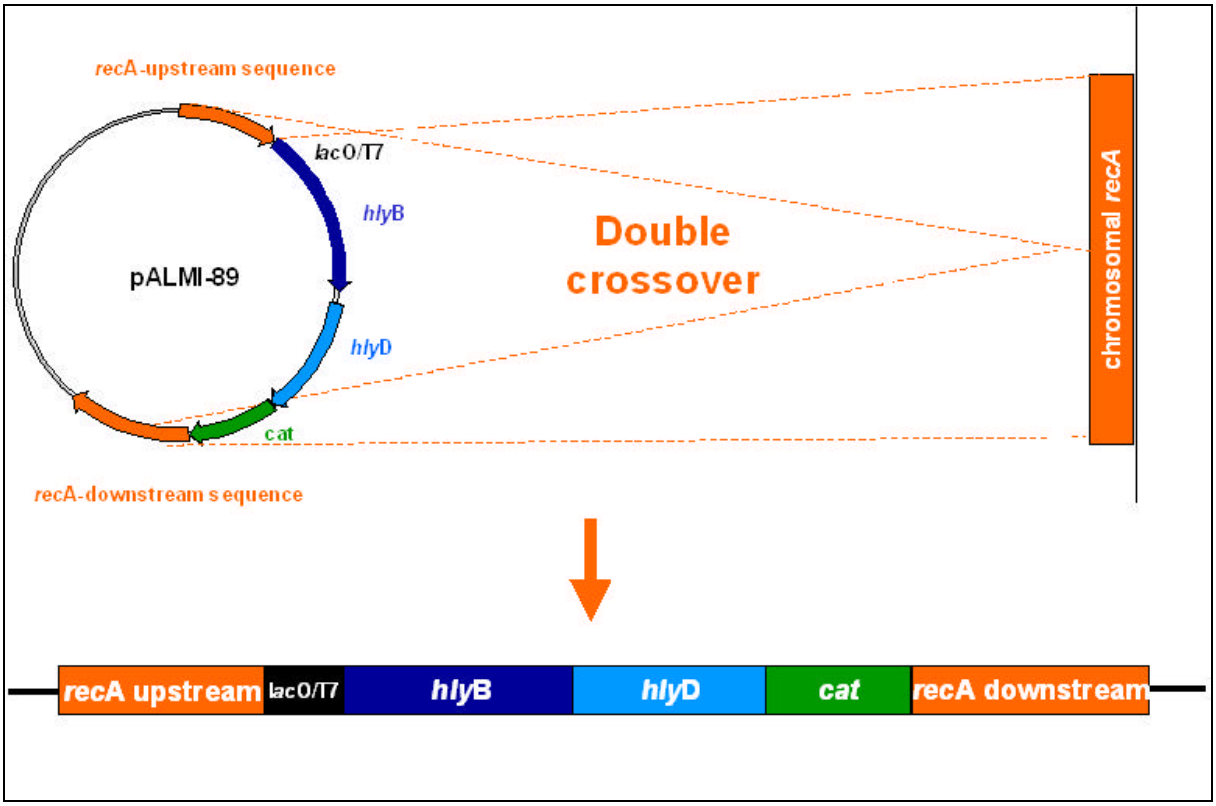


Figure 4.3: Integration of T7 promoter controlled *hly*BD into the chromosomal *rec*A gene of the *E. coli* BL21:DE3

4.2.18 Production of competent *E. coli*

4.2.18.1 Production of chemically competent E. coli

Following over night growth of the *E. coli* strains, 1% cultures were prepared in a final volume of 50 ml. Growth occurred in a shaker flask at 37°C until the logarithmic phase.

35 ml of this culture was centrifuged for 10 min at 4°C and 5000 rpm. The bacterial cell pellet was resuspended in 17,5 ml ice-cold 50 mM $CaCl_2$- solution and incubated for 30 min on ice. After centrifugation for 10 min at 4°C and 5000 rpm the pellet was resuspended in 2,5 ml ice-cold 50 mM $CaCl_2$-solution. The bacterial cells were used either immeditately for transformation or were frozen at –70°C after addition of 1,5 ml 50% glycerol and 150 µl 500 mM $CaCl_2$.

4.2.18.2 Production of electro-competent E. coli

Following overnight growth of the *E. coli* strains, 1% cultures were prepared in a volume of 1 l. Growth occurred in a shaker flask at 37°C until the logarithmic phase.

The culture was divided in 4 x 250 ml and was placed on ice. The cultures were centrifuged for 25 min at 4000 rpm and 4°C. After centrifugation, the bacterial cell pellet was resuspended in 200 ml 10% ice-cold sterile water. After second centrifugation, it was resuspended in 100 ml ice-cold sterile water. After a third centrifugation, it was resuspended in 20 ml ice-cold 10% glycerol. Consecutively, 40 ml of the bacterial suspension was centrifuged for 10 min at 4000 rpm and 4°C. The pellet was resuspended in 1 ml ice-cold 10% glycerol and 40 µl aliquots were frozen in liquid nitrogen and stored at –70°C.

4.2.19 Transformation

4.2.19.1 Chemical transformation

A: 50 µl chemically competent bacteria were placed on ice and mixed with 1-100 ng DNA. The bacterial cells were incubated on ice for 30 min. After heat shock for 90 sec at 42°C in a water bath, the bacteria were placed on ice for 1-2 min.

B: The bacterial cells were diluted in 5 ml medium and grown for 1 h or overnight in a shaker flask at 37°C. The incubation time depends on the resistence marker of the plasmid. For integration of plasmids into the chromosome, the bacterial cells were incubated over night.

50 -100 µl of the bacterial culture were plated onto selection plates.

4.2.19.2 Electroporation

40 µl of electro-competent bacteria were incubated on ice, mixed with 1-100 ng DNA and electroporated under the following conditions:

Capacity: 25 µF
Voltage: 1,5 kV
Resistance: 400 Ohm

After electroporation, the transformed bacterial cells were grown in an appropriate medium. The further steps were performed according to the protocol used for chemical transformation (see 4.2.13.1 B).

4.2.20 Protein detection

The BioRad Protein Assay (BioRad) was used for protein detection. Bovine Serum Albumin (BSA) was used as standard.

4.2.21 Production of bacterial cell lysates

Following liquid growth of *E. coli* and centrifugation for 10 min at 10000 rpm and 4°C, the bacterial pellet was heated for 10 min at 95°C in a sample buffer containing 2% SDS.

4.2.22 TCA-precipitation

For protein precipitation, the supernatants of *E. coli* cultures were mixed with 1/10 10% Trichloroacetic acid (w/v) and incubated on ice for 1 h. Consecutively, the samples were centrifuged for 30 min at 11000 rpm and 4°C. The bacterial cell pellet was washed with 70% (v/v) acetone for 15 min at 11000 rpm and 4°C and dried in a vacuum centrifuge.

4.2.23 Polyacrylamide gel electrophoresis (PAGE)

4.2.23.1 One-dimensional SDS-PAGE

The separation of proteins according to their molecular weight was carried out in a discontinuous buffer system (Laemmli, 1970). Electrophoresis occurred according to Schägger und Jagow for separation of proteins with low molecular weight. The composition of the stacking and separation gel of both gel systems is listed in Table 4.14.

Table 4.14: Composition of stacking and separation gel according to Laemmli; *stacking gel: 0,5 M Tris-HCl pH 6,8; **separation gel: 1,5 M Tris/HCl pH 8,8.

	Stacking gel	Separation gel			
	4%	5%	7,5%	10%	12,5%
Acrylamide-Bis	0,67 ml	1,67 ml	2,5 ml	3,33 ml	4,17 ml
Stacking gel buffer*	1,25 ml	-	-	-	-
Separation gel buffer**	-	1,25 ml	1,25 ml	2,5 ml	2,5 ml
10% SDS	0,1 ml	0,1 ml	0,1 ml	0,1 ml	0,1 ml
80% Glycerol	-	1,2 ml	1,2 ml	1,2 ml	1,2 ml
H_2O	3 ml	5,78 ml	4,95 ml	2,79 ml	1,95 ml
TEMED	3,8 µl	5 µl	7,5 µl	7,5 µl	7,5 µl
10% (w/v) APS	37,5 µl	50 µl	50 µl	75 µl	75 µl

**Table 4.15: Composition of stacking gel and separation gel at a 15% tricine gel according to Schägger und Jagow. ***Stacking gel buffer: 3 M Tris pH 8,45, 0,3% SDS, 4x; **separation gel buffer: 3 M Tris pH 8,45, 0,3% SDS, 3x

	Stacking gel	Separation gel
Acrylamide-Bis 46.513	-	1,52 ml
48% Acrylamide, 1,5% Bisacrylamide	0,078 ml	-
Stacking gel buffer*	0,31 ml	-
Separation gel buffer**	-	1,67 ml
80% Glycerol	-	833 µl
H_2O	0, 845 ml	0,95 ml
TEMED	1 µl	2,5 µl
10% (w/v) APS	15 µl	25 µl

The gel polymerized for 1 h at room temperature. The electrophoresis was carried out in an appropriate electrophoresis buffer for 15 min at 50 V and afterwards for 1 h at 150 V. Samples were put on the gel in tricine sample buffer or Laemmli buffer (BioRad).

Electrophoresis buffer (Laemmli):	0,025 M Tris
	0,192 M Glycine
	0,1% (w/v) SDS

Electrophoresis buffer (Schägger and Jagow):

Anode buffer:	0,2 M Tris-HCl pH 8,9
Cathode buffer:	0,1 M Tris
	0,1 M Tricin
	0,1% (w/v) SDS

Sample buffer:	4% (w/v) SDS
	50 mM Tris HCl pH 6,8
	12% glycerol
	0,01% (w/v) Coomassie Brillant Blue G-250
	4% (v/v) ß-mercapto ethanol (reducing)

4.2.23.2 Protein detection by Coomassie blue

Proteins separated by SDS-PAGE were visualized with GelCode Blue Staining Reagent (Pierce, USA): After electrophoresis, the gels were washed three times for 10 min in $H_2O_{bidest.}$, incubated in GelCode Blue Staining Reagent for 1 h at room temperature and washed in $H_2O_{bidest.}$ for 1-2 hours or overnight. For documentation, photos were taken with a video camera (Gene genius, Bio imaging system).

4.2.23.3 Protein detection by silver staining

The proteins were fixed for 20 min in fixing buffer 1 (10% acetic acid, 30% ethanol), for 30 min in fixing buffer 2 (30% ethanol, 0,5 M sodium acetate, 0,5%

glutaraldehyde, 0,2% sodium-thiosulfate) and washed three times for 30 min in H_2O_{bidest}. The staining was performed for 30 min in a staining solution (0,1% silver nitrate, 0,02% formaldehyde (37%). Then the gel was washed for 30 sec and was developed for 1-10 min in a solution consisting of 2,5% sodium carbonate and 0,01 % formaldehyde. The reaction was stopped with 50 mM EDTA. After 5 min, the gels were washed in $H_2O_{bidest.}$ for 10 min.

4.2.24 Immunological detection of proteins by Western blotting

For the immunological detection of proteins, which were separated by the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Protean BA85 Schleicher & Schüll) by a wet blotting procedure. Very hydrophobic proteins were transferred on 0,45 µM PVDF membranes (Immobilon, Millipore) previously incubated in methanol.

The transfer of proteins was done in transfer buffer for 60 min at maximal voltage and 950 mA.

Transfer buffer: 0,025 M Tris
0,192 M Glycine
0,01% SDS
20% (v/v) Methanol

After the transfer of the proteins, the unspecific binding sites were blocked with 5% BSA (in 1x TBS). The first antibody was diluted in 0,5% BSA in 1xTBS. The blot was incubated with the first antibody overnight with slight shaking. After three consecutive washes for 20 min in TBS-T (0,05% TWEEN 20 in 1xTBS), the blot was incubated for 1 h at room temperature with a second antibody in 1xTBS-T. After three consecutive washes for 20 min in 1xTBS-T, detection was performed using the Lumi Light Western Blotting Substrate from Roche. The signal was detected by application of the LUMINESCENT IMAGE ANALYZER LAS-1000plus (Fuji Film).

1xTBS: 0,02 M Tris HCl pH 7,5
0,5 M NaCl

4.2.25 Determination of alkaline phosphatase (PhoA) activity

The optical density OD_{600} of the bacterial culture was measured. One ml was centrifuged and the cell pellet retained. The cell pellet was washed three times with Tris-HCl pH 8 and then it was resuspended in 1 ml of the same buffer. Optionally, dilutions could be made. 100 µl Sigma 104 Phosphatase substrate (p-nitrophenyl-phosphate, disodium, 0,4% in 1 M Tris-HCl pH 8) were added to 1 ml of cell suspension or culture supernatant and incubation followed at 37°C until the yellow colour became visible. The reaction was stopped by adding 100 µl 1 M K_2HPO_4 and the reaction time was noted. The cells were centrifuged and the supernatant was retained for measuring the absorption values at ? = 420 nm and 550 nm. The alkaline phosphatase activity (units of phosphatase) was calculated according to the following formula:

$$\text{Activity (units)} = 1000 \times \text{dilution factor} \times \frac{OD_{420} - 1{,}75 \times OD_{550}}{\text{reaction time} \times OD_{600}}$$

4.2.26 Hemolysin assay (Goebel *et al*., 1982)

Bacterial cells were grown in brain heart infusion media (Difco Labaratories) at 37°C in a shaker flask. Then the cells were pelleted by centrifugation, the culture supernatant was removed and the cells were suspended in 1/25 of the original volume of hemolysin assay buffer 1. After 30 min incubation on ice, the bacterial cells were pelleted, the supernatant was removed, and the cell pellet was suspended in 2 ml of the buffer and lysed by sonication (five 10-s bursts with a Branson ultrasonifier). Hemolysin assay mixture contained: Hemolysin assay buffer, 10 µl of sonic extract (cytoplasmic) or 200 µl of culture supernatant. The hemolysin assay mixture was incubated at 37°C, samples were removed at different time points and chilled on ice, and the intact erythrocytes were removed by centrifugation (1 min). The adsorption of the supernatant at 530 nm was determined and plotted as a function of time.

Hemolysin assay buffer 1: Tris-hydrochloride (pH 7,4; 10 mM)
25% Sucrose
EDTA-sodium (40 mM)
Lysozyme (100 µg/ml)

Hemolysin assay buffer 2: $CaCl_2$ (20 mM)
Tris-hydrochloride (pH 7,4; 10 mM)
NaCl (160 mM)
2% washed sheep erythrocytes

4.2.27 Compartment systems

The protein binding capacities of different filter types were tested by filtration of the culture supernatant of UPEC 536.

Table 4.16: Filter material

Pore diameter (µm)	Filter material	Filter diameter (mm)	Company	Code number
0,2	Cellulose acetate	30	Schleicher&Schuell	10 462 205
0,45	Cellulose acetate	30		10 462 100
0,2	Regenerated cellulose	30		10 462 960
0,45	Regenerated cellulose	30		10 462 950
0,22	Polyethersulfone (PES)	25		SLGP R25 LS
0,1	Polyvinylidenefluoride (PVDF)	25	Millipore	SLVV R25 LS
0,22	PVDF	25		SLGV R25 LS
0,45	PVDF	25		SLHV R 25 LS
0,22	Mixed cellulose esters	25		SLGS 025 OS
0,45	Mixed cellulose esters	25		SLHA 025 OS
0,2	Polytetrafluoroethylene (PTFE)	25		SLLG 025 SS
0,45	Nylon membrane	14,7		SLHN 013 NL
0,2	Nylon membrane	14,7		SLGN 013 NL

4.2.27.1 Double filter system

The double filter system was established as follows: Two filters were placed on a blood agar plate (see Figure 3.4). The filter material that was examined in this study was listed in Table 4.17. The uropathogenic *E. coli* 536 and its negative mutant 536-21 grew on the upper filter by receiving their nutrients from the agar plate. The secreted proteins bound on the lower filter. After incubation for three hours at 37°C, the filter material was removed from the blood agar plate for detection of hemolysis. Consecutively, the lower filter containing the secreted proteins was put vice versa on a fresh sheep blood agar plate and incubated for 3 h at 37°C to find out, whether hemolysis became visible.

Table 4.17. Used Filters for establishment of double filter system

Pore diameter (µm)	Filter material	Filter diameter (mm)	Company	Code number
0,1	**Mixed cellulose esters, hydrophilic**	**90**	**Millipore**	**VCWP09025**
0,2	**Cellulose acetate**	**142**	**Sartorius**	**11107-142G**
0,45	**Cellulose acetate**	**142**	**Sartorius**	**11106-142G**
0,45	**PVDF, hydrophilic**	**90**	**Millipore**	**HVLP09050**

4.2.27.2 Stacked Microwell system

The stacked microwell system was established using the „MultiScreen Assay System“ provided by Millipore (Eschborn, Germany). The 0,45 µm Durapore PVDF membranes (MAHVS4510) were used as filtration plates and the Greiner Polyproylen-Microplates 651201 were used as the lower harvest plates.

5. Results

A novel Functional Protein Technology (FunProTec) was established. It is based upon the application of a secretion system and a compartment system. A suitable system for secretion of heterologous proteins in *E. coli* was constructed on basis of the *E. coli* hemolysin secretion system. Moreover, suitable compartment systems were established.

5.1 Construction of E. coli with a hemolysin based-secretion system

The aim was to construct a Gram-negative bacterial strain which stably expresses the hemolysin secretion apparatus and can therefore be used as a competent host strain for the secretion of any $HlyA_S$-protein fusion.

5.1.1 *E. coli* with a chromosomally encoded, T7 promoter controlled secretion apparatus

The suicide vector pALMI-89 (Figure 5.1) was constructed for integration T7 promoter controlled *hly*B and *hly*D genes into the *rec*A gene of the bacterial chromosome.

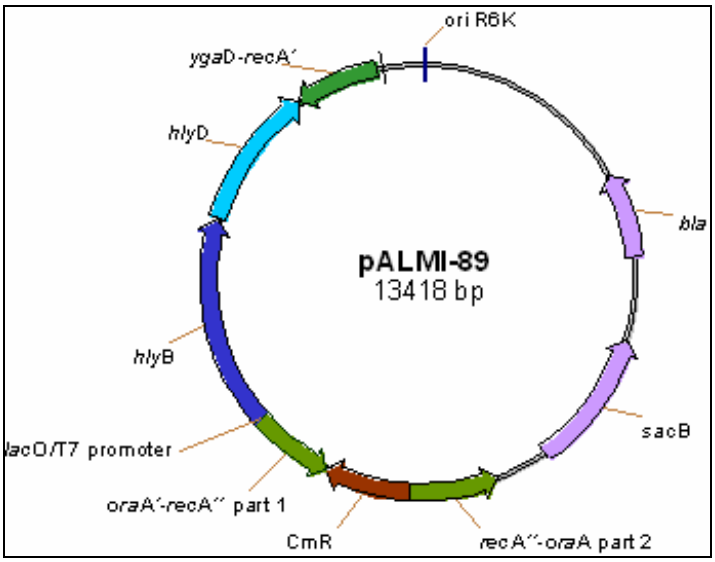


Figure 5.1 Vector map of plasmid pALMI-89

Plasmid pALMI-89 contains the T7 promoter controlled *hly*BD genes flanked by sequences corresponding with the chromosomal *rec*A gene and its neighbouring genes *ora*A and *yga*D. Moreover, plasmid pALMI-89 contains the chloramphenicol

acetyl transferase (CmR) and the ß-lactamase (*bla*) genes as selection marker genes.

The *E. coli* strain BL21:DE3 was transformed with pALMI-89 to integrate the T7 promoter controlled *hly*BD genes into the *rec*A gene by a double crossover event as described in chapter 4.2.17. However, this integration was not successful.

5.1.2 *E. coli* with a plasmid encoded, T7 promoter controlled secretion apparatus

A host strain expressing the hemolysin secretion apparatus on a single plasmid was constructed: *E. coli* BL21:DE3 (pALMI-84). Plasmid pALMI-84 contains the T7 promoter controlled *hly*BD genes, the *lac* I gene encoding the Lac repressor and the p15A origin (see Figure 5.2).

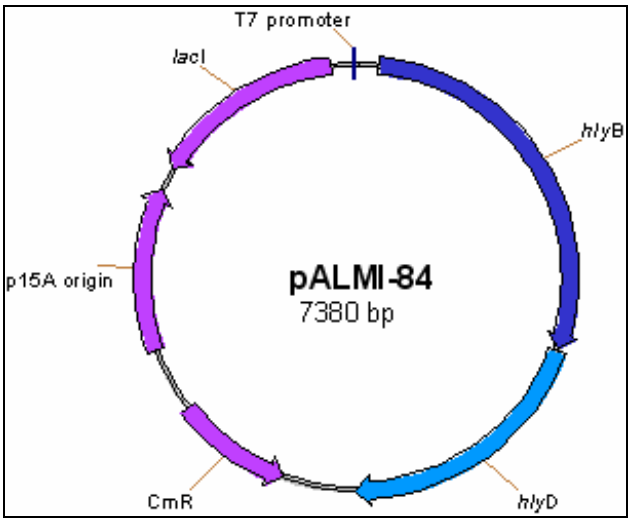


Figure 5.2: Vector map of plasmid pALMI-84

The p15A origin is compatible with pUC or pBR322 derived plasmids. Plasmid pALMI-84 was used for co-expression of the *hly*BD genes encoding the hemolysin secretion apparatus with genes encoding $HlyA_s$-protein fusions in *E. coli* BL21:DE3 cells.

5.1.3 *E. coli* with plasmid encoded, T7 promoter controlled gene fusions between gene X-*hly*A_s and *hly*BD

A plasmid containing a T7 promoter controlled gene fusions between gene X-*hly*A_s and *hlyBD* was constructed as outlined in Figure 5.3.

