The high-adhesive properties of the FimH adhesin of *Salmonella enterica* serovar Enteritidis are determined by a single F118S substitution

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The binding properties of low- and high-adhesive forms of FimH adhesins from *Salmonella enterica* serovars Enteritidis and Typhimurium (*S. Enteritidis* and *S. Typhimurium*) were studied using chimeric proteins containing an additional peptide that represents an N-terminal extension of the FimF protein. This modification, by taking advantage of a donor strand exchange mechanism, closes the hydrophobic groove in the fimbrial domain of the FimH adhesin. Such self-complemented adhesins (scFimH) did not form aggregates and were more stable (resistant to proteolytic cleavage) than native FimH. High-adhesive variants of scFimH proteins, with alanine at position 61 and serine at position 118, were obtained by site-directed mutagenesis of *fimH* genes from low-adhesive variants of *S. Enteritidis* and *S. Typhimurium*, with glycine at position 61 and phenylalanine at position 118. Direct kinetic analysis using surface plasmon resonance (SPR) and glycoproteins carrying high-mannose carbohydrate chains (RNase B, horseradish peroxidase and mannan-BSA) revealed the existence of high- and low-adhesive allelic variants, not only in *S. Typhimurium* but also in *S. Enteritidis*. Using two additional mutants of low-adhesive FimH protein from *S. Enteritidis* (Gly61Ala and Phe118Ser), SPR analysis pointed to Ser118 as the major determinant of the high-adhesive phenotype of type 1 fimbriae from *S. Enteritidis*. These studies demonstrated for the first time that the functional differences observed with whole fimbriated bacteria could be reproduced at the level of purified adhesin. They strongly suggest that the adhesive properties of type 1 fimbriae are determined only by structural differences in the FimH proteins and are not influenced by the fimbrial shaft on which the adhesin is located.

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INTRODUCTION

Fimbriae are proteinaceous filamentous structures present on the surface of many members of the *Enterobacteriaceae* (Duguid *et al.*, 1966; Thorns, 1995). An increasing amount of evidence suggests that type 1 fimbriae play an important role in attachment to and colonization of the gut mucosa by different serovars of *Salmonella enterica*, including *Salmonella* Enteritidis and *Salmonella* Typhimurium (De Buck *et al.*, 2003; Dibb-Fuller *et al.*, 1999; Dibb-Fuller &

Woodward, 2000; Ewen *et al.*, 1997; Naughton *et al.*, 2001). In addition, it has recently been shown that internalization of *S. Typhimurium* by murine dendritic cells can be initiated by a type 1 fimbriae-dependent mechanism (Guo *et al.*, 2007). However, their precise role in the pathogenesis of *Salmonella* is not clear (Allen-Vercoe & Woodward, 1999; Allen-Vercoe *et al.*, 1999; Rajashekara *et al.*, 2000).

Type 1 fimbriae are rod-like surface structures 7 nm thick and up to 3 µm in length, composed primarily of protein subunits called FimA (Jones *et al.*, 1995). The adhesive properties of type 1 fimbriae depend on a lectin-like FimH adhesin located on the tip of the fimbrial shaft (Krogfelt *et al.*, 1990). FimH binds to oligomannosidic structures present on many eukaryotic membrane-bound and secreted glycoproteins (Firon *et al.*, 1983, 1984).

Abbreviations: CD, circular dichroism; HRP, horseradish peroxidase; SPR, surface plasmon resonance.

Two supplementary figures, showing HPLC elution profiles of scFimH adhesins and SDS-PAGE of mutated EscFimH/L proteins, are available with the online version of this paper.

Based on the early studies, it was proposed that enterobacterial type 1 fimbriae are structurally related (Abraham *et al.*, 1988); however, recent data have shown significant heterogeneity among FimH adhesins from different genera and even within the same species or serovar with respect to binding properties for mannosylated ligands (Sokurenko *et al.*, 1994, 1995). On the serovar level we recently showed that inactive FimH adhesins from *Salmonella* Gallinarum biovars Gallinarum and Pullorum are characterized by the presence of isoleucine at position 78 instead of the threonine found in the active FimH adhesins of *S. Typhimurium* and *S. Enteritidis* (Kisiela *et al.*, 2005a, 2006). At the strain level, Boddicker *et al.* (2002) have found that two strains of *S. Typhimurium*, which express allelic variants of FimH protein differing by two amino acid residues (Gly61Ala and Phe118Ser), adhere weakly and strongly to human HEp-2 cells.

