Selection of peptide entry motifs by bacterial surface display

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Surface display technologies have been established previously to select peptides and polypeptides that interact with purified immobilized ligands. In the present study, we designed and implemented a surface display-based technique to identify novel peptide motifs that mediate entry into eukaryotic cells. An *Escherichia coli* library expressing surface-displayed peptides was combined with eukaryotic cells and the gentamicin protection assay was performed to select recombinant *E. coli*, which were internalized into eukaryotic cells by virtue of the displayed peptides. To establish the proof of principle of this approach, the fibronectin-binding motifs of the fibronectin-binding protein A of *Staphylococcus aureus* were inserted into the *E. coli* FhuA protein. Surface expression of the fusion proteins was demonstrated by functional assays and by FACS analysis. The fibro-

nectin-binding motifs were shown to mediate entry of the bacteria into non-phagocytic eukaryotic cells and brought about the preferential selection of these bacteria over *E. coli* expressing parental FhuA, with an enrichment of 100 000-fold. Four entry sequences were selected and identified using an *S. aureus* library of peptides displayed in the FhuA protein on the surface of *E. coli*. These sequences included novel entry motifs as well as integrin-binding Arg-Gly-Asp (RGD) motifs and promoted a high degree of bacterial entry. Bacterial surface display is thus a powerful tool to effectively select and identify entry peptide motifs.

Key words: fibronectin-binding protein (FnBP), FhuA, peptide library, *Staphylococcus aureus*.

INTRODUCTION

Phage display libraries have been widely used for the identification of high-affinity ligands for target molecules (for a review see [1]). Bacterial cell-surface display libraries are emerging as a powerful complementary system to phage display for the identification of ligand sequences, and expression systems for the display of proteins on Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus* have been developed [2–5]. Display systems have been used not only to identify ligands that bind to defined target molecules, but also to complex target mixtures and to intact eukaryotic cells [6,7]. However, due to the inability to selectively separate intracellular phage from extracellular phage, screening of phage display libraries for binding to eukaryotic cells cannot differentiate between peptide motifs mediating binding alone and those that bind and trigger entry into the cells. Bacterial surface display systems could have an advantage over phage display in this regard, as extracellular bacteria can be selectively eliminated by the application of antimicrobial agents, which do not penetrate into eukaryotic cells and do not adversely affect the viability or integrity of eukaryotic cells or intracellular bacteria [8]. The antibiotic gentamicin was first used in this way by Isberg et al. [8] to select for intracellular bacteria from an *E. coli* expression library of *Yersinia pseudotuberculosis* co-incubated with cultured mammalian cells and led to the discovery of the first, and perhaps the strongest, bacterial protein mediating invasion into eukaryotic cells. This protein was invasins of *Y. pseudotuberculosis*, which binds β 2 integrins on eukaryotic cells and so signals uptake of the bacteria by the cell [8]. The technique was termed a gentamicin protection assay and, since then, this method has been extensively used to measure bacterial uptake into eukaryotic

cells, as gentamicin is not detrimental to cells or intracellular bacteria when the appropriate concentration and time of incubation are used [9,10]. Moreover, the validity of this technique is strengthened further by the extremely good correlation between the results obtained when the degree of bacterial entry into eukaryotic cells is measured simultaneously by antimicrobial protection assays and by flow cytometry analysis of internalized fluorescently labelled bacteria [11]. By combining bacterial surface display with the gentamicin protection assay technique, we sought to identify novel peptide motifs, rather than full length surface proteins, which mediate uptake into eukaryotic cells.

Several eukaryotic cell interactive domains of viral proteins have been identified, and synthetic peptides corresponding to these domains have been shown to interact with eukaryotic cells and mediate the entry of associated molecules into these cells [12]. These carrier peptides include amphiphilic and cationic peptides, such as the protein transduction domain of Tat from HIV. The Tat domain mediates efficient cellular uptake of covalently attached molecules, including β -galactosidase, horseradish peroxidase and RNase A [13,14]. Another carrier peptide is the cell-binding Arg-Gly-Asp (RGD) motif, which is found in the adhesins of numerous viruses as well as in the adhesins of other pathogens [15]. The RGD motif is recognized by the integrin family of eukaryotic receptors that are expressed on numerous cell types and incorporation of the RGD sequence into recombinant proteins induces their uptake by eukaryotic cells [16].

One strategy for bacterial surface display involves fusing peptides to extracellular appendages, such as pili or flagellae, whereas another involves the fusion of peptides to *E. coli* outer membrane proteins (OMPs) [2,5,17]. Libraries of short peptides

Abbreviations used: cfu, colony-forming units; Cm^R, chloramphenicol-resistant; IPTG, isopropyl β -D-thiogalactoside; Kn^R, kanamycin-resistant; MFI, mean fluorescence intensity; moi, multiplicity of infection; OMP, outer membrane protein; ORF, open reading frame; pfu, plaque-forming units; RGD, Arg-Gly-Asp; TIGR, The Institute for Genomic Research; wt, wild type.

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inserted in the surface-exposed loops of *E. coli* OMPs, e.g. LamB and FhuA, can be screened successfully for binding to target molecules, and the availability of multiple display platforms allows a large range of peptide sequences to be presented on the bacterial surface, eliminating the potential platform bias of a single platform [18]. FhuA is the target of several bacteriophages, including T5 and Φ 80, and is also a target for bacteriocins, e.g. colicin M. It forms a β -barrel structure exposing 11 loops on the bacterial surface, of which the largest, loops 4 and 5, tolerate inserts of up to 200 amino acids in length without disturbing surface expression or sensitivity to phages T5 and Φ 80 or colicin M [18–20]. Thus the FhuA surface display is a powerful system with which to screen libraries for interactive peptides.

