Development of bispecific filamentous bacteriophages for the generation of a novel automated screening system based on phage display technology

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I Introduction

With the human genome project approaching completion (International Human Genom Sequencing Consortium 2004), there is a growing interest in functional analysis of gene products. Protein microarrays are being utilized for functional proteomic analysis, providing information not obtainable by DNA microarrays (Espina et al. 2004). This is not unexpected because DNA microarrays cannot give information about protein posttranslational modifications or protein-protein interactions. Protein microarrays, on the other hand, can be designed to monitor these molecular interactions and provide a means of differential display of a cell population’s proteome as well as high-throughput formats for drug discovery (Greenbaum et al. 2002; Ring and Ellis 2002; Wilson and Nock 2003). Within the past few years, researchers have begun to realize the goal of proteomic-scale analysis of protein-protein, protein-ligand, enzyme-substrate or protein-drug interactions and thus created a new scientific trend (Zhu and Snyder 2003). This scientific trend and the economically requests within the field of proteomics and protein drug development describe the need for novel microarray technologies, addressing the problems of current protein microarray platforms.

The two key problems for the construction of protein microarrays are the requirement for individual production of active proteins, which necessitates proteome-wide protein expression, and the immobilization of proteins onto surfaces, which can affect their activity (Lee and Mrksich 2002). Currently, specific capture molecules have to be designed for all possible proteins encoded by the genome. The classical capture molecules for proteins are antibodies, which are difficult to manufacture with known affinity and specificity. This requires individual validation of antibody specificity and sensitivity prior to use as a probe for protein microarrays (Templin et al. 2002; Liotta et al. 2003).

Within this thesis a novel protein microarray platform based on phage display technology is presented. Phage display is a powerful tool for the generation and screening of protein and peptide libraries in order to determine specific protein-protein interactions and allow the identification of novel therapeutic or diagnostic proteins as well as vaccines (Smith and Scott 1993; Smith and Petrenko 1997). Filamentous bacteriophages are capable of displaying foreign proteins on their surface and thus provide a universal linker for immobilization of these proteins on the surface of a protein microarray device. Proteins and peptides are functionally displayed on phage particles and by the establishment of bispecific filamentous bacteriophages, a side-directed immobilization of protein or peptide libraries on the surface of a microarray device without loss of activity becomes feasible. In addition, proteome-wide protein expression is possible by the generation and usage of cDNA phage libraries. The
development of bispecific filamentous bacteriophages and a suitable biochip platform represents the initial step toward the establishment of a new generation of protein microarrays allowing the analysis of protein-protein interactions on a single molecule level and in addition addressing the key problems of current protein microarrays.

I.1 Filamentous bacteriophages

Filamentous bacteriophages belong to a group of single-stranded DNA viruses encapsidating their circular genome in a long protein capsid cylinder. As the name implies, these bacteriophages are specific for F plasmid containing *Escherichia coli* bacteria and use the tip of there F pilus as a receptor. The most extensively studied filamentous bacteriophages are the nonlytic Ff class phages f1, fd and M13. The genome of these three phages shows 98% homology and consequently the protein sequence of their gene products are practically the same (Van Wezenbeek *et al.* 1980; Beck and Zink 1981; Hill and Petersen 1982).

I.1.1 Structure of filamentous bacteriophages

The Ff phage is approximately 8-10nm in diameter and 930nm in length (Fig.I-1). The phage genome is a single-stranded, covalently closed DNA molecule of about 6400 nucleotides and is protected by a somewhat flexible protein cylinder. The filamentous structure of the phage coat is comprised of 2600 copies of the major coat protein pVIII, each consisting of 50 amino acids sticking with their C-terminus to the ssDNA molecule. At one end of the particle, there are about five copies of the 33-residue minor coat protein pVII and five copies of the 32-residue minor coat protein pIX. The other end contains approximately five molecules each of the 406-residue minor coat protein pIII and the 112-residue minor coat protein pVI. The DNA is orientated within the virion such that a 78-nucleotide hairpin region called the packaging signal (PS) is always located at the end of the particle containing the pVII and pIX proteins.

The pVIII cylinder of the filamentous bacteriophage has been described in great detail (Marvin *et al.*1994; Overman and Thomas 1995; Williams *et al.* 1995; Marvin 1998). The pVIII monomers are present in the particle as an uninterrupted α-Helix except for their N-terminal 5 amino acids. The pVIII monomers are packed quite tightly, as only three residues are accessible by proteases (Terry *et al.* 1997). The C-terminal 10-13 residues of pVIII form the inner wall of the phage coat. This region possesses four positively charged lysine residues that reside on one face of an amphiphilic helix. These positive charges interact with the sugar phosphate backbone of the DNA that is present in the particle with the bases
pointed inwards (Greenwood et al. 1991; Marvin et al. 1994). The N-terminal portion of pVIII is present on the outside of the phage. The residues connecting N- and C-terminus of pVIII interact with the same region of other pVIII monomers in order to form the stable but flexible inner core of the protein cylinder. Most of this middle portion of pVIII spans the periplasmic membrane of bacterial host cells before being assembled into mature progeny phage.

Fig.I-1: Structure of filamentous bacteriophage. A: Electron micrograph of a filamentous bacteriophage (Kay et al. 1996); B: Schematic diagram of the structure of filamentous bacteriophage describing its coat morphology with the five coat proteins, pIII, pVI, pVIII, pVII and pIX encapsidating the circular single-stranded phage DNA.

The first part of the phage being assembled is the end containing the packaging signal. This end has approximately five copies each of the small hydrophobic pVII and pIX proteins (Houbiers et al. 1999; Houbiers et al. 2001A; Houbiers et al. 2001B). Currently it is not known how these minor coat proteins are arranged at the end of the phage and how they are interacting with the pVIII cylinder. Attempts to model this end of the phage suggest that one of these proteins must be sticking closely to the DNA, whereas the other one is exposed to the outside (Makowski 1992). The fact that antibodies specific for pIX but not for pVII are able to interact with one end of the phage indicate that only pIX is exposed and pVII is buried. Data obtained by immunoprecipitation experiments of detergent-disrupted phage
suggestion an interaction between pVII and pVIII in the intact particle and thus confirm the described model of this end of the phage (Endemann and Model 1995).

The other end of the filamentous bacteriophage consists of five copies each of the minor coat protein pIII and pVI, accounting for about 10-16 nm of the phage length (Specthrie et al. 1992). pIII consists of the three domains pIII-D1, pIII-D2 and pIII-CT separated by glycine-rich regions shown in Fig.I-2 (Stengle et al. 1990).

![Fig.I-2: Domain structure of the minor coat protein pIII. G1: glycine-rich linker region 1; G2: glycine-rich linker region 2; D1: domain 1 of pIII coat protein (pIII-D1); D2 domain 2 of pIII coat protein (pIII-D2); CT: C-terminal domain of pIII coat protein; pVI: minor coat protein pVI; pVIII: major coat protein pVIII.](image)

The first domain, pIII-D1, contains the N-terminal 68 amino acids and is responsible for the injection of the phage genome into the bacterial cytoplasm during the infection process. Amino acid residue 87-217 of pIII form the second domain, pIII-D2, which is responsible for binding to the bacterial F pilus (Deng et al. 1999). Both domains show specific 3D structures required for their function and are formed by intramolecular disulfide bonds within each domain (Kremser and Rasched 1994). The structure of these two domains has been analyzed by NMR spectroscopy and X-ray crystallography (Riechmann and Hollinger 1997; Lubkowski et al. 1998; Hollinger et al. 1999). pIII-D1 and pIII-D2 are exposed on the surface of the phage and are essential for phage infectivity. Removal of these two domains for example by proteolytic cleavage results in non-infectious phage (Gray et al. 1981; Armstrong et al. 1981). The third domain of pIII, pIII-CT, is built by the C-terminal 150 amino acid residues and is responsible for the stability of the phage (Crissman and Smith 1984; Kremser and Rasched 1994). The CT domain of pIII together with pVI interacts with the pVIII cylinder at the end of the assembly process before the phage is released from the bacterial membrane (Rakonjac et al. 1999). The interaction of pIII and pVI was also proven by
immunoprecipitation experiments of detergent-disrupted phages (Gailus and Rasched 1994; Endemann and Model 1995). Very little is known about the structure and the function of the minor coat protein pVI. However, as pVI is not accessible to anti-pVI antisera when incorporated in a complete phage (Endemann and Model 1995) it is proposed that the hydrophobic N-terminus of pVI is buried within the particle, whereas the C-terminus is located near the surface (Makowski 1992). This hypothesis was confirmed by the data of Jespers et al. who showed that filamentous bacteriophages can be produced displaying foreign proteins fused to the C-terminus of pVI (Jespers et al. 1995). Minor coat protein pVI seems to be the only phage coat protein orientated with the C-terminus outside of the phage.

I.1.2 Genome of filamentous bacteriophages

The M13, f1 and fd phage genomes have been completely sequenced (Van Wezenbeek et al. 1980; Beck and Zink 1981; Hill and Petersen 1982). Table I-1 gives an overview of the size and function of the eleven genes encoded by the phage genome.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>No. of amino acid</th>
<th>Protein MW (kDa)</th>
<th>Final location</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>replication of DNA</td>
<td>410</td>
<td>46.137</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>X</td>
<td>replication of DNA</td>
<td>111</td>
<td>12.672</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>V</td>
<td>ssDNA binding</td>
<td>87</td>
<td>9.682</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>VIII</td>
<td>major coat protein</td>
<td>50</td>
<td>5.235</td>
<td>virus cylinder</td>
</tr>
<tr>
<td>III</td>
<td>minor coat protein</td>
<td>406</td>
<td>42.522</td>
<td>virus tip</td>
</tr>
<tr>
<td>VI</td>
<td>minor coat protein</td>
<td>112</td>
<td>12.342</td>
<td>virus tip</td>
</tr>
<tr>
<td>VII</td>
<td>minor coat protein</td>
<td>33</td>
<td>3.599</td>
<td>virus tip</td>
</tr>
<tr>
<td>IX</td>
<td>minor coat protein</td>
<td>32</td>
<td>3.650</td>
<td>virus tip</td>
</tr>
<tr>
<td>I</td>
<td>assembly</td>
<td>348</td>
<td>39.502</td>
<td>inner membrane</td>
</tr>
<tr>
<td>IV</td>
<td>assembly</td>
<td>405</td>
<td>43.476</td>
<td>outer membrane</td>
</tr>
<tr>
<td>XI</td>
<td>assembly</td>
<td>108</td>
<td>12.424</td>
<td>inner membrane</td>
</tr>
</tbody>
</table>

According to their function in the life cycle of the filamentous bacteriophages, the genes are grouped within the phage genome (Fig.I-3). The first group of genes (geneII, V and X) encodes proteins which are required for the replication of the phage genome. The second group (geneVII, IX, VIII, III and VI) encodes phage coat proteins and the third group (geneI,
XI and IV) encodes proteins which are essential for the phage assembly. Gene product pX and pXI are the result of a translation starting at an internal methionine codon in gene II and I, which is in-frame and thus resulting in smaller proteins with the same amino acid sequence as the C-terminus of their larger counterparts (Model and Russel 1988; Guy-Caffey et al. 1992; Rapoza and Webster 1995). In addition, the phage genome possesses a short sequence called the intergenic region that does not encode for any protein. This intergenic region contains the origin for the synthesis of viral (+) and complementary (-) DNA as well as the packaging signal which is located near gene IV.

The (-) strand of the double-stranded intermediate in viral DNA replication is required for transcription. Therefore, the messages produced have the same sequence as the (+) strand. Transcription is starting from gene II through gene IV and is directed counterclockwise. Transcription is stopped by two strong terminators (T), a rho-independent one just behind gene VIII and a rho-dependent one located in the intergenic region. These terminators divide the phage genome into two main transcription regions. One is a frequently transcribed region containing gene II through gene VIII and the other one is an infrequently transcribed region containing gene III through gene IV.

**Fig.I-3: Genome organisation of filamentous bacteriophage.** This genomic map shows the relative positions of the genes, important promoters and terminators in the genome of filamentous bacteriophage. IR refers to the intergenic region; T, the two strong terminators; t, the weak terminator in gene I; G_A, G_B, G_H, the promoters of the frequently transcribed regions; P, the promoters for the infrequently transcribed regions; +/- ori, the relative position of the origin of replication for the viral (+) and the complementary (-) DNA strand.
The three strong promoters, \( G_A, G_B \) and \( G_H \), are responsible for initiation of transcription of the frequently transcribed region resulting in three different transcripts all terminated behind \( \text{geneVIII} \). The smallest and most stable RNA molecule is the not processed transcript starting from \( G_H \). The transcript starting from \( G_A \) and \( G_B \) are rapidly processed into five smaller and more stable mRNA molecules (Stump and Steege 1996). All six mRNAs are encoding \( \text{pVIII} \) because they utilize the same terminator at the end of \( \text{geneVIII} \). The four largest of the processed mRNAs also encode \( \text{pV} \). These two proteins, \( \text{pV} \) and \( \text{pVIII} \), are needed and produced in large amounts because \( \text{pVIII} \) is the major coat protein forming the virion cylinder and \( \text{pV} \) interacts with the ssDNA during replication. The single stranded binding protein \( \text{pV} \) is required for protection of the ssDNA in the bacterial cytoplasm and for their transport to the periplasmic membrane. In contrast, only small amounts of \( \text{pVII} \) and \( \text{pIX} \) are needed and produced, even though their genes are located on the same mRNA between \( \text{geneV} \) and \( \text{geneVIII} \). The translation of \( \text{geneVII} \) and \( \text{genelX} \) is coupled to the translation of \( \text{geneV} \), because the initiation site for translation of \( \text{geneVII} \) is inherently inactive and masked by the secondary structure of the mRNA (Ivey-Hoyle and Steege 1989; Ivey-Hoyle and Steege 1992; Stump and Steege 1996). Model and Russel showed that there is also translational regulation for the synthesis of \( \text{pII} \) and \( \text{pX} \) (Model and Russel 1988). In addition to the before mentioned ssDNA binding function, \( \text{pV} \) is also able to bind specifically to the mRNA of \( \text{genelII} \) and \( \text{genelX} \) and thus inhibiting their translation. Binding only occurs at high concentrations of \( \text{pV} \), when there is more \( \text{pV} \) than needed for binding to newly synthesized viral ssDNA.

The transcription initiation of the infrequently transcribed region is regulated by two major promoters (P) located before \( \text{genelII} \) and \( \text{genelV} \), respectively. Transcription starting from the promoter upstream of \( \text{genelII} \) gives two classes of mRNA because there is a weak rho-dependent terminator (t) in the beginning of \( \text{genel} \). The most abundant class encodes only \( \text{genelII} \) and VI, whereas there is less mRNA encoding \( \text{genelII} \), VI and I/XI. The resulting down-regulation of \( \text{pl} \) is important, as \( \text{pl} \) is able to form channels in the bacterial membrane and lethal in high concentration. Substantial amounts of \( \text{plV} \) mRNA are produced from the promoter located at the end of \( \text{genel} \) and XI.

There are only two regions within the phage genome that do not encode for proteins, the intergenic region and the small region between the end of \( \text{geneVIII} \) and the beginning of \( \text{genelII} \). Currently, only these two regions and the position between \( \text{genelII} \) leader and \( \text{genelII} \) are used for modifications of the phage genome, for example the insertion of an antibiotic resistance in the intergenic region (Zacher et al. 1980) or the insertion of a cloning site into \( \text{genelII} \) (McCafferty et al. 1990; Clackson et al. 1991). For insertion of DNA sequences between \( \text{geneVIII} \) and \( \text{genelII} \), the positions of the terminator and the promoter in
this region must be altered to maintain their function. In addition, care has to be taken not to interfere with the origins of replication or other control areas. Similarly, it is not recommended to disturb normal terminators or promoters. There appears to be a delicate balance in the synthesis of phage proteins that allow phage production without seriously affecting bacterial growth (Model and Russel 1988, Webster 1996). These observations and the fact that the transcription and translation of the phage genome is highly regulated by multiple control elements on a relatively small DNA molecule indicate that successful genetic modifications of the phage genome are difficult to achieve.

I.1.3 Life cycle of filamentous bacteriophages

I.1.3.1 Infection process of filamentous bacteriophages

Phage infection is a multistep process requiring interactions between the phage coat protein pIII and the bacterial F pilus as well as the cytoplasmic membrane proteins TolQ, R and A as shown in Fig.I-4. The bacterial F pilus is assembled and extended from pilin subunits in the cytoplasmic membrane of the bacterial cell. The proteins which are responsible for the structure, assembly and disassembly of the pilus are encoded by genes in the tra operon located on the F plasmid (Frost et al. 1994). The normal function of the F pilus is the conjugal transfer of DNA from a bacterial donor cell into a recipient cell (Firth et al. 1996). This DNA transfer, called conjugation, is initiated by the interaction of the tip of the F pilus with the envelope of the recipient bacterium. Then the F pilus retracts and draws the donor and recipient together to facilitate the DNA transfer.

This mechanism is used by filamentous bacteriophage in order to infect bacteria and inject their genome into the host cell. The infection is initiated by the binding of pIII-D2 domain (Fig.I-2) of the phage coat protein pIII to the tip of the bacterial F pilus (Stengle et al. 1990; Deng et al. 1999). The exact binding site of pIII-D2 is unknown. Probably, it is not the same one recognized by pIII-D1, because purified pIII fragments pIII-D2 and pIII-D1-D2 appear to compete equally well with wild type phages for binding to the F pilus (Krebbner et al. 1997; Deng et al. 1999). After the phage binds to the bacterium, the F pilus starts retracting and draws the pIII end of the phage to the periplasm. It is not known whether the retraction of the pilus is the result of a normal assembly-retraction cycle or whether the phage attachment is triggering the retraction process (Firth et al. 1996).
Fig. I-4: Infection process of filamentous bacteriophage. The membrane topology of the Tol proteins is shown. TolD1, tolD2 and tolD3 refer to the three domains of TolA. D1: domain 1 of pIII coat protein of the filamentous bacteriophage (pIII-D1); D2 domain 2 of pIII coat protein of the filamentous bacteriophage (pIII-D2); tolD1: domain 1 of bacterial TolA protein; tolD2: domain 2 of bacterial TolA protein; tolD3: domain 3 of bacterial TolA protein; OM: outer membrane; CM: cytoplasmic membrane.

After retraction of the F pilus, interaction between domain pIII-D1 of phage coat protein pIII and the bacterial Tol proteins is necessary for injection of the phage genome into the bacterial cytoplasm (Riechmann and Hollinger 1997). TolQ, R and A are bacterial proteins that appear to be required for maintaining the integrity of the bacterial outer membrane (Lazzaroni et al. 1999). Mutations in gene tolQ, R and A lead to hypersensitive bacteria that do not tolerate various detergents and drugs. In addition, these bacteria release periplasmic proteins into the medium and form outer membrane vesicles (Webster 1991; Bernadac et al. 1998). All three of these Tol proteins are located in the cytoplasmic membrane of the bacterial cell and appear to form a complex via interactions among their transmembrane domains (Lazzaroni et al. 1995; Germon et al. 1998). TolQ spans the cytoplasmic membrane three times, with most of its residues located in the cytoplasm (Kampfenkel and Braun 1993; Vianney et al. 1994). TolA and R just possesses one transmembrane domain located near their N-terminus and the bulk of their residues are exposed in the periplasmic space (Levengood et al. 1991; Muller et al. 1993). TolA consists of three domains, which are separated by glycine-rich regions. Domain 1 (tolD1) comprises the N-terminal 43 amino acid residues, which anchor the protein to the cytoplasmic membrane. Domain 3 (tolD3) is formed...
by the C-terminal portion of approximately 108 residues that appear to interact with the outer membrane (Levengood-Freyermuth et al. 1993). TolD1 and tolD3 are connected by the central domain (tolD2), which contains a α-helical structure long enough to span the periplasmic space.

These three Tol proteins, TolQ, R and A, are required during phage infection for translocation of the phage genome into the cytoplasm and translocation of the phage coat proteins into the cytoplasmic membrane (Russel et al. 1988; Click and Webster 1998). Binding of domain pIII-D2 of the phage coat protein pIII to the bacterial pilus releases pIII-D1 from pIII-D2 and allows pIII-D1 to interact with domain 3 of TolA (tolD3) after retraction of the pilus (Riechmann and Hollinger 1997). The tip of the F pilus is the receptor for phage attachment and tolD3 acts as the coreceptor. Structural analysis shows that both pIII-D2 and tolD3 are binding to the same region of pIII-D1, even though there is no topological similarity between pIII-D2 and tolD3 (Lubkowski et al. 1999). Therefore, the binding of the pilus tip to pIII-D2 must displace the pIII-D1, making it available for interacting with tolD3. Recently, it has been shown that binding of pIII-D1 to tolD3 is necessary for the initiation of translocation of the phage genome into the bacterial cytoplasm but the subsequent steps involved in phage infection remain unknown.

I.1.3.2 Replication and translation of the phage genome

After phage infection and translocation of the viral (+) strand DNA into the cytoplasm of the host cell, the complementary (-) strand is synthesized by the bacterial enzyme machinery. The resulting dsDNA is modified by gyrase, a type II topoisomerase that catalyzes the formation of negative supercoiled DNA. The final product is a covalently closed, supercoiled, dsDNA called the replicative form (RF). The (-) strand of the RF is the template for transcription and the resulting mRNAs are translated into all of the phage proteins. One of these phage proteins, pII, nicks the (+) strand of the RF at a specific position in the intergenic region. The nick allows the synthesis of a new viral strand by replication via “rolling circle”. After one round, pII circularizes the new viral (+) strand DNA, which is then converted again to a covalently closed, supercoiled, dsRF molecule. By this, a pool of RF molecules is produced that can direct the synthesis of the phage proteins. RF molecules are synthesized until the amount of phage protein pV reaches a critical concentration. As described above (I.1.1), pV is able to cooperatively bind newly synthesized viral ssDNA after forming dimers and thus prevents its conversion to additional RF molecules. Therefore, the presence of adequate amounts of pV switches most of the DNA replication to the synthesis of ss(+) viral DNA. pV is protecting the viral ssDNA by forming a pV-DNA structure about 800nm long and 8nm in diameter (Gray 1989; Skinner et al. 1994; Guan et al. 1995; Olah et al. 1995). The
packaging signal (PS) is exposed on one end of the pV/DNA complex and is not decorated with any pV protein (Bauer and Smith 1988). The pV/DNA particle is the substrate for the phage assembly, which is initiated at the end exposing the PS. The exact role of pX in the replication process is unclear, but it appears to function as an inhibitor of pII and thus is involved in the regulation of the amount of viral DNA produced (Fulford and Model 1984).

**Fig.I-5: Life cycle of filamentous bacteriophages.** Infection: sequential binding of phage pIII coat protein to the tip of the host F pilus followed by retraction of the pilus. Binding of phage pIII coat protein to the host TolA protein and injection of the ssDNA phage genome into the bacterial cytoplasm. Replication: ssDNA is converted by host enzymes to the double-stranded RF, which serves as template for gene expression. Assembly: coat proteins pVII, pX, pVIII, pIII and pVI are directed to the periplasmic membrane. Substrate for the assembly process is the pV-ssDNA complex. pV dimers are replaced by pVIII as the ssDNA is extruded through the bacterial membrane using the pI/pXI/pIV-channel. RF: replicative form; PS: packaging signal.

The only phage proteins remaining in the bacterial cytoplasm are pII, pX and pV, which are the three proteins being engaged in the replication process. All other phage proteins are synthesized and inserted into the cytoplasmic or outer membrane. The phage coat proteins, pVIII, pIII, pVI, pVII and pIX have been shown to reside in the cytoplasmic membrane (Endemann and Model 1995; Williams et al. 1996; Papavoine et al. 1997). The major coat protein pVIII and minor coat protein pIII are synthesized each with N-terminal signal-peptides, which are removed after membrane insertion. The remaining three minor coat protein, pVI, pVII and pIX are synthesized without signal peptides and their mechanism for
insertion into the bacterial membrane is unknown. It has been shown that pVIII and pIII are
inserted into the cytoplasmic membrane with their N-terminus toward the periplasmic space
(Davis et al. 1985; McDonnell et al. 1993) whereas the orientation of pVII and pIX is thought
to be the same as the one of pVIII and pIII. Currently no convincing data regarding the
topology of pVI in the cytoplasmic membrane of the bacterial cells are available.