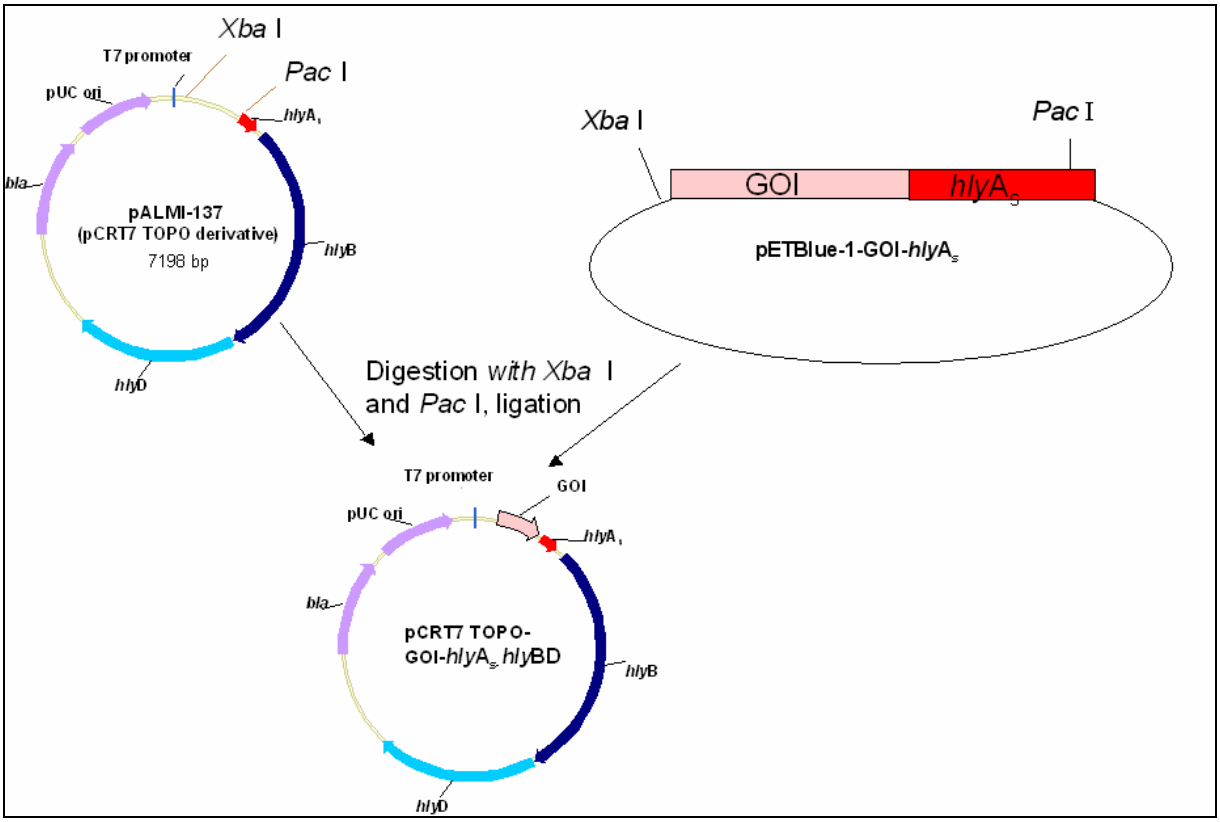


Figure 5.3: General construction scheme of a plasmid encoded gene fusions between a gene of interest (GOI), *hly*A$_S$ and *hly*BD. A pETBlue-1 derivative containing a gene fusion between a gene of interest and *hly*A$_S$ was digested with *Xba* I and *Pac* I. The resulting GOI-*hly*A$_S$ fragment with truncated *hly*A$_S$ was ligated with pALMI-137 cleaved with the same enzymes.

The resulting expression vector contains a T7 promoter controlled gene fusion between a gene of interest (GOI) and *hly*A$_S$*hly*BD by maintaining the genetic structure of the *hly* operon. It also contains the pUC origin. *E. coli* strain BL21:DE3 (pLysS) was used for the expression of the T7 promoter controlled genes. The additional plasmid pLysS reduces the basal level expression of these genes. Plasmid pLysS confers resistance to chloramphenicol and contains the p15A origin. This origin is compatible with the pUC derived expression vector.

5.1.4 *E. coli* with a plasmid encoded, *hly* promoter controlled secretion apparatus with *hly*C upstream sequences

E. coli strain Top 10 (pALMI-122) was constructed. Plasmid pALMI-122 (Figure 5.4) contains the *hly*BD genes encoding the secretion apparatus and 0,8 kb of the *hly*C upstream sequence. The *hly*C upstream sequence (Figure 7.8) contains the *hly* promoter and the JUMPStart sequence as putative promoter activating sequence (Hobbs and Reeves, 1994).

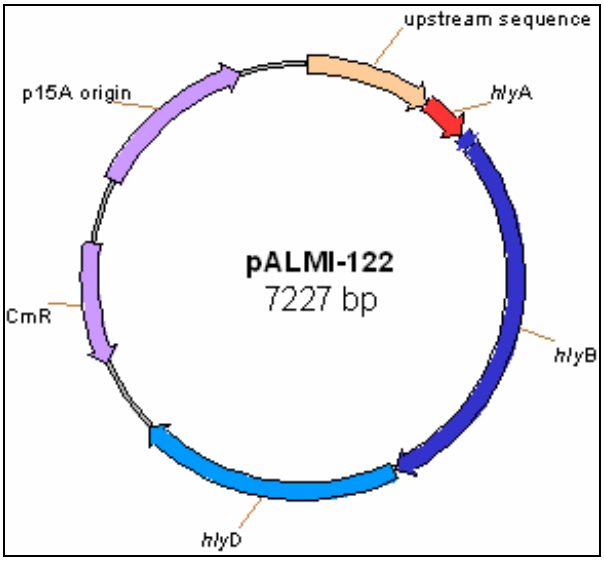


Figure 5.4: Vector map of pALMI-122

Plasmid pALMI-122 contains the p15A origin. This origin is compatible with pUC derived vectors. Thus, pALMI-122 is suitable for co-expression of the *hly*BD genes with genes encoding $HlyA_s$-fusion proteins localized on pUC derived expression vectors.

5.1.5 *E. coli* with a plasmid encoded, *hly* promoter controlled gene fusion between gene X-*hly*A_s and *hly*BD with *hly*C upstream sequences

E. coli strain Top 10 (pALMI-130) was generated. Plasmid pALMI-130 contains the 0,8 kb *hly*C upstream sequence and a fusion between a *hly* promoter controlled gene of interest, *hly*A_s and *hly*BD. The *hly*C upstream sequence (Figure 7.8) contains the *hly* promoter, the JUMPStart sequence as putative promoter activating sequence (Hobbs and Reeves, 1994) and the Shine Dalgarno sequence.

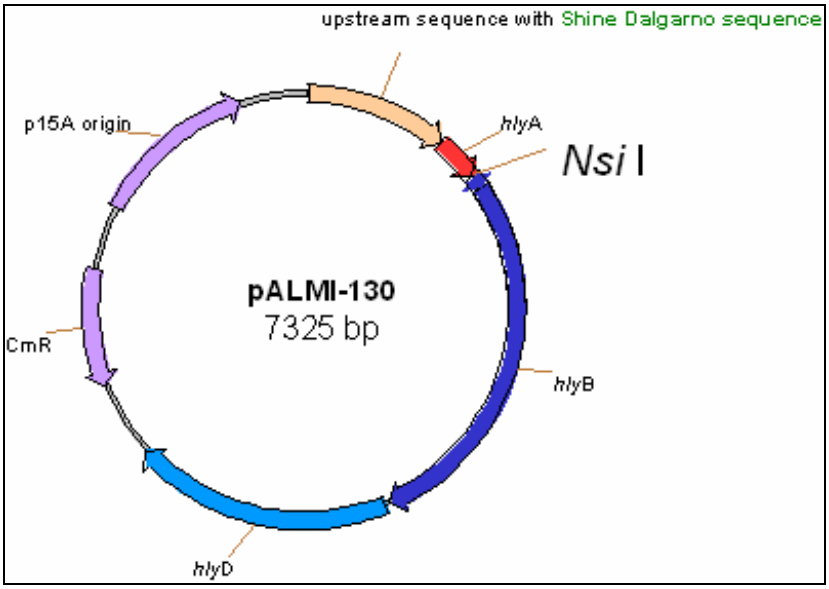


Figure 5.4: Vector map of pALMI-130

Site directed mutagenesis of the *Nsi* I site located between the *hly* promoter and the Shine Dalgarno sequence in the *hly*C upstream sequence was performed. The *Nsi* I site located between the Shine Dalgarno sequence and *hly*A_S could be used for insertion of a gene of interest, resulting in a gene fusion between a heterologous gene and the signal sequence *hly*A_S which is transcribed from the original *hly* promoter together with the *hly*BD genes.

5.1.6 Summary of the secretion constructs in *E. coli*

Different plasmid vectors for secretion studies of $HlyA_S$ fusion proteins in *E. coli* strains were generated. They differ in their promoters, origins and *hly* determinants (see Table 5.1).

Table 5.1: Vectors and appropriate *E. coli* expression strains generated for secretion experiments of $HlyA_S$-fusion proteins

Plasmid	Origin	Promoter	JUMPStart sequences	*hly*A_S	*hly*BD	Coexpression with $HlyA_S$ fusion proteins	*E. coli* expression strain
pALMI-84	p15A	T7	-	-	+	+	BL21:DE3
pCRT7 TOPO-GOI-*hly*A_S-*hly*BD	pUC	T7	-	+	+	-	BL21:DE3
pALMI-122	p15A	*hly*	+	-	+	+	Top 10 (Invitrogen)
pALMI-130	p15A	*hly*	+	+	+	-	Top 10 (Invitrogen)

5.2 Expression and secretion of proteins in E. coli harbouring different secretion constructs

5.2.1 Comparison of expression and secretion of HlyA in *E. coli* harbouring different secretion constructs

The *E. coli* strains described in Table 5.1 were compared in regard to their HlyA secretion efficiencies.

Secretion of HlyA was studied in the *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84) after induction of the T7 promoter.

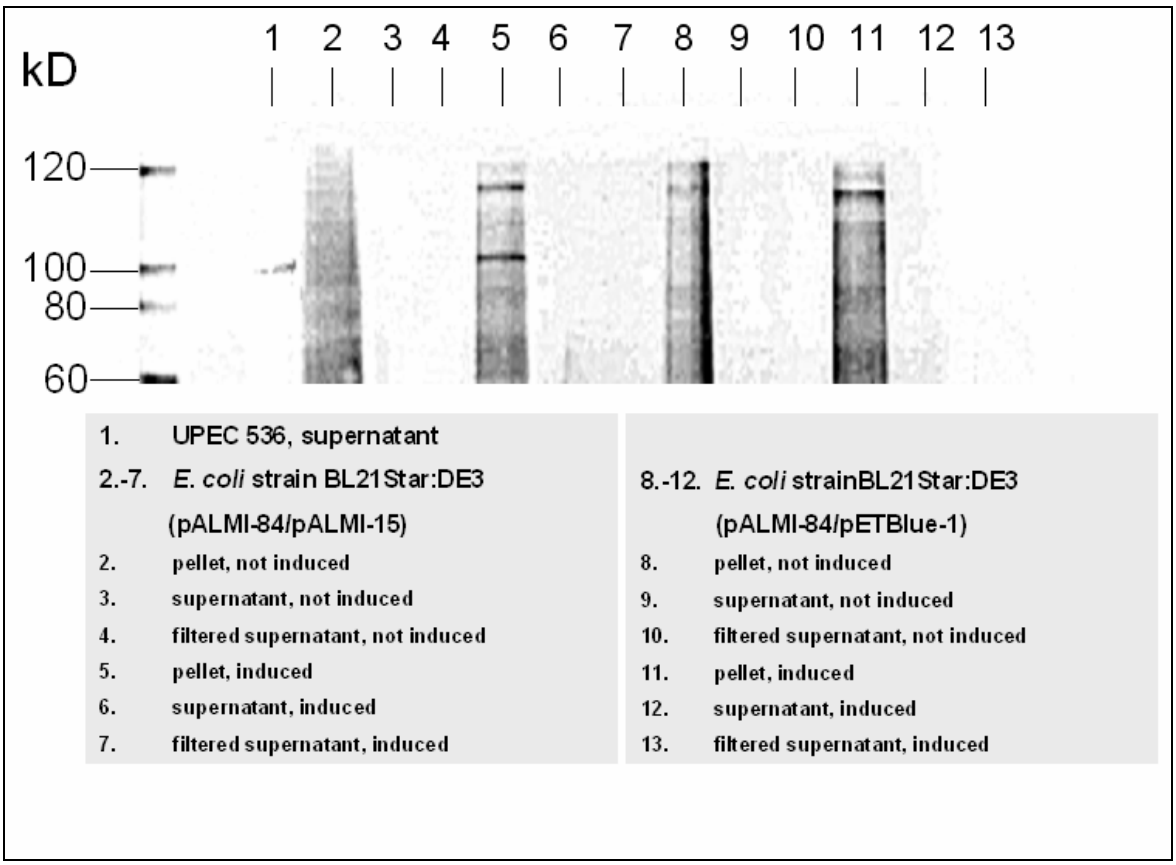


Figure 5.6: Western blot analysis. Polyclonal rabbit serum raised against $HlyA_s$ (1:1000) was used for detection. Concentrated culture supernatants (filtered with a 0,45 µM PVDF filter and unfiltered) and pellets of a 50 µl culture were separated in 10% sodium dodecyl sulfate-polyacrylamide Laemmli gel. The different *E. coli* strains are listed below the Western blot. The induction was performed with 1 mM IPTG for 2 h. The *E. coli* strain BL21:DE3 (pETBlue-1/pALMI-84) is the common host background. The supernatant of uropathogenic *E. coli* 536 was used as positive control for HlyA.

Western blot analysis revealed a protein corresponding to HlyA (110 kD) in the bacterial lysate of the induced *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84) (see Figure 5.6, lane 5), but not in the culture supernatant of this strain (see lane 6 and 7).

Secretion studies using the secretion construct pALMI-138 showed similar results. A protein corresponding to HlyA was detected in the pellet, but not in the culture supernatant of the induced *E. coli* strain BL21:DE3 (pLysS) (pALMI-138) (Figure 5.7).

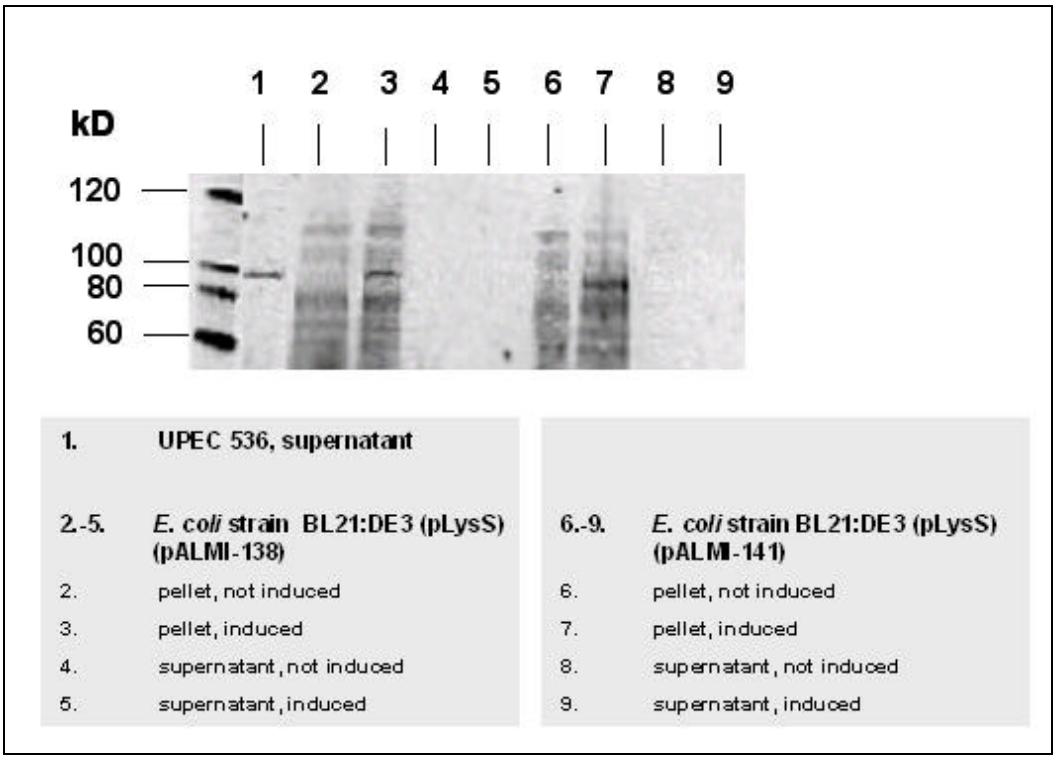


Figure 5.7: Western blot analysis. Polyclonal rabbit serum raised against $HlyA_s$ (1:1000) was used for detection. The different *E. coli* strains are listed below the Western blot. Concentrated culture supernatants and pellets of a 50 µl culture were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli. The culture supernatants were filtered with a 0,45 µM PVDF filter. The *E. coli* strain BL21:DE3 (pLysS/pALMI-141) is the common host background. The culture supernatant of UPEC 536 was used as positive control for HlyA. (The stripes were assembled in Paint Shop Pro to obtain this order.)

Secretion of HlyA was examined in the *E. coli* strain Top 10 (pALMI-122/pALMI-168). The hemolytic activity of the *E. coli* strain Top 10 (pALMI-122/pALMI-168) was determined following growth on agar plates containing sheep blood at 37°C. Hemolysis became visible by the formation of a clear lysis zone around the hemolysin secreting bacteria due to lysis of sheep erythrocytes (see Figure 5.8).

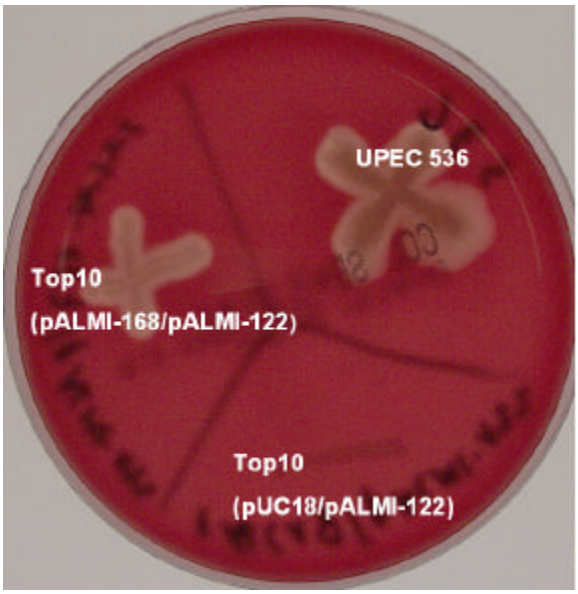


Figure 5.8: Determination of the hemolytic activity of the *E. coli* strain Top 10 (pALMI-168/pALMI-122) following over night growth on a sheep blood agar plate. The *E. coli* strain Top 10 (pUC18/pALMI-122) was used as negative control and UPEC 536 was used as a positive control for hemolysis.

The secretion of HlyA was confirmed by Western blot analysis (see Figure 5.9): A protein corresponding to HlyA (110 kD) was detected in the culture supernatant of the *E. coli* Top 10 (pALMI-168/pALMI-122).

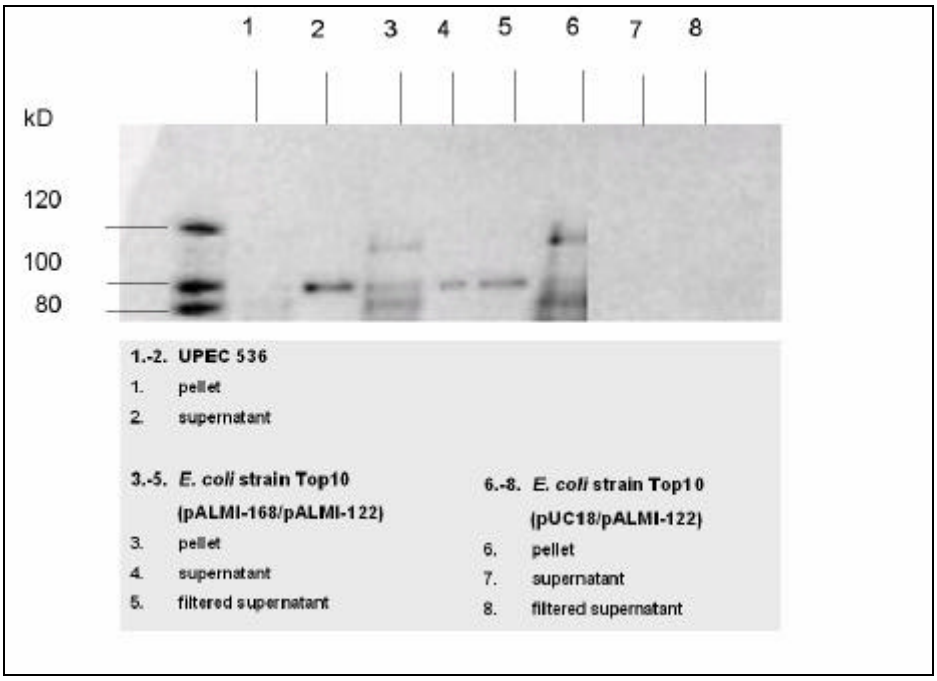


Figure 5.9: Western blot analysis. Polyclonal rabbit serum raised against $HlyA_s$ (1:1000) was used for detection. Concentrated culture supernatants (filtered with a 0,45 µM PVDF filter and unfiltered) and pellets of a 50 µl culture were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The different *E. coli* strains are listed below the Western blot. The *E. coli* strain Top 10 (pUC18/pALMI-122) is the common host background. The supernatant of UPEC 536 was used as positive control for HlyA. (The stripes were assembled in Paint Shop Pro to obtain this order.)

Further secretion studies of HlyA were performed with *E. coli* strain Top 10 (pALMI-140). The hemolytic activity of this strain was determined following over night growth

at 37°C on sheep blood agar plates, which became obvious by the formation of a clear lysis zone around the hemolysin secreting bacteria (see Figure 5.10).

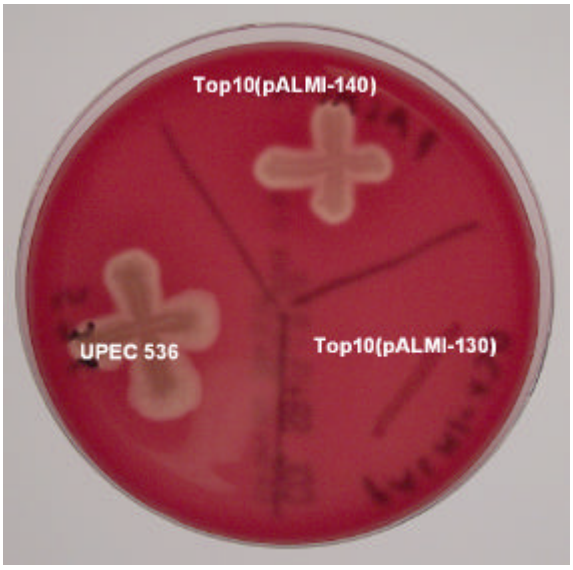


Figure 5.10: Determination of the hemolytic activities of the *E. coli* strain Top 10 (pALMI-140) following over night growth on a sheep blood agar plate. The *E. coli* strain Top 10 (pALMI-130) was used as a negative control, UPEC 536 was used as positive control for hemolysis.