We set out to identify novel carrier sequences and, in the present study, we describe a novel application of bacterial surface display libraries to identify peptide motifs that mediate entry into eukaryotic cells by selective elimination of extracellular bacteria while maintaining viable intracellular bacteria. Peptides are functionally displayed in the FhuA platform protein leading to *E. coli* uptake into eukaryotic cells, and selection of invasive clones can be achieved with multifold enrichment. Application of this procedure to a library of peptides led to the selection and identification of integrin-binding RGD motifs and of novel entry motifs. These motifs are candidates for carrier peptides to deliver molecules of medical importance into cells *in vivo*, e.g. vaccine antigens or antibiotics. This is the first time surface display libraries have been used to identify peptides mediating entry into eukaryotic cells and demonstrates a unique application of bacterial surface display systems.

MATERIALS AND METHODS

Bacterial strains and cell lines

Human epithelial HeLa cells were cultivated in growth medium [Dulbecco's modified Eagle's medium containing 5% (v/v) foetal calf serum, 1 mM L-glutamine, 40 μ g/ml gentamicin (Life Technologies, Lofer, Austria) and 1 mM sodium pyruvate] at 37 °C with 5% CO₂/95% air mixture. Unless otherwise stated all cell-culture media and reagents were obtained from PAA Laboratories (Lucerne, Switzerland).

The bacterial strains used in this study are shown in Table 1. *E. coli* was grown in liquid Luria broth (Life Technologies) or on Luria agar (Luria broth solidified with 1.5% agar) at 37 °C. Antibiotics, 50 μ g/ml kanamycin and 200 μ g/ml ampicillin, were added where appropriate.

Molecular biology techniques and plasmid constructions

All molecular biology techniques were performed as described previously [21] unless otherwise stated. Reagents, chemicals and other materials were obtained from Sigma–Aldrich (Vienna, Austria), except when indicated otherwise. Oligonucleotides were synthesized at the Molecular Services Section of the Institute of Molecular Pathology, Vienna, Austria. The plasmids used in this study are listed in Table 1.

Plasmid pHIE11 encodes FhuA with unique *FseI* and *NotI* restriction sites in the DNA encoding loop 5 and FhuA protein expression is under the control of the isopropyl β -D-thiogalactoside (IPTG)-inducible *lac-uv5* promoter [18]. The 360 bp DNA fragment encoding FnBP domains D1–D3 (D1–3; amino acids 709–823) and the 230 bp DNA fragment encoding FnBP domains D2–D3 (D2–3; amino acids 747–823) were amplified by PCR from the template pFNBA4 (Table 1) using primer ICC379 (5'-CGCGTTGCGGCCGCGCCGCTTACTTTTGGAAG-3') with ICC387 (5'-CTGGCCGCGCCGCAAGGTGGCCAAAATAGCG-3') and primer ICC379 with ICC380 (CGGCTACCGCGCCGCCCCTCAAATTCATGGTCAAAATAAA) respectively. The peptide sequence of these domains is shown in Figure 1(A). The PCR fragments were digested with *NotI* and *FseI* (sites underlined in the oligonucleotide sequences) and inserted into the corresponding restriction sites of pHIE11 to generate pST4 encoding FhuA–D1–3 and pST5 encoding FhuA–D2–3 (Table 1). All plasmids were verified by sequencing at the Molecular Services Section of the Institute of Molecular Pathology, Vienna, Austria.

The LSA50 library was constructed with genomic DNA of the methicillin-resistant *S. aureus* COL strain [supplied by The Institute for Genomic Research (TIGR), Rockville, MD, U.S.A.] and contains DNA inserts of approx. 50 bp (approx. 17 amino acids) in length with a complexity of 2×10^6 and a 50-fold coverage of the genome ([21a], and H. Etz, T. Henics and A. Meinke, unpublished work). Briefly, the bacterial genomic DNA was fragmented by sonication and fragments of approx. 50 bp in length were isolated following agarose-gel electrophoresis. These fragments were ligated into the *SmaI* site of the lacZ vector to select for in-frame inserts. The selected in-frame inserts were removed by *FseI* and *NotI* digestion and ligated into the corresponding restriction sites of pHIE11. Two types of chimera could result from this cloning strategy. Those from the first library construction generated by blunt-end ligation without any linker addition and those from the second transferred library generated by *FseI* and *NotI* ligation, which therefore have small linker sequences attached.

Table 1 Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source
<i>E. coli</i>		
DH5 α	F'/endA1 hsdR17 (r_k^- , m_k^+) supE44 thi-1 recA1, gyrA96 relA1 Δ (lacZYA-argF)U169 [ϕ 80dlac Δ (lacZ)M15]	[44]
DH10B	F' mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 endA1 recA1 deoR Δ (ara, leu)7697 ara Δ 139 galU galK nupG rpsL χ	Life Technologies
UL4	aroB tsx malT thi thuA recA Δ (srl)	[45]
Plasmids		
pEH1	<i>lacI</i> , <i>lacZ'</i> pT7/lac placUV5 flori pMB1ori Kn ^R	[46]
pEH3	<i>lacI</i> , <i>lacZ'</i> pT7/lac placUV5 flori pMB1ori Cm ^R	[46]
pHIE3	pEH1 expressing FhuA	[18]
pHIE10	pEH3 expressing FhuA	[18]
pHIE11	<i>FseI/XbaI/NotI</i> cloning site in pHIE3 (amino acid at position 405 of FhuA)	[18]
pFNBA4	<i>S. aureus</i> shuttle plasmid encoding <i>fnbA</i>	[47]
pST4	pHIE11 expressing FhuA–D1–3	This study
pST5	pHIE11 expressing FhuA–D2–3	This study
LSA50	Approx. 50 bp <i>S. aureus</i> genomic DNA fragments in <i>thuA</i> of pHIE11	[21a] and H. Etz, T. Henics and A. Meinke, unpublished work