The three assembly proteins, pIV, pI and pXI, are also integral membrane proteins. pIV is
synthesized with an N-terminal signal peptide and is translocated into the periplasm,
probably using the Sec system of the host bacterium (Raposa and Webster 1993; Russell
and Kazmierczac 1993). Twelve to fourteen pIV molecules are forming channels across the
outer membrane with a diameter of 6-8nm, which is large enough to accommodate an
extruding phage (Kazmierczak et al. 1994; Linderoth et al. 1997). This channel must be
gated in some way, as it does not let large molecules in or out of the periplasm in absence of
other phage proteins (Marciano et al. 1999). The other two assembly proteins, pI and pXI,
are synthesized without any signal peptide and are inserted into the cytoplasmic membrane
in a manner that requires SecA (Raposa and Webster 1993). It is assumed that pI and pXI
interact with each other in order to create channels in the cytoplasmic membrane, which
could then interact with the outer membrane pVI complex to form a channel through both
bacterial membranes during phage assembly (Guy-Caffey and Webster 1993; Rapoza and
Webster 1995).

I.1.3.3 Assembly process of filamentous bacteriophages

The assembly process of filamentous bacteriophages is a membrane-associated event,
which does not cause lysis of the host cell. The assembly requires the five phage coat
proteins, the three assembly proteins, ATP, a proton motive force and at least one bacterial
protein, which is thioredoxin (Model and Russel 1988; Feng et al. 1997). The initiator for the
assembly process is the pV/DNA complex. In principle the assembly is a process in which
the pV dimers are removed and the coat proteins are assembled around the viral ssDNA as it
is extruded through the bacterial membranes. The assembly process can be divided into
initiation, elongation and termination. The process is initiated by interaction of the PS, which
is exposed in the pV/DNA complex and the C-terminal portion of pI and the membrane-
associated proteins pVII and pIX together with the first set of pVIII molecules (Lopez and
Webster 1983; Russel and Model 1989). The order in which pVII, pIX and pVIII associate
with the PS is unknown. One hypothesis is that pI is able to direct an ordered assembly of
these three coat proteins around the PS to form the phage tip. Perhaps, this assembly
induces a conformational change in pI, allowing its periplasmic region to interact with pIV and
open the gate of the pIV channel (Russel et al. 1997; Marciano et al. 1999). After initiation,
the phage has to be **elongated** by removing pV dimers and replacing them with pVIII as the DNA is extruded through the bacterial membranes. The structure of the DNA in the pV/DNA complex is different from its structure in the assembled phage, suggesting that some conformational change in the DNA is required to allow the interaction with pVIII. Rapoza and Webster showed that the ten residues of pl and pXI adjacent to the cytoplasmic face of the cytoplasmic membrane have an amphiphilic character extremely similar to the C-terminal residues of pVIII that interact with the viral ssDNA (Rapoza and Webster 1995). These regions of pl and pXI may interact with the viral DNA that has just been stripped of the pV dimers, to facilitate the adoption of the DNAs’ conformation for interaction with pVIII. In addition the conformation of pVIII has to change before being assembled with the viral DNA. The structure of this protein is somewhat different in the assembled phage than in the bacterial membrane, where it tends to form dimers that are not suitable for packaging (Haigh and Webster 1998; Marvin 1998). When the end of the viral DNA is reached, assembly is **terminated** by the incorporation of pVI and pIII. In the absence of pIII, elongation continues with pVIII encapsulating another phage DNA. This process is resulting in the production of so called polyphage, which are not released from the bacterial membrane (Model and Russel 1988; Rakonjac and Model 1998). The exact mechanism by which pVI and pIII are added to the end of the bacteriophages and how the assembly process is terminated is unknown.

### I.2 Phage display

The generation of new drugs has long involved the screening of hundreds of thousands of compounds with well defined **in vitro** assays, seeking leads to mimic as closely as possible the desired **in vivo** activity of the new drug. New library methodologies offer many alternative routes that are at least as powerful as traditional approaches such as combining the generation of billions of compounds with a fast screening or selection procedure to identify interesting lead candidates. One of the most powerful tools and the most widely used library methodology is the so called phage display technology (Smith 1985). This technology is based on the use of filamentous bacteriophage for screening of libraries containing millions or even billions of different peptides or proteins. The most successful application of phage display has been the isolation of recombinant antibody fragments using large phage antibody libraries (Winter *et al.* 1994; Hoogenboom and Chames 2000). The principle of the phage display technology is shown in Fig.I-6:
I Introduction

Fig.I-6: Principle of phage display technology using a phagemid vector. Genes encoding for millions of variants of ligands or binding molecules are cloned into a phagemid vector carrying the gene encoding for one of the five phage coat proteins (pIII is shown here). Large phage libraries can be obtained by transforming *E. coli* with the phagemids and rescue of the phages with a helperphage. From these repertoires, phages displaying the specific-binding ligand can be isolated by a series of recursive cycles of selection on the target, each of which involves binding, washing, elution and amplification.

DNA encoding for millions of variants of certain ligands/binding molecules (e.g., peptides, proteins or protein fragments) is cloned into the phage genome as a fusion to one of the genes encoding the five phage coat proteins (pIII, pVI, pVII, pVIII or pIX). Upon expression, the chimeric coat protein fusion will be packaged into new progeny phages that are assembled in the bacterial host. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being displayed on the phage surface, whereas its genetic information resides within the phage. This connection between ligand genotype and phenotype allows the enrichment of specific binding phages, e.g., using selection on an immobilized target. Phages that display a relevant ligand will be retained, but nonadherent phages will be washed away. The specific binding phages can be recovered from the surface, used for re-infection of host bacteria and amplified again. The resulting phages are subjected to another round of affinity purification and multiple rounds of this procedure lead to an enrichment of phages containing specific binding ligands. This type of enrichment, using several rounds of binding between the displayed ligand and its immobilized target, is sometimes referred to as “panning” (Parmley and Smith 1988). Many variations of this procedure have been devised to reduce the enrichment of non-specific binding phages and to increase the specificity and kinetics of binding to desired targets.
Large libraries of peptides and proteins have been created using pIII as the display vehicle (Smith and Scott 1993; Winter et al. 1994), leading to the development of a number of techniques for selecting the molecules desired from such libraries (Clackson and Wells 1994; Hoogenboom 1997). Peptides and proteins have also been fused to the N-terminus of the major coat protein pVIII (Iannolo et al. 1995; Malik et al. 1996). In addition, there has been a report of phages displaying proteins fused to the C-terminus of pVI (Jespers et al. 1995). This allows the construction of full-length cDNA libraries because the stop codon of the cDNAs is not effecting the expression of the cDNA/pVI fusion proteins when cloned to the 3'-end of geneVI, although the efficiency of display appears to be lower. In addition, antibody heavy- and light-chain variable regions as well as peptides have been fused to the N-terminus of pVII and pIX and displayed on filamentous phages, showing that these two minor coat proteins can also be used for phage display (Gao et al. 1999; Gao et al. 2002a; Gao et al. 2002b; Gao et al. 2003). However, the majority of proteins or peptides displayed on filamentous bacteriophages are fused to either pIII or pVIII and selected by panning on solid phase, in solution or even on cells.

The first antibodies were successfully displayed on the surface of filamentous bacteriophages by McCafferty (McCafferty et al. 1990). This was achieved by fusing the coding sequence of the antibody variable (V) regions encoding a single chain Fv (scFv) to the N-terminus of the minor coat protein pIII using a phage vector based on the genome of fd-tet phage (Zacher et al. 1980). The scFv sequence was cloned in frame with geneIII and downstream of the geneIII signal sequence, which directs the fusion protein to the periplasmic space. In the periplasmic environment, the VH and VL domains fold correctly and form a functional scFv (Skerra et al. 1988; Better et al. 1988). Initially, phage vectors that carried all the genetic information required for the phage life cycle were used (Zacher et al. 1980; Skerra et al. 1988; Better et al. 1988; Clackson et al. 1991; McCafferty et al. 1990), but phagemid vectors have since become the most popular vector system for phage display.

Phagemids are small plasmid vectors that have high transformation efficiencies and are therefore ideally suited for the generation of large peptide or protein libraries (Bass et al. 1990; Breitling et al. 1991; Barbas et al. 1991; Hoogenboom et al. 1991; Marks et al. 1991). These phagemid vectors contain the filamentous phage intergenic region with the origin of replication for viral and complementary strand synthesis as well as the hairpin packaging signal. In addition, they contain a plasmid origin of replication and a gene encoding resistance to specific antibiotic. Phagemids also carry geneIII, geneVI, geneVII, geneVIII or geneIX with appropriate cloning sites for the construction of the peptide or protein libraries. The gene expression is under control of a specific inducible promoter. The phagemid can maintain itself as a plasmid, directing expression of the protein in bacteria if desired. Infection
of phagemid carrying bacteria with a filamentous helperphage activates the phage origin of replication, resulting in single-stranded phagemid DNA being encapsulated into recombinant phages using the helperphage encoding proteins. The commonly used helperphage contains a defective packaging signal so that the majority of produced phages contain the single-stranded phagemid DNA, which is necessary for connecting genotype and phenotype of the library proteins (Russel et al. 1986). Since the helperphage genome encodes the wild type coat proteins, typically over 90% of rescued phages do not display the library protein at all. In addition, the vast majority of the recombinant phages that do display the fusion product will only contain a single copy of the library protein, comparing to multivalent display of foreign proteins when using a phage vector (Marks et al. 1992; O’Connell et al. 2002). Ideally, phage vectors leading to multivalent display would therefore be preferable when selecting large antibody libraries to guarantee selection with a limited number of phages. Monovalent display by using phagemid vectors, on the other hand, may be essential when selecting antibodies of higher affinity (O’Connell et al. 2002). Therefore, the use of inducible promoters (Lutz and Bujard 1997) or the use of helperphages with deleted or truncated genelll (Duenas and Borrebaeck 1995; Rondot et al. 2001), which may be efficiently produced in bacterial cells containing genelll under control of the phage promoter (Rakonjac et al. 1997), may in future allow successful modulation of the valency of displayed library proteins. So far, these modified helperphages were only constructed for the pIII-display system.

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**Fig.I-7: Phage library versus phagemid library.** A: Use of phage vectors leading to multivalent display of the library proteins on the phage surface. B: Use of phagemid vectors leading to monovalent display of the library proteins on the phage surface.
I.3 Research objectives

I.3.1 Protein Nanochip

This doctoral thesis was part of an interdisciplinary Fraunhofer MAVO project entitled “Surface technology and photonic in proteomics”. MAVO (Markt-orientierte Strategische Vorlaufforschung) relates to “market oriented and precompetitive strategic research” and is based on internal Fraunhofer research projects. Seven Fraunhofer Institutes were involved in this project forming an interdisciplinary consortium called “Fraunhofer Allianz Proteinchip”. The main objective of this MAVO project is the development of a novel protein microarray enabling automation of phage display technology.

Phage display (I.2) is a powerful tool for screening large protein or peptide libraries in order to identify novel therapeutics, diagnostics or vaccines. The emerging arena of protein based drug development urgently requires high-throughput technologies for automated library handling and phage display selection. Currently, technologies have been developed by adapting the basic selection steps of the panning procedure to robotic platforms (Walter et al. 2001; Konthur and Walter 2002; Hallborn and Carlsson 2002; Bradbury 2003b). The Fraunhofer Institute for Molecular Biology and Applied Ecology IME and the Fraunhofer Institute for Laser Technology ILT (Aachen; Germany) developed an innovative strategy for the generation of a novel high-throughput screening system, called Protein Nanochip. This Protein Nanochip circumvents the entire panning procedure (I.2; Fig.I-6) by direct laser-based detection of individual binding partners, combined with fluorescent activated cell
sorting (FACS), and final identification of the interacting partners by Duplex-PCR and sequencing. This technology should be suitable for screening an immobilized phage library against a second phage library in solution to enable the identification of new targets and their specific ligands within one single screening procedure.

Fig.I-9: Principle of the Protein Nanochip. The first phage library is immobilized on the surface of the chip via a photosensitive linker and a second fluorescently labelled phage library is then applied in a fluidic system to the immobilized one. After suitable washing steps, the exact positions of all specific binding partners on the surface of the Protein Nanochip are determined by two detection lasers using the fluorescent dye of the bound phages from the second library. Knowing the x/y-coordinates of all specific binding partners, a delamination laser selectively cleaves their photosensitive linker and the phage pairs are released from the chip and sorted by FACS for subsequent analysis.

The principle of the Protein Nanochip is shown in Fig.I-9. The first phage library displaying e.g. a cDNA library is immobilized on the chip surface via a photosensitive linker, necessary for selective delamination of specific binding partners. The second fluorescent labelled phage library displaying e.g. a scFv antibody library is then added in a fluidic system to the immobilized library. Upon selective protein-protein interaction of the scFv-displaying phages with the cDNA-displaying phages the exact position of specific binding partners is detectable through a novel highly sensitive detection system (Currently under development at the Fraunhofer ILT). Detection was based on the usage of two lasers, detecting the fluorescent label of the captured phages from the second library. One laser scans the chip in the x-section and the other one in the y-section to determine the x/y-coordinate and thus the exact localization of the binding partners on the Protein Nanochip surface. The coordinates of the
x/y position of the binding partners should allow a delamination laser to selectively cleave a photosensitive linker thus releasing the specific phage pair. Released binding partners then might be separated via FACS using the fluorescent signal and analyzed by duplex PCR and DNA sequencing.

The approach developed and presented within this doctoral thesis provided the initial and most critical step towards the realization of the Protein Nanochip. For the development of this new screening devise **bispecific filamentous bacteriophages** were to be generated, displaying two functional moieties on opposite sites of the particle. One side should display a moiety for the side-directed immobilization onto the chip surface or for fluorescent labelling and on the other side a peptide or protein library should be displayed for interaction with specific binding partners in solution.

### I.3.2 Aim of this thesis

As described in I.3.1 the aim of this doctoral thesis was to use phage display technology in order to generate the biological entities of the Protein Nanochip (Fig.I-9). Therefore two main objectives needed to be achieved:

- Generation of **bispecific filamentous bacteriophages** allowing simultaneous display of a protein or peptide library and specific moieties for immobilization or labelling on the opposite site of the particle.
- Establishment of a novel technology for the side-directed immobilization of filamentous bacteriophages onto the surface of the Protein Nanochip.

#### I.3.2.1 Generation of bispecific filamentous bacteriophages

To fulfill the first aim a vector system suitable for the generation of bispecific filamentous bacteriophages was developed. The bispecific phages should display the protein libraries either as a fusion to the pIII or pVI coat protein and in parallel display functional peptides or proteins for the immobilization or labelling as a fusion to the pIX coat protein on the opposite site. The pIII-display system was chosen, because currently it is the best described phage display system and suitable for the screening of nearly all kinds of protein libraries (Smith and Petrenko 1997). However, display of full-length cDNA libraries fused to the N-terminus of pIII is not possible, because the stop codons of the full-length cDNAs will effect the expression of the related cDNA/pIII-fusion protein. Therefore, the pVI-display system was chosen in order to create bispecific phages allowing the screening of cDNA libraries (Jespers
et al. 1995). At the other end of the phage, the phage coat protein pIX was the display system of choice, because it had been shown that pIX-display is the more suitable screening system compared to pVII (Gao et al. 2003; Endemann and Model 1995).

Two different strategies were developed to generate bispecific bacteriophages.

I.3.2.1.1 Generation of a bispecific phage vector

A bispecific phage vector was designed based on a derivative of the fd-tet phage vector (Zacher et al. 1980). Currently, only phage vectors are available allowing the display of foreign proteins as a pIII-fusion by cloning the corresponding gene between the gene\_III signal sequence and gene\_III (Skerra et al. 1988; Better et al. 1988). Therefore, the challenging task was to modify the fd-tet phage vector by inserting:

- a cloning site and an appropriate linker in frame with gene\_III downstream of the gene\_III signal sequence or alternatively downstream of gene\_VI depending on the kind of library that had to be screened;

- a cloning site and an appropriate linker in frame upstream of gene\_IX.

The resulting bispecific phage vectors should allow the generation of bispecific phages displaying two different moieties in multivalent fashion on their opposite sites without the need of a helperphage.

I.3.2.1.2 Generation of a bispecific phagemid vector

The second strategy for the generation of bispecific phages describes the construction of a bispecific phagemid vector was based on the well-established pHEN phagemid (Hoogenboom et al. 1991). The insertion of a second cloning cassette containing gene\_IX should allow the construction of this bispecific phagemid vector. However, for generation of the bispecific phages an infection of the transformed bacteria with a helperphage is necessary. This system has the disadvantage, that only a small percentage of the produced phages will be bispecific and that these phages display only one copy of the two foreign proteins on their surface and thus are monovalent (O'Connell et al. 2002).

As proof of principle for the two vector systems a scFv antibody gene needed to be cloned into the gene\_III cloning site and a cDNA gene into the pVI cloning site. The functional display of these proteins on the surface of the resulting phages was to be confirmed by immunological analysis such as phage ELISA. The second specificity of the bispecific vector systems was to be determined by integration of a scFv antibody gene or a suitable peptide into the pIX cloning site. In addition, the bispecificity of the phages was to be proven using phage ELISA and immunogold labelling followed by subsequent electron microscopy based analysis.
I.3.2.2 Immobilization of filamentous bacteriophages

The second aim of this thesis was the establishment of a suitable technology for the side-directed immobilization of bispecific filamentous bacteriophages onto the surface of the Protein Nanochip. The prerequisite was the immobilization of filamentous bacteriophages on biotinylated surfaces. Therefore phages were to be generated, displaying functional streptavidin, avidin or other biotin binding motives as a pIX fusion. In order to reach this aim, a phagemid test system was to be developed allowing the display of foreign proteins or peptides on pIX coat protein. For the generation of this vector system gene III was to be replaced by gene IX using a modified pHEN phagemid vector (Hoogenboom et al. 1991). The resulting pIX phagemid vector was to be used for the screening of a suitable protein or peptide motif, which is able to bind biotinylated surfaces when displayed on phage pIX. The most suitable binding motif was to be determined by phage ELISA and subsequent immobilization assays using the Protein Nanochip, which were to be provided by the other project partners.

Finally the best binding motif needed to be integrated into the bispecific filamentous bacteriophages and side-directed immobilization on the Protein Nanochip needed to be demonstrated.

The different tasks, necessary to reach the main objectives of this doctoral thesis are shown in Fig.I-10. All developed phage and phagemid vectors are listed in V.2.1 and V.2.2.
Fig. I-10: Flow chart of this doctoral thesis.

I Introduction

**Generation of bispecific phages**
- fd-tet phage vector
- pHENHi phagemid vector

**Immobilization of phages**
- pHENHi phagemid vector

**Steps for Generation of Bispecific Phages**
1. Insertion of MCS and linker to geneIII/VI and to geneIX
2. Insertion of a second expression cassette fused to geneIX
3. Cloning of test genes into both cloning sites
4. Production of bispecific phages
5. Phage ELISA and Immunogold assay
6. Determination of suitable vector system for the generation of bispecific phages

**Steps for Immobilization of Phages**
1. Replacement of geneIII through geneIX
2. Cloning of genes encoding for biotin binding motifs into pIX phagemid vector
3. Binding analysis by Phage ELISA
4. Determination of a suitable biotin binding motif
5. Cloning of biotin binding motif into bispecific vector system
6. Immobilization of bispecific phages on the surface of the Protein Nanochip
II  Materials and Methods

II.1  Materials

II.1.1  Chemicals and consumables

All chemicals were purchased from the following companies: Becton Dickinson Bioscience Clontech (Heidelberg), Biochrom (Berlin), BioRad (München), ICN (Eschwege), Gibco BRL (Eggstein), IBA GmbH (Göttingen), Invitrogen (Karlsruhe), New England Biolabs (Schwalbach), Promega (Mannheim), Roche Diagnostics (Mannheim), Roth (Karlsruhe), Serva (Heidelberg), VWR (Darmstadt) and Sigma (Deisenhofen).

Consumables were purchased from the following companies: Amersham Pharmacia (Freiburg), Biozym (Oldendorf), Corning Inc. (Schiphol-Rijk, Niederlande), Eppendorf (Hamburg), Greiner (Solingen), Hewlett-Packard (München), IBA GmbH (Göttingen), Invitrogen (Karlsruhe), Kodak (Stuttgart), Millipore (Eschborn), Nunc (Biebrich), Qiagen (Hilden), Roth (Karlsruhe), Schott Glaswerke GmbH (Mainz), Starlab (Ahrensburg) and Whatman (Maidstone, England).

II.1.2  Enzymes and reaction kits

DNA was digested with restriction enzymes obtained from New England Biolabs. Expand™ high fidelity Taq DNA polymerase from Roche Diagnostics was used for SOE-PCR (II.2.1.2.2). Taq DNA polymerase from Invitrogen was used for standard (II.2.1.2.1) and colony (II.2.1.2.3) PCR.

The following kits were used:

<table>
<thead>
<tr>
<th>Kits</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAprep® Plasmid Isolation Kits (Mini, Maxi)</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick® Gel Extraction Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick® PCR Purification Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>SuperScript™ First-Strand Synthesis Kit</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Quick Ligation™ Kit</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>
II.1.3 Primary and secondary antibodies

The following antibodies were used for detection and analysis of recombinant filamentous bacteriophages by ELISA (II.2.2.5) or immunogold assay (II.2.2.6):

Table II-1: Primary and secondary antibodies used throughout this work.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope specificity</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti-penta-His, (Qiagen)</td>
<td>N-terminal, C-terminal and internal 6xHis tags</td>
<td>monoclonal, unconjugated</td>
</tr>
<tr>
<td>rabbit anti-6xHis, (Acris Antibodies, Hiddenhausen)</td>
<td>N-terminal, C-terminal and internal 6xHis tags</td>
<td>polyclonal, unconjugated</td>
</tr>
<tr>
<td>Strep-tag® II specific mouse antibody, (IBA GmbH)</td>
<td>N-terminal, C-terminal and internal Strep-tag® II</td>
<td>monoclonal, unconjugated</td>
</tr>
<tr>
<td>mouse anti-pIII (MoBiTec, Göttingen)</td>
<td>bacteriophage M13 minor coat protein pIII</td>
<td>monoclonal, unconjugated</td>
</tr>
<tr>
<td>HRP-conjugated Fc specific goat anti-mouse IgG (GAM&lt;sup&gt;HRP&lt;/sup&gt;), (Sigma)</td>
<td>Fc of mouse IgG</td>
<td>polyclonal, HRP (horseradish peroxidase) conjugated</td>
</tr>
<tr>
<td>HRP-conjugated mouse anti-M13, (Amersham Pharmacia)</td>
<td>bacteriophage M13 major coat protein pVIII</td>
<td>monoclonal, HRP-conjugated</td>
</tr>
<tr>
<td>15nm gold-conjugated H&amp;L specific goat anti-mouse IgG (Biocell, Cardiff, England)</td>
<td>Heavy and light chain (H&amp;L) of mouse IgG</td>
<td>polyclonal, gold-conjugated (15nm particle)</td>
</tr>
<tr>
<td>10nm gold-conjugated H&amp;L specific goat anti-rabbit IgG (Biocell, Cardiff, England)</td>
<td>Heavy and light chain (H&amp;L) of rabbit IgG</td>
<td>polyclonal, gold-conjugated (10nm particle)</td>
</tr>
<tr>
<td>10nm gold-conjugated goat anti-HRP (Plano, Wetzlar)</td>
<td>horseradish peroxidase</td>
<td>polyclonal, gold-conjugated (10nm particle)</td>
</tr>
</tbody>
</table>

All ELISA assays (II.2.2.5) were detected with secondary HRP-conjugated antibodies and developed with ABTS substrate (Roche Diagnostics). Color development was measured at 405 nm against ABTS solution as a blank by photometric analysis.
II Material and Methods

II.1.4 Vectors

II.1.4.1 Phage vectors

II.1.4.1.1 fd-tet

Fd-tet was the first phage vector being developed by Zacher et al. (1980) and was used as a basic vector for generating novel phage vectors within this thesis. Fd-tet is a derivative of the filamentous bacteriophage fd genome and differs by the insertion of a tetracycline resistance cartridge into the origin of “-”strand replication, which has the effect of “de-tuning” the phage and making it far more tolerant of loss-of-function mutations (Parmley et al. 1988). Therefore fd-tet tolerates for example frame shifts and deletion of geneIII whereas the same lesion causes death in the parental fd infected host.