Sequencing of the *fimH* genes from different avian and human *S. Enteritidis* isolates (Kisiela *et al.*, 2005b) shows that all the analysed strains represent the low-adhesive variant of *S. Typhimurium* described by Boddicker *et al.* (2002). The functional characterization of low-adhesive variants of FimH proteins from *S. Enteritidis* and *S. Typhimurium* reveals that FimH adhesins bind equally to human colon carcinoma and bladder cancer cells (Kisiela *et al.*, 2006). In contrast, the high-adhesive variant of *S. Typhimurium* FimH adhesin binds to mouse enterocytes, but not to mouse uroepithelial cells, as shown by Thankavel *et al.* (1999). To explain these discrepancies we hypothesized that only the high-adhesive variants of FimH proteins are able to discriminate between different eukaryotic cells, in contrast to the low-adhesive forms of FimH proteins (Kisiela *et al.*, 2006). As in our study the purified FimH proteins were used, the other possible explanation is that the binding specificity of FimH adhesins is modulated by the fimbrial shaft on which this adhesin is presented (Duncan *et al.*, 2005). In the present study we addressed this problem by constructing low- and high-adhesive variants of FimH proteins from *S. Enteritidis* and *S. Typhimurium*, using site-directed mutagenesis. Their binding properties were studied at the molecular level using purified recombinant protein.

METHODS

Construction of EextfimH.LA and TextfimH.LA vectors containing variants of the *fimH* gene with an N-terminal extension of the *fimF* gene and myc epitope- and His-tags. ssDNA oligonucleotides with sequences corresponding to the first 17 amino acids of the *S. Enteritidis* FimF protein [N-terminal extension containing additional sequences corresponding to a four amino acid linker (Glu-Lys-Asp-Asn) (Barnhart *et al.*, 2000) and *Xba*I restriction sites at the 5' and 3' termini (5'-CTAGGTCCACCACGTTACCGCGAGTTCAATATTCACCTCGCCAGAGATGAGTTCTGTTTATTAT-3')] were obtained from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. They were hybridized to make dsDNA and ligated to the corresponding restriction sites in the vectors T.fimH/pTrcHis2b or E.fimH/

pTrcHis2b containing *fimH* genes from *S. Typhimurium* and *S. Enteritidis*, respectively (Kisiela *et al.*, 2005a). The new constructs were used as templates to amplify, by PCR, *fimH*-N-terminal *fimF* extension fusion genes containing a myc epitope-tag with primers 5'-extfimH/myc (5'-GCATCCATGGCGACGGTTTGC-3') and 3'-extfimH/myc (5'-CAGAAGAGGATCTGAATAAGCTTCGC-3'). The PCR primers contained additional sequences corresponding to the *Nco*I restriction site in the primer 5'-extfimH/myc and to *Hind*III in 3'-extfimH/myc. The gene constructs were amplified as follows: hot start (94 °C for 5 min), then 24 cycles of denaturation (94 °C for 50 s), annealing (59 °C for 50 s), elongation (72 °C for 90 s) and terminal elongation at 72 °C for 5 min. The PCR amplicons were digested with the respective enzymes and ligated to the corresponding sites of plasmid pET-22b (Novagen). The resulting vectors contained the low-adhesive variants of *fimH* genes from *S. Enteritidis* and *S. Typhimurium* linked to the N-terminal extension of the *fimF* gene from *S. Enteritidis*, a myc-tag and a His-tag, and were named EextfimH.LA and TextfimH.LA, respectively.

Site-directed mutagenesis of *fimH* genes. To construct high-adhesive variants of the *fimH* genes from *S. Enteritidis* and *S. Typhimurium*, mutagenesis of their respective low-adhesive variants of the *fimH* genes linked to the N-terminal extension of the *fimF* gene from *S. Enteritidis* was performed using a PCR-based method. The mutations of interest were generated by two separate PCRs using two pairs of primers. PCR I was carried with the common 5' primer (fimH-1, 5'-CGCGGATCCAATGAAATATACTCAGC-3') and 3' primers with the desired Gly61Ala (primer fimH-2, 5'-GCTGGGTTCGTCTAAAGAGGCCGTCG-3') or Phe118Ser (primer fimH-3, 5'-CGCCTATTCTATGCGGTCGCTGTGATGC-3') mutations and the plasmids E.fimH/pTrcHis2b and T.fimH/pTrcHis2b as templates (Kisiela *et al.*, 2005a). PCR II was performed with the common 3' primer (fimH-4, 5'-GCGTCTAGAGCATCATAATCGACTCG-3') and 5' primers with the desired Gly61Ala (fimH-5, 5'-GCTGCCGGAGAAATCTGCTTGGGTCG-3') and Phe118Ser (fimH-6, 5'-CGATAGTGTGCTGGCGTATCTTATCCGC-3') mutations. The first and second PCRs were performed as follows: (I) hot start (2 min, 94 °C), then 20 cycles of denaturation (50 s, 94 °C), annealing (50 s, 57 °C) and elongation (1 min, 72 °C) for the Gly61Ala mutation; and (II) hot start (2 min, 94 °C), then 20 cycles of denaturation (50 s, 94 °C), annealing (50 s, 55 °C) and elongation (72 °C, 1 min) for the Phe118Ser mutation. The resulting DNA fragments were used as templates for a third PCR with primers fimH-1 and fimH-4. The reaction was performed as follows: hot start (2 min, 94 °C), then 20 cycles of denaturation (1 min, 94 °C), annealing (1 min, 52 °C) and elongation (1 min, 72 °C). The mutated *fimH* genes were cloned into plasmid pET-22b and the expression vectors containing the high-adhesive variants of the *fimH* genes from *S. Enteritidis* and *S. Typhimurium* were designated EextfimH.HA and TextfimH.HA, respectively.