Figure 1 Surface expression of FhuA, FhuA-D2-3 and FhuA-D1-3 in *E. coli*

(A) Amino acid sequence of *S. aureus* FnBPA fibronectin-binding domains D1–D3. Identical amino acids in the three domains are indicated in bold. Amino acids flanking the D domains 1 and 3 as shown were also included in the FhuA fusions. Amino acids of domain D1 present in FhuA–D1-3 and absent in FhuA–D2-3 are underlined. (B) Expression of FhuA, FhuA–D2-3 and FhuA–D1-3 in *E. coli*. Whole-cell preparations of *E. coli* UL4 in the absence or presence of plasmids pHIE11 (expressing FhuA), pST4 (expressing FhuA–D1-3), pST5 (expressing FhuA–D2-3) were separated by SDS/PAGE. After transfer on to a nitrocellulose membrane, FhuA protein was detected with rabbit anti-FhuA primary antibody, secondary antibody conjugated to horseradish peroxidase and visualized by ECL[®]. Detection of OmpA with anti-(OmpA) antibodies was included to verify that an equal amount of protein was loaded in each sample. (C) Surface localization of FhuA, FhuA–D2-3 and FhuA–D1-3. Intact *E. coli* UL4 in the absence or presence of plasmids pHIE11 (FhuA), pST4 (FhuA–D1-3), pST5 (FhuA–D2-3) were incubated with rabbit anti-FhuA primary antibody and then FITC-conjugated secondary antibody. Samples were then analysed using a FACS Calibur flow cytometer.

Analysis of expression and surface exposure of FhuA fusion proteins

Single *E. coli* colonies were inoculated into Luria broth and grown overnight at 37 °C. The overnight culture was diluted in Luria broth to a $D_{600} = 0.1$, grown at 37 °C to $D_{600} = 0.5$ and then protein expression was induced for 3 h by the addition of IPTG to a final concentration of 1 mM.

For protein expression analysis, bacterial lysates were loaded on to SDS/PAGE gels (10% acrylamide) and, following electrophoresis, stained with Coomassie Blue or transferred on to

nitrocellulose membranes for Western blotting with rabbit anti-FhuA antibodies (10 mg/ml) at a dilution of 1:100 000 [18]. After the membrane was incubated with the secondary goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase (DAKO Diagnostics, Vienna, Austria) at a dilution of 1:5000, FhuA proteins were revealed with a chemiluminescent detection system (ECL[®]; Amersham Biosciences, Little Chalfont, Bucks., U.K.). Detection of OmpA with anti-(OmpA) antibodies at a dilution of 1:10 000 [22] was included to verify that an equal amount of protein was loaded in each sample.

For analysis of the conformational activity of FhuA and FhuA fusion proteins, sensitivity to FhuA-specific phage and colicin was tested. The phage and colicin were prepared as described previously [18]. The bacteriophages T5 and Φ 80 use the FhuA protein as a receptor and were propagated on *E. coli* XL1-Blue MRF' (Stratagene, Cedar Creek, TX, U.S.A.). Colicin M was produced from *E. coli* M57T containing plasmid pTO4 [18]. For the plaque assay, bacteria were mixed with Luria top agar and poured on to Luria agar plates. Once the top agar had solidified, 1 μ l of phage T5 [6.8×10^9 plaque-forming units (pfu)/ml], phage Φ 80 (2.6×10^{11} pfu/ml) or the antimicrobial agent colicin M was dropped on to the top agar. The plates were incubated for 16 h at 37 °C and assessed for inhibition of bacterial growth.

FACS analysis

Presentation of the FhuA fusion protein on the bacterial surface was determined by FACS analysis with polyclonal rabbit anti-FhuA antibodies [18]. A single bacterial colony was inoculated into Luria broth supplemented with 50 μ g/ml kanamycin and grown at 37 °C until mid-log phase before protein expression was induced with 1 mM IPTG for 30 min. Bacteria (1×10^6) were harvested by centrifugation and washed with 500 μ l of PBS/0.5% BSA. After centrifugation at 1800 *g* for 8 min, cells were resuspended in 50 μ l of PBS/0.5% BSA and the anti-FhuA antibody (10 mg/ml) was added at a dilution of 1:5000. Following incubation for 30 min on ice, 450 μ l of PBS/0.5% BSA was added and cells pelleted once again by centrifugation as above. The bacteria were then resuspended in 50 μ l of PBS/0.5% BSA and FITC-labelled goat anti-(rabbit IgG) antibodies (DAKO Diagnostics) were added at a dilution of 1:500. After incubation for 30 min on ice, cells were washed once with 450 μ l of PBS/0.5% BSA and then resuspended in 1 ml of PBS/1% paraformaldehyde. The fluorescence intensity was analysed using a FACS Calibur flow cytometer (BD Biosciences, Schwenh, Austria). A total of 10 000 bacteria were counted and analysed with the WinMDI software (Dr Joe Trotter, Flow Cytometry Core Facility, Scripps University, La Jolla, CA, U.S.A.).

Invasion/adhesion assay

HeLa cells were seeded at 80% confluency in growth medium and bacteria were inoculated into Luria broth. After overnight incubation, bacterial cultures were diluted to a final $D_{600} = 0.2$, grown to mid-log phase and, where appropriate, induced with 0.1 mM IPTG. After growth for a further 1 h, bacteria were harvested, washed and resuspended in PBS. After washing the cells in growth medium lacking both foetal calf serum and antibiotics, bacteria were added at a multiplicity of infection (moi) of 5:1. Bacteria were centrifuged on to cells at 200 *g* for 5 min. The remainder of the bacterial samples were serially diluted and plated on to agar plates to determine the colony-forming units (cfu). Bacteria and cells were co-cultivated for 1 h and then non-adherent bacteria were removed by washing.

