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BACTERIOLOGICAL APPLICATIONS OF RANDOM PHAGE DISPLAY LIBRARIES

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thesises

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1. Introduction

Phage display is a rapidly growing technology first described in 1985 by G. P. Smith in Science. It is a very effective way for producing a large number of diverse peptides and proteins and isolating molecules that perform specific functions. The method relies on two simple concepts. 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. The major coat protein pVIII and the minor coat protein pIII of the filamentous bacteriophage M13 have been the platform of choice both from historical perspective as the first and best characterised library display vehicles and as the source of the majority of successful screenings. The most commonly used selection procedure is called biopanning. After capturing the target on a solid surface a big mixture of phage expressing random peptides are added to the target protein, unbound phage removed by washes in an appropriate buffer and bound sequences eluted by a low pH buffer e.g. glycine (pH 2.2), neutralised and propagated in a host *E. coli* strain. The eluted propagated and concentrated phage is used as a source of input phage for the next round of biopanning. During several rounds specific tight binder sequences emerge sharing common amino acid motifs which can reveal on the interaction site of the partner protein of interest. Several factors might influence the character of the emerged sequences as the length of incubation and elution times, concentration of targets, concentration of detergents in wash buffers, number of washes. Still due to the relatively simple screening procedure and sequencing possibilities phage display is widespreadly used in epitope and drug discovery. Microbiological utilisations are less established. In my thesis I want to present two application possibilities of phage display 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. These agents genuinely are zoonotic pathogenes 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. 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. Aims of the study

- 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.
- Characterisation of selected sequences, assessment of structural criteria for binding.
- Development of fluorescent labelling methods for tagging the selected phage or the chemically synthesised peptide sequences.
- Improvement of flow cytometry assays suitable for discrimination of different spore types.
- Performance of sequence similarity searches with the selected peptide sequences to recognise protein sequences mimicked by the phage-displayed peptides.

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

peptides.

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

3. Materials and methods

3.1. Phage display and associated methods

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 and a heptamer library for mapping the laminin binding epitope of Pla. For propagation of eluted phage F-pilus positive derivatives of *E. coli* laboratory strains were used.

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 sterile Tris buffered saline-Tween 20[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, and the supernatant was removed. Spore-phage complexes were washed ten times in TBST with alternating resuspension and centrifugation. After the final wash, spore-phage complexes were resuspended in 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 1 M Tris-HCl (pH 9.1) 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.

Four rounds of biopanning were performed against laminin from Engelbreth-Holm-Schwarm murine sarcoma tumor on a microtiter plate with a random heptamer Ph.D. 7- library. Steps of the provided bulletin were followed.

3.1.2.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. Three rounds of biopanning were performed and pools of the individual eluates were sequenced after the eluate fractions had been propagated in the *E. coli* host strain and single stranded phage DNA had been extracted.

3.1.3. Concentration of propagated phage

Host cell cultures (usually 20 ml) were centrifuged twice and the supernatant was precipitated overnight with 1/6 volume of PEG/NaCl. The precipitate was collected by centrifugation nd the pelleted precipitate was dissolved in TBS and re-precipitated in 1/6 volume of PEG/NaCl. The formed precipitate was microfuged and re-suspended in TBS-0.2% NaN_3. This phage stock was used for titration.

3.1.4. Phage titration

From phage stocks tenfold dilutions were made in Luria-Bertani broth. Aliquots were incubated with mid-log phase culture of host strains at room temperature and then plated with Agarose Top onto pre-warmed LB -agar plates. After overnight incubation at 37°C phage plaques were counted and the titre was calculated and given in pfu/ml.

3.2. *Bacillus* strains and growth conditions

3.2.1. *Bacillus* strains

The following *Bacillus* strains were used: *B. subtilis* (*trp*C2) 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 ΔAmes ($pXO1^-$) strains of *B. anthracis* were from the U.S. Army

Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA. *B. thuringiensis* Al Hakum, *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.

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) 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 Renographin 60 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 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. ΔAmes spores of *B. anthracis* were prepared by Dr. Joanie Jackman at USAMRIID and killed by gamma irradiation before use.