Two additional mutants of the FimH protein were made using a method based on the QuikChange Site-Directed Mutagenesis kit (Stratagene). The Gly61Ala mutant was made by a single PCR using the primers 5'-GCCGGAATAATCTGCTTGGGTCGGCG-3' and 5'-CGCCGACCAAGCAGATTTCCGGCGC-3' and the EextfimH.LA vector as template. The Phe118Ser mutant was obtained by a single PCR using the primers 5'-CGCTGGCGTATCTTATCCGCCCGGTAAAC-3' and 5'-CGGGGCGGATAAGATACGCCAGGACAC-3' and the same vector as template. The mutants were amplified as follows: hot start (2 min, 95 °C), then 14 cycles of denaturation (45 s, 95 °C), annealing (1 min, 55 °C) and elongation (6 min 40 s, 68 °C). The reaction mixture contained high-fidelity OptiTaQ DNA polymerase (EURx, Gdańsk, Poland) and *Dpn*I restriction enzyme to remove the original template. The mutations introduced into the *fimH* gene were confirmed by DNA sequencing. As above, the mutated *fimH* genes

were cloned into plasmid pET-22b, and the resulting vectors were designated EextfimH.Gly61Ala and EextfimH.Phe118Ser.

Expression and purification of recombinant self-complemented FimH proteins (scFimHs). FimH proteins self-complemented with the N-terminal extension of the FimF protein (scFimH) and representing the high- and low-adhesive variants were named, respectively, EscFimH/H and EscFimH/L for *S. Enteritidis*, and TscFimH/H and TscFimH/L for *S. Typhimurium*. *Escherichia coli* BL21 cells (Novagen), transformed with the appropriate expression vector, were grown at 37 °C in 250 ml Luria-Bertani (LB) medium containing 25 mM MgCl₂, 0.4% glycerol (v/v) and ampicillin (100 µg ml⁻¹). When the OD₆₀₀ of the growing culture reached 0.6, IPTG (Fermentas) was added to a final concentration of 0.5 mM. The temperature was lowered to 26 °C and bacterial growth was continued for another 12 h. Bacteria were centrifuged and lysed with 20 mM Tris/HCl, pH 7.8, containing 0.1 M NaCl and 8 M urea. The presence of urea in lysed buffer increased the yield of the final purification by about 10 times. The recombinant proteins were purified using Ni-NTA affinity chromatography (Sigma-Aldrich), concentrated on Centricon 10 filters (Millipore) and quantified by a bicinchoninic acid (BCA) protein assay kit (Sigma).

Biotinylation of FimH proteins was performed as previously described (Kisiela *et al.*, 2005a).

Synthesis of mannosylated BSA (mannan-BSA). Mannan-BSA was synthesized according to the method of Kanska *et al.* (2008). Briefly, mannan (Sigma) was oxidized by 0.5% sodium periodate in 0.1 M acetate buffer, pH 5.4. The reaction was carried out for 1 h at 4 °C in the dark. Four milligrams of oxidized mannan was mixed with 12 mg BSA (Sigma) (1:3, w/w). The samples were frozen in an acetone/dry ice bath and lyophilized, then heated at 118 ± 2 °C for 20 min and dissolved in Tris-buffered saline (TBS; 10 mM Tris/HCl, 150 mM NaCl, pH 7.4). The efficiency of mannan-BSA conjugation was confirmed by SDS-PAGE.