Extracellular bacteria were killed by incubation with 50 µg/ml gentamicin for 90 min. At this concentration of gentamicin and incubation time, complete killing of accessible bacteria was achieved (results not shown). Cells were washed, lysed by the addition of double-distilled water and, afterwards, serially diluted in PBS and the cfu were determined. A number of parameters were analysed for optimization of assay conditions including, moi of 5:1, 50:1 or 500:1; preinduction of bacteria with 0, 0.1 or 1 mM IPTG; co-incubation of bacteria and eukaryotic cells for 30 min, 1 h or 2 h; inclusion or omission of the centrifugation step; and lysis with distilled water, PBS/0.1 % Triton X-100 or PBS/0.5 % Triton X-100.

For the competition assays, peptides (200 µg/ml) were added to the cells 30 min before the addition of bacteria and were present throughout incubation. Peptides p1 [RGDS, which inhibits fibronectin binding to platelets (A9041)] and p2 [GRGDTP, which inhibits cell attachment of fibronectin (G5646)] were obtained from Sigma-Aldrich. Peptide pS3 (YEV-KRRGDMEEVHYRYLNS) was synthesized at InterCell (Vienna, Austria). In each peptide sequence the RGD motif is underlined and the single-letter amino-acid notation has been used here and throughout.

After selection of invasive bacteria from the *E. coli* library, clones were sequenced, trimmed and assigned to *S. aureus* open reading frames (ORFs) at TIGR. Additional preliminary sequence data was obtained from TIGR website at <http://www.tigr.org>. All ORF numbers given in this paper are TIGR designations.

RESULTS

Bacterial surface display of FhuA fusion proteins

One of the most well studied bacterial adhesins is the fibronectin-binding protein A (FnBP_a) of *S. aureus*. The interaction between FnBP_a and cell-associated fibronectin represents the dominant pathway for the adherence of *S. aureus* to human cells *in vitro* and this interaction is required for subsequent bacterial internalization [9,11,23–27]. The fibronectin-binding domain of the protein has been identified as a 38-amino-acid unit (Figure 1A) that is repeated three times (D1–D3), and synthetic peptides pD1, pD2 and pD3 inhibit binding of *S. aureus* to intact fibronectin [28–30]. The C-terminal 20 amino acids of each D motif possess a pattern of amino acids defined by GG_{3/4}(I,V)DF, which is required for fibronectin binding, and the sequence SVDFEED of the D3 motif is essential, but not sufficient, for binding to fibronectin. As these domains are known adhesive peptides and potential invasive motifs, they were used to construct adherent and invasive *E. coli* strains with which we could establish and optimize invasion assay conditions for bacterial surface display selection. To this end, *E. coli* producing FhuA fusions with the fibronectin-binding domains D1–D3 (expressed from plasmid pST4) or D2–D3 (expressed from plasmid pST5) of the *S. aureus* FnBP_a protein were generated (see the Materials and methods section). After introduction of the constructs into the FhuA-deficient *E. coli* strain UL4, expression of the FhuA fusion proteins was analysed by Western blot with anti-FhuA antibodies. As shown in Figure 1(B), the FhuA fusion proteins were expressed at levels comparable with parental FhuA (expressed from plasmid pHIE11). Lower-molecular-mass bands, probably corresponding to degradation products, which also react with the anti-FhuA antibodies, were also detected. Western-blot analysis with anti-(OmpA) antibodies verified that equivalent protein levels were present in each sample (Figure 1B). Similar expression patterns and levels were obtained with the *E. coli* strain DH5α (results not shown), indicating that co-expression of

chromosomally encoded wild-type (wt) FhuA did not interfere with the expression of the plasmid-encoded FhuA fusion proteins.

Presentation of the inserted adhesive peptides on the bacterial surface is a necessary requirement for bacterial–host cell interaction, thus the surface location of the FhuA fusion proteins was analysed. To examine the relative surface expression of these proteins, FACS analysis with anti-FhuA antibodies was performed with intact bacteria. As shown in Figure 1(C), each protein was clearly exposed on the bacterial surface. The mean fluorescence intensity (MFI) for each strain was 2.08 for UL4, 8.67 for UL4(pHIE11), 15.53 for UL4(pST4) and 16.16 for UL4(pST5), indicating that greater amounts of the FhuA fusion proteins were expressed on the bacterial surface than wt FhuA.

FhuA is the natural target for the phages T5 and Φ80 and the antimicrobial agent colicin M and therefore the correct conformational folding of FhuA fusion proteins was tested by sensitivity assays to these molecules. UL4(pST4), UL4(pST5) and UL4(pHIE11) were sensitive to colicin M and phages T5 and Φ80, in contrast with UL4, which remained resistant to these agents (results not shown). This retention of FhuA function with regards to phage and colicin sensitivity suggested that the conformation of FhuA on the bacterial surface was not greatly altered by the inserted D domain amino acids.

Together, these results show that FhuA–D1-3 and FhuA–D2-3 were expressed efficiently and displayed on the surface of *E. coli*.

FhuA displaying entry peptides mediate *E. coli* entry into eukaryotic cells: establishment and optimization of an internalization selection method for bacterial surface display

As a prerequisite for screening of bacterial surface display libraries for entry peptides, it was necessary to optimize several conditions of the invasion assay. First, invasion into the human epithelial HeLa cell line of three *E. coli* strains (UL4, DH5α, and DH10B) expressing FhuA, FhuA–D2-3 or FhuA–D1-3 was tested. Each of the three *E. coli* strains gave similar results and DH10B was chosen for further assays (Figure 2). Both FhuA–D1-3 (0.050–0.221 % recovery of input bacteria) and FhuA–D2-3 (0.357–0.830 % recovery of input bacteria) mediated significant entry of *E. coli* into these cells, whereas a low level of non-specific uptake was seen with *E. coli* expressing parental FhuA (0.002–0.016 % recovery of input bacteria). These results demonstrated that the fibronectin-binding domains of *S. aureus* FnBP_a are sufficient to trigger entry of bacteria into eukaryotic cells. In comparison with FhuA, FhuA–D2-3 induced a 50–330-fold increase in bacterial uptake, whereas FhuA–D1-3 induced a 14–25-fold increase. This result may represent differences in the conformation and accessibility of the fibronectin-binding domains in the two fusion proteins. DH10B(pST5) *E. coli* expressing FhuA–D2-3 were chosen for use in further experiments because of their higher invasive ability.