3.3. Strains used in the Pla study.

E. coli TB1 pC4006 and TB1 pC4004 are derivatives of *E. coli* TB1 harbouring the pUC19 plasmid or the pK18 plasmid respectively with cloned determinant for Pla. TB1 Inv bore the plasmid pJS-1-pUC6.2. expressing *Yersinia pseudotuberculosis* invasin that was a gift of Prof Jürgen Heesemann, Munich, Germany.

3.4. Fluorescent labelling techniques and flow cytometry

3.4.1. Fluorescent labelling of anti M13 antibody

1 mg batches of monoclonal anti M13 (anti pVIII) antibodies were labelled with the amine-reactive Alexa 488 Protein Labelling Kit according to the instructions of the supplier.

3.4.2. Fluorescent labelling of M13 phage

Samples of M13 phage displaying a particular peptide were labelled using the amine-reactive

Alexa Fluor® 488 Protein Labelling Kit 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. Labelled phage were precipitated from the reaction mixture by PEG/NaCl.

3.4.3. Fluorescent labelling of synthetic peptide

Peptide molecules were attached to R-phycoerythrin (RPE) by using the heterobinfunctional crosslinker sulfosuccinimidyl-4- (N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) following the manufacturer´s instructions. SMCC is able to bind lysine residues of RPE through its reactive N-hydroxy-succimimide-ester group and the C-terminal free SH-groups of the peptides through its reactive maleimide group.

3.4.4. Flow cytometry

Spores were mixed either with unlabelled (for fluorescently labelled anti M13 labelling) or Alexa 488-labelled M13 phage or with a peptide-RPE conjugate in various concentrations and incubated at room temperature for 1 hour to ensure complete binding. Unbound conjugate molecules were removed by washing spores three times in PBST (0.5% Tween 20). Spores were collected after each wash by centrifugation In case of using fluorescently labelled antibodies spore-phage conjugates were resuspended fluorescently labelled anti M13 antibody and incubated for one more hour at room temperature. Unbound antibodies were removed by centrifugation as described above. Spore conjugate complexes were re-suspended in PBS and fluorescence was measured using a BD FACSCalibur instrument and analysed with CellQuest Pro software. Spores were identified by their light-scattering properties, and 20,000 spores were analysed for associated fluorescence.

3. 5. Molecular biological methods

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. For DNA-extraction either steps of the QIAGEN M13 Spin Kit bulletin were followed or DNA was extracted once with equal volumes of phenol: chloroform: isoamyl-alcohol (25:24:1, precipitated with 95% ethanol plus 3 M Na-acetate (pH 5.2) and washed once in 70% ethanol. DNA concentration was estimated spectrophotometrically and samples were sequenced using the dideoxy-chain termination method either manually or automatically using appropriate primers.

3.5.2. Construction of recombinant phage

To construct recombinant M13 phage displaying a specific peptide, we prepared the double-stranded replicative form of the genomic DNA with QUIAGEN Plasmid Purification Kit. The peptide-encoding *Kpn*I-*Eag*I) fragment in the RF DNA of the library phage was excised and replaced with synthetic and annealed oligonucleotides encoding the specified peptides. he recombinant M13 RF DNA was transformed into the *E. coli* host strain and plated with Top Agarose and mid-log phase *E. coli* host strain culture. Plaques were propagated in host cell cultures, single-stranded DNA was extracted and sequenced as described at 3.5.1.

3.5.3. Site-directed mutagenesis of Pla

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. For each mutation a single polymerase chain-reaction was performed. The whole pACYC177 plasmid construct was used as template. PCR products were treated with T4 polymerase to produce blunt ends for self-ligation and with *Dpn*I 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.

3.6. Microtitre plate-based assays

3.6.1. Phage-ELISA tests for laminin binding

A microtitre plate was sensitised with laminin. Following blockage with BSA wells were incubated with phage and then with horseradish peroxidase (HRPO) conjugated monoclonal anti-M13 antibody. A_{492} values were determined on an automated plate reader (Metertech, Taiwan). Random phage were applied as negative control. Wells with laminin, anti-M13 antibody and OPD-reagent without phage served as blank.

