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BACTERIOLOGICAL APPLICATIONS OF RANDOM PHAGE DISPLAY LIBRARIES

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„A kis jércét nem irigylem én,
mikor kapar az udvar szemetén.
Irigylem a kotlóst: lázban, egyedül
az áthevülő tojásokon ül,
s a változás titka körülkerengi.
A boldogság útszéli szemét,
szedhet eleget, ki fenyújtja kezét,
az érlelő kint kell megérdemelni."

(Weöres Sándor: Gnómák- A boldogságról)

CONTENTS

LIST OF ABBREVIATIONS

A, Ala-alanine
N,Asn-asparagine
D,Asp-aspartate
R,Arg-arginine
C,Cys-cysteine
Q,Gln-glutamine
E,Glu-glutamate
G,Gly-glycine
H,His-histidine
I, Ile-isoleucine
L,Leu-leucine
K,Lys-lysine
M,Met-methionine
F,Phe-phenyl-alanine
P,Pro-proline
S,Ser-serine
T,Thr-threonine
Y,Tyr-tyrosine
W,Trp-tryptophane
V,Val-valine

DNA-deoxy-ribonucleic acid
cDNA-cloned DNA
ssDNA-single-stranded DNA
RNA-ribonucleic acid
IG-intergenic region
RF-replicative form
PS-packaging signal
HIV-human immunodeficiency virus
Fab- fragment antigen binding
Fv- fragment variable
scFv-single chain fragment variable
V_L-variable region of immunoglobulin light chain
V_H-variable region of immunoglobulin heavy chain
C_L-conservative region of immunoglobulin light chain
C_H-conservative region of immunoglobulin heavy chain
Ph. D.-phage display
DSM-Difco Sporulation Medium
TBS-Tris buffered saline
PBS-phosphate buffered saline
Tris- tris(hydroxymethyl)aminomethane

PA-plasminogen activator
tPA-tissue-type plasminogen activator
uPA-urokinase-type plasminogen activator
PlgR-plasminogen receptor
SK-streptokinase
SAK-staphylokinase
Pla-*Yersinia pestis* plasminogen activator
ddH_2O-double distilled water
RPMI-Roswell Park Memorial Institute Medium

BM-basement membrane
HRPO-horse-radish-peroxidase
OPD-ortho-phenylenediamine dihydrochloride
TMB-tetra-methyl-benzidine
ECM-extracellular matrix
LD_{50}-50 % lethal dose
MMP-matrix metalloproteases
A_λ-absorbance at λ wavelength
NDGA-nordihydro-guaretic acid

PK-protein kinase

PKC-protein kinase C

TPK-tyrosine protein kinase

GEF-guanine-nucleotide-exchange factor

GTP-guanosine-triphosphate

PtIns-4-kinase phopshatidyl inositol-4-kinase

phosphatidyl-inositol-3 kinase PI 3 kinase

PLA_2- phospholipase A_2

IC_{50}- 50% inhibitory concentration

cAMP-cyclic adenosine-monophosphate

kDa-kilodalton

TRITC- tetramethyl-rhodamine-isothiocyanate

EGTA-ethylene glycol tetra acetic acid

ELISA-enzyme-linked-immunosorbent-assay

BSA-bovine serum albumine

1.INTRODUCTION TO PHAGE DISPLAY

1.1. THE PRINCIPLE OF PHAGE DISPLAY

Phage display is a rapidly growing technology first described in 1985 by G. P. Smith (Smith, 1985), which is a very effective way for producing a large number of diverse peptides and proteins and isolating molecules that perform specific functions. It relies on two simple concepts (Rodi and Makowski, 1999). Firstly an insertion mutation at an appropriate location within a structural gene of a virus will lead- as long as it does not interrupt essential functions of the gene product- to the display of the mutation-encoded peptide on the surface of the viral particle. Secondly, if the insert is a random oligonucleotide, the resulting particles will comprise a library of peptides - each one displayed on a viral scaffold which bears mutated coat proteins surrounding the enclosed mutant DNA. It is this physical linkage that is the basis of the broad utility of phage display libraries. Large-scale growth of that viral particle and sequencing of the inserted nucleic acid can easily determine a single peptide sequence selected for some specific property by an appropriate screening technique. Instead of having to genetically engineer proteins or peptide variants one-by-one and then express, purify and analyse each variant, phage display libraries containing several billion of variants can be constructed simultaneously. Biological vehicles that have been utilised as platforms for the presentation of random peptide, gene fragment, cDNA and antibody libraries on a genetic package include λ and T4 phages, baculovirus or even bacterial flagella, pili and cell-surface proteins (Rodi and Makowski, 1999). Nonetheless, the filamentous bacteriophage M13 has been the platform of choice both from historical perspective as the first and best characterised library display vector and as the source of the majority of successful screenings.

1.2. STRUCTURE AND REPLICATION OF M13 BACTERIOPHAGE

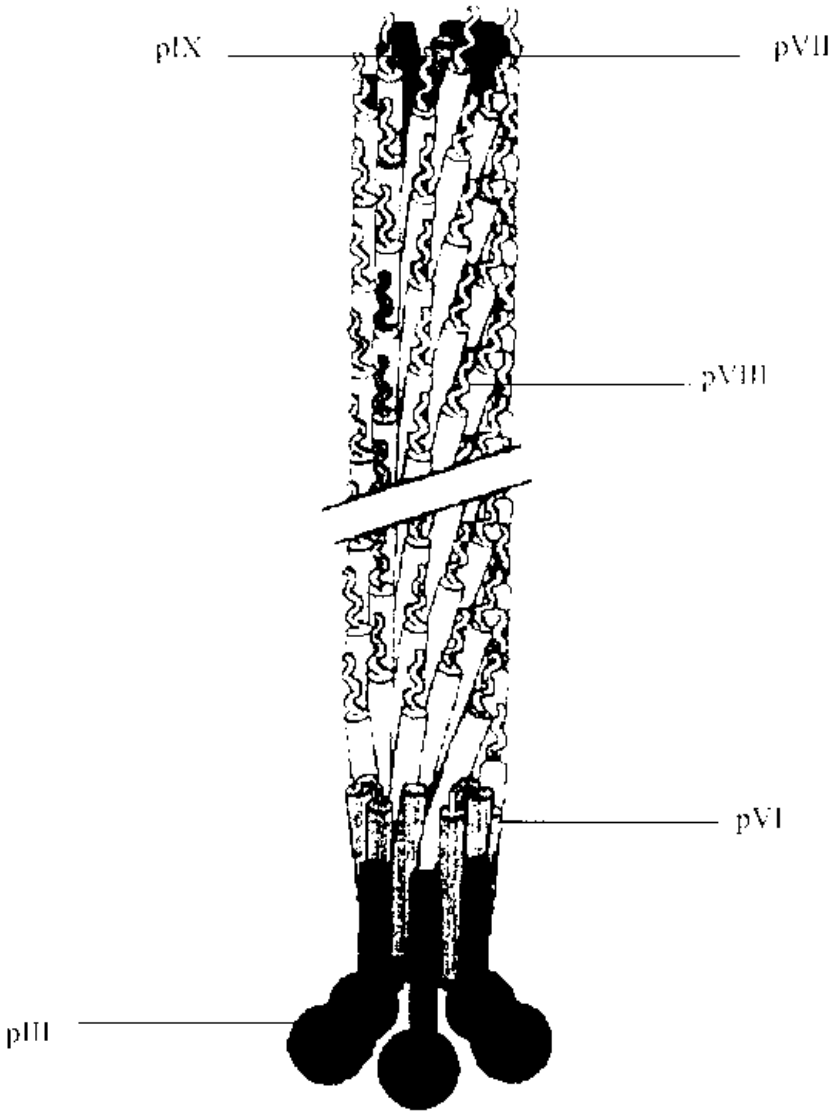
1.2.1. Capsid structure

The M13 filamentous phage is a member of the family *Inoviridae*, contains single-stranded DNA and displays the simplest helical viral capsid. It is closely related to phage fd. They are about 900 nm long and 9 nm in diameter and the particles contain 5 proteins. All are similar and are known collectively as Ff phage- they require the *E. coli* F pilus for infection. The major coat protein is the product of phage gene 8 (pVIII) and there are 2,700 - 3,000 copies of this protein per particle, together with approximately 5 copies each of four minor capsid proteins, pIII, pVI, pVII and pIX which are located at the ends of the filamentous particle. They are indistinguishable in electron

micrographs. It is the protein pIII, which interacts with the bacterial pilus. Hence the pointed capsid end expressing pIII along with pVI-is called the proximal end. Both proteins are needed in order to detach the phage from the cell membrane; pVI is degraded in cells that lack pIII, which suggests that these proteins assemble in the cell membrane before their incorporation into phage particles (Russel, Model and Clackson, 2004). The 406 residue pIII is the most commonly used coat protein for display. Its N-terminal domain, which is necessary for phage infectivity, is surface exposed and forms the small "knobs" that can often be seen in electron micrographs. Three pIII domains have been determined, the two N-terminal domains (N1 and N2) probably interact intramolecularly, based on crystallographic analysis (Russel, Model and Clackson, 2004). The three domains are separated by two long, presumably flexible linkers characterised by repeats of a glycine-rich sequence. The final 132 residues within the C-terminal CT domain are necessary and sufficient for pIII to be incorporated into the phage particle and to mediate termination of assembly and release of phage from the cell; this domain is possibly buried in the particle (Russel, Model and Clackson, 2004). The other distal (blunt) end of the phage expresses pVII and pIX, two of the smallest ribosomally translated proteins (33 and 32 amino acid long respectively). Neither the structure nor the disposition of pVII and pIX in the particle is known. However, immunological evidence indicates that at least some of pIX are exposed and antibody variable regions have been successfully displayed on the amino termini of pVII and pIX (Russel, Model and Clackson, 2004). Phage assembly begins at the pVII-pIX end, and in the absence of either protein, no particle is formed. The primary structure of the major coat protein pVIII explains many of the properties of the particle. Mature molecules of pVIII consist of approximately 50 amino acid residues (a signal sequence of 23 amino acids is cleaved from the precursor protein during its translocation into the outer membrane of the host bacterium), and is almost entirely alpha-helical in structure so that the molecule forms a short rod. There are three distinct domains within this rod: A negatively-charged region at the amino terminal end which contains acidic amino acid residues and which forms the outer, hydrophilic surface of the virus particle, a basic, positively-charged region at the carboxy-terminal end which lines the inside of the protein cylinder adjacent to the negatively-charged DNA genome and a hydrophobic region which is responsible for interactions between the pVIII subunits which allow the formation of and stabilise the phage particle. Ff phage particles are held together by the hydrophobic interactions between the coat protein subunits and this is demonstrated by the fact that the particles fall apart in the presence of chloroform, even though they do not contain any lipid component. The pVIII protein subunits in successive turns of the helix are tilted at an angle of

approximately 20° to the long axis of the particle and have been described as overlapping one another like the scales of a fish (Russel, Model and Clackson, 2004)

Figure 1. Structure of the M13 filamentous phage. Modified after Rodi and Makowski, 1999



1.2.2. Phage genome

The 6400 nucleotide Ff genome contains nine genes, one major non-coding region (IG), which includes the replication origins for (+) and (-) strand synthesis and the packaging signal (PS) determining the orientation of the phage genome (R). PS, an imperfect but stable hairpin is positioned at the pVII pIX end of the particle and is necessary and sufficient for efficient encapsidation of circular ssDNA into phage particles. Two of the phage genes (I and II) have internal translational initiation sites from which in-frame restart proteins, (pIX and pX, respectively) are produced. In each case, both the full-length and the restart protein (whose sequence is identical to the carboxy terminal third of the full-length protein) are necessary for successful phage production. Of the 11 phage-encoded proteins, three (pII, pX, pV) are required to generate ssDNA, three (pI, pXI, pIV) are required for phage assembly, and five (pIII, pVI, pVII, pVIII, pIX) are components of the phage particle. Since the phage DNA is packaged inside the core of the helical particle, the length of the particle is dependent on the length of the genome. In all Ff phage preparations the following forms occur: Polyphage contain more than one genome length of DNA, miniphage contain deleted forms- 0.2-0.5 phage genome lengths- of DNA, maxiphage are also genetically defective forms but they comprise more than one phage genome length of DNA. This plastic property of these filamentous particles has been exploited by molecular biologists to develop the M13 genome as a cloning vector - insertion of foreign DNA into the non-essential intergenic region results in recombinant phage particles, which are longer than the wild-type filaments. Unlike most viruses, there is no sharp cut-off genome-length at which the genome can no longer be packaged into the particle. However, as M13 genome size increases, the efficiency of replication declines such that while recombinant phase genomes 1-10% longer than the wild-type do not appear to be significantly disadvantaged, those 10-50% longer than the wild-type replicate significantly more slowly and above 50% increase over the normal genome length it becomes progressively more difficult to isolate recombinant phase. This property has been exploited in M13 cloning vectors (Russel, Model and Clackson, 2004).

1.2.3. Phage replication

1.2.3.1. Attachment and penetration

The consecutive steps of replication start with viral attachment to and penetration into the host *E. coli* bacterium. Ff phage are 'male-specific', i.e. they require the F pili on the surface of *E. coli* for infection. Infection normally begins when the N2 domain of pIII binds to the tip of a pilus. Pili normally assemble and disassemble continuously, and this, possibly stimulated by phage binding, brings the phage close to the cell surface. Upon pilus binding to N2, the N1 domain is

released from its normal interaction with N2, making it available to bind to the host TolA protein, which extends into the periplasm from the cytoplasmic membrane (Russel, Model and Clackson, 2004). How the phage penetrates the outer membrane and the underlying peptidoglycan layer is not known. Three Tol proteins (Q, R, and A), all integral cytoplasmic membrane proteins, are absolutely required for phage infection (Russel, Model and Clackson, 2004). They mediate depolymerisation of the phage coat proteins into the cytoplasmic membrane and the translocation of the viral ssDNA into the bacterial cytoplasm. The gpIII-TolA interaction causes a conformational change in pVIII: Initially, its structure changes from 100% alpha-helix to 85% alpha helix and this causes the filament to shorten. The end of the particle attached to the F pilus flares open, exposing the phage DNA. Subsequently, a second conformational change in the pVIII subunits reduces its alpha-helical content from 85% to 50%, causing the phage particle to form a hollow spheroid about 40 nm in diameter and expelling the phage DNA, thus initiating the infection of the host cell. Then pVIII is stripped off and ends up in the inner cell membrane, where it may possibly be stored and reused to produce new particles.

1.2.3.2. Genome replication and transcription

During the genome replication the infecting (+) strand DNA is converted in double-stranded RF (replicative form) by host cell enzymes, which together with pII build up a pool of RF DNA in the cell. PII is a site-specific nicking-closing enzyme necessary for further replication. Virus proteins are synthesised from this pool of DNA. Through a rolling circle mechanism, pII nicks the (-) strand of the RF at a specific site in the non-coding IG region of the phage genome, and the 3'end of the nick is elongated by host DNA polymerase III using the (-) strand as template. The original (+) strand is displaced by Rep helicase as the new (+) strand is synthesised, and when a round of replication is complete, the displaced (+) strand is recircularised by the nicking-closing activity of pII and again converted to RF. Synthesis of the (-) strand requires an RNA primer. The primer is generated by RNA polymerase, which initiates synthesis at an unusual site in the IG region of the (+) strand consisting of two adjacent hairpins including promoter-like -35 and -10 motifs detached by a single-stranded region (Russel, Model and Clackson, 2004). All phage proteins are synthesised simultaneously, although diverse mechanisms ensure that each is produced at an appropriate rate. There are differences in promoter and ribosome binding site strength or accessibility. At the beginning of gene I a weak rho-dependent termination signal limits its transcription, and the large number of infrequently used codons reduces its rate of translation. Proteins pV and pVIII required in greatest quantities have overlapping transcripts from multiple promoters (there are only two terminators) and multiple RNA processing events

increase the abundance of RNAs. During the early phase of infection, when the concentration of the phage ssDNA-binding protein (pV) is low, newly synthesised single strands are immediately converted to RF, and both RF and phage proteins increase exponentially. As its concentration increases, pV binds co-operatively to newly generated (+) strands, preventing polymerase access and blocking their conversion to RF. Protein pX is required for the stable accumulation of single strands at this stage (Russel, Model and Clackson, 2004). pV is a dimeric protein, with the interaction surface of the subunits opposite the DNA-binding surface. The pV:ssDNA complex is the substrate for phage assembly.

1.2.3.3. Viral assembly

The final viral assembly occurs at the inner membrane of the host cell. It has five stages: preinitiation, initiation, elongation, pretermination, and termination. Preinitiation is defined as the formation of an assembly site, a region visible by electron microscopy where the cytoplasmic and outer membranes are in close contact (Russel, Model and Clackson, 2004). Assembly sites are composed of the three morphogenetic proteins, pI, pXI, and pIV, which interact via their periplasmic domains (N-terminal for pIV and C-terminal for pI and, presumably, for pXI); the sites form independently of any other phage proteins. pIV is a cylindrical structure with a central cavity. In the cryoelectronmicroscopical structure of pIV there is some density within the cavity explaining why the normal state of the pIV channel is closed. Certain mutant forms of pIV, however, open frequently and allow entry of foreign substances into the bacterial periplasm. The interior of the pIV channel can accommodate an occasional pVIII subunit carrying a large N-terminal extension or pVIII uniformly substituted with short (6-8 residue-) N-terminal extensions, as in phage display. The dimensions and/or properties of the channel may be a factor limiting the size of polypeptides that can be displayed on pVIII. pI and pXI also form a multimeric complex composed of about 5-6 copies of each. In the absence of the other phage proteins, pI/pXI causes membrane depolarisation, which suggests that the complex may also be a channel. Thus the assembly site may be an extremely large channel that traverses both bacterial membranes. The cytoplasmic N-terminal domain of pI (absent from pXI) contains a conserved nucleotide-binding motif, which is essential for phage assembly. Phage assembly requires ATP hydrolysis (Feng, Russel, and Model, 1997) therefore pI is likely to be an ATP-ase. Initiation takes place only if the assembly site, the two minor coat proteins (pVII and pIX) located at the distal tip of the particle, and the ssDNA substrate are present. In the membrane pVII and pIX interact with the PS, which protrudes from one end of the pV-ssDNA complex (in the cytoplasm) and associates with the cytoplasmic domain of pI. Host-encoded thioredoxin, a small, cytoplasmic protein known as a

potent reductant of protein disulfides, also interacts with pI. Although phage assembly does not utilise this redox activity, thioredoxin appears to be part of the initiation complex, and may confer processivity to the elongation reaction. Elongation involves the successive replacement of pV dimers that cover the viral DNA by membrane embedded pVIII and translocation of the DNA across the membrane. The process continues until the end of the viral DNA has been coated by pVIII. If either pIII or pVI is absent, the largely extracellular phage particle remains tethered to the cytoplasmic membrane where it remains competent to resume elongation when another pV-ssDNA complex enters the assembly site; ultimately, tethered phage filaments of more than 10 times unit length accumulate. Even in normal infections when pIII and pVI are present, about 5% of progeny phage particles are double length. Pretermination is the incorporation of the membrane-embedded pIII-pVI complex at the proximal end of the nascent phage particle. A fragment containing only the C-terminal 83 residues of pIII is sufficient to mediate this step, but cannot affect detachment of the phage from the cell. Termination or release of the phage, which requires a 93 residue C-terminal segment of pIII, has been proposed to consist of a conformational change in the pIII-pVI complex that detaches the complex (and the phage) from the cytoplasmic membrane. A still longer portion of pIII (the 132 C-terminal residues) is required for the formation of stable virus particles.

1.3. COAT PROTEINS USED FOR DISPLAY

All five capsid proteins have been used to display proteins or peptides, to varying degrees. One report has described the fusion of antibody fragments to the amino termini of both pVII and pIX (Russel, Model and Clackson, 2004). The pVI protein, which interacts with pIII has also been used to display polypeptides through a carboxy-terminal fusion (Russel, Model and Clackson, 2004). C-terminal linkage is particularly desirable for display of polypeptides encoded by cDNA fragments, since the inclusion of the stop codon at the end of the cDNA will not prevent display. However, by far the most commonly used virion proteins for phage display are pVIII and pIII.

1.3.1. pVIII

pVIII, the major coat protein, is present in several thousand copies in phage particles. Sequences for display are typically inserted at the N-terminus, between the signal sequence and the beginning of the mature protein coding sequence. However, only short peptide sequences (6-8 residues) can be displayed on every copy of pVIII because larger sizes prevent packaging of the virions. It might be due to the size restrictions of the pIV channel through which phage pass during extrusion. Display of larger polypeptides on pVIII requires expression of the fusion

protein from a phagemid vector, yielding hybrid virions bearing mainly wild-type pVIII. Recently engineered pVIII proteins have been described that permit the display of large polypeptides at high copy number, or the display of proteins fused to the C-terminus (Russel, Model and Clackson, 2004).

1.3.2. pIII

PIII, present in five copies at the proximal end of the capsid, is the protein of choice for most phage display fusions due to its tolerance for large insertions and the wide availability of suitable vectors. Although pIII is more tolerant than pVIII to substantial insertions, infectivity of the resulting phage can be reduced, sometimes dramatically. As with pVIII, this can be overcome by using phagemid constructs, resulting in the production of hybrid virions that also bear wild-type pIII. Since such virions no longer rely on the infectivity of the pIII fusion protein, proteins can instead be fused to truncated pIIIs designed with the structure of the protein in mind. These can confer more efficient display, by reducing or eliminating proteolysis of the fusion protein, as well as reducing the size of the phagemid vector. Potential disadvantages include the possibility of sterically hindering access to the displayed protein. C-terminal pIII display through fusion to a linker at the C-terminus of the pIII is also possible (Russel, Model and Clackson, 2004)

1.4. CONSTRUCTION OF LIBRARIES

1.4.1. Feasibility

The first step in a phage display project is establishing that display of the polypeptide of interest is feasible (Russel, Model and Clackson, 2004). It was supposed that the limitations of virion extrusion and infection would restrict display to small peptides and proteins, and only proteins that are normally extracellular would be suitable, since display involves secretion of the fusion protein into the bacterial periplasm (Russel, Model and Clackson, 2004). Both concerns have proved false, and intracellular and extracellular proteins of a wide range of sizes and structures have been functionally displayed. For proteins that are normally intracellular, precautions can be taken to try to preserve the native structure of the molecule-for example, addition of zinc during preparation of phage displaying zinc finger proteins. If necessary, displayed proteins can even be refolded prior to selection by exposure of the particles to denaturants followed by dialysis. It has also proved feasible to display multi-subunit proteins. Homo-oligomeric proteins can also be displayed by relying on proteolysis of the displayed fusion protein to release sufficient soluble protein, or by invoking interactions between displayed proteins, either inter- or intra-phage.

1.4.2. Display systems

Proteins can be displayed using vectors based on the natural Ff phage sequence-phage vectors-or using plasmid-based phagemid vectors that contain only the fusion protein gene, and no other phage genes. In phage vectors, the heterologous sequence for display is inserted directly into the coding sequence for pIII or another coat protein. When introduced into *E. coli*, phages will be produced in which all copies of the coat protein display the heterologous protein, in other words the protein is displayed polyvalently. Examples of pIII phage display vectors include the fUSE vectors constructed by Smith and coworkers (Parmley and Smith, 1988), and the M13KE vectors commercially available from New England Biolabs. Libraries constructed in such a way are also called 'type 3' for pIII, or 'type 8' for pVIII. In type '88' and type '33' vectors the single phage genome bears two genes of pVIII and pIII, respectively, encoding two different types of pVIII or pIII molecule: one is ordinarily recombinant and the other wild-type (Smith and Petrenko, 1997). The resulting virion is a mosaic; its coat comprised of both wild type and recombinant pVIII molecules (the former usually predominating). This allows hybrid pVIII proteins with quite large foreign peptides to be displayed on the virion surface, even though the hybrid protein by itself cannot support phage assembly. Similarly, a type '33' vector bears two genes of pIII, one of which is recombinant. Mosaic proteins are produced using '3+3'and '8+8'systems as well (Smith and Petrenko, 1997). In these cases the wild type and the recombinant gene 3 or gene 8 are present in the distinct genomes of a helper phage and a phagemid, respectively. In phagemid vectors, the displayed protein fusion gene is cloned into a small plasmid under the control of a weak promoter. In addition to a plasmid origin of replication, the vector also has a Ff origin to allow production of single-stranded vector and subsequent encapsidation into phage particles. To produce such particles, *E. coli* cells harbouring the plasmid are infected with helper phage, which is a Ff phage with a compromised origin that leads to its inefficient packaging. The infected cells express all the wild-type phage proteins from the helper phage genome, as well as a small amount of the fusion protein encoded by the phagemid. Because the helper phage genome is poorly packaged, nearly all the phage particles contain the phagemid genome, preserving the linkage between the displayed protein and its gene. Phagemid vectors have been described for both pIII and pVIII display. A major advantage of phagemid vectors is their smaller size and ease of cloning, compared to the difficulties of cloning in phage vectors without the disruption of the complex structure of overlapping genes, promoters, and terminators. This generally translates into much higher library sizes for phagemid vectors. For pVIII display, use of phagemids is generally required to achieve display of sequences longer than 6-8 amino acids.

1.4.3. Valency

The choice of monovalent display (display of a single recombinant capsid protein) or polyvalent display of more than one copies of the recombinant capsid protein) is associated to the choice of vector type. Conventional phage vectors with natural phage promoters will generally produce polyvalent display unless there is extensive proteolysis of the displayed proteins. Phagemid vectors for pVIII display, in which only a fraction of the ~ 2700 copies are fusion proteins, will typically still display polyvalently. On the other hand, use of phagemid vectors to display protein on pIII under the control of a weak (or uninduced) promoter will typically lead to monovalent display (Russel, Model and Clackson, 2004). The valency of display is important principally because of its impact on the ability to discriminate binders of differing affinities. It has been shown that polyvalent display prevented the highest-affinity clones in a selection from being identified, because multivalency conferred a high apparent affinity (avidity) on weak-binding clones (Russel, Model and Clackson, 2004). Monovalent display allows selection based on pure affinity, and is therefore generally preferred for the many studies where the aim is to identify the tightest binding variant(s) from a library. Conversely, in applications where the initial selectants are of very low affinity target polyvalency increases the chances of isolating rare and weakly binding clones. A frequent experimental strategy in such projects is to start with polyvalent display, and then move to monovalent display as the affinity of the displayed polypeptide matures.

1.5. SELECTION PROCEDURES

1.5.1. General principles

Selection consists of adding an initial sizeable population of phage-borne peptides to give a subpopulation with increased fitness according to user-defined criterions (Smith and Petrenko, 1997). In most cases, the input to the first round of selection is a very large initial library (10^9 different sequences represented individually on average in about 100 individual copies) and the selected subpopulation is a very small fragment of the initial population. Selected sequences can be amplified by infecting fresh bacterial host cells, so that each individual phage in the subpopulation is represented by millions of copies in the amplified stock. Then the amplified population can be subjected to further rounds of selection (perhaps accompanied by mutagenesis) to obtain an ever-fitter subset of the starting peptides.

1.5.2. Affinity selection

The most common selection pressure imposed on phage-displayed peptide populations is their affinity for a target receptor. Throughout a procedure called biopanning (instead of gold you search for biologically valuable molecules) it is possible to select for sequences and common motifs for interest. The receptor is tethered to a solid support, and the phage mixture is passed over the immobilised receptor. Those phage whose displayed peptides bind the receptor are captured on the surface or matrix, unbound phage are washed away. Then the bound phage are eluted in a solution that loosens receptor-peptide bonds, yielding an eluate population of phage that is greatly enriched (often a million fold or more) for receptor-binding clones. The eluted phage are still infective and are propagated by infecting fresh bacterial host cells, yielding an amplified eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2-3 rounds of selection) are propagated and characterised individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined by uncovering the corresponding coding sequence in the viral DNA.