SDS-PAGE and Western blotting. The purified scFimH proteins were analysed in Coomassie Brilliant Blue-stained SDS-PAGE gels (12%). They were identified by Western blotting using primary anti-myc mAb 9E10 (ATCC, no. CRL-1729) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Dako) or rabbit polyclonal antibodies directed against FimH (Kisiela *et al.*, 2005b) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (Sigma). The binding of the scFimH proteins to RNase B (Sigma), HRP (Sigma) and mannan-BSA was analysed by far-Western blotting. High-mannose glycoproteins, after SDS-PAGE

Fig. 1. (a) Schematic representation of *fimH* gene constructs with an N-terminal extension of the *fimF* gene and a myc epitope-tag. (b) (i) Coomassie blue-stained SDS-PAGE, and immunostaining with (ii) rabbit polyclonal anti-FimH antibodies and (iii) anti-myc mAb 9E10 of FimH proteins with an N-terminal extension of the FimF protein from *S. Enteritidis* (scFimH). Lanes 1 and 3, low-adhesive variants of scFimH adhesins from *S. Enteritidis* and *S. Typhimurium*, respectively; lanes 2 and 4, high-adhesive variants of scFimH proteins from *S. Enteritidis* and *S. Typhimurium*, respectively. scFimH proteins (10 µg), purified on Ni-NTA resin, were subjected to SDS-PAGE under reducing conditions in a 12% gel. The migration positions of protein standards (in kDa) are indicated on the left.

in a 10 % gel, were transferred to nitrocellulose (Bio-Rad), blocked overnight with 5 % fat-free dry milk in TBST (TBS supplemented with 0.2 % Tween 20) and incubated with scFimH proteins (0.2 mg ml⁻¹) for 12 h. Immunodetection was performed using anti-myc mAb 9E10 and alkaline phosphate-conjugated goat anti-mouse immunoglobulins.

HPLC. A Waters Alliance HPLC system equipped with a 2996 PDA detector and a Superdex 75 10/300 GL column (GE Healthcare) was used to analyse the recombinant scFimH proteins. Each protein sample was injected into an HPLC column previously equilibrated in TBS and run at a solvent flow rate of 0.8 ml min⁻¹ for 25 min at 20 °C. For molecular mass determination the column was calibrated with the protein markers BSA (66 kDa), HRP (40 kDa) and RNase B (18 kDa) (Sigma). The eluted proteins were detected by UV absorbance at 280 nm.

Circular dichroism (CD) spectroscopy. The secondary structures of proteins were analysed by CD measurements in a JASCO J-715 spectropolarimeter (JASCO International). The proteins were dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 2 mM K₂HPO₄, pH 7.4) and CD spectra were measured at 25 °C using a thermostat-controlled 0.1 cm cell. Spectra were recorded with a response time of 1.0 s and with 1.0 nm resolution. Each spectrum is the result of five spectra accumulated and averaged.

Real-time interaction analysis by surface plasmon resonance (SPR). The immobilization of high-mannose glycoproteins to CM5 (GE Healthcare) sensor chips has been described previously (Kisiela *et al.*, 2005a), and the binding of the scFimH analytes was analysed using a BIAcore 1000 SPR instrument. All binding experiments were carried out at 25 °C with a 5 µl min⁻¹ flow rate. To determine FimH affinity to the immobilized proteins, 40 µl of six different concentrations (1 µM, 500 nM, 250 nM, 125 nM, 62.5 nM and 31.25 nM) of scFimH adhesive variant solutions, as well as sample buffer blank, were passed over the ligand-immobilized chip surface (association phase), followed by 8 min of dissociation with the running buffer. The same samples were passed over a control chip surface without immobilized ligand. Three replicates of each analyte concentration were injected. The resulting sensorgrams were obtained first by subtracting the buffer blank from curves recorded for the interactions of FimH proteins with these glycoproteins. Then the curves recorded when adhesins were passed over the blank surface were subtracted from such sensorgrams. The equilibrium constants were determined using BIAevaluation 3.1 software. For global fitting a 1:1 Langmuir binding model with an included mass transport step was applied based on criteria provided by the BIAevaluation handbook.