Secretion of HlyA by the *E. coli* strain Top 10 (pALMI-140) in the culture supernatant was confirmed by Western blot analysis (see Figure 5.11).

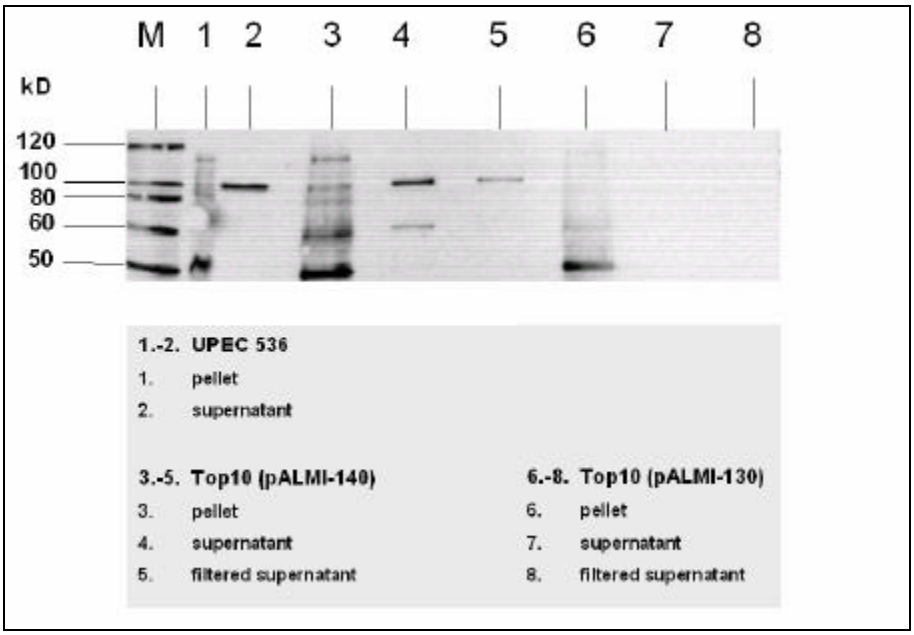


Figure 5.11: Western blot analysis. Polyclonal rabbit serum raised against $HlyA_s$ (1:1000) was used for detection. The different *E. coli* strains are listed below the Western blot. The *E. coli* strain TOP 10 (pALMI-130) is the common host background. The culture supernatant of UPEC 536 was used as positive control for HlyA. Concentrated culture supernatants (filtered with a 0,45 µM PVDF filter and unfiltered) and pellets of a 50 µl culture were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (The stripes were assembled in Paint Shop Pro to obtain this order.)

A protein corresponding with HlyA (110 kD) was detected in the culture supernatant and in the pellet of the *E. coli* strain Top 10 (pALMI-140) by Western blot analysis. The amount of this protein was lower in the pellet than in the culture supernatant. Western blot analysis revealed a 60 kD protein in the culture supernatant of the *E. coli* strain Top 10 (pALMI-140).

The hemolysin secretion efficiencies of the *E. coli* strains Top 10 (pALMI-140), Top 10 (pALMI-168/pALMI-122) and UPEC 536 were compared. The pellet and filtered culture supernatants of these strains were incubated with human erythrocytes. The release of hemoglobin as a consequence of hemolysis was measured by the adsorption at 530 nm (see Figure 5.12).

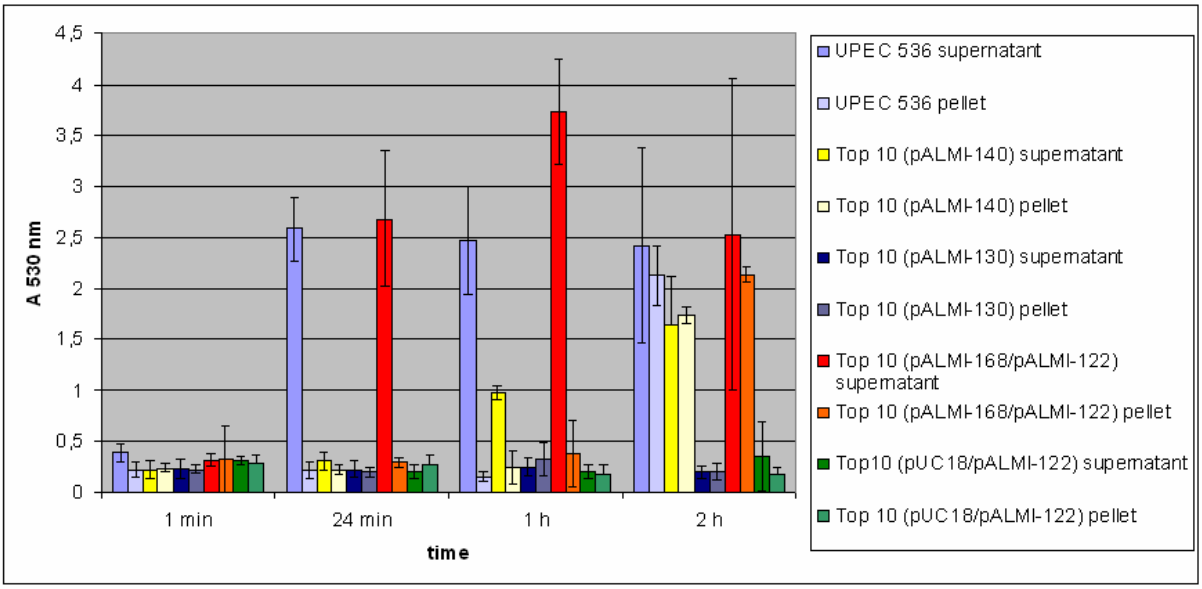


Figure 5.12: Determination of hemolysin secretion. The released hemoglobin was measured at an adsorption of 530 nm at different time points. The values given are the relative rate of hemolysis (Goebel *et al*., 1982). The different *E. coli* strains are listed on the right of the plot. The *E. coli* strain Top 10 (pALMI-130) was the negative control for the *E. coli* strain Top 10 (pALMI-140). The *E. coli* strain Top 10 (pUC18/pALMI-122) was the negative control for the *E. coli* strain Top 10 (pALMI-168/pALMI-122). UPEC 536 was used as positive control for HlyA in the culture supernatant.

The culture supernatant of UPEC 536 and the *E. coli* strain Top 10 (pALMI-168/pALMI-122) caused hemolysis of erythrocytes after 24 min incubation, while the culture supernatant of the *E. coli* strain Top 10 (pALMI-140) caused hemolysis of erythrocytes after 1 h incubation. Erythrocytes incubated with the culture supernatant of the *E. coli* strain Top 10 (pALMI-168/pALMI-122) released 3-4 times more hemoglobin than the erythrocytes incubated with supernatant of the *E. coli* strain Top 10 (pALMI-140) and about 1,1 times more hemoglobin than the erythrocytes incubated with UPEC 536. After two hours of incubation, the pellets and culture

supernatants of all hemolytic strains showed similar values of hemolysis (Figure 5.12).

5.2.1.1 Summary: Expression and secretion of the wild-type protein HlyA

The *E. coli* strains Top 10 (pALMI-122/pALMI-168) and Top 10 (pALMI-140) secreted active HlyA into the culture supernatant. HlyA was not secreted into the culture supernatant of the *E. coli* strains BL21:DE3 (pALMI-84/pALMI-15) and BL21:DE3 (pALMI-138). The hemolytic activity of the *E. coli* strain Top 10 (pALMI-122/pALMI-168) was 3-4 times higher than hemolytic activity of the *E. coli* stain Top 10 (pALMI-140) and about 1,1 times higher than the hemolytic activity of the uropathogenic *E.coli* strain 536.

Table 5.2: Comparison of the *E. coli* strains harbouring different secretion constructs in relation to their secretion of the wild-type protein HlyA

Plasmid	*E. coli* Expression strain	Coexpression with	HlyA Expression	HlyA Secretion
pALMI-84	BL21:DE3	pALMI-15	+	-
pALMI-138	BL21:DE3	-	+	-
pALMI-122	Top 10	PALMI-168	+	+
pALMI-140	Top 10	-	+	+

5.2.2 Comparison of transcription of the *hly*A, *hly*B, *hly*C and *hly*D gene in *E. coli* harbouring different secretion constructs

A relative quantitation of the *hly*A, *hly*B, *hly*C and *hly*D transcripts of the above described hemolytic and ahemolytic *E. coli* strains (see Table 5.2) was carried out using TaqMan PCR (see chapter 4.2.12). The relative quantitation of mRNA was determined by the comparative Ct method. The Ct value of the target, the *hly*A, *hly*B, *hly*C or *hly*D mRNA, is normalized to the Ct value of the enodogenous reference, the *pho*A mRNA. The threshold Ct is defined as the cycle number arbitrarily selected from the logarithmic phase of the PCR curve where an increase of fluorescence can be detected above background. It is defined as 10 times the standard deviation optical devices, measured between cycle 3 and 15. The Ct values are summarized in Table 5.3 and Table 5.4.

Table 5.3

Target gene	Ct values				
	UPEC 536	**Top 10 (pALMI-130)**	**Top 10 (pALMI-140)**	**BL21:DE3 (pLysS) (pALMI-138) not induced**	**BL21:DE3 (pLysS) (pALMI-138) induced**
***pho*A**	28,44+/-0,09	27,93+/-0,29	31,94+/-0,26	32,03+/-0,38	27,23+/-0,7
***hly*A**	23,10+/-0,25	40	23,39+/-0,51	29,47+/-0,31	30,79+/-0,20
***hly*B**	28,23+/-0,25	23,00+/-0,26	27,23+/-0,28	32,92+/-0,10	35,04+/-0,39
***hly*C**	25,78+/-0,08	40	24,56+/-0,12	29,85+/-0,23	31,51+/-0,33
***hly*D**	28,71+/-0,19	23,82+/-0,31	27,50+/-0,17	24,53+/-0,32	17,92+/-0,005

Table 5.4

Target	Ct values					
	UPEC 536	**Top 10 (pUC18/ pALMI-122)**	**Top 10 (pALMI-168/ pALMI-122)**	**BL21:DE3 (pETBlue-1/ pALMI-84) induced**	**BL21:DE3 (pALMI-15/ pALMI-84) not induced**	**BL21:DE3 (pALMI-15/ pALMI-84) induced**
***pho*A**	29,2+/-0,37	30,86+/-0,03	29,83+/-0,47	31,92+/-0,2	31,58+/-0,38	31,58+/-0,38
***hly*A**	24,09+/-0,13	40	18,18+/-0,33	40	14,03 +/-0,11	17,28+/-0,23
***hly*B**	28,89+/-0,26	23.91+/-0,55	23,17+/-0,23	18,21+/-0,18	17,10+/-0,49	22,17+/-0,2
***hly*C**	26,46+/-0,44	40	18,42+/-0,42	40	14,88+/-0,2	20,42+/-0,2
***hly*D**	29,55+/-0,28	24.98+/-0,38	23,43+/-0,06	20,42+/-0,20	18,66+/-0,16	23,44+/-0,39

Table 5.3 and Table 5.4: Summary of the Ct values (middle value with standard deviation) of a TaqMan PCR reaction for comparison of transcription of *hly*A, *hly*B, *hly*C and *hly*D. *pho*A was used as a reference gene. The TaqMan PCR was performed as described in chapter 4.2.12. The total cDNA of each strain was used as a template.

The Ct value of the target was normalized to the Ct value of the reference *pho*A by calculating the ?Ct value. It was determined by subtracting the Ct of *pho*A from the Ct of the target (?Ct= Ct target-Ct *pho*A).

Table 5.5 and table 5.6: Summary of the ?Ct values for the relative quantification of the *hly*A, *hly*B, *hly*C and *hly*D transcripts.

Table 5.5

Target gene	?Ct=Ct target-Ct *pho*A			
	UPEC 536	**Top 10 (pALMI-140)**	**BL21:DE3 (pLysS)(pALMI-138) not induced**	**BL21:DE3 (pLysS)(pALMI-138) induced**
***hly*A**	-5,34	-8,55	-2,56	3,56
***hly*B**	-0,21	-4,01	0,89	7,81
***hly*C**	-2,66	-7,38	-2,17	4,28
***hly*D**	0,27	-4,44	-7,50	-9,31

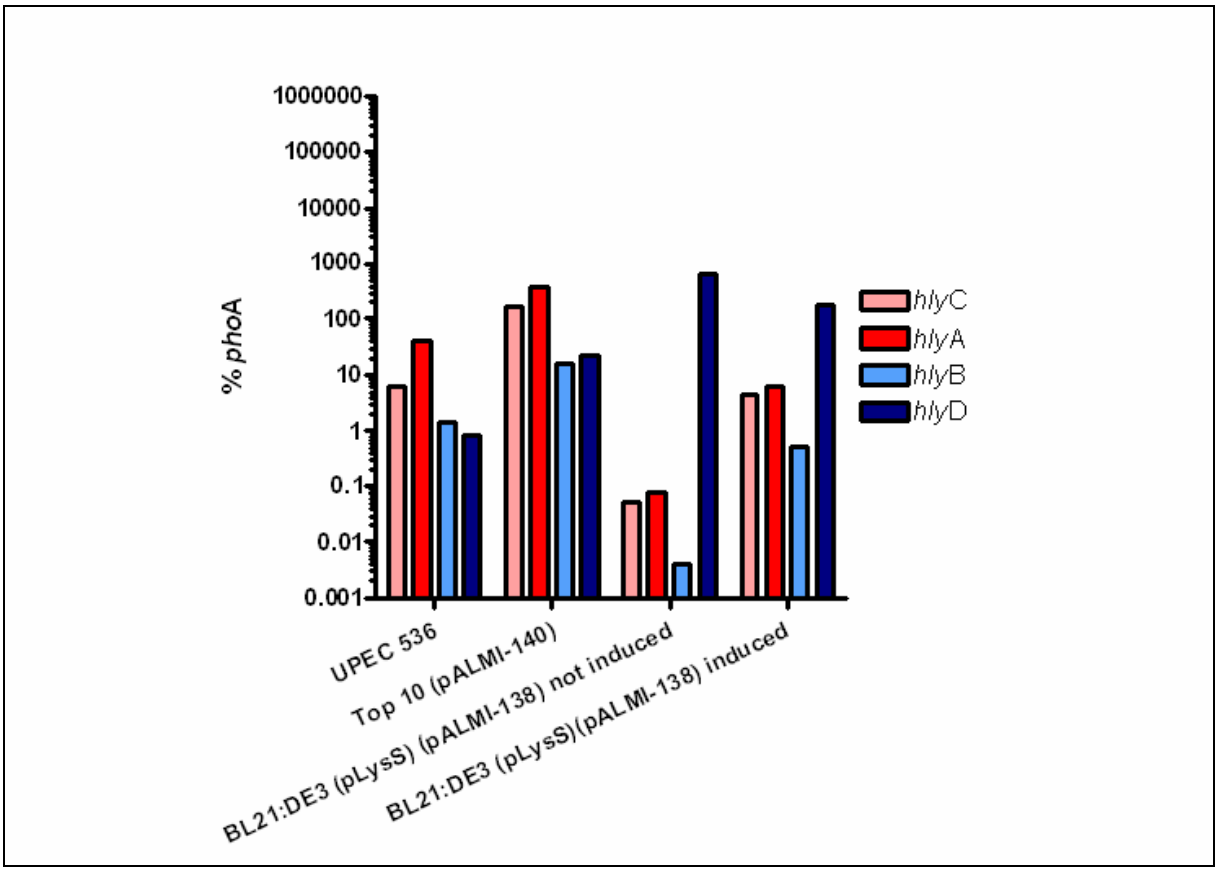
Table 5.6

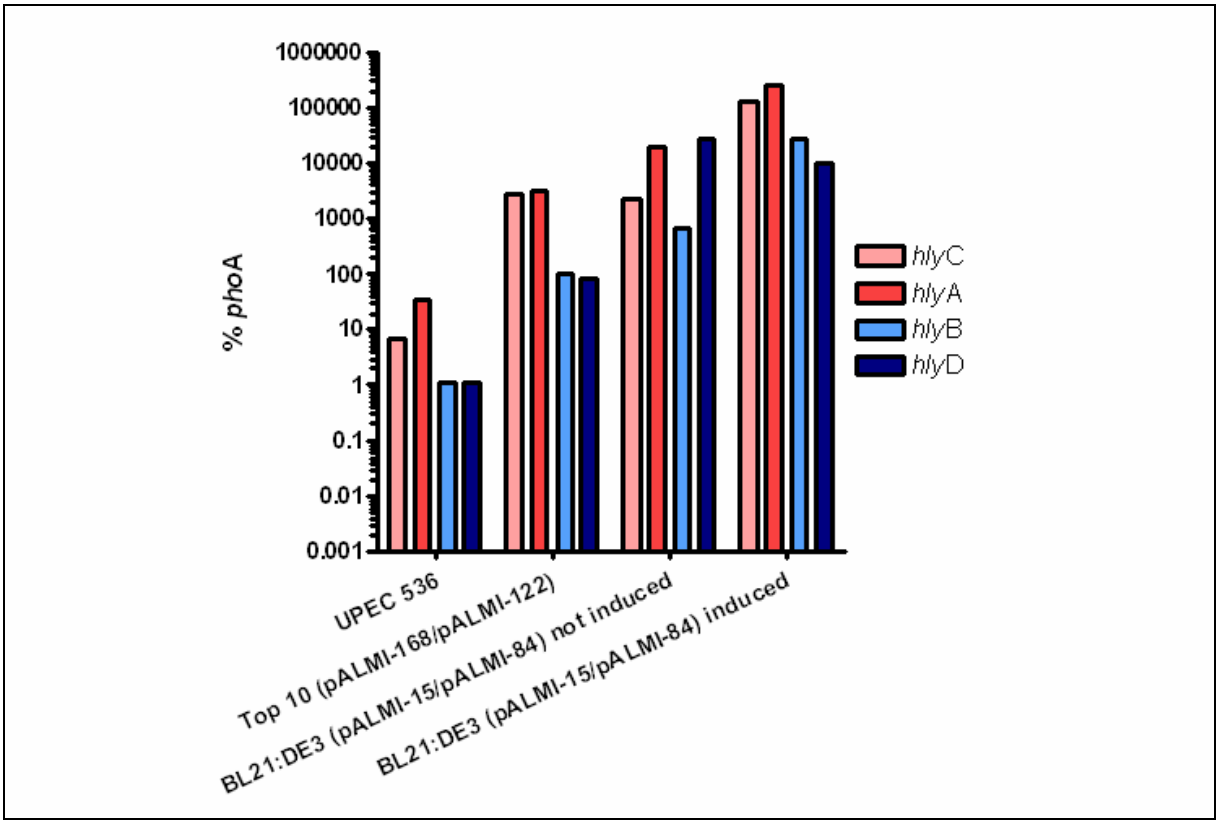
Target gene	?Ct=Ct target gene-Ct *pho*A			
	UPEC 536	**Top 10 (pALMI-168/pALMI-122)**	**BL21:DE3 (pALMI-15/pALMI-84) not induced**	**BL21:DE3 (pALMI-15/pALMI-84) induced**
***hly*A**	-5,11	-11,64	-14,3	-17,89
***hly*B**	-0,21	-6,66	-9,41	-14,73
***hly*C**	-2,74	-11,41	-11,16	-17,04
***hly*D**	-0,15	-6,4	-8,14	-13,26

The relative gene expression of target gene to reference gene (*pho*A) was defined as $2^{-?Ct\ target}$ =% reference (*pho*A). Figures 5.13 and 5.14 describe the relative gene expression of *hly*A, *hly*B, *hly*C and *hly*D mRNA in different *E. coli* strains.

As shown in Figures 5.13 and 5.14, the relative gene expressions rates of *hly*C, *hly*A, *hly*B and *hly*D mRNA to endogenous *pho*A mRNA were very different in all *E. coli* strains. The highest gene expression rate of the *hly* genes was determined in the induced *E. coli* strain *E. coli* BL21:DE3 (pALMI-15/pALMI-84).

Figure 5.13 and Figure 5.14: Diagram of the relative gene expression of *hly*A, *hly*B, *hly*C, *hly*D mRNA to endogenous *pho*A mRNA in different *E. coli* strains.





The *hly*A expression rate was higher than the *hly*C expression rate in all *E. coli* strains: The amount of *hly*C transcript was about five or six fold higher than the amount of *hly*A transcript in UPEC 536. However, it is only 1-2 fold higher than the amount of *hly*A transcript in the *E. coli* strains Top 10 (pALMI-140), Top 10 (pALMI-168/pALMI-122), BL21:DE3 (pLysS) (pALMI-138) and in the induced BL21:DE3 (pALMI-15/pALMI-84). The *hly*A expression rate was even eight fold higher than the *hly*C expression rate in the uninduced *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84).

The *hly*B gene was expressed in similar amounts as *hly*D in the *E. coli* strain Top 10 (pALMI-140), UPEC 536 and in the *E. coli* strain Top 10 (pALMI-168/pALMI-122). The amount of *hly*A transcript was 30 or 40 fold higher than the amount of *hlyB*D transcript in these *E. coli* strains.

However, the expression rate of *hly*B was very different to the expression rate of *hly*D in the *E. coli* BL21:DE3 (pALMI-15/pALMI-84) and *E. coli* strain BL21:DE3 (pLysS) (pALMI-138): The amount of *hly*D transcript was about 350 fold higher than the amount of *hly*B transcript in induced *E. coli* BL21:DE3 (pLysS) (pALMI-138). The amount of *hly*B transcript was about three fold higher than the amount of *hly*D transcript in the *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84).

5.2.2.1 Summary: Relative gene expression of the hly genes

The relative gene expression rates of *hly*C, *hly*A, *hly*B and *hly*D mRNA in comparison with endogenous *pho*A mRNA were very different in all *E. coli* strains, but the *hly*A expression rate was higher than the *hly*C expression rate in all *E. coli* strains. The *hly*B gene was expressed in similar amounts as *hly*D in the hemolytic *E. coli* strains Top 10 (pALMI-140), UPEC 536 and in the *E. coli* strain Top 10 (pALMI-168/pALMI-122). The expression rate of *hly*B was different from the *hly*D expression rate in the ahemolytic *E. coli* strains BL21:DE3 (pALMI-15/pALMI-84) and BL21:DE3 (pLysS) (pALMI-138). Moreover, 30-40 fold more *hlyA* transcript than *hly*BD transcript was determined in the hemolytic *E. coli* strains.

5.2.3 Expression and secretion of heterologous proteins in *E. coli*

Several heterologous proteins of different size, organisms and cellular compartments were cloned and expressed in strains harbouring the hemolysin secretion apparatus (see Table 5.9).