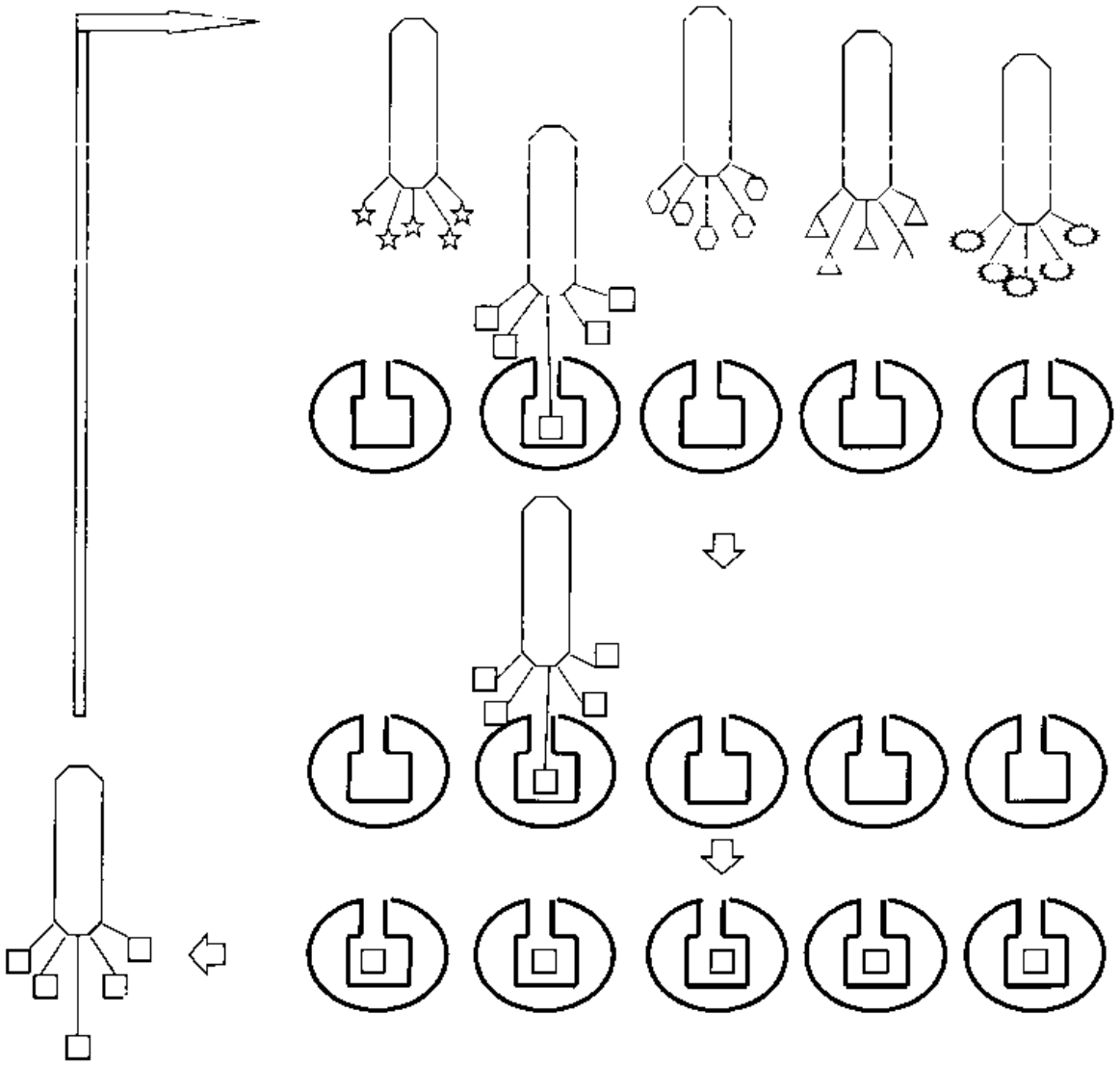


Figure 2. Main steps of affinity selection

1.5.2.1. Capturing the target

The solid supports to which target receptors are captured are usually surface supports like polystyrene dishes (Smith, 1985), impermeable plastic beads (Bass, Greene, and Wells, 1990), nitrocellulose membranes (Dyson and Murray, 1995), paramagnetic beads (Fowlkes et al., 1992). Permeable agarose beads are convenient to use (McCafferty et al., 1990) and have a very high capacity per unit volume. Nevertheless it seems unlikely that phage particles, whose long dimension (~1 μm) is orders of magnitude larger than the average diameter of the pores of an agarose gel, can diffuse far into the interior of a bead; for this reason, only receptors tethered at the very surface of a bed may actually be effective at capturing phage. Receptors can be directly attached to the solid support by chemical coupling (Smith and Petrenko, 1997) or non covalent

adsorption to a hydrophobic plastic surface (Smith, 1985). Alternatively, receptor molecules can be biotinylated and allowed to bind to a surface that has already been coated with avidin or streptavidin, thereby attaching them indirectly through the super strong biotin-avidin or biotin-streptavidin bond (Parmley and Smith, 1988). This allows a two-step mode of capture. In the first step, the phage mixture is reacted with biotinylated receptor in homogeneous solution, which overcomes issues of conformational changes of coating proteins to solid surfaces. In the second step, the mixture is reacted with streptavidin-coated solid support in order to capture those phage whose displayed peptide bound the biotinylated receptor during the first step. In principle, at least, two-step capture allows the kinetics of the binding reaction to be controlled without the complications of surface reactions. A promising new variant of affinity selection does not rely on physical capture on a solid support (Duenas and Borrebaeck, 1994). Here, the peptide is displayed on a mutant version of coat protein pIII that is missing its N-terminal domain. Since this domain is required for infectivity, these particles are non-infective. Attaching the missing N-terminal domain to a receptor that binds the phage-borne peptide can restore infectivity. Therefore, only phage displaying peptides that bind the receptor are infective and are thus amplified. Another alternative possibility is an *in vivo* selection, when phage repertoires are directly injected into animals and then tissues are collected and examined for phage bound to tissue-specific cell markers (Smith and Petrenko, 1997). *In vivo* panning has several further advantages: an inherent blocking step is included where most of the phage-displayed peptides that recognise ubiquitous plasma and cell surface proteins are eliminated; these peptides may be useful for the functional analysis of new receptors and potential identification of novel drug target candidates.

1. 5. 2. 2. Washes and elution

After the capture step, the solid support is washed to remove unbound phage and eluted under conditions that release the bound phage without impairing their infectivity. Non specific elution conditions weaken receptor-peptide interactions without regard to their specificity. They exploit the high resistance of filamentous phage to denaturation by acidic buffers with pH's down to 2. 2 (Smith, 1985), alkaline buffers such as 0. 1 M triethylamine, proteases such as trypsin and factor Xa (Smith and Petrenko, 1997). Specific elution releases phage bound to the target receptor's binding site without releasing phage that are bound for some other reason-for example, by interaction with a contaminant, or with the carrier protein that is often used to block non specific adsorption sites on the solid support after the target receptor itself has been immobilised. In competitive elution, a known soluble ligand for the receptor competes with phage for binding to

immobilised receptor (Smith and Petrenko, 1997). This is a two-stage process: the phage-borne peptides must first dissociate spontaneously from the solid support, after that the competitor binds the receptor binding site reducing its availability for rebinding phage-borne peptide. Thus if the time course of dissociation is long on the scale of the experiment, competitive elution will fail. Non-competitive elution, in contrast, relies on a compound that specifically loosens binding by the receptor without binding to its binding site, and without weakening binding interactions in general. For instance, phage bound to a calcium dependent receptor can be eluted with the calcium cheater EGTA (Smith and Petrenko, 1997) increasing the specificity of elution, since only rarely would a non-specifically bound phage happen to be held in a calcium-dependent fashion. It is not inevitably necessary to elute the captured phage at all. Adding fresh bacterial host cells to the solid support allows the captured phage to infect cells and thus be propagated. The yield is generally low, but the first round of selection is probably sufficient to ensure retention of binding clones. The progress of affinity selection through succeeding rounds is ordinarily reflected in increasing affinity of individual phage clones or of entire eluate populations for the target receptor. The affinity of individual clones or entire eluate populations can be assessed quantitatively by standard enzyme-linked immunosorbent assay (ELISA).

1.5.2.3. Yield and stringency

Affinity selection is a very sensitive method and depending on the adjustment of its different phases a completely different cluster of sequences can be collected. There are two pivotal parameters of selection, which can often be manipulated to some extent in order to enhance the efficacy of selection (Smith and Petrenko, 1997). Stringency is the degree to which peptides with higher fitness are favoured over peptides with lower fitness; yield is the fraction of particles with a given fitness that survive selection. The ultimate goal of selection is usually to isolate peptides with high fitness. Stringency is most often controlled by target concentration though other means such as increasing wash time are also effective. High target concentrations (low stringency) ensure the survival of the best ligands, but at the cost of reducing differential enrichment of the better species and hence requiring many rounds of good selection. Low target concentrations (high stringency) yield differential enrichment, but also risk losing some of the best ligands due to their initially very low concentration. As a compromise to allow relatively rapid enrichment of the best species with low risk of their loss, laboratories have various schedules for starting with low stringency that are increased in later generations (Levitan, 1998). The more stringent selection favours higher affinity phage. However, even though mole fraction of the high affinity peak increases each round, this mole fraction is always much lower than that for the

corresponding generation at constant stringency. This happens because decreasing target concentrations lessen the total number of phage that bind target but does not lessen the degree of background binding (Levitan, 1998). As a result, the mole fraction of low-affinity phage is much higher than that in the constant stringency case. Thus while an increasing stringency strategy allows higher affinity ligands to be found, more rounds will be needed to find them with the same probability. For higher rates of stringency increase, the peak migrates to higher affinities more rapidly, but the background contribution decreases very slowly. The best strategy for low background binding is using a large initial target concentration followed by a rapid increase in stringency. Washes in general have only a relatively little effect on the selection process-except of the case when there is very little non specific binding (Levitan, 1998). Long washes can also increase stringency and may be of greater benefit than using lower target concentrations. They affect both target-bound and non-specifically bound ligands. As a result, long washes will not drastically reduce the mole fraction of high affinity ligands but will still remove low affinity ligands compared to high affinity ligands. Including detergents like Tween 20 in wash buffers can also have a similar effect. When too many rounds of biopanning are performed, there is a point, when it becomes progressively more difficult to find a consensus as the best few phage compete the other best phage out of the library and measurement error can cause the continual loss of the best ligands in the library. During amplification between the individual rounds there is the possibility that phase not expressing a real binder sequence but showing extreme good growth properties can overgrow phase of interest therefore the yielded peptide sequence will not be valuable for analysis. Identical peptide sequences with completely identical nucleotide sequences are suspicious of selecting for a phase with favourable growth but not with beneficial affinity. Valence of display has also an effect on stringency as it is described at 1.4.3. Monovalent display is the one, which allows selection of ligands with really high affinity. Furthermore shorter incubation and longer elution times between ligands and targets ensure the selection of high affinity targets.

1.5.3. Effect of conformational constraints

Unlike natural proteins or protein domains, random peptides do not generally fold into a well-defined three-dimensional structure. However, constraints can be artificially imposed on the peptide in order to greatly reduce the range of available conformation. In general, a library of constrained peptides will represent far fewer three-dimensional shapes than a library of unconstrained peptides. The probability that a clone will possess the target activity-affinity for a receptor is correspondingly reduced (Smith and Petrenko, 1997). On the other hand, a

constrained peptide whose accessible conformations happen to overlap extensively with active conformations may possess far higher activity than any unconstrained peptide. The most common constraint on displayed peptides is a disulfide bond between two half-cystine residues at fixed positions in an otherwise random sequence. Because the phage coat proteins are secreted into the oxidising milieu of the periplasm and ultimately secreted into extracellular medium with abundant dissolved oxygen, cysteine residues within a single displayed peptide can be expected to form intrapeptide disulfides in at least a portion of the displayed peptides. Interchain disulfides are much less likely, since the distance between neighbouring coat-protein subunits is at least 10 times longer than a disulfide bond. The disulfide bond has been shown to be required for the ability of the displayed peptide to bind a target receptor (Smith and Petrenko, 1997). In general, the closer the half-cystines, the tighter the constraint imposed on the amino acids lying between them. Thus, disulfides spanning different numbers of amino acid positions would be expected to interrupt very different, mutually exclusive conformational constraints when the numbers are small. In contrast, disulfides spanning more than about six residues probably impose relatively weak constraints that are compatible with a great diversity of conformations. Co-ordination bonds between histidine residues and metal ions can constrain peptides in much the same way as disulfide bonds. A second way of constraining peptides is to present them in the context of a protein scaffold. In this case, random peptides can be presented not only as loop structures, but also as parts of helices, sheets, turns, and other elements of secondary structure (Smith and Petrenko, 1997)

1.5.4. Enrichment and analysis of specific sequence motifs

Increasing fitness is typically accompanied by emergence of a common motif in the amino acid sequences of the selected peptides (sometimes more than one motif). Hence the selected peptides can provide insight into the minimum size and composition requirements for binding to a protein or to a drug. (Rodi, Makowski, and Kay, 2002) Though subsets of peptide ligands share a consensus the clear-cut identification of that consensus may not be easy. For example, SH3 domains, where a single, well-defined, peptide-binding cleft is involved in the peptide-protein interaction, a relatively small number of sequences can lead to the definition of a motif (Rood, Makowski, and Kay, 2002). On the other hand, for a protein target such as actin, which is known to bind to several proteins along different surfaces, such a simple result would not be expected, and many peptides need to be sequenced and sorted in order to identify these multiple binding motifs. Using oligopeptide libraries there is usually a small chance that the peptide with the highest affinity for the target is in that library. However, there is a relatively high chance that

peptides with similar sequences are present and will be selected. Although there are well documented cases where conservative amino acid substitutions alter affinities by orders of magnitude, in many cases, conservative and even semi-conservative substitutions may not greatly alter affinity (Rodi, Makowski, and Kay, 2002).

1.6. APPLICATIONS OF PHAGE-DISPLAY

1.6.1. Epitope mapping and mimicking

An epitope is the small determinant on the surface of a ligand with which the receptor makes close, geometrically and chemically specific contact. If the ligand is a protein, the epitope is sometimes continuous, comprising a few adjacent critical amino acids in the primary sequence. For instance, antibodies specific for continuous epitopes on protein antigens typically contact three to four critical amino acids over a six-residue segment. More often, however, protein epitopes are more complex. Many are discontinuous comprising critical binding residues that are distant in the primary sequence but close in the folded native conformation. Several epitopes, including discontinuous ones, are conformation-dependent because they require the context of the overall protein structure to constrain them in a binding conformation (Smith and Petrenko, 1997).

It is often desirable to map the epitope to a confined portion of the natural protein ligand. If the epitope is (or might be) continuous and not conformation dependent, random peptide libraries provide an easy approach to this goal. The receptor is used to affinity select random peptide ligands, and the sequence motif in the selected peptides is compared to the amino acid sequence of the natural ligand. In these cases the motif frequently matches clearly the critical binding amino acids in the natural protein ligand, thereby mapping the epitope to a very narrow part of the overall natural ligand structure. Only rarely will a random peptide library contain a binding motif extending to more than about six amino acids or adequately represent conformation-dependent or discontinuous epitopes. Although receptors recognising such epitopes often select ligands from random peptide libraries, these artificial ligands seldom bear a recognisable similarity to any part of the natural protein ligand at the amino acid sequence level. Alternatively gene-specific libraries displaying 15-100 amino acid segments of the natural amino acid sequence can be constructed, which are long enough to occasionally include small elements of secondary structure from the native protein (Smith and Petrenko, 1997). Such libraries sometimes contain good ligands for receptors that fail to select ligands from random peptide libraries. Because it requires construction of a specific library for each new ligand gene, however, this approach is much more demanding than use of all-purpose random peptide

libraries. Affinity selection from random peptide libraries often reveals entirely unexpected ligands, which do not match any linear epitope and which could not have been anticipated from even extensive knowledge of the receptor and/or its natural ligand. This is especially so when the receptor's natural epitope is non-proteinaceous or is a discontinuous or conformation-dependent protein epitope. Geysen and his colleagues introduced the term "mimotope"(Geysen et al., 1987) to refer to small peptides that specifically bind a receptor's binding site (and in that sense mimic the epitope on the natural ligand) without matching the natural epitope at the amino acid sequence level. The definition includes cases where the natural ligand is non-proteinaceous.

1.6.2. Identifying new receptors and natural ligands

A ligand for a receptor can be used as a probe to identify new receptors that bind the same ligand. In a few very favourable cases, identifying peptide ligands from a random peptide library may suffice to find the natural ligand for an orphan receptor - a receptor whose natural ligand is unknown (Smith and Petrenko, 1997).

1.6.3. Drug discovery

Many of the receptors used in affinity selection are targets of drug discovery programs, and the peptide ligands selected by them are therefore potential leads to new drugs (Smith and Petrenko, 1997). Such peptides might act as receptor agonists or antagonists (for example, of enzymes or hormone receptors) or otherwise modulate the receptor's biological effect. Affinity selection resembles in essence the traditional approach to drug discovery: screening libraries of synthetic compounds or natural products for substances that bind the target receptor and that might therefore be leads to new agonists, antagonists, or modulators. However affinity selection has the key advantage that the scale of the search is many orders of magnitude greater than is feasible when chemical libraries must be screened compound by compound - billions of peptides versus tens of thousands of chemicals. On the other hand, for most pharmaceutical applications, peptides have poor pharmacological properties, being generally orally unavailable and subject to rapid degradation in the body by naturally occurring enzymes. The most important contribution of phage display to drug discovery will be confined to applications where peptides themselves can serve as plausible therapeutics.

1.6.4. Epitope discovery in vaccine development and diagnostic

When the receptor used for affinity selection is an antibody, the peptides it selects from random peptide libraries are called antigenic mimics of the corresponding natural epitope. When these

peptides are used in turn to immunise naive animals, some are able to elicit new antibodies that cross-react with the natural epitope, even though the naive animals have never been directly exposed to it (Smith and Petrenko, 1997). Such peptides are immunogenic mimics as well as antigenic mimics. However, not all antigenic mimics are immunogenic mimics. Most small peptides are flexible, and they might adopt one conformation when it binds the selector antibody but myriad other conformations when it elicits new antibodies, few if any of which would therefore cross-react with the authentic epitope. On the other hand a peptide may be an antigenic mimic without being a true structural mimic. Such a peptide would bind the selector antibody in an entirely different way than does the original authentic epitope. Such a peptide would be expected to elicit new antibodies that fit it in an altogether different way than does the original selector antibody. Only rarely and coincidentally would these antibodies cross-react with the authentic epitope. Antigenic and immunogenic mimicry are the basis of epitope discovery, a new approach to disease diagnosis and vaccine development (Smith and Petrenko, 1997). Most diseases- particularly infectious ones - leave their imprint on the complex mixture of antibody specificities that comprises the total serum immunoglobulin population. Included in this population are disease-specific antibodies - some elicited directly by antigens on a pathogen, others possibly recognising antigens that reflect the disease process more indirectly. When total serum antibody from a patient is used to affinity select clones from a random peptide library, therefore, some of the selected ligands will correspond to disease-specific antibodies. Of course the patient's pool of antibodies will contain many non-disease-specific antibodies, too, so it may require extensive counter-selection or screening with antibodies from control subjects (not suffering from the disease) to identify those peptides that correspond to authentic disease-related antibody specificities and that therefore can be considered diagnostic for the disease. Peptides obtained through epitope discovery have two main uses (Smith and Petrenko, 1997). First, as antigenic mimics they serve as specific probes for antibodies that are diagnostic for the diseases, much as natural viral proteins serve in current tests for HIV. Their advantages over natural antigens as diagnostic reagents include that they are easier and cheaper to discover and manufacture, that they can focus on a few particularly diagnostic specificities and exclude potentially confusing signals from non-diagnostic determinants, and that they can be discovered and used even when the natural antigens associated with the disease are entirely unknown. The second possible use of peptides obtained through epitope discovery is as components of synthetic vaccines (Smith and Petrenko, 1997). Only antigenic mimics that are also immunogenic mimics are useful in this regard, of course, since in order to be protective an antibody must react with a natural epitope on the actual pathogen. Small segments of different proteins displayed on M13

virus particles were used to elicit antibodies against the coat proteins of parasites and viruses. The immunological response to injected M13 is T-cell dependent and does not require an adjuvant.

1.6.5. Single-chain antibody libraries

One of the most powerful applications of phage display has been the isolation of recombinant antibodies with a unique specificity (Hoogenboom *et al*., 1991). In this regard, phage display technology can be used to 1.) generate human monoclonal antibodies or humanise mouse antibodies, significant for cancer immunotherapy, 2.) to isolate human antibodies from patients exposed to certain viral pathogens to better understand the immune response during infection and how protective antibodies are generated, and 3.) to elucidate the specificity of autoimmune antibodies. Both Fab (consisting V_H-C_H and V_L-C_L segments linked by a disulphide bond) and the variable fragment (Fv) were expressed on the surface of M13 viral particles with no apparent loss of the antibody's specificity and affinity. To construct a single chain variable fragment (scFv) library using phage display, genes of variable heavy (V_H) and variable light (V_L) chains of antibodies are prepared by reverse transcription of mRNA obtained from B-lymphocytes. The heavy and light chain gene products are amplified and assembled into a single gene using a DNA linker fragment. The assembled scFv DNA fragment is inserted into a phagemid vector and the recombinant phagemid is introduced into competent *E. coli* by $CaCl_2$ transformation or electroporation. Ligation and bacterial transformation are crucial, as they directly influence the size of the library. Phagemid-containing bacterial cells are grown and then infected with a helper phage to yield recombinant phage that display scFv antibody fragments as fusion to one of the phage coat proteins.

1.6.6. Selection of DNA-binding proteins

Phage display may help to design proteins that specifically bind a given target DNA sequence. A promising approach is to construct a library of randomised variants of a parent DNA binding domain (e.g., one of the zinc-finger domains, a common DNA-binding motif in eukaryotic nuclei) displayed on a filamentous phage; randomisation is concentrated on positions that are thought to make sequence-specific contacts with the target DNA in the parent domain. From this library, clones that bind a new target DNA sequence, different from that recognised by the parent domain, are then affinity-selected (Rebar and Pabo, 1994).

1.6.7. Landscape libraries

The surface landscape of a filamentous virion is a cylindrical array of thousands of repeating subunits composed of the exposed parts of the major coat protein pVIII accounting for about half the weight of the particle. When a random peptide is displayed on every copy of this protein, it subtends a major fraction (20% or more) of the repeating unit and thus of the entire particle surface. Unless the random peptide is loosely tethered to the bulk of the major coat protein, it is forced to interact with residues in its immediate neighbourhood, and may therefore be constrained in a definite three-dimensional conformation that differs markedly from the surface conformation of wild-type particle and of clones displaying other random peptides {Smith and Petrenko, 1997}. A large population of such clones can therefore be regarded as a library of organic landscapes. The ensemble of a random peptide in a landscape library with its surrounding wild-type residues may have emergent properties that are lost when the peptide is excised from its context. Such peptides are analogous to the complementarity-determining regions of antibodies: oligopeptide loops that in the context of the intact protein make most of the specific contacts with antigen but as free peptides seldom have appreciable antigen-binding propensities. Localisable emergent properties are present even when the foreign peptide is displayed on only an occasional pVIII molecule, as in type 88 and 8·8 systems. Nevertheless, the high-density display in landscape phage may greatly enhance overall effectiveness in some applications. For instance, if a single target receptor complex can bind two or more neighbouring peptides on the phage surface, the overall effective affinity may be enhanced many orders of magnitude compared to monovalent binding. Some emergent properties are not localisable to a single subunit but seem instead to be a global property of the entire surface landscape.

1.6.8. Ribosomal display

A major limiting step of constructing a large-size phage display library is the transformation efficiency. Libraries smaller than 10^9 independent members prepared in *E. coli* are smaller by several orders of magnitude due to less efficient transformation of bacterial cells.

Ribosome display was developed to overcome this limitation (Schaffitzel *et al.*, 1999). This *in vitro* technology aims for simultaneous selection and evolution of proteins from diverse libraries without any bacterial transformation. In ribosome display, DNA (which encodes a protein library) is first transcribed to mRNA that is then purified and used for in vitro translation. *In vitro* translation of mRNA is designed in such a way to prevent dissociation of mRNA, ribosomes, and the translated peptide. Such mRNA-ribosome-peptide complexes are then used

for affinity selection on an immobilised target where only the complexes that do not encode a binding polypeptide, that specifically recognises the target antigen, are removed by washing. The mRNA, that encodes a polypeptide cognate for the target, is then dissociated from the ribosomal complexes and reverse transcribed into cDNA. The prepared cDNA is then amplified by PCR and used for the next cycle of enrichment and PCR and/or analysed by sequencing. Because no transformation is necessary, large libraries can be constructed and used for selection. Additionally, library diversification is suitably introduced either before starting or in between cycles of ribosome display via DNA shuffling or error-prone PCR. Ribosome display has been successfully applied to both peptides and folded proteins.

1.6.9. Diagnostic applications.

Phage display is a newcomer in the detection area, which expertise in the development of molecular probes for targeting of various biological structures. Specific proline-rich peptide ligands binding to the coat protein of cucumber mosaic virus were selected from a nonamer pVIII library. Binding peptides were also selected against botulinum neurotoxin. Phage antibodies were isolated against several viruses and spores of the genus *Bacillus* (Petrenko and Vodyanoy, 2003).

1.6.10. Applications of phage display in this thesis work

After describing the main aspects of phage display I want to present two application possibilities in two different fields of microbiology. The first section deals with identification of *Bacillus* spores, the second one with mapping the laminin binding site of *Yersinia pestis* plasminogen activator. The actuality of these subjects is underlined by the fact that both *Bacillus anthracis* and *Yersinia pestis* are re-emerging pathogens with global significance. It is pertinent to note that these agents genuinely are zoonotic pathognes but they also have the potential to be weaponised. A further remarkable common feature of the two pathogens is that both can spread through inhalational route. In this way these bacteria impose a threat as possible or even factual means of bioterrorism. In June 1999, an American meeting of national experts was convened to review agents that might be utilised as biological weapons or tools of bioterrorism. A list of agents for public health preparedness to respond against attacks of bioterrorism was developed and placed into three categories. Both *B. anthracis* and *Y. pestis* were placed into Category A including agents considered to have the greatest potential for adverse public health impacts (Rotz *et al.*, 2002).

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Concerns about the use of *B. anthracis* as a biological weapon have re-emerged in the United States when spore containing envelopes were sent to selected individuals via the postal systems (CDC, 2001).

Plague – probably because of its success in devastating Europe in the 14th century – is generally regarded as a disease of the Middle Ages. In fact, *Yersinia pestis* is known to have caused at least three pandemics, and there have been a steady increase in reported plague morbidity since the early 1990s (Dennis, 1999). The potential of plague to induce panic and economic loss was demonstrated during the 1994 outbreak of pneumonic plague in Surat, India. The international response resulted in increased control at ports and airports including people being placed in quarantine, and embargoes were placed on the import of goods and movement of people from India. The final death toll was 54 people, and the total cost of this outbreak to India was estimated at 2 billion US $ (Cash and Narasimhan, 2000). Interestingly enough, in spite of the public and media alertness and the public health concern no positive sputal culture was achieved.