RESULTS

Construction of scFimH variants of *S. Enteritidis* and *S. Typhimurium*

To fill the hydrophobic groove exposed in the fimbrial domain of FimH proteins in the absence of FimF protein (Jones *et al.*, 1995; Hancox *et al.*, 1997; Choudhury *et al.*, 1999), the strategy described by Barnhart *et al.* (2000) was used. The N-terminal extension of FimF, preceded by a short linker, was linked to the C-terminal end of FimH to allow the donor strand to supplement the hydrophobic groove in the fimbrial domain with the missing C-terminal

Fig. 2. (a) Far-UV CD spectra of scFimH adhesins with an N-terminal extension of FimF (0.1 mg ml⁻¹); short-dashed line, low-adhesive variant of *S. Enteritidis*; heavy solid line, high-adhesive variant of *S. Enteritidis*; long-dashed line, low-adhesive variant of *S. Typhimurium*; light solid line, high-adhesive variant of *S. Typhimurium*. (b) Far-UV CD spectra of the low-adhesive variant of the scFimH adhesin of *S. Enteritidis* with an N-terminal extension from FimF (0.1 mg ml⁻¹) purified in (solid line) denaturing (presence of 8 M urea) and (dashed line) non-denaturing (without urea) conditions. Spectra were recorded with a response time of 1.0 s and a data point resolution of 1 nm, using a cuvette with 0.1 cm path length. $[\theta]_{MRE}$ indicates mean residue ellipticity (in degrees cm² dmol⁻¹). Five scans were averaged to obtain final spectra.

extension (Fig. 1a). These fusion proteins were named self-complemented FimHs (scFimHs).

The low- and high-adhesive variants of the *S. Enteritidis* and *S. Typhimurium* *fimH* genes extended by the N-terminal extension of the *fimF* gene from *S. Enteritidis* were constructed as described in Methods and cloned into plasmid pET-22b containing a His affinity tag. Then, *E. coli* BL21 cells were transformed with the respective plasmids and the resulting recombinant proteins EscFimH/L (low-adhesive variant) and EscFimH/H (high-adhesive variant) of *S. Enteritidis* and TscFimH/L (low-adhesive variant) and TscFimH/H (high-adhesive variant) of *S. Typhimurium* were purified by chromatography on an Ni-NTA affinity matrix. The identity of the scFimH proteins was confirmed first by SDS-PAGE

Fig. 3. Far-Western blot analysis of scFimH adhesins with an N-terminal extension from the FimF protein binding to glycoproteins carrying high-mannose-type oligosaccharide chains. Binding of the (a) low- and (b) high-adhesive variants of scFimH from *S. Enteritidis* and (c) low- and (d) high-adhesive variants of scFimH from *S. Typhimurium* to RNase B (lane 1), HRP (lane 2) and mannan-BSA (lane 3). Each glycoprotein (4 µg) was subjected to SDS-PAGE under reducing conditions in a 10% gel and transferred to nitrocellulose. The migration positions of protein standards (in kDa) are indicated on the left.

and then by Western blotting with rabbit polyclonal anti-FimH antibodies and anti-myc mAb 9E10 (Fig. 1b).

Molecular characterization of scFimH proteins

To confirm that the EscFimH/L, EscFimH/H, TscFimH/L and TscFimH/H proteins did not form aggregates, they

were subjected to analytical HPLC using a Superdex 75 10/300 GL column. As expected, for each of the analysed proteins over 95% of the applied sample was present in monomeric form (Supplementary Fig. S1). The structural organization of the scFimH proteins was analysed using CD. It was found that all four analysed adhesins (EscFimH/L, EscFimH/H, TscFimH/L and TscFimH/H) possessed

Fig. 4. Interaction of scFimH proteins of *S. Enteritidis* and *S. Typhimurium* possessing an N-terminal extension from the FimF protein with immobilized RNase B analysed with an SPR biosensor. (a) Low-adhesive variant of *S. Enteritidis*; (b) high-adhesive variant of *S. Enteritidis*; (c) low-adhesive variant of *S. Typhimurium*; (d) high-adhesive variant of *S. Typhimurium*. Grey lines represent different concentrations of analyte (1 µM, 500 nM, 250 nM, 125 nM, 62 nM and 31 nM) in TBS. Binding data were collected at a flow rate of 5 µl min⁻¹. Black solid lines represent the best global fit of the experimental data to a 1 : 1 Langmuir reaction model with an included mass transport step (BIAevaluation 3.1 software). RU, relative units.

Fig. 5. Interaction of scFimH proteins of *S. Enteritidis* and *S. Typhimurium* possessing an N-terminal extension from the FimF protein with immobilized HRP analysed with an SPR biosensor. (a) Low-adhesive variant of *S. Enteritidis*; (b) high-adhesive variant of *S. Enteritidis*; (c) low-adhesive variant of *S. Typhimurium*; (d) high-adhesive variant of *S. Typhimurium*. Grey lines represent different concentrations of analyte (1 μ M, 500 nM, 250 nM, 125 nM, 62 nM and 31 nM) in TBS. Binding data were collected at a flow rate of 5 μ l min⁻¹. Black solid lines represent the best global fit of the experimental data to a 1 : 1 Langmuir reaction model with an included mass transport step (BIAevaluation 3.1 software). RU, relative units.

essentially identical, extensive secondary structure (Fig. 2a). No differences in CD spectra were found between scFimH proteins purified in denaturing (presence of 8 M urea) conditions and those purified in non-denaturing (without urea) conditions (Fig. 2b).