II.1.4.1.2 fd-tet-DOG1

Fd-tet-DOG1 (McCafferty et al. 1990) is a derivative of fd-tet (Zacher et al. 1980) and was used as another basic vector for the generation of novel phage vector. Fd-tet-DOG1 was designed to allow the insertion of single chain antibody cDNA sequences as fusions to the N-terminus of geneIII product. Therefore an ApaLI-NotI-cloning site was inserted between geneIII leader and geneIII. These two enzymes were chosen because they are cutting rarely in antibody heavy and light chain variable antibody domains.

II.1.4.2 Phagemid vectors

II.1.4.2.1 pHEN4II

pHEN4II phagemid (Zhang et al. 2001) is a derivative of the pHEN1 phagemid (Hoogenboom et al. 1991) and was used as a basic vector for generation of novel phagemids within this thesis. pHEN4II contains SfiI/BstEII- and AscI/NotI-cloning sites for cloning of heavy and light chain variable domains of antibodies spaced by a modified 218 poly-linker based on the 218-Whitlow linker (Whitlow et al. 1993), which allows the easy construction of single chain antibody fragments.

II.1.4.2.2 pHENHi

pHENHi phagemid (Peschen et al. 2004) is a derivative of the pHEN4II phagemid (Zhang et al. 2001) and was also used as a basic vector for generation of novel phagemids. The pHENHi phagemid possesses an additional C-terminal 6xHis tag inserted between the C-terminal myc-tag and the amber stop codon of pHEN4II.
II.1.5 Biological material

II.1.5.1 Bacterial strains

All intermediate gene constructs generated within this thesis are based on a phage vector and were transformed into *E. coli* XL1-Blue MR. All intermediate constructs based on a phagemid vector were transformed into *E. coli* DH5α. For phage assembly all final phage vectors as well as phagemid vectors were transformed into *E. coli* TG1.

Table II-2: Names and genotypes of bacterial strains used throughout this thesis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>Ausubel <em>et al.</em> 1994</td>
<td>F' endA hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF) φ80d lacZΔM15</td>
</tr>
<tr>
<td>XL1-Blue MR</td>
<td>Stratagene</td>
<td>F' Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</td>
</tr>
<tr>
<td>TG1</td>
<td>Stratagene</td>
<td>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (n'c m'c) [F' traD36 proAB lacI^ZΔM15]</td>
</tr>
</tbody>
</table>

II.1.5.2 Helperphage

II.1.5.2.1 M13KO7

M13KO7 helperphage is an M13 derivative, which carries the mutation Met40Ile in genell. The p15A origin of replication and the kanamycin resistance gene from Tn903 were inserted within the M13 origin of replication (Vieira *et al.* 1987).

II.1.5.2.2 Hyperphage M13KO7ΔpIII

Hyperphage M13KO7ΔpIII is a genelll-deleted helperphage derivative of M13KO7. M13KO7ΔpIII increases the percentage of recombinant phages carrying foreign proteins on their surface (Rondot *et al.* 2001).

II.1.5.3 L540Cy cell line

Human Hodgkin Lymphoma cell line L540Cy (Kapp *et al.* 1992) (DSMZ-Nr.: ACC 72) were used for isolation of total RNA (II.2.1.11.1) and for the amplification of the CD30 receptor encoding cDNA (II.2.1.11.2).
II Material and Methods

II.1.6 Buffer, media and solutions

All standard solutions, buffers and media were prepared according to Sambrook et al. (1996), Ausubel et al. (1995) and Coligan et al. (1995). The non-standard solutions or buffers are listed at the end of the corresponding method description. Media for bacterial cultivation were sterilized by autoclaving (20min/121°C/1bar). Thermo labile components were filter sterilized by passing through a 0.2µm filter and added to the autoclaved media or buffer after they were cooled down to 50°C.

II.1.7 Oligonucleotides

Oligonucleotides used for the amplification or sequencing analysis are listed below. All oligonucleotides were synthesized by MWG Biotech (Ebersberg) or Biomers.net (Ulm) and HPLC purified.

II.1.7.1 Oligonucleotides for construction and analysis of fd-g6m

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-BamHI</td>
<td>5’- ctg gct tta atg agg atc cat tcg -3’</td>
</tr>
<tr>
<td>p-3’-pVISfiI</td>
<td>5’- gga tgg ccg act tgg ccg atc cgc ctg agc ctc cac ctt tat ccc aat cca aat aag aaa cg -3’</td>
</tr>
<tr>
<td>p-5’-pVINotI</td>
<td>5’- gat cgg cca agt cgg cca tcc cat atc acg cgg ccg cgt aaa tat ggc tgt tta ttt tgt aac tg -3’</td>
</tr>
<tr>
<td>p-3’-PacI</td>
<td>5’- gta aat cgt cgc tat taa tta att ttc c -3’</td>
</tr>
<tr>
<td>p-5’-TS1-Seql</td>
<td>5’- gcc atg tat gac gct tact g -3’</td>
</tr>
<tr>
<td>p-3’-TS1-SeqII</td>
<td>5’- gaa tta tca ccc tca ccc ac -3’</td>
</tr>
<tr>
<td>p-5’-TS1-SeqIII</td>
<td>5’- ctg cta tcg atg ttc tca ttc -3’</td>
</tr>
<tr>
<td>p-3’-TS1-SeqIV</td>
<td>5’- caa taa taa gag cca gaa aca atg -3’</td>
</tr>
<tr>
<td>p-5’-TS1-SeqV</td>
<td>5’- gag gtt cgc taa aac gcc tc -3’</td>
</tr>
<tr>
<td>p-3’-TS1-SeqVI</td>
<td>5’- ctg gaa aaa gcc tgt tta tgt -3’</td>
</tr>
<tr>
<td>p-5’-TS1-SeqVII</td>
<td>5’- cct act gtt gag cgt tgt c -3’</td>
</tr>
<tr>
<td>p-3’-TS1-SeqVIII</td>
<td>5’- gta cat aac tca ata tat tgt atg -3’</td>
</tr>
</tbody>
</table>
II Material and Methods

II.1.7.2 Oligonucleotides for construction and analysis of fd-g6/9m

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-BsrGI</td>
<td>5’- cgt atg cgc ctg tgt aca c -3’</td>
</tr>
<tr>
<td>p-3’-pIXm</td>
<td>5’- acc gag cca ccg cca cca gta gcc gag ccc tgc aga ctc atc ttg gac ccc cag c -3’</td>
</tr>
<tr>
<td>p-5’-pIXm</td>
<td>5’- tag cgg tgg cgg tgg ctc ggt ttt agt gta ttc ttt cgc ctc ttc c -3’</td>
</tr>
<tr>
<td>p-3’-BamHlib</td>
<td>5’- ctt gat att cac aaa cga atg gat c -3’</td>
</tr>
<tr>
<td>p-5’-BS2-Seql</td>
<td>5’- gtc agg gca agc ctt ttt cac -3’</td>
</tr>
<tr>
<td>p-3’-BS2-SeqlII</td>
<td>5’- ctt aaa cag ctt gat acc gat ag -3’</td>
</tr>
<tr>
<td>p-5’-BS2-SeqlIII</td>
<td>5’- ctc agc gac cga ata tat cgg -3’</td>
</tr>
<tr>
<td>p-3’-BS2-SeqlIV</td>
<td>5’- ggt gta tca ccg tact ca gg -3’</td>
</tr>
<tr>
<td>p-5’-TS1-SeqlII</td>
<td>5’- gaa tta tca ccg tca ccc cag ac -3’</td>
</tr>
</tbody>
</table>

II.1.7.3 Oligonucleotides for amplification of CD30R cDNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-CD30SfiI</td>
<td>5’- gtc ctc gca act gcg gcc agg tgc ctc ctc gcc gcg c -3’</td>
</tr>
<tr>
<td>p-3’-CD30NotI</td>
<td>5’- cat gcg gcc gcc tta ctt ccc cgt gga gag agc -3’</td>
</tr>
<tr>
<td>p-5’-Seq-p6</td>
<td>5’- ctt ctg gta act ttc ttc gcg -3’</td>
</tr>
<tr>
<td>p-3’-Seq-p6</td>
<td>5’- cat gaa gaa acc aat caa taa tc -3’</td>
</tr>
</tbody>
</table>

II.1.7.4 Oligonucleotides for amplification of Ki-4(scFv) DNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-pIXKi4</td>
<td>5’- gtc ctc gca act gcg ctc gcg ctc gcg cag ctg gag cag -3’</td>
</tr>
<tr>
<td>p-3’-pIXKi4</td>
<td>5’- gtc ctc gca act gcg ctc gcg ctc gcg cag ctg gag cag -3’</td>
</tr>
</tbody>
</table>
II Material and Methods

II.1.7.5 Oligonucleotides for construction and analysis of pHEN9

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-p9NotIa</td>
<td>5'- ata aga atg cgg ccg cag aac aaa aac tca tct cag aag agg atc tga atg ggg -3'</td>
</tr>
<tr>
<td>p-5’-p9NotIb</td>
<td>5'- ctc aga aga gga tct gaa gga tct gaa tgg ggc cgc ata gat gag ttt tgt ttt agt gta ttc ttt ggc-3'</td>
</tr>
<tr>
<td>p-3’-p9EcoRI</td>
<td>5'- ccg gaa ttc tta tga gga agt ttc cat taa acg gg -3'</td>
</tr>
<tr>
<td>p-5’-pHENx</td>
<td>5'- cac aca gga aac agc tat gac c -3'</td>
</tr>
<tr>
<td>p-3’-pHENx</td>
<td>5'- caa ggc gat taa gtt ggg taa cg -3'</td>
</tr>
</tbody>
</table>

II.1.7.6 Oligonucleotides for construction of intermediate pHEN9-Bi

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-HindIII</td>
<td>5'- cgc caa gct tgc atg caa att ct -3'</td>
</tr>
<tr>
<td>p-3’-HEN39a</td>
<td>5'- gcc atc gcc ggc tga ggc ggg agt gtt ttt gag ttt tcg aat ggt ggg cgg cgg g -3'</td>
</tr>
<tr>
<td>p-3’-HEN39b</td>
<td>5'- gat atc tct aga ctc gag ggc cat cgc cgg ctg agc gg -3'</td>
</tr>
<tr>
<td>p-5’-HEN39a</td>
<td>5'- gat atc ctg cag cca tgg gaa caa aaa ctc atc tca gaa gag gat c -3'</td>
</tr>
<tr>
<td>p-5’-HEN39b</td>
<td>5'- ggc cct cga gtc tag aga tat cct gca gcc atg gga ac -3'</td>
</tr>
<tr>
<td>p-3’-EcoRI</td>
<td>5'- cag tga att ctt atg agg aag ttt c -3'</td>
</tr>
</tbody>
</table>

II.1.7.7 Oligonucleotides for construction and analysis of pHEN3+9

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-HEN9Eco</td>
<td>5'- ccg gaa ttc gaa tgt gag tta gct cac tca tta g -3'</td>
</tr>
<tr>
<td>p-3’-HEN9Eco</td>
<td>5'- cgt gaa ttc tta gga gga agt ttc c -3'</td>
</tr>
<tr>
<td>p-5’-H39Seq</td>
<td>5'- cc acct tta tgt atg tat ttt cga c -3'</td>
</tr>
</tbody>
</table>

II.1.7.8 Oligonucleotides for sequencing of pHEN3CWP+9

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-biHEN3</td>
<td>5'- cga ctg gaa agc ggg cag tg -3'</td>
</tr>
<tr>
<td>p-3’-biHEN3</td>
<td>5'- ctg tgt gag tgt cta aac aac -3'</td>
</tr>
</tbody>
</table>
II Material and Methods

II.1.7.9 Oligonucleotides for construction and analysis of pHEN3+9Strep

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-5’-StrepIX</td>
<td>5’- ccc gca gtt cga aaa acc atg gga aaa act cat ctc -3’</td>
</tr>
<tr>
<td>p-3’-StrepIXa</td>
<td>5’- gtg gct cca gct agc ggc cat cgc cgg ctg agc -3’</td>
</tr>
<tr>
<td>p-3’-StrepIXb</td>
<td>5’- gtt ttt cga act gcg ggt ggc tcc agc tag cgg cc -3’</td>
</tr>
<tr>
<td>p-3’-biHEN9</td>
<td>5’- gcg aaa gaa tac act aaa aca ctc -3’</td>
</tr>
</tbody>
</table>

II.1.7.10 Oligonucleotides for construction and analysis of pHEN9-Strep

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-3’-StrepI</td>
<td>5’- ctg cgg gtg gct cca gct agc ggc cat ggc cgg ctg ggc cgc -3’</td>
</tr>
<tr>
<td>p-3’-StrepII</td>
<td>5’- gtt ctg cgg cgg ctt ttt cga act gcg ggt ggc tcc agc tag -3’</td>
</tr>
<tr>
<td>p-5’-HENSeq</td>
<td>5’- gta tgt tgt gtg gaa ttg tga gcg -3’</td>
</tr>
</tbody>
</table>

II.1.8 Equipment

Centrifuges: Avanti™ 30, Allegra™ 6KR and Microfuge™ (Beckman, California, USA), Eppendorf 5415D (Eppendorf, Hamburg). Rotors: Ja-10, Ja-30.50, JLA-16.250 (Beckman), F45-24-11 (Eppendorf).

DNA gel electrophoresis apparatus: wide mini and mini cells for DNA agarose electrophoresis and power suppliers (BioRad).

DNA-sequencing: “ABI Prism 3700” Capillary-sequencer (Perkin-Elmer, Applied Biosystems, Foster City, USA).


Electroporation apparatus: Gene pulser TM and Pulse controller unit (BioRad).

ELISA-Reader: ELISA-Reader SpectraMax 340 (Molecular Devices, München).

Laminar flow: Hera Safe HS12 (Kendro, Hanau).
**II Material and Methods**

**PCR Thermocycler:** Primus 96 Plus (MWG-Biotech); „Programmable Thermal Controller“ PTC-200™ (MJ Research Inc, Watertown, USA).

**Shaker incubator:** Innova™ 4430 (New Brunswick Scientific, Nürtingen); 37°C incubator (Heraeus Instruments, Hanau); Eppendorf Thermomixer compact (Eppendorf).

**UV-Transilluminator:** 302nm wavelength and UVT-20M (Herolab); UV-chamber (BioRad).

**Vortex:** Vortex Genie II (Scientific Industries Inc., Bohemia, USA).

**II.1.9 Approbation for the conducted work**

Permits for conducted work at security level S1 was given by the “Landesumweltamt NRW”. Number of registration is: 64-K-1.19/02.
II.2 Methods

II.2.1 Recombinant DNA techniques

General recombinant DNA techniques including DNA precipitation, restriction enzyme digest, DNA ligation and DNA agarose gel electrophoresis were performed according to the standard protocols described by Sambrook et al. (1996) or according to the manufacturer’s protocol when using a kit.

II.2.1.1 Isolation of plasmid, phage and phagemid DNA from *E. coli*

Plasmid, phagemid and phage DNA was purified using QIAprep® Plasmid Isolation Mini or Maxi Kit (II.1.2) according to the manufacturers’ manual. Quality and quantity of DNA was confirmed by spectrophotometric analysis (II.2.1.5) or analytical agarose gel electrophoresis (II.2.1.3). Isolated DNA samples were stored at –20°C.

II.2.1.2 PCR amplification

II.2.1.2.1 Standard PCR amplification of DNA fragments

Polymerase chain reaction (PCR), a procedure for rapid *in-vitro* enzymatic amplification of a specific segment of DNA, was used for the amplification and modification of genes of interest Saiki et al. 1988) as well as for the insertion or deletion of restriction sites or short nucleotide sequences. The reactions were performed in 0.2ml PCR tubes (Biozym, Oldenburg), using a PCR thermocycler (MWG Biotech or MJ Research Inc.). PCR reactions were carried out in a total volume of 25µl as described in the following table:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>0.5-1µl</td>
<td>1-10ng</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>2.5µl</td>
<td>1x</td>
</tr>
<tr>
<td>2,5M betain</td>
<td>2.5µl</td>
<td>250mM</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>1µl</td>
<td>0.4mM each</td>
</tr>
<tr>
<td>100pmol/µl forward primer</td>
<td>1µl</td>
<td>10pmol/µl</td>
</tr>
<tr>
<td>100pmol/µl backward primer</td>
<td>1µl</td>
<td>10pmol/µl</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (5U/µl) destilled water</td>
<td>0.25µl</td>
<td>1.25 units</td>
</tr>
<tr>
<td></td>
<td>added to 25µl</td>
<td></td>
</tr>
</tbody>
</table>
Amplification was carried out under the following conditions:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5min</td>
<td>initial denaturation</td>
</tr>
<tr>
<td>95°C</td>
<td>30sec</td>
<td>denaturation</td>
</tr>
<tr>
<td>TP or gradient</td>
<td>30sec</td>
<td>primer annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>1min/1kb</td>
<td>elongation</td>
</tr>
<tr>
<td>72°C</td>
<td>7min</td>
<td>final elongation</td>
</tr>
</tbody>
</table>

25x

The optimal annealing temperature (TP) of the primer was experimentally optimized (temperature gradient) or calculated by the empirical formula (WU et al. 1991).

\[ TP = \{22 + 1.046[2x(G +C) + (A + T)]\} \]

PCR products were resolved on a 1-1.2% (w/v) agarose gel (II.2.1.3) with appropriate DNA markers to confirm the successful amplification and integrity of the amplified product.

II.2.1.2.2 SOE-PCR

Splice overlap extension (SOE) PCR was used for insertion of the leader sequence, linker sequence, cloning sites or tags into the phage or phagemid vectors. SOE PCR is a modified PCR method in which the primers are designed in a way that the coding region of the 3' end of one primer is complementary to the 5' region of the next primer (Horton et al. 1989).

All modifications of phage or phagemid vectors were constructed by SOE PCR. First, two standard PCR’s (II.2.1.2.1) were carried out in order to amplify the DNA sequence upstream and downstream of the region, which had to be modified. By using appropriate primer the modified DNA sequence was fused to the 3’ end of the first (upstream) PCR product and to the 5’ end of the second (downstream) PCR product, respectively. The two resulting PCR products were assembled by SOE PCR using the inserted complementary sequences at their ends. Subsequently, the final SOE PCR constructs were cloned into the vectors of interest.

SOE PCR was carried out with equimolar amounts of the two overlapping PCR fragments. Two reaction mixtures were prepared and SOE PCR was performed as described below:
Reaction mixture I:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product 1</td>
<td>1-3µl</td>
<td>~5ng</td>
</tr>
<tr>
<td>PCR product 2</td>
<td>1-3µl</td>
<td>~5ng</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>2.5µl</td>
<td>1x</td>
</tr>
<tr>
<td>2,5M betain</td>
<td>2.5µl</td>
<td>250mM</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>2µl</td>
<td>0.8mM each</td>
</tr>
<tr>
<td>Taq DNA polymerase (5U/µl)</td>
<td>0.25µl</td>
<td>1.25 units</td>
</tr>
<tr>
<td>destilled water</td>
<td>added to 25µl</td>
<td></td>
</tr>
</tbody>
</table>

Reaction mixture II:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer</td>
<td>2.5µl</td>
<td>1x</td>
</tr>
<tr>
<td>2,5M betain</td>
<td>2.5µl</td>
<td>250mM</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>1µl</td>
<td>0.4mM each</td>
</tr>
<tr>
<td>100pmol/µl forward primer (PCR1)</td>
<td>1µl</td>
<td>10pmol/µl</td>
</tr>
<tr>
<td>100pmol/µl backward primer (PCR2)</td>
<td>1µl</td>
<td>10pmol/µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5U/µl)</td>
<td>0.25µl</td>
<td>1.25 units</td>
</tr>
<tr>
<td>destilled water</td>
<td>added to 25µl</td>
<td></td>
</tr>
</tbody>
</table>

Amplification was carried out under the following conditions:

**Reaction mixture I**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5min</td>
<td>initial denaturation</td>
</tr>
<tr>
<td>95°C</td>
<td>30sec</td>
<td>denaturation</td>
</tr>
<tr>
<td>72°C or gradient</td>
<td>30sec</td>
<td>annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>1min/1kb</td>
<td>elongation</td>
</tr>
<tr>
<td>60°C</td>
<td>forever</td>
<td></td>
</tr>
</tbody>
</table>

**Reaction mixture II was added**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>45sec</td>
<td>denaturation</td>
</tr>
<tr>
<td>T&lt;sub&gt;p&lt;/sub&gt;</td>
<td>45sec</td>
<td>annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>1min/1kb</td>
<td>elongation</td>
</tr>
<tr>
<td>72°C</td>
<td>5min</td>
<td>final elongation</td>
</tr>
</tbody>
</table>
II.2.1.2.3 PCR based analysis of recombinant bacterial clones

Bacterial clones harboring plasmid DNA containing the gene of interest were identified by PCR as described by Jesnowski et al. (1995). Single colonies were picked with sterile toothpicks and dipped each in a PCR tube containing 10µl of sterile water. 15µl of the PCR mix (II.2.1.2.1) were added to each 10µl bacterial suspension giving a final volume of 25µl. Specific primers annealing to the 5’ and 3’ ends of the cloned gene or primers specific for the vector backbone were used for PCR reaction (II.2.1.2.1). Thermocycler conditions were used as described in II.2.1.2.1 but for effective lysis of the bacterial cells the time for initial denaturation was increased to 10 min. 10µl of the PCR product were analyzed on a 1.2% (w/v) agarose gel (II.2.1.3).

II.2.1.3 Analytical agarose gel electrophoresis

Undigested DNA (II.2.1.1), restriction enzyme digested DNA (II.2.1.6) and PCR fragments (II.2.1.2) were electrophoretically separated on 0.8-1.2% (w/v) agarose gels prepared in TAE buffer containing 0.1µg/ml ethidium bromide.

Known amount of DNA molecular marker such as PstI-digested lambda DNA were used for evaluation of sample size, integrity and determination of DNA concentration. The DNA was visualized on an UV transilluminator at 302nm and documented by a black and white E.A.S.Y 429K camera (Herolab).

II.2.1.4 Preparative gel electrophoresis

Preparative gel electrophoresis was used for isolation of PCR amplified DNA (II.2.1.2) or DNA fragments after restriction enzyme digestion (II.2.1.6) prior to cloning in the appropriate vectors. After electrophoresis the desired DNA fragments were excised from the gel and purified using the “QIAquick Gel Extraction Kit” (Qiagen) (II.2.1) according to the manufacturers’ protocol. The concentration of the recovered DNA was measured spectrophotometrically (II.2.1.5) or determined by analytical agarose gel electrophoresis (II.2.1.3).

II.2.1.5 Quantification of nucleic acids

The amount of RNA or DNA in a sample was estimated by measuring the OD$_{260nm}$. The OD$_{260nm}$ of 1 corresponds to ~50µg/ml of dsDNA or ~40µg/ml of ssDNA and RNA. Purity of the nucleic acid was ascertained by the OD$_{260nm}$/OD$_{280nm}$ ratio of the measured optical density, which is 1.8 for pure DNA and 2.0 for pure RNA.
II.2.1.6 Restriction digest of DNA

Restriction endonucleases, corresponding buffers and BSA solution were obtained from New England Biolabs. Restriction digest and double restriction digest of DNA were performed according to the manufacturers’ protocol.

II.2.1.7 Ligation of DNA

Restriction enzyme digested DNA (II.2.1.6) was ligated by using the Quick Ligation™ Kit from New England Biolabs. Ligation reactions were performed as recommended in the manufacturers' protocol.

II.2.1.8 Growth and maintenance of bacterial strains

*E. coli* DH5α and XL1-Blue MR bacterial cells were grown at 37°C over night either in liquid LB medium in a shaker incubator or plated on solidified LB-agar [1.5% (w/v) agar] plates. *E. coli* TG1 were grown in 2xTY medium under the same conditions. The LB or 2xTY media were supplemented with appropriate antibiotics and 1% (w/v) glucose for the growth of recombinant bacteria. The plates were stored at 4°C for not more than two weeks. For long-term storage of bacterial strains, glycerol was added to a final concentration of 15% (v/v) to the liquid culture and frozen at –80°C.