All the above-presented data support the assumption that an intensive research aiming at the fast and specific diagnosis, control and prevention of these infections is justified. Basic research on the spore surface proteins of *Bacillus spp.* including *B. anthracis* and the virulence associated outer membrane protein Pla of *Y. pestis* might supply data applicable in those practical fields listed above. As phage display is a well-established method for the identification and functional investigation of peptide domains we applied this assay system to analyse spore coat proteins and Pla.

2. UTILISATION OF RANDOM PHAGE DISPLAY LIBRARIES FOR IDENTIFICATION OF *BACILLUS* SPORES

2.1. INTRODUCTION

2.1.1. Formation and structure of *Bacillus subtilis* endospores

The genus *Bacillus* includes a diverse collection of gram-positive, rod-shaped, aerobic, spore-forming bacteria. The best studied of the spore forming bacteria is one with no special powers other than the ability to be readily manipulated in the laboratory: *Bacillus subtilis*. Spore formation, referred to as sporulation, occurs in a series of stages that can be monitored by light and electron microscopy and, once initiated, requires approximately 8 h to complete. The morphological process of sporulation is driven by a temporally and spatially controlled program of gene expression (Driks, 2002). Commencement of this program requires that the cell has reached a certain stage in the cell cycle that the tricarboxylic acid cycle is intact, that at least one extracellular pheromone is present in the appropriate amount, and that an unknown environmental stimulus has activated a complex phosphorylation cascade. When these conditions are met (as they are during starvation), sporulation ensues and the pattern of vegetative gene expression is largely replaced with the specialised program of sporulation gene expression. The rapid series of morphological changes that ensue during sporulation is due, in large part, to the sequential appearance of a series of transcription factors, called sigma factors, which bind to core polymerase and direct it to transcribe only from specific promoters (Driks, 2002). An early and dramatic morphological event is the formation of the asymmetrically placed sporulation septum, which divides the cell into the forespore and mother cell compartments, at about the second hour of sporulation. The smaller compartment (the forespore) will go on to become the spore. The larger compartment (the mother cell) will serve to nurture the spore until its development is complete. After the sporulation septum is laid down, the sporulation gene expression program splits and two distinct programs become active, one in each of the resulting cellular compartments. These two divergent programs of gene expression, in the mother cell and in the forespore, result in the spore being built from the outside (as a result of protein synthesis in the mother cell) and from the inside (as a consequence of the proteins produced in the forespore). At about the third hour, the edge of the septum migrates in the direction of the forespore pole of the cell, pinching the forespore compartment off to become a protoplast which sits free in the mother-cell cytoplasm and is surrounded by a double layer of membrane. After engulfment, a cell wall-like material is deposited between the membrane layers that surround the forespore.

This cell wall has two layers: an inner layer, called the germ cell wall, which will become the peptidoglycan layer of the nascent cell after germination, and an outer layer, called the cortex, which participates in maintenance of the dehydrated state of the spore. This is followed by formation of the coat from proteins synthesised in the mother cell, which then assemble around the forespore. The coat is evident by about the fifth hour of sporulation. Two major coat layers can be discerned in the electron microscope: a darkly staining outer coat and a more lightly staining lamellar inner coat. The final step in sporulation is lysis of the mother cell and release of the fully formed spore. Coat assembly in *B. subtilis* occurs in stages that are tightly coupled to the well-described developmental program driving spore formation (Driks, 2002). The interior-most compartment of the spore houses the chromosome. This compartment is surrounded by two membrane layers derived from the septum formed at the beginning of sporulation. A specialised peptidoglycan (the cortex) resides between these membranes, the outermost of which is the site of coat protein deposition. In *B. subtilis*, electron microscopy reveals a darkly staining outer layer (the outer coat) and a more lightly staining inner layer (the inner coat). The coat comprises approximately 30 protein species that do not resemble one another or, in general, any other proteins in the databases. In most cases, the deletion of any one coat protein in *B. subtilis* has only a subtle effect on the coat beyond the absence of that protein, suggesting that important functions of the coat remain undetected by current assays. Exceptions to this pattern are the morphogenetic proteins SpoIVA and CotE, which have especially striking effects on coat assembly. SpoIVA designates the spore surface as the site of all subsequent deposition of coat protein and connects the coat to the spore (Driks, 2002). The function of SpoIVA is more complex than this, however; in its absence, the thick specialised peptidoglycan residing just beneath the coat never accumulates to wild-type level. SpoIVA resides at the membrane separating the coat and the peptidoglycan, the two structures whose synthesis it co-ordinates. CotE forms a shell around the spore well before most coat proteins are synthesised. In its absence, the outer coat fails to form (Driks, 2002). Consistent with this, CotE directs the deposition (but not the synthesis) of at least eight proteins, most of which reside in the outer coat. The inner coat forms as a layer underneath the CotE shell and, like the outer coat, is not evident until after CotE is in place (Driks, 2002). Unlike the outer coat, however, the formation of the inner coat is largely CotE independent. The carboxy-terminal 28 amino acids of CotE, which possess a preponderance of acidic residues, are crucial for coat protein deposition (Driks, 2002). The amino acid differences between CotE homologues in *B. subtilis*, *B. anthracis*, *B. stearothermophilus* and *Bacillus halodurans* are particularly pronounced in the carboxy-terminal

28 residues. Thus, the differences in coat protein composition between these species could, to a large degree, be a function of sequence variation at the CotE carboxyl terminus.

Two interacting proteins, SpoVID and SafA, also play important morphogenetic roles in coat assembly. Intriguingly, like CotE, both SpoVID and SafA appear to have a carboxy-terminal module that varies among the spore formers (Driks, 2002). In contrast to CotE, these varying regions extend across the majority of the SpoVID and SafA sequences. Thus, the varying regions of these proteins probably comprise domains. Carbohydrates are also important components of spore structure. At least some of the coat proteins undergo modification of several types including also glycosylation. Loss of the gene products of the spore polysaccharide biosynthesis determinants (*spsA-K*) resulted in a hydrophobic spore coat. SpsA is the only well characterised member of the cluster. It is a glycosyltransferase implicated in the synthesis of the spore coat of *Bacillus subtilis*, whose homologues include cellulose synthase and many lipopolysaccharide and bacterial O-antigen synthases. Mutations in the *cgeAB* and *cgeCDE* operons of *B. subtilis*, produce spores with altered surface properties leading to the hypothesis that proteins encoded by the cge operons influence maturation of the outermost layer of the spore, most likely by glycosylation of coat proteins at the spore surface (Roels and Losick, 1995).

If conditions are suitable, the spore can germinate and thereby convert back into a growing cell. When this occurs, first the spore core rehydrates and swells and then the coat cracks, releasing the nascent cell.

2.1.2. Formation and structure of *B. anthracis* spores

In spite of its importance to spore survival, germination, and pathogenesis, the spore coat assembly and protein composition of the *B. anthracis* coat has received a broader study only recently when gene expression during growth and sporulation was monitored with DNA microarrays and tandem mass spectrometry (Liu et al., 2004). The majority of what is thought to be true about the *B. anthracis* spore and how is made is based on the assumption that the large body of experimental data accumulated through the characterisation of sporulation in *B. subtilis* will apply to *B. anthracis*. The complex genomical and proteomical analysis revealed a similar cascade of sigma factor expression as it was already described in *B. subtilis*. Furthermore, it seems likely that gene expression during sporulation may be mainly related to the physical construction of the spore rather than synthesis of eventual spore events. Comparison of the *B. subtilis* and *B. anthracis* genomes also shows that coat proteins with key roles in morphogenesis are present in both organisms and therefore, it is plausible that coat assembly follows largely the

same program in the two species (Liu *et al.*, 2004). Given the current understanding of coat assembly in *B. subtilis*, comparison of its genome with that of *B. anthracis* is particularly revealing. Both SpoIVA and CotE have unambiguous homologues in *B. anthracis* suggesting that coat assembly in the pathogen follows a similar overall program to that of its benign relative (Lai et al., 2003). This supports the view that *B. anthracis* possesses inner and outer coat layers, although the morphological distinction between them is less evident than in *B. subtilis*. On the other hand only a small fraction of the late assembled or synthesised coat and coat associated proteins in *B. subtilis* have *B. anthracis* homologues. Thus, the major differences between these species could be among the outer coat proteins. For *B. anthracis*, as for many *Bacillus* species the spore is enclosed by a prominent, loose-fitting, balloon-like layer called the exosporium, which is composed of a basal layer and an external hair-like nap and serves as a primary permeability barrier (Gerhardt, 1967). The aforementioned comprehensive genomic study identified the CotZ and CotJ proteins also described in *B. subtilis* as structural components of the *B. anthracis* exosporium.

2.1.3. Diagnostic methods for *Bacillus* identification

The gram-positive soil bacterium *Bacillus anthracis*, the causative agent of anthrax, has been developed into a weapon of mass destruction by numerous foreign governments and terrorist groups (Turnbough, Jr., 2003). The use of *B. anthracis* as a biological weapon, with severe consequences, was demonstrated in the fall of 2001 in the United States. *B. anthracis* is an effective agent for biological warfare and terrorism primarily because it forms spores being resistant to extreme temperatures, noxious chemicals, desiccation, and physical damage. These properties make them suitable for incorporation into explosive weapons and for concealment in terrorist devices (Turnbough, Jr., 2003). Spores enter the body through three routes: by skin abrasions, by ingestion or inhalation. Once exposed to internal tissues, the spores germinate and vegetative cell growth ensues, often resulting in the death of the host within several days (Turnbough, Jr., 2003). Natural strains of *B. anthracis* are sensitive to common antibiotics that can be used to treat anthrax. However, to ensure a successful outcome, treatment must begin within a day or two after exposure to spores (Turnbough, Jr., 2003). Thus, rapid detection of *B. anthracis* spores is critical in responding to the anthrax threat. Several detection systems are currently used to identify *B. anthracis*. The most accurate systems employ either PCR-based assays or traditional phenotyping of cultured bacteria (Turnbough, Jr., 2003). However, these methods are complex, expensive, cumbersome, and slow, typically requiring spore germination

and outgrowth of vegetative cells. Other systems are based on antibody binding to spore surface antigens. These systems are relatively fast because they detect spores directly. However, current antibody-based detectors suffer from a lack of accuracy and limited sensitivity, which result in an unacceptably high level of both false-positive and false-negative responses, according to federal government trial in the United States and other, independent tests (King et al., 2003). The lack of accuracy with these systems is compounded by the normal presence in the environment of *Bacillus* spores that resemble (and share surface antigens with) *B. anthracis* spores. Particularly problematic are spores of the opportunistic human pathogen *B. cereus* and the insect pathogen *B. thuringiensis*, species which, based on genome sequence comparisons, are the most similar to *B. anthracis* (Read et al., 2003). These three species, along with *B. mycoides*, comprise the phylogenetically similar *B. cereus* group (Turnbough, Jr., 2003). Therefore, due to the aforementioned limitations and deficiencies, all currently available systems for detecting *B. anthracis* are inadequate for frontline use by emergency workers and soldiers on the battlefield and for routine monitoring of public areas. Clearly, there is an urgent need for a better detector that can be used where the threat of *B. anthracis* spore exposure is the greatest. The desired detector will, in all probability, require simple and hardy ligands capable of tight and specific binding to *B. anthracis* spores. Because it is impractical to use pathogenic spores (or in some cases even non pathogenic variants of these spores) in the early stages of detector development, we constructed a model detection system involving a safe prototypical spore and a standard small-molecule ligand capable of binding this spore: peptide ligands that bind tightly to spores of *B. subtilis*. The peptide ligands were identified by biopanning a library of phage-displayed peptides against *B. subtilis* spores.

2.2. AIMS OF THE STUDY

1. Selection and identification of heptamer and dodecamer peptides or potential heptamer and dodecamer peptide families binding tightly to *B. subtilis* and *B. anthracis* spores with the utilisation of a commercially available random phage-display library
2. Characterisation of selected sequences, assessment of structural criteria for binding.
3. Development of fluorescent labelling methods for tagging the selected phage or the chemically synthesised peptide sequences.
4. Improvement of flow cytometry assays suitable for discrimination of different spore types.

5. Performance of sequence similarity searches with the selected peptide sequences to recognise protein sequences mimicked by the phage displayed peptides.

2.3. MATERIALS AND METHODS

2.3.1. Phage display and associated methods

2.3.1.1. Phage display libraries

Commercially available random phage display libraries expressing heptamer and dodecamer peptides (Ph.D.-7 and Ph.D.-12, New England BioLabs, MA, USA) were utilised for screening spore ligands. The Ph.D.-7- and Ph.D.-12 libraries contain random heptamer and dodecamer peptides, respectively, fused to the amino terminus of the M13 minor phage capsid protein pIII. This protein is represented in five copies at one end of the filamentous phage particle. Therefore each phage displays five copies of an individual peptide whose sequence is encoded in the recombinant pIII gene. For propagation of eluted phage *Escherichia coli* strains ER2537 [F'lacI^q Δ(lacZ) M15 proA$^+$B$^+$ fhuA2 supF thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 ($r_k^-m_k^-$ McrBC$^-$)] (New England Biolabs) and XL1 Blue MRF'(recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB, lacI^q ZΔM15, Tn10 (Tet^r), Cam^r] (Stratagene) were used. Strain ER2537 was maintained on minimal agar (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1g/l NH_4Cl, 0.5 g/l NaCl, 0.3 mg/ml $CaCl_2$, 1 mM $MgSO_4$, 2g/l glucose, 0.5 mg/l thiamine, 15 g/l agar), strain XL1 Blue MRF' was maintained on Luria-Bertani agar (10 g/l Bacto trypton, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) supplemented with 20 μg/ml tetracycline. Both strains were grown in Luria-Bertani broth for XL1 Blue MRF' supplemented with tetracycline to a final concentration 20 μg/ml.

2.3.1.2. Biopanning

Biopanning of spores was performed in Eppendorf tubes. 10^9 spores were mixed with 10^{11}phage from the library in 1 ml of sterile Tris buffered saline-Tween 20 (TBST) [50 mM Tris-HCl (pH 7.5), 150mM NaCl, 0.5% Tween 20] for 10 minutes at room temperature. Tween 20 was included to break non-specific interactions and being able to form a compact spore pellet. The spore-phage complexes were collected by centrifugation (12,000×g) at 4°C for 10 min, and the supernatant was removed. Spore-phage complexes were washed ten times in 1-1 ml of TBST with alternating resuspension and centrifugation. After the final wash, spore-phage complexes were resuspended in 1 ml of elution buffer [0.2 M glycine-HCl (pH 2.2), 1 mg/ml bovine serum albumine (BSA)] and then mixed gently for 5 min at room temperature. This sample was

centrifuged as above for 5 min. The supernatant, which contained eluted phage, was quickly removed and neutralised by the addition of 150 µl of 1 M Tris-HCl (pH 9.1) to prevent phage killing. During biopanning small fractions of the input phage, supernatants from the initial collection of spore-phage complexes and selected washes and eluted phage were saved for titering. Eluted phage were amplified by infecting *E. coli* strain ER2537. The resulting phage stock was used for a second round of biopanning, which was performed exactly as described above. A total of four rounds of biopanning were performed, after which the final eluted phage were plated to obtain single plaques. These plaques were used to prepare phage stocks, from which genomic DNA was extracted and the peptide-encoding region of DNA determined.

2.3.1.3.Competitive biopanning

Competitive biopannings were performed in a similar way as the original starter biopanning described in the previous section except of the following modifications: Besides the original library a phage stock with a unique sequence of interest was also added. This unique sequence represented only 0.1 % of the random library sequence, which practically meant the usage of 10^{10} random phage from the library and 10^{7} unique sequences as input. Three rounds of biopanning were performed and pools of the individual eluates were sequenced after the eluate fractions had been propagated in the ER2537 host strain and single stranded phage DNA had been extracted.

2.3.1.4. Concentration of propagated phage

Host cell cultures (usually 20 ml) were centrifuged twice at 4000 rpm, 4°C for 10 minutes. The upper 80% of the supernatant was precipitated overnight with 1/6 volume of PEG/NaCl (20% polyethylene-glycol 8000, 2.5 M NaCl). The precipitate was collected by centrifugation under the same conditions as before and the pelleted precipitate was dissolved in 1 ml of TBS and re-precipitated in 1/6 volume of PEG/NaCl (167 µl) for an hour on ice. The formed precipitate was microfuged at 12,000×g at 4°C for 10 min and resuspended in 200 µl TBS-0.2% NaN_3. This phage stock was used for titration.

2.3.1.5. Phage titration

From phage stocks tenfold dilutions were made in Luria-Bertani broth. 10 µl aliquots were incubated with 200 µl of mid-log phase culture of either ER2537 or XL1 Blue MRF' strains for 10 minutes at room temperature and then plated with 3 ml of Agarose Top (10 g/l Bacto-

tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l $MgCl_2.6H_2O$, 7 g/l agarose) onto - to 37°C-prewarmed LB -agar plates. After overnight incubation at 37°C phage plaques were counted and the titre was calculated and given in pfu/ml.

2.3.2. Bacillus strains and growth conditions

2.3.2.1. Bacillus strains

The following Bacillus strains were used: *B. subtilis* (*trpC2*) 1A700 (originally designated 168), *B. amyloliquefaciens* 10A1 (originally H), *B. licheniformis* 5A36 (originally ATCC 14580), *B. pumilus* 8A3 (originally ATCC 7061) from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA. *B. globigii* (also called *atrophaeus* and *subtilis* variety „niger") *B. thuringiensis* subsp. kurstaki, *B. thuringiensis* B8, *B. cereus* T and non-enapsulated Sterne (pXO2⁻) and non-toxigenic AAmes (pXO1⁻) strains of *B. anthracis* were from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA. *B. thuringiensis* Al Hakam, *B. thuringiensis* 3A FRI-41, *B. thuringiensis* USDA HD-571, *B. cereus* ATCC 4342, *B. cereus* F1-15 FRI-43, *B. cereus* D17 FRI-13, and *B. cereus* S2-8 FRI-42 from Los Alamos National Laboratory, NM, USA. *B. mycoides* ATCC 10206 and *B. megaterium* ATCC 14581 were from the American Type Culture Collection, Manassas, VA, USA.

2.3.2.2. Sporulation

Spores were produced except for *B. pumilus* using the medium exhaustion method by cells grown in liquid Difco sporulation medium (DSM) at 37°C (8 g/l Difco nutrient broth, 2.5 g/l $MgSO_4.7H_2O$, 1 g/l KCl, 0.01 mM $MnCl_2$, 0.001 mM $FeSO_4$, and 10 mM $CaCl_2$, pH 7.1) with shaking at 150 rpm or on solid DSM (1.5%) agar until sporulation was essentially complete, usually in 48-72 hours. *B. pumilus* spores were prepared by growing cells on solid DSM at 30°C. Spores were collected and washed with cold, sterile, distilled water by centrifugation and purified by sedimentation through two-step gradient of 20% and 50% Renographin 60 (Bracco Diagnostics, NJ, USA) and then washed again extensively in cold, sterile, distilled water. Spores were stored protected from light in sterile distilled water at 4°C and washed every other week to prevent germination. Then they were quantitated microscopically using a Petroff-Hauser counting chamber (Sigma, NJ, USA) and were checked for the absence of germinating spores. Only freshly prepared spores of *B. globigii* were used in the studies shown because these spores gradually loose their capacity for peptide binding over several months. AAmes spores of *B. anthracis* were prepared by Dr. Joanie Jackman at USAMRIID and killed by gamma irradiation before use.

2.3.3. Fluorescent labelling techniques and flow cytometry

2.3.3.1. Fluorescent labelling of anti M13 antibody

1 mg batches of monoclonal anti M13 (anti pVIII) antibodies (Amersham Biosciences, USA) were labelled with the amine-reactive Alexa 488 Protein Labelling Kit (Molecular Probes, USA) according to the instructions of the supplier. Concentration of the antibody-dye conjugate was determined by the following equation: protein concentration (M) =[A_{280}- (A_{494}×0.11)]. dilution factor /203,000 where 203,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of a typical IgG and 0.11 is a correction factor to account for absorption of the dye at 280 nm. Degree of labelling was calculated with the following equation: moles dye per mole protein = A_{494}× dilution factor/[71,000 ×protein concentration (M)] where 71,000 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of the Alexa Fluor 488 dye at 494 nm.

2.3.3.2. Fluorescent labelling of M13 phage

Samples of M13 phage displaying a particular peptide were labelled using Alexia Fluor® 488 Protein Labelling Kit (Molecular Probes, OR, USA) as well. Labelling conditions were those essentially provided by Molecular Probes except that 4×10^{12} phage particles were labelled instead of 1-mg protein sample. This amount of phage corresponds to 1mg of protein based on the known molecular weight of viral capsid proteins. Labelled phage were precipitated from the reaction mixture by adding 1/6 volume of PEG/NaCl, mixed thoroughly and allowing the sample to stand for 1 hour at room temperature. The labelled phages were collected by centrifugation (10,600 g) at 4°C for 15 minutes, and the supernatant was removed. The phage pellet was suspended in 1ml of PBS (phosphate buffer saline - 135 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 pH 7.4), and the phage were precipitated again with PEG/NaCl for 20 minutes and collected as above. The phage were resuspended in 0.4 ml of PBS and stored in the dark at 4°C. The phage concentration and the degree of labelling were determined by measuring the absorbance of a diluted labelled phage sample at 494 nm and at 280 nm and employing conversion factors equivalent to those provided by Molecular Probes. Phage/ml = [A_{280}-(A_{494}×0.11)] × dilution factor× (5×10^{12}phage/ml). Dye molecules/phage = A_{494}×dilution factor× (8.5×10^{15} molecules/ml)/ (phage/ml).

2.3.3.3. Fluorescent labelling of synthetic peptide

Peptides were chemically synthesised with a three-glycine-spacer and a C-terminal cysteine

group to allow conjugation to the fluorochrome R phycoerythrine (Molecular Probes, OR, USA) through a crosslinker molecule and purified by HPLC (UAB Peptide Synthesis Core Facility, AL, USA). Peptide molecules were attached to R-phycoerythrin (RPE) (Prozyme, CA, USA) by using the heterobifunctional crosslinker sulfosuccinimidyl-4- (N-maleimidomethyl)-cyclohexane 1-carboxylate (SMCC) (Pierce) following the manufacturer's instructions. SMCC is able to bind lysine residues of RPE through its reactive N-hydroxy-succinimide-ester group and the C-terminal free SH-groups of the peptides through its reactive maleimide group.

2.3.4. Flow cytometry

10^7 spores were mixed either with unlabelled (for fluorescently labelled anti M13 labelling) or Alexa 488-labelled M13 phage (10^{10} or 10^{11}) or with a peptide-RPE conjugate in various concentrations [4, 40 400 and 4000 nM for testing *B. anthracis* binders, 0.135 mg/ml (»500 nM) for *B. subtilis* binders] in 20 µl of PBS and incubated at room temperature for 1 hour to ensure complete binding. Unbound conjugate molecules were removed by washing spores three times in PBST (0.1 % Tween 20). Spores were collected after each wash by centrifugation at 8200×g for 5 min at 4°C. In case of using fluorescently labelled antibodies spore-phage conjugates were resuspended in 0.5 µg/ml solution of fluorescently labelled anti M13 antibody and incubated for one more hour at room temperature. Unbound antibodies were removed by centrifugation as desribed above. Spore conjugate complexes were resuspended in 200 µl of PBS and fluorescence was measured using a BD FACSCalibur instrument and analysed with CellQuest Pro software (Beckton Dickinson Biosciences, CA, USA). Spores were identified by their light-scattering properties, and 20,000 spores were analysed for associated fluorescence.

2.3. 5.Molecular biological methods

2.3.5.1. Extraction of single-stranded phage DNA, sequencing

Single-stranded phage DNA was extracted either from the concentrated phage stocks or from the supernatant of the propagated host cell cultures because these fractions contain mature phage particles. For DNA-extraction steps of the QIAGEN M13 Spin Kit (QIAGEN, CA, USA) bulletin were followed. The DNA concentration was estimated either spectrophotometrically and samples were sequenced using the dideoxy-chain termination method either manually or automatically using the -28 gIII or the -96gIII primers (provided by New England BioLabs), respectively. Manual sequencing was done with ^{35}S labelling after the instruction of the

Sequenase Version 2.0 DNA Sequencing Kit (Amersham Biosciences, NJ, USA) and run on polyacrylamide gels with 6% urea to detect ladders.

2.3.5.2. Construction of recombinant phage

To construct recombinant M13 phage displaying a specific peptide, we prepared the double-stranded RF of the genomic DNA, which can be treated as a medium-copy number plasmid and therefore can be extracted from the pelleted host cells of a propagated host culture. For this purpose a QUIAGEN Plasmid Purification Kit (QIAGEN, CA, USA) was used. The peptide-encoding *Kpn*I-*Eag*I (New England BioLabs, MA, USA) fragment in the RF DNA of the library phage was excised and replaced with synthetic and annealed oligonucleotides (synthesised by UAB Oligonucleotide Core Facility, AL, USA) encoding the specified peptides. These primers also bear respective ends suitable for ligation into the cut RF form. The recombinant M13 RF DNA was transformed into *E. coli* strain ER2537 and plated with 3 ml of Top agarose to produce phage plaques. Plaques were propagated in host cell cultures, single-stranded DNA was extracted and sequenced as described at 2.3.5.1.

Oligonucleotide sequences used for recombinant phage cloning are listed in Table 1.

Table 1. List of oligonucleotides used for phage cloning.