3.6.2. Assaying of Pla mediated laminin binding of bacteria

Plates were first sensitised overnight with laminin. After blockage with BSA adjusted suspensions from overnight cultures were added to the wells and the plates were incubated 37°C or at 25°C. Adhered bacteria were fixed with formalin, stained with crystal violet solution and lysed with 1% SDS-solution. A_{595} absorbance of the released crystal violet was measured.

3.6.3. Phage mediated inhibition of laminin binding

The test was performed basically in the same way as at 3.6.2. except that 10^{10} phage were co-incubated with bacterial cells on the laminin-coated plate at 37°C. Random phage were used as negative control. After incubation the number of adhered bacteria was determined not by staining but by detaching bacterial cells from the wells with Triton-Trypsin solution, serial dilutions were plated, incubated at 37°C overnight and colonies were counted on the next day.

3.6.4. Peptide mediated inhibition of bacterial laminin binding

Instead of co-incubation laminin sensitised were pre-incubated with twofold serial dilutions of peptides with a starting concentration of 2 mM. Bound bacteria were detected with polyclonal anti-TB1 antibody and with anti-rabbit immunoglobulin G conjugated to HRPO and TMB (tetra-methyl-benzidine) chromogen substrate. Absorbance was read at λ=450 nm. One row was used as negative control, when neither peptides nor bacteria were added to the wells. 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)]$\}\times 100$.

3.7. Peptide pattern search and homology modelling

Peptide patterns of the two inhibitory phage 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.

3.8. Plasminogen activation

Bacteria and Glu-plasminogen were incubated on a microtitre plate with or without the chromogen substrate S-2251 (negative control). At different time-points the absorbance was measured at λ=405 nm. Absorbance values of the negative control were always subtracted from the respective values of the test wells.

3.9. SDS-polyacrylamide gelelectrophoresis

Total protein extracts of the strains were run on a 12% denaturing polyacrylamide gel 10^9 bacteria from an overnight culture were boiled in 5×sample, spun briefly and fractions of the supernatant corresponding to 2.5×10^8 bacteria were loaded on the gel. After running the gel was stained in Coomassie blue and destained in Coomassie destaining solution for overnight.

3.10. Cell culture conditions

HeLa cells were grown in RPMI 1640 medium supplemented with 1 mM $NaHCO_3$ and 10% foetal calf serum. Cells were seeded a day before the experiments in 35×10 mm Petri dishes to reach either confluency or semiconfluency by next day. For fluorescent staining HeLa cells were seeded onto 22 mm glass coverslips in 12-well tissue culture plates to reach semiconfluency by next day.

3.11. Preparation of signal transduction inhibitors and cytochalasin D

Signal transduction inhibitors were all dissolved in dimethyl-sulfoxide except of the C3 exoenzyme, which was dissolved in ddH_20 at recommended concentrations. For the experiments stocks were diluted in RPMI 1640.

3.12. Adhesion assays

3.12.1. Microscopic assay

Semiconfluent HeLa cell cultures were infected with aliquots of adjusted suspensions from overnight LB cultures of bacterial strains and incubated at 37°C in 5% CO_2. HeLa cells were washed, fixed with and stained with Giemsa stain at room temperature. Cell cultures were examined by light microscopy using a Zeiss Axioskop 40

3.12.2. Quantitative adhesion assay

After removing unbound bacteria HeLa cells were lysed with Triton-Trypsin solution. Aliquots of serial dilutions of the lysates were plated onto LB agar for overnight at 37°C and colony forming units were counted. 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.

3.12.3. HeLa cell invasion assay

Principally the gentamicin protection assay 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.

3.12.4. Inhibitor 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. 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.

3.12.5. Fluorescence staining

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. Cells were then fixed with paraformaldehyde and permeabilised with Triton X-100 (in PBS). Cells were stained for 1 hour at room temperature with TRITC-phalloidin and mounted with glycerol-PBS (9:1), covered with glass slips and closed with nail polish. Specimens were examined under an Olympus BX 61 epifluorescence microscope.

3.12.6. 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 with the negative control strain on semiconfluent or confluent HeLa cell cultures. Preincubation was an hour long at 37°C, 5% CO_2. Phage were added in RPMI. Following 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. The effect of 5×10^{11}, 10^{11} and 5×10^{10} phage particles was tested.