II.2.1.9 Bacterial transformation

II.2.1.9.1 Preparation of competent *E. coli* cells for heatshock transformation

*E. coli* competent cells were prepared for CaCl₂-mediated heatshock transformation as described by Hanahan (1983). Briefly, 5ml of LB broth were inoculated with a single bacterial colony and cultured at 37°C over night. 500µl of the over night culture was transferred into 50ml of LB broth containing 20mM MgSO₄ and 10mM KCl. The cells were cultured at 37°C for 3-4 h until the OD₆₀₀nm reached 0.5 and then transferred to an ice-cold tube. After cooling on ice for 10min, the cells were recovered by centrifugation (2,000g/4°C/10min). The pellet was resuspended in 15ml ice-cold TfB-I solution by gentle vortexing and stored on ice for 10min. The cells were sedimented down by centrifugation as described above and resuspended in 2ml ice-cold TfB-II. 100µl aliquots of the suspension were dispensed into prechilled 1.5ml Eppendorf tubes, frozen immediately in liquid nitrogen and stored at –80°C.

TfB-I (pH 5.8): 30mM potassium acetate; 50mM MnCl₂; 10mM CaCl₂; 15% (v/v) glycerol

TfB-II (pH 5.8): 30mM MOPS; 75mM CaCl₂; 10mM RbCl; 15% (v/v) glycerol
II Material and Methods

II.2.1.9.2 Transformation of E. coli by heatshock

Competent cells (II.2.1.9.1) were thawed on ice and ice-cold plasmid DNA (~80ng) or ligation product (II.2.1.7) were mixed gently with the competent cells and placed on ice for 30min. A heatshock of 42°C was given for 90sec and the sample was cooled on ice for two minutes. 900µl SOC media, pre-heated at 37°C, were added to the sample and cells were incubated for regeneration at 37°C for 30min. 10µl and 100µl were plated onto LB or 2xTY agar plates supplemented with appropriate antibiotics and incubated at 37°C over night.

II.2.1.9.3 Preparation of electrocompetent E. coli cells

Electrocompetent E. coli cells were prepared for DNA transformation as described by Dower et al. (Dower et al. 1988). A single colony was cultured in LB broth at 37°C until the mid-log phase (OD600nm = 0.5-0.8). The cells were placed on ice for 30min prior to centrifugation (3,000g/4°C/10min). The harvested cells were subsequently washed thrice with ice-cold sterile water and resuspended in ice-cold 10% (v/v) glycerol resulting in a 300-fold concentration of the original culture volume (at >10^10 cells/ml). 100µl aliquots were frozen immediately in liquid nitrogen and stored at –80°C.

II.2.1.9.4 Transformation of E. coli by electroporation

Electrocompetent cells (II.2.1.9.3) were thawed on ice and the cells were mixed with 80ng ice-cold DNA in sterile water. The cell/DNA mixture was transferred into a pre-chilled electroporation cuvette (0,1cm, BioRad) and assembled into the safety chamber of the electroporation apparatus. After application of a pulse (25µF, 1.7kV, 200Ω), the cells were diluted in 900µl pre-heated (37°C) SOC media and incubated for regeneration at 37°C for 30min. 10µl and 100µl were plated onto LB or 2xTY agar plates supplemented with appropriate antibiotics and incubated at 37°C over night.

II.2.1.9.5 Determination of transformation efficiency of competent cells

Efficiency of transformation of each new batch of competent cells was determined by test transformation with known concentration of supercoiled DNA of pUC18. The following transformation rates were obtained: heatshock competent cells >10^8/µg pUC18 and electrocompetent cells >10^9/µg pUC18.

II.2.1.10 DNA sequencing and sequencing analysis

DNA sequencing reactions were based on the dideoxy chain termination method described by Sanger et al. (1977). Fluorescent labelled dideoxynucleotide were used for sequencing of genes or DNA fragments of interest. Sequencing analysis was performed by using an “ABI Prism 3700” Capillary-Sequencer (Applied Biosystems) and BigDye™ cycle sequencing
terminator chemistry. The extension products were detected by exciting the unique dyes attached to each dideoxynucleotide with a laser, following by a measurement of fluorescent emission with a CCD camera. Subsequently, the signals were interpreted by the Applied Biosystems Sequencing Analysis Program in order to determine the nucleotide sequence of the DNA template. Chromas software package was used for displaying the chromatogram files. For evaluation of sequence data the sequences were exported to the DNAsis and GCG software packages.

**II.2.1.11 RNA isolation and synthesis of first-strand cDNA**

**II.2.1.11.1 Total RNA extraction**

TRIzol® reagent (Invitrogen) was used for extraction of total RNA according to the manufacturers' protocol. Briefly, $10^7$ L540 cells (II.1.5.3) were lysed by resuspension in 1ml TRIzol® reagent followed by incubation of the homogenized cells at room temperature for 5min. 0.2ml of chloroform (VWR) was added, followed by 15sec of vigorous shaking by hand and subsequent incubation for 3min at room temperature. After centrifugation (12,000g/4°C/15min) the colorless aqueous phase containing the RNA was transferred to a fresh tube. RNA was precipitated by incubation with 0.5ml of isopropanol (Roth) for 10min at room temperature and centrifuged (12,000g/4°C/10min). The RNA pellet was washed once with 75% (v/v) ethanol (Roth). The pellet was air dried for 5min and redissolved in 50µl RNAase free water. The purity and the amount of total RNA were estimated by spectrophotometric analysis (II.2.1.5).

**II.2.1.11.2 cDNA synthesis**

First-strand cDNA was synthesized by using the SuperScript™ First-Strand Synthesis Kit (Invitrogen) and oligo-dT primer according to the manufacturers' protocol. The cDNA primer p-5’-CD30Sfi and p-3’-CD30Not (II.1.7.3), which specifically anneal to the CD30-receptor (CD30R) encoding region were used for the amplification of first-strand cDNA (II.2.1.2.1).
II.2.2 Methods for phage analysis

II.2.2.1 Phage amplification

II.2.2.1.1 Phage amplification using phage vectors

A 50ml 2xTY culture supplemented with tetracycline at a final concentration of 15µg/ml was inoculated with recombinant *E. coli* TG1 containing the appropriate phage vector and incubated over night at 30°C while shaking. The over night culture was centrifuged for 20min at 4,000g and the supernatant containing the assembled recombinant phages was used for phage preparation (II.2.2.2).

II.2.2.1.2 Phage amplification using phagemid vectors

A 50ml 2xTY culture supplemented with 1% (v/v) glucose and 100µg/ml ampicillin was inoculated with 500µl of an over night culture of recombinant *E. coli* TG1 containing the appropriate phagemid vector. The culture was inoculated at 37°C for 1.5-2.5 h while shaking. After OD_{600}\text{mm} of 0.5 was reached, 5ml of the exponentially grown bacteria were transferred to a 50ml Falcon tube and 200µl helperphage M13KO7 (10^{11} phage/ml; II.1.5.2.1) or hyperphage M13KO7ΔpIII (10^{11} phage/ml; II.1.5.2.2) were added immediately. For infection of the recombinant bacteria, the culture was incubated for 30min in a 37°C pre-heated water bath, following by 30min incubation at 37°C while shaking. After the infection process, the cells were spun down at 4,000g for 10min. The bacterial pellet was resuspended in 50ml (Midi preparation) or 500ml (Maxi preparation) 2xTY media supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin and incubated at 30°C over night while shaking. The over night culture was centrifuged for 20min at 4,000g and the supernatant containing the assembled recombinant phages was used for phage preparation (II.2.2.2).

II.2.2.2 Phage preparation

1/6 volume of PEG/NaCl solution was added to the phages containing supernatant (II.2.2.1.1 or II.2.2.1.2) and the solution was incubated for 4 h on ice. The precipitated recombinant phages were separated from the supernatant by centrifugation for 15min at 4,000g and 4°C. The phage pellet was resuspended in 1ml PBS (for Midi and Maxi preparation) and transferred to a fresh 1.5ml eppendorf tube. Remaining bacteria were removed by an additional centrifugation steps (14,000g/RT/2min). After the final centrifugation no bacterial pellet should be visible at all. The corresponding supernatant was transferred to a fresh eppendorf tube and stored at 4°C until further experiments.

PEG/NaCl solution: 20% (w/v) polyethylene glycol 8000; 2.5M NaCl
II.2.2.3 Phage titration

Phage titration as well as phage titration ELISA (II.2.2.5.1) were used for quantification of prepared recombinant phages (II.2.2.2). The phage concentration was determined by transduction of *E. coli* TG1 with serial dilutions of the appropriate phage stock and subsequent counting of the colony forming units (cfu) of infected bacteria. Serial dilutions of the phage stock were titrated in 500µl of 2xTY media. To each dilution (10^{-2} – 10^{-12}) 500µl exponentially growing *E. coli* TG1 were added and incubated at 37°C for 30min. After transduction of *E. coli* TG1, 100µl of the bacterial cells were plated on 2xTY plates supplemented with 100µg/ml ampicillin (for phagemid vector based phages, II.2.2.1.2) or 15µg/ml tetracycline (for phage vector based phages, II.2.2.1.1). Plates were incubated over night at 30°C and after determination of the number of cfu the average phage titer was calculated.

\[
\text{phage titer [phage/ml]} = \frac{\text{number of cfu} \times 10}{\text{dilution}} \times 200
\]

II.2.2.4 Purification of recombinant phages

II.2.2.4.1 Purification of recombinant Strep-tag® displaying phages

A new method was developed for the purification of recombinant phages displaying the Strep-tag® based on the Strep-tag® technology of IBA GmbH in Göttingen. IBA GmbH offers a well-established platform technology for expression, purification and detection of Strep-tag® fusion proteins (http://www.iba-bioTAGnology.com). The Strep-tag® is a short peptide consisting of 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) binding specifically to the biotin binding pocket of streptavidin when fused to recombinant proteins. To optimize the binding properties IBA GmbH also engineered streptavidin to obtain Strep-Tactin®.

Freshly prepared recombinant phages (Maxi prep; II.2.2.2) displaying the Strep-tag® either on pIII or pIX coat protein were purified by using Strep-Tactin® Superflow® cartridges (1ml column bed volume, IBA GmbH). The Strep-Tactin® cartridges were equilibrated by applying 5ml Buffer W using a 10ml syringe at a flow rate of 1 drop/sec. Stock solution of freshly prepared phages was centrifuged (14,000g/RT/2min) and the cleared supernatant diluted 1:5 with PBS before applying to the equilibrated Strep-Tactin® cartridge. Diluted supernatant was applied with a flow rate of not more than 0.5 drops/sec by using a 10ml syringe. Cartridges were washed with 5ml Buffer W and a flow rate of 1 drop/sec. Strep-tag® displaying phages were eluted by applying 3ml Buffer E containing the specific competitor desthiobiotin with a flow rate of not more than 0.5 drops/sec. The purified Strep-tag® displaying phages were stored at 4°C until further experiments.
For detection and analysis of these phages via Streptavidin or Strep-Tactin<sup>®</sup> Phage ELISA (II.2.2.5.3), the phages were dialyzed over night at 4°C in 2 liter Buffer E without desthiobiotin because remaining desthiobiotin is affecting the binding activity to streptavidin or Strep-Tactin<sup>®</sup> coated microtiter plates.

For regeneration of the Strep-Tactin<sup>®</sup> cartridges 2x 5ml Buffer W were applied with a flow rate of 1 drop/sec, followed by two injections of 5ml Buffer R using the same flow rate. Finally, the cartridges were washed with 2x 4ml Buffer W and stored at 4°C overlayed with 1ml Buffer W.

Buffer W: 100mM Tris-HCl; 150mM NaCl; 1mM EDTA; pH 8
Buffer E: 100mM Tris-HCl; 150mM NaCl; 1mM EDTA; 2.5mM desthiobiotin; pH 8
Buffer R: 100mM Tris-HCl; 150mM NaCl; 1mM EDTA; 1mM HABA (hydroxy-azophenylbenzoic acid), pH 8

II.2.2.4.2 Purification of recombinant 6xHis-tag displaying phages

According to II.2.2.4.1, a method for purification of recombinant phages displaying a 6xHis-tag was developed. Freshly prepared recombinant phages (II.2.2.2) displaying a 6xHis-tag were purified using Ni-NTA Superflow<sup>®</sup> cartridges (1ml column bed volume, IBA GmbH). The Ni-NTA cartridges were equilibrated by applying 5ml Ni-NTA Lysis Buffer using a 10ml syringe at a flow rate of 1 drop/sec. Stock solution of freshly prepared phages was centrifuged (14,000g/RT/2min) and the cleared supernatant diluted 1:5 with PBS before applying to the equilibrated Ni-NTA cartridge. Diluted supernatant was applied with a flow rate of not more than 0.5 drops/sec using a 10ml syringe. Cartridges were washed with 5ml Ni-NTA Wash Buffer at a flow rate of 1 drop/sec. 6xHis-tag displaying phages were eluted by applying 3ml Ni-NTA Elution Buffer at a flow rate of not more than 0.5 drops/sec. The purified 6xHis-tag displaying phages were stored at 4°C until further experiments.

For regeneration, the Ni-NTA cartridges were washed as described by the manufacturer.

Ni-NTA Lysis Buffer: 50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, 10mM imidazol, pH 8
Ni-NTA Wash Buffer: 50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, 20mM imidazol, pH 8
Ni-NTA Elution Buffer: 50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, 250mM imidazol, pH 8

II.2.2.4.3 Purification of bispecific phages

According to II.2.2.4.1 and II.2.2.4.2, a method for purification of bispecific phages displaying a 6xHis-tag on pIII phage coat protein and a Strep-tag<sup>®</sup> on pIX phage coat protein was developed. Freshly prepared bispecific phages (II.2.2.2) displaying both tags were purified by
using a Ni-NTA Superflow® and a Strep-Tactin® Superflow® cartridge (each 1ml column bed volume, IBA GmbH). The Strep-Tactin® purification was carried out as described in II.2.2.4.1. The purified Strep-tag® displaying phages were collected in a sterile petri dish and directly applied to a pre-equilibrated Ni-NTA cartridge as described in II.2.2.4.2. The purified bispecific phages were stored at 4°C until further experiments.

For detection and analysis of these phages via Streptavidin or Strep-Tactin® phage ELISA (II.2.2.5.3), the phages were dialyzed overnight at 4°C in 2 liter Buffer E (II.2.2.4.1) without desthiobiotin.

Cartridges were regenerated as described in II.2.2.4.1 and II.2.2.4.2.

**II.2.2.5 Phage ELISA**

**II.2.2.5.1 Phage Titration ELISA**

In addition to standard phage titration (II.2.2.3) the phage titration ELISA was used for quantification of prepared recombinant phages (II.2.2.2), especially to determine the concentration of phages prepared with the hyperphage M13KO7ΔpIII (II.1.5.2.2) as the infectivity of these phages is highly decreased (Rondot et al. 2001 and O’Connell et al. 2002). In contrast to the commercially available phage titration ELISA Kit from Progen Biotechnik GmbH (Heidelberg), the assay used within this thesis is based on a direct phage ELISA. Serial dilutions of the phage stock were directly coated on a 96-well high binding microtiter plate (Greiner) and detected by HRP-conjugated monoclonal antibodies specific for the phage major coat protein pVIII (II.1.3). The phage titer was determined by comparing the measured titration curves with the titration curve of a standard M13KO7 stock solution (10^{11} phage/ml). The following conditions were used for phage titration ELISA:

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Vol. (µl/well)</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating</td>
<td>serial dilution of phages in CB</td>
<td>150</td>
<td>over night</td>
<td>RT</td>
</tr>
<tr>
<td>Blocking</td>
<td>2% (w/v) BSA in PBS</td>
<td>300</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>HRP-antibody</td>
<td>1:2,000 in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Substrate</td>
<td>ABTS (Roche)</td>
<td>100</td>
<td>15</td>
<td>RT</td>
</tr>
</tbody>
</table>

CB (coating buffer, pH 9.6): 1.7ml of 0.2 M Na₂CO₃,

0.8ml of 0.2 M NaHCO₃

7.5ml dest. H₂O
All incubation steps were done while shaking the microtiter plates. After each incubation the plates were washed three times with PBS-T and PBS.

### II.2.2.5.2 Cell wall protein (CWP) phage ELISA

Cell wall protein (CWP) phage ELISA was used for detecting recombinant phages displaying functional CWPD2 scFv antibody fragment (Peschen et al. 2004) either on the minor coat protein pIII or on pIX. CWP is a cell wall protein extract of fusarium and acts as antigen for the chicken scFv antibody fragment CWPD2 (Peschen et al. 2004). The following conditions were used for the CWP Phage ELISA:

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Vol. (µl/well)</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating</td>
<td>CWP antigen 1:100 in PBS</td>
<td>150</td>
<td>over night</td>
<td>RT</td>
</tr>
<tr>
<td>Blocking</td>
<td>2% (w/v) BSA in PBS</td>
<td>300</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Phages</td>
<td>serial dilution in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>HRP-antibody</td>
<td>1:2,000 in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Substrate</td>
<td>ABTS (Roche)</td>
<td>100</td>
<td>15</td>
<td>RT</td>
</tr>
</tbody>
</table>

CWP antigen was kindly provided by Dr. Dieter Peschen (RWTH Aachen, Department of Molecular Biology). All incubation steps were done while shaking the microtiter plates. After each incubation the plates were washed three times with PBS-T and PBS.

### II.2.2.5.3 Streptavidin or Strep-Tactin® phage ELISA

Streptavidin or Strep-Tactin® phage ELISA were used for detection of Strep-tag® displaying recombinant phages. Streptavidin coated microtiter plates were obtained from Roche Diagnostics (Mannheim), whereas Strep-Tactin® coated ones were ordered from IBA GmbH (Göttingen). The following conditions were used for Streptavidin or Strep-Tactin® Phage ELISA:

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Vol. (µl/well)</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking</td>
<td>2% (w/v) BSA in PBS</td>
<td>300</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Phages</td>
<td>serial dilution in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>HRP-antibody</td>
<td>1:2,000 in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Substrate</td>
<td>ABTS (Roche)</td>
<td>100</td>
<td>15</td>
<td>RT</td>
</tr>
</tbody>
</table>
All incubation steps were done while shaking the microtiter plates. After each incubation the plates were washed three times with PBS-T and PBS.

II.2.2.5.4 Biotin phage ELISA

In contrast to the Streptavidin or Strep-Tactin® phage ELISA (II.2.2.5.3), the Biotin phage ELISA is an indirect assay used for detection of Strep-tag® displaying recombinant phages. Biotinylated microtiter plates were obtained from Pierce/Perbio (Bonn) and specific binding of Strep-tag® displaying phages was detected by using soluble unconjugated streptavidin (1mg/ml, Roche Diagnostics) or Strep-Tactin® (1mg/ml, IBA GmbH) as a linker molecule. The following conditions were used for Biotin phage ELISA:

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Vol. (µl/well)</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking</td>
<td>2% (w/v) BSA in PBS</td>
<td>300</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Streptavidin or</td>
<td>1:1,000 in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Strep-Tactin®</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phages</td>
<td>serial dilution in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>HRP-antibody</td>
<td>1:2,000 in PBS + 0.5% (w/v) BSA</td>
<td>100</td>
<td>60</td>
<td>RT</td>
</tr>
<tr>
<td>Substrate</td>
<td>ABTS (Roche)</td>
<td>100</td>
<td>15</td>
<td>RT</td>
</tr>
</tbody>
</table>

All incubation steps were done while shaking the microtiter plates. After each incubation the plates were washed three times with PBS-T and PBS.

II.2.2.6 Electron microscopy

Electron microscopy is a tool for structural (II.2.2.6.1) and immunological (II.2.2.6.2) analysis of recombinant filamentous bacteriophages. Samples for electron microscopy were prepared using Cu-grids (II.2.2.6.1) or Ni-grids (II.2.2.6.2) laminated with a pioloform-carbon support film. For electron microscopic analysis a transmission electron microscope EM-400 (Philips) was used.

II.2.2.6.1 Adsorption compounds

Adsorption compounds were prepared for morphological studies of the recombinant filamentous bacteriophages and their structural analysis by transmission electron microscopy. Freshly prepared recombinant phages (II.2.2.2) were diluted 1:10,000 in PBS and Cu-grids were floated for 15min on drops of this solution. The grids were washed with 20
II Material and Methods drops PBS-T and blocked by floating on a drop of 0.5% (w/v) BSA in phosphate buffer (PB; pH7.2) for 30min at RT. Afterwards the grids were extensively washed with 40 drops PBS-T and 40 drops distilled water and stained with 6 drops of 1% (w/v) uranyl acetate for transmission electron microscopy.

II.2.2.6.2 Immunogold assay

Immunogold labelling (Louro & Lesemann, 1984) was used for detection and visualization of foreign proteins displayed on the surface of filamentous bacteriophages. Freshly prepared recombinant phages (II.2.2.2) were used to prepare adsorption compounds with Ni-grids instead of Cu-grids as described in II.2.2.5.3. After blocking the grids with 0.5% (w/v) BSA in PB (pH 7.2) primary antibodies (II.1.3) or binding proteins were bound to the samples by incubating the grids in a 1:500 PBS-T dilution of the appropriated antibody or *Strep*-Tactin® for 2 h at RT. The grids were washed twice with 20 drops PBS-T and incubated over night at RT with secondary gold-labelled antibodies (II.1.3) by floating the grid on a 1:50 PBS-T dilution. The grids were extensively washed with 40 drops PBS-T and 40 drops distilled water and stained with 6 drops of 1% (w/v) uranyl acetate for transmission electron microscopy. Two different set ups were used for immunogold labelling.

First set up:

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
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<td>15</td>
<td>RT</td>
</tr>
<tr>
<td>Blocking</td>
<td>0.5% (w/v) BSA in PB (pH 7.2)</td>
<td>30</td>
<td>RT</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>1. <em>Strep</em>-tag® II specific mouse antibody (II.1.3; IBA GmbH)</td>
<td>120</td>
<td>RT</td>
</tr>
<tr>
<td></td>
<td>2. polyclonal rabbit anti-6xHis (II.1.3; Acris Antibodies)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>1. 15nm gold-conjugated goat anti-mouse IgG (II.1.3; Biocell)</td>
<td>over night</td>
<td>RT</td>
</tr>
<tr>
<td></td>
<td>2. 10nm gold-conjugated goat anti-rabbit IgG (II.1.3; Biocell)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contrast</td>
<td>6 drops 1% (w/v) uranyl acetate</td>
<td>-</td>
<td>RT</td>
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Second set up:

<table>
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<td>Adsorption</td>
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<td>Blocking</td>
<td>0.5% (w/v) BSA in PB (pH 7.2)</td>
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<td>RT</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>1. mouse anti-penta-His, (II.1.3; Qiagen)</td>
<td>120</td>
<td>RT</td>
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<tr>
<td></td>
<td>2. horseradish peroxidase conjugated</td>
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<td><em>Strep</em>-Tactin® (IBA GmbH)</td>
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<td>Secondary antibody</td>
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<td>2. 10nm gold-conjugated goat anti-HRP (II.1.3; Plano)</td>
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<tr>
<td>Contrast</td>
<td>6 drops 1% (w/v) uranyl acetate</td>
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</table>
III Results and Discussion

This doctoral thesis was part of an interdisciplinary research project funded by the Fraunhofer Gesellschaft and was initiated to develop a novel protein microarray platform, called Protein Nanochip (Fig.I-9). As described in I.3.1, the Protein Nanochip should allow the automated screening of two phage libraries against each other in a high-throughput manner. A suitable chip surface structure and a laser detection system, both essential for the realization of the Protein Nanochip, had to be developed by other research partners involved in this project. Within this thesis a novel vector system for the generation of bispecific filamentous bacteriophages was developed. On the one hand these bispecific phages should allow the side-directed immobilization of a primary library onto the surface of the Protein Nanochip and on the other hand allow the binding of a secondary fluorescently labelled library, followed by detection, separation and analysis of specific binding partners.