Table 5.9: List of heterologous proteins used for secretion experiments.

Protein	*Size (kD)*	*Compartment*	*Organism*	*Role*
Ccl-21 (SLC) (mature)	12,3	extracellular	Human (placenta)	-Ligand for CCR-7 receptor -Homing and trafficing of lymphocytes into secondary lymphoid tissues (Bardi *et al*., 2001).
PhoA * (alkaline phosphatase)	51	periplasm	*E. coli*	Non-specific phosphomonoesterase (Holtz *et al,* 1999)
PhoA* (without 23 C-terminal aa)	47			
OmpA * (outer membrane protein A)	35,8	periplasm/ membrane	*E. coli*	-bacterial conjugation -structural stability of the outer membrane (Pautsch *et al*., 2000)
Pde1B1 (phosphodiesterase 1B1)	63	cytoplasm	Human (brain)	Interaction between the cyclic nucleotide and Ca^{2+} messenger systems (Sharma *et al*., 1994, Rajenda *et al*., 1997).

***The secretion of the periplasma proteins by the *hly* secretion system was studied without their own N-terminal signal sequence being important for secretion by the *sec* secretion pathway (Gentschev *et al*., 1997)**

Secretion of different alkaline phosphatase (PhoA)-HlyA_s fusion proteins by the *hly* secretion systems was examined: Expression and secretion of PhoA-HlyA_s containing either full-length PhoA or 23 aa C-terminal truncated PhoA were compared. Each gene fusion was expressed in cis and in trans with the *hly*BD genes.

The *pho*A-*hly*A_s gene fusion containing full-length *pho*A was expressed in cis with *hly*BD using the *E. coli* strain Top 10 (pALMI-139). Plasmid pALMI-139 contains a *hly* promoter controlled gene fusion between *pho*A, *hly*A_s and *hly*BD. The enzyme activities of alkaline phosphatase in the pellets and filtered culture supernatants of *E.*

coli Top 10 (pALMI-139) were measured using an alkaline phosphatase activity assay. The results from this assay are summarized in Table 5.10.

***E. coli* strain**	**Pellet (Units/min)**	**Supernatant (Units/min)**
***E. coli* Top 10 (pALMI-139)**	5,58 (+/- 0,61)	0
***E. coli* Top 10 (pALMI-130)**	3,64 (+/- 0,17)	0

Table 5.10: The alkaline phosphatase activity was measured as described in materials and methods. The mean activity with the standard deviation of one representative experiment is shown. *E. coli* Top 10 (pALMI-130) shows the background activity of the endogenous PhoA protein.

Alkaline phosphatase activity was detected in the cell pellet of *E. coli* Top 10 (pALMI-139), but not in the culture supernatant. The detection of alkaline phosphatase-HlyA_s by Western blot analysis using an anti-alkaline phosphatase or anti-HlyA_s antibody failed.

A gene fusion encoding the 23 aa C-terminal truncated PhoA-HlyA_s fusion protein was also expressed in cis with *hly*BD using the *E. coli* strain Top 10 (pALMI-198). Plasmid pALMI-198 contains a *hly* promoter controlled gene fusion between the truncated *pho*A gene, *hly*A_s and *hly*BD. The truncated PhoA-HlyA_s fusion protein was not detected in the pellet of the *E. coli* strain Top 10 (pALMI-198) by Western blot analysis using an anti-alkaline phosphatase or anti-HlyA_s antibody.

A gene fusion encoding PhoA-HlyA_s fusion protein using full-length PhoA was co-expressed with *hly*BD using the *E. coli* strain BL21:DE3 (pALMI-135/pALMI-122). PhoA activity was measured in the pellet, but not in the culture supernatant of the induced *E. coli* BL21:DE3 (pALMI-135/pALMI-122) (Table 5.11).

The *E. coli* strain BL21:DE3 (pALMI-135/pALMI-122) showed more PhoA activity than the *E. coli* strain Top 10 (pALMI-139) in comparison with the corresponding negative control.

E. coli strain	Pellet (Units/min)		Supernatant (Units/min)	
	Not induced	Induced	Not induced	Induced
***E. coli* BL21: DE3 (pALMI-135/ pALMI-122)**	1,01 (+/-0,03)	5,35 (+/-0,72)	0	0
***E. coli* BL21: DE3 (pCRT7 TOPO/ pALMI-122)**	0,97 (+/-0,03)	1,13 (+/-0,13)	0	0

Table 5.11: The alkaline phosphatase activity was measured as described in materials and methods. The mean activity with the standard deviation of one representative experiment is shown. The strains were induced for 1 h with 1 mM IPTG. *E. coli* BL21:DE3 (pCRT7 TOPO/pALMI-122) shows the background activity of the endogenous PhoA protein.

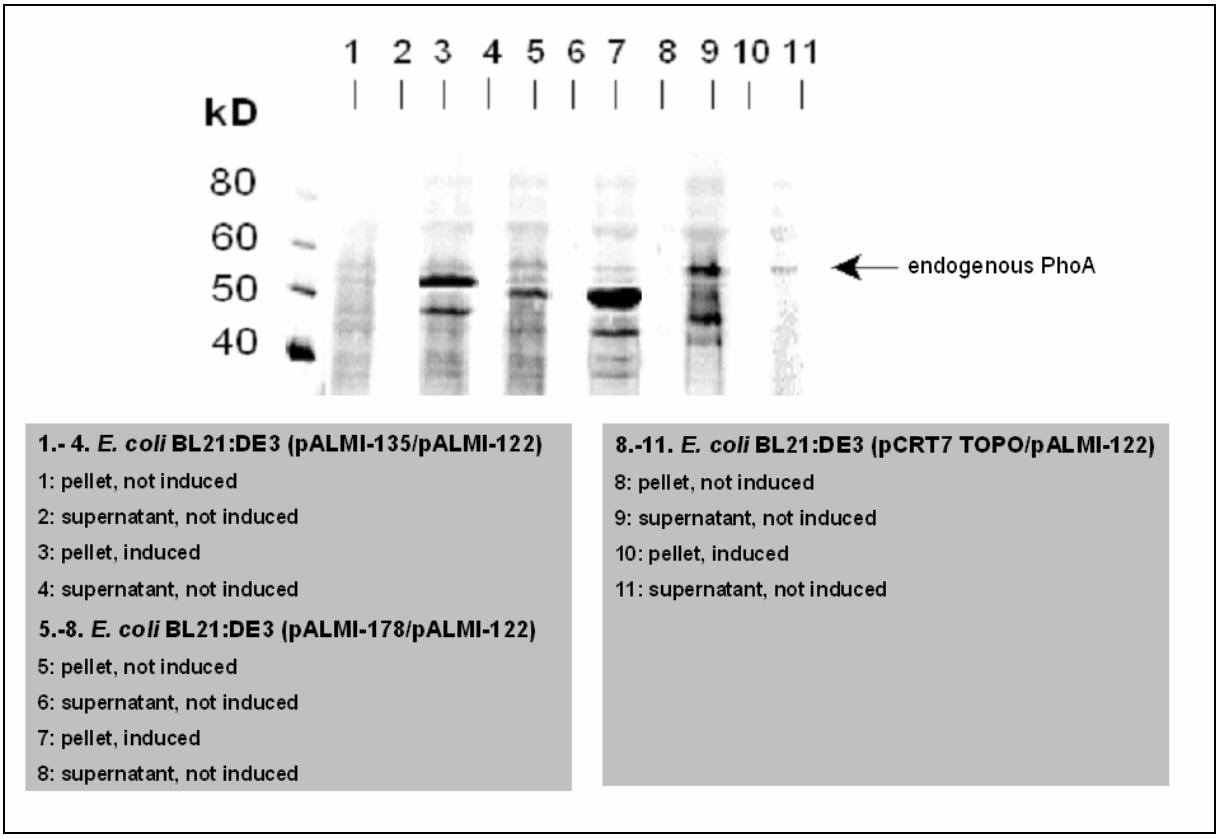


Figure 5.15: Western blot analysis. Polyclonal rabbit serum raised against PhoA (1:10 000) was used for detection. The different *E. coli* strains are listed below the Western blot. The strains were induced 2 h with 1 mM IPTG. The *E. coli* strain BL21:DE3 (pCRT7 TOPO/pALMI-122) is the common host background. The antibody also recognized endogenous PhoA. Concentrated culture supernatants (filtered with a 0,45 µM PVDF filter) and pellets of a 50 µl culture were subjected to 12,5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (The stripes were assembled in Paint Shop Pro to obtain this order.)

The results of the alkaline phosphatase assay were confirmed by Western blot analysis: A protein corresponding with the size of the PhoA-HlyA$_s$ (56 kD) fusion protein was detected in the pellet of the *E. coli* strain BL21:DE3 (pALMI-135/pALMI-

122) (Figure 5.15, lane 3), but not in the culture supernatant. Moreover, a protein that was smaller than 50 kD was also detected in the pellet of this strain.

The PhoA-HlyA$_s$ fusion protein containing 23 aa C-terminal truncated PhoA was co-expressed with HlyBD in the *E. coli* strain BL21:DE3 (pALMI-178/pALMI-122). Western blot analysis revealed a protein corresponding with the size of the truncated PhoA-HlyA$_s$ (54 kD) in the pellet of the *E. coli* strain BL21:DE3 (pALMI-178/pALMI-122) (Figure 5.15, lane 7), but not in the culture supernatant. Another protein with a size ranging from 40-50 kD was also detected in the pellet of this strain.

Secretion of an OmpA-HlyA$_s$ fusion protein by the Hly secretion apparatus was examined. In this study, the construction of a plasmid that contains a *hly* promoter controlled *omp*A-*hly*A$_s$-*hly*BD gene fusion with an intact *omp*A gene failed. A vector with a T7 promoter controlled *omp*A-*hly*A$_s$ gene fusion was generated. The resulting vector, designated pALMI-177, was used for co-expression of *omp*A-*hly*A$_s$ with *hly*BD in *E. coli* BL21:DE3 (pALMI-177/pALMI-122).

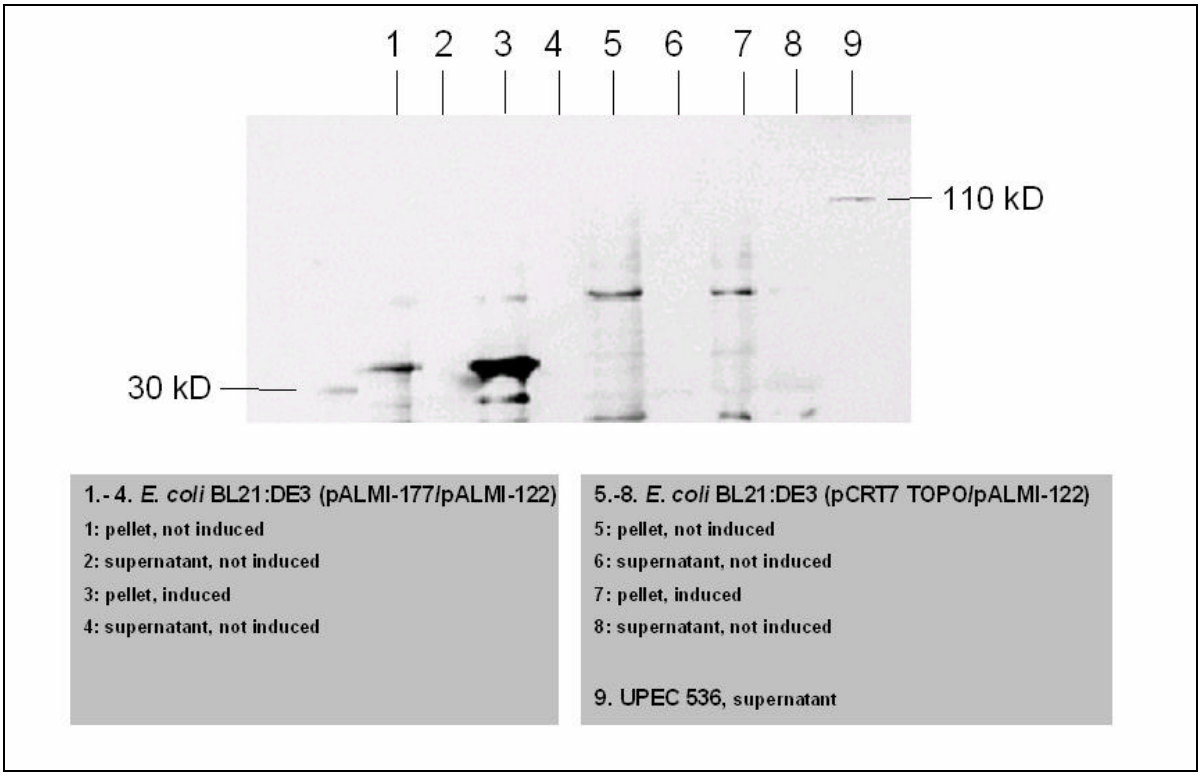
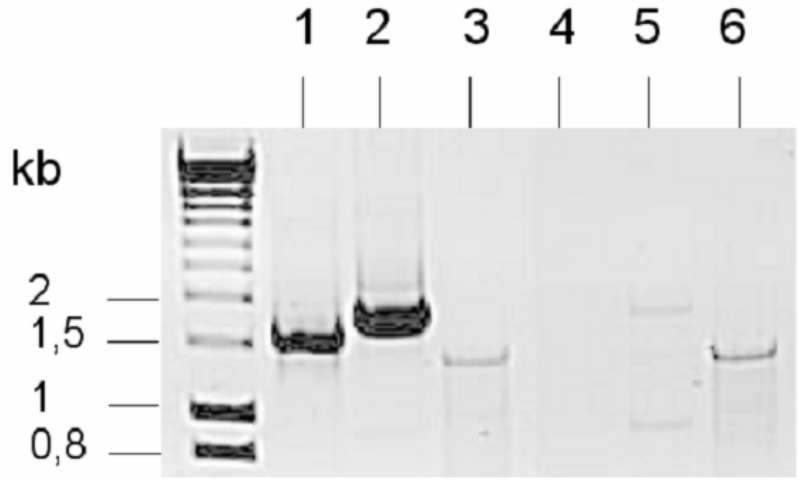


Figure 5.16: Western blot analysis. Polyclonal rabbit serum raised against HlyA$_s$ (1:1000) was used for detection. The different *E. coli* strains are listed below the Western blot. The strains were induced 2 h with 1 mM IPTG. The *E. coli* strain BL21:DE3 (pCRT7 TOPO/pALMI-122) is the common host background. Concentrated supernatants (filtered with a 0,45 µM PVDF filter) and pellets of a 50 µl culture were subjected to 12, 5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The expression and secretion of a PDE 1B1-HlyA_s fusion protein was examined in the *E. coli* strain Top 10 (pALMI-186). Plasmid pALMI-186 contains a *hly* promoter controlled *pde* 1B1-*hly*A_s-*hly*BD gene fusion. The PDE 1B1-HlyA_s fusion protein was neither detected in the culture supernatant nor in the pellet of *E. coli* Top 10 (pALMI-186) by Western blot analysis using either an anti-PDE 1B1 antibody or an anti-HlyA_s antibody.

The transcription of *pde* 1B1-*hly*A_s was examined by RT-PCR analysis of RNA extracted from *E. coli* Top 10 (pALMI-186) (Figure 5.17).



Lane	Template	Primer pair	Gene to be amplified	Expected fragment size	PCR product
1	cDNA of Top 10 (pALMI-186)	Mibi 258/ Mibi 292	*pde* 1B1	1608 bp	1,5 kb
2		Mibi 258/ Mibi 145	*pde* 1B1-*hlyA*$_s$	1794 bp	1,5-2 kb
3		Mibi 597/ Mibi 148	*pho*A without signal sequence	1266 bp	1-1,5 kb
4	cDNA of Top 10 (pALMI-130)	Mibi 258/ Mibi 292	-	-	-
5		Mibi 258/ Mibi 145	-	-	-
6		Mibi 597/ Mibi 148	*pho*A without signal sequence	1266 bp	1-1,5 kb

Figure 5.17: 1% agarose gel electrophoresis (1xTBE) of products from PCR reaction listed in the table below. The cDNAs used as templates for PCR reaction in lane 1, 2, 4, 5, derived from RT-PCR using the primer Mibi 258 and the cDNA used as templates in lane 3 and 6 derived from RT-PCR using the primer Mibi 148. The cDNA of *E. coli* Top 10 (pALMI-130) shows the common host background, the endogenous *pho*A-gene was used as positive control for PCR reaction.

The *pde* 1B1-*hly*A_s gene fusion was transcribed in *E. coli* Top 10 (pALMI-186), *pde* 1B1 and *pde* 1B1-*hly*A_s were amplified from cDNA of this strain.

The expression and secretion of a Ccl-21-HlyA$_s$ fusion protein were studied in *E. coli* Top 10 (pALMI-176). Plasmid pALMI-176 contains a *hly* promoter controlled gene fusion between mature *ccl*-21 and *hly*A$_s$,separated by a sequence encoding factor Xa protease cleavage site, and *hly*BD. The Ccl-21-HlyA$_s$ fusion protein was not detected by Western blot analysis using either an anti-Ccl-21-antibody or an anti-HlyA$_s$ antibody. A smear of small proteins between 4 and 7 kD was visualized in the silver stained tricine gel containing the proteins of the cell pellet or of the culture supernatant of *E. coli* Top 10 (pALMI-176) (see Figure 5.18). These proteins were absent in the pellet and supernatant of the negative control *E. coli* Top 10 (pALMI-130) that does not contain the *ccl*-21-*hly*A$_s$ gene fusion.

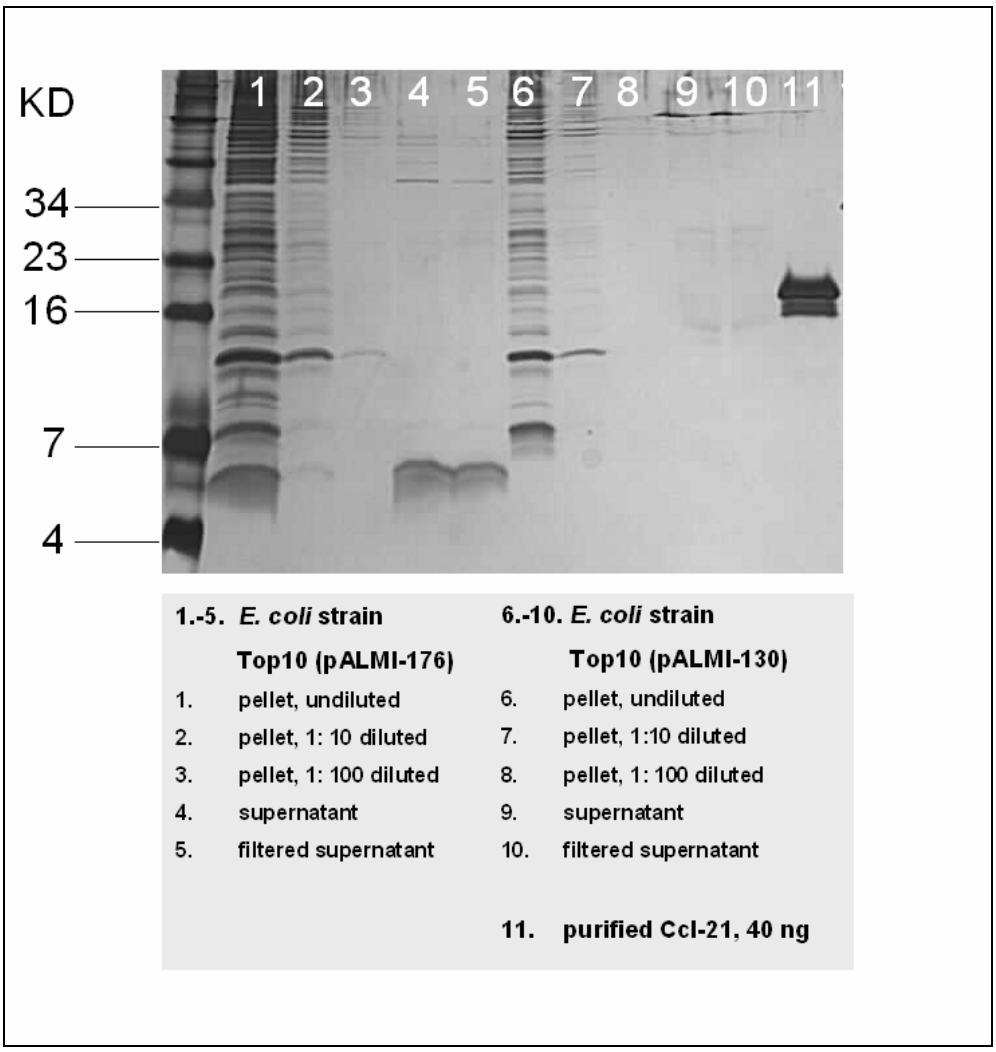


Figure 5.18: 15% tricine gel after silver staining. Supernatants and undiluted and diluted pellets of 50 µl cultures were subjected to SDS gel electrophoresis. The *E. coli* strain Top 10 (pALMI-130) is the common host background. The purified 12 kD Ccl-21 is usually visible at 16 kD in SDS gels.

5.2.3.1 Summary: Expression and Secretion of heterologous proteins

Enzyme activity of the alkaline phosphatase-HlyA$_s$ fusion protein was detected in the cell pellets of *E. coli* Top 10 (pALMI-139) and *E. coli* BL21:DE3 (pALMI-135/pALMI-122), but not in the culture supernatants of these strains. It was higher in the pellet of *E. coli* BL21:DE3 (pALMI-135/pALMI-122) than in the pellet of *E. coli* Top 10 (pALMI-139). A protein >60 kD corresponding with the alkaline phosphatase-HlyA$_s$ fusion protein (56 kD), was detected in the cell pellet of the *E. coli* BL21:DE3 (pALMI-135/pALMI-122), but not in the pellet of *E. coli* Top 10 (pALMI-139). A protein <50 kD was also detected in the pellet of the *E. coli* BL21:DE3 (pALMI-135/pALMI-122).

The PhoA-HlyA$_s$ fusion protein containing a 23 aa C-terminal truncated PhoA was not detected in the culture supernatant and pellet of *E. coli* Top 10 (pALMI-198). However, a protein (about 50 kD) that corresponded with the truncated PhoA-HlyA$_s$ fusion protein (54 kD) was present in the pellet of *E. coli* BL21:DE3 (pALMI-178/pALMI-122), but not in the culture supernatant. A protein >50 kD was also detected in the pellet of this *E. coli* strain.