Binding properties of G61A and F118S double mutants of scFimHs

The ability of the low- and high-adhesive variants of scFimH proteins to bind mannose-rich carbohydrate structures was studied by Western blotting with RNase B, HRP and the synthetic glycoconjugate mannan-BSA (Fig. 3). The binding was completely inhibited by preincubation with 0.2 M D-mannose (data not shown). There was no difference in binding between the low- and high-adhesive variants of the FimH adhesins from *S. Enteritidis* and *S. Typhimurium*.

The binding of *S. Enteritidis* and *S. Typhimurium* low- and high-adhesive variants of the scFimH adhesins to RNase B (Fig. 4), HRP (Fig. 5) and mannan-BSA (Fig. 6) was analysed by SPR. The sensorgrams were obtained first by subtracting the buffer blank from curves recorded for the

interactions of FimH proteins with these glycoproteins. Then the curves, recorded when adhesins were passed over the blank surface, were subtracted from such sensorgrams. Association (k_a) and dissociation (k_d) rate constants were calculated using a 1 : 1 Langmuir model with an included mass transport step, based on the criteria provided by the BIAevaluation handbook. The SPR study revealed profound differences in K_D values, estimated as k_d/k_a ratio, between the high- and low-adhesive variants of the scFimH proteins from both *Salmonella* serovars studied (Table 1).

Binding properties of Gly61Ala or Phe118Ser single mutants of *S. Enteritidis* scFimH

To address the question of which amino acid substitution (Gly61Ala or Phe118Ser) was mainly responsible for the differences in binding affinity of the low- and high-adhesive variants of the FimH adhesins, *fimH* gene mutants of the low-adhesive variant of *S. Enteritidis* with single Gly61Ala or Phe118Ser mutations were constructed as described in Methods. The resulting plasmids were used to transform *E. coli* BL21 cells, and the recombinant scFimH

Fig. 6. Interaction of scFimH proteins of *S. Enteritidis* and *S. Typhimurium* possessing an N-terminal extension from the FimF protein with immobilized mannan-BSA analysed with an SPR biosensor. (a) Low-adhesive variant of *S. Enteritidis*; (b) high-adhesive variant of *S. Enteritidis*; (c) low-adhesive variant of *S. Typhimurium*; (d) high-adhesive variant of *S. Typhimurium*. Grey lines represent different concentrations of analyte (1 μ M, 500 nM, 250 nM, 125 nM, 62 nM and 31 nM) in TBS. Binding data were collected at a flow rate of 5 μ l min⁻¹. Black solid lines represent the best global fit of the experimental data to a 1 : 1 Langmuir reaction model with an included mass transport step (BIAevaluation 3.1 software). RU, relative units.

adhesins, named EscFimH/L(Gly61Ala) and EscFimH/L(Phe118Ser), after chromatography on an Ni-NTA affinity matrix, were analysed by SDS-PAGE and Western blotting. The same pattern of Coomassie blue staining and immunostaining with anti-FimH antibodies as the low- and high-adhesive variants of other scFimH adhesins was observed (Supplementary Fig. S2a).

Both mutants of the *S. Enteritidis* scFimH low-adhesive variant carrying the single mutations Gly61Ala or Phe118Ser bound to glycoproteins carrying high-mannose glycoproteins to similar extents, as shown by far-Western blotting (Supplementary Fig. S2b). However, striking differences in the association (k_a) and dissociation (k_d) rate constants were found between the EscFimH/L(Gly61Ala) and EscFimH/L(Phe118Ser) adhesins when their interactions with RNase B, HRP and mannan-BSA were studied by SPR (Fig. 7). The mutant identical to the low-adhesive variant of the FimH protein from *S. Enteritidis* except at position 118, where phenylalanine was replaced by serine, bound to all three analysed high-mannose glycoproteins with much higher affinity than the parental scFimH protein (Table 1). Interestingly, the

second mutant, identical to the low-adhesive variant except at position 61, where glycine was exchanged by alanine, bound to the same glycoproteins with K_D values similar to those of the non-mutated protein (Table 1).