4.Results

4.1. Biopanning results

4.1.1. Signs of sequence enrichment and selection during biopanning

Emergence of tight-binders was indicated by a titre increase in the elution fractions, which was 10^4 fold for *Bacillus subtilis* (from 10^4 eluted phage to 10^8) and 200 fold (from 10^4 to 2×10^6) for *Bacillus anthracis* ΔAmes strain. There was a 40 fold enrichment during laminin biopanning in the eluate fraction.

4.1.2. Nature of the selected tight-binder sequences

13 unique heptamer and 8 unique dodecamer peptide sequences were selected during the *B. subtilis* biopanning. 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 prolines and basic amino acids in position 5-7. 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. From biopanning of ΔAmes and Sterne spores a family of peptides with the consensus of Thr-Tyr-Pro-X-Pro-X-Arg emerged. From the laminin biopanning a consensus motif of His-X-X-His in changing position and a longer consensus sequence X-Ser-X-Leu-Thr-X-Ala was selected.

4.2. Confirmation of the high affinity of a characteristic *B. subtilis* tight binder sequence

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^{10} 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. Peptide sequence requirements for *B. subtilis* spore binding.

4.3. Comparison of binding affinities of the *B. subtilis* tight-binder heptamer sequences

A competitive biopanning experiment was performed. 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 with *B. subtilis* spores, and four rounds of biopanning were performed. Eluted phage from round 4 were plaque purified, and 31 plaque sequences were analysed. Comparing frequencies of phage appearance 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 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.

4.4. 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. We then performed three-three rounds of 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. 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 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. 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.

4.5. Spore binding peptides as a developmental targeting sequence

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 hit was a perfect match in the SpsC coat protein where the Asn-His-Phe-Leu-Pro sequence is located near the amino terminus, at positions 6 to 10. 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. 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.

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

To determine the spore specificity of peptide binding, we fluorescently labelled the 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) or synthetic peptides. Under standard conditions for assaying peptide binding, spores of *B. subtilis* and nine other phylogenetically similar *Bacillus* species were analysed. 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*, which are the closest relatives of *B. subtilis*. No binding of peptide 4 was detected with the rest of the spore species.

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

A representative TYP peptide with the sequence TYPLPIRGGGC was fluorescently labelled with R-phycoerythrin. Peptide binding to *B. anthracis* (Sterne and ΔAmes) spores was then measured by incubating spores with different concentrations of the conjugate. The results showed essentially identical, concentration-dependent binding of the peptide-PE conjugate to spores of the Sterne and ΔAmes strains on the other hand they did not bind to 15 of the other *Bacillus* strains. 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. 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.

4.8. Pla-mediated laminin binding

Our data indicat 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.

4.9. Phage mediated inhibition of laminin binding

Two phage sequences (WSLLTPA and YPYIPTL) interfered strongly with Pla mediated laminin binding of *E. coli*. 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.

4.10. Laminin binding ELISA with inhibitory phage

The laminin binding capacity of the inhibitory phage 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

4.11. 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.Peptide YPYIAAA served as negative control displaying an inhibitory capacity around zero.

4.12. Localisation of the laminin binding motifs

The program PATTINPROT 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 phage WSLLTPA and YPYIPTL. We found the S-X-L-T motif of peptide WSLLTPA at amino acids S63, L65 and T66. WSLLTPA represents another pattern, S-X-L-X-A, which is localised at amino acids S3, L5, and P7. The P-Y-I pattern of peptide YPYIPTL was localised at P175, Y176, and I177 of Pla. Motifs were also positioned in the three-dimensional model of Pla. The model was built with the help of the program DeepView. 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

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

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. 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. 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.

4.14. 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. On the other hand the Pla expressing recombinant TB1 pC4006 strain heavily colonised the HeLa cells. The absence of microbes at the HeLa cell free areas of the semiconfluent culture points to a real bacterium-HeLa cell interaction.

4.15. Time-course of Pla mediated adhesion and invasion

In a four-hour experiment we determined the dynamics of Pla mediated bacterial adhesion 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 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.