Sequence of oligonucleotide	Name	Expressed peptide sequence
5'-CTT TCT ATT CTC ACT CTA ATC ATT TTC TTC ggT ggA ggT TC-3'	Tetramer-down	NHFL
5'-ggC CgA ACC TCC ACC AAg AAA ATg ATT AgA gTg AgA ATA gAA Agg TAC-3'	Tetramer-up	NHFL
5'-CTT TCT ATT CTC ACT CTA ATC ATT TTC TTC CTg gTg gAg gTT C-3'	Pentamer-down	NHFLP
5'-ggC CgA ACC TCC ACC Agg AAg AAA ATg ATT AgA gTg AgA ATA gAA Agg TAC-3'	Pentamer-up	NHFLP
5'-CTT TCT ATT CTC ACT CTA Tgg TgC AgA AgA ggA ATC ATT TTC TTC CTg gTg gAg gTT C-3'	M-decamer-down	MVQKRNHFLP
5'-ggC CgA ACC TCC ACC Agg AAG AAA ATg ATT CCT CTT CTg CAC CAT AgA gTg AgA ATA gAA Agg TAC-3'	M-decamer-up	MVQKRNHFLP
5'-CTT TCT ATT CTC ACT CTA ggA ATC ATT TTC TTC CTT ATT CTT TgC Cgg gTg gAg gTT C-3'	R-decamer-down	RNHFLPYSLP
5'-ggC CgA ACC TCC ACC Cgg CAA AgA ATA Agg AAg AAA ATg ATT CCT AgA gTg AgA ATA gAA Agg TAC-3'	R-decamer-up	RNHFLPYSLP
5'-CTT TCT ATT CTC ACT CTA ATC ATT TTC TTC CTT ATT CTT TgC Cgt Tgg gTg gAg gTT C-3'	N-decamer-down	NHFLPYSLPL
5'-ggC CgA ACC TCC ACC CAA Cgg CAA AgA ATA Agg AAg AAA ATG ATT AgA gTg AgA ATA gAA Agg TAC-3'	N-decamer-up	NHFLPYSLPL

2.4. RESULTS

2.4.1. Biopanning results

2.4.1.1. Signs of sequence enrichment and selection during biopanning of *Bacillus subtilis* and *Bacillus anthracis* spores

A total of four rounds of biopanning were performed with both the heptamer and the dodecamer libraries. Samples of input phage, initial supernatants, supernatants of selected washes, and

eluted phage were titered in each performed biopanning (data not shown). Emergence of tight-binders was indicated by a titre increase in the elution fractions, which was 10^5 fold for *Bacillus subtilis* (from 10^4 eluted phage to 10^9) and 200 fold (from 10^4 to $2 \cdot 10^6$) for *Bacillus anthracis* ΔAmes stram, and by a twofold decrease of the supernatant fraction revealing that after some rounds more members of the input phage population stay bound to the spore surface

2.4.1.2. Nature of the selected *B. subtilis* tight-binder sequences

We used a sample of the eluted phage from the fourth round of biopanning to purify plaques of individual phage, thirty of which were used to prepare genomic DNA for sequence analysis of the peptide-encoding regions. The results showed that these phage genomes encoded 13 unique peptide sequences. (Table 2A) Phage displaying the same peptide also contained the same peptide-encoding genomic DNA sequence, indicating that they were probably siblings. All of the peptides contained the sequence Asn-His-Phe-Leu-Pro at the amino terminus. Although the sequences at positions 5 through 7 were not identical, there were clear preferences for amino acids. For example, 6 of 13 (46%) position 5 residues and 12 of 39 (31%) position 5-7 residues were Pro. In addition, 10 of 39 (26%) position 5-7 residues were basic amino acids, while no acidic amino acids were found at these positions. We also biopanned a Ph.D.-12 phage display library (containing $2.7 \cdot 10^9$ different sequences) for dodecamer peptides that bind spores of *B. subtilis* as described for the Ph.D.-7 library. Ten plaques from the 4^{th} round eluate were used to determine peptide-encoding genomic sequences. Eight unique peptide sequences were found and again they contained the sequence Asn-His-Phe-Leu at the amino terminus (Table 2B). Also, the 0amino acids found at positions 5-7 were similar to those of the heptamer peptides (Table 2A), with at least one Pro residue found in this region in all but 1 sequence. The latter sequence had a Pro residue at position 8. One dodecamer sequence included Glu at position 6 (between 2 Pro residues), the only acidic residue found in positions 5-7 of a spore-binding peptide. The sequences in positions 8-12 were not highly restricted. The failure to find the Asn-His-Phe-Leu sequence internally in the dodecamer (or heptamer) peptides strongly indicated that this sequence must be present at the amino terminus of the peptide to permit spore binding.

Table 2. Peptide sequences of phage from the fourth round eluate using a heptamer (A) or a dodecamer (B) library. Numbers in parentheses indicate the number of phage with the given peptide sequence.

(A)

Sequence #	1	2	3	4	5	6	7
1(6)	Asn	His	Phe	Leu	Ile	Lys	Pro
2 (3)	Asn	His	Phe	Leu	Arg	Ser	Pro
3 (3)	Asn	His	Phe	Leu	Pro	Arg	Trp
4 (8)	Asn	His	Phe	Leu	Pro	Lys	Val
5	Asn	His	Phe	Leu	Leu	Pro	Pro
6	Asn	His	Phe	Leu	Pro	Pro	Arg
7	Asn	His	Phe	Leu	Pro	Thr	Gly
8	Asn	His	Phe	Leu	Met	Pro	Lys
9	Asn	His	Phe	Leu	Lys	Gly	Thr
10	Asn	His	Phe	Leu	Pro	Gln	Asn
11	Asn	His	Phe	Leu	Leu	Trp	Arg
12 (2)	Asn	His	Phe	Leu	Ile	Lys	Arg
13	Asn	His	Phe	Leu	Pro	Thr	Ala

(B)

Sequence #	1	2	3	4	5	6	7	8	9	10	11	12
1 (2)	Asn	His	Phe	Leu	Lys	Ser	Gln	Pro	Gly	Val	Val	Thr
2	Asn	His	Phe	Leu	Asn	Arg	Pro	Ala	Gln	Ser	Gln	Val
3	Asn	His	Phe	Leu	Pro	Pro	Lys	Met	Gly	Pro	Thr	Asp
4	Asn	His	Phe	Leu	Pro	Glu	Pro	Arg	Leu	Val	Met	Pro
5 (2)	Asn	His	Phe	Leu	Ala	Pro	Gln	Pro	Pro	Val	Lys	Pro
6	Asn	His	Phe	Leu	Met	Pro	Asn	Pro	Leu	Leu	Ala	Met
7	Asn	His	Phe	Leu	Ile	Pro	Pro	Glu	Pro	Leu	Arg	Glu
8	Asn	His	Phe	Leu	Pro	Leu	Asn	Pro	Pro	Ala	Pro	Ile

2.4.1.3. Confirmation of the high affinity of a characteristic tight binder sequence

To confirm that this family of peptides bound to *B. subtilis* spores and did not arise because of preferential amplification of phage displaying these peptides, we examined phage enrichment without amplification. A phage mixture was prepared containing 99.9% phage from the Ph.D.-7 library and 0.1% phage displaying peptide #4 (Asn-His-Phe-Leu-Pro-Lys-Val). A sample of this

mixture containing 10^{11} total phage was mixed with 10^{9} spores and a single round of biopanning was performed. The eluted phage were plaque purified and ten plaques were used to determine the sequences of peptide-encoding genomic DNA. Seven of the ten phage examined contained the sequence for peptide #4, indicating a 700-fold enrichment of the peptide #4 phage. This large enrichment was almost certainly due to binding of peptide #4 to spores.

2.4.1.4. Nature of *B. anthracis* tight-binders

27 plaques were amplified and sequenced from the fourth round eluate and 11 different peptide sequences were identified as listed in Table 3. Three peptide sequences were repeated several times. The most common peptide, sequence #4 (Thr-Ser-Gln-Asn-Val-Arg-Thr) was represented thirteen times. However because the peptide was encoded by the same codons in each isolate it is possible that this sequence emerged due its excellent growth properties and not because of expressing a motif with high affinity. Two of the repeated sequences, peptide #5 (Thr-Tyr-Pro-Ile-Pro-Ile-Arg) and peptide #6 (Thr-Tyr-Pro-Ile-Pro-Phe-Arg) along with the single sequence #9 (Thr-Tyr-Pro-Val-Pro-His-Arg) formed a family of peptides with the consensus of Thr-Tyr-Pro-X-Pro-X-Arg. Biopanning of Sterne spores also yielded similar sequences: Thr-Tyr-Pro-Leu-Pro-Ile-Arg 11 times from 35 sequences and the single sequence Thr-Tyr-Pro-Pro-Pro-Thr-Arg. Thr-Tyr-Pro-Leu-Pro-Ile-Arg was encoded by two different nucleotide sequences as permitted by the degeneracy of the genetic code. Although the Thr-Tyr-Pro consensus sequence was variable at positions 4 and 6, the residues at these positions were typically similar. For example, apolar amino acids, Leu, Ile, or Val occupied position 4 in all but one unique peptide sequence.

Table 3. Sequence of tight-binder peptides from the *Bacillus anthracis* ΔAmes spore biopanning. Consensus labelled with bold letters. Numbers in parentheses indicate the number of phage with the given peptide sequence.

Sequence	1	2	3	4	5	6	7
1	Asn	Ser	Val	Thr	Leu	Glu	Pro
2	Lys	Pro	Arg	Gln	Pro	Gly	Leu
3	Ser	Thr	Pro	Ala	Trp	Leu	Ser
4 (13)	Thr	Ser	Gln	Asn	Val	Arg	Thr
5 (3)	**Thr**	**Tyr**	**Pro**	Ile	**Pro**	Ile	**Arg**
6 (3)	**Thr**	**Tyr**	**Pro**	Ile	**Pro**	Phe	**Arg**
7	Ser	Tyr	Pro	His	Gly	Gln	Ile
8	Phe	Thr	Gly	Thr	Leu	Asn	Pro
9	**Thr**	**Tyr**	**Pro**	Val	**Pro**	His	**Arg**
10	Arg	Thr	Pro	Ser	Leu	Ser	Pro
11	Phe	Ser	Val	Pro	Arg	Met	Pro

2.4.2. Peptide sequence requirements for *B. subtilis* spore binding

2.4.2.1. Comparison of binding affinities of tight-binder heptamer sequences

To identify the tightest binding heptamer peptide(s) listed in Table 2A, we performed a competitive biopanning experiment. A phage pool containing equal amounts of each of the 13 phage displaying a unique heptamer peptide sequence was prepared, and its approximate composition was confirmed by DNA sequence analysis of 33 plaques from the pool. A sample of the phage pool containing a total of 1.3×10^{12} phages was mixed with 10^8 *B. subtilis* spores, and this mixture was used in a first round of biopanning following the standard protocol in Materials and Methods. The eluted phage were amplified, and 1.3×10^{12} of these phage were mixed with 10^8 spores for a second round of biopanning. Two more rounds (3 and 4) of biopanning were performed following this procedure. Eluted phage from round 4 were plaque purified, and 31 plaques were used to analyse peptide-encoding genomic sequences for phage identification. Comparing frequencies of phage appearance (Table 4) in the original phage pool with those in the round 4 eluted phage, there were no statistically significant differences for 10 of the unique phage. Phage displaying peptides #5, #7, and #9 were not detected in the final eluted phage, although 2 or 3 copies of each were present in the 33 phage identified in the original pool. Statistically, this result may not be meaningful, and inspection of the missing peptide sequences

did not reveal a common feature. Taken together, these results indicate that the spore binding properties of most (and perhaps all) of the 13 unique peptides are similar under the conditions examined here. In addition, repeated washing of complexes of spores and phage displaying the spore-binding peptides were highly resistant to repeated washing, indicating tight binding.

Table 4. Comparison of sequence frequencies between the first round input and the fourth round eluate fractions in a competitive biopanning experiment with tight-binder heptamers *of B. subtilis*.

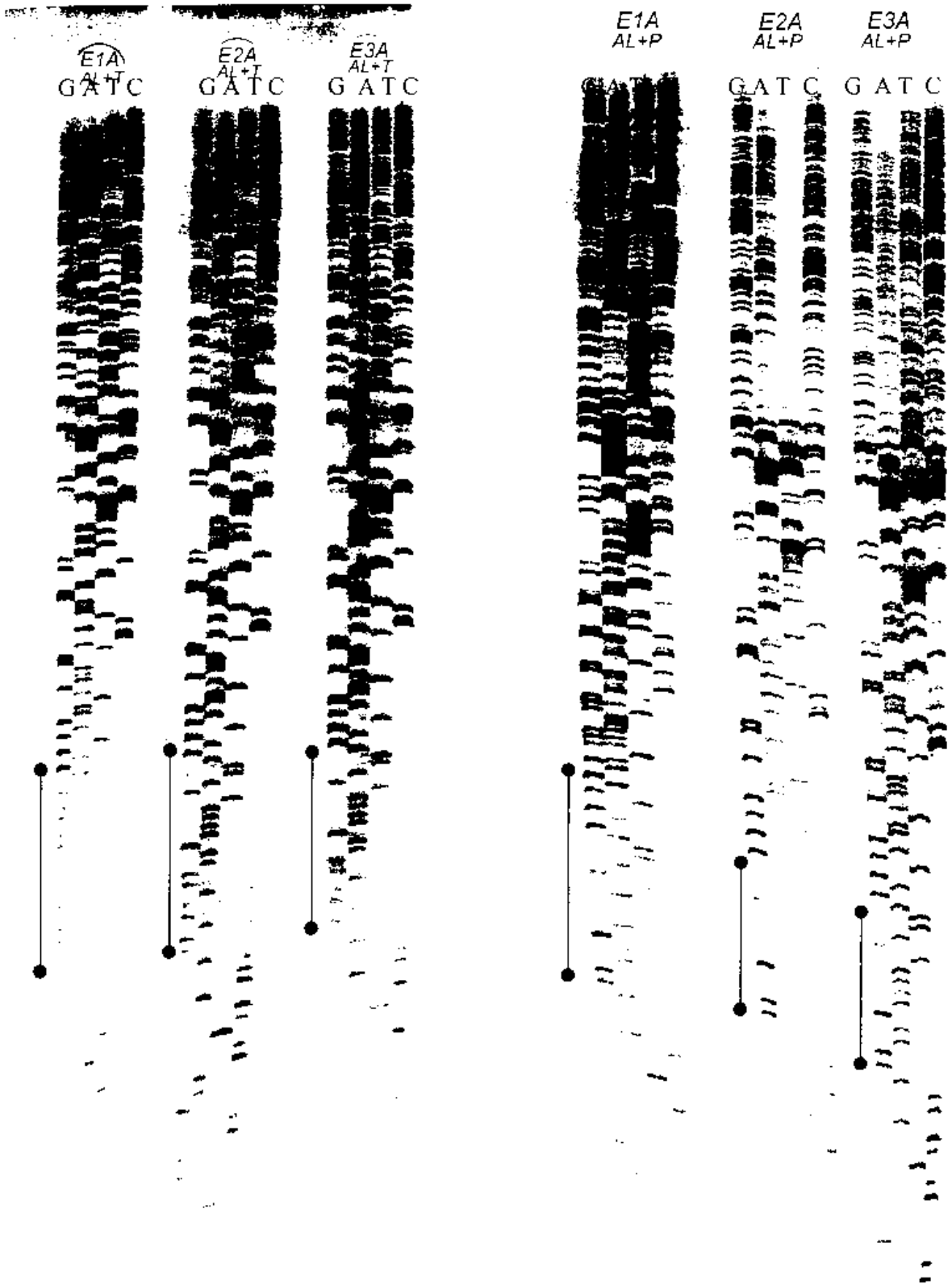
Serial number of tight-binder heptamer phage	**Number of plaques in the input fraction of first round of biopanning**	**Number of plaques in the eluate fraction of the fourth round of biopanning**
1	2	2
2	3	3
3	2	6
4	5	8
5	2	0
6	1	2
7	3	0
8	1	3
9	3	0
10	5	1
11	2	3
12	1	1
13	3	2
Σ:	33	31

2.4.2.2. Analysis of binding affinities of shorter (consensus) sequences

To determine whether shorter peptides can efficiently bind *B. subtilis* spores we constructed two recombinants that display either the tetrapeptide Asn-His-Phe-Leu or the pentapeptide Asn-His-Phe-Leu-Pro instead of a heptamer sequence. (Note that the same linker sequence, Gly-Gly-Gly-Ser, follows the tetramer, pentamer, and heptamer sequences.) We then performed two competitive biopanning experiments using a phage mixture containing 99.9% phage from the Ph.D.-7 library and 0.1% phage displaying either the tetramer or pentamer peptide. A sample of this mixture containing 10^{10} total phage was mixed with 10^{8} spores for the first round of

biopanning. Amplified eluted phage (10^{11}) were mixed with 10^{9} spores for two additional rounds of biopanning. Amplified eluted phage from each round were analysed as a mixture (i.e., no plaque purification). Genomic DNA was extracted from the eluted phage mixtures (each round separately), and the sequences of the peptide-encoding regions were examined as an aggregate. In the sequencing ladder of each round (Figure 3), it was possible to identify and roughly quantitate (within the mixed sequences) phage displaying different length peptides and also to identify dominant phage species. In the case of the Ph.D-7/tetramer phage mixture, the results showed that phage displaying the shorter peptide were undetectable in round 1 (i.e., only random heptamer sequences were observed). In rounds 2 and 3, phage displaying heptamer peptides with the sequence Asn-His-Phe-Leu- (Pro- or Xxx) emerged as major species, with no indication of tetramer phage. Thus, it appeared that phage displaying only the Asn-His-Phe-Leu sequence were relatively poor ligands. The results with the Ph.D-heptamer/pentamer phage mixture were strikingly different. After round 1, eluted phage contained a mixture of phage displaying the pentamer peptide and random heptamer sequences. After rounds 2 and 3 phage displaying the pentamer were the predominant or only phage species. Therefore, the Asn-His-Phe-Leu-Pro peptide appeared to bind spores as well as a heptamer containing the Asn-His-Phe-Leu- (Proxx or Xxx) sequence.

Figure 3. Emergence of heptamer peptides and a pentamer peptide from competitive biopannings with random heptamers and tetramers or random heptamers and pentamers respectively. Sequencing ladder of eluate mixtures are shown. Peptide displaying regions are labelled with line



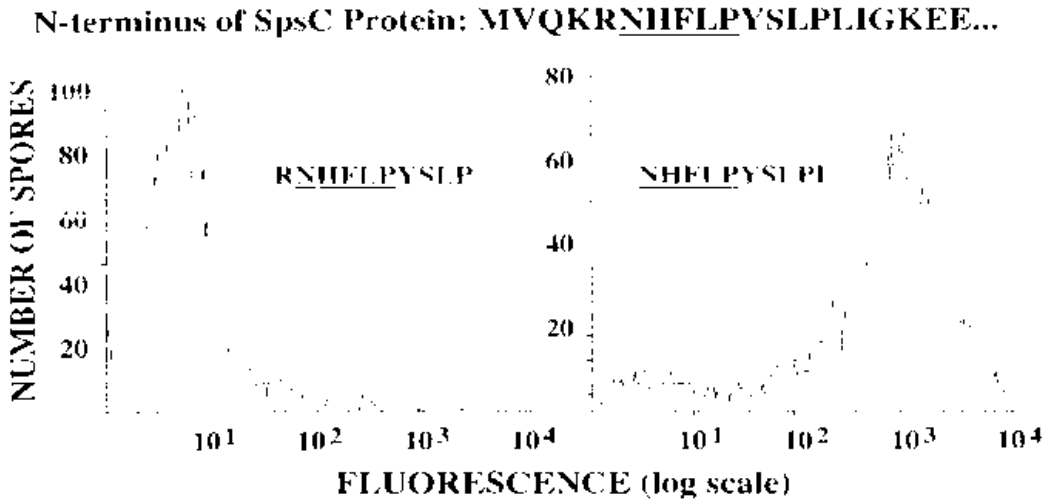


Figure 4. Phage expressing the segment NHFLP show a strong association with *B. subtilis* spores only if the amino acid string is at the very N-terminus of the peptide

2.4.2.3. Spore-binding peptides as a developmental targeting sequence

Conceivably, the spore-binding peptide sequences identified in this study could be used to direct mother cell proteins to a receptor on the surface of the developing forespore. To test this hypothesis, we used the sequence Asn-His-Phe-Leu-Pro as a probe to search the sequenced *B. subtilis* genome for exact or close matches within known or possible spore surface proteins. The most interesting hits were a perfect match in the SpsC protein and close matches in the SpsA (Asn-His-Phe-Tyr-Pro) and CotA (Thr-His-Pro-Leu-Pro) proteins. The SpsC and SpsA proteins are reported to be spore coat proteins involved in polysaccharide biosynthesis, and CotA is a pigmented outer spore coat protein with copper-dependent laccase activity. A problem with these matches, however, is that the putative spore binding sequence is not present at the amino terminus of the proteins – a requirement indicated by our biopanning results. This is a particularly serious problem for SpsA and CotA, where the putative spore binding sequence is located near the carboxy terminus of the protein. In the case of SpsC, the Asn-His-Phe-Leu-Pro sequence is located near the amino terminus, at positions 6 to 10. Interestingly, this sequence is preceded by a basic amino acid (actually two, Lys-Arg), a possible cleavage site for a trypsin-like protease. If cleavage occurred between residues 5 and 6, perhaps as a timing event during spore formation, then presumably the truncated SpsC protein could bind the spore surface and participate in polysaccharide synthesis. To examine more directly the requirements for SpsC

binding to the *B. subtilis* spore surface, we constructed three recombinant M13 phage displaying either SpsC residues 1 to 10 (Met-Val-Gln-Lys-Arg-Asn-His-Phe-Leu-Pro), 5 to 14 (Arg-Asn-His-Phe-Leu-Pro-Tyr-Ser-Leu-Pro), or 6 to 15 on coat protein pIII (Asn-His-Phe-Leu-Pro-Tyr-Ser-Leu-Pro-Leu). These phages were labelled equally with the fluorochrome Alexa 488 and used to measure spore binding by FACS under standard assay conditions. This assay measures the increase in spore-associated fluorescence caused by ligand binding. Phages displaying residues 1 to 10 and 5 to 14 failed to bind spores, while phage displaying residues 6 to 15 (with an amino terminal Asn-His-Phe-Leu-Pro) readily bound spores. Only results for phages displaying residues 5 to 14 and 6 to 15 are shown. These results confirm that the Asn-His-Phe-Leu-Pro sequence must be present at the amino terminus of a peptide or protein for spore binding. The results also support the hypothesis that SpsC is proteolytically processed prior to participating in spore maturation. Figure 4 shows the fluorescence shift of *Bacillus subtilis* spores detectable in the presence of the fluorescently labelled tight-binder ligand Asn-His-Phe-Leu-Pro-Tyr-Ser-Leu-Pro-Leu on the right side. In the presence of the protected Asn-His-Phe-Leu-Pro segment (Arg-Asn-His-Phe-Leu- Pro-Tyr-Ser-Leu-Pro) spores display only their usual weak autofluorescence as it is demonstrated on the left side.

2.4.3. Discriminatory power of *B. subtilis* tight-binder.

To determine the spore specificity of peptide binding, we first fluorescently labelled M13 phage displaying either heptamer peptide #4 (Asn-His-Phe-Leu-Pro-Lys-Val) or a control peptide (i.e., Asp-Pro-Leu-Lys-Val-His-Glu). Both phage contained approximately 500 molecules of the fluorochrome Alexa 488 per phage particle. Under standard conditions for assaying peptide binding, spores of *B. subtilis* and nine other phylogenetically similar *Bacillus* species were individually mixed with fluorescently labelled phage for 10 min, unbound phage were removed by washing, and the spores were analysed by FACS. The results show that peptide 4 binds well to spores of *B. subtilis*, nearly as well to spores of *B. amyloliquefaciens*, and somewhat weaker to spores of *B. globigii* (Figure 5, left panels). No binding of peptide 4 was detected with spores of selected strains of *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. mycoides*, and *B. megaterium* (Figure 5, left side; only *B. licheniformis* and *B. pumilus* results are shown).

No binding of the phage displaying the control peptide was detected with any spore species (Figure 5, left side, and data not shown). In addition, phage displaying peptide 4 did not bind to vegetative cells of *B. subtilis* (data not shown). Binding of peptide 4 appears to be restricted to spores of species that occupy a single, three-member branch of the *Bacillus* phylogenetic tree

(Figure 6). To demonstrate that peptide binding was independent of the attached fluorochrome (i.e., Alexa-labelled M13), we prepared conjugates of peptide 4 or control peptides (e.g., Leu-Phe-Asn-Lys-His-Val-Pro) with phycoerythrin. Each peptide included a (Gly)$_3$-Cys carboxy-terminal extension, and approximately 10 peptide molecules were attached through the carboxy-terminal Cys residue to the ε-amino groups of dispersed lysine residues on one molecule of phycoerythrin (240 kDa). Under standard assay conditions, spores of *B. subtilis* and seven other *Bacillus* species (i.e., all of the species described above except *B. anthracis* and *B. mycoides*) were individually mixed with a peptide-phycoerythrin conjugate for 60 min, unbound conjugate molecules were removed by washing, and the spores were analysed by FACS. The results show that peptide #4 binds equally well to spores of *B. subtilis* and *B. amyloliquefaciens* and somewhat weaker to spores of *B. globigii* (Figure 5, right panels). No binding of peptide 4 was detected with spores of *B. licheniformis*, *B. pumilus*, and *B. megaterium* (Figure 5, right side; only *B. licheniformis* results are shown). No binding of the phage displaying the control peptide was detected with spores of *B.subtilis*, *B. amyloliquefaciens*, *B. globigii*, *B. licheniformis*, *B.pumilus*, or *B. megaterium* (Figure 5, right side, and data not shown). Spores of *B. thuringiensis* and *B. cereus* showed slight and equal binding of the peptide 4 and control peptide conjugates, indicating non specific association (Figure 5, right side; only *B. cereus* results are shown). This non specific binding appears to be related to the prominent exosporium present on spores of *B. thuringiensis* and *B. cereus*. Selective removal of the exosporium from these spores by passage through a French press essentially eliminates this nonspecific binding (data not shown). A longer binding time (i.e., 60 min) was used with peptide-phycoerythrin conjugates to enhance labelling of spores. Approximately five times more peptide 4 conjugate bound at 60 min than at 10 min of incubation with *B. subtilis*, *B. amyloliquefaciens*, and *B. globigii* spores. The longer incubation time had no effect on the spores. The large and small peaks seen in the histograms of peptide 4 conjugate binding to *B. subtilis* and *B. amyloliquefaciens* spores (Figure 5 right side) apparently represent single spores and pairs (or small aggregates) of spores, respectively, as judged by light-scattering properties analysed by FACS.

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Finally, the peptide 4-phycoerythrin conjugate was shown to bind germinated spores of *B subtilis* prior to outgrowth but not to vegetative cells of *B. subtilis* (data not shown).

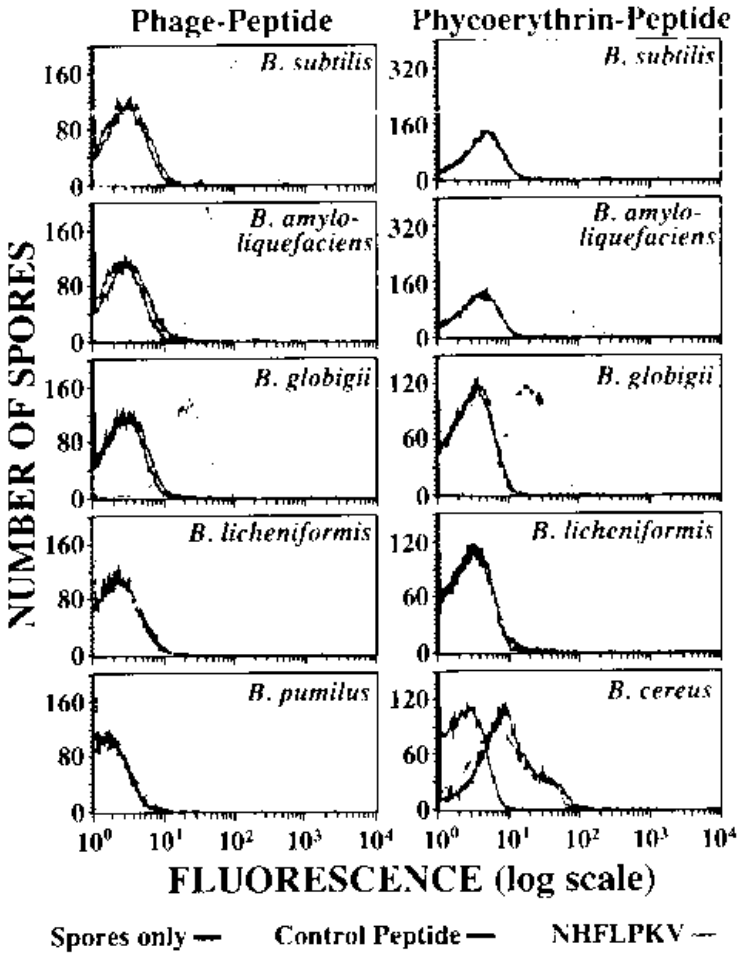


Figure 5. Species-specific binding of fluorescently labelled affinity selected peptide NHFLPKV. Binding was assessed both by using fluorescently labelled phage expressing NHFLPKV and random control phage (left side) and fluorescently labelled synthetic synthetic peptides.

