BINDING SPECIFICITY OF SALMONELLA PLASMID-ENCODED FIMBRIAE ASSESSED BY GLYCOMICS

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The Salmonella enterica serotype Typhimurium genome encodes 12 intestinal colonization factors of the chaperone/usher fimbrial assembly class, however, the binding specificity is known for only one of these adhesins known as type 1 fimbriae. Here we explored the utility of glycomics to determine the carbohydrate binding specificity of plasmid-encoded fimbriae from S. Typhimurium. A cosmid carrying the pef operon was introduced into Escherichia coli and expression of fimbrial filaments composed of PefA confirmed by flow cytometry and immune-electron microscopy. Plasmid-encoded fimbriae were purified from the surface of E. coli and the resulting preparation was shown to contain PefA as the sole major protein component. Binding of purified plasmid-encoded fimbriae to a glycanarray suggested that this adhesin specifically binds the trisaccharide Galβ1-4(Fucα1-3)GlcNAc, also known as the Lewis X (Le^a) blood group antigen. Results from the glycanarray were validated by enzyme-linked immunosorbent assay (ELISA) in which plasmid-encoded fimbriae bound Le^a-coated wells in a concentration-dependent manner. Binding of plasmid-encoded fimbriae to Le^a-coated wells could be inhibited by co-incubation with soluble Le^a antigen. Our results establish glycomic analysis as a promising new approach for determining the carbohydrate binding specificity of bacterial adhesins.

The S. enterica serotype Typhimurium (S. Typhimurium) genome contains 12 operons encoding type II secretion systems of the chaperone/usher assembly class (1). These include eight members of the γ-Fimbriae (fim, bcf, sti, sth, lpf, saf, stc and stb), one member of the β-Fimbriae (stj), and two members of the π-Fimbriae (std and stf) and one member of the κ-Fimbriae (pef) (2). However, the binding specificity is known only for type 1 fimbriae, which are encoded by the S. Typhimurium fim operon (3,4). Type 1 fimbriae mediate mannose-sensitive agglutination of erythrocytes or yeast cells (5) because the FimH fimbrial tip adhesin binds terminal a-D-mannose residues present in host glycoproteins (6-10). The paucity of data on the binding specificities of chaperone/usher systems is partly due to the fact that, with the exception of type 1 fimbriae, these surface structures are poorly expressed during growth of S. Typhimurium under standard laboratory conditions (11,12).

The pef operon is located on the virulence plasmid of S. Typhimurium (13) but plasmid-encoded fimbriae cannot be detected after growth under standard laboratory growth conditions (11,12), because expression is controlled negatively by the histone-like protein (H-NS), the stationary-phase sigma factor (RpoS) and the presence of type-1 fimbrial biosynthesis genes (fimACDHF) (2,14). Although production of the major fimbrial subunit PefA is not detected under laboratory growth conditions, seroconversion of mice infected with S. Typhimurium (12) and detection of PefA on bacteria recovered from bovine ligated ileal loops (11) provide evidence for in vivo expression of the pef operon. Introduction of the cloned pef operon into Escherichia coli results in expression of thin (2-5 nm in diameter) flexible fibrillae composed of the major fimbrial subunit PefA (13,15). The ability to express plasmid-encoded fimbriae under laboratory growth conditions makes them well-suited for experiments aimed at characterizing their binding specificity. The goal of this study was to explore the utility of using glycan arrays...
for determining the binding properties of plasmid-encoded fimbriae.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. E. coli strains TOP10 and BL21(DE3) were obtained from Stratagene and Invitrogen, respectively. E. coli strain ORN172 carries a ∆fimBEACDFGH::Km allele and is phenotypically non-fimbriate (16). Bacteria were routinely grown at 37°C in Luria Bertani (LB) broth (with shaking) or on LB agar plates. For expression of fimbriae, bacteria were grown statically in LB broth at 37°C over night.

Generation of anti-PefD serum. A DNA region encoding an internal portion of PefD was amplified with primers 5’-CGGGATCCGGCACGCGTGTAGTCCTGGG AAGG-3’ and 5’-GGAATTCTCAGCCTAGGTGATCCGTGGG TG-3’ and cloned into plasmid vector pGEX4T-2 (17)(Amersham Pharmacia) to generate pCWD41, which encodes a PefD-GST fusion protein. The PefD-GST fusion protein was purified from E. coli strain BL21(DE3) using affinity chromatography as described previously (11). Anti-PefD serum was raised in a female New Zealand White rabbit as previously described (11). Serum was pre-absorbed with E. coli BL21 pGEX4T-2 expressing GST) using a protocol described previously (18).