Two different strategies were developed for the generation of the bispecific filamentous bacteriophages. The first one was based on a phage vector system and the challenging task was to modify the fd-tet phage vector (Zacher et al. 1980) by inserting two cloning sites with appropriate linkers upstream of geneIX and downstream of geneVI. The results achieved with this strategy are shown and discussed in III.1. The second strategy was based on a phagemid vector system and describes the construction of a bispecific phagemid vector by modifying the well-characterized pHEN1 phagemid vector (Hoogenboom et al. 1991). The insertion of a second cloning cassette downstream of geneIII should allow the construction of the bispecific phagemid vector and the generation of bispecific phages. The results achieved using this strategy are shown and discussed in III.2.

All phage and phagemid vectors created within this thesis are listed in V.2.1 and V.2.2.

In addition a suitable technology for the side-directed immobilization of these bispecific phages onto the surface of the Protein Nanochip had to be developed. Appropriate strategies and the corresponding results are described in III.3.

III.1 Construction and analysis of bispecific phage vectors

The development of a bispecific phage vector system based on the fd-tet phage vector (Zacher et al. 1980) describes the first strategy for the generation of bispecific phages. This strategy was very challenging, because currently there are only phage vectors available which allow the display of foreign proteins as a pIII-fusion by cloning the corresponding gene
between the geneIII signal sequence and geneIII within the phage genome (Skerra et al. 1988; Better et al. 1988). As described in I.1.2 there are only three regions, which were successfully used for modifications of the phage genome, leading to the correct assembly of functional phages. Modifications of the phage genome were tolerated within the intergenic region, within the small region between the end of geneVIII and the beginning of geneIII as well as between geneIII leader and geneIII (Zacher et al. 1980; McCafferty et al. 1990; Clackson et al. 1991). In addition there appears to be a delicate balance in the synthesis of modified phage proteins that allows phage production without seriously affecting bacterial growth (Model and Russel 1988; Webster 1996). These observations indicate that the transcription and translation of the phage genome is highly regulated and can be interpreted in a way that successful genetical modifications of the phage genome are challenging.

Nevertheless, the construction of a bispecific phage vector system had been one of the objectives of the presented work. This strategy was chosen because phage vectors allow multivalent display of foreign proteins on their surface, whereas phagemid vectors lead to monovalent display (Marks et al. 1992; O’Connell et al. 2002). Multivalent display of a repertoire of proteins induces an avidity effect resulting in higher binding activity between two binding phages and thus would facilitate the separation and sorting of these phage pairs. In addition, the tetrameric streptavidin molecule needed to be used for phage immobilization on biotinylated chip surfaces and thus multivalent display of streptavidin monomers was necessary. A disadvantage of this system is the decreased infectivity of rescued phages, due to the fact that currently only pIII phage vectors are available and that at least one incorporated wild type pIII copy is necessary for each phage to be infectious (I.1.3). Therefore a novel pVI phage vector had to be designed based on the data shown by Jespers et al. (Jespers et al. 1995). Previously, coat protein pVI had not been described to be involved in the infection process and therefore the modification of geneVI should not affect the infectivity of the corresponding phages. Originally, the pVI phagemid system was developed for the screening of full-length cDNA libraries, because the stop codon of the cDNAs is not affecting the expression of the cDNA-pVI fusion proteins when cloned to the 3’-end of geneVI. Therefore, establishing a bispecific phage vector based on the pVI-display system would not only circumvent the infectivity problem but also allow the library display on the same side of the phage as in the pIII-system. In addition, it should be possible to use pVI for the display of different kinds of protein libraries including cDNA libraries. For integration of the second specificity the phage coat protein pIX was the display system of choice, because it had been shown that pIX-display is the better screening system compared to pVII (Gao et al. 2003; Endemann and Model 1995).
III.1.1 Construction of bispecific phage vector fd-g6/9m

The bispecific phage vector fd-g6/9m was cloned by insertion of two cloning sites and appropriate linker sequences. For the construction of this bispecific phage vector the intermediate fd-g6m was cloned first. The corresponding cloning strategy is shown in Fig.III-1. According to Jespers et al. (Jespers et al. 1995) a G3SG2-linker sequence as well as a SfiI/NotI-restriction site were inserted downstream of geneVI within the fd-tet phage vector (Zacher et al. 1980) using SOE-PCR.

Fig.III-1: Cloning strategy for the construction of intermediate fd-g6m. p-5’-BamHI: forward primer for the amplification of amplicon A; p-3’-pVISfi: backward primer for the amplification of amplicon A and for insertion of the G3SG2-linker sequence (Jespers et al. 1995) and a SfiI-restriction site at the 3’-end of geneVI; p-5’-pVINot: forward primer for the amplification of amplicon B and for insertion of a G3SG2-linker sequence and a SfiI/NotI-cloning site at the 5’-end of geneI/X; p-3’-PacI: backward primer for the amplification of amplicon B. Amplicon A and B were assembled using SOE-PCR (II.2.1.2.2) and subsequently cloned into BamHI/PacI-digested fd-tet phage vector (Zacher et al. 1980).
Fd-g6m intermediate was used for the construction of the bispecific phage vector fd-g6/9m and the corresponding cloning strategy is shown in Fig.III-2. According to Gao et al. (Gao et al. 1999) a G₄S-linker sequence as well as a XhoI/Nhel-restriction site were inserted downstream of the second triplet of geneIX within the fd-g6m intermediate (Fig.III-1) using SOE-PCR (II.2.1.2.2). Insertion at this position was necessary because the 3'-end of geneVII and 5'beginning of geneIX are overlapping (Blumer et al. 1987; Ivey-Hoyle and Steege 1989) and any alteration of regulatory elements had to be avoided.

Fig.III-2: Cloning strategy for the construction of the bispecific phage vector fd-g6/9m. p-5’-BsrGI: forward primer for the amplification of amplicon A; p-3’-pIXm: backward primer for the amplification of amplicon A and for insertion of a G₄S-linker sequence (Gao et al. 2002b) and a XhoI/Nhel-cloning site downstream the second triplet of geneIX; p-5’-pIXm: forward primer for the amplification of amplicon B and for insertion of a G₄S-linker sequence and a XhoI/Nhel-cloning site behind the second triplet of geneIX; p-3’-BamHIIb: backward primer for the amplification of amplicon B. Amplicon A and B were assembled using SOE-PCR (II.2.1.2.2) and subsequently cloned into BsrGI/BamHI-digested intermediate phage vector fd-g6m (Fig.III-1). Sequencing analysis (II.2.1.10) was done using primers p-5’-BS2-Seql, p-3’-BS2-Seql, p-5’-BS2-SeqlII, p-3’-BS2-SeqlIV, p-5’-BS2-SeqV and p-3’-TS1-Seql (II.1.7.1 and II.1.7.2).
III.1.2 Analysis of bispecific phage vector fd-g6/9m

The CD30R cDNA encoding the extracellular domain of the human CD30 receptor was cloned as fusion to geneVI in order to verify the functionality of the bispecific phage vector fd-g6/9m (Fig.III-2). The cloning strategy for the construction of fd-g6CD30/9m is shown in Fig.III-3. CD30R cDNA was amplified using total RNA extracted from the human cell line L540Cy (II.1.5.3) and primers p-5’-CD30SfiI and p-3’-CD30NotI (II.1.7.3; II.2.1.11.2). Restriction sites were fused to the ends of the CD30R cDNA and cloned in the SfiI/NotI-cloning site upstream of geneVI.

**Fig.III-3: Cloning strategy for the construction of fd-g6CD30/9m.** Total RNA (II.2.1.11.1) isolated from L540Cy cells (II.1.5.3) were used for first-strand cDNA synthesis (II.2.1.11.2). First-strand cDNA was used as a template for the amplification of the DNA sequence encoding for the open reading frame of the extracellular domain of the human CD30 receptor (CD30R). p-5’-CD30SfiI (II.1.7.3): forward primer for the amplification of CD30R cDNA and for insertion of a SfiI-restriction site at the 5’-end of this sequence; p-3’-CD30NotI (II.1.7.3): backward primer for the amplification of CD30R cDNA and for insertion of a NotI-restriction site at the 3’-end of this sequence. Amplified CD30R cDNA was cloned into SfiI/NotI-digested fd-g6/9 phage vector (Fig.III-2). Sequencing analysis (II.2.1.10) was done using primers p-5’-Seq-p6 and p-3’-Seq-p6 (II.1.7.3).
The fd-g6CD30/9m phage vector was transformed into *E. coli* TG1 and freshly prepared phage particles (II.2.2.2) were analyzed in order to prove their infectivity. As described above, the infectivity should not be affected in any way by multivalent display of the CD30R on the pVI coat protein. However, due to the fact that pVI is located at the same end of the phage as pIII, there was a chance that phage infectivity would decrease due to steric problems and therefore the infectivity had to be investigated. Phages were used to infect *E. coli* TG1 and subsequently twelve tetracycline resistant colonies were analyzed for the presence of the CD30R cDNA by colony PCR (II.2.1.2.3) using primers p-5'-Seq-p6 and p-3'-Seq-p6. The results are shown in Fig.III-4.

**Fig.III-4: Colony PCR of *E.coli* TG1 infected with fd-g6CD30/9m phages.** In order to prove the infectivity of fd-g6CD30/9m phages, *E. coli* TG1 were infected with phages as described in II.2.2.3 and tetracycline resistant clones were used for PCR analysis (II.2.1.2.3) using primers p-5'-Seq-p6 and p-3'-Seq-p6 (II.1.7.3). *λ*: *PstI*-digested lambda DNA; fd-tet: *E. coli* TG1 containing fd-tet phage vector were used as a control for the colony PCR; fd-g6CD30/9m: 12 clones of *E. coli* TG1 infected with fd-g6CD30/9m phages used for colony PCR analysis; pc: fd-g6CD30/9m DNA used as a positive control for PCR analysis, showing the size of PCR products containing the CD30 receptor cDNA (1811bp); nc: fd-g6/9m DNA used as a negative control for PCR analysis, showing the size of PCR products not containing the CD30 receptor cDNA (684bp).

Ten out of the twelve clones tested, contained fd-g6CD30/9m and thus confirmed the presence of the CD30R cDNA within the phage vector. Therefore, the infectivity of the fd-g6CD30/9m phages was clearly demonstrated. Moreover, they were displaying five copies of the large extracellular domain of the human CD30R on pVI. In conclusion phage vectors can be designed for pVI-display of foreign proteins without affecting their infectivity.

To confirm the correct assembly and therefore the right morphology of the CD30R displaying phages an electron microscopical analysis (II.2.2.6) was performed and the corresponding results are shown in Fig.III-5.
III Results and Discussion

Fig.III-5: Electron microscopical analysis of fd-g6CD30/9m phages: The morphology of CD30R-displaying phages (fd-g6CD30/9m) was analyzed by electron microscopy. Phages (II.2.2.2) were immobilized on Cu-grids (II.2.2.6.1) and analyzed using an EM-400 electron microscope (Philips) with 60kV and a magnification of 130,000x.

In comparison to wild type fd phages (Fig.I-2) the morphology of fd-g6CD30/9m phages seems to be homologous. However, phage preparations for the infectivity assay (Fig.III-4) and for the electron microscopy (Fig.III-5) indicated decreased assembly efficiency, although all phages were infectious and showed the “correct” morphology. To further investigate this observation the assembly efficiency of fd-g6CD30/9m phages was determined by phage titration (Fig.III-6).

<table>
<thead>
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<th>phages</th>
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</tr>
</thead>
<tbody>
<tr>
<td>fd-tet</td>
<td>$8.31 \times 10^{14}$</td>
</tr>
<tr>
<td>fd-g6m</td>
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</tr>
<tr>
<td>fd-g6/9m</td>
<td>$1.02 \times 10^{8}$</td>
</tr>
<tr>
<td>fd-g6CD30/9m</td>
<td>$4.47 \times 10^{7}$</td>
</tr>
<tr>
<td>no phages</td>
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</tr>
</tbody>
</table>

Fig.III-6: Assembly efficiency of the modified phage vectors. Assembly efficiencies of all modified phage vectors were determined by a phage titration assay (II.2.2.3). The data were acquired in triplicate and the mean assembly efficiency (cfu/ml) calculated. Fd-tet: original phage vector (Zacher et al. 1980); fd-g6m: intermediate phage vector (Fig.III-1); fd-g6/9m: bispecific phage vector (Fig.III-2); fd-g6CD30/9m: bispecific phage vector containing CD30R cDNA fused to geneVI (Fig.III-3); no phages: E. coli TG1 incubated with 2xTY media without phages.
The results shown in Fig. III-6 confirmed that the assembly efficiency of the fd-g6CD30/9m phages was dramatically decreased. Twenty million times less of these phages were produced in comparison to unmodified fd-tet phages. Surprisingly, the decreased assembly efficiency was mainly due to gene VI modification itself and not to the insertion of the CD30R cDNA into the bispecific fd-g6/9m phage vector, as expected. Inserting the G3SG2-linker sequence and the SfiI/NotI-restriction site downstream of gene VI (Fig. III-1) decreased the assembly efficiency about \(7 \times 10^6\) times. Nevertheless, \(4 \times 10^7\) CD30R-displaying phages were produced, maintaining their infectivity and featuring the “correct” morphology.

In conclusion the gene VI modification of the fd-tet phage vector, which was done according to Jespers et al. (1995), dramatically affected the assembly efficiency of the corresponding phages. The most obvious reason might be a steric problem that occurs by multivalent display of the large CD30R cDNA as fusion to the pVI coat protein. The resulting protein cluster at the end of the phages could prevent the release of the phage particles from the bacterial membrane because the channels, which are formed by phage protein pIV, pI and pXI, are only 8nm in diameter and therefore could be clogged by the phage. pVI coat protein is much smaller than pIII and therefore the higher flexibility of pIII could explain its capability for multivalent display of foreign proteins on the surface of filamentous bacteriophages causing just slight decreased assembly efficiency (O’Connell et al. 2002). But the insertion of the CD30R cDNA did not seem to be the main reason for the assembly problem of the bispecific phage vector fd-g6/9m. The insertion of the G3SG2-linker sequence and the SfiI/NotI-restriction site to gene VI might have caused this problem. If insertion of these few amino acids to the 3'-end of gene VI was enough to cause a steric problem or if there are any other reasons for the loss of assembly efficiency can only be speculated on and has not been experimentally determined due to time constrains. Nevertheless, \(4 \times 10^7\) CD30R-displaying phages were produced, maintaining their infectivity and featuring the “correct” morphology. In conclusion, gene VI modification can be used for multivalent display of foreign proteins and for the generation of small libraries with a maximum diversity of about \(10^7\).

Modification of gene IX and insertion of the CD30R cDNA seemed not to be responsible for the decrease of the assembly efficiency, because bispecific fd-g6/9m phage vector showed the same efficiency as fd-g6m.
III.1.3 Construction and analysis of phage vector fd-g9m

III.1.3.1 Construction of phage vector fd-g9m

Although the above mentioned results show a decreased assembly efficiency resulting in protein libraries with a limited diversity, the use of a phage vector system could still be feasible by using geneIII instead of geneVI. GeneIII has already been described in the literature to be a suitable display system (Zacher et al. 1980; Smith et al. 1985; Clackson et al. 1991; O’Connell et al. 2002). In addition, it should be possible to display full-length cDNA’s on pIII coat protein by employing the Fos/Jun technology described by Crameri and Suter (Crameri and Suter 1993). According to their strategy, the described Jun-sequence had to be fused to geneIII and in addition an expression cassette had to be inserted into e.g. the intergenic region of the phage vector containing a pelB signal sequence, the Fos-sequence and a cloning cassette for insertion of the cDNA sequences. The pelB/Fos/cDNA fusion proteins would be directed to the periplasm of the bacterial host and should be incorporated into the phage by forming a leucin zipper with the Jun/pIII fusion protein.

Before establishing this cDNA display system it had to be proven that geneIX can be used for multivalent display of foreign proteins. Especially the modification of geneIX was expected to be critical because the 3’-end of geneVII and 5’-end of geneIX are overlapping and transcription, RNA processing and translation of these genes are somehow coupled to geneV and geneVIII. These four adjacent genes are transcribed to a polycystronic mRNA encoding pV, pVII, pIX and pVIII coat proteins and the different expression levels of the highly expressed pV and pVIII coat proteins and the rarely expressed pVII and pIX coat proteins have been explained due to local secondary structures of the mRNA at the transition from geneV to geneVII and by translation initiation sites showing differences in their activity (Blumer et al. 1987; Ivey-Hoyle and Steege 1989; Ivey-Hoyle and Steege 1992; Stump and Steege 1996). Whereas, the ribosome binding sites of geneVII appears to be limited mainly due to the local mRNA structure, no interaction of ribosomes with the corresponding binding site of geneIX has been proven at all and the appropriate regulatory elements have not been described up to now. Knowing at least some of these regulatory elements, the position for modification of geneIX was determined as the one between its second and third triplet, hopefully not destroying any regulatory mechanism within the transition of geneVII and geneIX. To verify this assumption the phage vector fd-g9m was constructed carrying the geneIX modification of fd-g6/9m without modification of geneVI. The corresponding cloning strategy is shown in Fig.III-7. According to Gao et al. (1999) fd-g9m was generated by inserting a G4S-linker sequence as well as an Xhol/Nhel-restriction site downstream of the second triplet of geneIX within the fd-tet phage vector (Zacher et al. 1980).
III Results and Discussion

**III.1.3.2 Analysis of phage vector fd-g9m**

The Ki-4(scFv) cDNA encoding the CD30 receptor specific single chain antibody Ki-4 (Barth et al. 2000) was cloned as a fusion upstream of *genelX* in order to verify the functionality of the phage vector fd-g9m (Fig.III-7; Fig.III-8). Ki-4(scFv) DNA was amplified using the
prokaryotic expression vector pMT-Ki4-ETA (Barth et al. 2000) as a template and primers p-5’-pIXKi4 and p-3’-pIXKi4 (II.1.7.4; II.2.1.11.2). Suitable restriction sites were incorporated for subsequent cloning into the XhoI/Nhel-cloning site of geneIX.

**Fig.III-8: Cloning strategy for the construction of fd-g9Ki4.** Ki-4(scFv) DNA was amplified using the prokaryotic expression vector pMT-Ki4-ETA as a template (provided by Dr. M. K. Tur, Fraunhofer IME Aachen; Barth et al. 2000) and primer p-5’-pIXKi4 as well as primer p-3’-pIXKi4. p-5’-pIXKi4: forward primer for the amplification of Ki-4(scFv) open reading frame and for insertion of a XhoI-restriction site at the 5’-end of this sequence; p-3’-pIXKi4: backward primer for the amplification of Ki-4(scFv) open reading frame and for insertion of a Nhel-restriction site at the 3’-end of this sequence. Amplified Ki-4(scFv) DNA was cloned into XhoI/Nhel-digested fd-g9m phage vector (Fig.III-7). Sequencing analysis (II.2.1.10) was done using primers p-5’-BS2-SeqI and p-3’-BS2-SeqII (II.1.7.2).

The influence of the geneIX modification on the assembly efficiency of fd-g9m and fd-g9Ki4 phages was determined by phage titration (Fig.III-9).
Results and Discussion

<table>
<thead>
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<th>phages</th>
<th>assembly efficiency [cfu/ml]</th>
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<tr>
<td>fd-tet</td>
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</tr>
<tr>
<td>fd-g9m</td>
<td>$5.84 \times 10^{11}$</td>
</tr>
<tr>
<td>fd-g9Ki4</td>
<td>0</td>
</tr>
<tr>
<td>no phages</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig.III-9: Assembly efficiency of modified phage vectors. Assembly efficiencies of geneIX modified phage vectors were determined by a phage titration assay (II.2.2.3). The data were acquired in triplicate and the mean assembly efficiency (cfu/ml) was determined. Fd-tet: original phage vector (Zacher et al. 1980); fd-g9m: pIX-display phage vector (Fig.III-7); fd-g9Ki4: pIX-display phage vector containing Ki-4(scFv) DNA within the pIX-cloning site (Fig.III-8); no phages: E. coli TG1 incubated with 2xTY media without phages.

Contrary to all expectations, the modification of geneIX by inserting a G4S-linker sequence and a XhoI/NheI-restriction site behind the second triplet within phage vector fd-tet leads only to a 1000x decreased assembly efficiency (Fig.III-9) compared to fd-tet, which would be tolerable for a library construction possessing a sufficient diversity of about $10^{11}$.

Surprisingly, no phages could be produced at all after insertion of the Ki-4(scFv) cDNA (Barth et al. 2000) into the geneIX cloning site because fd-g9Ki4 (Fig.III-8) transformed E.coli TG1 bacteria could not be cultivated in liquid media. It was not possible to inoculate liquid media by picking these bacteria from plates. Nevertheless colony PCR (II.2.1.2.3) and sequencing analysis (II.2.1.10) confirmed the integrity of these fd-g9Ki4 transformed E. coli TG1 bacteria.

This lethal effect of expressed Ki-4(scFv)/pIX fusion proteins for the bacterial host could be explained by the observations of Model and Russel as well as by Webster, who claim that there is a delicate balance in the synthesis of modified phage proteins that allow phage production without seriously affecting bacterial growth (Model and Russel 1988, Webster 1996). Due to the fact that the Ki-4(scFv)/pIX fusion proteins did not cause a lethal effect when being encoded by a phagemid vector, there could be just a limitation in the size of the foreign protein or peptide being multivalently displayed on pIX coat protein. Nevertheless, Gao et al. (Gao et al. 2002) and the results shown in Fig.III-12 prove functional monovalent display of single chain antibody fragments on pIX coat protein using a phagemid vector, a lethal effect may occur when the same scFv/pIX-fusion proteins is encoded by a phage vector. The incorporation of the 30kDa scFv/pIX-fusion protein during the assembly process in the absence of wild type pIX coat proteins may lead to this lethal effect. Perhaps, the pl/pIX/pIV-channels are damaged within the host membrane due to the size of the five scFv/pIX-fusion proteins that have to be incorporated within one phage particle in comparison to the incorporation of just one of these fusion proteins together with four wild type pIX proteins in a phagemid system (Guy-Caffey and Webster 1993; Webster 1995; Kazmiercak et al. 1994; Linderoth et al. 1997). Thus the essential membrane potential could not be maintained causing the death of the bacterial host. This lethal effect will not occur by
multivalent display of small peptides because the display of the nine amino acids encoded by the G$_2$S-linker sequence and the XhoI/Nhel-restriction site on pIX coat protein only leads to a 1000-fold decrease of the assembly efficiency and no lethal effect was observed (fd-g9m; Fig.III-7; Fig.III-9). In conclusion, geneIX modification can be used for multivalent display of foreign peptides on the surface of filamentous bacteriophages and for the generation of peptide libraries encoding up to nine amino acid residues to provide a diversity of about $10^{11}$.

Summary of the results obtained with modified phage vectors:

- **GeneVI** modified phages showed six million times decreased assembly efficiency caused by the insertion of the G$_3$SG$_2$-linker sequence and the Sfil/NotI-restriction site.

- Modification of geneVI and insertion of the CD30R cDNA into its cloning site resulted in $4.47 \times 10^7$ infectious phages per ml all featuring the “correct” morphology, which would be suitable for construction of immunized protein libraries.

- Modification of geneIX by insertion of a G$_2$S-linker sequence and an XhoI/Nhel-restriction site downstream of its second triplet decreases the assembly efficiency 1000-fold, which would be suitable for construction of peptide libraries.

- Insertion of the Ki-4(scFv) DNA into the geneIX cloning site abolished the phage assembly due to a lethal effect on the bacterial host, which cannot be grown in liquid culture.

There might be an option for generating bispecific filamentous bacteriophages using a pVI/pIX-phage vector system but with a limitation in the size of corresponding libraries displayed on pVI coat protein. The construction of a pIII/pIX-phage vector based on for example phage vector fd-tet-DOG1 (Clackson et al. 1991) might be a better alternative. Phage vector fd-tet-DOG1 possesses the capability for multivalent display of foreign proteins on pIII coat protein and by insertion of DNA sequences downstream of the second triplet of geneIX encoding for small peptides which are no longer than nine amino acids it should be possible to generate bispecific phages. These bispecific phages could display a protein library fused to pIII coat protein and any peptide motif of interest on the opposite site of the phage as fusion to the pIX coat protein. Unfortunately, due to time constrains it was not possible to verify this hypothesis.