4.16. Effect of several signal transduction inhibitors on Pla mediated adhesion and invasion.

Cells were pre-treated first with 100 nM wortmannin, which caused 50% inhibition. Then 10, 25 and 50 nM concentrations of wortmannin were used to discriminate which of the above enzymes might have a role in invasion. 10 and 25 nM concentrations altered neither invasion nor adhesion. A 50 nM concentration of wortmannin elicited about a 50% decrease in invasion but did not affect adhesion. NDGA in 15 μg/ml concentration completely blocked Pla mediated invasion but not adhesion. 0.5μM staurosporin decreased Pla mediated invasion to a relative 14% without altering adhesion Genistein does not affect adhesion either while

50% decrease in internalisation was detected after Cytochalasin D in 2 or 5 μg/ml concentrations elicited a thirty-fold decrease in invasion of HeLa cells by the Pla-positive strain but did not affect bacterial adhesion. Treatment of cells with 5 μg/ml C3 exoenzyme did not affect Pla mediated adhesion but decreased the internalisation to 20%.

4.17. 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.

4.18. Fluorescence staining

During a time course-experiment we were able to detect punctuate accumulation of actin 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.

5. Discussion

Phage display is a popular method in biotechnology and suitable for a broad range of application. In these two projects we worked on the development of a fast, non-nucleic acid based method for the identification of *Bacillus* spores and on mapping the laminin binding epitope of *Yersinia pestis* plasminogen activator (Pla). 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. 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. 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. 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 ΔAmes ($pXO1^-$). They lack virulence plasmids whose products are unlikely to be involved in the formation of the spore surface therefore these strains might suitably mimic the wild-type *B. anthracis*. *B. anthracis* strains are highly monomorphic as well, with genes from different isolates typically having greater than 99% nucleotide sequence identity. 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* T 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. 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 and differentially labelled with assayable tags, such as luminescent quantum dots that provide a signal sufficient to detect a single spore. 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.

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. 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. 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. 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 laminin. Pla shows a high degree of homology with the *E. coli* outer membrane protein OmpT whose crystal structure has been resolved by. 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 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. The YPYIPTL pattern P175-Y176-I177 is close to loop 3. 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. It was shown 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 SM**PYI**GLA 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. 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. 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. First we analysed cell adhesion and invasion conferred on the non-invasive *E. coli* K-12 strain TB1 by the cloned Pla determinant to set up a proper test system for phage inhibition studies. 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. 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. Our studies applying different concentrations of wortmannin enabled us to discriminate between the effects of specific enzymes, which are inhibited by this enzyme. 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 Ptln 4-kinase in internalisation. Staurosporin and genistein treatment is known to block invasion of enteropathogenic *Yersiniae* 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. Rac, Rho and Cdc 42 belong to this family of enzymes having a central role in cytoskeletal rearrangement 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. 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 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. 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.

In our phage inhibition studies we were not able to detect any kind of disturbance of Pla mediated adhesion and invasion 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.

7. Major conclusions

- Phage display of *Bacillus subtilis* and *anthracis* spores enables the selection of tight-binder peptides with consensus motifs.
- Emerged sequences can be used for the development of short, discriminatory flow cytometry assays, which may serve as an alternative of quick, DNA-based identification.
- Peptide sequence analysis, sequence similarity searches and different competitive biopanning strategies support the determination of requirements for tight and specific binding.
- Phage display enabled the indentification of amino acids involved in the laminin binding of *Yersinia pestis* plasminogen activator (Pla), an unique virulence factor and outer membrane protease characteristic for the species.
- For the study of Pla and eukaryotic cell interactions another approaches are necessary.

List of publications

1. **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

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

4. ***O. Benedek*, G.Nagy and L.Emődy** (2004) Intracellular signalling and cytoskeletal rearrangement in *Yersinia pestis* plasminogen activator Pla mediated HeLa cell invasion. Microbial Pathogenesis. 37: 47-54. (IF: 1.708)

5. ***O. Benedek*, A.S.Khan, Gy. Schneider, G. Nagy, R. Autar, R.J. Pieters, J. Hacker and L. Emődy** (2004) Identification of laminin binding sites of *Yersinia pestis* plasminogen activator via phage display (submitted)

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