DISCUSSION

Our previous study of FimH sequences from different avian and human isolates of *S. Enteritidis* revealed that all the analysed isolates express the same allelic variant of FimH (Kisiela *et al.*, 2005b), which was first found in the *S. Typhimurium* SL1344 strain by Boddicker *et al.* (2002). This strain of *S. Typhimurium* bound weakly to HEp-2 cells, in contrast to the high-adhesive strain LB5010, which attached strongly to those cells. The authors claimed that the different properties of the low- and high-adhesive variants of FimH proteins depend mostly on two amino acid residues located at positions 61 and 118. A glycine residue at position 61 and phenylalanine at position 118 were found in the low-adhesive variant of the FimH adhesin, and the high-adhesive variant of this protein had alanine at position 61 and serine at position 118. Therefore,

Table 1. Dissociation constants of scFimH adhesins determined by SPR analysis

FimH variant	Ligand	K _D (M)
EscFimH/L	RNase B	$5.60 \times 10^{-8} \pm 1.06 \times 10^{-8}$
	HRP	$2.30 \times 10^{-8} \pm 6.30 \times 10^{-9}$
	Mannan-BSA	$3.23 \times 10^{-6} \pm 9.10 \times 10^{-7}$
EscFimH/H	RNase B	$6.33 \times 10^{-9} \pm 1.25 \times 10^{-9}$
	HRP	$2.60 \times 10^{-9} \pm 7.80 \times 10^{-10}$
	Mannan-BSA	$2.00 \times 10^{-7} \pm 3.30 \times 10^{-8}$
TscFimH/L	RNase B	$3.48 \times 10^{-8} \pm 7.92 \times 10^{-9}$
	HRP	$4.94 \times 10^{-8} \pm 5.23 \times 10^{-9}$
	Mannan-BSA	$9.00 \times 10^{-6} \pm 6.03 \times 10^{-7}$
TscFimH/H	RNase B	$8.70 \times 10^{-9} \pm 1.10 \times 10^{-9}$
	HRP	$8.36 \times 10^{-9} \pm 1.07 \times 10^{-9}$
	Mannan-BSA	$9.50 \times 10^{-7} \pm 2.11 \times 10^{-8}$
EscFimH/L(Gly61Ala)	RNase B	$3.91 \times 10^{-8} \pm 3.68 \times 10^{-9}$
	HRP	$2.50 \times 10^{-8} \pm 6.59 \times 10^{-9}$
	Mannan-BSA	$3.50 \times 10^{-6} \pm 8.82 \times 10^{-7}$
EscFimH/L(Phe118Ser)	RNase B	$5.67 \times 10^{-9} \pm 1.24 \times 10^{-9}$
	HRP	$7.40 \times 10^{-9} \pm 1.33 \times 10^{-9}$
	Mannan-BSA	$2.44 \times 10^{-7} \pm 7.70 \times 10^{-8}$

we asked whether the same amino acid substitutions (Gly61Ala and Phe118Ser) could be responsible for similar changes in the binding properties of the low-adhesive FimH from *S. Enteritidis*. In contrast to the earlier study, the binding properties of the FimH adhesins were studied at the molecular level using purified recombinant wild-type and mutated proteins. As the purified native FimH proteins have a high tendency to form aggregates, which is a major problem when they are analysed by SPR (Van der Merwe & Barclay, 1996), we constructed fusion FimH proteins containing an additional amino acid sequence representing an N-terminal extension of the FimF protein. Such a modification, by taking advantage of a donor strand exchange mechanism, enabled the completion of the immunoglobulin fold in the FimH protein, closing the hydrophobic groove in the fimbrial domain (Jones *et al.*, 1995; Barnhart *et al.*, 2000). As expected, self-complemented adhesins (scFimH) did not form aggregates and were more stable (resistant to proteolytic cleavage) than native FimH.

As shown by SPR, the low-adhesive FimH variant of *S. Enteritidis* bound with a much higher K_D (about 10 times) to RNase B, HRP and mannan-BSA than its high-adhesive counterpart. Less profound differences in K_D values were found between the low- and high-adhesive variants from *S. Typhimurium*. The interactions of the low-adhesive variants with RNase B and HRP gave dissociation constants about five to six times higher than the high-adhesive proteins. The distinctly higher K_D (about 10 times) was found only for mannan-BSA. The K_D values obtained in this study for the interactions of the low-adhesive variants with all three high-mannose glycoproteins are much lower than those previously published (Kisiela *et al.*, 2006). These discrepancies are most probably the result of the different approach applied in the present study. Instead of native FimH protein, with a high tendency to form aggregates,