However, in addition to this first phage vector based strategy for the generation of bispecific filamentous bacteriophages a second strategy was developed based on a bispecific phagemid vector system. The cloning strategies and results are described in III.2.
III.2 Construction and analysis of bispecific phagemids

Due to the assembly problems of phages produced using modified phage vectors (III.1), a new strategy for the generation of bispecific bacteriophages was developed. A bispecific phagemid vector was generated based on the pHENHi phagemid vector (Peschen et al. 2004) a derivative of the well-characterized pHEN1 phagemid vector (Hoogenboom et al. 1991) containing an additional 6xHis-tag and a modified 218 poly-linker (II.1.4.2.2). This bispecific phagemid vector should allow the monovalent display of:

a. library proteins on pIII coat protein

b. proteins or peptides for immobilization on biotinylated surfaces or labelling as a fusion to the pIX coat protein

Coat protein pIII was chosen because of the assembly problems encountered by the pVI-display system (III.1), which might also be the case when using a phagemid vector. So far only Jespers (Jespers et al. 1995) published data on the pVI-display system. For the construction of full-length cDNA libraries, pIII can be used by adapting the Fos/Jun-system as described by Crameri and Suter (Crameri and Suter 1993).

For the integration of the second specificity the phage coat protein pIX was selected as the display system of choice, because pIX-display has been shown to be the better screening system compared to pVII (Gao et al. 2003; Endemann and Model 1995). The final bispecific phagemid vector pHEN3+9 (Fig.III-14) was generated by insertion of a complete second expression cassette containing geneIX downstream of the already existing one within the pHENHi phagemid vector (Peschen et al. 2004). This second expression cassette was designed in a way that it is compatible to the first one. Therefore, a new multi cloning site (MCS, Fig.III-14) was synthesized and fused to geneIX using a G4S-linker sequence in between. Transcription and translation of the additional expression cassette was regulated by a lac-promoter/operator and a ribosome binding site, according to the regulatory elements within the first expression cassette of phagemid vector pHENHi in order to achieve an equimolar expression level of the pIII- and pIX-fusion proteins. This vector design was chosen following the principle of the commercially available and well-characterized pETDuet™-1 vector (Novagen) generated for prokaryotic coexpression of two different target genes. This pETDuet™-1 vector also contains two different multi cloning sites, each of which is proceeded by the same regulatory elements, which in particular are two T7-promoters and lac-operators combined with the same ribosome binding site. The final bispecific pHEN3+9 phagemid (Fig.III-14) should allow the bacterial expression of pIII- and pIX-fusion proteins in parallel and thus the assembly of bispecific bacteriophages by incorporation of these
recombinant proteins into the phage particles after infection of the bacteria with a helperphage.

Due to the lethal effect observed on bacterial cells transformed with the fd-g9Ki4 phage vector (Fig.III-9), the integrity of the pIX-display system was investigated before assembling the bispecific phagemid vector pHEN3+9

### III.2.1 Construction and analysis of phagemid vector pHEN9

#### III.2.1.1 Construction of phagemid vector pHEN9

According to Gao et al., who is the only one publishing data on the pIX-display system (Gao et al. 1999; Gao et al. 2002a; Gao et al. 2002b; Gao et al. 2003) and in consideration of the experiences with the modified phage vectors (III.1), the pIX-system had to be investigated before being used for cloning the bispecific phagemid vector. Therefore, the pIX phagemid vector pHEN9 was generated by replacing geneIII with geneIX within the phagemid vector pHEN4II (Zhang et al. 2001). The cloning strategy for the construction of pHEN9 is shown in Fig.III-10.
Fig. III-10: Cloning strategy for the construction of pHEN9 phagemid vector. Gene III of pHEN4II phagemid vector (II.1.4.2.1) was replaced by gene IX in order to create pHEN9 phagemid vector. Phage gene IX was amplified by PCR (II.2.1.2.1) using fd-tet (Zacher et al. 1980) and primers p-5'-p9NotIa, p-5'-p9NotIb, and p-3'-p9EcoRI (II.1.7.5). p-5'-p9NotIa/b: forward primers for the amplification of phage gene IX open reading frame and for insertion of a NotI-restriction site and a c-myc tag at the 5’-end of this sequence; p-3'-p9EcoRI: backward primer for the amplification of phage gene IX open reading frame and for insertion of an EcoRI-restriction site at the 3’-end of this sequence. Amplified gene IX was cloned into NotI/EcoRI-digested pHEN4II phagemid vector (Zhang et al. 2001). Sequencing analysis was done using primers p-5’-pHENx and p-3’-pHENx (II.1.7.5).

### III.2.1.2 Analysis of phagemid vector pHEN9

In order to prove the functionality of the newly synthesized pIX-display system, the scFvCWPD2 cDNA encoding for the single chain antibody scFvCWPD2 (Peschen et al. 2004) was cloned into the pHEN9 phagemid vector. ScFvCWPD2 specifically binds to a cell wall protein extract of fusarium (CWP antigen) and was chosen because a well-established phage ELISA set-up was available. The cloning strategy of pHEN9-CWP is shown in Fig.III-11.
III Results and Discussion

Fig.III-11: Cloning strategy for the construction of pHEN9-CWP phagemid vector. The DNA sequence encoding for the single chain antibody scFvCWPD2 (Peschen et al. 2004), which binds specifically to fusarium cell wall proteins, was cloned into pHEN9 phagemid vector (Fig.III-10). Gene scFvCWPD2 and upstream pelB-signal sequence were excised from pHEN-CWP (Peschen et al. 2004) using HindIII/NotI-restriction sites and cloned into HindIII/NotI-digested phagemid vector pHEN9 (Fig.III-10). Sequencing analysis was done using primers p-5’-pHENx and p-3’-pHENx (II.1.7.5).

The binding capacity of the pHEN9-CWP phages (Fig.III-11) and the original pHEN-CWP phages (Peschen et al. 2004) to CWP antigen was determined by CWP phage ELISA (II.2.2.5.2). The results shown in Fig.III-12 allow the direct comparison of the pIII- and pIX-display system.
In order to investigate the functional display of scFvCWPD2 as a pIX-fusion using pHEN9-CWP a CWP phage ELISA (II.2.2.5.2) was performed. Binding activity of pHEN9-CWP phages to CWP antigen was determined in comparison to pHEN-CWP phages, displaying scFvCWP as a fusion to the pIII coat protein. Serial dilutions of the phages (stock = $10^{12}$ phages/ml) were applied to a microtiter plate coated with CWP antigen and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS (Roche) substrate was used and OD$_{405\text{nm}}$ was measured. Phages prepared by using pHENHi (II.1.4.2.2) phagemid vector were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD$_{405\text{nm}}$ was calculated and is shown above.

The results confirm the data of Gao et al. (Gao et al. 1999; Gao et al. 2002a; Gao et al. 2002b; Gao et al. 2003) and prove the integrity of the pIX-display system. However, pIX-displayed scFvCWP fragments showed weaker binding activity to the CWP antigen than the same antibody fragment fused to pIII coat protein. Nevertheless, geneIX was chosen for the construction of the bispecific phagemid vector pHEN3+9 (Fig.III-14).

### III.2.2 Construction of bispecific phagemid vector pHEN3+9

The bispecific phagemid vector pHEN3+9 was generated by inserting a second expression cassette encoding for geneIX into the pHENHi phagemid vector (Peschen et al. 2004) as described above. A SfiI/NotI-restriction site and the linker sequence 218 of phagemid vector pHEN9 (Fig.III-10) were replaced by a mUlti cloning site (MCS). The cloning strategy for the generation of pHEN9-Bi is shown in Fig.III-13.
Fig.III-13: Cloning strategy for the construction of intermediate pHEN9-Bi. p-5'-HindIII (II.1.7.6): forward primer for the amplification of amplicon A; p-3'-HEN39a and p-3'-HEN39b (II.1.7.6): backward primer for the amplification of amplicon A and for insertion of a XhoI/XbaI/EcoRI-restriction sites at the 3'-end of pelB leader sequence and for removal of the SfiI-restriction site; p-5'-HEN39a and p-5'-HEN39b (II.1.7.6): forward primer for the amplification of amplicon B and for insertion of a XhoI/XbaI/EcoRI/PstI/NcoI-restriction sites (MCS) at the 5'-end of the c-myc tag and for removal of the NotI-restriction site; p-3'-EcoRI (II.1.7.6): backward primer for the amplification of amplicon B. Amplicon A and B were assembled using SOE-PCR (II.2.1.2.2) and subsequently cloned into HindIII/EcoRI-digested phagemid vector pHEN9 (Fig.III-10). Sequencing analysis was done using primers p-5'-pHENx and p-3'-pHENx (II.1.7.5).

The newly designed expression cassette of pHEN9-Bi was used as template for the generation of the bispecific phagemid vector pHEN3+9 and the cloning strategy is shown in Fig.III-14.
III Results and Discussion

III.2.3 Analysis of bispecific phagemid vector pHEN3+9

III.2.3.1 Construction and analysis of pHEN3CWP+9

Firstly, the scFvCWPD2 DNA was cloned as fusion to geneIII into the bispecific phagemid vector pHEN3+9 (Fig.III-14), in order to confirm that the insertion of a second expression cassette (for geneIX-fusion) did not affect the functionality of the first one (for geneIII-fusion). The resulting pHEN3CWP+9 phages were analyzed by CWP phage ELISA (II.2.2.5.2). The cloning strategy of pHEN3WP+9 is shown in Fig.III-15.
Fig.III-15: Cloning strategy for the construction of pHEN3CWP+9 phagemid vector. Gene scFvCWPD2 encoding for the single chain antibody scFvCWPD2 (Peschen et al. 2004) was cloned into the bispecific phagemid vector pHEN3+9 (Fig.III-14). Gene scFvCWPD2 was excised from pHEN9-CWP (Fig.III-11) using the SfiI/NotI-restriction sites and cloned into SfiI/NotI-digested bispecific phagemid vector pHEN3+9 (Fig.III-14). Sequencing analysis was done using primers p-5’-biHEN3 and p-3’-biHEN3 (II.1.7.8).

The binding efficiency of the pHEN3CWP+9 phages (Fig.III-15) to CWP antigen was determined by CWP phage ELISA (II.2.2.5.2) and compared to pHEN-CWP phages (Peschen et al. 2004), displaying the scFv as a fusion to geneIII but lacking the second expression cassette. The results are shown in Fig.III-16.
III Results and Discussion

Fig. III-16: CWP phage ELISA with pHEN3CWP+9. In order to analyze if the insertion of the second expression cassette for construction of the bispecific phagemid vector pHEN3+9 affects the functionality of the first one, scFvCWPD2 gene was cloned into the first cassette and the binding activity of the corresponding phages was checked by CWP phage ELISA (II.2.5.2). Binding activity of pHEN3CWP+9 phages to CWP antigen was determined in comparison to pHEN-CWP phages, lacking the second expression cassette. A serial dilution of the phages (stock = 10^{12} phages/ml) were applied to microtiter plates coated with CWP antigen and specific binding phages were detected using a 1:2000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD_{405nm} was measured. pHENHi phages (II.1.4.2.2; Peschen et al. 2004) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD_{405nm} was calculated and is shown above.

No significant difference in the binding activity of pHEN3CWP+9 and pHEN-CWP phages was observed. Both phagemid vectors allow functional display of the scFvCWPD2 antibody fragment as fusion to phage coat protein pIII. Thus, insertion of the second expression cassette (for genelX-fusion) downstream of genelIII within the pHENHi phagemid vector does not affect the functionality of the scFv fused to genelIII.

III.2.3.2 Construction and analysis of pHEN3CWP+9Strep

The next step to reach the projects aim was the generation of a second binding moiety fused to pIX coat protein. Therefore, a protein or peptide motif had to be determined, suitable for immobilization on the biotinylated surface of the Protein Nanochip. Tetrameric streptavidin, the most commonly used biotin binding protein, cannot be used because phagemid vectors lead to monovalent display and the binding affinity of monomeric streptavidin is dramatically decreased in comparison to its tetrameric form. Recombinant monomeric avidin (Airenne et al. 1994; Airenne et al. 1999) might have been a suitable binding moiety but unfortunately the DNA sequence was not accessible and there was no time for repeating Airenne’s work.
Therefore, the Strep-tag® (IBA GmbH, Göttingen) was cloned as a fusion to geneIX within the bispecific phagemid pHEN3+9. The Strep-tag® is an eight amino acid streptavidin binding peptide and should allow the indirect immobilization of phages to the biotinylated chip surface via soluble tetrameric streptavidin as this consists of four binding domains. The Strep-tag® technology has been developed by IBA GmbH in Göttingen and provides a tool for expression, purification and detection of Strep-tag® fusion proteins. Recombinant streptavidin has been engineered by IBA GmbH and the resulting Strep-Tactin® possesses an optimized binding efficiency to Strep-tag® fusion proteins. Based on Strep-Tactin® IBA GmbH provides coated columns, cartridges, microtiter plates as well as magnetic beads for purification and detection of recombinant Strep-tag® proteins. This technology was adapted to filamentous bacteriophages in order to allow purification (II.2.2.4.1; Fig.III-32), detection by phage ELISA (II.2.2.5.3; Fig.III-30), and indirect immobilization on biotinylated surfaces (II.2.2.5.4; Fig.III-31). The adjustment of this technology to filamentous bacteriophages is described in detail in III.3. The results discussed in III.3 indicate that the Strep-tag® is a suitable peptide motif for the side-directed immobilization of phages when displayed as a fusion to geneIX. For the cloning of the Strep-tag® DNA into the bispecific phagemid pHEN3CWP+9 (Fig.III-15) the intermediate pHEN3+9Strep phagemid vector was generated. The two step cloning strategy was necessary because the scFvCWP DNA possesses an internal EcoRI-restriction site and EcoRI had to be used for subsequent cloning of the Strep-tag®/geneIX PCR product. Therefore, the tag-PCR product was cloned into the “empty” bispecific phagemid vector pHEN3+9 (Fig.III-17) followed by integration of the scFvCWPD2 DNA, which was excised from the pHEN3CWP+9 phagemid vector and cloned into the intermediate pHEN3+9Strep using the SfiI/NotI-restriction sites (Fig.III-18). The cloning strategy of the intermediate pHEN3+9Strep is shown in Fig.III-17 and the construction of the final bispecific phagemid vector in Fig.III-18.
Fig.III-17: Cloning strategy for the construction of intermediate pHEN3+9Strep. p-5'-HEN9Eco (II.1.7.7): forward primer for the amplification of amplicon A; p-3'-StreplXa and p-3'-StreplXb (II.1.7.9): backward primer for the amplification of amplicon A and for insertion of the Strep-tag® sequence at the 3’-end of the pelB leader sequence; p-5'-StreplX (II.1.7.9): forward primer for the amplification of amplicon B and for insertion of the Strep-tag® sequence at the 5’-end of the c-myc tag; p-3'-HEN9Eco (II.1.7.7): backward primer for the amplification of amplicon B. Amplicon A and B were assembled using SOE-PCR (II.2.1.2.2) and subsequently cloned into EcoRI-digested bispecific phagemid vector pHEN3+9 (Fig.III-14); Strep: DNA sequence encoding for the Strep-tag® (IBA GmbH). Sequencing analysis was done using primers p-5’-H39Seq (II.1.7.7), p-3’biHEN9 (II.1.7.9) and p-3’-pHENx (II.1.7.5).
Fig.III-18: Cloning strategy for the construction of pHEN3CWP+9Strep phagemid vector. Gene scFvCWPD2 encoding for the single chain antibody scFvCWPD2 (Peschen et al. 2004), was cloned into the intermediate phagemid vector pHEN3+9Strep (Fig.III-17). Gene scFvCWPD2 was excised from pHEN3CWP+9 (Fig.III-15) using the SfiI/NotI-restriction sites and cloned into SfiI/NotI-digested phagemid vector pHEN3+9Strep (Fig.III-17). Strep: DNA sequence encoding for the Strep-tag® (IBA GmbH). Sequencing analysis was done using primers p-5’-biHEN3 and p-3’-biHEN3 (II.1.7.8) as well as p-5’-H39Seq (II.1.7.7) and p-3’-biHEN9 (II.1.7.9).

The functional display of the Strep-tag® on pIX coat protein of bispecific pHEN3CWP+9Strep phages was confirmed by Strep-Tactin® phage ELISA (II.2.2.5.3) in direct comparison to the pHEN9-Strep (Fig.III-19). An additional CWP phage ELISA (II.2.2.5.2) was performed to confirm these results, and exclude a negative effect on the scFvCWPD2/pIII fusion protein expression by insertion of the Strep-tag® into the second expression cassette. The results are shown in Fig.III-20.
III Results and Discussion

Fig. III-19: Strep-Tactin® phage ELISA with pHEN3CWP+9Strep. In order to confirm the functional display of the Strep-tag® on pIX coat protein of bispecific pHEN3CWP+9Strep phages (Fig. III-18) a Strep-Tactin® phage ELISA (II.2.2.5.3) was performed. Binding activity of pHEN3CWP+9Strep phages to Strep-Tactin® coated microtiter plates (IBA GmbH) was determined in comparison to pHEN9-Strep (Fig. III-28) phages. A serial dilution of the phages (stock = 10^14 phages/ml) were applied to the Strep-Tactin® coated microtiter plates and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD\textsubscript{405nm} was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD\textsubscript{405nm} was calculated and is shown above.

Fig. III-20: CWP phage ELISA with pHEN3CWP+9Strep. In order to verify the functional display of the scFvCWPD2 on pIII coat protein of bispecific pHEN3CWP+9Strep phages a CWP phage ELISA (II.2.2.5.2) was performed. Binding activity of pHEN3CWP+9 phages to CWP antigen was determined in comparison to pHEN-CWP phages. A serial dilution of the phages (stock = 10^12 phages/ml) were applied to microtiter plates coated with CWP antigen and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD\textsubscript{405nm} was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD\textsubscript{405nm} was calculated and is shown above.
Insertion of the Strep-tag® into the second expression cassette of phagemid vector pHEN3CWP+9Strep did not alter the binding activity of the scFvCWPD2/pIII-fusion proteins encoded by the first expression cassette. In addition binding activity of pHEN3CWP+9Strep phages to Strep-Tactin® was shown proving the functionality of the second expression cassette. These results clearly demonstrated that the bispecific phagemid vector pHEN3CWP+9Strep allows the production of phages displaying either functional scFvCWPD2 antibody fragments on pIII coat protein or the Strep-tag® on pIX coat protein. The integrity of both expression cassettes of the pHEN3CWP+9Strep phagemid vector was proven. However, the simultaneous display of both proteins cannot be verified employing this set-up.

III.2.3.3 Analysis of bispecific filamentous bacteriophages

III.2.3.3.1 Analysis of bispecific phages by Phage ELISA

Employing the phagemid system for the generation of bispecific phages requires the usage of a helperphage for the initiation of the assembly process. This leads to the competition of wild type pIII and pIX coat proteins encoded by the helperphage genome with the fusion proteins encoded on the bispecific phagemid vector for the assembly into the final phage particles. Thus, over 90% of rescued phages do not display the foreign proteins at all and in addition the display is monovalent (Marks et al. 1992; O'Connell et al. 2002). In conclusion less than 10% of the produced pHEN3CWP+9Strep phages display either the scFvCWPD2 antibody fragment or the Strep-tag® on their surface and even less phages will display both foreign proteins and are therefore bispecific. The percentage of bispecific phages within the rescued phage population had to be investigated in order to prove the integrity of the phagemid vector pHEN3+9 as a suitable tool for the Protein Nanochip.

Therefore, additional phage ELISA set ups were tested:

I. Modified Strep-Tactin® phage ELISA (II.2.2.5.3)

Strep-Tactin® binding phages were detected using a mouse anti-6xHis antibody (Qiagen; II.1.3) and an HRP-conjugated goat anti mouse antibody (II.1.3) instead of the HRP-conjugated mouse anti-M13 antibody (II.1.3). The mouse anti-6xHis antibody was used in order to determine the display of 6xHis-tagged scFvCWPD2 antibody fragment on the surface of Strep-Tactin® binding phages. However, after applying the ABTS substrate, it was not possible to obtain specific signals measuring the OD405nm. Moreover, Strep-Tactin® Phage ELISA set ups using different anti-6xHis antibodies and different HRP-conjugated goat anti-mouse antibodies or using directly HRP-conjugated anti 6xHis antibodies failed.
II. Modified CWP Phage ELISA (II.2.2.5.2)

It was attempted to reveal CWP-binding phages by Strep-tag® specific antibodies from IBA GmbH (II.1.3) as well as from Qiagen but no binding could be detected. Finally, recombinant HRP-conjugated Strep-Tactin® (IBA GmbH) was used for detection of CWP-binding phages and the results obtained are shown in Fig.III-21.

![Graph showing CWP phage ELISA results](image)

**Fig.III-21: CWP phage ELISA with pHEN3CWP+9Strep for detection of bispecificity.** In order to determine bispecific pHEN3CWP+9Strep phages a CWP phage ELISA (II.2.2.5.2) was performed using horseradish peroxidase (HRP) conjugated Strep-Tactin® (IBA GmbH) instead of anti-M13 antibody for detection of CWP antigen binding phages. Binding activity of pHEN3CWP+9Strep phages to CWP antigen was determined in comparison to pHEN-CWP phages not displaying the Strep-tag®. Serial dilution of each phages (stock = 10^{14} phages/ml) were applied to microtiter plates coated with CWP antigen and specific binding phages were detected using 1:2000 diluted HRP-conjugated Strep-Tactin® (IBA GmbH). For detection ABTS substrate (Roche Diagnostics) was applied and OD_{405nm} was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD_{405nm} was calculated and is shown above.

The usage of recombinant HRP-conjugated Strep-Tactin® caused high background signals within this modified CWP phage ELISA. The obvious reason for this background signal was the binding activity of Strep-Tactin® to fusarium biotin that is a natural component of the CWP extract which was coated on the microtiter plates. Despite this high background signal, a positive tendency was observed for the concentrated pHEN3CWP+9Strep phages in comparison to the control phages. Therefore, the ELISA was repeated by adding soluble unconjugated streptavidin (Roche Diagnostics) to the blocking solution in order to saturate the coated fusarium biotin and thus reduce the unspecific background signal.

The results obtained with this ELISA set up are shown in Fig.III-22.
III Results and Discussion

Fig.III-22: CWP phage ELISA with pHEN3CWP+9Strep for detection of bispecificity. In order to determine bispecific pHEN3CWP+9Strep phages a CWP phage ELISA (II.2.2.5.2) was performed using horseradish peroxidase (HRP) conjugated Strep-Tactin® (IBA GmbH) for detection of CWP antigen binding phages. For decrease of unspecific binding of HRP-conjugated Strep-Tactin®, soluble recombinant streptavidin (Roche Diagnostics) were added 1:5,000 diluted to the blocking solution. Binding activity of pHEN3CWP+9Strep phages to CWP antigen was determined in comparison to pHEN-CWP phages not displaying the Strep-tag®. A serial dilution of the phages (stock = 10^{14} phages/ml) were applied to microtiter plates coated with CWP antigen and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated Strep-Tactin® (IBA GmbH). For detection ABTS substrate (Roche diagnostics) was used and OD_{405nm} was measured. pHENHi phages (II.1.4.2.2; Peschen et al. 2004) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD_{405nm} was calculated and is shown above.

The corresponding results obtained (Fig.III-22) confirmed the assumption that fusarium biotin caused the high background signal because the unspecific signal was significantly decreased. Again the positive tendency of pHEN3CWP+9Strep phages was visible and even became stronger, giving a first hint for “real” bispecific phages. Nevertheless, the extinction of the pHEN3CWP+9Strep phages did not provide significant positive signals in comparison to the control phages. Further supporting these results another strategy for detection of bispecific phages was developed

III. CWP Phage ELISA using Strep-Tactin® purified pHEN3CWP+9Strep phages

In order to verify this positive tendency another strategy for detection of bispecific phages was developed. As described above, only a small percentage of the prepared pHEN3CWP+9Strep phages are theoretically bispecific indicating the need for an enrichment of these phages before applying them to the microtiter plates and thus perhaps allow reaching the detection limit. Therefore, a novel purification method was established for the enrichment of bispecific phages. As described in II.2.2.4.1 and shown in Fig.III-32 Strep-
Tactin® cartridges (IBA GmbH) provide a suitable tool for the purification of Strep-tag® displaying phages. This technology was used to enrich bispecific phages by purifying the Strep-tag® displaying ones and removing the phages only displaying the single chain Fv antibody or none of the foreign proteins. Finally, binding activity to the CWP antigen of these Strep-Tactin® affinity purified pHEN3CWP+9Strep phages was verified by CWP phage ELISA (Fig.III-23).