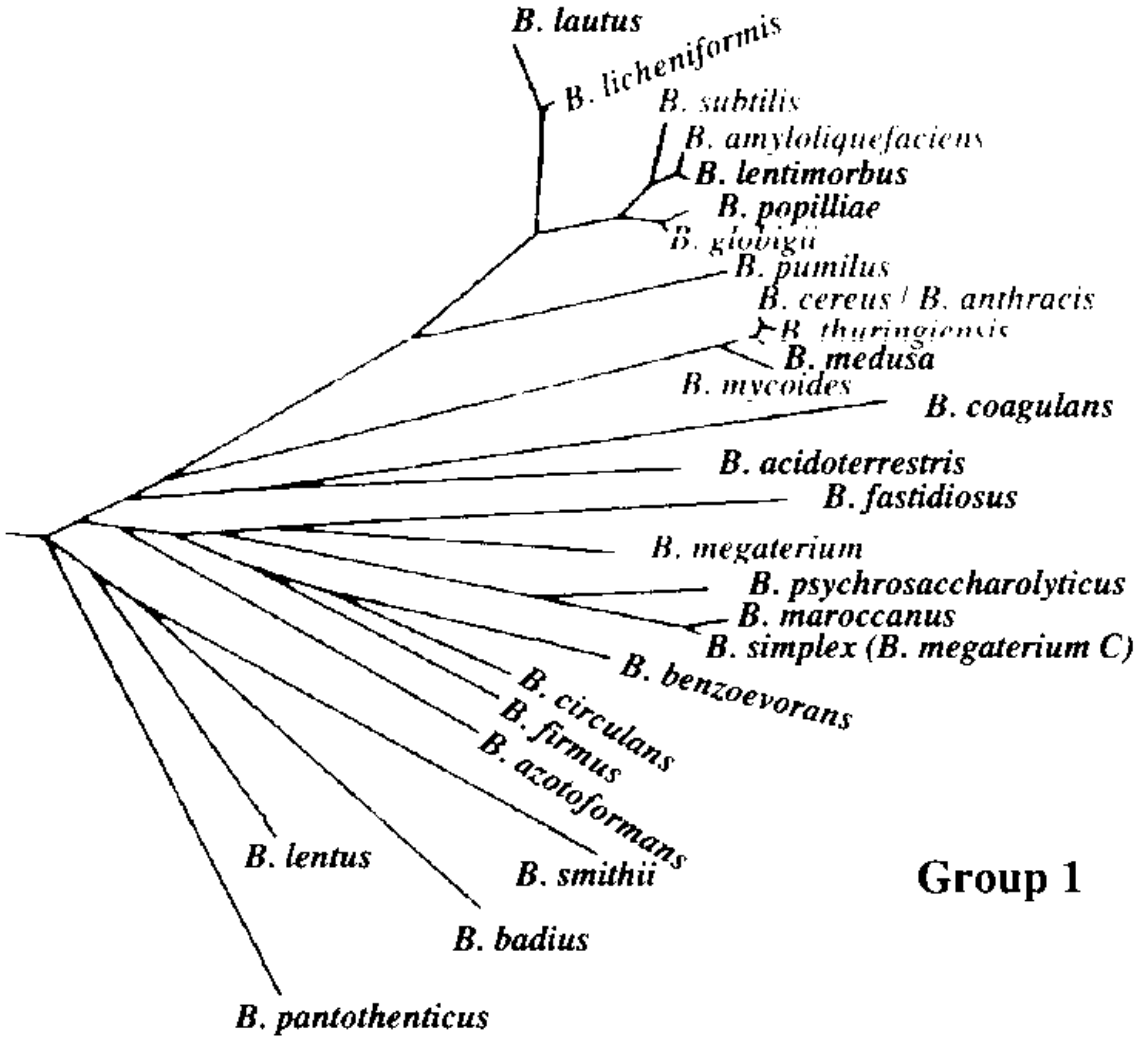
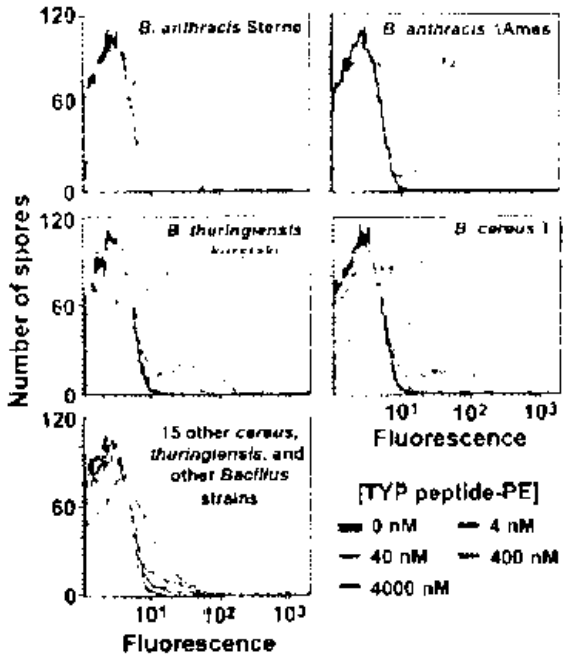


Figure 6. Phylogenic tree of group 1 (of five) members of the genus *Bacillus* based on 16S rRNA sequence analysis. Species of spores that were examined for binding by the Asn-His-Phe-Leu-Pro-Lys-Val peptide are coloured (red- or blue-), and those that showed peptide binding are displayed in red.

A



B

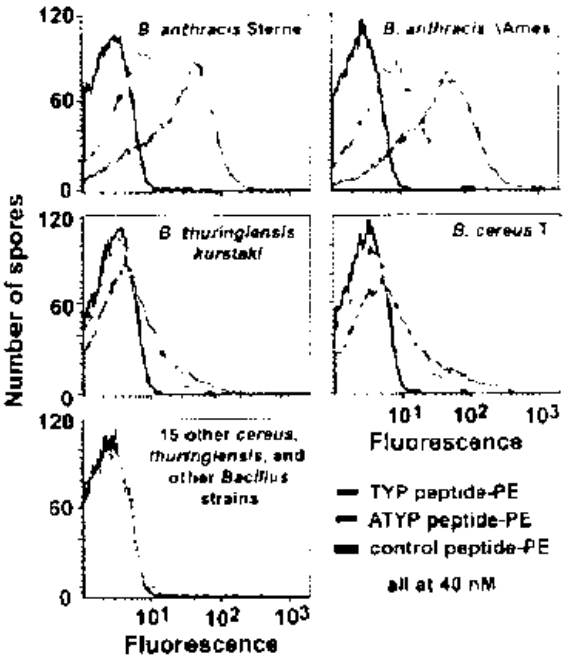


Figure 7. Selective binding of *B. anthracis* tight-binder TYPILPR to *B. anthracis*, *B. cereus* T and *B. thuringiensis kurstaki* strains (A) Selectivity was improved by attaching a single alanine residue to the N-terminus of the peptide (B) hence the modified peptide bound uniquely to *B. anthracis* spores.

2.4.4. Binding requirements of *B. anthracis* spores and discriminatory power of tight-binder ligands

To this end we chemically synthesised a representative TYP peptide with the sequence TYPLPIRGGGC; the GGGC extension was included as a carboxy-terminal linker for fluorochrome attachment. Approximately 10 peptide molecules were then attached (using a cross-linker) through their terminal cysteine residues to the ε-amino groups of dispersed lysine residues on one molecule of PE, a 240-kDa highly fluorescent protein. Peptide binding to *B. anthracis* (Sterne and ΔAmes) spores was then measured by incubating spores with from 4 to 4,000 nM peptide-PE conjugate, removing unbound conjugate by washing, and analysing spore-peptide complexes by FACS. The results showed essentially identical, concentration-dependent binding of the peptide-PE conjugate to spores of the Sterne and ΔAmes strains (Figure 7, first panels on the left and right side). To examine the specificity of peptide binding, we measured (as described above) the binding of the TYPLPIRGGGC-PE conjugate to spores of 17 other *Bacillus* strains, including 6 strains of *B. cereus* (T, ATCC 4342, D17/FRI-13, 3A/FRI-41, S2-8/FRI-42, and F1-15/FRI-43), 4 strains of *B. thuringiensis* (subsp. *kurstaki*, B8, Al Hakum, and USDA HD-571), and 1 strain each of *B. mycoides*, *B. pumilus*, *B. globigii*, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. megaterium*. These strains were all members of *Bacillus* group 1 (of 5), within which *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. mycoides* comprise the closely related *B. cereus* group. Seven of these strains – i.e., *B. thuringiensis* strains Al Hakum and USDA HD-57 and all *B. cereus* strains except T – are human pathogens and nearest neighbours to *B. anthracis* as determined by amplified fragment length polymorphism analysis. The binding assays showed that the peptide-PE conjugate did not bind to 15 of the other *Bacillus* strains (Figure 7). Minimal binding at a conjugate concentration of 4,000 nM was due to non-specific entrapment. Peptide binding was detected for spores of *B. cereus* T and *B. thuringiensis* subsp. *kurstaki*, but this binding was weaker (or less extensive) than that observed with *B. anthracis* Sterne and ΔAmes spores. These results indicated a high degree of specificity in TYPLPIR binding to spores but revealed that binding was not absolutely restricted to *B. anthracis* spores.

To control for non-specific binding in each experiment shown in Figure 7A, several dissimilar undecamer peptides (for example, HWHHHGHGGGC and HLPRPYTGGGC, the latter being a scrambled version of a TYP peptide) were attached to PE as described above. These conjugates were tested for spore binding and no significant binding was detected. In a related control experiment, we showed that binding of the TYPLPIRGGGC-PE conjugate to *B. anthracis* spores was not inhibited by inclusion of bovine serum albumin at 10 mg/ml in the binding and wash

buffers. Furthermore, we demonstrated that the TYPLPIRGGGC-PE conjugate did not bind to vegetative cells of the Sterne and ΔAmes strains (data not shown).

When analysing the binding requirements of *B. subtilis* we have shown that they require an N-terminal NHFLP consensus for spore attachement. Hence it was reasonable to test the *B. anthracis* ligands for N-terminus requirements. When a single alanine was present at the N-terminus in front of the threonine moiety it was demonstrated that a peptide ligand with improved species discriminatory power was developed (Figure 7B).

2.5. DISCUSSION

Phage display is a popular method in biotechnology and suitable for a broad range of application. However its role in microbiological diagnostics has not yet been shown. In this project we worked on the development of a fast, non-nucleic acid based method for the identification of *Bacillus* spores. As a model system first we developed a phage-display-based assay for the detection of harmless *B. subtilis* spores. From a random phage display library, we identified a family of short heptamer and dodecamer peptides that bind tightly to spores of *B. subtilis*. These peptides contain the consensus sequence Asn-His-Phe-Leu-Pro and displayed a similar binding affinity as it was confirmed by a competitive biopanning experiment. Using a representative peptide, we demonstrated that binding was restricted to spores of three *Bacillus* species. We observed nearly equal binding to spores of *B. subtilis* and its most closely related species. *B. amyloliquefaciens*, and slightly weaker binding to spores of the closely related species *B. globigii*. These three species comprise one branch on the *Bacillus* phylogenetic tree. The representative peptide did not bind to spores of several *Bacillus* species located on adjacent and nearby branches of the phylogenetic tree or to vegetative cells of *B.subtilis*. Proper presentation of the peptide on a large carrier as a phage or large fluorchrome molecule was essential for being able to collect such data. Conjugation to small molecular weight fluorochromes caused a non specific entrapment. These results show that short peptides can be used as species-specific ligands and suggest that other short peptides can be isolated as specific ligands for different spore species and perhaps for any cell type. Theoretically, the binding site for such ligands can be a fortuitous or physiological receptor on the cell surface. Our approach of reiteratively biopanning a phage display peptide library yielded a single consensus sequence, presumably the best binding peptide under the conditions employed. Possibly, a different peptide could be recovered by employing different biopanning conditions (e.g., buffer, number of washes, etc.), or by using a library displaying peptides in a different context (e.g., on the M13 major coat protein, pVIII) (Smith and Petrenko, 1997). For all of the peptides isolated in our study, the Asn-His-

Phe-Leu sequence is located at the amino terminus, and this location was shown to be essential for spore binding. Typically, the spore-binding heptamer peptides contain at least one Pro residue at positions 5 to 7. In addition, extension of the non-binding Asn-His-Phe-Leu peptide by a single Pro residue (when displayed on pIII of phage M13) enables tight spore binding. These results indicate an important but somewhat flexible role for the Pro residue in spore binding. Because of its unique ability to limit polypeptide chain rotation, the Pro residue may stabilise or allow a peptide conformation that permits proper orientation of the four amino-terminal residues with respect to the spore surface receptor. In the case of *B. subtilis*, the Asn-His-Phe-Leu-Pro peptide apparently binds to a receptor on the outer surface of the spore. This location for the receptor is based on the fact that molecules as large as the peptide-fluorochrome conjugates used in this study do not penetrate the outer coat of the spore. In addition, this receptor may have a physiological role in spore development. This possibility is indicated by the discovery that the spore surface protein SpsC contains the Asn-His- Phe-Leu-Pro sequence near its amino terminus, and this sequence is immediately preceded by a possible cleavage site for a trypsin-like protease. Indeed, trypsin-like proteolytic activity is apparently involved in the temporal processing and activation of mother cell proteins (e.g., the coat proteins CotF and CotT) during the latter stages of sporulation (Bourne, FitzJames, and Aronson, 1991;Cutting, Zheng, and Losick, 1991). Such a processing event would enable the SpsC protein, directed by its amino-terminal Asn-His-Phe-Leu-Pro sequence, to bind to its forespore receptor at the appropriate time during spore formation and maturation. Once bound to the forespore, the SpsC protein would participate in the synthesis of surface polysaccharides, which gives *B. subtilis* spores their hydrophilic character (Koshikawa *et al.*, 1989). Identification of the receptor still needs to be accomplished however serious efforts were made to find the receptor. Utilising UV-crosslinking experiments between the peptide ligand and the spore coat along with a biotinylated heterobifunctional, reducable crosslinker followed by the extraction of the spore coat proteins and search for a biotinylated signal on Western blots did not give any useful results. It was probably due to the non-specific binding of the tight-binder peptide ligand when it was coupled to a low-molecular-weight molecule as it was detected in flow cytometry experiments applying Alexa-488-peptide conjugates. It is also possible that the receptor is a modified glycoprotein and therefore a different strategy should be employed for the identification.

A family of heptamer peptides expressing the TYPXPXR consensus sequence was identified with a similar phage display of the avirulent *B. anthracis* strains Sterne (pXO2$^-$) and AAmes (pXO1$^-$). They lack virulence plasmids whose products are unlikely to be involved in the formation of the spore surface (Read *et al.*, 2003) therefore these strains might suitably mimic

the wild-type *B. anthracis*. There is a superficial difference in the length of the hair-like nap on the spore surface (Kramer and Roth, 1968), (Mikesell *et al.*, 1983), (Sylvestre, Couture-Tosi, and Mock, 2003). *B. anthracis* strains are highly monomorphic as well, with genes from different isolates typically having greater than 99% nucleotide sequence identity (Price *et al.*, 1999). Performing FACS-analysis with a representative member of the consensus-bearing family on a spore set of different *Bacillus* species the labelled peptide bound to *B. cereus* I and *B. thuringiensis* kurstaki, closely related to *B. anthracis* as well. Attachement of a single alanine residue to the N-terminus of the peptide in front of threonine increased the discriminatory power of the system. The modified peptide bound well exclusively to *B. anthracis*, with the exception of weaker binding to spores of an apparently small subset of the *B. cereus* group. Apparently, spores of this group contain species-specific surface features (e.g., peptide receptors), which may reflect the different ecological niches and/or hosts of these species (Read *et al.*, 2003)

B. anthracis specific peptides however have a probable lower affinity than the *B. subtilis* binders because FACS-analysis did not work well when peptides were displayed and presented on the bacteriophage but only when used in a much higher copy number of synthetic, fluorochrome-conjugated peptides. The sites on the *B. anthracis* spore surface to which ATYP and TYP peptides bind have not yet been identified. However, the most likely binding sites are components of the exosporium, a prominent, loose-fitting, balloon-like layer that encloses the spores of *B. cereus* group strains. The exosporium, which is composed of a basal layer and an external hair-like nap, serves as a primary permeability barrier that would exclude the M13 phage (Gerhardt, 1967).

If the peptides identified in this study are indeed generally useful in identifying *B. anthracis* spores, they offer several advantages in detector design. They bind directly to the spore, eliminating the need for extracting spore components or for growing vegetative cells. They can be easily incorporated, covalently if necessary into detectors presently employing antibodies or into detection platforms that cannot accommodate antibodies because of size limitations or denaturing conditions. They can be easily and differentially labelled with assayable tags, such as luminescent quantum dots that provide a signal sufficient to detect a single spore. They can be produced rapidly and inexpensively. Finally, the use of two peptides should eliminate or greatly reduce the incidence of false-positive signals. We expect that the peptides for *B. anthracis* spores can be utilised in simple, inexpensive, and portable detectors based on an assortment of analytical platforms. In our study we employed assays based on increased fluorescence, but binding of peptides to spores can also be detected by many other analytical techniques (Turnbough, Jr., 2003)

2.6. NEW RESULTS PRESENTED IN CHAPTER 2

1. Identification of thirteen heptamer and eight dodecamer tight-binder peptides for *Bacillus subtilis* expressing a consensus NHFLP terminus.
2. Determination of sequence requirements for binding:
    - assessment of similar affinities of heptamer sequences with competitive biopanning
    - assessment of binding capacities of truncated pentamer (NHFLP) and tetramer (NHFL) sequences: NHFLP is a minimum necessary sequence for successful binding.
    - Peptides need the N-terminal presence of the consensus sequence: similarity searches revealed a homology with the N-terminus of the SpsC protein. Expression of the SpsC N-teminal amino acids yielded binding ligands only when the N6H7F8L9P10...L15 string of amino acids were displayed on the phage surface.
3. Development and adjustment of flow cytometry assays with fluorescent anti-M13 antibody and phage labelling. Involvement in the development of peptide-labelling.
4. Identification a TYPXPXR consensus sharing family of tight-binders peptide ligands to *Bacillus anthracis* AAmes spores.

5. Outlining of a simple and quick flow cytometry test for detection of *B. anthracis* spores.

3. MAPPING OF THE LAMININ BINDING SITE OF *YERSINIA PESTIS* PLASMINOGEN ACTIVATOR

3.1. INTRODUCTION

3.1.1. Structure of mammalian tissue barriers and strategies of pathogenic bacteria to cross them

Within the mammalian body, tissue barriers are mainly formed by extracellular matrices (ECM) (Bosman and Stamenkovic, 2003). In electron micrograph the two main domains of the extracellular matrix are clearly identifiable: the basement membrane, a condensed matrix layer that is formed adjacent to epithelial cells, covering cell sheets (mesothelium, meningothelium, and synovia), muscle and Schwann cells, adipocytes, and the interstitial matrix. The main characteristic these two domains have in common is that their basic structure is defined by a collagen scaffold, although the collagens that make up the scaffold are different, as are their three-dimensional architecture. Adhesive glycoproteins, including laminin and tenascin, and proteoglycans adhere to the scaffold and interact with the cells in or adjacent to the matrix. Interaction with these cells is conducted through matrix receptors, of which the integrins constitute the most important class. The extracellular matrix is not static: it is remodelled constantly, which implies constant breakdown by proteases, notably the family of matrix metalloproteases. Collagens are ubiquitous proteins responsible for maintaining the structural integrity of vertebrates and many other organisms. More than 20 genetically distinct collagens have been identified (Bosman and Stamenkovic, 2003). In tissues that have to resist shear, tensile, or pressure forces, such as tendons, bone, cartilage, and skin, collagen is arranged in fibrils, with a characteristic axial periodicity providing tensile strength. Only collagen types I, II, III, V, and XI self-assemble into fibrils consisting a triple helix of approximately 300 nm in length and 1.5 nm in diameter. Some collagens form networks (types IV, VIII, and X). A typical example of such a network is the basement membrane, which is mostly made of collagen IV. Collagens are mostly synthesised by the cells comprising the extracellular matrix: fibroblasts, myofibroblasts, osteoblasts, and chondrocytes. Some collagens are also synthesised by adjacent parenchymal or covering (epithelial, endothelial, mesothelial) cells. A typical example is type IV collagen, which is synthesised in a cooperative effort between the stromal cell and the parenchymal/covering cell.

Laminin, together with type IV collagen, nidogen and perlecan, is one of the main components ofthe basement membrane(Bosman and Stamenkovic, 2003). What is now known as laminin was first discovered over 20 years ago in the matrix formed in a murine sarcoma (the mouse Engelbreth-Holm-Schwarm sarcoma). The molecule appeared to be between 200 and 400 kDa,

was composed of three disulphide linked chains, and had a characteristic cross shape. Molecular cloning of the three chains (now known as α1, β3, and γ 2) of laminin led to the discovery of a variety of homologues. As yet, five α chains, three β chains and three γ chains have been identified(Colognato and Yurchenco, 2000). Not all possible combinations of the three chains appear to be used: 12 distinct laminin isoforms have been isolated. All laminin chains have certain structural features in common. They share small globular domains. One of them is involved in chain polymerisation. They also have common epidermal growth factor-like repeats which host the nidogen binding site. Nidogen links laminin to type IV collagen. Some structures are more chain specific: α chains have a large C-terminal globular domain, which hosts many of the binding sites for integrins. Laminin isoforms are synthesised by a wide variety of cells in a tissue-specific manner. Notably, virtually all epithelial cells synthesise laminin, as do smooth, skeletal, and cardiac muscle, nerves, endothelial cells, bone marrow cells, and the neuroretina. Epithelial cells express α1, α3, β3, and γ 2 chains. The pattern of expression of α5, β1, β2, and γ 1 chains is less specific. The synthesising cells deposit laminin mostly but not exclusively in basement membranes. Laminins have a variety of effects on adjacent cells, including cell adhesion, cell migration, and cell differentiation. They exert their effects mostly through integrins, many of which recognise laminins, the integrin binding domain residing predominantly in the α chain. The primary role of laminins is mediating the interaction between cells and the basement membrane. This pleiotropic function is dependent on proteolytic processing of laminin by plasmin or matrix metalloproteinases. Given the wide range of roles laminins play in tissue structure and cell function, it is not surprising that laminins are significantly involved in a variety of disease processes. Disregulation of the interaction between cancer cells and the extracellular matrix is accompanied by aberrant synthesis, chain composition, and proteolytic modification of laminins. Proteoglycans have a protein core which is richly decorated with glycosaminoglycans. They function as organisers of collagen networks, are involved in signal transduction through the EGF receptor, which participates in modulation and differentiation of epithelial and endothelial cells (Bosman and Stamenkovic, 2003).

The ECM is subject to constant remodelling, a process that involves breakdown of existing, and synthesis and deposition of new ECM proteins. Numerous classes of proteolytic enzymes are believed to participate in ECM degradation, but one class that appears to play a dominant role is that of matrix metalloproteinases (MMPs) (Bosman and Stamenkovic, 2003). MMPs play an essential role in physiological events, including development, hormone-dependent tissue remodelling, and tissue repair. However, they also play a key role in pathological conditions such as inflammation, tumour invasion, and metastasis. MMPs promote normal and malignant

cell invasion of the ECM and participate in controlling normal and tumour cell responses to growth factors, cytokines, and chemokines, as well as cell–cell and cell–ECM interactions.

Invasive bacteria must penetrate the ECM in order to reach the circulation (Lahteenmaki, Kuusela, and Korhonen, 2001). Degradation of and penetration through these barriers represents a major problem in migration of bacterial and eukaryotic cells. Production of ECM-degrading proteases is, however, limited to a restricted number of bacterial pathogens and infectious diseases. Degradation of collagens, elastin, and fibronectin by secreted bacterial proteases leads to massive tissue destruction seen in diseases like corneal keratitis (caused by *Serratia marcescens* or *Pseudomonas aeruginosa*), periodontitis (*Porphyromonas gingivalis*), cystic fibrosis (*P. aeruginosa*) and gangrene (*Clostridium perfringens*). Most enteric bacteria as well as major agents causing bacterial meningitis (*Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Escherichia coli* K1) produce low level of proteinases (Lahteenmaki, Kuusela, and Korhonen, 2001). Some of these bacteria are intracellular pathogens and their penetration through cellular layers apparently involves an intracellular phase. However, a number of invasive bacteria are extracellular pathogens that obviously must rely on other mechanisms for invasiveness. Pathogenic bacteria are known to interact with proteinase-dependent cascade systems of the hosts, including coagulation, fibrinolyis, complement activation, phagocytosis and the kallikrein-kinin cascade (Lahteenmaki, Kuusela, and Korhonen, 2001). These pathways are tightly regulated by host protein activators or inhibitors. Bacteria may activate or inactivate these cascades directly through their proteases or other surface components, or indirectly by causing release of effector molecules from epithelial or endothelial cells or proteolytic enzymes or their precursors from phagocytic cells. Some of these protease-dependent pathways can be utilised by bacteria to ensure growth or spread within the host.

3.1.2. Physiological roles of the plasminogen-plasmin system and its connections to mammalian barriers

Due to the high concentration of the serine protease precursor plasminogen (Plg) in plasma and the broad proteolytic activity of the enzymatic form plasmin, the mammalian Plg system offers a highly potential proteolytic system that could be utilised by pathogenic bacteria (Lahteenmaki, Kuusela, and Korhonen, 2001). Plasmin has been proposed to play a role in several physiological processes in mammals: it is a key enzyme in fibrinolysis, degrades various ECM components due to the activation of procollagenases as well as latent macrophage elastase and is involved in activation of certain prohormones and growth factors as well as in tumor cell metastasis(Lahteenmaki, Kuusela, and Korhonen, 2001). Bacteria interact with the Plg system in

various ways (Lahteenmaki, Kuusela, and Korhonen, 2001). They have been found to produce Plg activators (PAs) or Plg receptors (PlgRs), they influence the production of host PAs and their inhibitors and also might have an effect on the host plasmin inhibitors. Bacterial PAs can be divided in two functional groups (Lahteenmaki, Kuusela, and Korhonen, 2001). 1.) Streptokinase (SK) (produced by *Streptococcus pyogenes*) and staphylokinase (produced by *Staphylococcus aureus*) (SAK) are not enzymes themselves but form 1:1 complexes with Plg and plasmin, leading to changes in conformation and specificity of plasmin(ogen). In contrast to plasmin alone, the SK-plasmin and SAK-plasmin complexes acquire a remarkable efficiency to activate Plg. SK and SAK share a little sequence homology but their crystal structures reveal that they have adopted a similar fold. The mechanism of Plg activation BY SAK and SK is basically similar but differs in some essential aspects, such as the fibrin dependence of the activation in human plasma. 2.) The Pla surface protease of *Yersinia pestis* resembles the mammalian PAs in function and activates Plg by limited proteolysis at the same Arg_{560}-Val_{561} bond as do the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA).