The generation of a *hly* promoter controlled gene fusion between *omp*A-*hly*A$_s$-*hly*BD failed. Therefore, a T7 promoter controlled *om*pA-*hly*A$_s$ gene fusion was constructed, resulting in plasmid pALMI-177, which was used for co-expression of OmpA-hlyA$_s$ with HlyBD in *E. coli* BL21:DE3 (pALMI-177/pALMI-122). A protein >30 kD was detected in the pellet of this strain. It correlates with the protein size of OmpA-HlyA$_s$ (42 kD). However, a protein <30 kD was also found in the cell pellet of this strain.

The *hly* promoter controlled *pde* 1B1-*hly*A$_s$ gene fusion was transcribed. However, the fusion protein was not detected in the cell pellet or the culture supernatant of *E. coli* Top 10 (pALMI-186).

The Ccl-21-HlyA$_s$ fusion protein could not be detected in the cell pellet or the culture supernatant of *E. coli* Top 10 (pALMI-176). However, proteins between 4 and 7 kD were visualized in the pellet of this strain, but not in the pellet of the negative vector control strain *E. coli* Top 10 (pALMI-130) in a silver stained tricine gel. These proteins were not detected by Western blot analysis using either an anti-Ccl-21-antibody or an anti-HlyA$_s$ antibody.

5.3 Establishment of compartment systems

The compartment system allows the preservation of the proteins of interest following their secretion by the above described Gram-negative bacteria. The following compartment systems were optimized: A modified double filter system and a so-called “stacked microwell system” (see Figure 3.4 and Figure 3.5).

The protein binding capacities of different filter types were tested using ?-hemolysin. For this reason, the culture supernatant of UPEC 536 was subjected to filtration by different filter types and consecutively incubated with human erythrocytes. The hemolytic activities of the filtered supernatants are compared in Figure 5.19.

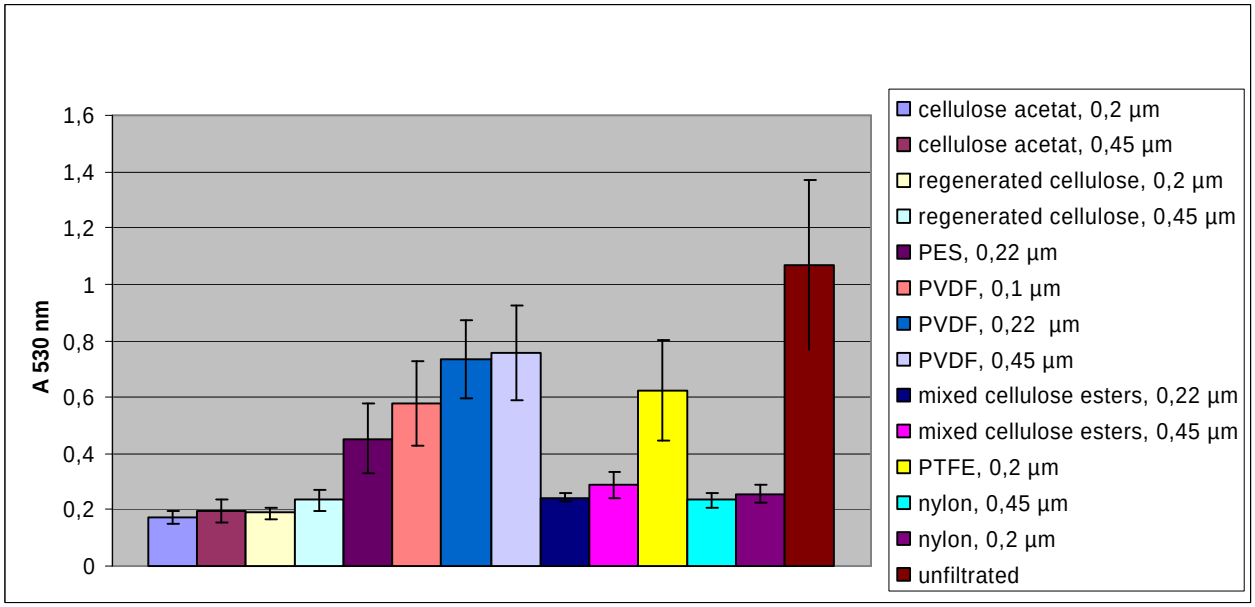


Figure 5.19: Determination of the hemolytic activities of the filtered and unfiltered culture supernatants of UPEC 536 as described in chapter 4.2.26. The released amount of hemoglobin was measured at an adsorption of 530 nm.

Erythrocytes which had been incubated with culture supernatants from UPEC 536 filtered over PVDF membranes (pore diameter of 0,22 nm and 0,45 nm) showed the highest mean value of the adsorption of hemoglobin, indicating that these filter types have a low protein binding capacity. The erythrocytes incubated with culture supernatants filtered with membranes consisting of cellulose acetate, regenerated cellulose, mixed cellulose esters or nylon showed a low adsorption of hemoglobin, indicating that these filters have a high protein binding capacity.

For establishment of the stacked microwell system, bacterial cultures of UPEC 536 and its hemolysin negative mutant 536-21 were subjected to filtration over 0,45 µm Durapore PVDF membranes using the Multi-Screen Assay system (Millipore) (see chapter 4.2.27.2). For measurement of their hemolytic activities, the filtered bacterial cultures were incubated with human erythrocytes. The release of hemoglobin as a consequence of hemolysis was measured by the adsorption at a wavelength of 530 nm.

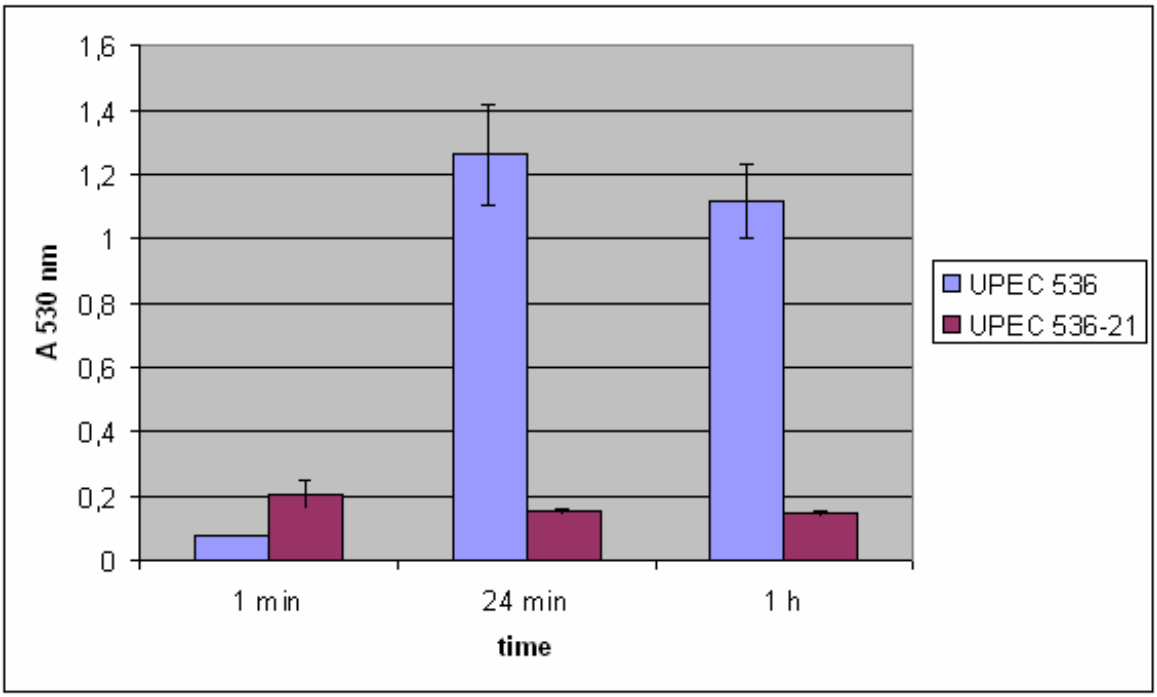


Figure 5.20: Determination of the hemolytic activity of the filtered culture supernatants of UPEC 536 and its negative mutant UPEC 536-21. The released amount of hemoglobin was measured at an adsorption of 530 nm.

It was demonstrated that the filtered culture supernatants of UPEC 536 caused hemolysis of the erythrocytes (Figure 5.20) indicating that the hemolytic activity of the hemolysin protein secreted by UPEC 536 remained stable after the filtration process.

The double filter system (see chapter 4.2.27.1) was established as follows: The *E. coli* strain UPEC 536 and its *hly*-negative mutant 536-21 were subjected to the double filter technology: Six different filter combinations (see Table 5.12, No. 1-6) were placed onto sheep blood agar plates. It was examined, which filter combination prevents the flow of hemolysin secreted by UPEC 536 onto the underlying blood agar plate during bacterial growth.

It was demonstrated that no hemolysis was visible on the blood agar plates which were placed underneath the filter combination 1 and 4 (see Table 5.12).

Consecutively, each of the lower filters of the filter combination 1-6 were placed vice versa on a fresh blood agar plate to examine whether active hemolysin has bound on the lower filter. Hemolytic zones became visible on the blood agar plates underneath the protein spots derived from *E. coli* 536 after incubation with the lower filter of the filter combination 1, 2, 3 and 6.

Table 5.12: Establishment of double filter system by using different filter types.

Two filters were placed on top of each on a sheep blood agar plate. The bacteria were picked onto the top filter and growth was allowed to occur for 3 h at 37°C. Then the corresponding lower filters were placed vice versa on a fresh blood agar plate and incubated for 3 h at 37°C.

No.	Upper filter	Lower filter	Hemolysis during 3 h growth of bacteria	Hemolysis after 3 h incubation with lower filter
1	PVDF, 0,45 µM	Mixed cellulose esters, 0,1 µM	-	+
2	PVDF, 0,45 µM	Cellulose acetate, 0,2 µM	+	+
3	PVDF, 0,45 µM	Cellulose acetate, 0,45 µM	+	+
4	Mixed cellulose esters, 0,1 µM	PVDF, 0,45 µM	-	-
5	Cellulose acetate, 0,2 µM	PVDF, 0,45 µM	+	-
6	Cellulose acetate, 0,45 µM	PVDF, 0,45 µM	+	+

All of secreted hemolysin had bound onto the lower filter of filter combination 1, indicating that the separation of active hemolysin protein from the producing uropathogenic *E. coli* 536 was achieved using the 0,45 µM PVDF membrane filter as a top filter and a 0,1 µM mixed cellulose esters membrane as a lower filter.

6. Discussion

The aim of my Ph.D thesis was the establishment of a novel „Functional Protein Technology" (FunProTec) which allows a quick and easy high yield synthesis of native protein solutions or protein arrays consisting of functionally active proteins. Functional proteins are required for a wide range of applications in pharmaceutical industry, e.g. for the characterization of structure and function of target proteins and their ligands. In this context, FunProTec may serve for the identification of protein binding partners of different chemical nature, e.g. other proteins (including antibodies), nucleic acids and lipids. FunProTec also allows mechanism of action studies of pharmaceutical compounds (see Introduction). The principle of "FunProTec" is the application of a pro- or eukaryotic expression and secretion system which allows the synthesis and secretion of heterologous proteins of interest and a compartment system which preserves the respective proteins and concomitantly separates them from the background proteins of the producing cell.

In this study, FunProTec was established on the basis of the hemolysin secretion system of uropathogenic *E. coli*. The synthesis and secretion of a-hemolysin is encoded by an operon consisting of four genes, designated *hly*C, *hly*A, *hly*B and *hly*D (Ludwig and Goebel, 1991). The *hly*A gene encodes the hemolysin protein (HlyA), HlyC is required for HlyA activation and HlyB and HlyD are components of the hemolysin secretion apparatus. The hemolysin secretion apparatus consists of the membrane proteins HlyB, HlyD and TolC. A heterologous protein will be secreted by the secretion apparatus, if a fusion is generated between the genes of interest and *hly*A_S, encoding the C-terminal signal sequence of hemolysin (HlyA) (Gentschev *et al*., 1994).

In order to create an *E. coli* strain with high secretion efficiency, the following *E. coli* strains were constructed and compared in regard to their capacities to secrete hemolysin. These *E. coli* strains harbour plasmids, in which the genetic structure of the *hly*CABD operon was either maintained (case A), or the *hly*A gene and the activator gene *hly*C were separated from the *hly*BD genes (case B).

The following *E. coli* strains were constructed:

Case A)

1. *E. coli* strain Top 10 (pALMI-140) contains an 0,8 kb *hly*C sequence located upstream of the *hly* operon. This sequence is also located upstream of the wild-type hemolysin gene cluster in the chromosome of uropathogenic *E. coli*. It contains the *hly* promoter and a JUMPstart activator sequence (Hobbs and Reeves, 1994).

2. *E. coli* strain BL21:DE3 (pLysS) (pALMI-138) contains a T7 promoter instead of a 0,8 kb *hly*C upstream sequence upstream of the plasmid encoded *hly* determinants.

Case B)

1. *E. coli* strain Top 10 (pALMI-168/pALMI-122) contains an 0,8 kb *hly*C sequence located upstream of the *hly* operon.

2. *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84) contains a T7 promoter instead of a 0,8 kb *hly*C upstream sequence upstream of the plasmid encoded *hly* determinants.

Another aim was the construction of an *E. coli* strain with a chromosomally encoded hemolysin secretion apparatus to be used as a host stain for plasmid encoded fusions between heterologous proteins and $HlyA_S$. However, the generation of an *E. coli* strain with a chromosomally encoded T7 promoter controlled *hly*BD genes failed. The high amount of *hly*BD mRNA that may have been produced following the integration of the *hly*BD genes into the bacterial chromosome, may have been toxic for the *E. coli* cell. It was reported that the extremely high activity of T7 RNA polymerase and the increased stability of mRNAs may cause basal level expression of the T7 promoter controlled genes before induction of the promoter (Invitrogen, 2002). Basal expression of the T7 RNA polymerase and consequently also the expression of the T7 promoter controlled *hly*BD genes were reduced in the above described *E. coli* strains BL21:DE3 (pLysS) (pALMI-138) and BL21:DE3 (pALMI-15/pALMI-84). The Lac repressor reduced the expression of T7 RNA polymerase in *E. coli* BL21:DE3 (pALMI-15/pALMI-84). Lysozyme expressed by the pLysS plasmid degraded the T7 RNA polymerase in BL21:DE3 (pLysS) (pALMI-138).

It was demonstrated in this thesis, that the *E. coli* strains habouring plasmids with the above mentioned 0,8 kb *hly*C upstream sequence, containing the *hly* promoter and the JUMPstart sequence upstream of the *hly* determinants, were hemolytic. The displacement of this sequence with the heterologous T7 promoter led to loss of the hemolytic activity by the respective *E. coli* strains. Nieto and Leeds demonstrated that the deletion of the JUMPstart region caused a reduction of all *hly* transcripts, leading to a reduced HlyA expression and secretion (Nieto *et al*., 1996, Leeds *et al*., 1997). In contrast to the secretion constructs used in our study, Nieto and Leeds used plasmids which contain an *hly*C upstream region that is larger than 0,8 kb. This region may contain further sequences that are important for hemolysin secretion. Consequently, the deletion of the JUMPstart sequence caused only reduced HlyA secretion in these strains, whereas the exchange of the 0,8 kb *hly*C upstream sequence by the T7 promoter led to a complete loss of HlyA secretion in the present thesis.

It is known that the JUMPstart sequence that is present in all above mentioned hemolytic strains is involved in transcriptional regulation of the *hly* genes (Leeds *et al*., 1997, Nieto *et al*., 1996). Nieto and Leeds described transcript elongation at the stem loop structure between *hly*A and *hly*B by the RNA polymerase and increased transcription of all *hly* determinants. It has been demonstrated in this thesis that the presence of this sequence may cause a fixed stoichiometry between the *hly*A, *hly*B and *hly*D transcripts that is an assumption for secretion of HlyA: In contrast to the ahemolytic strains, the amount of *hly*B mRNA was similar to the amount of *hly*D mRNA in the hemolytic *E. coli* strains containing the JUMPstart sequence.

It should be further investigated, whether the amount of transcript of *tolC* is a critical factor in the HlyA secretion process. If the over-expression of the *hly*BD genes is expected to confer the formation of a higher frequency of intact channels, it may be necessary to also over-express *tol*C. The present study demonstrates that in spite of high-level expression of the *hly*BD genes in the *E. coli* strain BL21:DE3 (pALMI-15/pALMI-84), this strain remained ahemolytic. This fact may be due to too low levels of the TolC protein.

This study demonstrated a 30-40 fold excess of *hly*A transcript over *hly*BD transcripts in all hemolytic *E. coli* strains, leading to the conclusion that the amount of

hemolysin protein may be higher than the amount of the secretion apparatus proteins in these *E. coli* cells. Benabdelhak, 2003, published that one HlyA molecule may interact with one HlyB molecule of the secretion apparatus. Taking this model into account, our results indicate that an excess of hemolysin molecules is produced inside the cell that will be transported step by step out of the cell (Oropezka-Wekerle *et al*., 1989).

This thesis demonstrated, that the transcription of the *hly* genes is regulated by the JUMPstart sequence in a complex manner. Every genetic manipulation may imbalance the stoichiometry of the *hly* transcripts that is responsible for efficient secretion of HlyA. The expression of the *hly* genes is also influenced by various regulation factors, e.g. temperature (Madrid *et al*., 2002) and oxygen as shown by Hahn *et al*., 2003.

In addition to transcriptional regulation, translational regulation was postulated by Guzman-Verri *et al*., 2001, who reported that a protease system for degradation of the HlyA activating HlyC protein may regulate the protein amount of hemolysin in the bacterial cell. However, heterologous proteins secreted by the *hly* secretion system are not activated by HlyC and are therefore not regulated by this protease system. In conclusion, their protein amount may be too high for efficient secretion by the secretion apparatus.

Another critical parameter that may influence the expression and secretion of heterologous proteins by the *hly* secretion system is the codon usage of *E. coli*. The fact that a fusion between a gene encoding the human phosphodiesterase PDE 1B1 and $hlyA_S$ did not result in the expression of the corresponding fusion protein, may be due to the presence of rare codons in the human gene. It was demonstrated that too many rare codons may hamper gene expression (Li *et al*., 2002), while too few rare codons may reduce the secretion rate of a heterologous protein by the *hly* secretion system (Lee *et al*., 2004). Thus, the codon usage has to be optimized in some heterologous genes to achieve not only a high-level gene expression, but also an efficient secretion of heterologous proteins by the *hly* secretion system.

The structure of the protein seems to be an important factor for secretion by the *hly* secretion system. It became obvious that a fusion protein between the human

cytokine receptor ligand Ccl-21 and the *hly* signal sequence $HlyA_s$ is degraded within the *E. coli* cell, while Ccl-21 that was not fused with the *hly* signal sequence was not degraded in the same *E. coli* strain (Karsten Keldermann, ALTANA Pharma, 2004, personal communication). Li *et al*., 2002 and Nakano *et al*., 1992, also described the degradation of heterologous proteins fused with the signal sequence. Nakano argued that the changed protein folding after fusion with $HlyA_s$ may render these proteins more sensitive to intracellular proteolysis (Nakano *et al*., 1992).

It is also conceivable that heterologous proteins will become insoluble in the culture supernatant after secretion. In the present study, a fusion protein between the *E. coli* outer membrane protein A (OmpA) and $HlyA_s$ was detected in the cell pellet of the respective *E. coli* strain. However, it remains to be investigated, whether the fusion protein was not secreted by the secretion apparatus or whether it precipitated in the culture supernatant after secretion. Mackmann *et al*., 1987, published the secretion of an *E. coli* outer membrane protein OmpF by the *hly* secretion system. However, this protein was not secreted as a full-length protein and was fused with ten amino acids of the LacZ protein at the N-terminus. This indicates that the truncation or the fusion with the LacZ protein may prevent the OmpF protein to become insoluble after its secretion into the culture supernatant**.** Insoluble proteins may be truncated and/or fused with different tags to achieve solubility in the culture supernatant after their secretion.

Moreover, the transmembrane domains of OmpA may hamper the secretion of the OmpA-$HlyA_s$ fusion protein by the *hly* secretion system (Gomez-Duarte *et al*., 2001, Sugawara *et al*., 1996). It may be possible that the OmpA-$HlyA_s$ fusion protein inserts itself into the outer membrane during the transport by the *hly* secretion apparatus. However, other studies described that the outer membrane protein PagC of *Salmonella typhimurium* also contains transmembrane segments, but was efficiently secreted into culture supernatant (Mollenkopf *et al*., 1996, Cirillo *et al*., 1996). In contrast to OmpA, the N-terminus of PagC was fused with the N-terminus of HlyA. It was shown that the N-terminus of HlyA may be important for secretion of hemolysin, because it translocates the protein in and across the bacterial membrane (Erb *et al*., 1987, Ludwig and Goebel, 1991). It may therefore be concluded that the secretion of $HlyA_s$-fusion proteins, whose N-termini are fused with the HlyA N-terminus, may be facilitated.

Many heterologous proteins which are secreted by the *hly* secretion system were fused with more than 60 amino acids of the HlyA C-terminus (Table 7.1). The secretion of heterologous proteins may be facilitated by fusion of the heterologous genes with a gene sequence encoding at least 150 amino acids of the HlyA C-terminus. Bingle *et al.,* 1997, described that the sequence localized about 150-250 amino acids upstream of the HlyA C-terminus contains aspartate-containing glycine rich regions which act as a spacer between the secretion signal and the heterologous protein and may be important for presentation of the secretion signal to the secretion apparatus.

There may be elements in the protein structure of heterologous proteins that influence their secretion efficiency. In this thesis, it was demonstrated that a fusion protein between alkaline phosphatase (PhoA) and $HlyA_s$ was not secreted by the *hly* secretion system, while the secretion of this protein by this system was published by Gentschev *et al*., 1990. However, in the latter case, the PhoA protein was truncated by 17 N-terminal amino acids and 23 C-terminal amino acids. Gentschev *et al*., 1990, described that the C-terminus of PhoA protein may be the critical factor for secretion, because it contains a potential interaction site with the chaperon SecB. SecB folds the protein into a water-soluble confirmation that may hamper the secretion by the *hly* secretion system (Gentschev *et al*., 1990). Therefore, we examined the secretion of a PhoA-$HlyA_s$ fusion protein lacking the 23 C-terminal amino acids of the PhoA protein. This fusion protein was not secreted into the culture supernatant of *E. coli*, indicating that the N-terminus of PhoA may be critical for transport of the PhoA-$HlyA_s$ fusion protein. It remains to be investigated, whether a PhoA-$HlyA_s$ fusion protein lacking solely the 17 N-terminal amino acids of the mature PhoA will be secreted by the *hly* secretion apparatus.