Flow cytometry. Approximately 5x10⁸ cells were incubated with an equal volume of 4% paraformaldehyde (EM Science) at room temperature for 20 minutes. Cells were washed twice with 0.5ml 0.02% gelatin in PBS (PBS-gel). To block non-specific binding, cells were harvested and resuspended in 0.5ml filter sterilized 2% Normal Goat Serum (NGS, SIGMA) and incubated at room temperature for 30 minutes on a tabletop rotator. Polyclonal rabbit anti-PefA serum (11) was added to the cells at a final dilution of 1:250 for detection of PefA and cells were incubated at room temperature for 60 minutes on a tabletop rotator. After washing cells three times in PBS-gel, bacteria were resuspended in 0.5ml of a solution of 0.04mM propidium iodide in 2%NGS with secondary antibody (fluorescein isothiocyanate (FITC) conjugated Goat-anti-rabbit IgG, Jackson ImmunoLabs) added at a dilution of 1:250. The mixture was rotated at room temperature for 1 hour in the dark. Samples were washed 3 times with PBS-gel and bacteria were resuspended in PBS to a final concentration of approximately 5x10⁶ cells/ml. For each sample the fluorescence of 10,000 particles (bacterial cells) was measured by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).

Purification of fimbriae. A non-fimbriate E. coli strain (ORN172) carrying the cloned pef operon (pFB11) (15) or the cloned fim operon (pISF101) (19) was grown in 2 liters of LB broth, harvested by centrifugation, and resuspended in 10ml of 0.5mM Tris 75mM NaCl. Plasmid-encoded fimbriae were separated from the cells by mechanical shearing in a blender for three 1-min periods, after which cells and cellular debris were removed by centrifugation (3,500 rpm 30 min, 4°C). The supernatant was collected and passed through a 0.45mm filter (Millipore), and (NH₄)₂SO₄ (60% final concentration) was added to precipitate the fimbriae. Precipitated fimbriae were recovered by centrifugation (14,000 rpm 30 min, 4°C). The pellet was resuspended in 50ml of sterile water and was analyzed by SDS-PAGE, Western blot and electron microscopy.

Western blot analysis. Polyclonal rabbit anti-PefA serum has been previously described (11). The serum was diluted 1:5 in phosphate-buffered saline (PBS) pH 7.4 containing 0.2% sodium azide and pre-absorbed 8 times (18) with ORN172 carrying plasmid pGEX-4T-2 (17) and 4 times with ADH19 (SR11 ∆pefBACDFl::Km). Purified fimbriae were disassembled into individual subunits by boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer for 10 minutes. Bacterial cultures were resuspended by OD₆₀₀ to a concentration of approximately 2x10⁸ colony forming units (cfu)/10µl in SDS-PAGE loading buffer and boiled for 5 minutes to generate cell lysates. Cell lysates were then separated by 15% SDS-PAGE, transferred to Immobilon-P (Millipore) membranes using a Trans-Blot semidry transfer cell (Bio-Rad), incubated with rabbit anti-PefA serum diluted 1:500 final, detected with goat anti-rabbit alkaline phosphatase conjugate and the Immun-Star chemiluminescent substrate (Bio-Rad), and visualized with X-Omat Blue Film (Kodak).
N-Terminal Sequencing. Proteins separated by SDS-PAGE were transferred to a PVDF membrane (Millipore) in 10mM CAPS, 10% methanol pH 11.0 using a Trans-Blot SD semi-dry electrophoretic transfer cell according to standard protocols. Following electrophoretic transfer, the PVDF membrane was washed two times in sterile water. Transferred protein was visualized by staining with Amido Black solution (Sigma Aldrich) for two minutes followed by destaining with 1% acetic acid. N-terminal sequencing of the Pef major subunit was performed by the Protein Chemistry Lab at Texas A&M University using automated Edman chemistry in a Hewlett Packard G1000A Automated Protein Sequencer.

Electron microscopy. For immune electron microscopy, bacteria were grown in a static culture, washed twice in PBS and resuspended in EM grade water (EM Science) at a titer of approximately 1x10^9 CFU/ml. The bacterial culture (10 µl) was allowed to adhere to a formvar/carbon coated grid (EM Science) for 2 minutes and grids were incubated for 20 min in a primary antiserum (rabbit) diluted 1:250 in PBS containing 1% bovine serum albumen (BSA). Grids were washed 5 times for 1 minute in PBS containing 1% BSA. Grids were incubated for 20 min in goat anti-rabbit 10nm gold conjugate (EM Science). Grids were washed 3 times for 1 minute in PBS containing 1% BSA and 3 times for 1 minute in EM grade water (EM Science) before they were analyzed by electron microscopy.