3.1.3. The plasminogen activator of *Yersinia pestis*.

The genus *Yersinia* comprises three human pathogenic species, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. *Y. pestis* is the causative agent of plague responsible for three large pandemics in the past and recent endemics in Africa, Middle Asia, Latin-America, and the Southern states of the USA. The last epidemic was reported from Surat, India, in 1994. *Y. enterocolitica* and *Y. pseudotuberculosis* trigger much milder gastroenteritis, pseudoappendicitis and several connective tissue sequelae (e.g., reactive arthritis). The three species show a high degree of chromosomal homology, and share a 70 kbp plasmid required for expression of virulence (Ben Gurion and Shafferman, 1981). Despite its highly invasive character *Y. pestis* expresses neither the inv gene product (Simonet et al., 1996) nor the YadA outer membrane protein (Skurnik and Wolf-Watz, 1989) described in the other two human pathogenic *Yersinia* species as important factors of invasiveness and mediators of matrix protein binding (Emödy et al., 1989). On the other hand, *Y. pestis* harbours two unique plasmids not present in the enteropathogenic *Yersiniae* (Ferber and Brubaker, 1981). The 110-kbp pFra plasmid encodes a phospholipase D required for *Y. pestis* survival in the flea midgut (Hinnebusch et al., 2002) and the F1 antiphagocytic capsule protein (Cavanaugh and Randall, 1959). The small, 9.5-kb plasmid (pPCP1) codes for a plasminogen activator (Pla) protein (Sodeinde and Goguen, 1988), which behaves as a fibrinolysin at 37°C and as a weak coagulase of rabbit plasma at 25°C presumably due to temperature induced conformational changes

(McDonough and Falkow, 1989). The role of Pla in invasiveness was first realised when it was shown that Pla$^+$ *Y. pestis* mutants injected into mice subcutaneously presented with a million fold increase in LD_{50} value compared to the isogenic Pla$^-$ strain (Sodeinde et al., 1992). Pla mutants remained localised to the site of injection being unable to spread to the liver and spleen. On the other hand, there was no difference in the LD_{50} values for the two variants when injected intravenously. Pla may be involved in several virulence functions like eukaryotic cell adhesion (Kienle et al., 1992) and invasion (Lahteenmaki, Kukkonen, and Korhonen, 2001) (Cowan et al., 2000), and matrix protein binding (Kienle et al., 1992) (Lahteenmaki et al., 1998). Effective plasmin formation by the action of Pla enables bacterial spread through tissue barriers like basal membranes since plasmin is a multi-substrate protease and able to degrade non collagenous extracellular matrix proteins (Lahteenmaki et al., 1998), and to activate latent procollagenases. The active site involved in plasminogen activation has been determined but it is still not known which parts of the molecule are responsible for matrix protein binding. Laminin is the best known member of a family of basement membrane proteins being able to affect also adhesion and colonisation of bacteria. Therefore it is of particular interest to characterise how it interacts with Pla. It is also worth investigating whether the same regions are also involved in internalisation into eukaryotic cells. Localisation of binding site(s) may perspectively initiate studies on selection of specific peptides being utilised as antigenic determinants for active immunisation to prevent plague.

3.2. AIMS OF THE STUDY

1. Affinity selection of peptide sequences binding to laminin with a random heptamer peptide library for biopanning.
2. Assessing the ability of selected phage to interfere with Pla-mediated laminin binding of a Pla-positive *E. coli* K-12 strain TB1.
3. Confirmation of laminin binding by the phage, which caused interference in Pla mediated bacterial laminin binding.
4. Performance of sequence similarity searches between Pla and the interfering peptide sequences.
5. Localisation of consensus peptide patterns in the three-dimensional model of Pla.
6. Investigation of interference with Pla-mediated bacterial laminin binding by synthetic peptides.

7. Performing alanine-scanning mutagenesis at the defined homologous sites and determination of its effect on laminin binding.
8. Studying Pla mediated *E. coli* internalisation into HeLa cells with the utilisation of several signal transeduction inhibitors, actin staining and its potential inhibition by the selected phage sequences.

3.3. MATERIALS AND METHODS

3.3.1. Phage display related methods.

3.3.1.1. Biopanning against laminin

Four rounds of biopanning were performed against laminin from Engelbreth-Holm-Schwarm murine sarcoma tumor (Sigma) on a microtiter plate (Nalge Nunc International, Denmark) with a random heptamer Ph.D. 7- library (New England BioLabs, MA, USA). Four rounds of biopanning were performed using approximately 10^{11} input phages in 100 µl volume per round. In each round a well of a microtiter plate (Nalge Nunc International, Denmark) was sensitised overnight at 4°C with 100 µl of 20 µg/ml laminin in 100mM $NaHCO_3$ (pH 8.6). Next day the well was washed three times with TBST [Tris buffered saline (TBS) (50 mM Tris, pH 7.5, 150 mM NaCl) pH 7.5 supplemented with 0.5% Tween 20 to increase stringency]. It was blocked with 2% BSA for two hours at room temperature and then washed six times with 100 µl-100 µl TBST. Laminin and phages were incubated for ten minutes at room temperature with gentle rocking. Then the liquid was removed and labelled as a supernatant fraction. Ten washes followed in TBST (100 µl volumes). Supernatant and wash fractions were kept in microfuge tubes at 4°C till usage. Tight-binder phages were eluted with 100 µl 0.2 M glycine pH 2.2 for 5 min at room temperature and then neutralised by the addition of 150 µl of 1 M Tris-HCl (pH 9.1) to prevent phage killing. 98 µl from the neutralised eluate was used for the next round of amplification. Instead of the ER2738 *E. coli* strain, XL1-Blue MRF (Kay et al., 1996) was used as F' host of coliphages. From each round of biopanning inputs, eluates, supernatants, first, fifth and tenth washes were titered.

3.3.1.2. Phage titration, propagation and concentration

Phage titration, propagation and concentration were performed as described in sections 2.3.1.4. and 2.3.1.5. except that *E. coli* XL1 Blue MRF was used as host cell.

7. Performing alanine-scanning mutagenesis at the defined homologous sites and determination of its effect on laminin binding.
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3.3.1.3. Phage DNA extraction and sequencing

From phage stocks single stranded DNA was extracted once with equal volumes of phenol: chloroform: isoamyl-alcohol (25:24:1) (Sigma, Germany). After spinning in microcentrifuge with 12,000×g at 4°C for 5 min the aqueous upper phase was transferred into a new tube and extracted with an equal volume of chloroform. Spinning followed as before, and the upper phase was transferred into a new tube and precipitated with 2.5 volume of 95% ethanol plus 0.1 volume of 3 M Na-acetate pH 5.2. After keeping at -70°C for 20 minutes the mixture was centrifuged at 12,000× g, 4°C for 10 minutes, and the precipitate was washed once in 1 ml of 70% ethanol. The pellet was dried in vacuum and dissolved in 20 µl of sterile ddH_2O. The DNA content was checked on 1% agarose gel and the sequence was determined using the dideoxy-chain-termination method with an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA).

3.3.1.4. Phage-ELISA tests for laminin binding

A microtitre plate was sensitised with 100 µl aliquots of 100 µg/ml laminin in phosphate buffered saline (PBS) (135 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4), pH 7.4 overnight at 4°C. Next day wells were washed three times with 200 µl of PBST (PBS, 0.5% Tween 20) and blocked with 100 µl of 2% BSA for 2 hours at room temperature. BSA was removed and three washes followed in PBST, 10^{11} phage particles were added in PBST for 1.5 hours at 37°C or at 25°C. After four washes in PBST the wells were incubated with 100 µl of 1:5000 diluted horseradish peroxidase (HRPO) labelled monoclonal anti-M13 antibody (AP Biotech, CA, USA). The plate was then incubated for 1 hour at room temperature. Unbound antibody was removed with three washes in PBST, and 150 µl ortho-phenylene-diamine (OPD)-reagent (Fluka AG, Germany) was added. Colour development was stopped by addition 50 µl of 4 M H_2SO_4. A_{492} values were determined on an automated plate reader (Metertech, Taiwan). Phage samples were tested in triplicates and random phages were applied as negative control. Wells with laminin, anti-M13 antibody and OPD-reagent without phages served as blank.

3.3.2. Bacterial strains and plasmids used in laminin-Pla interaction study

E. coli XL1-Blue MRF (Stratagene, USA) (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac [F' proAB, lacI^{q}ZΔM15, Tn10 (Tet^r), Cam^r]) was used as phage host strain. It was grown in Luria Bertani broth (LB broth) complemented with 20 µg/ml tetracycline for keeping it F-pilus positive. *E. coli* TB1 pC4006 is a derivative of *E. coli* TB1 harbouring the pUC19 plasmid with cloned determinants for *Y. pestis* plasminogen activator (Kienle et al., 1992) *E. coli* TB1 pUC19

served as control strain. These two strains were cultivated also in Luria Bertani broth supplemented with 100 µg/ml ampicillin. TB1 pC4004 (Kienle et al., 1992) and the low-copy-number vector pACYC177 (MBI Fermentas, Lithuania) was utilised for site directed mutagenesis. TB1 pC4004 was a pK18 derivative and grown also in Luria-Bertani broth supplemented with 50 µg ml kanamycin. TB1 Inv bore the plasmid pJS-1-pUC6.2, expressing *Yersinia pseudotuberculosis* invasin that was a gift of Prof. Dr. Dr. Jurgen Heesemann, Ludwig-Maximilians-University, Faculty of Medicine, Max von Pettenkofer-Institute for Hygiene and Medical Microbiology, Department of Bacteriology, Munich, Germany.

3.3.3. Assaying of Pla mediated laminin binding of bacteria

For laminin binding experiments overnight cultures of *E. coli* TB1 pC4006 and *E. coli* TB1 pUC19 were spun down on a Hettich centrifuge (Hettich, Germany) at 4000 rpm, 4°C for 10 minutes and washed once with an equal volume of PBS. Concentration of the suspension was adjusted to 10^9/ml in PBS (with a spectrophotometer at A_{690} (Spekol, Gemany) and 100 µl aliqots of the suspension (10^8 bacteria) were added to microtiter plates (Nalge Nunc International, Denmark). Plates were first sensitised overnight at 4°C with 20 µg/ml of laminin in PBS. Wells were then washed three times with PBST and blocked with 2% BSA for two hours at room temperature. BSA was removed and wells were washed again with PBST. 10^8 bacterial cells were added to the wells and the plates were incubated for 1.5 hours at 37°C or at 25°C. Wells were washed then four times in PBST. Adhered bacteria were fixed with PBS containing 2% formalin. Wells were washed once again with PBST and stained with 0.13% crystal violet (Reanal, Hungary) solution. Unbound stain was then removed and wells were washed three times in PBST. Adhered bacterial cells were lysed with 1% SDS-solution and the A_{595} absorbance of the released crystal violet was measured on an automated plate reader (Metertech, Taiwan). Samples were tested in triplicates at least two times.

3.3.4. Phage mediated inhibition of laminin binding

The test was performed basically in the same way as described for assaying Pla-mediated bacterial laminin binding except of the following modifications: 10^{10} phages were co-incubated with bacterial cells on the laminin-coated plate at 37°C for 1.5 hours. Random phages were added as negative control. After this incubation the number of adhered bacteria was determined not by staining but by detaching bacterial cells from the wells with Triton-Trypsin solution (0.1

% Triton X-100, 0.25% Trypsin), serial dilutions were plated, incubated at 37°C overnight and colonies were counted on the next day.

3.3.5. Peptide mediated inhibition of bacterial laminin binding

Twofold serial dilutions of peptides were prepared in PBST (0.1% Tween 20) with a starting concentration of 2mM. Aliquots were added to a microtitre plate which had previously been sensitised overnight at 4°C with 100-100 µl 10 µg ml laminin in PBS and blocked for two hours at room temperature with 150-150 µl 3% BSA. After 90 minute incubation at 37°C peptides were removed and 10^7 bacteria were added to each well. After another 90-minute-long incubation at 37°C unbound bacteria were removed and 1:2000 dilution of a polyclonal anti-TB1 antibody raised in rabbit was added in PBST (0.1% Tween 20) for another 1.5 hours at 37°C. After removal 1:1000 dilution of anti-rabbit immunoglobulin G conjugated to horse-radish peroxidase (Dako, Germany) was incubated on the wells for 35 minutes at 37°C. Secondary antibodies were discarded and the TMB (tetra-methyl-benzidine) chromogen substrate (Pierce, USA) was added to the wells. Colour development was stopped after 2 minutes by the addition of 2 M H_2SO_4 and absorbance was read at λ 450 nm with an automated plate reader (Multiscan Ascent, Thermo Labsystems, Finland). After each step wells were washed three times in PBST (0.5 % Tween 20) except after peptide pre-incubation when wells were washed only once in PBST (0.5% Tween 20). Two rows were used as positive control preincubated only with PBST. One row was used as negative control, when neither peptides nor bacteria were added to the wells. Test was performed in duplicates and repeated three times. Inhibition was tested for both the Pla$^+$ and the Pla$^-$ vector control strain. The A_{450} values of the control strain were subtracted from the ones of the Pla$^+$ strain. The percentage of inhibition was determined as follows: {1-[(A_{450} of the test well- A_{450} of the negative control well)/(A_{450} of the positive control well-A_{450} of the negative control well)]}×100.

3.3.6. Peptide pattern search and homology modelling

Peptide patterns of the two inhibitory phages were compared to the amino acid order of Pla. Similarities were identified with the program PattinProt constructed and supplied by the Institute of Protein Biology and Chemistry at the University Claude-Bernard, Lyon, France at the Expasy website of the Swiss Institute of Bioinformatics, Geneva. Three-dimensional modelling was performed with the Swiss PDB Viewer/ DeepView program, another free software created and distributed by Glaxo-Smith-Kline R&D on the Expasy website. The structure of Pla was modelled basing on its closest homologue OmpT and the two structures were also superimposed.

3.3.7. Site-directed mutagenesis of *Yersinia pestis* plasminogen activator

For introducing mutations into the proposed laminin binding sites the pla gene was subcloned from the construct pC4004 by a single Hind III digestion into the low copy number vector pACYC177. Transformants resistant to ampicillin but sensitive to kanamycin due to the kanamycin cassette disruption appearing along with the integration of the pla gene were selected and sequenced. Primers for mutagenesis (Table 5) were from Sigma Genosys, U.K. and were all phosphorylated. In each case a single polymerase chain reaction was performed by the utilisation of the KlenTaqu Long Accuracy Polymerase (Sigma, Germany). Mutant L65AT66AL67A was created with primers Pla LTL and Pla LTL reverse, mutant G178A with primers Pla GA and Pla GL reverse, mutant L179A with primers Pla LA and Pla GL reverse and mutant G178AL179A with Pla GL and Pla GL reverse. The whole pACYC177 plasmid construct was used as template. Reaction conditions in an Eppendorf Thermocycler (Mastercycler Personal) were the following: After a two-minute initial denaturation at 95°C 35 cycles of amplification were carried as follows: denaturation at 95°C for 30 sec, annealing at 58°C for 45 sec, extension at 68°C for 5 min. Then a final elongation step at 68°C for 10 min was performed. Reaction mixtures had a total volume of 50 µl containing the template DNA, 10 pmol of each primer, 200 µM dNTP, 5 µl of 10 × KlenTaqu LA buffer and 2.5 U of KlenTaqu LA Polymerase. After finishing the PCR, the total reaction mixture was loaded onto a 1% agarose gel, run, stained with 0.1 % ethidium-bromide and the PCR product was cut and eluted into ddH_2O with QIAQuick Gel Purification Kit (QIAGEN, Germany). The eluted fragment was treated with T4 Polymerase (MBI Fermentas, Lithuania) to produce blunt ends and methylated template strands were removed with Dpn I (MBI Fermentas, Lithuania) digestion. The final product was self-ligated with T4 Ligase (MBI Fermentas, Lithuania) for overnight at 16°C and then transformed into competent TB1 cells. Plasmid DNA from transformants were extracted with the alkali-lysis method, cleaned up on QIAGEN columns and sequenced with an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA) using either the GL reverse primer or the Pla LTL mutagenic primer. PCR reactions were performed with several primer pairs. The primer pairs were constructed in such a way that the whole plasmid of TB1 pC4006 encoding the Pla gene could be amplified through the PCR reaction (Table 5). PCR products were run on 1% agarose gel and isolated with QIAquick Spin Kit. Fragments were treated with T4 polymerase to produce blunt ends for self-ligation and with DpnI to remove template strands. Ligation was performed overnight at room temperature and inactivated ligation mixes were transformed into competent TB1 cells. Transformant clones were tested for protein expression with a simple fibrinolytic assay because

mutations should not affect this activity of Pla. Clones were inoculated into a fibrin film composed of 0.5 % fibrinogen and 50 U/ml thrombin, and fibrinolytic activity was checked after incubation at 37 °C for 4 hours.

Table 5. Primers for alanine-scanning mutagenesis

Sequence of primer	Introduced mutation
5'-TCC Tgg gAT CCA TAC TCA TTT gCT gCC gCg AAT Agg ggg Tgg ACg-3'	L65AT66AL67A
5'-TAT ATC ACC TTT CAg gAT AgC gAC-3'	L65AT66AL67A
5'-CCA TAT ATT gCT CTT gCA ggC CAg TAT CgC-3'	G178A
5'-CAT-AgA AAA gCg CTg gTT-ATA A-3'	G178A
5'-CCA TAT ATT ggA gCT gCA ggC CAg TAT CgC-3'	L179A
5'-CAT-AgA AAA gCg CTg gTT ATA A-3'	L179A
5'-CCA TAT ATT gCT gCT GCA ggC CAg TAT CgC-3'	G178AL179A
5'-CAT-AgA AAA gCg CTg GTT ATA A-3'	G178AL179A

3.3.8. Plasminogen activation

Kinetic measurement of plasminogen activation was performed in a similar way as described by (Kukkonen et al., 2001) with some modifications. 8×10^6 bacteria and 4 µg Glu-plasminogen (Sigma, Germany) were used per well on a microtitre plate in 200 µl of PBS and 45 µl from the 1.65 mg/ml stock of the chromogen substrate S-2251 (Chromogenix, Germany) was added to them and incubated at 37°C for 3.5 hours. At different time-points the absorbance was measured at λ=405 nm with an ELISA-reader (Multiscan Ascent, Thermo Labsystems, Finland). Control wells contained no Glu-plasminogen but only bacteria and substrate. Samples were tested in duplicates and experiments repeated three times.

3.3.9. SDS-polyacrylamide gelelectrophoresis

Total protein extracts of the strains TB1 pUC19 and TB1 pC4006 were run on a 12% denaturing polyacrylamide gel at 200 V for 45 minutes. 10^9 bacteria from an overnight culture were boiled in 32 µl 5×sample buffer [60mM Tris-HCl (pH 6.8), 2%SDS, 14.4 mM 2-mercaptoethanol, 0.1%

bromophenol blue, 25% glycerol] for 10 minutes, spun briefly and 8 µl fractions of the supernatant corresponding to 2.5×10^5 bacteria were loaded on the gel. After running the gel was stained for 40 minutes in Coomassie blue and destained in Coomassie destaining solution for overnight.

3.3.10. Cell culture conditions

HeLa cells were grown in RPMI 1640 medium (Gibco BRL, UK) (pH 7.1) supplemented with 1 mM $NaHCO_3$ and 10% foetal calf serum (Gibco BRL, UK). Cells were seeded a day before the experiments in 35×10 mm Petri dishes (Greiner, Germany) to reach either confluency (1×10^6 cells/dish) or semiconfluency (3×10^5 cells/dish) by next day. For fluorescent staining HeLa cells were seeded onto 22 mm glass coverslips (Biocoat™, BD Biosciences, Becton Dickinson, USA) in 12-well tissue culture plates (Greiner, Germany) to reach semiconfluency (1×10^5cells /well) by next day.

3.3.11. Preparation of signal transduction inhibitors and cytochalasin D

Signal transduction inhibitors were all dissolved in dimethyl-sulfoxide (Sigma, Germany) except of the C3 exoenzyme, which was dissolved in ddH_20 at the following concentrations: wortmannin (Sigma, Germany) 10 mM, genistein (Sigma, Germany) 100 mM, nordihydroguaretic acid (NDGA) (Sigma, Germany) 100 mg/ml, staurosporin (Sigma, Germany) 10 mM, cytochalasin D (Sigma, Germany) 1 mg/ml, C3 exoenzyme (Sigma, Germany) 0.5 mg/ml. For the experiments stocks were diluted in RPMI 1640 (Gibco BRL, UK) to the following working concentrations: wortmannin 10, 25, 50 and 100 nM, genistein 250 µM, NDGA 17 µg/ml, staurosporin 0.5 µM, cytochalasin D 0.5, 1, 2 and 5 µg/ml, C3 exoenzyme: 5 µg/ml.

3.3.12. Adhesion assays

3.3.12.1. Microscopic assay

Overnight LB cultures of bacterial strains were washed in phosphate buffered saline (PBS), pH 7.4. Suspensions of Pla positive and Pla negative isogenic strains were spectrophotometrically adjusted to a concentration of 10^8cells/ml in PBS. The suspensions were diluted 1:1 in PBS, washed once, and resuspended in an equal volume of RPMI 1640 (Gibco BRL, UK) supplemented with 1 mM $NaHCO_3$. Semiconfluent HeLa cell cultures were infected with 2 ml

aliquots of the suspensions and incubated at 37°C for 3 hours in 5% CO_2. Previously, we checked that keeping bacteria in RPMI medium for three hours did not significantly increase the number of bacterial cells. After incubation unbound bacteria were removed with eight washes in PBS. HeLa cells were fixed with methanol for 1 minute, washed once in PBS and stained for 25 minutes with Giemsa stain at room temperature. The dye was removed then with three washes in dd H_2O, and cell cultures were examined by light microscopy using a Zeiss Axioskop 40 (Zeiss, Germany) apparatus. Tests were prepared in triplicates and repeated at least twice.

3.3.12.2. Quantitative adhesion assay

In alternative experiments the number of cell associated bacteria was evaluated on confluent cell cultures as follows: after removing unbound bacteria with eight washes HeLa cells were lysed with Triton-Trypsin [0.1% Triton X-100 (Sigma, Germany), 0.25% Trypsin (Difco, USA)] solution. This treatment had no influence on bacterial viability. 10 µl aliquots of serial dilutions of the lysates were plated onto LB agar supplemented with 100 µg/ml ampicillin. Plates were incubated overnight at 37°C and colony forming units were counted. Tests were prepared in triplicates and repeated at least twice. Pla-positive and negative bacteria were also added to HeLa cell free Petri dishes to assess the level of binding of bacteria to the plastic surface.

3.3.13. HeLa cell invasion assay

Principally the gentamicin protection assay (Isberg and Falkow, 1985) was applied to kill cell associated but not internalised bacteria. Steps of the experiment were the same as in the adhesion assay but after removing unbound bacteria the dishes were incubated with 100 µg/ml gentamicin in RPMI for one hour at 37°C in 5% CO_2. Then cells were washed three times in PBS, lysed by Triton-Trypsin, and the number of internalised bacteria was determined as described in section 3.3.13.2.

3.3.14. Inhibitor assays

First we checked whether the inhibitors exhibited a harmful effect on bacteria or HeLa cells. Bacteria were incubated for four hours (time interval of the presence of inhibitors in the assays) with the signal transduction inhibitors diluted in RPMI to working concentration. Bacterial counts were determined by plating and colony counting before and after the four-hour incubation period.

HeLa cell cultures grown in dishes were also incubated with the inhibitors for four hours. Then they were washed eight times carefully with PBS, and stained with Trypane blue to detect exclusion.

In the inhibition assays confluent HeLa cell cultures were pre-treated for an hour with the inhibitor diluted to working concentration in RPMI (without foetal calf serum since it might inhibit invasion) at 37 °C in 5 % CO_2 atmosphere. The only exception was the pretreatment with the slowly diffusible C3 exoenzyme. When HeLa cells were incubated with the exoenzyme for 24 hours before starting bacterial invasion. Control cultures were preincubated with RPMI alone. Then cultures were washed once in PBS and bacteria were added. During the three-hour-long bacterial incubation RPMI media was supplemented with the inhibitors at the same concentration as used for pre-treatment. Bacterial adhesion and invasion were evaluated as described above. Due to the highly reversible nature of genistein, this inhibitor was also present during the gentamicin treatment in the cell culture medium.

3.3.15. Fluorescence staining

Strains TB1 pUC19 and TB1 pC4006 were used throughout the experiment. 5×10^{7} bacteria in RPMI were incubated on semiconfluent HeLa cultures for 4 hours at 37°C, 5% CO_2. The invasion was stopped at several time points: every seven minutes during the first hour and then at the end of each hour bacteria were removed with eight washes in PBS. Cells were then fixed for 20 minutes with 4% paraformaldehyde (in PBS) at room temperature. Thereafter they were washed three times in PBS and permeabilised with 0.1%Triton X-100 (in PBS) for 30 minutes at room temperature. Cells were washed again three times with PBS and stained for 1hour at room temperature with 0.65 µg/ml TRITC-phalloidin (Sigma, Germany). Coverslips were washed then three times in PBS and three times in ddH_2O. Finally they were mounted with glycerol-PBS (9:1), covered with glass slips and closed with nail polish. Staining was always performed in duplicates. Specimens were examined under an Olympus BX 61 epifluorescence microscope (Olympus Japan) equipped with the following filter set: excitation: 557 nm, emission: 576 nm and analysed with the AnalySIS software (Soft Imaging System, Germany). Cell cultures without bacteria were also stained in the same way and used as negative control.

3.3.16. Studying the effect of inhibitory phage on Pla mediated internalisation

In these tests phage #5, #14 and random phage were either coincubated or preincubated with the Pla$^+$recombinant strain TB1 pC4006 and with the negative control strain TB1 pUC19 on semiconfluent or confluent HeLa cell cultures. Preincubation was an hour long at 37°C, 5% CO_2.

Phages were added in RPMI. Following three washes in PBS a regular three-hour-long invasion assay was performed. In case of coincubation phage and bacteria were added together in RPMI onto the HeLa cell cultures for a three- hour-incubation at 37°C, 5% CO_2. Additional steps were performed as described in sections 3.3.12. and 3.3.13. The effect of 5×10^{12}, 10^{12} and 5×10^{11} phage particles was tested

3.4. RESULTS

3.4.1. Biopanning against laminin

Four rounds of biopanning were performed against immobilised laminin with a library of recombinant random heptamer peptides expressed as a fusion with M13 minor coat protein gpIII. The titre of eluates increased thirty fold by the end of the fourth round (data not shown) demonstrating selection and enrichment of tight-binder peptide sequences to laminin. Twenty plaques were isolated and sequenced (Table 6). Two of them, NSELTTA and FKNYEQP occurred twice. Among the eighteen different sequences only NSELTTA and WSLLTPA showed a conserved motif.

Table 6. Peptide sequence of phage were isolated from the fourth round eluate from a biopanning against laminin with a random heptamer phage display library. Consensus sequences with Pla are given in bold.

No. of phage	Heptamer amino acid sequences						
1	V	V	S	V	S	P	I
2	H	T	W	H	P	G	Q
3	S	E	K	S	M	F	Q
4	A	P	L	V	L	N	I
5	W	**S**	L	**L**	**T**	P	**A**
6	H	P	S	H	N	H	R
7	Y	S	G	F	P	N	A
8	N	**S**	E	**L**	**T**	T	**A**
9	A	Q	S	W	A	M	A
10	S	H	S	A	H	I	F
11	V	H	Q	R	A	P	N
12	W	D	A	P	P	V	D
13	F	K	N	Y	E	Q	P
14	Y	P	Y	I	P	T	L
15	N	A	K	H	S	P	P
16	H	M	H	Y	R	I	H
17	A	I	P	Q	R	L	I
18	G	L	A	A	R	W	L

3.4.2. Pla-mediated laminin binding

Involvement of Pla in laminin binding was investigated both at the body temperature of the mammalian host and at ambient temperature present in the arthropod vector. Our data indicate (Figure 8) that laminin binding by bacteria is 50% less effective at room temperature (25°C) than at 37°C for both control and test strains. In the presence of Pla laminin binding increases about ten times at 37°C and almost twenty times at 25°C.