A number of other parameters were also optimized to increase specific uptake and decrease non-specific uptake of *E. coli* into HeLa cells (results not shown). The final optimal conditions were preinduction of bacteria with 0.1 mM IPTG for 1 h, an moi of 5:1, centrifugation of bacteria on to cells, co-incubation of bacteria and eukaryotic cells for 1 h and, finally, lysis of eukaryotic cells with distilled water for 10 min.

E. coli expressing bacterial surface-displayed invasive peptides are greatly enriched from mixed cultures after selection

For the entry assay to be successfully applied to bacterial surface display libraries, it is important that it has the capacity to effectively recover a significant number of true positive invasive

Figure 2 The fibronectin-binding domains of *S. aureus* FnBP mediate entry of *E. coli* into eukaryotic cells

E. coli UL4, DH5α and DH10B expressing FhuA, FhuA-D1-3 and FhuA-D2-3 were added to HeLa cells at an moi of 5:1 and incubated with cells for 1 h before addition of gentamicin. The cells were washed and lysed and the number of internalized bacteria determined by serial dilution on agar plates and counting the cfu. Internalization was measured as a percentage of the number of input bacteria recovered from the cells. The results shown are means $\pm$ S.D. and are representative of those obtained in separate experiments performed in duplicate.

bacteria while minimizing the number of bacteria that are taken up non-specifically by the cells. Therefore we tested the ability of the assay to select invasive *E. coli* from a mixture composed of a large number of non-invasive bacteria and a relatively small number of bacteria with an invasive-positive phenotype. These experiments were performed to test the system and not to mimic a library situation in terms of complexity. Starting with an input mixture of kanamycin-resistant (Kn^R) DH10B(pST5) *E. coli* expressing FhuA-D2-3 and chloramphenicol-resistant (Cm^R) DH10B(pHIE10) *E. coli* expressing wt FhuA at a ratio of 1:38 000, Kn^R *E. coli* were enriched 9500-fold to a ratio of 1:4 in the output mixture after one round of selection with the invasion

assay (Table 2). In order to increase the enrichment for invasive clones, a separate experiment was performed with two rounds of selection. A duplication of the ratio of input bacteria used in the first experiment (1:38 000) could not be performed as the numbers of input bacteria are determined only approximately at the start of the experiment and afterwards exact bacterial numbers are determined by cfu counts. When two rounds of selection were performed the enrichment of a 1:570 FhuA-D2-3/FhuA starting mixture was enhanced 1100-fold in the first round and 110-fold in the second round to yield a total enrichment of 130 000-fold. Thus, with this method, we were able to greatly enrich for true positive clones and so proceeded to apply this procedure to an *E. coli* surface display FhuA library.

Identification of invasive peptide motifs

Following successful optimization of the invasion assay an *S. aureus* genome-derived library displaying in the *E. coli* platform protein FhuA was screened for peptides that mediate entry of the bacteria into the target eukaryotic cells. Library LSA50 contains inserts of an average size of 30–50 bp in length, which are derived from the genomic DNA of *S. aureus* ([21a], and H. Etz, T. Henics and A. Meinke, unpublished work). This library therefore encodes peptides derived from *S. aureus* proteins and, in addition, peptides unrelated to proteins expressed by *S. aureus*. These latter peptides are encoded by *S. aureus* DNA fragments inserted in-frame with *fhuA* in such a way that the resulting reading frame of the insert is not that of an *S. aureus* ORF. The library was thus a mixture of a random and genomic *S. aureus* peptide library containing peptides of 10–30 amino acids in size. Thus novel invasive sequences could potentially be identified or sequence motifs delineated within *S. aureus* protein adhesins, which are involved in the attachment to eukaryotic cells and extracellular matrix proteins.

In an initial trial, invasive clones were recovered from the LSA50 library in one round of selection for entry into HeLa cells. A total of 22 clones were chosen and tested individually in an invasion assay. One invasive clone (L19) was identified (Figure 3) and, upon sequencing, was found to contain a triplet of inserts resulting in a total insert size of 42 amino acids (Table 3). Each insert was of *S. aureus* genomic origin, as determined by exact

Table 2 Selection and enrichment of invasive *E. coli* expressing FhuA-D2-3 from a bacterial mixture of invasive and non-invasive bacteria

Cm^R DH10B(pHIE10) expressing wt FhuA and Kn^R DH10B(pST5) expressing FhuA-D2-3 were mixed and added as input bacteria to HeLa cells. A gentamicin protection assay was performed as described in the Materials and methods section and the internalized bacteria were recovered. The input and recovered bacteria were plated on to agar plates containing either chloramphenicol [to determine the number of DH10B(pHIE10)] and/or kanamycin [to determine the number of DH10B(pST5)]. From these cfu, the ratio of the bacteria and the fold enrichment of the invasion-positive DH10B(pST5) bacteria were calculated. The results from two experiments using either one (a) or two (b) rounds of selection are shown.

(a) Experiment 1

Selection	DH10B(pHIE10) cfu	DH10B(pST5) cfu	pHIE10:pST5 ratio	Fold enrichment
First round				
Input bacteria	4.6×10^5	12	38 000:1	
Recovered bacteria	22	5	4:1	9.5×10^3

(b) Experiment 2

Selection	DH10B(pHIE10) cfu	DH10B(pST5) cfu	pHIE10:pST5 ratio	Fold enrichment
First round				
Input bacteria	2.8×10^6	4.9×10^3	570:1	
Recovered bacteria	1.9×10^2	4.3×10^2	1:2	1.1×10^3
Second round				
Input bacteria	9.6×10^5	2.0×10^6	1:2	
Recovered bacteria	8.7×10^1	2.0×10^4	1:230	1.1×10^2
Total				1.3×10^5

Figure 3 Invasion efficiencies of individual recovered clones

The 29 clones obtained from the selection procedure were analysed for their ability to invade HeLa cells. Black bars indicate clones that invade HeLa cells at least 100 times more efficiently than bacteria expressing wt FhuA. Grey bars indicate clones with a 10–100-fold increase in invasion compared with DH10B(pHIE11). The invasion of clones represented by white bars was not significantly different from that of the negative control. DH10B expressing wt FhuA and FhuA–D2-3 (D2-3) were used as negative and positive controls respectively. The experiment was carried out as described in the legend to Figure 2. The results are representative of those obtained in separate experiments performed in duplicate.