The fact that the full-length or C-terminally truncated alkaline phosphatase was not secreted, but the C- and N-terminally truncated protein was secreted may be explained by an alteration in the protein folding that may hamper secretion efficacy.

This and other secretion studies of heterologous proteins demonstrated, that the folding of the proteins may play a key role in the secretion process. With the exception of toxins or peptides and very small proteins up to 200 amino acids, only two full-length proteins, the ß-lactamase and the cholesterinesterase/lipase, were

secreted by the *hly* secretion apparatus (Table 7.1). This indicates that the degree of folding of the proteins may influence their secretion. Hemolysin itself may remain in an unfolded state during its secretion (Gentschev *et al*., 1990). The diameter of the TolC channel may also be insufficient for the entry of a folded protein (Fraile *et al*., 2004). It was reported that the intact lipopolysaccharide core influences the tertiary structure of hemolysin during or after secretion (Vakharia *et al*., 2001). It remains to be investigated whether the application of chaperons may improve the secretion of proteins by the *hly* secretion system by changing their folding behaviour.

Sheps *et al*., 1995, described sites in HlyB that are specific for its interaction with HlyA. These sites may be not recognized by some heterologous proteins and hamper their secretion. An alteration of these sites by mutagenesis may improve the secretion of some heterologous proteins (Sugamata *et al.,* 2005).

In summary, a variety of parameters are critical in the secretion process of heterologous proteins and its efficiency. E.g. some $HlyA_S$ fusion proteins were secreted into the culture supernatant with an efficiency of 20-100 fold lower than that of HlyA (Hahn *et al.*, 2003), while other fusion protein were secreted in yields of 50-100 fold higher than that of HlyA (Blight and Holland, 1994).

There are other bacterial secretion systems which may be exploited for the development of FunProTec.

Hahn *et al.,* 2003, described a type I secretion system from *Caulobacter crescentus* which is involved in the export of (S)-layers, for the heterologous expression and secretion of 109 amino acids of an envelope glycoprotein from infectious hematopoietic necrosis virus. The secretion apparatus has a higher transport efficiency than the Hly system. In contrast to the Hly secretion system, secretion occurs not only during the logarithmic growth phase, but during the whole period of cell growth and division (Hahn *et al*., 2003).

Moreover, Majander *et al*., 2005, used a modified type III secretion apparatus from an *E. coli* K12 derivative for the secretion of heterologous proteins into the culture supernatant of *E. coli*. Four different pro- and eukaryotic proteins with a length

of up to 400 amino acids were secreted in concentrations of 1-15 mg protein per litre culture. However, it has been mentioned by the authors that there are some proteins, which were not further described, were not secreted by this secretion system.

Bacillus spp. is the major representative of Gram-positive bacterial secretion systems often used for commercial production of extracellular proteins (van Wely *et al*., 2001; Westers *et al*., 2004). Those proteins are secreted across the cell membrane by a Sec or Tat translocase after fusion with a signal peptide (Westers *et al*., 2004).

In general, prokaryotic secretion systems are well adapted to their naturally secreted protein and vice versa. In consequence, secretion of heterologous proteins which resemble the natural substrate, e.g. in respect to its protein structure, may be facilitated, while the secretion of certain heterologous proteins with no similarities to the natural substrate may be hampered.

More than 200 heterologous proteins of different organisms, compartments and cell localization have already been secreted by the secretion system of *Pichia pastoris* (Cereghino *et al*., 2002; Cereghino and Cregg, 2000; Cregg *et al.,* 2000). The proteins are fused with a signal sequence (alpha factor) for their transport across the endoplasmatic reticulum and the Golgi apparatus. However, it was shown that this secretion system also has its limits, i. e. not all proteins are secreted by the *Pichia pastoris* secretion system, may be due to an improperly processed signal sequence or certain structural protein features. The critical parameters are under investigation (Cereghino *et al*., 2002, Cereghino and Cregg, 2000).

As mentioned above, in addition to a suitable secretion system, FunProTec requires a suitable compartment system which preserves the secreted proteins and concomitantly separates them from the background proteins of the producing cell. The double filter system should be used for the production of protein arrays, while the stacked microwell system should be applied for the production of protein solutions in micro-scale.

In this thesis, the double filter system and the stacked microwell system were validated as suitable compartment systems, using alpha hemolysin producing *E. coli* strains. The separation of the hemolysin protein from the secreting *E. coli* strains without loss of its activity was achieved in these compartment systems by the application of suitable filters.

In conclusion, this thesis showed that the *E. coli* hemolysin secretion system is not applicable as a general system for the secretion of heterologous proteins. However, it may be suitable for the high-yield synthesis and secretion of unfolded proteins, bacterial toxins, small proteins or peptides. It suggests a defined stoichiometry between the heterologous proteins and the secretion apparatus proteins HlyBD and TolC to be important for efficient secretion.

Each pro- and eukaryotic secretion system described in the literature has its pros and cons. In order to gain a deeper insight into the applicabilities of the various systems for FunProTec, a variety of proteins from different origin and structure should be expressed in the various systems and compared for their secretion efficiencies. The specific protein features should be considered.

As mentioned above, both, the stacked microwell and the double filter system, have been validated in this thesis as suitable compartment systems, applicable for FunProTec.

In conclusion, FunProTec might be a suitable technology for the production of either arrays or protein solutions with either unfolded proteins, or native bacterial toxins, small proteins or peptides.

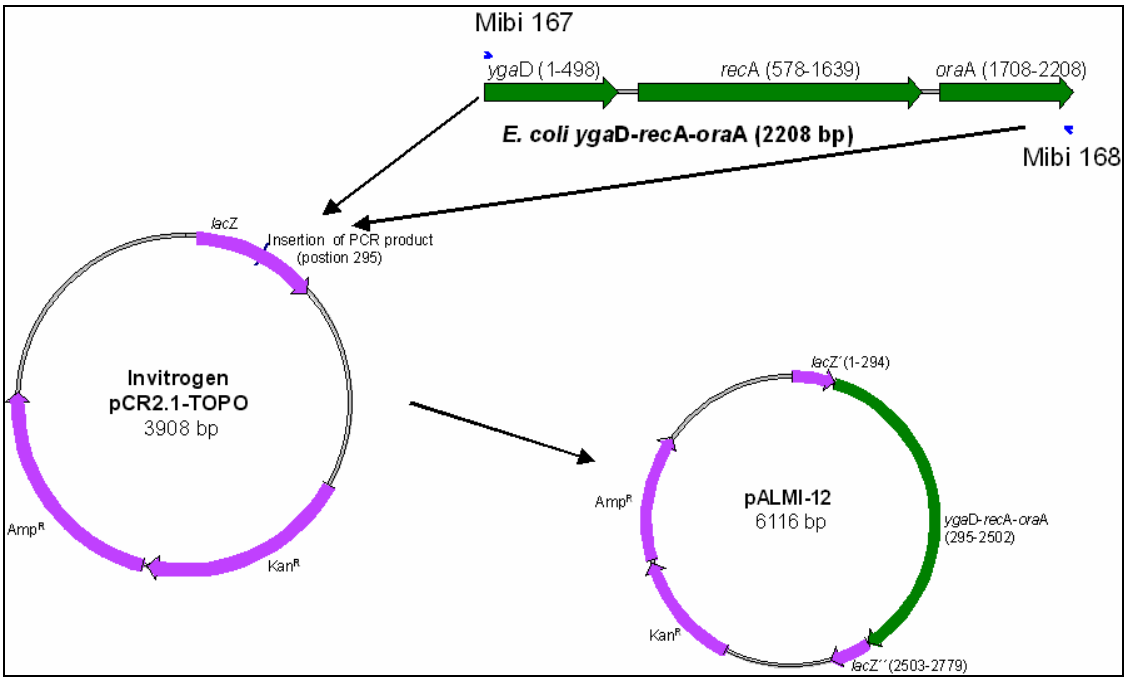


Figure 7.1: Construction of pALMI-12: *yga*D-*rec*A-*ora*A (2208 bp) was amplified using the primer pair Mibi 167 and Mibi 168 and the *E. coli* strain J53 as a template. The PCR product was ligated with pCR2.1-TOPO vector to create pALMI-12.

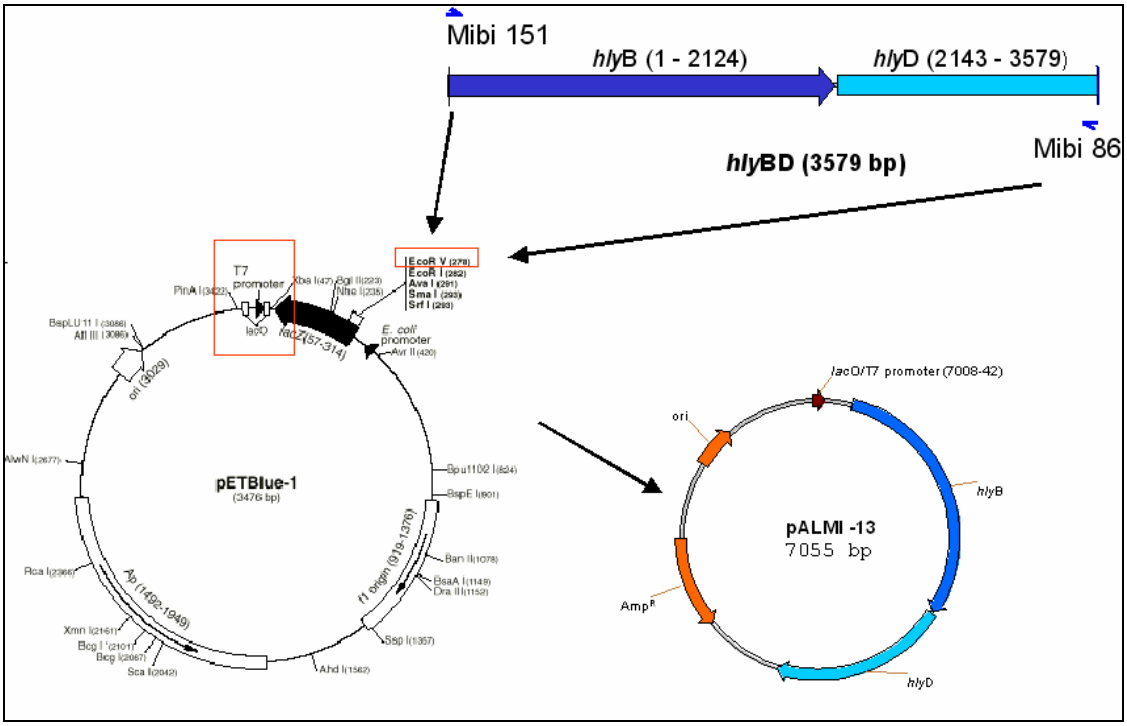


Figure 7.2: Construction of pALMI-13: *hly*BD (3579 bp) was amplified using the primer pair Mibi 151 and Mibi 86 and pCOS10 as a template. The PCR product was ligated with the pETBlue-1 vector restricted with *Eco* RV to create pALMI-13.

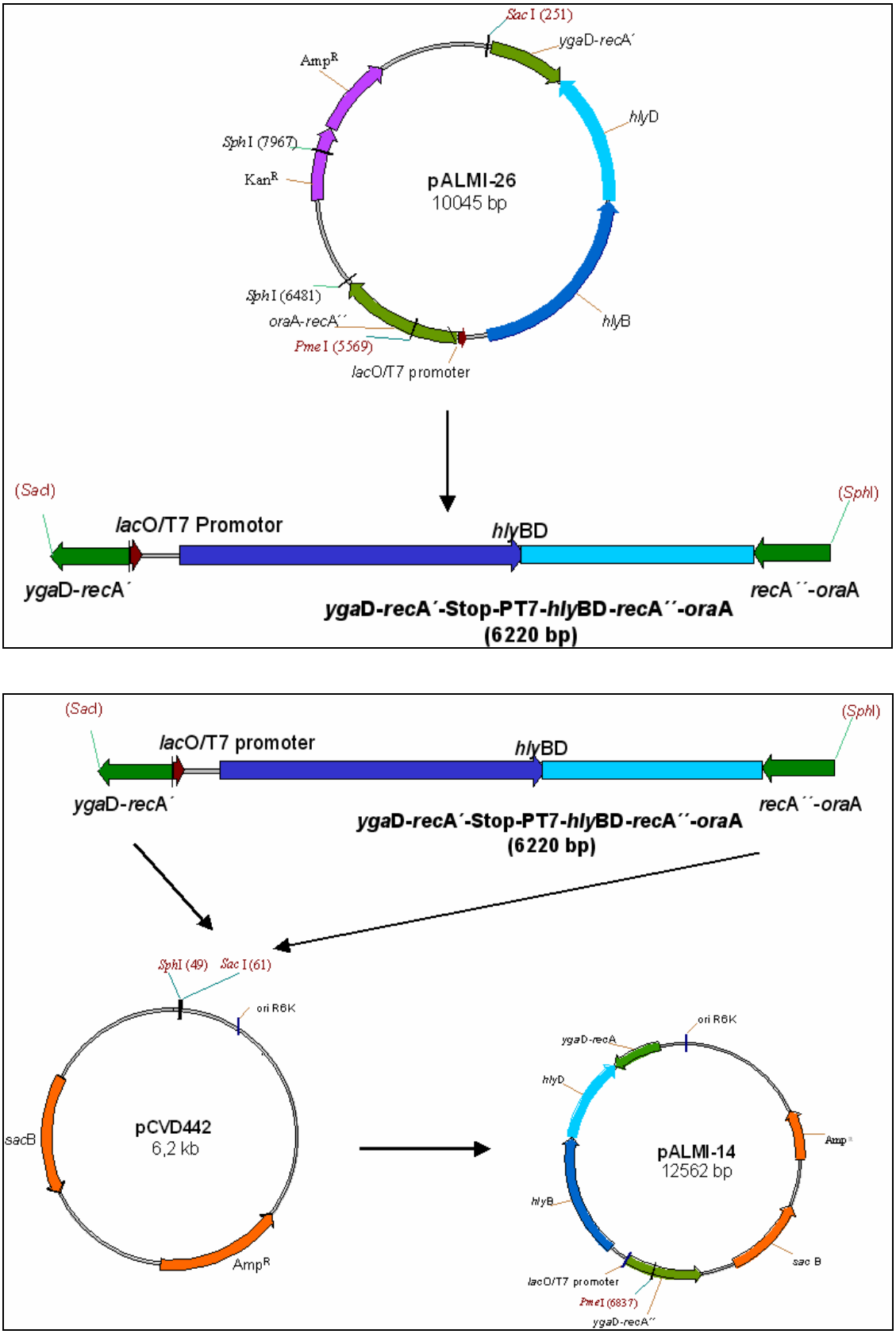


Figure 7.3: Construction of pALMI-14: Plasmid pALMI-26 was restricted with *Sac* I and *Sph* I. The resulting 6220 bp fragment was ligated with pCVD442 cleaved with the same enzymes.

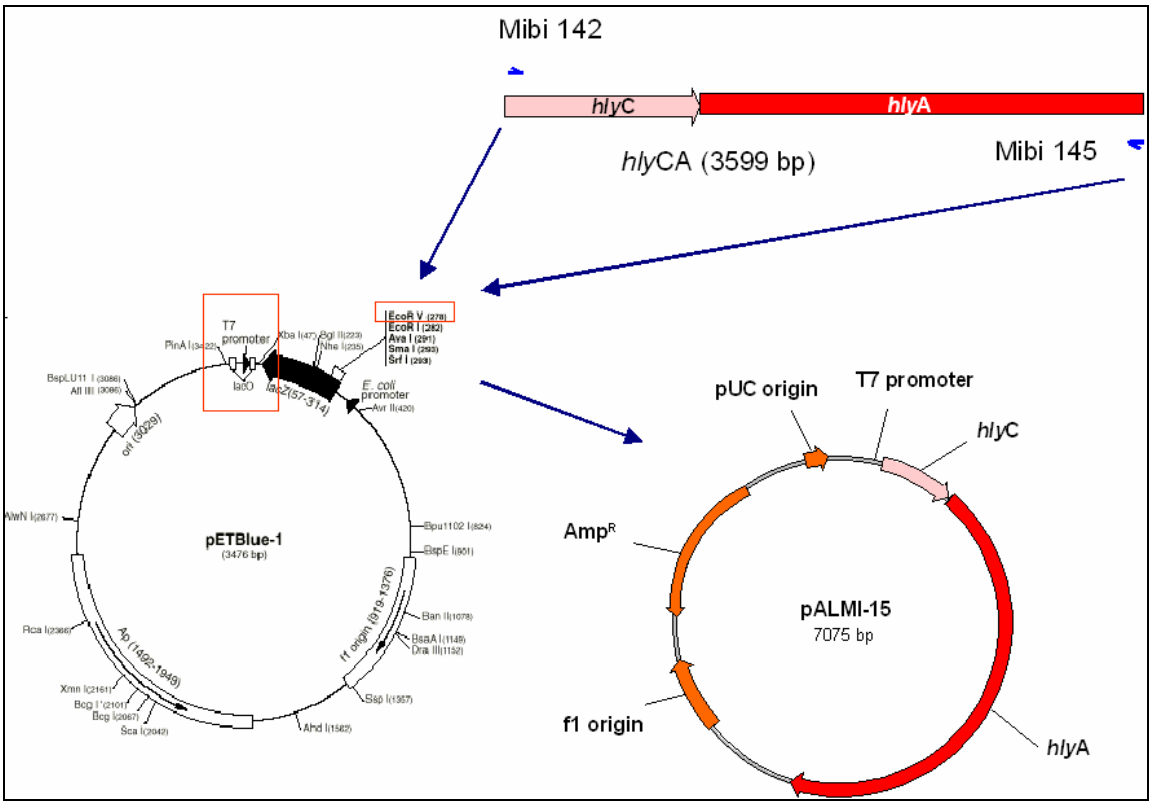


Figure 7.4: Construction of pALMI-15: The *hly*CA genes (3599 bp) were amplified using the primer pair Mibi 142 and Mibi 143 and pCOS10 as a template. The PCR product *hly*CA was ligated with pETBlue-1 restricted with *Eco*RV to create pALMI-15.

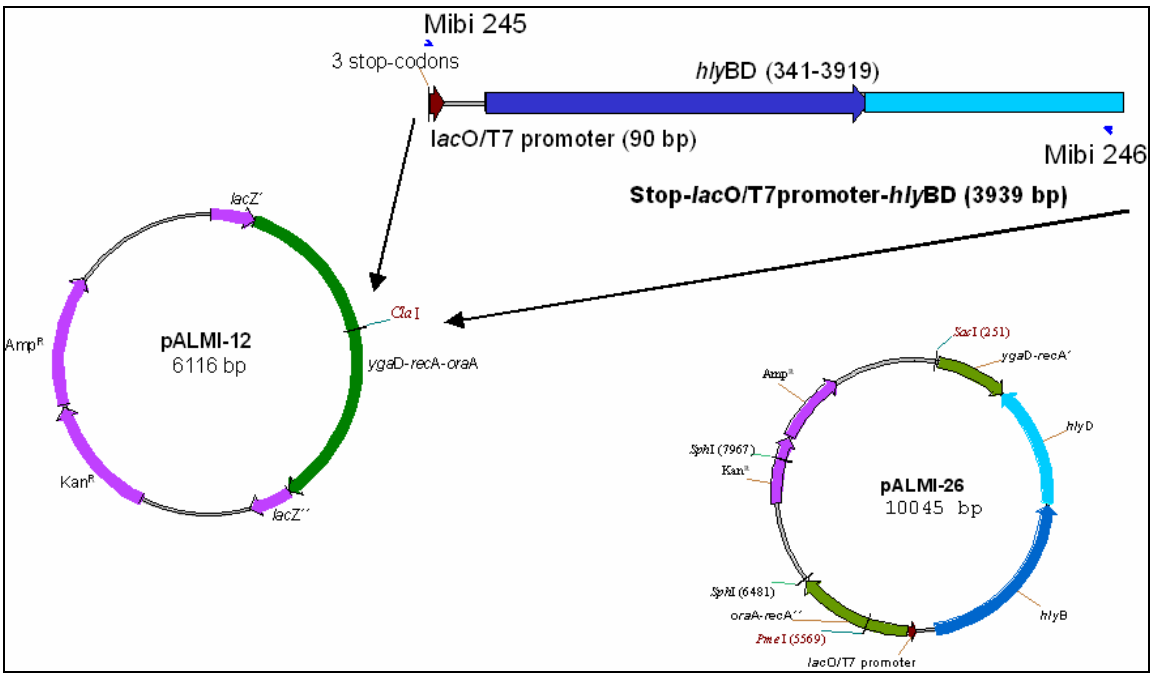


Figure 7.5: Construction of pALMI-26: The *hly*BD genes (including three stop codons, *lac* operator and T7 promoter) were amplified from pALMI-13 using the primer pair Mibi 245 and Mibi 246. The PCR product was restricted with *Cla* I and ligated with *Cla* I- restricted pALMI-12.

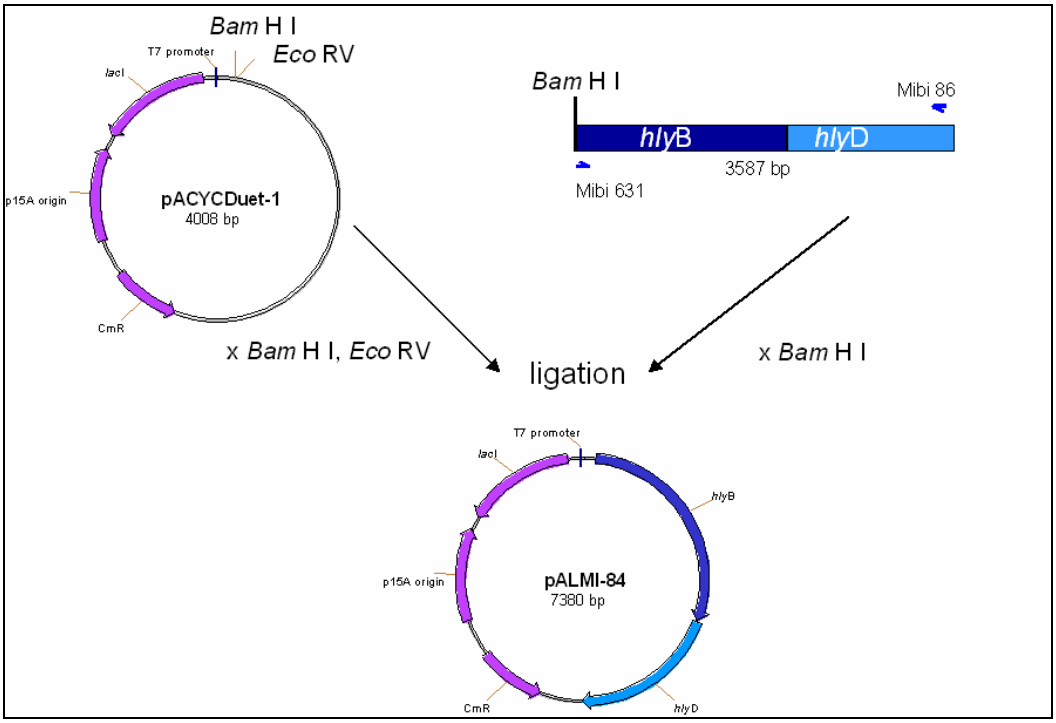


Figure 7.6: Construction of plasmid pALMI-84: The *hly*BD genes were amplified from pCOS10 using the primer pair Mibi 631 and Mibi 86. Pfu polymerase was used to generate blunt ends. The PCR product containing a 5` *Bam* H I restriction site was restricted with *Bam* H I and ligated with pACYCDuet-1 cleaved with *Bam* H I and *Eco* RV.