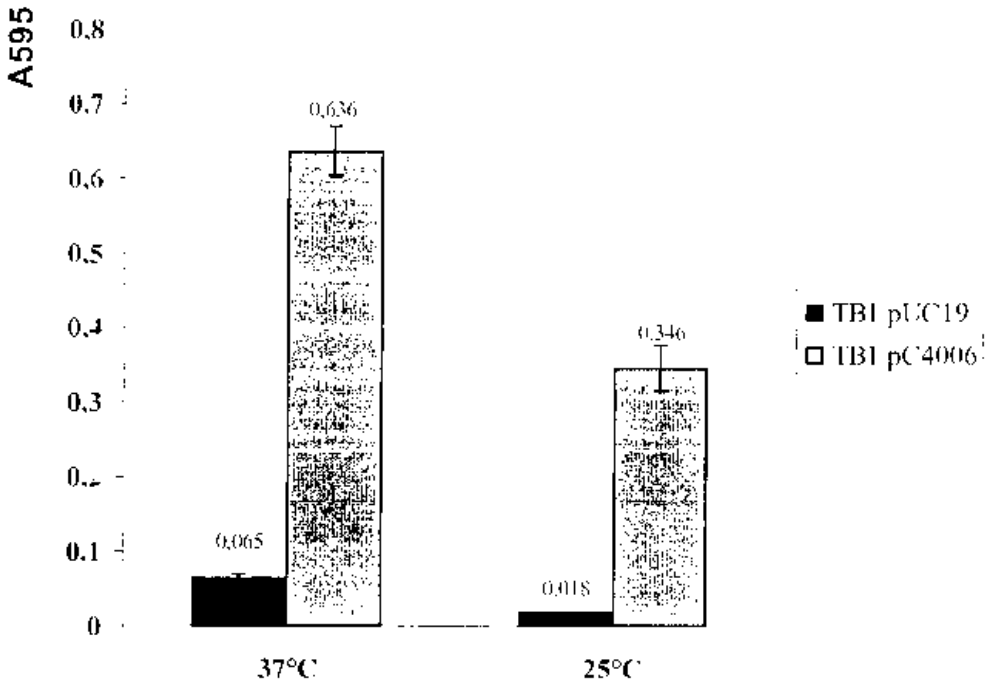


Figure 8. Pla enhances the laminin binding of non-adherent *E. coli* tenfold at 37°C and twentyfold at room temperature.

3.4.3. Phage mediated inhibition of laminin binding

Two phage sequences (WSLETPA and YPYHPTL) interfered strongly with Pla mediated laminin binding of *E. coli* (Figure 9). They decreased laminin binding by the Pla$^+$ *E. coli* strain TB1 pC4006 to the level of the background vector control, which means a complete interference. This effect should be due to a competition of the phage and Pla for the same binding site(s) on laminin since the random phage and all the other sequences exerted no significant effect on the

bacterium-laminin interaction. Though WSLLTPA and NSELTTA show homology only WSLLTPA but not NSELTTA was able to inhibit the Pla-mediated laminin binding. This might mean that not only the consensus motif accounts but also the neighbouring amino acids might have an influence on the interaction site. In the third position the non polar leucine of WSLLTPA and the acidic glutamate of NSELTTA, and in the sixth position the non polar proline and the polar threonine of the peptides respectively, have a different side-chain character.

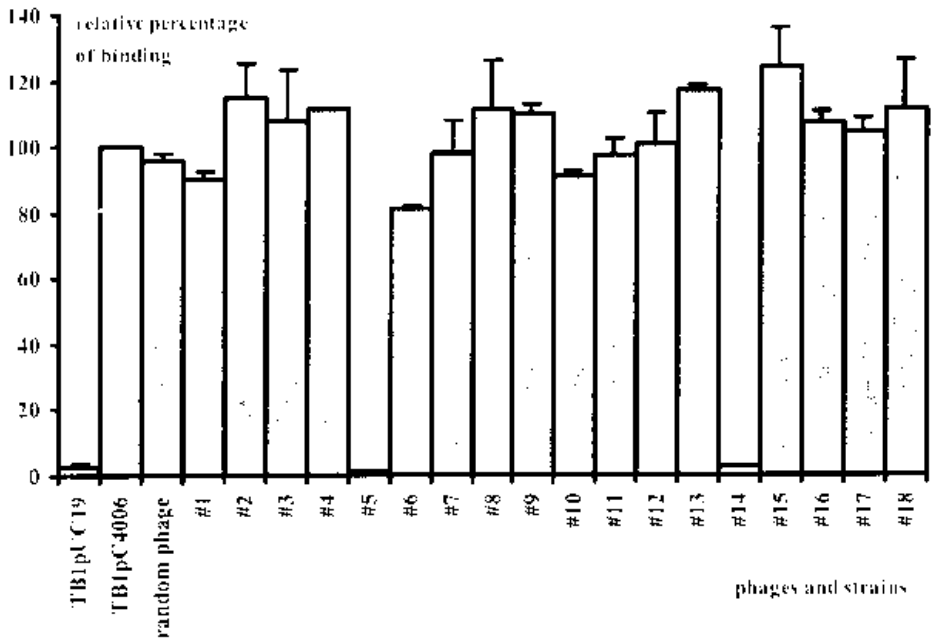


Figure 9. Interference with Pla-mediated laminin binding of recombinant *E. coli*. Number of bound bacteria is expressed as relative percentage of the bacterial binding without phage (second column). Phage #5 and phage #14 completely inhibited Pla mediated laminin binding back to the level of the background strain TB1 pUC19.

3.4.4. Laminin binding ELISA with inhibitory phage

To disprove that inhibition of bacterial laminin binding might be due to any kind of non-specific interactions of phage and the plasminogen activator molecule we needed to verify that selected phages inhibiting Pla mediated laminin binding of *E. coli* efficiently bind themselves to laminin. The laminin binding capacity of these phages was tested in an ELISA assay using horseradish

peroxidase (HRPO)-conjugated monoclonal anti-M13 antibody. Phage WSLLTPA bound about seventeen times and phage YPYIPTL about thirteen times stronger to laminin than the random phage used as negative control (Figure 10). These results also suggest that inhibitory phage and Pla compete for the same binding site on laminin.

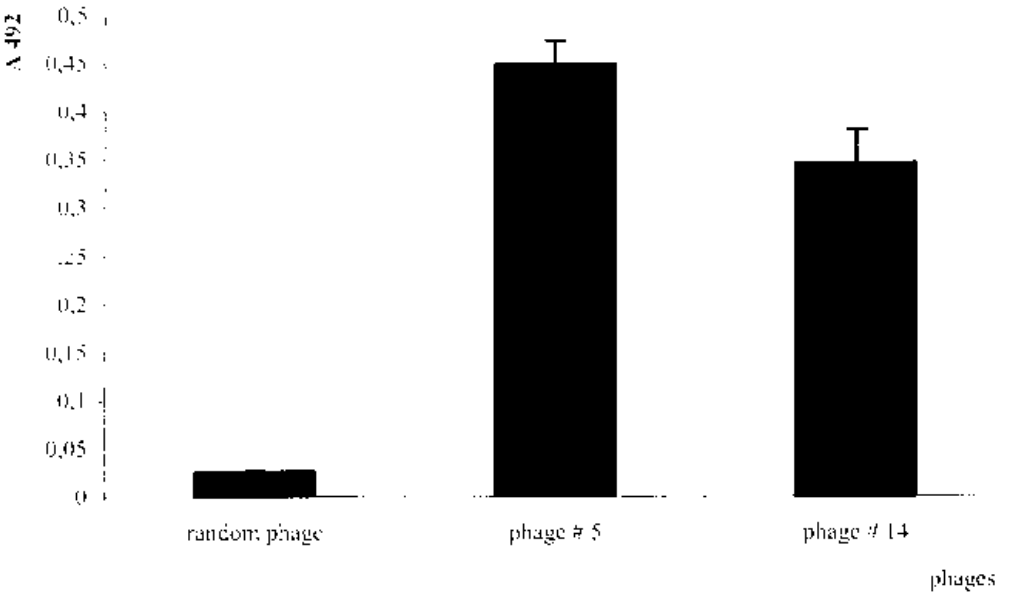


Figure 10. Laminin binding capacitites of the two eluted phage sequences, which were able to interfere with Pla mediated laminin binding. Adhered phages were detected with HRPO-labelled monoclonal anti M13 phage and OPD-substrate at λ=492 nm. Phage #5 bound seventeen times stronger, phage #14 thirteen times stronger than the random phage control.

3.4.5. Inhibition of Pla mediated laminin binding with synthetic peptides

The inhibitory effect of synthetic peptides WSLLTPA and YPYIPTL was investigated with using serially diluted fractions of them. The highest tested concentration was 2 mM due to the poor water solubility of peptide WSLLTPA. They showed a more moderate inhibition than the respective phages displaying these sequences. While WSLLTPA showed a maximum of 55% inhibition YPYIPTL reached only a 33% maximal interference (Figure 11) Peptide YPYIAAA

served as negative control displaying an inhibitory capacity around zero.

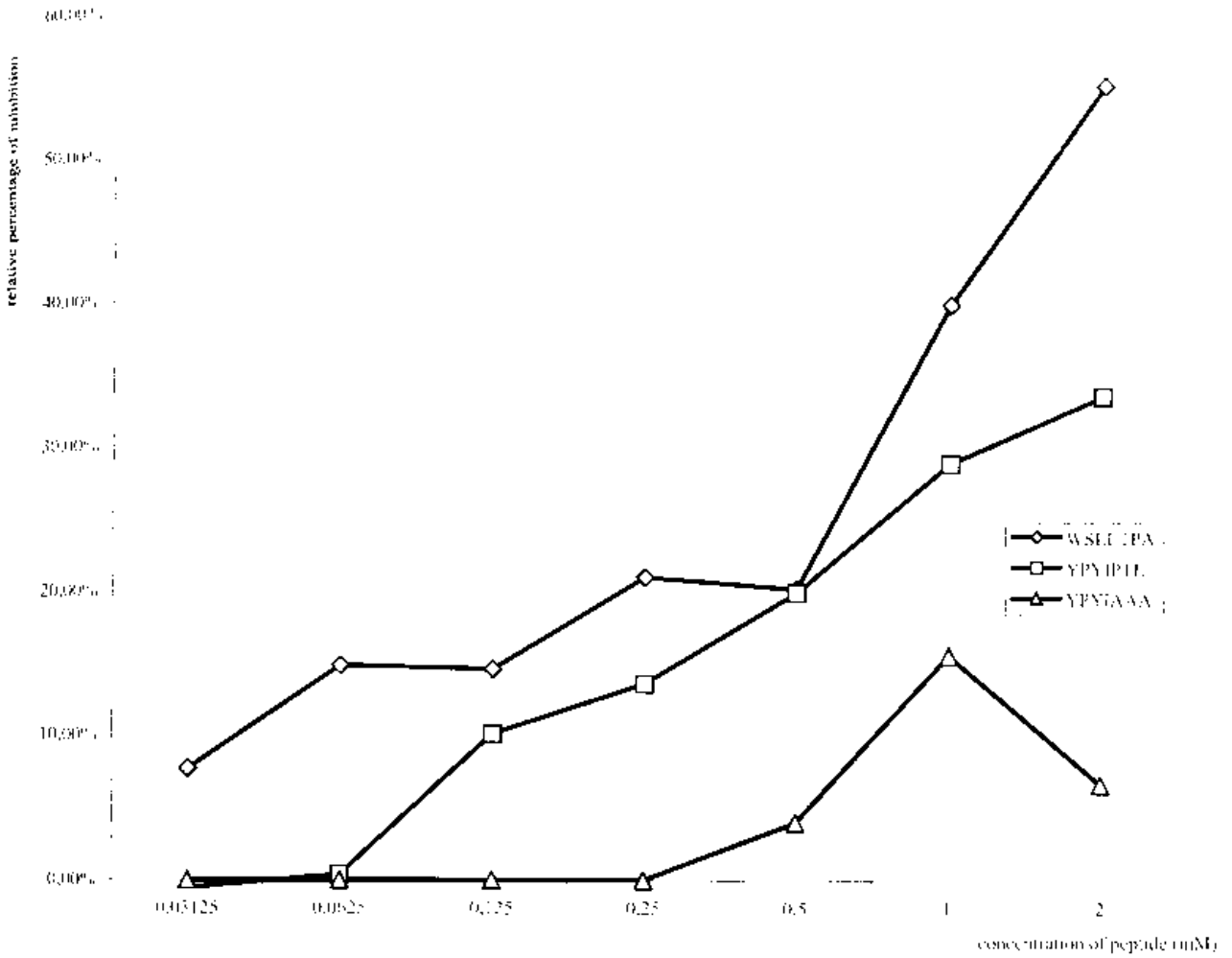


Figure 11. Inhibition of Pla mediated laminin binding by synthetic peptides. A moderate but gradual inhibition was detectable with peptides showing a marked inhibitory capacity when displayed on the phage surface. Peptide YPYIAAA was used as negative control showing no or a very limited inhibition.

3.4.6. Localisation of the laminin binding motifs

For localisation of the two inhibitory peptide sequences we used two programs available at the ExPASy Molecular Biological Server. PATTINPROT was created at the Institute of Protein

Biology and Chemistry of the University Claude-Bérnard, Lyon. It enables searching for peptide motifs with several degrees of similarity inside shorter and longer protein sequences. We compared the amino acid sequence of Pla from the SWISSPROT database (accession number: P17811) with the ones of the two inhibitory phages WSLLTPA and YPYIPTL. Figure 12 shows the phage sequences and their localisation inside the amino acid sequence of Pla along with the pair wise alignment of Pla with E. coli OmpT. This latter protein shows a 60% homology with Pla (Sodeinde and Goguen, 1989) and its crystal structure has been determined (Vandeputte-Rutten et al., 2001). We found the S-X-L-T motif of peptide WSLLTPA at amino acids S63, L65 and T66 (numbering reveals the position of the amino acids in the mature protein). The whole string aligned to the heptamer is ...YSFLTLN... Comparing the two patterns it is obvious that the first and the last amino acids are non-conservatively substituted. Both tryptophane and tyrosine have aromatic side chains, however tryptophane has an apolar but tyrosine a polar side-chain character. The last leucine residue of the peptide has an apolar, and the last asparagine residue of the protein fragment has a polar side chain. The five inner amino acids either show identity or similarity by conservative substitutions in the character of the side-chain. WSLLTPA represents another pattern, S-X-L-X-A, which is localised at amino acids S3, L5, and P7. These three and the neighbouring amino acids are arranged in the string SSQLIPN. The P-Y-I pattern of peptide YPYIPTL was localised at P175, Y176, and I177 of Pla. The whole corresponding amino acid environment was ...MPYIGLA.... Comparing to YPYIPTL two non-conservative differences in the amino acid characters are apparent. Tyrosine and methionine in the first position, threonine and leucine in the sixth position bear distinct features. In the pairwise alignment surface-exposed loops and periplasmic turns of OmpT were also indicated based on the structure provided by Kramer et al. (2001). Motifs were also positioned in the three-dimensional model of Pla. The model was built with the help of the DeepView program provided by Glaxo SmithKline R&D Geneva based on the already determined crystal structure of OmpT. Considering the high degree of homology with OmpT Pla also has an assumed beta-barrel structure like OmpT (Kukkonen et al., 2001). The localisation of peptide patterns is labelled on different views of modelled Pla (Figure 13). The two WSLLTPA patterns localise periplasmically, SXLXP close to the N-terminus, SXLT at the first turn. PYI is close to loop 3, and this string appears also in OmpT.

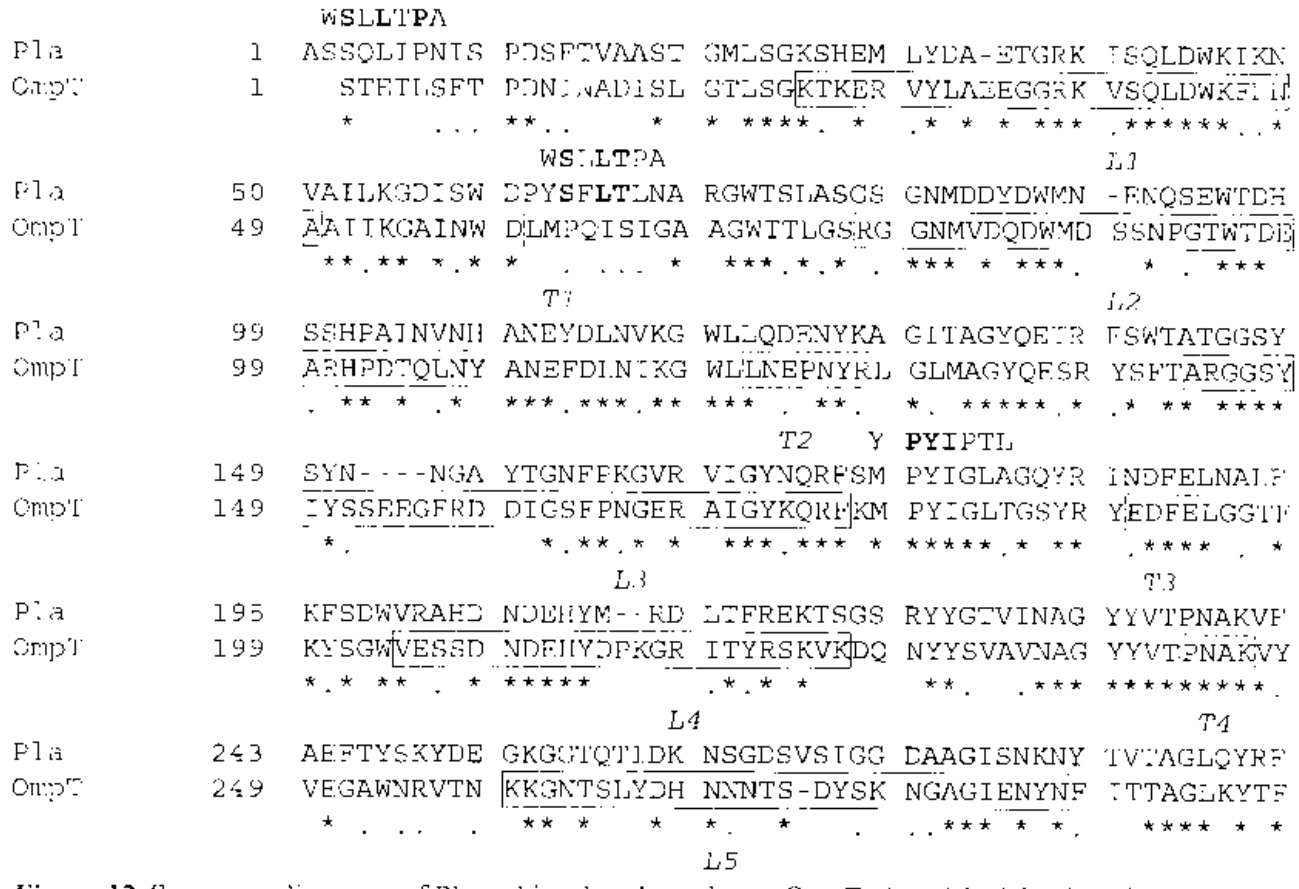
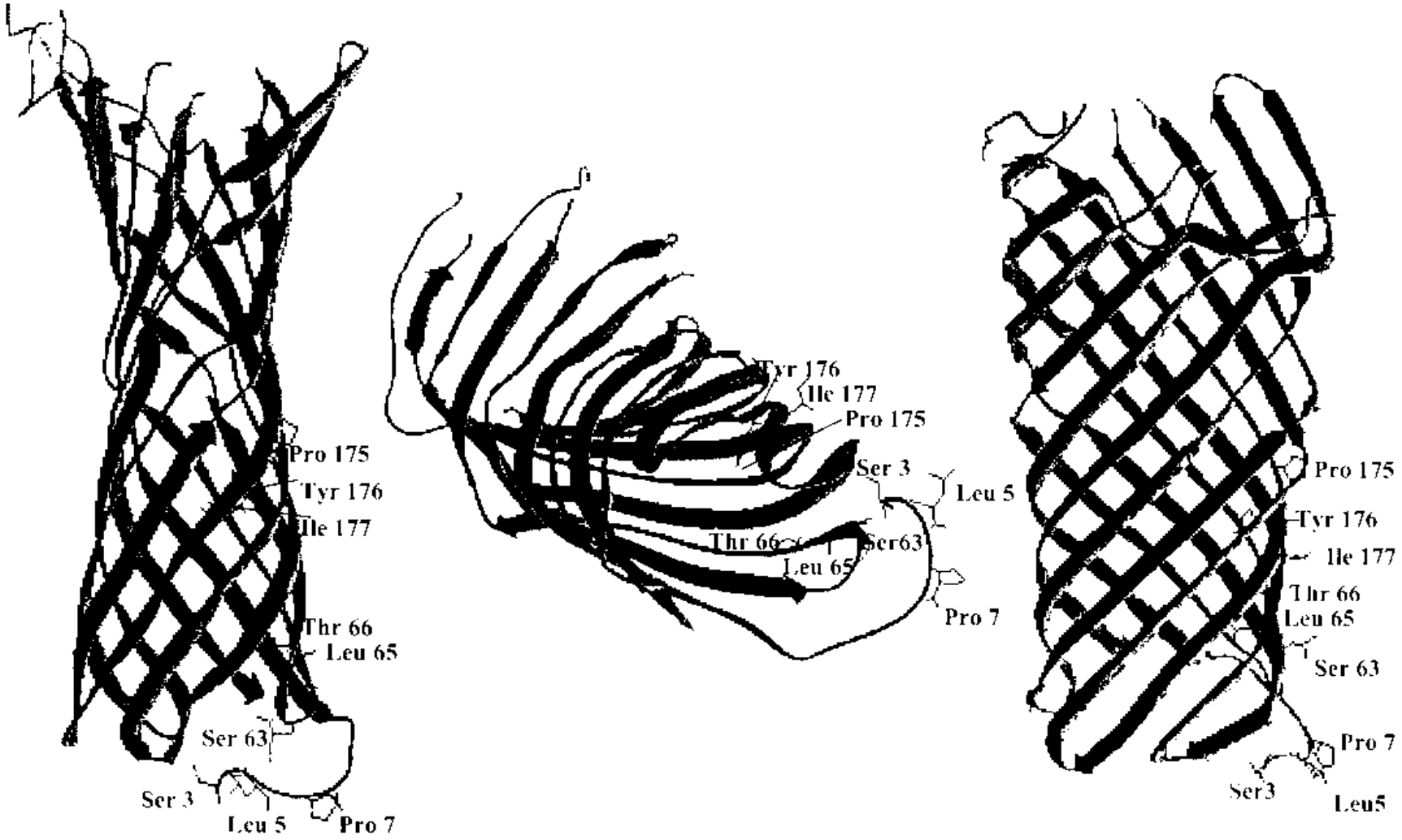


Figure 12. Sequence alignment of Pla and its close homologue OmpT. Asterisks label identity, dots label amino acids with similar side-chain character. Peptides #5 and#14 are also aligned. L reveals on loops, T on periplasmic turns. Loops are framed with continuous, periplasmic turns with a dotted line.

Figure 13. Three-dimensional model of Pla built with the help of the DeepViewer Program. The protein is shown from different position and the aligned amino acids from peptide #5 (WSLLTPA) and #14 (YPYIPTL) are also labelled.



3.4.7. Investigation of protein expression, laminin binding and plasminogen activation of recombinant mutagenic Pla

Pla expression of mutagenic clones was detected on a 12% denaturing polyacrylamide gel with Coomassie blue staining. A strong band corresponding to Pla was detected also in the mutagenic strains whereas no band was visible in the extract of the negative vector control strain, TB1 pACYC177 (Figure 14A).

Laminin binding capacity was assessed by counting the adhered bacteria after removing them with 0.1% Triton X-100 from the microtitre plate. Wild type Pla bound more than ten times stronger than the negative vector control in accordance with the previously presented data with TB1 pC4006. The triple mutant L65AT66AL67A, and the double mutant G178AL179A showed a decreased laminin binding: about half as many bacteria adhered to laminin as in the wild-type strain. The single mutants G178A and L179A displayed an affinity comparable to the wild-type strain (Figure 14B).

Plasminogen activation was detected by the colour development of the chromogenic plasmin substrate S-2251 at λ= 405 nm after the addition of Glu-plasminogen. The introduced alanine substitutions did not substantially alter the plasminogen activating ability of Pla (Figure 14C).

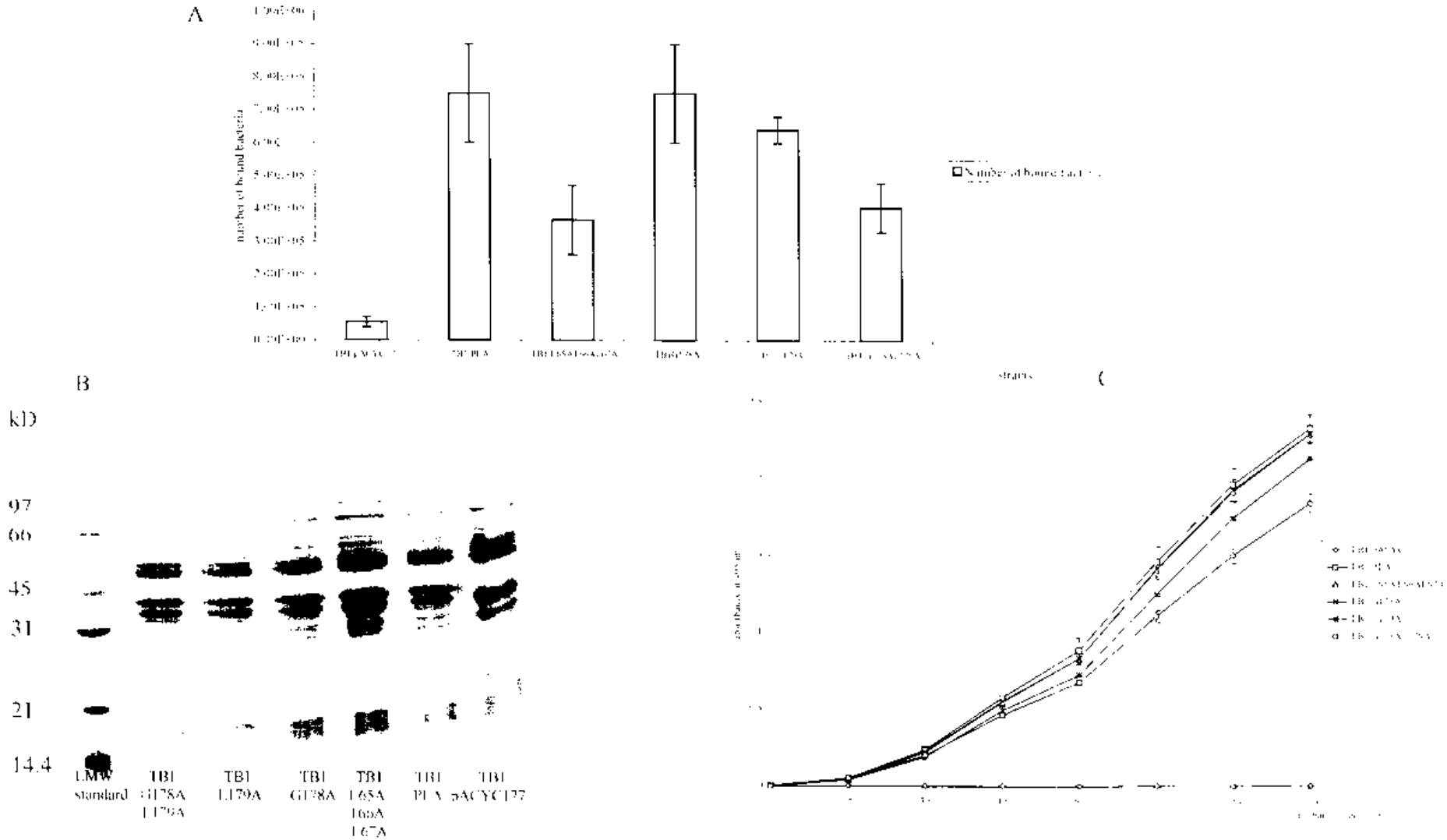


Figure 14. Effect of alanine scanning mutagenesis of Pla on laminin-binding (A), protein expression (B) and plasminogen activator (C) of recombinant *E. coli*. Pla is labelled with an asterisk.