Fig.III-23: CWP phage ELISA with Strep-Tactin® purified pHEN3CWP+9Strep for detection of bispecificity. In order to check the bispecificity of the pHEN3CWP+9Strep phages a combinatorial assay was created. Freshly prepared phages (Maxi Phage Preparation; II.2.2.2) were purified (II.2.2.4.1) using Strep-Tactin® cartridges (IBA GmbH) and subsequently a CWP phage ELISA (II.2.2.5.2) was performed using the unpurified and the purified Strep-tag® displaying phages. Binding activity of purified and unpurified pHEN3CWP+9Strep phages to CWP antigen was determined in comparison to purified and unpurified pHEN-CWP (Peschen et al. 2004) and pHEN-Strep (Fig.III-29) phages. A serial dilution of the phages (stock = 10^{12} phages/ml) were applied to microtiter plates coated with CWP antigen and specific binding phages were detected using a 1:2000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS (Roche) substrate was used and OD_{405nm} was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD_{405nm} was calculated and is shown above.

Strep-Tactin® affinity purified pHEN3CWP+9Strep phages showed significant binding activity to the CWP antigen compared to Strep-Tactin® purified pHEN-Strep (Fig.III-29) phages not displaying the scFvCWPDP2 antibody fragment. Thus the presence of “true” bispecific phages displaying the single chain antibody fragment and the Strep-tag® could be confirmed. In addition it was clearly demonstrated that only Strep-tag® displaying phages were specifically purified using Strep-Tactin® cartridges, because unpurified pHEN-CWP phages lead to high
extinction signals whereas no binding activity to CWP antigen could be observed with Strep-Tactin® purified pHEN-CWP phages.

This combinatorial assay provides the first evidence for the integrity of the bispecific phagemid vector pHEN3+9 to be a suitable tool for the envisaged application, which is the immobilization of protein libraries on the surface of the Protein Nanochip.

**III.2.3.3.2 Analysis of bispecific phages by immunogold assay and EM**

The phage ELISA results were confirmed by electron microscopical analysis performing an immunogold assay. According to the phage ELISA analysis, several set ups of the immunogold assay had to be investigated to determine the optimal set up. Finally, pHEN3CWP+9Strep phages (Maxi prep; II.2.2.2) were affinity purified using Strep-Tactin® cartridges (II.2.2.4.1) and immobilized on Ni-grids (II.2.2.6.1) for subsequent immunological detection of the 6xHis-tagged scFvCWPD2 fragment and the Strep-tag® displayed on the phage surface. Parallel detection of both foreign proteins on the surface of the immobilized phages was tried to be achieved by incubation with two different first antibodies and two different secondary gold-conjugated antibodies (II.2.2.6.2; first set up). A Strep-tag® specific mouse antibody (II.1.3) and a polyclonal rabbit anti-6xHis antibody (II.1.3) were chosen for the first incubation step and for visualization of these first antibodies, a 15nm gold-conjugated goat anti-mouse antibody and a 10nm gold-conjugated goat anti-rabbit antibody (II.1.3) were used. The results of the subsequent electron microscopical analysis of these samples are shown in Fig.III-24 and Fig.III-25.
Fig. III-24: Immunogold based analysis of bispecific pHEN3CWP+9Strep phages:

pHEN3CWP+9Strep phages (Fig. III-18; II.2.2.2) were purified via Strep-Tactin® cartridge (II.2.2.4.1), immobilized on Ni-grids (II.2.2.6.1) for subsequent immunological detection with gold-labelled antibodies (II.2.2.6.2, first set up) and analyzed by electron microscopy (II.2.2.6). Detection and visualization of the His6-tagged scFvCWPD2 and the Strep-tag® on the surface of the pHEN3CWP+9Strep phages was realized using two different primary antibodies. A polyclonal rabbit anti-6xHis antibody (II.1.3) was used for detection of the 6xHis-tagged scFvCWPD2 displayed on phage pIII coat protein and a monoclonal Strep-tag® specific mouse antibody (II.1.3) was used for detection of the Strep-tag® displayed on phage pIX coat protein. The two primary antibodies were detected by using two different secondary, gold-labelled antibodies. A 10nm gold-conjugated goat anti-rabbit IgG antibody and 15nm gold-conjugated goat anti-mouse IgG antibody (II.1.3) were used, allowing the specific detection of the His6-tagged scFvCWPD2 and the Strep-tag® on the phage surface in parallel. The following magnifications were used in electron microscopy: A: 130,000x; B: 80,000x; C: 100,000x.
Fig. III-25: Immunogold based analysis of bispecific pHEN3CWP+9Strep phages: pHEN3CWP+9Strep phages (Fig.III-18; II.2.2.2) were purified via Strep-Tactin® cartridge (II.2.2.4.1), immobilized on Ni-grids (II.2.2.6.1) for subsequent immunological detection with gold-labelled antibodies (II.2.2.6.2, first set up) and analyzed by electron microscopy (II.2.2.6). Detection and visualization of the His6-tagged scFvCWPD2 and the Strep-tag® on the surface of the pHEN3CWP+9Strep phages was realized using two different primary antibodies. A polyclonal rabbit anti-6xHis antibody (II.1.3) was used for detection of the 6xHis-tagged scFvCWPD2 displayed on phage pIII coat protein and a monoclonal Strep-tag® specific mouse antibody (II.1.3) was used for detection of the Strep-tag® displayed on phage pIX coat protein. The two primary antibodies were detected by using two different secondary, gold-labelled antibodies. A 10nm gold-conjugated goat anti-rabbit IgG antibody and 15nm gold-conjugated goat anti-mouse IgG antibody (II.1.3) were used, allowing the specific detection of the His6-tagged scFvCWPD2 and the Strep-tag® on the phage surface in parallel. The following magnifications were used in electron microscopy: A: 100,000x; B: 80,000x; C: 130,000x.
The electron microscopical pictures presented in Fig.III-24 and Fig.III-25 show a selection of specifically gold-decorated pHEN3CWP+9Strep phages. The results of this electron microscopical analysis clearly demonstrate the functional display of either the scFvCWPD2 antibody fragment or the Strep-tag®. The six phages depicted in Fig.III-24 and Fig.III-25 were either decorated with 10nm or with 15nm gold particles and in addition to these selected ones, several equally labelled phages have been determined. The immunological gold-labelling of all phages was focussed at the display area of the recombinant proteins and only observed on their tip and none of the analyzed phages show gold-labelling on their middle portion. These observations confirm the model of the structure of filamentous bacteriophages shown in Fig.I-1 and prove that pIII and pIX coat proteins are incorporated at the tip of each phage.

In order to facilitate the separation between 10nm and 15nm gold particles, phages were shown separately. Fig.III-24 shows three different pHEN3CWP+9Strep phages decorated with 10nm gold-conjugated antibodies at their tip, proving the functional display of the 6xHis-tagged scFvCWPD2 antibody fragment on pIII coat protein. To facilitate the distinction between 10nm and 15nm gold particles, picture C of Fig.III-24 shows a decorated phage in close proximity to unspecifically adsorbed 15nm gold-conjugated antibodies, clearly demonstrating that the bacteriophage is decorated with 10nm gold particles. All phages shown in Fig.III-25 were decorated with 15nm gold-conjugated antibodies, proving the functional display of the Strep-tag® on pIX coat protein. Again one picture (picture C) shows a decorated phage in close proximity to unspecifically adsorbed gold-conjugated antibodies but this time the unspecific adsorbed antibodies are conjugated with 10nm gold particles and the bacteriophages shown are decorated with 15nm gold-conjugated antibodies.

Interestingly, more 10nm gold particles per phage tip could be observed in comparison to the 15nm gold particles. Whereas up to seven 10nm gold particles were observed to bind to one phage tip, just three copies of 15nm gold particles were maximum found to bind to one tip. A reason for this phenomenon might be the higher flexibility of the pIII-fusion proteins and thus a better accessibility of the 6xHis-tagged scFvCWPD2 due to the length of the pIII coat protein in comparison to pIX. Phage pIII is a 42.5kD protein and pIX is a 3.6kD protein. An additional reason might be a low affinity of the Strep-tag® specific antibody (IBA GmbH, Göttingen) that was already observed by the results of the modified CWP Phage ELISA set up. The fact, that only one in a hundred phages was specifically decorated with 15nm gold particles additionally confirms this hypothesis. Theoretically all phages should have been decorated at least with one 15nm gold-conjugated antibody because the phages were affinity purified via Strep-Tactin® cartridges and thus should all display the Strep-tag® on their surface. These observations leaded to the development of another detection set up for this
immunogold assay, which finally allows the visualization of a "real" bispecific filamentous bacteriophage. Again Strep-Tactin® (IBA GmbH) was chosen for the detection of the Strep-tag® displaying phages but for the immunogold assay a HRP-conjugated version was used. These HRP-conjugated Strep-Tactin® molecules were visualized by applying a 10nm gold-conjugated goat anti-HRP antibody (II.1.3). For detection of the 6xHis-tagged scFvCWPD2 antibody fragments on the other side of the phages a monoclonal mouse anti-penta-His antibody (II.1.3) and a 15nm gold-conjugated goat anti-mouse antibody (II.1.3) were utilized. In order to avoid unspecific binding of the HRP-conjugated Strep-Tactin® to hydroxybiotin that had to be used for competitive elution of Strep-tag® phages from the Strep-Tactin® cartridges, an additional purification method was developed for the enrichment of bispecific phage particles. Ni-NTA cartridges (IBA GmbH) were used instead of the Strep-Tactin® ones and the protocols (II.2.2.4.2) were adapted for the purification of 6xHis-tag displaying phages and thus for enrichment of bispecific phages. These Ni-NTA purified pHEN3CWP+9Strep bacteriophages were immobilized on Ni-grids and analyzed using the described second set up of the immunogold assay (II.2.2.6.2; second set up) and electron microscopy. The results of the electron microscopical analysis are shown in Fig.III-26.

Fig.III-26: Immunogold based analysis of bispecific pHEN3CWP+9Strep phages:
PHEN3CWP+9Strep phages (Fig.III-18; II.2.2.2) were purified via Ni-NTA cartridge (II.2.2.4.2), immobilized on Ni-grids (II.2.2.6.1) for subsequent immunological detection with gold-labelled antibodies (II.2.2.6.2) and analyzed by electron microscopy (II.2.2.6). Detection and visualization of the 6xHis-tagged scFvCWPD2 and the Strep-tag® on the surface of the pHEN3CWP+9Strep phages was realized using two different primary binding proteins. A monoclonal mouse anti-penta-His antibody (II.1.3) was used for detection of the 6xHis-tagged scFvCWPD2 displayed on phage pIII coat protein and horseradish peroxidase (HRP) conjugated Strep-Tactin® (IBA GmbH) was used for detection of the Strep-tag® displayed on phage pIX coat protein. The two primary binding molecules were detected by using two different secondary, gold-labelled antibodies. A 15nm gold-conjugated goat anti-mouse IgG antibody (II.1.3) and a 10nm gold-conjugated goat anti-HRP antibody (II.1.3) were used, allowing the specific detection of the 6xHis-tagged scFvCWPD2 and the Strep-tag® on the phage surface in parallel. Magnification of 130,000x.
The electron micrograph presented in Fig.III-26 shows a bispecific pHEN3CWP+9Strep phage decorated with 10nm and with 15nm gold-conjugated antibodies. This picture clearly demonstrates bispecificity because on the left side 10nm gold-conjugated antibodies visualized the Strep-tag® fused to geneIX and on the right side 15nm gold-conjugated antibodies visualized the scFvCWPD2 fused to geneIII.

These results prove the integrity of the bispecific phagemid vector pHEN3+9 (Fig.III-14) as a suitable tool for the realization of the Protein Nanochip. This phagemid vector allows the side-directed immobilization of protein libraries by displaying a suitable peptide motif (e.g. Strep-tag®) on the pIX coat protein and simultaneously displaying functional library proteins (e.g. scFv antibody fragments) fused to pIII coat protein at the opposite side of the filamentous bacteriophages. These results fulfilled the main milestones of the research project.

With the establishment of the bispecific phagemid vector pHEN3CWP+9Strep and the proven generation of bispecific phages simultaneously displaying a functional scFv antibody on pIII and the Strep-tag® on the opposite side on pIX, immobilization of all kind of protein libraries on biotinylated or Strep-Tactin® coated surfaces becomes feasible. The functional display of the scFv antibody on the surface of these bispecific filamentous bacteriophages indicates that all other classes of proteins, as described by Smith and Petrenko (1997), could also be functionally displayed using the bispecific phagemid vector pHEN3+9. In conclusion, the immobilization of all kind of protein libraries, like genomic libraries (Jacobsson et al. 1995), peptide libraries (Cwirla et al. 1990), different kinds of antibody libraries (Hoogenboom et al. 1991; Griffiths 1993; Winter et al. 1994), cDNA libraries (Crameri et al.1994; Crameri and Blaser 1996) and even full-length cDNA libraries using the Fos/Jun-system (Crameri and Suter, 1993; Palzkill et al. 1998) became possible and might be adapted to automated screening systems as e.g. the envisaged Protein Nanochip (Fig.I-9).

Based on currently available literature these data confirm for first time that a bispecific filamentous bacteriophage was generated simultaneously displaying two different foreign proteins on its pIII and pIX coat protein. However, in August 2004 Chen et al. also published data describing a bispecific filamentous bacteriophage displaying an alpha integrin-targeting motif as a fusion to the pIII coat protein and a streptavidin binding peptide fused to the major pVIII coat protein (Chen et al. 2004). They did not use a phagemid vector for the construction of this bispecific vector system but a hybrid phage vector (Perham et al. 1995) or type-88 phage vector (Zhong et al. 1994; Roberts et al. 1996; Bonnycastle et al. 1996), which had been used previously for displaying peptides longer than eight amino acids on the major coat protein pVIII. All these phage vectors are derivatives of the f88-4 phage vector (Zhong et al. 1994) bearing two copies of geneVIII, encoding two different types of pVIII proteins, the
original wild type pVIII protein and a pVIII-fusion protein encoded by an additional recombinant geneVIII expression cassette inserted into the intergenic region of the phage vector. The resulting hybrid phages are mosaic and their coat is comprised both of wild type pVIII proteins and recombinant pVIII-fusion proteins. This provided pVIII fusion with larger foreign peptide sequences (up to 20mers) to be displayed on the phage surface, even though the fusion protein itself cannot support phage assembly. Although only 1% of the incorporated pVIII proteins are recombinant pVIII-fusion products it was possible to achieve a multivalent peptide display on the middle portion of the phage. Chen et al. combined a derivative of this f88-4 vector with a derivative of the fd-tet phage vector (Zacher et al. 1980) possessing a cloning site between geneIII signal sequence and geneIII in order to generate a kind of bispecific phage vector, which they call chimeric phage vector (Chen et al. 2004). Next to their specific therapeutic application, it should be possible to generate phages multivalently displaying library proteins on pIII coat protein at the tip and in parallel displaying peptides (up to 20mers) on 1% of the major coat protein pVIII on the middle portion of the phage. This kind of phages are not suitable for screening protein libraries via standard phage display, because the multivalent pIII-display leads to highly decreased infectivity of the phages and thus complicates the panning procedure. In addition, this chimeric phage vector cannot be used for the Protein Nanochip set up, although circumventing the panning procedure because the second specificity can only be displayed on the major coat protein pVIII and thus will not allow the side-directed immobilization of protein libraries.

In addition, Chen’s results confirm the phagemid vector based strategy for the generation of bispecific filamentous bacteriophages allowing simultaneously display of two different proteins on pIII and pIX coat protein. The application for the set up described by Chen’s publication (Chen et al. 2004) shows an approach for finding novel carriers, which are targeting tumor cells and thus allow the specific delivery of therapeutics proteins. This interesting research project was supported by grants from the National Institutes of Health, the National Cancer Institute and the Department of Defence and some of the renowned scientists in the field of phage display worked on this project and George P. Smith the inventor of the original phage display technology was scientific adviser. Even these brilliant scientists did not provide a novel phage vector that is modified within the encoding regions of the phage genome, indicating that indeed it is very challenging to modify for example the original geneVI or geneIX within the phage genome and thus confirm the phagemid vector based strategy for the generation of bispecific phages allowing the display of two different foreign protein on pIII and pIX coat protein. Due to the project objective it was essential to combine exactly this pIII- and pIX-display system in order to allow the side-directed
immobilization and therefore it is the pHEN3+9 phagemid vector making the realization of the Protein Nanochip possible at all.

III.3 Immobilization of phages using *Strep*-tag\textsuperscript{®} technology

III.3.1 Adjustment of the *Strep*-tag\textsuperscript{®} technology to phages

Side-directed immobilization of phage libraries on the surface of the Protein Nanochip (Fig.1-9) was one research objective of this doctoral thesis. Due to the fact that the surface of the Protein Nanochip is biotinylated, determined by the technical research partner, a protein or peptide had to be found possessing specific binding activity to biotin when displayed on filamentous bacteriophage. Commonly used biotin-binding proteins are avidin or streptavidin, both tetrameric molecules with four biotin-binding domains per molecule. Native streptavidin is a prokaryotically expressed protein and therefore the better display motif for the envisaged application in comparison to the eukaryotically expressed avidin. But for functional display of the streptavidin molecule on the surface of filamentous bacteriophage a multivalent display of at least four streptavidin monomers is necessary to form a functional tetrameric streptavidin molecule. Unfortunately, it was not possible to achieve this multivalent display on pIX coat protein within the time of this thesis. A phagemid vector system had to be used for the generation of bispecific filamentous bacteriophages and the immobilization of protein libraries on the surface of the Protein Nanochip. Therefore, a monomeric biotin-binding protein or peptide had to be found suitable for functional display on the phage coat using this phagemid vector system. Firstly, recombinant monomeric avidin (Airenne *et al.* 1994; Airenne *et al.* 1999) was thought to be an appropriate protein because this engineered avidin is functionally expressed in prokaryotes and shows good biotin-binding capabilities even in the monomeric form (Laitinen *et al.* 2003). Unfortunately, it was not possible to get access to the corresponding DNA sequence and due to a lack of time Airenne’s work was not repeated. Thus, the so called *Strep*-tag\textsuperscript{®} (IBA GmbH) was chosen for the side-directed immobilization of bispecific phages on the biotinylated surface of the Protein Nanochip. The *Strep*-tag\textsuperscript{®} is an eight amino acid peptide binding specifically to streptavidin or its engineered form *Strep*-Tactin\textsuperscript{®} and had been chosen as a suitable peptide motif for indirect immobilization of the corresponding phages to the chip surface. *Strep*-tag\textsuperscript{®} displaying phages should be able to bind soluble tetrameric *Strep*-Tactin\textsuperscript{®}, which in parallel is able to bind biotinylated surfaces by using one of its other three biotin binding domains. The *Strep*-tag\textsuperscript{®} technology has been developed by IBA GmbH in Göttingen and provides a tool for expression, purification and detection of *Strep*-tag\textsuperscript{®} fusion proteins. Recombinant streptavidin has been engineered by
III Results and Discussion

IBA GmbH and the resulting Strep-Tactin® possesses an optimized binding capability to Strep-tag® fusion proteins. Based on Strep-Tactin® the IBA GmbH provides coated columns, cartridges, microtiter plates as well as magnetic beads for purification and detection of recombinant Strep-tag® proteins.

The DNA and amino acid sequence of the Strep-tag® is shown in Fig.III-27.

![Fig.III-27: DNA and amino acid sequence of the Strep-tag® (IBA GmbH, Göttingen): The DNA sequence of the eight amino acid Strep-tag® is shown in the codon view. Below the DNA sequence the corresponding amino acid sequence of the Strep-tag® is shown.]

III.3.1.1 Generation of Strep-tag® phagemid vectors

This Strep-tag® technology was adapted to the phagemid vector system in order to allow the side-directed immobilization of the phages to biotinylated surfaces. Therefore, the Strep-tag® DNA sequence was cloned into the phagemid vector pHENHi (Peschen et al. 2004) as a fusion to geneIIII and into pHEN9 (Fig.III-10) as a fusion to geneIX. Subsequently, the streptavidin- and Strep-Tactin®-binding efficiency of the resulting phages was evaluated. The cloning strategies for the construction of the phagemid vectors pHEN-Strep and pHEN9-Strep are shown in Fig.III-28 and Fig.III-29. These two cloning strategies were developed to determine differences in the binding properties of the Strep-tag® when being displayed either on pIII- or pIX coat protein.
Fig.III-28: Cloning strategy for the construction of phagemid vector pHEN9-Strep. p-5'-HindIII (II.1.7.6): forward primers for the amplification of pelB signal sequence using phagemid vector pHEN9 (Fig.III-10) as template; p-3'-StrepI and p-3'-StrepII (II.1.7.10): backward primers for the amplification of pelB signal sequence and for insertion of the Strep-tag\textsuperscript{®} (Strep) DNA sequence and a NotI-restriction site to the 3’-end of pelB signal sequence. Amplified pelB-Strep\textsuperscript{®} sequence was cloned into HindIII/NotI-digested phagemid vector pHEN9 (Fig.III-10). For sequencing primers refer to Fig.III-29.

Fig.III-29: Cloning strategy for the construction of phagemid vector pHEN-Strep. C-myc tag, 6xHis tag and geneIII were cloned into the phagemid vector pHENH9-Strep (Fig.III-28). C-myc tag, 6xHis tag and genelll were excised from pHENHi (II.1.4.2.2) using the NotI/EcoRI-restriction sites and cloned into NotI/EcoRI-digested phagemid vector pHEN9-Strep (Fig.III-28). Sequencing analysis of pHEN9-Strep (Fig.III-28) and pHEN-Strep was done using primers p-5’HENSeq (II.1.7.10) and p-3’pHENx.
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III.3.1.2 Analysis of Strep-tag® displaying phages

Functional display of the Strep-tag® on pIII- and pIX coat protein was analyzed by Streptavidin and Strep-Tactin® Phage ELISA (II.2.2.5.3)

![Graphs showing binding of streptavidin and Strep-Tactin® phages](image)

**Fig.III-30: Streptavidin and Strep-Tactin® phage ELISA with pHEN-Strep and pHEN9-Strep phages.** In order to check the functional display of the Strep-tag® on pIII and pIX phage coat protein a Streptavidin (A) and a Strep-Tactin® (B) phage ELISA (II.2.2.5.3) was performed using pHEN-Strep (Fig.III-29) and pHEN9-Strep (Fig.III-28) phages. Binding activity of these phages to Streptavidin (Roche Diagnostics) and Strep-Tactin® (IBA GmbH) coated microtiter plates was determined. A serial dilution of both phages (stock = $10^{12}$ phages/ml) were applied to the coated microtiter plates and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD$_{405nm}$ was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD$_{405nm}$ was calculated and is shown above.

Surprisingly, the binding of the phages to streptavidin and Strep-Tactin® was only sustained when the Strep-Tag® was fused to the pIII phage coat protein. The same Strep-Tag® displayed on pIX phage coat protein looses the binding capability to recombinant streptavidin, whereas the Strep-Tactin® binding activity is not affected. Therefore, Strep-Tactin® coated surfaces have to be employed to achieve side-directed immobilization of...
bispecific phages on biochips or other matrices. However the projects aim was the immobilization on biotinylated surfaces and therefore the indirect binding activity of the pHEN9-Strep phages to biotin via soluble Streptavidin and Strep-Tactin® was determined. The results of this Biotin Phage ELISA (II.2.2.5.4.) are shown in Fig.III-31.