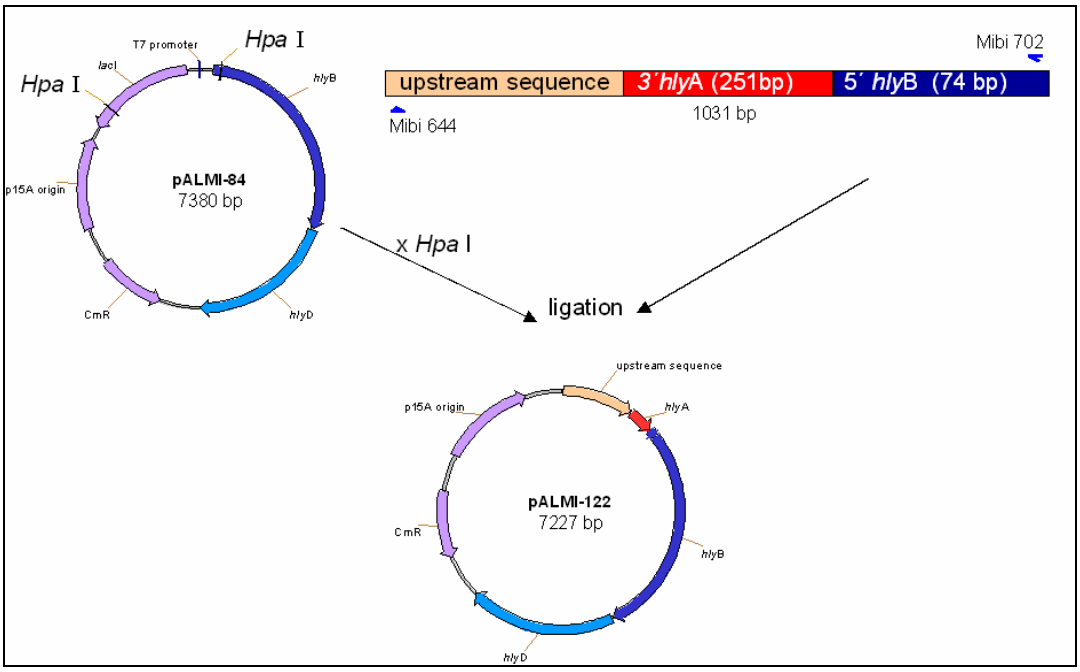


Figure 7.7: Construction of pALMI-122. Amplification of a PCR product containing 795 bp upstream of *hly*C, 251 bp of the 3´end of *hly*A and 74 bp of the 5´end of *hly*B using the 5´phosphorylated primer pair Mibi 644 and Mibi 702 and pALMI-121 as a template. The PCR product was blunted with Klenow to remove the 3`overhanging A and ligated into *Hpa* I restricted pALMI-84.

```
CCC GGG TTC ATT GTG CGA AGG CAT GGC ATA TTT GTT CCC
GGT GTC GTC AGA CAG CAT AAT TCG ACT CTC CAT CTG CTG
TGT GGC CAG ACA AAA GAT GGC CTT GTT TGC CGC GGT GGA
AAT GGA GGG AGG ACG AGC CCA CAA GCA TGA AAA ACT TAG
CCC AGG CTG TGG AAG TGC AAC TTT TGC TCC GGT TGC TGC
AGT AAA ACA CGA GTA TAC GTC TGC CGT ACA CAT TCA TAA
GCG ATT CTG ATG GCT CAC TTA CGT GGA GAA ATG GAG TGC
CGT CTG TTC TTT ATT TAT TGT TTG TGT TAT GTT ATA ACA
TAT AAA AGA GTA TTG TTT GGC ATC TGT AAC TTA TTG AGA
AGG CAA ACT GCA GAG TGG TTA ATG CAG TAA TTG CAT TAG
CTA AAA TTT TGT TAA TTA AAA TTT GTT TGA TTG CTA ATG
GTT TGT GCT GGT TGA TGA CTG TTA ATT CCA GAA GGC GGT
AGT CTG CAT TAA TAT TAG CAT TAC GGT GAC CAG CTT TTA
TTC CGG CCC CTT CTT TCA TGA AAC AAC GTA TTC CGA AGA
ATA AGT TTG AGA ATG GCA GAC GAA CTT TTA TTA TTC TGC
TGA ATA CAG AAG ATA TGA ATT GTT CTT GTT TAT GTC AGA
TAT TCA TAA CAC AGG TAT TAT GGT TAA CTC ATA ACA TTA
ATT CCT GTA TTT TTC TGC TCA ATG GCA GCG TCG TAT GCA
TAT GTT TTT ATT TCA AAT GAA GCA AGG TGC AGG AAA TAA
AAA TAA CCA TTT CTT TAT GTG ATC TTT TTA TCA ATG GAG
ATA GTA TCA TTT ATA Start hlyC
```

Figure 7.8: Amplified *hly*C upstream sequence of uropathogenic *E. coli* J96.

The green lines show the differences to the sequence of UPEC J96 published by Felmlee *et al*., 1985 (insertion of A, nucleotide change A to G, deletion of C, nucleotide change T to C). The red lines of sequence show the promoter region of the *hly*CABD operon as described by Welch *et al*., 1988. The start of translation is shown in blue. The yellow co lour depicts the JUMPStart and extended sequences as defined by Hobbs and Reeves, 1994.

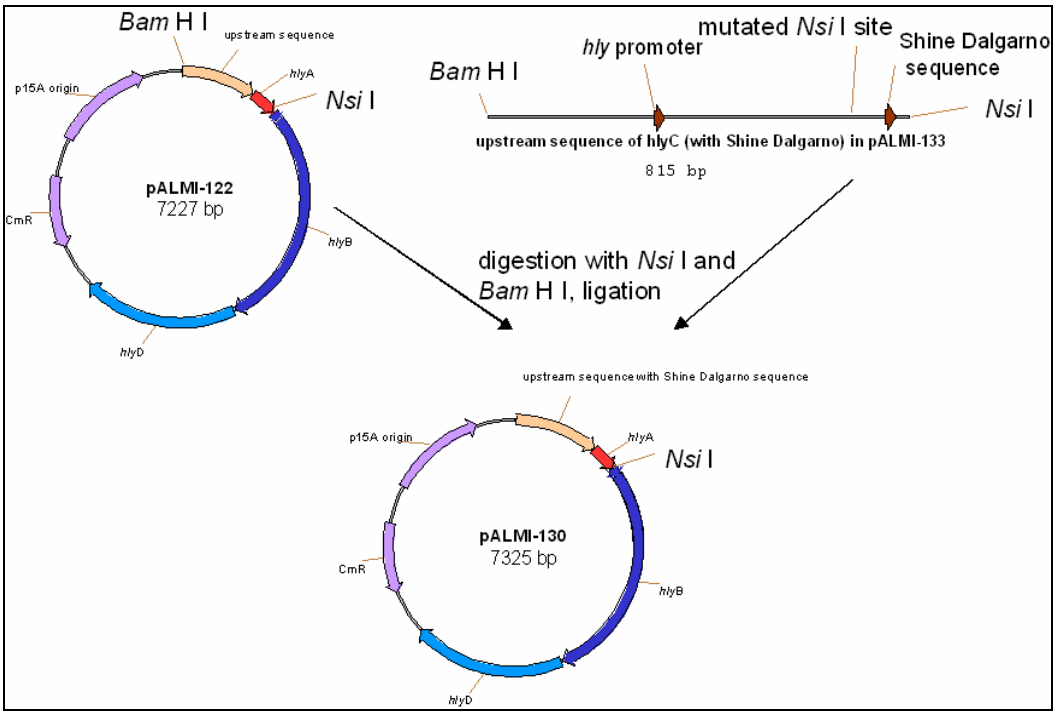


Figure 7.9: Construction of pALMI-130: The *hly*C upstream sequence was cut out of pALMI-133 with *Bam* H I and *Nsi* I and ligated with pALMI-122 restricted with *Bam* H I and *Nsi* I to construct pALMI-130.

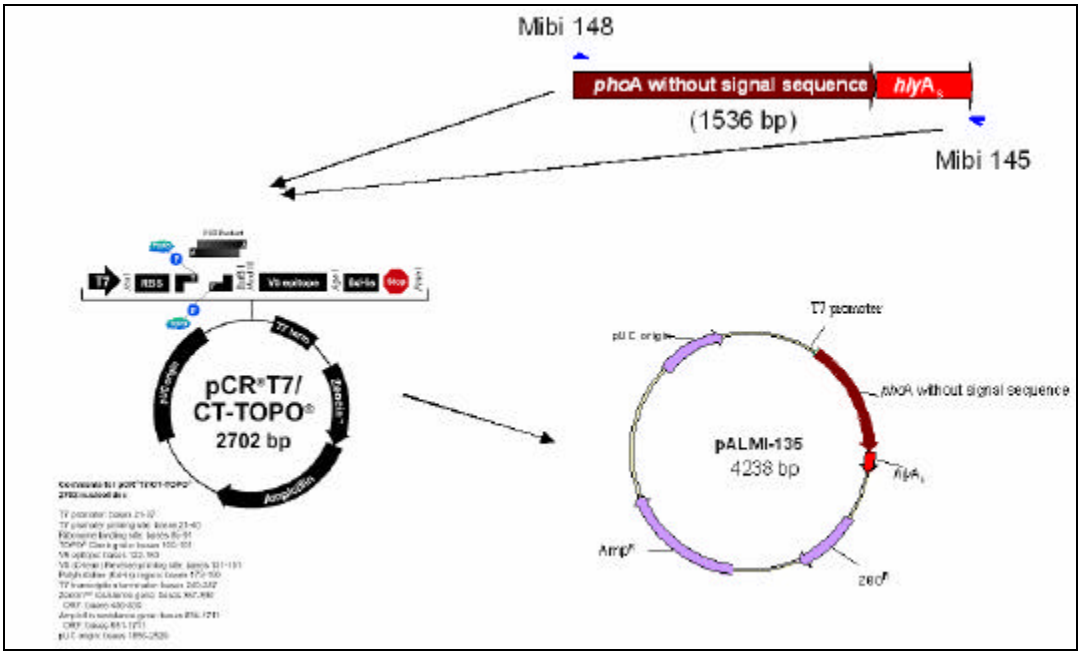


Figure 7.10: Construction of pALMI-135: *pho*A-*hly*A$_s$ was amplified using the primer pair Mibi 148 and Mibi 145 and pALMI-19 as a template. The PCR product was ligated with pCRT7/CT TOPO to construct pALMI-135.

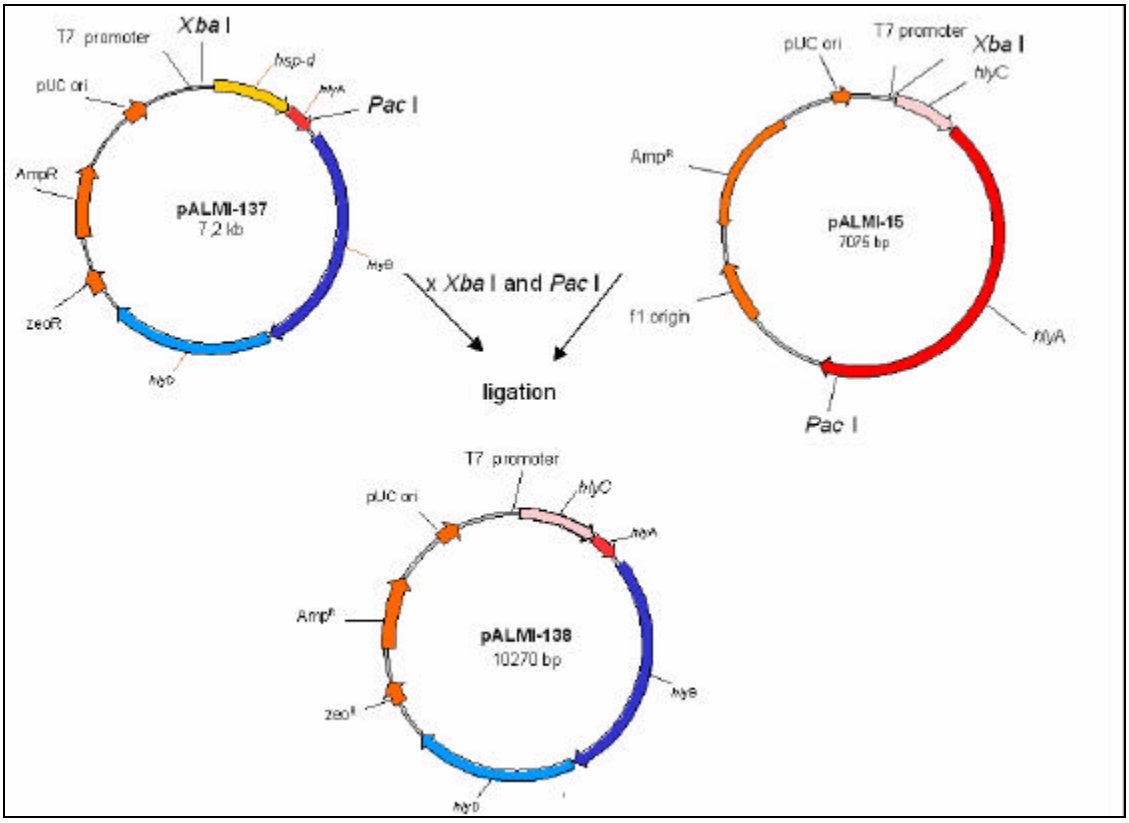


Figure 7.11: Construction of pALMI-138: pALMI-15 was restricted with *Xba* I and *Pac* I. The *Xba* I-*hly*CA-*Pac* I fragment was ligated with pALMI-137 restricted with *Xba* I and *Pac* I.

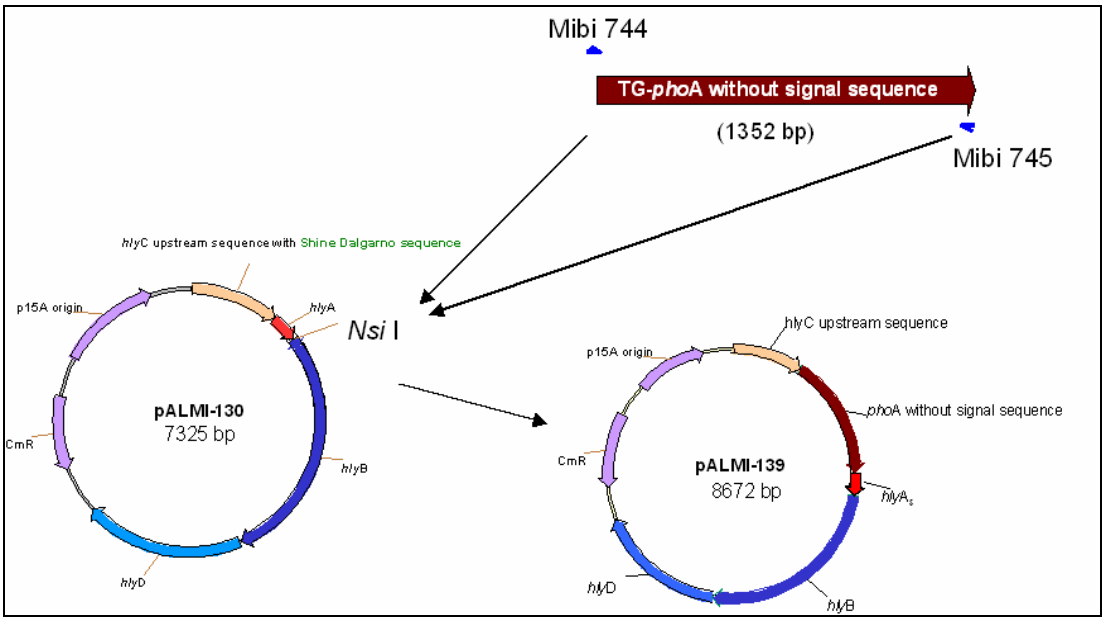


Figure 7.12: Construction of pALMI-139: TG-*pho*A was amplified using the 5´phosphorylate d primer pair Mibi 744 and Mibi 745 and pALMI-19 as a template. The PCR product was blunted with Klenow to remove the 3`overhanging A. Plasmid pALMI -130 was restricted with *Nsi* I and blunted with Klenow. The PCR product was ligated with restricted pALMI -130 to construct pALMI-139.

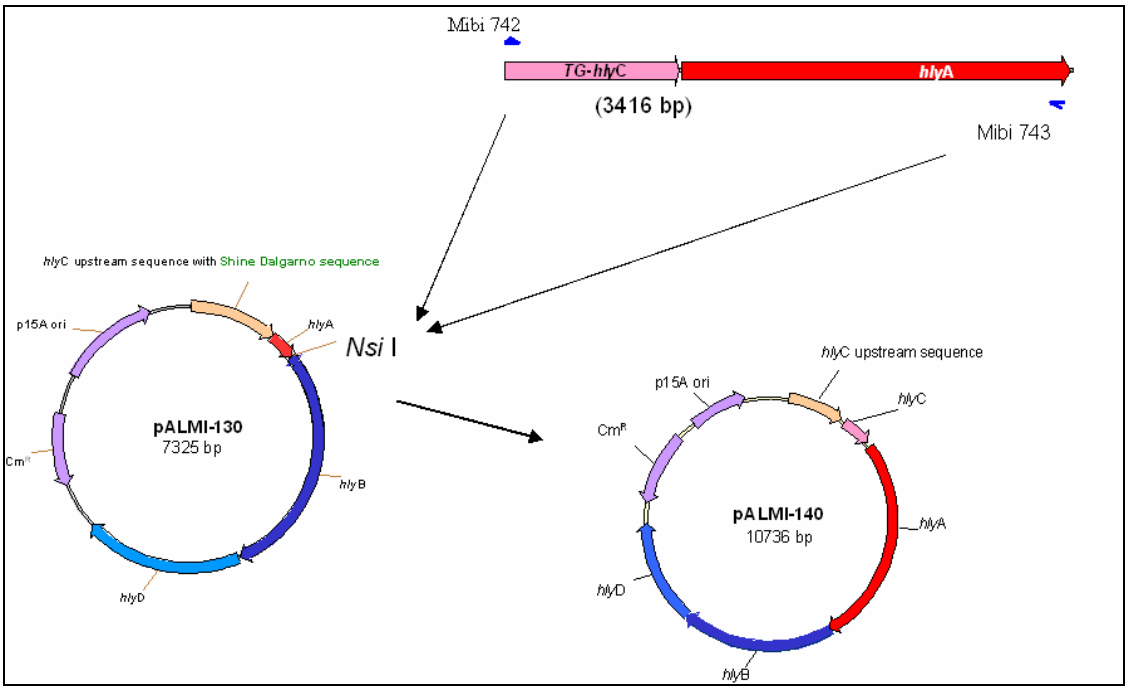


Figure 7.13: Construction of pALMI-140: TG-*hlyC*A was amplified using the 5´phosphorylated primer pair Mibi 742 and Mibi 743 and pALMI-14 as a template. The PCR product was blunted with Klenow to remove the 3`overhanging A. Plasm id pALMI-130 was restricted with *Nsi* I and blunted with Klenow. The PCR product was ligated with restricted pALMI -130 to construct pALMI-140.

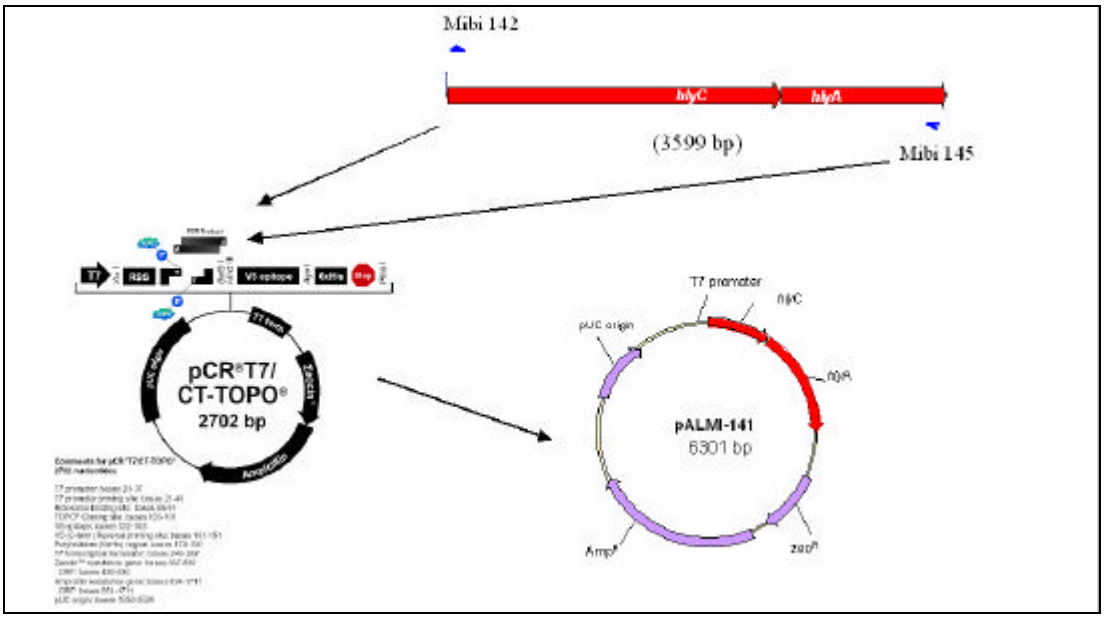


Figure 7.14: Construction of pALMI-141: The *hly*CA genes were amplified using the primer pair Mibi 142 and Mibi 145 and pCOS10 as a template. The PCR product was ligated with pCRT7/CT-TOPO vector to construct pALMI-141.