3.4.8. Light-microscopy of Pla mediated adhesion

On semiconfluent HeLa cell cultures the Pla negative TB1 pUC19 strain exhibited practically no visible adhesion as shown by light microscopy (Figure 15A). On the other hand the Pla expressing recombinant TB1 pC4006 strain heavily colonised the HeLa cells (Figure 15B). The absence of microbes at the HeLa cell free areas of the semiconfluent culture points to a real bacterium-HeLa cell interaction.

Figure 15. Investigation of Pla-mediated adherence by *E. coli* TB1 on semiconfluent HeLa cell cultures. Pla-positive (TB1 pC4006) and Pla-negative (TB1 pUC19) *E. coli* strains were incubated with HeLa cultures for three hours. Unbound bacteria were removed by thorough washes. Cells were then fixed with methanol, stained by Giemsa and visualised by light microscopy using a Zeiss Axioskop 40 at a magnification of 400×. (A) Numerous Pla+ bacteria attach to the surface of HeLa cell. (B) No adherence is detectable for the Pla- strain.

3.4.9. Time-course of Pla mediated adhesion and invasion

In a four-hour experiment we determined the dynamics of Pla mediated bacterial adhesion (Figure 16A) and internalisation (Figure 16B) on confluent HeLa cell cultures. Throughout the experiments TB1 pC4006, a recombinant *E. coli* strain expressing Pla, and TB1 pUC19 as negative vector control have been used. There were considerable changes detectable already by the end of the first hour. Approximately 2×10^{7} Pla-positive bacteria adhered to the cell layer by the end of the first hour and about 2.5×10^{5} (1%) of them also entered the HeLa cells. During the next three hours there was a slight 1.5 fold increase in cell-association and a fourfold increase in internalisation revealing the relative time-dependency of the latter event. On the other hand the Pla-negative control strain (TB1 pUC19) showed a forty times weaker adhesion and no internalisation at all. Addition of 10^{8} or 10^{9} bacteria did not substantially

change the dynamics of adhesion and invasion or the number of adhered and internalised bacteria. Results also reveal that cloned Pla rendered the originally non-adhesive *E. coli* K-12 strain adhesive and invasive on epithelial cells. Adhesion of TB1 pC4006 and TB1 pUC19 did not show any difference when background binding was assessed by adding them to HeLa cell free Petri dishes

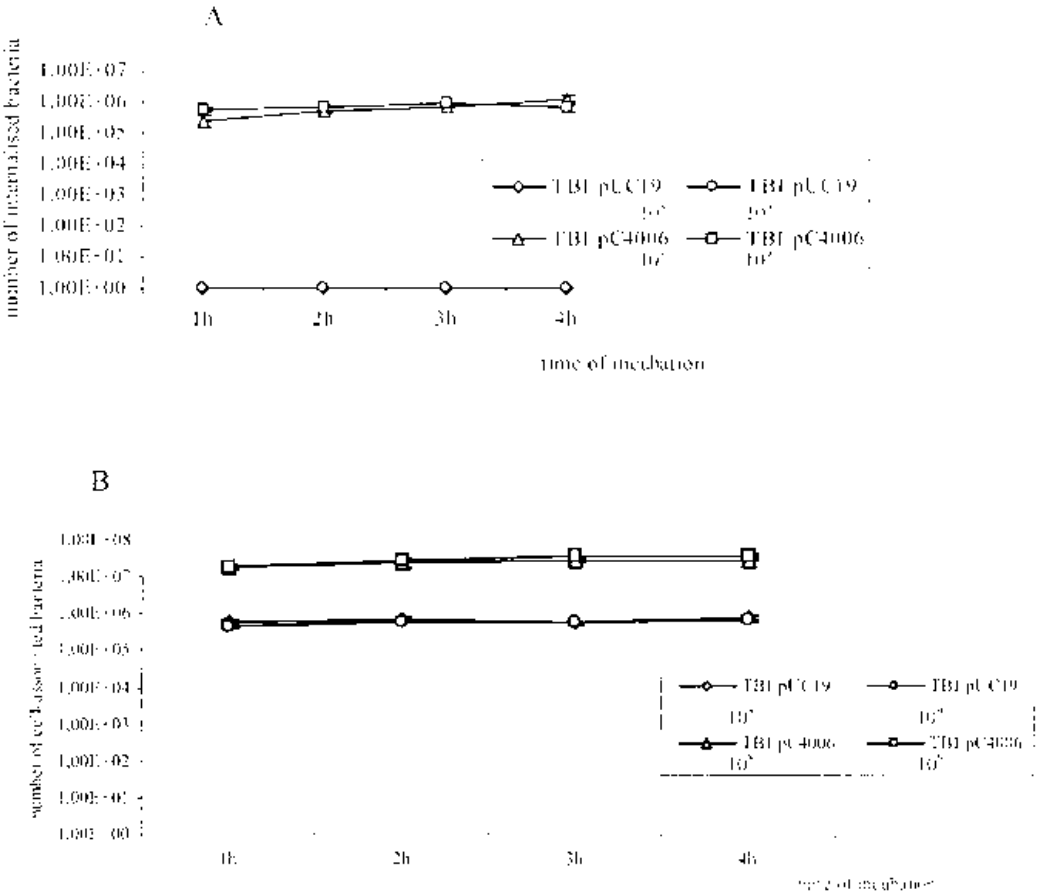


Figure 16. Time-course of Pla-mediated cell-association (A) and internalisation (B). Pla$^+$ and Pla$^-$ strains were incubated on confluent HeLa cell cultures for 4 hours. Unbound bacteria were removed by washes. Eukaryotic cells were lysed and cell-associated bacteria plated for overnight.

3.4.12. Effect of cytochalasin D and C3 exoenzmye of *C. botulinum* on Pla mediated invasion by *E. coli*

Cytochalasin D, which disrupts actin filaments, was tested in different concentrations. Concentrations 0.5 and 1 µg/ml did not interfere with cell-association or invasion. Higher concentrations elicited a thirty-fold decrease in invasion of Hela cells by the Pal-positive stram but did not affect bacterial adhesion (Figure 17B). The effect was not dose dependent as increasing amounts of cytochalasin D (2 and 5 µg ml) exhibited the same level of interference (data not shown). C3 exoenzyme of *C. botulinum* is a specific ADP-ribosylating inhibitor of the small Rho GTPase RhoA and its isoforms. Treatment of cells with 5 µg/ml C3 exoenzyme did not affect Pla mediated adhesion but decreased the internalisation to 20% (Figure 17B).

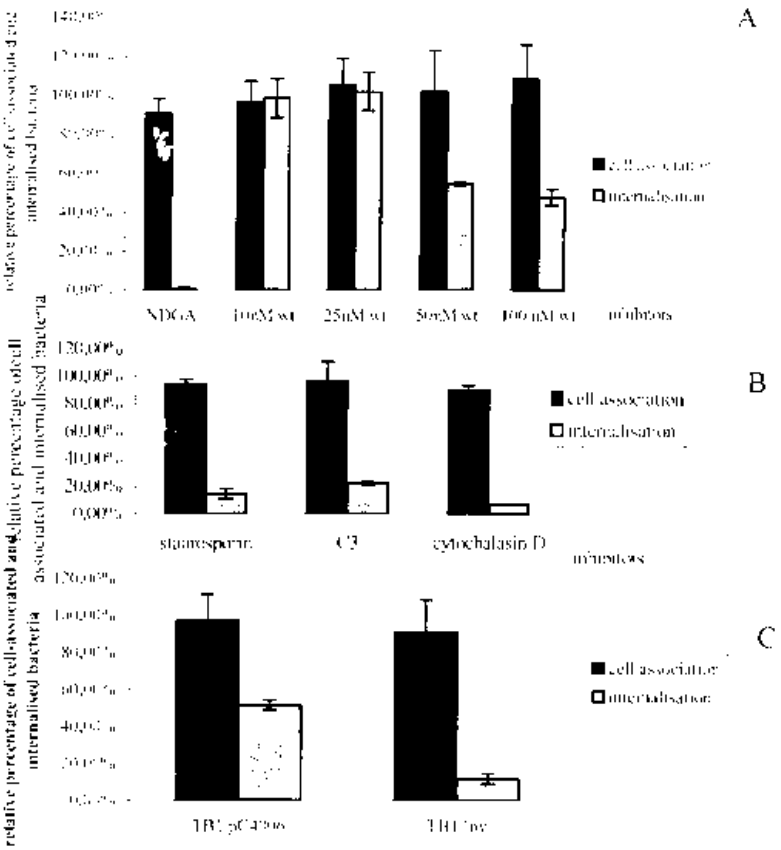


Figure 17. Effect of several signal transduction inhibitors on the cell association and internalisation of recombinant, Pla expressing *E.coli* TB1 pC4006 on confluent HeLa cell cultures. The number of cell-associated and internalised bacteria was expressed as a relative percentage of the untreated control tests. (A) shows the strong inhibitory activity of 15 µg/ml NDGA and the 50% internalisation decrease induced by wortmannin at 100 nM and at the differentiating 50 nM concentration. (B) displays internalisation inhibition initiated by 0.5 µM staurosporin, 5 µg/ml C3 exotoxin and 2 µg/ml cytochalasin D. (C) compares the inhibitory activity of 250 µM genistein on Pla (TB1 pC4006) and invasin (TB1 Inv) mediated internalisation.

3.4.13. Effect of phage inhibiting Pla mediated laminin binding on Pla mediated adhesion and internalisation into HeLa cells

Co- or preincubating Pla-expressing bacteria with phage #5 and #14 did not affect either Pla-mediated internalisation or adhesion in the tested phage concentrations. There was no difference in the number of colony forming units regained after HeLa cell lysis or in the Giemsa stained preparates. (data not shown).

3.4.14. Fluorescence staining

During a time course-experiment we were able to detect punctuate accumulation of actin (Figure 18B) after 42 minutes in the case of the Pla$^+$ recombinant *E. coli* strain TB1 pC4006. The changes were most dramatic after one hour and less intense after the further hours of incubation. This is in accordance with the quantitative time-course assay showing the most dynamic increase of internalisation during the first hour of incubation. We have not detected any cytoskeletal change with the incubation of the negative control strain TB1 pUC19 (Figure 18A).

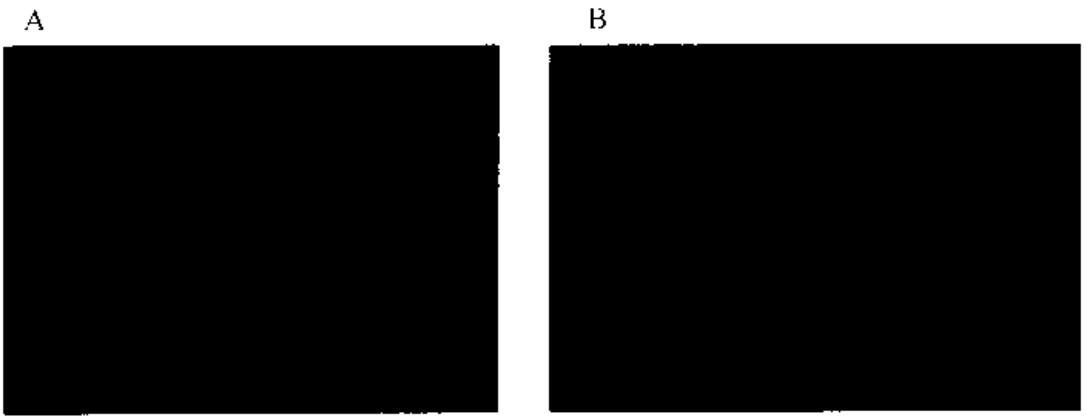


Figure 18. Pla induced actin rearrangement in HeLa cells. The reaction was monitored in a time-course experiment when the Pla$^+$ (TB1 pC4006) and the Pla$^-$ strain (TB1 pUC19) were incubated for four hours on semiconfluent HeLa cell cultures. The invasion process was interrupted at several time points: every seven minutes in the first hour and then at the end of each hour. Unbound bacteria were removed. HeLa cells were fixed, permeabilised and actin was stained with TRITC-phalloidin. Infection with TB1 pUC19 showed a regular pattern of cellular actin during the whole infection (A) whereas a punctuate accumulation of actin was detectable from the 42nd minute of infection with TB1 pC4006 (B).
Accumulation was most intense after the first hour but was still visible during the entire infection (data not shown).

3.5. DISCUSSION

Yersinia pestis plasminogen activator (Pla) is a unique outer membrane protein with protease activity characteristic for this species. Being responsible for several virulence functions this molecule might be a major determinant of the highly invasive character of the plague bacillus (Sodeinde et al., 1992) In our phage display study we focused on the identification of the binding site of Pla to laminin, one of the matrix proteins which is also a major constituent of basal membranes separating several human tissue compartments. Plasmin, the product of plasminogen activation is a protease of wide substrate specificity including the capability to degrade extracellular matrix proteins (Lahteenmaki et al., 1998). Hence Pla is able to activate an enzyme involved in bacterial dissemination. Pla is not the only example of bacterial products being able to capture plasminogen and laminin. Invasive enterobacteria express laminin-binding fimbriae functioning also as plasminogen receptors (Kukkonen et al., 1998) (Parkkinen, Hacker, and Korhonen, 1991)

Amino acid motifs involved in plasminogen activation by Pla have been determined (Kukkonen et al., 2001), nonetheless the timing of plasminogen activation and laminin binding, and the laminin binding site of Pla have not been clarified yet. It is a question whether different active sites are responsible for these two different actions.

We applied a phage display assay to identify the possible motifs of Pla involved in laminin binding. All the eighteen different phage yielded by the fourth round eluate of biopanning were checked for their ability to prevent Pla mediated laminin binding of *E. coli* TB1 pC4006. Phage with WSLLTPA or YPYIPTL heptapeptides showed complete blocking of Pla mediated laminin binding, and in addition these phage themselves exhibited a strong laminin binding capacity in an enzyme-linked immunosorbent assay. Using synthetic heptamer peptides we were able to show a relatively moderate 50% and 33% inhibition with WSLLTPA and YPYIPTL respectively. Due to the bad solubility of WSLLTPA a 2mM maximum concentration was utilised. From these results we assumed that the two phage compete with Pla for the same binding site in laminin and they might mimic the region of Pla being involved in capturing of laminin. (McDonough and Falkow, 1989) proposed that the coagulase activity at 25°C and the fibrinolytic activity at 37°C of Pla were due to posttranslational conformational changes. On the other hand, our results support the idea that these conformational changes do not basically affect the lamina binding site(s) as assays for Pal mediated bacterial lamina binding, and binding of the two inhibitory phase to lamina

yielded comparable results when performed at both temperatures. Pal-mediated lamina binding at environmental temperature might have a physiological role in colonisation of the flea midgut. However, (Hinnebusch, Fischer, and Schwan, 1998)) did not find any difference in the course of flea infection by wild type and pPCP1 plasmid cured *Y. pestis* strains.

Pla shows a high degree of homology with the *E. coli* outer membrane protein OmpT whose crystal structure has been resolved by (Vandeputte-Rutten et al., 2001). OmpT is a ten-stranded, vase-shaped, anti-parallel ß-barrel containing long, flexible surface-exposed loops at the extracellular part, and short turns at the periplasmic site. Proposing a similar model for Pla (Kukkonen et al., 2001) demonstrated that certain amino acid motifs located in the surface exposed loops had a key role in plasminogen activation.

We localised the peptide patterns expressed by phage inhibiting Pla mediated bacterial laminin binding in the amino acid sequence and in the three-dimensional model of Pla. Patterns displayed by phage WSLLTPA (S63-L65-T66) are situated periplasmically at the N-terminus and at the first turn, respectively. Amino acids of the S63-L65-T66 string show conservative substitutions except of W>Y and A>N. The YPYIPTL pattern P175-Y176-I177 is close to loop 3, however this string is also represented in OmpT whose role in laminin binding has not yet been proved. Nonetheless, the last amino acid in the motif has a different character in OmpT (T, polar) than in the YPYIPTL peptide (L, apolar) or in Pla (A, apolar). The peptides WSLLTPA and YPYIPTL share the common string of three amino acids in opposite order: proline, threonine and leucine. We performed alanine-scanning mutagenesis with amino acids of the Pla molecule, which are corresponding to the LTP/PTL motif following the peptide/protein alignment. Four mutants were created: a triple mutant changing the L65T66L67 string to A65A66A67, two single mutants changing G178 and L179 to alanine and a double mutant, which is the combination of the latter two. While the triple mutant displayed a 50% decrease in laminin binding, the double mutant showed a 40% decrease in laminin binding compared to the wild-type strain. The two single mutants did not change substantially the laminin binding ability of Pla. On the other hand plasminogen activation was only slightly affected by the introduced mutations. This indicates that the conformational change induced by the mutations slightly affected the active site organisation of Pla. Due to the absence of an exact crystal structure of Pla it is complicate to explain these data. (Kukkonen et al., 2004) showed that the lack of O-antigen is a prerequisite of Pla mediated matrix-protein adhesion, which indicates that amino acid motifs involved also in laminin binding

are hidden from laminin in the presence of O-antigen. The motifs we localised are not surface-exposed and the SMPYGI A motif might be better exposed in the absence of O-antigen. Neither the triple nor the double alanine-change caused a complete loss in laminin binding, which might indicate that other regions of the molecule also might be involved in the interaction. It is also possible that the proposed regions support the optimal structure (especially the periplasmically involved motifs of WSLLTPA) of Pla for sufficient laminin binding, however the efficient laminin binding of the two phage sequences, which interfered with Pla mediated laminin binding contradicts this assumption. The localisation of laminin during the interaction is also a remaining question because Pla is possibly not a porine - like outer membrane protein. It is related to OmpT, which is monomeric and its inner polar core forms a hydrogen bonding network (Vandeputte-Rutten et al., 2001). Consequently, it still remains a question how these motifs form a binding site and interact with laminin.

Another unique function of Pla is its ability to mediate adhesion and internalisation into eukaryotic cells (Kienle et al., 1992) (Lahteenmaki, Kukkonen, and Korhonen, 2001). Motifs of Pla involved in this interaction have not yet been identified. Therefore after studying some aspects of Pla mediated adhesion and internalisation into HeLa cells we tested the ability of phage #5 (WSLLTPA) and phage #14 (YPYIPTL) to interfere with these processes. Entry of bacteria into mammalian cells usually involves exploitation of existing signal transduction pathways whose major outcome is the rearrangement of the actin cytoskeleton with consequent remodelling of the host cell surface (Ireton and Cossart, 1998). First we analysed cell adhesion and invasion conferred on the non-invasive *E. coli* K-12 strain TB1 by the cloned Pla determinant. As expected the background strain presented with a low level of adhesive capacity, as the bacterial counts practically did not differ in tissue culture dishes with or without HeLa cells and no bacteria could be visualised on the Giemsa stained preparations either. The above data point to the role of Pla in the complicated series of events involved in cell adherence and invasion by *Y. pestis*. To characterise signalling pathways involved in Pla mediated invasion we examined the activity of several signal transduction inhibitors whose inhibitory effect on *Y. pseudotuberculosis* and *Y. enterocolitica* invasion has already been described. Since bacterial and HeLa cell viability was not affected by the concentration of inhibitors used we were able to conclude on exploitation of signalling pathways. Our studies applying different concentrations of wortmannin enabled us to discriminate between the effects of specific enzymes, which are

inhibited by this agent (Meesas, Raupach, and Falkow, 1998). The non-differentiating 100 nM concentration of wortmannin elicited a 50% decrease in invasion but did not alter bacterial adhesion. Consecutively, when differentiating concentrations were used on HeLa cell cultures only 50 nM but not lower concentrations of wortmannin evoked an effect comparable to the potency of 100 nM concentration. From these results we assume a partial involvement of PIth 4-kinase in internalisation. Staurosporin and genistein treatment is known to block invasion of enteropathogenic *Yersiniae* (Rosenshine, Duronio, and Finlay, 1992) hence it seemed worth studying whether they had an influence also on Pla mediated internalisation. Similarly to wortmannin staurosporin and genistein did not affect adherence of the Pla-positive derivative but staurosporin at 0.5 μM concentration decreased the invasion rate to 14% and genistein to 50% of that of the non-treated control. This might imply that internalisation exploits the action of several protein kinase classes like PKC, cAMP-dependent PK and also TPKs, specifically inhibited by genistein. These kinases play a major role in transducing extracellular signals into eukaryotic cells. They are also involved in cytoskeletal rearrangements localised at the site of bacterial attachment. PKC and TPKs are able to activate guanine nucleotide exchange factors (GEFs) which induce formation of active Rho guanosine-triphosphatases, central organisers of cytoskeletal rearrangement (Schoenwaelder and Burridge, 1999). Rac, Rho and Cdc 42 belong to this family of enzymes having a central role in cytoskeletal rearrangement (Schoenwaelder and Burridge, 1999).

Involvement of RhoGTPases was further and more exactly proved with the utilisation of NDGA and C3 exoenzyme as inhibitors. NDGA inhibits 5-lipoxygenase, which converts arachidonic acid into leukotrienes after Rac activation. Leukotrienes induce stress fibre formation by activating Rho protein (Peppelenbosch et al., 1995). Inhibition of Pla mediated internalisation indicates that Rho activation and concomitant actin stress fibre formation play a role in the invasion process. The role of Rho in Pla mediated invasion is further supported by the finding that the Rho specific inhibitor, *C. botulinum* exoenzyme C3 (Wilde and Aktories, 2001) also inhibited the internalisation process fivefold.

The contribution of actin rearrangement was confirmed by treating cells with several concentrations of cytochalasin D. Although no change was detected when 0.5 or 1 μg/ml concentrations were applied, higher concentrations of cytochalasin D decreased Pla mediated invasion thirty fold. On the other hand adhesion of bacteria was unaffected by the treatment.

(Cowan et al., 2000) found that epitheloid cell invasion by *Y. pestis* was strongly inhibited by cytochalasin D. As Pla negative *Y. pestis* preserved a portion of its invasive capacity it remained a question whether the inhibitory action of cytochalasin D was completely directed on the Pla associated component of internalisation. Our findings with isogenic *E. coli* derivatives strongly suggest that cytoskeletal reorganisation is a major event in Pla mediated epithelial cell invasion. We proved the involvement of Pla in cytoskeletal rearrangement also with fluorescence microscopy. Experiments were conducted to visualise polymerised actin by fluorescent labelling with TRITC-phalloidin. We detected punctuate actin accumulation in HeLa cells first after 42 minutes of Pla mediated internalisation of recombinant *E. coli*. Changes were most dramatic after one-hour-incubation and turned to be less intense later on. This seems to be in concert with the quantitative time-course experiment revealing the most intense invasion during the first hour of incubation.

We studied whether the two phage, which presented strong inhibition of Pla mediated laminin binding are also able to interfere with Pla mediated adhesion and internalisation of *E. coli*. We were not able to detect any kind of disturbance of these processes in the used phage concentration (maximum of 2.5×10^{12} phage/ml). Steric hindrance due to the large phage size might be excluded with using purified peptides for inhibition in future experiments but it is also possible that these two different functions of Pla do not share a common epitope at all. The eukaryotic receptor for Pla has not yet been identified but it is known that Pla has a lectin-like behaviour. It binds to glycolipid extracts from several cell lines as well as neutral extracts from cells rich in globo tetraosylceramide (globoside) and to purified globoside (Kienle et al., 1992). Utilisation of a random phage display library might help in mapping the involved amino acids of Pla by adding random input phage to HeLa cells and eluting them in a competitive way with Pla-expressing recombinant bacteria. This would also demonstrate the widespread employment of phage display libraries in microbiological studies.

3.6. New results presented in chapter 3

1. Selection of 18 different heptamer sequences after performing a biopanning with a random phage display library.
2. Development of a simple in vitro assay to measure Pla mediated laminin binding in recombinant *E. coli*. Collected data reveal a twenty fold and ten fold increase in binding

capacity of non-adhesive *E. coli* in the presence of Pla at room temperature and at 37°C, respectively.

3. Investigation of the potential interference of the selected heptamer peptides with Pla-mediated laminin binding. Two sequences: WSLLTPA and YPYIPTL displayed complete inhibition.
4. Development of a simple ELISA-test for checking the laminin binding of phage. WSLLTPA showed a 17-fold, YPYIPTL a 13-fold stronger binding than random phage.
5. Demonstrating interference with Pla mediated laminin binding by the utilisation of synthetic peptides. Inhibition was moderate and gradual with a maximum of 55% using WSLLTPA and 33% using YPYIPTL.
6. Following alignment of peptides and the Pla protein alanine-scanning mutagenesis was performed to examine the effect of mutations on the laminin binding capacities of Pla. A triple mutation of the amino acids I65T66I67 and a double mutation of amino acids G178L179 decreased the laminin binding capacity of Pla about twofold. This indicates the involvement of the mutagenised amino acids in laminin binding.
7. On the other hand mutations did not substantially affect the plasminogen activation function of Pla revealing a possible dissection of the two different functions.
8. Phage WSLLTPA and YPYIPTL had no effect on Pla-mediated adhesion and internalisation, indicating the involvement of a different protein region in these functions.
9. Pla-mediated internalisation of non-invasive *E.coli* exploits the host HeLa cell's signal transduction system by utilisation of protein kinases including tyrosine protein kinases, the small Rho GTP-ases, RhoA and Rac, the phosphatidyl-inositol-4-kinase and consecutive rearrangement of the actin cytoskeleton.

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1. O. Benedek, J. Knurr, C.L. Turnbough Jr. (2001) Characterisation of tight-binder peptide ligands to *Bacillus subtilis* spores. Acta Microbiol. Immunol. Hung. 48: 226

2. J. Knurr, O Benedek., J.L. Heslop, R.B. Vinson., J.A. Boydston, J. McAndrew, J.F. Kearney and C.L. Turnbough Jr. (2003) Peptide Ligands That Bind Selecitvely to Spores of *Bacillus subtilis* and Closely Related Species. Appl. Env. Microbiol. 69: 6841-6847.

3. D.D. Williams, O.Benedek and C.L. Turnbough Jr. (2003) Detection of *Bacillus anthracis* Spores Using Species-specific Peptide Ligands. Appl. Env. Microbiol. 69: 6288-6293.

4. O. Benedek, J. Bene, B. Melegh and L. Emödy (2003) Mapping of possible laminin binding sites of *Yersinia pestis* plasminogen activator via phage display. Adv. Exp. Med. Biol. 529:101-104.

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