Purified plasmid-encoded fimbriae (10 µl) were visualized by transmission electron microscopy by allowing attachment to formvar coated copper TEM grids for 1 minute. The purified fimbriae were negatively stained for 1 minute using 1% aqueous ammonium molybdate. The grids were allowed to dry before they were analyzed by electron microscopy.

Glycan Array Analysis. Glycan array analysis was performed by the Consortium for Functional Glycomics (CFG) (http://www.functionalglycomics.org/static/index.shtml). Streptavidin Coated High Binding Capacity Black 384-Well plates (Pierce #15513) were coated at saturating density (60pmoles/well) with 99 different biotinylated carbohydrate ligands in triplicate (Glycan-array version 1.2) and washed 3x (Embla 96/384 Well Washer from Molecular Devices Corporation) with 0.1ml wash buffer (PBS + 0.05% Tween 20). Purified plasmid-encoded fimbriae (0.05ml of a solution containing 0.03g/ml) were added and incubated for 1 hour at room temperature. After 3 washes (washing buffer), plates were incubated with rabbit anti-PefA serum (1:23.5) for 1 hour at room temperature. After 3 washes, plates were incubated with goat anti-rabbit IgG-Alexa488 conjugate (0.025ml/well of a solution containing 0.05mg/ml) and incubated for 1 hour at room temperature. After three washes, wells were loaded with 0.05ml PBS and fluorescence measured at 485/535nm using Core H plate reader (Wallac Victor^2 1420 Multi-label Counter from Perkin Elmer Corporation). Background signal was determined in wells treated with primary (rabbit anti-PefA) and secondary antibody (goat anti-rabbit IgG-Alexa488), but not with plasmid-encoded fimbriae (control wells). The average reading from three wells coated with one biotinylated carbohydrate ligand was divided by the average reading from three control wells. Binding that was increased at least two-fold above background levels was considered significant.

Enzyme-linked immunosorbent assay (ELISA). EvenCoat™ Streptavidin Microplates (R&D Systems) were coated with Lewis X (Le^4)-PAA-biotin, D-Mannose-PAA-biotin or HOCH_2(HOCH)_3CH_2NH-PAA-biotin (GlycoTech). Streptavidin coated high binding capacity 96-well plates were coated for 30 min at room temperature with 100 µl per well of 2 µg/l biotin-conjugated sugar diluted in PBS. Serial dilutions of a plasmid-encoded fimbrial preparation were added and incubated for 30 minute at room temperature. After washing with PBS plus 0.05%(w/v) Tween 20 (PBS/T), the plate was incubated for 20 min at room temperature with PBS/T containing 0.25% (w/v) bovine serum albumin (BSA). Then, the plate was incubated one hour at room temperature with anti-PefA serum (1:500). After washing, the plate was incubated another hour at room temperature with goat anti-rabbit alkaline phosphatase conjugate (1:1000). The reaction was developed with Sigma 4-nitrophenyl phosphate disodium salt hexahydrate. The resulting color reaction was read at 415 nm
with an ELISA microplate reader (Bio Rad Model 680).

Competition assays for determining binding specificity were performed by coating Streptavidin Microplates (R&D Systems) with 2 µg/l Le3-PAA-biotin (GlycoTech) in a 100 µl volume as described above. Wells were incubated with 2 µg/l of a preparation of plasmid-encoded fimbriae in the presence of increasing concentrations of Le3-PAA or D-Mannose-PAA (GlycoTech) in a 100 µl volume for 30 minute at room temperature. Binding of plasmid-encoded fimbriae to wells was performed with anti-PefA serum and goat anti-rabbit alkaline phosphatase conjugate as described above.