DNA sequence matches in TIGR *S. aureus* genome sequence database. Linker regions between these inserts result from the cloning strategy used to construct the library. There are two types of possible chimaera in the LSA50 library. Those from the first library construction generated by blunt-end ligation without any linker addition and those from the second transferred library generated by *FseI*–*NotI* ligation, which therefore have small linker sequences attached. L19 is of the latter type. The separate inserts in this clone are connected, as predicted, by a stretch of 16 bp each, which would consistently convert into either 5 or 6 amino acids.

Table 3 Sequences identified as carrier peptides

RGD sequences are underlined in the insert sequence column. In the invasion column, ++ indicates clones that invade HeLa cells at least 100-fold more efficiently than bacteria expressing wt FhuA. +, indicates clones with a 10–100-fold increase in invasion compared with DH10B(pHIE11). The selected sequences were analysed for their correlation with the predicted ORFs of the *S. aureus* genome and for similarity with amino acid sequences in the GenBank® database. Where an exact match was found, this is indicated in the ORF correspondence column by the name of the organism, the ORF designation/protein name and reference.

Clone	Length (amino acids)	Insert sequence	Number of colonies with this sequence/ total number colonies sequenced	Invasion	ORF correspondence
L19	42	TIKSNLNTRFCMGGGPPCDSRRGDSTITREAGLPICHEEFFG TIKSNLNTRFCM GGGPP CDSRRGDSTIT REAGLP ICHEEFFG	1/22	++	None Linker None Linker None
S3	19	YEVKR <u>RGD</u> MEEVHYRYLNS YEVKR <u>RGD</u> MEE VHYRYLNS	48/96	++	<i>S. aureus</i> ORF01297 [34] None
I12	7	PHPITAT	2/96	+	<i>M. marinum</i> Mag24-3 [33]
I07	45	DLSDFLFSGGGRPH ¹ TL ² LN ³ YQ ⁴ DRR ⁵ GQ ⁶ DEG ⁷ RP ⁸ PL ⁹ VV ¹⁰ Q ¹¹ VLD ¹² M ¹³ NI ¹⁴ KL ¹⁵ S DLSDFLFS GGGRPH TL ² LN ³ YQ ⁴ DRR ⁵ GQ ⁶ DE GGRP ⁸ PL VV ¹⁰ Q ¹¹ VLD ¹² M ¹³ NI ¹⁴ KL ¹⁵ S	1/96	+	None Linker <i>S. aureus</i> ORF00172 [35] Linker None

Due to the low number of true positives recovered after one round of selection, the *S. aureus* genomic library was then subjected to two rounds of HeLa cell entry selection. A total of 118 clones of the LSA50 library were chosen for sequencing and, among the 96 that were successfully sequenced, one sequence predominated representing 48 of these clones (S3 in Table 3). Of the remaining 48 clones, 28 different sequences were obtained (named as in Figure 3). The invasive ability of the 28 *E. coli* strains were tested individually alongside *E. coli* expressing wt FhuA as a negative control and *E. coli* expressing FhuA–D2-3 and clone L19 as positive controls (Figure 3). One highly invasive clone (S3) was identified with a 100-fold increase in invasion as compared with the FhuA control and contained the sequence selected with highest frequency. Both the S3 and L19 sequences contain the RGD motif, a well-known integrin-binding sequence. Sequences I07 and I12, displaying intermediate invasive properties (10-fold increase as compared with the FhuA control), were recovered at lower frequencies in the selections. Like L19, the insert present within FhuA of the I07 clone is a hybrid of three separate genomic fragments separated by linker regions. S3 is representative of the other type of chimaera formed at the initial stage of library construction, where no linker regions are present between the inserts. In this assay, a 10-fold increase in invasion, as compared with the FhuA control, was chosen as a cut-off point for a clear indication of an invasive clone.

To address the differences in the invasive abilities of the clones, the expression and surface localization of these FhuA proteins in the FhuA mutant strain UL4 was examined by Western blotting and FACS analysis with FhuA-specific antibodies. As shown in Figure 4, the FhuA fusion proteins expressed from clones S3 (MFI = 14.88) and L19 (MFI = 14.18) were displayed on the bacterial surface at levels even greater than wt FhuA (MFI = 8.67). However, the FhuA fusion proteins expressed by clones I07 (MFI = 2.74) and I12 (MFI = 2.49) were expressed at much lower levels. These results correlate with the degree of internalization of these clones as reflected by their recovery in the screen, as clones with higher invasion efficiency express higher levels of the entry peptide in FhuA on the

Figure 4 Surface expression of selected FhuA entry peptides

(A) Expression of FhuA entry peptide fusions in *E. coli*. Whole-cell preparations of *E. coli* UL4 expressing FhuA entry peptide fusions were separated by SDS/PAGE. FhuA and OmpA were detected as described in Figure 1. (B) Surface localization of FhuA entry peptide fusions. Intact *E. coli* UL4 expressing FhuA entry peptide fusions were incubated with rabbit anti-FhuA primary antibody and then FITC-conjugated secondary antibody. Samples were then analysed using a FACS Calibur flow cytometer.

bacterial surface than those clones with lower invasion efficiency. The selection of invasive clones thus seems to reflect a combination of the FhuA fusion protein expression level on the bacterial surface and invasive ability. The clones selected in the invasion assay which fell below the cut-off point in the individual invasion assay could possibly be clones expressing weakly invasive peptides or, alternatively, clones expressing low levels of stronger invasive peptides on the bacterial surface.