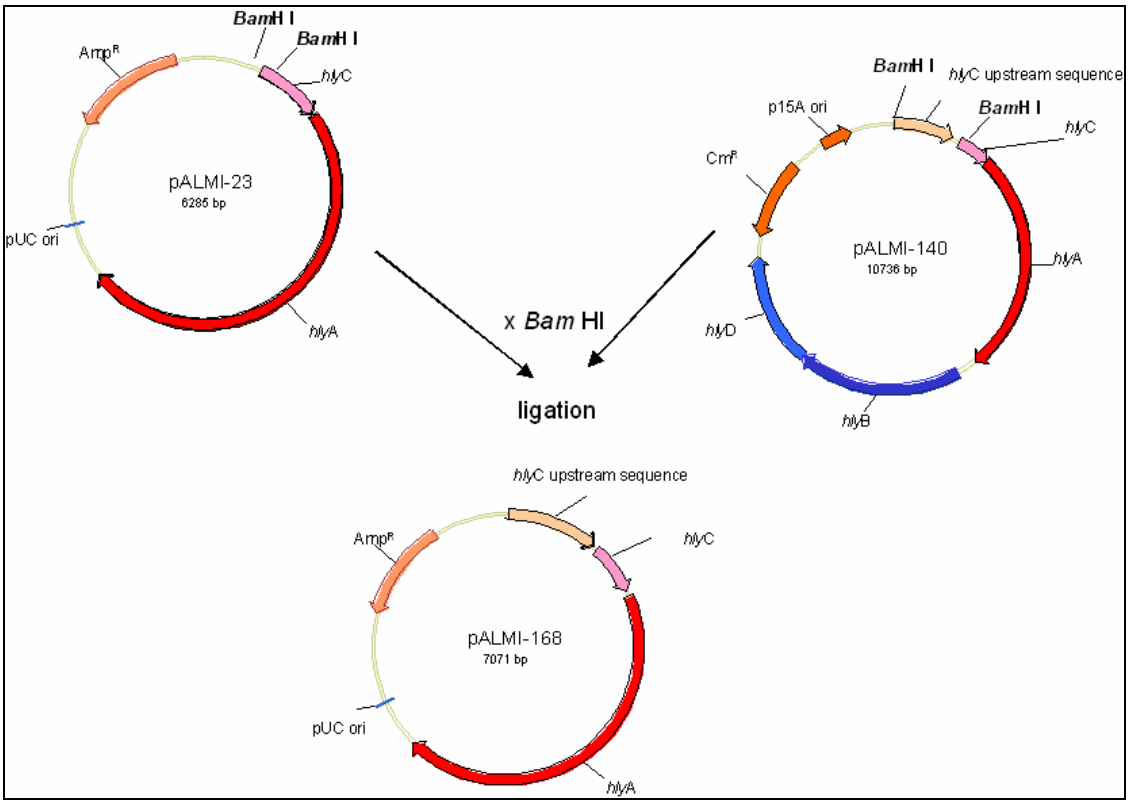


Figure 7.15: Construction of pALMI-168: pALMI-140 was restricted with *Bam*H I. The smaller fragment containing a *hly*C upstream sequence and a part of *hly*C was ligated with pALMI-23 restricted with *Bam* H I.

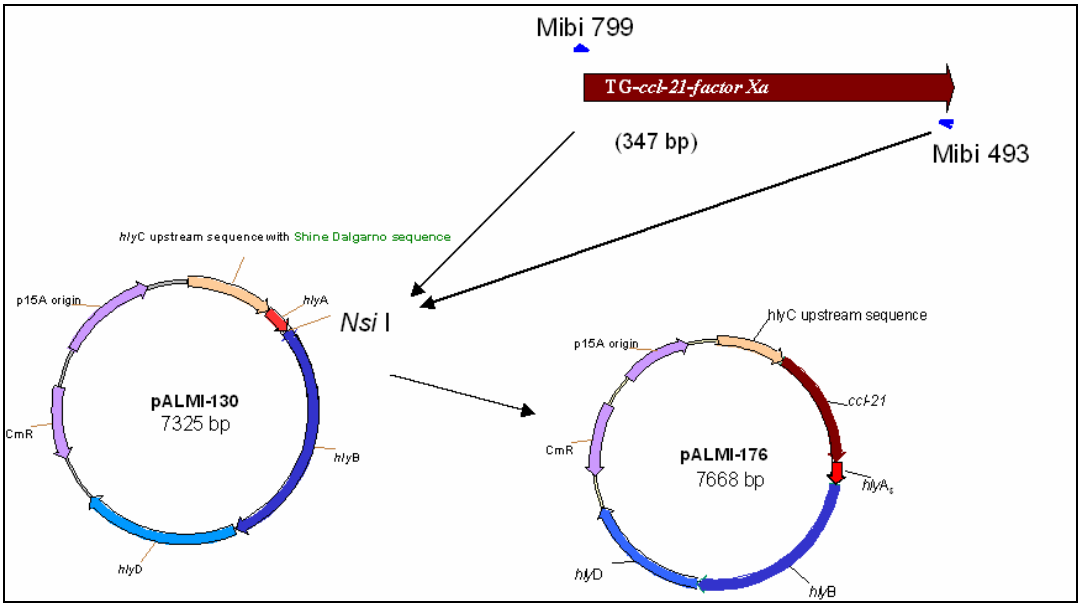


Figure 7.16: Construction of pALMI-176: TG-*ccl*-21 including a sequence encoding factor Xa was amplified from pALMI-76 using the 5´phosphorylated primer Mibi 799 and Mibi 493. The PCR product was blunted with Klenow to remove the 3`overhanging A. Plasmid pALMI -130 was restricted with *Nsi* I and blunted with Klenow. The PCR product was ligated with restricted pALMI-130 to construct pALMI-176.

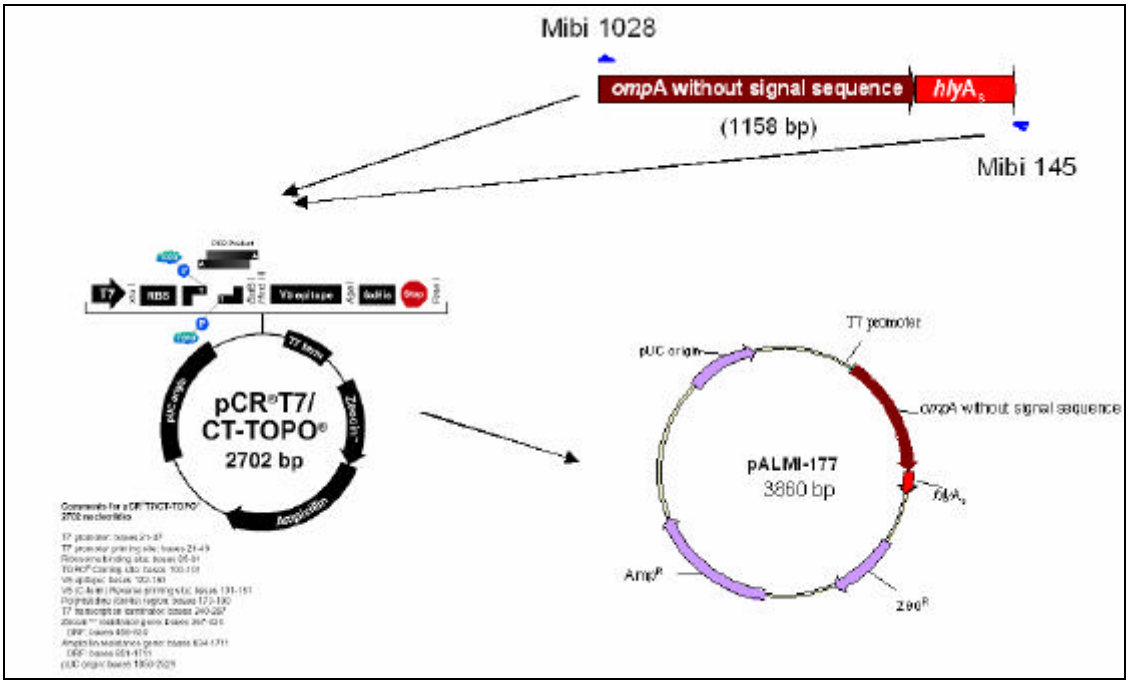


Figure 7.17: Construction of pALMI-177. *omp*A-*hly*A$_s$ was amplified using the primer pair Mibi 1028 and Mibi 145 and pALMI-17 as template. The PCR product was ligated with pCRT7/CT -TOPO vector to construct pALMI-177.

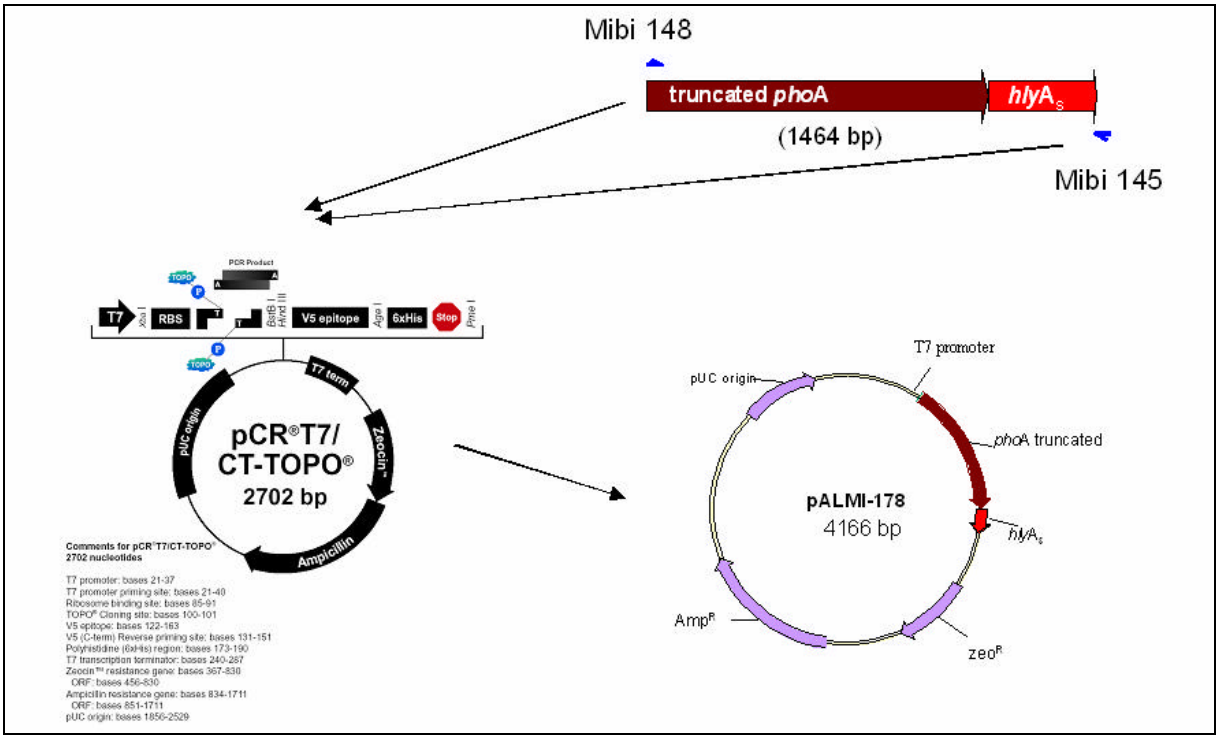


Figure 7.18: Construction of pALMI-178. *pho*A (truncated)-*hly*A$_s$ was amplified using the primer pair Mibi 148 and Mibi 145 and pALMI-198 as a template. The PCR product was ligated with pCRT7/CT-TOPO vector to construct pALMI-178.

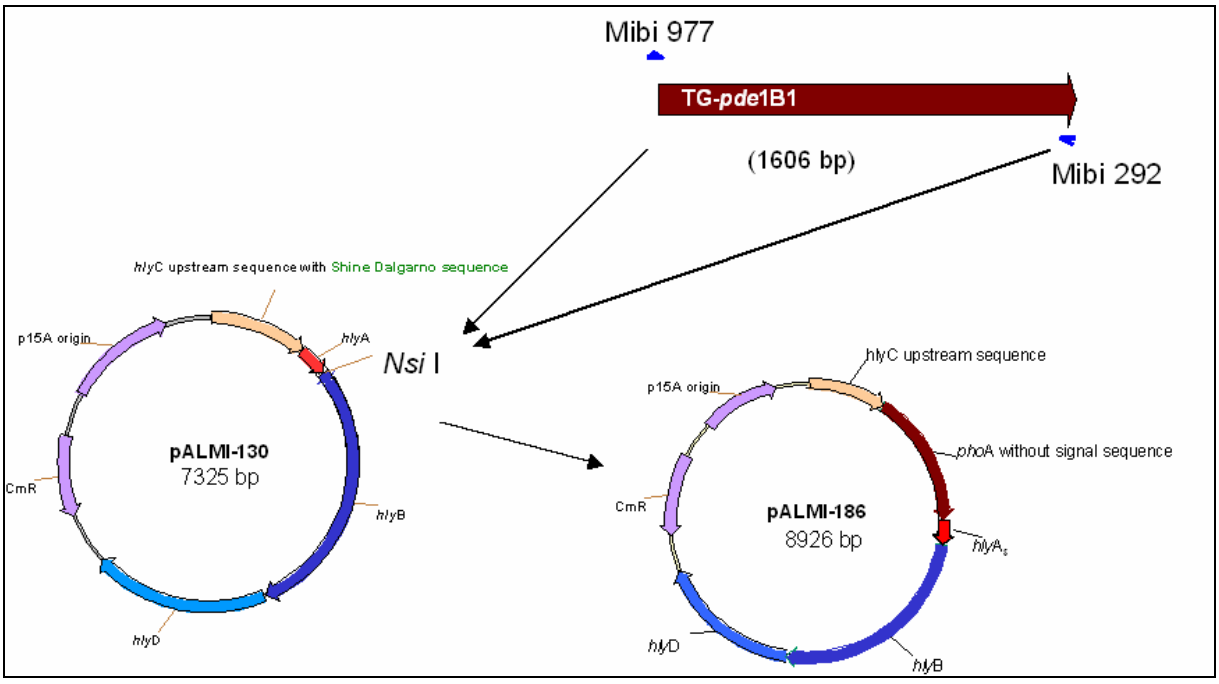


Figure 7.19: Construction of pALMI-186. TG-*pde* 1B1 was amplified from pZ132 using the 5´phosphorylated primer Mibi 977 and Mibi 292. The PCR product was blunted with Klenow to remove the 3`overhanging A. Plasmid pALMI-130 was restricted with *Nsi* I and blunted with Klenow. The PCR product was ligated with restricted pALMI -130 to construct pALMI-186.

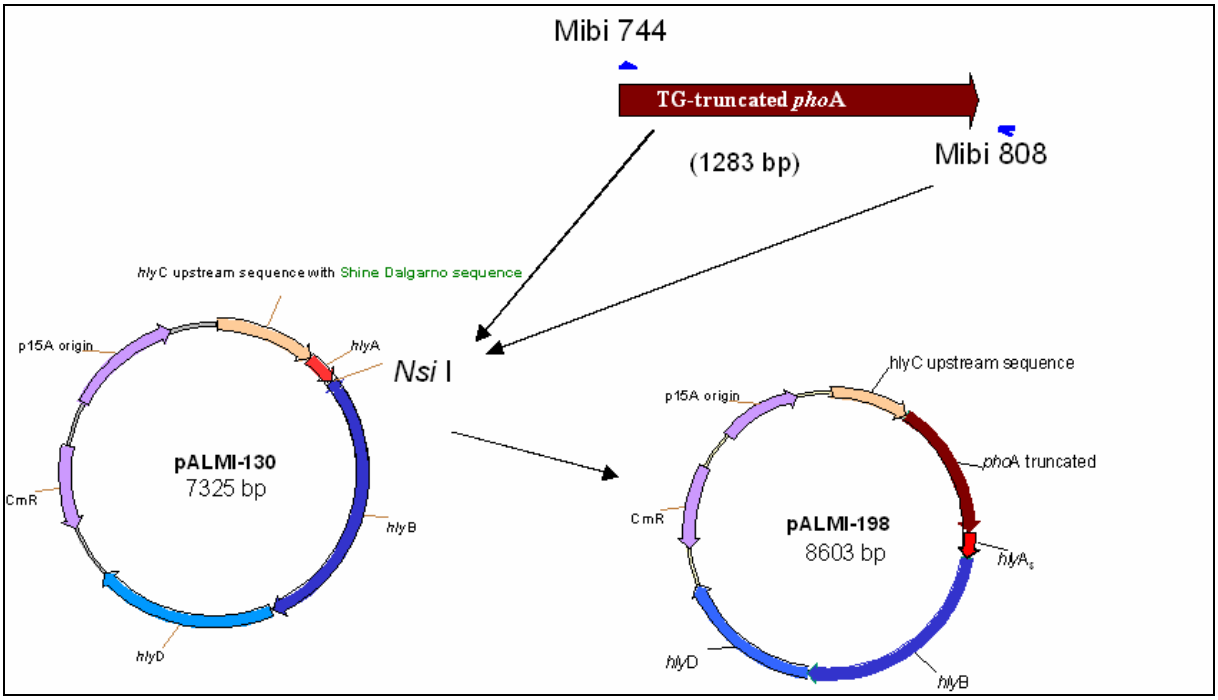


Figure 7.20: Construction of pALMI-198. TG-*pho*A (truncated) was amplified from pZ132 using the 5´phosphorylated primer pair Mibi 744 and Mibi 808. The PCR product was blunted with Klenow to remove the 3`overhanging A. Plasmid pALMI-130 was restricted with *Nsi* I and blunted with Klenow. The PCR product was ligated with restricted pALMI-130 to construct pALMI-198.

Table 7.1: Heterologous proteins secreted by the *hly* secretion system

The secretion constructs differ in the length of the 3´part of *hly*A that was fused with the gene encoding a heterologous protein of interest. Some proteins are additionally fused with the N - terminus of HlyA.

Protein	Organism/ localisation	Truncation	HlyA C-terminus (aa)	HlyA N-terminus (aa)	Secretion	Reference
LacZ (10aa)-OmpF	*E. coli*/ membrane	+	218	-	+	Mackmann *et al*., 1987
Pag C (mature)	*S. typhimurium*/ membrane	-	61	30	+	Mollenkopf *et al*., 1996
p67	*Theileria parva*/ membrane	+	60	30	+	Gentschev *et al*., 1997
Beta-lactamase (mature)	*E. coli*/ periplasm	-	218	-	+	Blight and Holland, 1994, Chervaux *et al*., 1995
Alkaline phosphatase (PhoA)	*E. coli*/ periplasm	+	60	-	+	Gentschev *et al*., 1990
antibody single chain Fv	Vertebrats/ cytoplasm	Antibody chain	218	-	+	Fernandez *et al*., 2000
Chloramphenicol acetyl transferase	*E. coli*/ cytoplasm	+	218	-	+	Kenny *et al*., 1991
Dehydrofolate reductase	*E. coli*/ cytoplasm	+	61	-	+	Nakano *et al*., 1992
Dehydrofolate reductase	*E. coli*/ cytoplasm	-	61	-	-	Nakano *et al*., 1992
ß-galactosidase	*E. coli*/ cytoplasm	+	745	-	+	Kenny *et al*., 1991
ß-galactosidase	*E. coli*/ cytoplasm	-	745	-	-	Kenny *et al*., 1991
Larvicidal toxin	*Bacillus spaericus*/ cytoplasm	?	218	-	+	Unpublished, cited by Blight and Holland, 1994
Prochymosin	*Bos taurus*/ cytoplasm	+	39/113/218	-	+	Kenny *et al*., 1991
SlyA regulator protein	*S. typhimurium*/ cytoplasm	-	60	30	+	Ludwig *et al*., 1995

Protein	Organism/ localisation	Truncation	HlyA C-terminus (aa)	HlyA N-terminus (aa)	Secretion	Reference
Superperoxid dismutase	*L. monocytogenes*/ cytoplasm	?	?	?	+	Unpublished, cited by Gentschev *et al*., 1997
30 kD antigen	*Mycobacterium bovis Bacille Calmette-Gluerin*/ extracellular	-	193	58	+	Hess *et al*., 2000
Cholesterinesterase/lipase	*Pseudomonas spp.*/ extracellular	-	60	-	+	Hanke *et al*., 1992
Colizin	Gram-negative bacteria/ extracellular	-	-	-	+	Fath *et al*., 1991
Diphterietoxin (mutated)	*Corynebacterium diphteriae*/ extracellular	-	60	30	+	Orr *et al*., 1999
Esat-6 antigen (6kD)	*Theileria parva*/ extracellular	-	60	30	+	Mollenkopf *et al*., 2001
Glycoprotease	*Pasteurella hemolytica*/ extracellular	+	141	-	+	Lo *et al*., 1994
Interleukin 1 a	*Homo sapiens*/ extracellular	-	218	-	-	Blight and Holland, 1994
Interleukin 6	*Homo sapiens*/ extracellular	-	60	30	+ low	Hahn *et al*., 1998
Lipase	*Homo sapiens*/ extracellular	+	218	-	-	Blight and Holland, 1994
Listeriolysin	*L. monocytogenes*/ extracellular	-	60	30	+	Gentschev *et al*., 1995
Measles virus epitopes	Measles virus/ extracellular	epitope	60	30	+	Spreng *et al*., 2000
Outer surface protein (OspA)	*Borrelia burgdorferi*/ extracellular	-	60	30	+	Chang et. al.,1993
P60 (murein hydrolase) (without signal sequence)	*L. monocytogenes*/ extracellular	+	60	30	+	Gentschev *et al*., 1992 I

Protein	Organism/ localisation	Truncation	HlyA C-terminus (aa)	HlyA N-terminus (aa)	Secretion	Reference
PA (protective antigen)	*Bacillus anthracis*/ extracellular	part of protein toxin	60	30	+	Garmory *et al*., 2003
Shiga Toxin B subunit	*Shigella spp.*/ extracellular	subunit	218	-	+	Tzschaschel *et al*., 1996
Sporozoite surface protein 2	*Plasmodium falciparum*/ extracellular	-	60	30	-	Gomez-Duarte *et al*., 2001
Sporozoite surface protein 2	*Plasmodium falciparum*/ extracellular	+	60	30	+	Gomez-Duarte *et al*., 2001
Toxin A	*Clostridium difficile*/ extracellular	+	60	30	+	Ryan *et al*., 1997
YopB	*Yersinia spp.*/ extracellular	?	?	?	low	Unpublished, cited by Gentschev *et al*., 1997

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