![Biotin phage ELISA with pHEN9-Strep](image)

**Fig.III-31: Biotin phage ELISA with pHEN9-Strep.** In order to check indirect binding activity of pHEN9-Strep (Fig.III-28) phages to biotinylated surfaces, a Biotin phage ELISA (II.2.2.5.4.) was performed. Indirect binding activity of the Strep-tag® displaying phages to biotinylated microtiter plates (Pierce/Perbio; Bonn) was determined using soluble Streptavidin (Roche Diagnostics) and Strep-Tactin® (IBA GmbH) as a linker molecule. Soluble, unconjugated Streptavidin (A) or Strep-Tactin® (B) was applied to the biotinylated microtiter plate and binding activity of the pHEN9-Strep phages to bound Streptavidin or Strep-Tactin® was determined. A serial dilution of the phages (stock = 10^{12} phages/ml) were applied to the microtiter plates and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD_{405nm} was measured. pHENHi phages (II.1.4.2.2) were used to determine unspecific binding activity. The data were acquired in triplicate and the mean OD_{405nm} was calculated and is shown above.

Side-directed immobilization to biotinylated surfaces of phages displaying the Strep-tag® on pIX coat protein was possible using soluble Strep-Tactin® (IBA GmbH) as a linker molecule. Again, no binding activity to recombinant streptavidin (Roche) was be observed using
phages displaying the *Strep*-tag® on pIX. Perhaps, again it's the higher flexibility of the longer pIII coat protein that facilitates binding of the displayed *Strep*-tag® also to its weaker binding partner streptavidin and not only to *Strep*-Tactin®. However, it could be clearly demonstrated that the *Strep*-tag® provides the tool for side-directed immobilization of bispecific filamentous bacteriophages. Considering these results and the functional display of the *Strep*-tag® on pIX coat protein of pHEN3CWP+9Strep phages (Fig.III-26), an immobilization of protein libraries on the surface of the Protein Nanochip becomes feasible. Unfortunately, it was not possible to prove this hypothesis because no prototype of the Protein Nanochip is available yet.

### III.3.2 Purification of phages using *Strep*-tag® technology

In addition to the immobilization assays it was considered to use the *Strep*-tag®/*Strep*-Tactin® technology for purification and consequently for the enrichment of bispecific pHEN3CWP+9Strep phages as already mentioned in III.2. Therefore, a suitable purification protocol was established based on the *Strep*-Tactin® tools provided by IBA GmbH:

- *Strep*-Tactin Sepharose® cartridge
- *Strep*-Tactin Superflow® cartridge
- *Strep*-Tactin MacroPrep® cartridge

The established protocol for purification of *Strep*-Tactin® phage particles and enrichment of bispecific pHEN3CWP+9Strep phages is described in II.2.2.4.1. The successful enrichment was evaluated by *Strep*-Tactin® phage ELISA (II.2.2.5.3). Purified phages were dialyzed before being applied to the *Strep*-Tactin® coated microtiter plates. Elution of *Strep*-tag® phages from the *Strep*-Tactin® cartridges was achieved by competitive elution with hydroxybiotin and this component had to be removed before ELISA analysis because otherwise no binding activity of *Strep*-tag® phages could be determined. The results of this assay are shown in Fig.III-32.

The *Strep*-Tactin MacroPrep® cartridge clogged and thus no ELISA results are shown in Fig.III-32. pHEN-Strep phages (Fig.III-29) purified by *Strep*-Tactin Sepharose® or *Strep*-Tactin Superflow® cartridges were applied to *Strep*-Tactin® coated microtiter plate in comparison to unpurified pHEN-Strep phages using stock solutions with the same phage concentration. The results shown in Fig.III-32 clearly demonstrate the successful enrichment of *Strep*-tag® displaying phages because 1:128 diluted purified phages show the same OD$_{405nm}$ as 1:8 diluted unpurified phages. Therefore, sixteen-fold enrichment was achieved by using either a *Strep*-Tactin Sepharose® or a *Strep*-Tactin Superflow® cartridge. Finally, the Superflow cartridges (II.2.2.4.1) were used for purification of *Strep*-tag® displaying phages because the flow rate was higher than with the Sepharose cartridge. In conclusion, the *Strep-
tag®/Strep-Tactin® technology provides a very useful tool for the purification and enrichment as well as side-directed immobilization of Strep-tag® displaying phages.

**Fig.III-32: Strep-Tactin® Phage ELISA with Strep-Tactin® cartridge purified pHEN-Strep phages.** Freshly prepared (Maxi prep; II.2.2.2) pHEN-Strep phages (Fig.III-29) were purified using Strep-Tactin® cartridges (IBA GmbH) as described in II.2.2.4.1. Three different Strep-Tactin® cartridges (Cartilage Evaluation Set; IBA GmbH) were tested for the most suitable matrix. Purification was done using a Sepharose, a Superflow and a MacroPrep cartridge. After elution, the purified pHEN-Strep phages were dialysed to remove remaining hydroxybiotin and the binding activity of unpurified and purified phages to Strep-Tactin® was determined by Strep-Tactin® Phage ELISA (II.2.2.5.3). A serial dilution of the purified and unpurified phages (stock = 10^{12} phages/ml) were applied to Strep-Tactin® coated microtiter plates and specific binding phages were detected using a 1:2,000 diluted HRP-conjugated mouse anti-M13 antibody. For detection ABTS substrate (Roche) was used and OD_{405nm} was measured. MacroPrep results are not shown because cartridge clogged and thus it wasn’t possible to elute any phages.

Based on the Strep-Tactin® purification protocol (II.2.2.4.1) a second purification procedure was established using Ni-NTA cartridges also provided by IBA GmbH (data not shown). This Ni-NTA purification protocol (II.2.2.4.2) was used as an alternative for the enrichment of bispecific pHEN3CWP+9Strep phages in order to facilitate their detection in immunogold assays (Fig.III-26).
III.4 Final discussion

III.4.1 Automation and miniaturization of phage display

With the human genome project approaching completion (International Human Genom Sequencing Consortium; 2004), there is a growing interest in functional analysis of gene products. The characterization of large numbers of proteins, their expression pattern and in vivo localization demand the use of automated and minituarized technologies that maintain a logistic link to the encoding genes. As a complementary approach, phage display is used for recombinant protein expression and the selection of specifically interacting molecules. Cloning of libraries of genes into phage or phagemid vectors provides a physical link between expressed protein and its encoding DNA sequence. Therefore, phage display describes a powerful tool for proteomic applications, especially for screening of novel therapeutic and diagnostic proteins as well as for epitope mapping or for the identification and validation of novel targets. In the emerging field of proteomics, there is an urgent need for high-throughput technologies for automated library handling and phage display selection. Currently, suitable technologies have been developed by adapting the basic selection steps of the panning procedure to robotics (Walter et al. 2001; Konthur and Walter 2002; Hallborn and Carlsson 2002; Bradbury 2003b). All panning steps, from selection, washing, elution of bound phages, infection spreading, colony-picking, arraying clones into multi-well microtiter plates through to screening by phage ELISA can be ran automatically on commercial robotics. These high-throughput technologies for automated library handling have been shown to combine efficiency with low consumption of the precious library (Hust and Dübel 2004).

This scientific trend and the economically requests within the field of proteomics and protein drug development describe the need for a novel highly innovative technology, that provides an even faster and more efficient automated high-throughput screening system based on phage display technology. Therefore, an interdisciplinary project was initiated by the Fraunhofer Gesellschaft in order to develop a novel protein microarray platform, called Protein Nanochip (Fig.I-9). This new chip-based platform technology should lead to miniaturisized devices, which enable the detection and characterization of before unknown protein-protein-interactions on a single molecule level. The concept of the Protein Nanochip circumvents the entire panning procedure because single binding partners can be directly selected by a novel laser-based detection system, FACS separation and subsequent analysis by duplex-PCR. This would make the multiple rounds of incubation, washing, elution and amplification of the recombinant phages superfluous and dramatically accelerate the
screening process. An additional advantage of the Protein Nanochip is the possibility for screening two phage libraries against each other and therefore allowing the identification of new targets and their specific ligands within one single screening step. For the realization of such a device a nano-structured chip surface was to be developed by one of the partners allowing the immobilization of phage libraries by side-directed binding of single phages on a 10nm activated dot within a well-defined pattern on the chip surface. This "organized" immobilization pattern of the single members of a first phage library on the chip allows the exact localization of each specific binding pair after incubation with a second library using the specific labelling of these secondary phage particles. The second library has to be applied in solution to the immobilized one and specific binding partners are detected after a suitable washing procedure. To obtain exact localization a sensitive laser detection system was to be developed because the determination of the exact position is essential for a selective delamination of single binding partners. Specific delamination was to be achieved by using photosensitive linkers for immobilization of the phage library, which than might be selectively cut by an appropriate laser impulse. Finally, released binding partners should be separated by flow cytometry and analyzed by duplex-PCR using the genetic information incorporated within the two phage particles. The biological content mainly the generation of bispecific filamentous bacteriophages, suitable for the envisaged application, described the focus of this doctoral thesis. The establishment of a novel vector system for the generation of bispecific filamentous bacteriophages allowing the side-directed immobilization of protein libraries onto the surface of the Protein Nanochip was successfully done.

The data generated within this thesis showed that the Strep-tag®/Strep-Tactin® technology in combination with bispecific filamentous bacteriophages makes the generation of the Protein Nanochip possible and thus allows the automated screening of two different protein libraries against each others in a high-throughput manner. The main obstacle of this project was the generation of bispecific filamentous bacteriophages and two different approaches were successfully developed within this thesis.

The first approach describes the usage of a pVI/pIX phage vector system, that seems to allow the generation of bispecific phages simultaneously displaying library proteins as a fusion to pVI proven by the functional CD30R-display and a peptide fused to pIX as proven by the successful insertion of a G4S-linker and a XhoI/NheI-restriction site downstream of the second triplet of geneIX. However, the decreased assembly efficiency of CD30R-displaying phages indicates that the size of the libraries created with a pVI/IX phage vector will be limited to a maximum diversity of $10^7$. The second approach describes the usage of a bispecific phagemid vector system and the production of functional bispecific filamentous bacteriophages simultaneously displaying a scFv antibody fused to pIII and the Strep-tag®
fused to pIX, which was confirmed by Phage ELISA and immunogold assay. However, a purification procedure was necessary for the detection of these bispecific phages. Both strategies lead to the production of bispecific filamentous bacteriophages that allows the side-directed immobilization of protein libraries onto the surface of the envisaged Protein Nanochip and thus provide the main tool for the generation of this highly innovative protein microarray device.

Considering the assembly problem of the bispecific phage vector system and the need for a purification procedure for the bispecific phagemid vector system, an additional strategy was developed describing an alternative way for the generation of bispecific phages combining pIII- and pIX-display. The functional display of peptides of at least nine amino acids on pIX coat protein using a genetically modified phage vector (fd-g9m, Fig.III-9) leads to the idea to create a bispecific phage vector based on fd-tet-DOG1 (Clackson et al. 1991). Phage vector fd-tet-DOG1 possesses the capability for multivalent display of foreign proteins on pIII coat protein and by insertion of a DNA sequence directly downstream of the second triplet of geneIX it should be possible to produce bispecific filamentous bacteriophages. These bispecific phages would multivalently display the library proteins on pIII coat protein and any interesting peptide motif on the opposite site fused to all pIX coat protein. Using this kind of phage vector no enrichment would be necessary, because all rescued phages would display the foreign proteins on their surface due to the multivalent display. In addition, there is no limitation in the size of the created protein libraries as shown with the pVI-system because the library proteins are displayed on pIII coat protein. However, as mentioned above these phages will show a decreased infectivity. This would not be a problem for the Protein Nanochip, which circumvents the entire panning procedure but perhaps for alternative interesting applications. Therefore an additional strategy was thought to provide the best solution for the generation of bispecific filamentous bacteriophages allowing the automation of the phage display screening procedure.

The generation of a novel helperphage in combination with standard pIII phagemid vectors could provide the best solution. The insertion of a DNA sequence directly downstream of the second triplet of geneIX within the helperphage genome encoding for small peptides not longer than nine amino acids (e.g. the Strep-tag®) should allow the generation of bispecific filamentous bacteriophages. This system would lead to phages monovalently displaying the library protein on pIII, encoded by the phagemid vector and simultaneously displaying an appropriated peptide motif on all five pIX coat proteins encoded by the helperphage genome. The pIX-modified helperphage would be compatible with all pIII phagemid vectors and thus allows the automated screening even of already existing protein libraries without sub-cloning the libraries into another vector system. All rescued phages would multivalently display the
foreign peptide e.g. the Strep-tag® on pIX coat protein and no purification procedure would be necessary. In addition, all phages would be infectious due the monovalent library display on pIII coat protein. Next to the automation of the screening procedure using a pIX-modified helperphage it should be possible to accelerate the rescue of the bispecific phage particles. The construction of a helperphage encoding for Strep-tag®/pIX fusion proteins would allow purifying the phages by using Strep-Tactin® columns as described and thus circumvents the standard rescue procedure, which is a PEG precipitation. In conclusion, these pIX-modified helperphages describe an interesting tool for fast rescue of all pIII phagemid libraries and would allow the automated screening of these phage libraries if a suitable device like the Protein Nanochip will be offered in addition. There will be a great market for this pIX-modified helperphage system if an interesting product portfolio is established for this system. A phage library purification kit could be based on the usage of Strep-Tactin® columns and a Strep-tag® helperphage. In addition, the same helperphage could be offered in combination with an automated screening device like the Protein Nanochip allowing the screening of the corresponding phage libraries in a high-throughput manner using the bispecific characteristics of the final phages.

However the bispecific filamentous bacteriophages will be produced at the end, they describe the main tool necessary for the generation of any kind of screening device allowing the automation of the phage display technology. Finally, these bispecific filamentous bacteriophages in combination with the Strep-tag®/Strep-Tactin® technology makes the generation of the Protein Nanochip possible and thus allows the automated screening of protein libraries. Even if the Protein Nanochip itself will never be achieved, bispecific filamentous bacteriophages will be used for the establishment of other automated screening systems allowing the screening of two different protein libraries against each other. A fluidic screening system for example could be envisaged using two differently labelled phage libraries. The integration of fluorescent dyes should be possible by using bispecific phages displaying a Strep-tag® in addition to the protein library and label them by applying fluorescent Strep-Tactin® conjugates to the rescued phages. Alternatively a genetical integration of fluorescent proteins is feasible. Finally, it should be possible to distinguish between single phages and specific binding phage pairs by a read-out of double signals or usage of fluorescence resonance energy transfer (FRET). Specific binding phages should be separable by FACS and might be analyzed by duplex-PCR according to the principle described for the Protein Nanochip.
III.4.2 Alternative applications of bispecific bacteriophages

In addition to the automation of the phage display technology and the establishment of novel high-throughput screening systems, it is also envisaged to use bispecific filamentous bacteriophages for therapeutic applications. Chen et al. published an interesting therapeutic application within the field of cancer treatment (Chen et al. 2004). The described principle to specifically target therapeutic proteins within the human body by using bispecific phages as a vehicle will definitely be expanded in the future. In addition to several other interesting drug delivery applications it should be possible to use the same principle for example for novel vaccination strategies. Bispecific phages may direct vaccines displayed on their surface to antigen presenting cells and induce the appropriate immune response. This would advance the methodology described by Bastien, who already showed specific immune responses caused by filamentous bacteriophages (Bastien et al. 1997). Another interesting application was published by Carrera (Carrera et al. 2004). He used filamentous bacteriophages as a vector-mediated delivery system to the brain (Frenkel and Solomon 2002) and developed a novel approach for treating cocaine addiction. An adjustment of bispecific filamentous bacteriophages to this application would provide a more specific delivery system and might reduce side effects. According to these applications, several other therapeutic or diagnostic approaches as well as vaccination strategies might be feasible using bispecific filamentous bacteriophages.
Phage display is a powerful tool for screening large protein libraries in order to identify novel therapeutics, diagnostics or vaccines. In the emerging field of protein based drug development, there is an urgent need for high-throughput technologies for automated library handling and phage display selection. Currently, suitable technologies have been developed by adapting the basic selection steps of the phage display panning procedure to robotics. This thesis presents an innovative strategy for the generation of an even faster high-throughput screening system, called Protein Nanochip. The basis for this novel protein microarray device was established within this thesis circumventing the entire panning procedure through direct laser-based detection of singular binding partners, combined with FACS, and final identification of the interacting partners by duplex-PCR and sequencing. This technology is suitable for screening of an immobilized phage library against a second fluorescent labelled phage library and therefore allows the identification of new targets and their specific ligands within one single automated screening step. For the development of this highly innovative screening devise a bispecific filamentous bacteriophage and a suitable technology for side-directed immobilization of these phages onto the surface of the Protein Nanochip had to be developed.

Two different strategies were investigated for the generation of bispecific filamentous bacteriophages. First of all, a modified phage vector based on the well-characterized fd-tet vector was established. The final bispecific phage vector was generated by insertion of a cloning site and an appropriate linker upstream of geneVI and another cloning site and an appropriate linker downstream of the second triplet of geneIX. Multivalent display of the extracellular domain of the human CD30 receptor fused to the C-terminus of the pVI coat protein was shown to be feasible but reduced the assembly efficiency and therefore would decrease the diversity of corresponding protein libraries. Multivalent display of single chain Fv antibodies fused to pIX coat protein showed a lethal effect on the bacterial host, whereas multivalent display of small peptides on the same coat protein was shown to be feasible and provides a tool for the generation of recombinant phages not inhibiting the propagation of the bacterial host. These results indicate that there might be an option for the generation of bispecific filamentous bacteriophages using a pVI/pIX-phage vector system. The final phage particles would multivalently display peptides as a fusion to pIX coat protein, which could be used for the side-directed immobilization and simultaneously display library proteins as a fusion to all five pVI coat proteins. However, there will be a limitation in the diversity of the corresponding library displayed on pVI coat protein.
Therefore, a novel phagemid vector system for the generation of bispecific filamentous bacteriophages was developed. The final phagemid vector was generated by insertion of a second expression cassette encoding geneIX into the well-established pHENHi phagemid vector. This bispecific phagemid vector was designed for library display on pIII coat protein and simultaneous display of functional peptides or proteins for side-directed immobilization or labelling on pIX coat protein. Finally, a single chain Fv antibody encoding DNA sequence was fused to geneIII and the Strep-tag® encoding DNA to geneIX. Functional coexpression of the resulting scFvCWP/pIII- and Strep-tag®/pIX-fusion proteins as well as their incorporation into correctly assembled phage particles was verified by phage ELISA and immunogold assay with subsequent electron microscopy. These results provided evidence for functional display of both the single chain Fv antibody on pIII coat protein and the Strep-tag® on pIX coat protein and prove the concept of the novel phagemid vector system as a suitable tool for the generation of bispecific filamentous bacteriophages displaying two different foreign proteins on their opposite sides.

The Strep-tag® technology was shown to provide a universal tool for purification, detection and side-directed immobilization of Strep-tag®-displaying filamentous bacteriophages. These phages could be enriched 16-fold using Strep-Tactin® cartridges and thus allowed the enrichment of bispecific phages that represented less than 1% of the initially rescued phage population, using the bispecific phagemid vector system. In addition, side-directed immobilization of Strep-tag®-displaying phages was shown by phage ELISA either directly on Strep-Tactin® coated surfaces or indirectly on biotinylated surfaces via soluble Strep-Tactin®.

In conclusion, a novel phagemid system for the generation of bispecific filamentous bacteriophages, and suitable technologies for purification, detection and side-directed immobilization of these phages were successfully developed allowing the immobilization of any kind of phage libraries on the biotinylated surface of the Protein Nanochip or comparable devices. In addition, labelling of protein libraries with a fluorescent dye can be realized using this novel phagemid vector system. Fluorescent proteins might be recombinantly incorporated within the phage particles by fusing the corresponding DNA to geneIX or by applying fluorescent Strep-Tactin® conjugates to Strep-tag®-displaying phages. Therefore, the bispecific phagemid vector system in combination with the adapted Strep-tag® technology represents an initial step toward the establishment of the Protein Nanochip allowing the automated screening of two different protein libraries against each other.
V Appendix

V.1 List of abbreviations

(+)  viral (coding) strand of DNA
(-)  complementary strand of viral DNA
°C   degree Celsius
%    percentage
A    adenine
aa   amino acid(s)
Ab   antibody
ABTS 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)]
Amp  ampicillin
Asn  asparagines
AP   alkaline phosphatase
bp   base pair
BSA  bovine serum albumin
C    cytosine
cDNA complementary DNA
cfu   colony-forming unit(s)
DNA  deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
dsDNA double-stranded DNA
E. coli  Escherichia coli
ELISA enzyme-linked immunosorbent assay
Fc   fragment crystalline
G    guanine
GAM  goat anti-mouse antibody
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>GAR</td>
<td>goat anti-rabbit antibody</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>h</td>
<td>hours(s)</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>l</td>
<td>liter(s)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>M</td>
<td>molarity (mol/l)</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mol</td>
<td>molar</td>
</tr>
<tr>
<td>mmol</td>
<td>millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>µl</td>
<td>mikroliter</td>
</tr>
<tr>
<td>µm</td>
<td>mikrometer</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate buffered saline containing 0.05% (v/v) Tween 20</td>
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PCR    polymerase chain reaction
PEG    polyethylene glycol
Phe    phenylalanine
Pro    proline
rAb    recombinant antibody
RNA    ribonucleic acid
rpm    rotations per minute
RT     room temperature
scFv   “single chain Fragment variable”
Ser    serine
SOE    splicing by overlap extension
ssDNA  single-stranded DNA
T      thymine
Taq    Thermus aquaticus
Tet    tetracycline
Tris   trishydroxymethylaminomethane
Trp    tryptophan
U      unit(s)
UV     ultraviolet
V      Volt
V_{H}  variable region of heavy chain
V_{L}  variable region of light chain
v/v    volume per volume
v/w    volume per weight
w/v    weight per volume
V.2 Vectors

V.2.1 Phage vectors

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<tr>
<th>Phage vector</th>
<th>geneI modification</th>
<th>geneX modification</th>
<th>geneX insert</th>
<th>geneVI modification</th>
<th>geneVI insert</th>
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<tr>
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<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>fd-g6m</td>
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<td>no</td>
<td>G3SG2</td>
<td>Sfi/NotI</td>
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<tr>
<td>fd-g6/9m</td>
<td>XhoI/NheI</td>
<td>G3S</td>
<td>no</td>
<td>G3SG2 and Sfi/NotI</td>
<td>no</td>
</tr>
<tr>
<td>fd-g6CD30/9m</td>
<td>XhoI/NheI</td>
<td>G3S</td>
<td>CD30R</td>
<td>G3SG2 and Sfi/NotI</td>
<td>CD30R cDNA</td>
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<td>Ki-4(scFv)</td>
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<td>no</td>
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<td>XhoI/NheI</td>
<td>G3S</td>
<td>Ki-4(scFv) DNA</td>
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</table>
V.2.2 Phagemid vectors
<table>
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<th>geneIII insert</th>
<th>geneIX cassette</th>
<th>geneIX insert</th>
</tr>
</thead>
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<tr>
<td>pHEN4II</td>
<td>present</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>pHENHi</td>
<td>present + 6xHis tag</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>pHEN-Strep</td>
<td>present</td>
<td>Strep-tag®</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>pHEN-CWP</td>
<td>present</td>
<td>scFvCWP</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>pHEN9</td>
<td>no</td>
<td>no</td>
<td>present</td>
<td>Strep-tag®</td>
</tr>
<tr>
<td>pHEN9-Strep</td>
<td>no</td>
<td>no</td>
<td>present</td>
<td>Strep-tag®</td>
</tr>
<tr>
<td>pHEN9-CWP</td>
<td>no</td>
<td>no</td>
<td>present</td>
<td>scFvCWP</td>
</tr>
<tr>
<td>pHEN9-Bi</td>
<td>no</td>
<td>no</td>
<td>present + new MCS</td>
<td>no</td>
</tr>
<tr>
<td>pHEN3+9</td>
<td>present + 6xHis tag</td>
<td>no</td>
<td>present + new MCS</td>
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<tr>
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<td>present + 6xHis tag</td>
<td>no</td>
<td>present + new MCS</td>
<td>Strep-tag®</td>
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<td>present + 6xHis tag</td>
<td>scFvCWP</td>
<td>present + new MCS</td>
<td>Strep-tag®</td>
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