Thus two peptide sequences conferring a highly invasive phenotype on clones S3 and L19 and two sequences mediating a medium invasive phenotype on clones I12 and I07 were selected from the bacterial surface display libraries.

RGD motifs presented by bacterial surface display trigger entry of bacteria into eukaryotic cells

To verify that entry of clones S3 and L19 into eukaryotic cells was indeed due to the RGD motif, competition assays were performed in the presence of exogenous peptide containing the

Figure 5 Competitive inhibition of bacterial invasion of *E. coli* bearing RGD motifs by RGD-containing peptides

The invasion assay was performed as described previously with the addition of 200 μ g/ml of the following competing peptides: p1 (RGDS), p2 (GRGDTP) and pS3 (YEVKRRGDMEEVHYRYLNS). The RGD motif is underlined. Results are expressed as the percentage of invasion of each clone in the presence of peptide, where 100% is the invasion efficiency of the clone in the absence of peptide. The results shown are the means $\pm$ S.D. and are representative of those obtained in separate experiments performed in duplicate.

RGD sequence (RGD peptide). An invasion assay was performed in which HeLa cells were preincubated with three different peptides: p1 (RGDS, which inhibits fibronectin binding to platelets), p2 (GRGDTP, which inhibits cell attachment of fibronectin) and pS3 (YEVKRRGDMEEVHYRYLNS, which is the insert sequence of clone S3). p1 and p2 decreased the invasion of the RGD-containing clones L19 and S3 by a factor of 10–100, whereas the invasive ability of the non-RGD-containing clone I12 was not inhibited (Figure 5). pS3 efficiently inhibited invasion of clones L19 and S3, although to a lower extent than p1 or p2, and caused a moderate increase in the invasion of I12. The differences observed between the extent of inhibition of each of the RGD-containing peptides for each of the clones could result from the differences in the bordering amino acids, which have been shown previously [31,32] to affect the affinity of binding of RGD to its receptor, and from the differences in conformation of the synthetic peptides and the stretch of amino acids inserted within FhuA, as constrained RGD motifs have a higher potency than linear peptides. These results showed that the invasive property of the clones L19 and S3, identified by selection from the FhuA bacterial surface display libraries for entry into eukaryotic cells, was conferred by the RGD motif present within the insert sequence.

Selection of invasive clones in the presence of competing RGD peptides

The above-mentioned competition experiments showed that it was possible to reduce the entry of clones expressing RGD entry peptides by performing the invasion assay in the presence of exogenous peptides containing an RGD motif. In order to remove the bias towards the selection of RGD entry motifs, the invasion selection experiments were repeated in the presence of the p1 and p2 RGD peptides. Two rounds of selection were performed with the LSA50 library for entry into HeLa cells and, of the 42 recovered clones chosen for sequencing, three in-frame sequences were identified multiple times (C4, C30 and C32;

Table 4 Selection of invasive clones in the presence of competing RGD peptides

The selected sequences were analysed for their correlation with the predicted ORFs of the *S. aureus* genome and for similarity to amino acid sequences in the GenBank® database. Where an exact match was found this is indicated in the ORF correspondence column by the name of the organism, the ORF designation and reference.

Clone	Length (amino acid)	Insert sequence	Number of colonies with this sequence/ total number of colonies sequenced	ORF correspondence
C4	42	VTCFKCSITNMRCGGREPPNLLAPDETIVLGRPASQSITQLVQ VTCFKCSITNMRC GGREPP NLLAPDETIVL GRPAS QSITQLVQ	7/42	None Linker <i>S. aureus</i> ORF03010 [48] Linker None
C30	9	KYFAIARQS	5/42	None
C32	7	EAAEYFS	2/42	<i>S. aureus</i> ORF02365 [49]

Table 4). None of these clones contained an RGD sequence, indicating that the entry and subsequent selection of clones expressing RGD entry motifs had been suppressed by inclusion of competitive RGD peptides during the selection process. Therefore selection can be modulated by adding inhibiting peptides and may lead to an increase in the number of potential novel entry motifs identified.

Sequence analysis of selected clones

The selected sequences were analysed for their correlation with the predicted ORFs of the *S. aureus* genome. All DNA sequences identified were of *S. aureus* genomic origin as determined by the presence of identical sequences within the TIGR *S. aureus* database. The sequences were classified into two groups. The first class included sequences with no similarity to *S. aureus* proteins. These were the sequences from clones L19 (an RGD-containing sequence) and I12. This indicates that, as predicted, some of the sequences recovered are sequences encoded by *S. aureus* DNA, but not corresponding to *S. aureus* proteins, suggesting that, to a certain extent, the LSA50 library encodes quasi-random peptides. Interestingly, the sequence of clone I12 shows 100% identity with a region in the *Mycobacterium marinum* protein Mag24-3, which is in turn homologous with the *M. tuberculosis* PE-PGRS [proteins consisting of a Pro-Glu (PE) motif found near the N-terminus, followed by a C-terminal extension with multiple tandem repeats of Gly-Gly-Ala or Gly-Gly-Asn encoded by the polymorphic GC-rich repetitive sequences ('PGRS')] [33]. The surface location of Mag24-3 and its importance for replication of *Mycobacterium* in macrophages correlates with the possibility that the I12 sequence could act as an invasive domain for this protein.