fully soluble chimeric adhesin with an N-terminal extension from FimF was used. In addition, this SPR study was performed in the absence of 0.2 M guanidine hydrochloride. Taken together, our study revealed on the level of isolated FimH proteins the existence of low- and high-adhesive variants of the FimH adhesins not only for *S. Typhimurium* but also for *S. Enteritidis*, although their binding characteristics to high-mannose structures were slightly different. Therefore, these data suggest that, in addition to amino acid residues at positions 61 and 118, other amino acid substitutions could influence the specificity of FimH variants for different ligands. The low- and high-adhesive variants of *S. Enteritidis* and *S. Typhimurium* differ at four amino acid positions (R126L, S131Y, M137K and N317I). The same number of differences in amino acid sequences was also found between high-adhesive variants of these two *Salmonella* serovars (R126L, S131Y, M137K and N317I). As the low- and high-adhesive allelic variants of FimH adhesin from *S. Enteritidis* and *S. Typhimurium* extracted from fimbrial shafts bound with distinctly different affinities to glycoproteins carrying high-mannose structures, our data strongly suggest that variations in binding specificity are dependent only on the structure of the FimH protein itself, and not on the fimbrial shaft, as has been proposed by several authors (Thankavel *et al.*, 1999; Duncan *et al.*, 2005; Madison *et al.*, 1994). Based on the results obtained with the FimH proteins of *E. coli* and *Klebsiella pneumoniae*, which show that the affinity of the adhesin for mannosylated ligands can be directly regulated by the pilin domain by the use of the allosteric catch bond mechanism (Aprikian *et al.*, 2007; Stahlhut *et al.*, 2009), we speculate that a similar mechanism could be responsible for the differences in K_D values obtained for high- and low-adhesive variants of FimH adhesins from *Salmonella*.

Fig. 7. Interaction of (a, c, e) EscFimH/L(Gly61Ala) and (b, d, f) EscFimH/L(Phe118Ser) mutants with immobilized RNase B, HRP and mannan-BSA analysed with an SPR biosensor. The EscFimH(Gly61Ala) mutant, constructed by site-directed mutagenesis, was identical to the low-adhesive variant of the scFimH adhesin of *S. Enteritidis* except at the position corresponding to amino acid position 61, where glycine was replaced by alanine (Gly61Ala); the EscFimH(Phe118Ser) mutant was identical to the low-adhesive variant of the scFimH adhesin of *S. Enteritidis* except at the position corresponding to amino acid 118, where phenylalanine was replaced by serine (Phe118Ser). Grey lines represent different concentrations of analyte (1 μ M, 500 nM, 250 nM, 125 nM and 31 nM) in TBS. Binding data were collected at a flow rate of 5 μ l min⁻¹. Black solid lines represent the best global fit of the experimental data to a 1 : 1 Langmuir reaction model with an included mass transport step (BIAevaluation 3.1 software). RU, relative units.

Our data on the involvement of amino acid residues 61 and 118 in determining the binding properties of low- and high-adhesive variants of FimH adhesin differ from those of Boddicker *et al.* (2002), who found that Ala61 is mostly responsible for supporting the attachment of *S. Typhimurium* LB5010 to HEp-2 cells. Mutated low-

adhesive FimH protein from *S. Enteritidis*, in which the glycine at position 61 was replaced by the alanine found in the high-adhesive variant of *S. Typhimurium*, bound with almost the same association (k_a) and dissociation (k_d) rate constants to all analysed glycoproteins as the native adhesin. In contrast, when phenylalanine at position 118

was replaced by the serine present in the high-adhesive variant of FimH, the mutated form of the low-adhesive protein from *S. Enteritidis* interacted with higher affinity with mannan-BSA (K_D about 10 times lower) as well as with RNase B and HRP (K_D about five to seven times lower). Therefore, using mutated forms of low-adhesive FimH from *S. Enteritidis* (Gly61Ala and Phe118Ser), our SPR study points to Ser118 as the major determinant of the high-adhesive phenotype of type 1 fimbriae from *S. Enteritidis*.

In summary, we have obtained for the first time functional full-size FimH adhesins as stable and highly soluble proteins which can be used to study structure–function relationships with their respective ligands at the molecular level. SPR analysis with highly purified fusion FimH proteins revealed the existence of high- and low-adhesive allelic variants not only in *S. Typhimurium* but also in *S. Enteritidis*. Our studies are believed to be so far the only ones in which the functional differences observed with whole fimbriated bacteria can be reproduced at the level of purified adhesin. They strongly suggest that the adhesive properties of type 1 fimbriae are determined only by structural differences in the FimH proteins and are not influenced by the fimbrial shaft on which the adhesin is located. Site-directed mutagenesis of the low-adhesive variant from *S. Enteritidis* also indicated the critical role of phenylalanine versus serine substitution at position 118 in the binding properties of the FimH adhesin.

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