The second class of sequences were those that contained multiple *S. aureus* sequences, one of which corresponded to a *S. aureus* protein, whereas the others did not. The S3 sequence is a hybrid with the first 11 amino acids, including the RGD motif, being located in ORF01297, which has similarity to proteins of the Nudix family of enzymes that hydrolyse nucleoside diphosphate derivatives [34]. Although this protein is not predicted to be secreted or to have a surface location in *S. aureus*, it does interact with other proteins within the cell. Amino acids 16–27 of the I07 sequence correspond to the C-terminal of ORF00172, which has similarity to multi-transmembrane protein transporters of *Bacillus* and *Staphylococcus* species [35]. Thus these amino acids may be exposed on the surface of *S. aureus* and be involved in the interaction and entry of this bacterium into eukaryotic cells. The remaining amino acids of S3 and I07 do not correspond to *S. aureus* proteins.

Thus sequences I12 and I07 are entry peptides that are identical with surface-located proteins of pathogenic bacteria and they merit further investigation for their role in bacterial virulence.

DISCUSSION

In the present study, we designed a procedure to identify carrier peptides presented on the bacterial surface as fusions with OMPs by selecting invasive *E. coli* displaying entry peptides that mediate internalization of the bacteria into eukaryotic cells. Proof of principle experiments were performed with *E. coli* expressing the fibronectin-binding domains of the FnBP_A of *S. aureus* fused to surface located FhuA. The expression and surface location of these proteins was determined by Western-blot and FACS analyses with anti-FhuA antibodies. The experiments showed clearly that peptides were displayed in a functionally active way leading to *E. coli* uptake into eukaryotic cells and that selection of invasive clones could be achieved with multifold enrichment. This procedure was applied to a surface display library of peptides derived from genomic DNA of *S. aureus* origin and led to the selection and identification of four entry motifs. Two novel entry peptides were isolated with similarity to surface-located proteins of bacterial pathogens. Sequences containing the integrin-binding RGD motif were also recovered and the essential role of the RGD motif in internalization was shown by competition studies. In the present study, surface display libraries have been used for the first time to identify novel peptides mediating entry into eukaryotic cells and demonstrates a unique application of bacterial surface display systems.

Bacterial surface display systems have been used previously [17,36,37] to study the interactions of specific peptide sequences with immobilized extracellular matrix proteins and with eukaryotic cells. For example, OmpS fused to the N-terminal of P-fimbrial PapG mediates *E. coli* binding to globoside and the OMP LamB has been used to display peptides of the cellular adhesive glycoprotein gp82 of *Trypanosoma cruzi* and thus mediate *E. coli* adhesion to HeLa cells [17,36]. A non-functional *Yersinia* invasin protein was used as a bacterial surface display platform in *E. coli* for a random library of CX₁₀C (where X₁₀ is any 10 amino acids) peptides to select clones that adhere to a human fibroblast cell line [7]. This latter selection identified four peptides, which bore no similarity to those selected in the present study, and that did not contain RGD motifs. Phage display too has been used for selection of adhesive epitopes to eukaryotic cells and for the identification of polypeptides of *S. aureus* binding to mammalian proteins [6,38–40]. For example, the use of human IgG as a target ligand led to the selection of the IgG-

binding domains of Protein A and to the identification of Sbi as a second IgG-binding protein of *S. aureus*.

In the present study, invasive rather than adhesive sequences of short length were the prime target of our search, and for this reason bacterial surface display was used in preference to phage display as it allows the selective elimination of extracellular bacteria. We performed the selections with a library derived from *S. aureus* genomic DNA, although known *S. aureus* adhesins were not identified, suggesting that short stretches of these proteins are not sufficient to mediate bacterial entry into eukaryotic cells. It has been shown previously [17,41] that long stretches of amino acids are required for binding of some *S. aureus* adhesins, e.g. the fibrinogen-binding domain of ClfA, or that short binding motifs need to be present in multiple copies for efficient binding, e.g. the fibronectin-binding motifs of FnBP [41]. Primarily clones were isolated that contained novel sequences and RGD sequences. A large family of cell attachment peptides and proteins share the RGD motif, which is necessary and sufficient for cell adhesion. This motif was first described in the early 1980s by Pierschbacher and Ruoslahti [15] and its main function is the binding of integrins to extracellular matrix proteins containing the RGD sequence, such as fibronectin. Many micro-organisms exploit the RGD motif for binding and invasion into target cells, e.g. either directly, via the envelope E protein of flaviviruses or the capsid protein of the foot and mouth disease VP1 virus, or indirectly, via the *S. aureus* FnBPs, which bind to $\alpha_5\beta_1$ integrins on the cell surface via fibronectin as a bridging molecule [42,43]. The fact that we recovered sequences containing the RGD motif not only proves the validity of the selection system, but also demonstrates the importance of this motif in invasion as well as in adhesion.

Sequences I12 and I07 are entry motifs that have not been described previously. They are of interest for development as carrier peptides to deliver conjugated substances into host cells, e.g. carrying antigens into the MHC pathways of antigen-presenting cells or antimicrobial agents into infected cells. Such carrier peptides, like the Tat peptide, can transport an array of components into cells [12]. This capacity may be enhanced by presenting the peptides in a conformationally restricted manner and/or in multiple copies, as for RGD motifs [34]. In addition sequences I12 and I07 correspond to amino acids within surface-located proteins of the virulent bacteria *M. marinum* and *S. aureus*. Thus these could be motifs utilized by the bacteria to mediate invasion into eukaryotic cells during infection. Studies on the role of these motifs in virulence could aid the development of treatments to counteract the strategies employed by pathogenic bacteria.

The selection system described in the present study very efficiently led to the identification of invasion-mediating peptides expressed as FhuA fusion proteins on the surface of *E. coli*. For the first time, display systems were employed for the identification of short invasive peptides. The procedure described in the present study can be applied to multiple bacterial surface display systems, as its use is not limited to any one particular platform protein. The range of entry peptide sequences could be expanded and/or be selected for cell specificity with screens performed with eukaryotic cells of different origins. Likewise, libraries derived from DNA of diverse sources could be employed. Thus bacterial surface display systems provide a versatile tool to identify novel entry motifs.

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