RESULTS

Expression and purification of plasmid-encoded fimbriae – An E. coli Δfim mutant (ORN172) carrying a cosmid (pFB11) containing the S. Typhimurium pef operon was used to express plasmid-encoded fimbriae (15). Expression of plasmid-encoded fimbriae by ORN172(pFB11) was confirmed using flow cytometry. Bacteria were labeled with propidium iodide for detection of DNA and with rabbit anti-PefA serum and goat anti-rabbit IgG FITC conjugate for detection of plasmid-encoded fimbriae. After gating for bacteria (i.e. for propidium iodide positive counts) (Figure 1A), the gate for detection of PefA was set using E. coli strain ORN172 as a negative control. Cells of E. coli strain ORN172(pFB11) were considered positive for expressing plasmid-encoded fimbriae when their FITC fluorescence intensity exceeded that of all but a small fraction (less than 1%) of the control population of the non-fimbriated parent (ORN172). Using this gate, expression of plasmid-encoded fimbriae was detected on the surface of 29% of cells in the ORN172(pFB11) culture (Figure 1B). Expression of plasmid-encoded fimbriae is regulated by phase variation (14), which provides a plausible explanation as to why expression of PefA was only detected in a fraction of cells in the population. A fraction of cells in cultures of ORN172(pFB11) carried thin flexible fibrillae on their surface (Figure 1C), which were not detected in cultures of the parent strain ORN172 (data not shown). The thin flexible fibrillae expressed by ORN172(pFB11) could be labeled with rabbit anti-PefA serum and goat anti-rabbit 10nm gold conjugate (Figure 1D), but no labeling was observed with the ORN172 negative control (Figure 1E). Furthermore, no labeling was detected in a control experiment detecting surface expression of PefD, a periplasmic chaperone involved in fimbrial assembly, in strain ORN172(pFB11) using rabbit anti-PefD serum and goat anti-rabbit 10nm gold conjugate (Figure 1F). Collectively, these data suggested that the thin flexible fibrillae detected on the surface of strain ORN172(pFB11) were plasmid encoded fimbriae of S. Typhimurium.

Fimbriae were removed from the surface of strain ORN172(pFB11) by mechanical shearing and concentrated by ammonium sulfate precipitation. Inspection of the fimbrial preparation by electron microscopy revealed the presence of fimbrial filaments (Figure 2A). The presence of the PefA fimbrial subunit in the fimbrial preparation was demonstrated by Western blot with anti-PefA serum (Figure 2B). Analysis of proteins present in the fimbrial preparation by SDS-PAGE revealed the presence of two major bands with apparent molecular masses of 17kDa and 10kDa (Figure 2C). N-terminal sequencing of the 17kDa band yielded the sequence ANEVTF, which corresponded to residues 22-27 in the PefA primary structure (Figure 2D). Analysis of the PefA primary structure using the SignalP software (20) predicted the presence of a 21 amino acid signal peptide, whose cleavage would give rise to a mature protein with a molecular mass of 15.3kDa. N-terminal sequencing confirmed cleavage of the predicted signal peptide and identified the 17kDa band as the mature form of PefA. The 10kDa protein band detected in the fimbrial preparation (Figure 2C) contained the N-terminal sequence PNAQGC, which corresponded to residues 75-80 in the PefA primary structure (Figure 2D). A truncated fragment of PefA containing amino acids 75-172 has a predicted molecular mass of 9.8kDa, which corresponded well to the apparent molecular mass of 10kDa detected by SDS-PAGE. Thus, N-terminal sequencing provided evidence that the 10kDa protein band represented a truncated form of PefA. Interestingly, the 10kDa band was not detected by Western blot analysis with anti-PefA serum (Figure 2B). Collectively, our data suggested that
preparations of plasmid-encoded fimbriae were composed predominantly of a single protein, PefA. These results identified PefA as the major fimbrial subunit of plasmid-encoded fimbriae. Like other members of the κ-Fimbriae, the pef operon does not encode a tip adhesin (2).

**Screening for carbohydrate ligands of plasmid-encoded fimbriae using glycanarrays** – The preparation of plasmid-encoded fimbriae was allowed to bind to streptavidin coated 384-well plates, in which triplicate wells had each been coated with one of 99 different biotinylated carbohydrate ligands. Carbohydrates present on the glycanarray are shown in Table 1. Binding of plasmid-encoded fimbriae to wells was detected using rabbit anti-PefA serum and goat anti-rabbit IgG-Alexa488 conjugate. Background signal was determined in wells treated with primary (rabbit anti-PefA) and secondary antibody (goat anti-rabbit IgG-Alexa488), but not with plasmid-encoded fimbriae (control wells). For each biotinylated carbohydrate ligand, the average signal from three wells was determined and divided by the average background noise detected in three control wells (average S/N ratio). By analogy to procedures commonly used during analysis of DNA microarrays, we used a two-fold increase in the average S/N ratio as an arbitrary cutoff for scoring binding to a biotinylated carbohydrate ligand as positive.

Glycanarray analysis revealed that binding of plasmid-encoded fimbriae to wells scored positive for only a single biotinylated carbohydrate ligand, Galβ1-4(Fucα1-3)GlcNAc-O(CH$_2$)$_3$NH(O)(CH$_2$)$_3$NHC(O)(CH$_2$)$_3$NHC(O)$_2$-Biotin (average S/N ratio=2.41) (Figure 3). These data suggested binding to the Galβ1-4(Fucα1-3)GlcNAc moiety, also known as the Lewis X (Le$^a$) blood group antigen, because various carbohydrates present on the glycanarray were conjugated to biotin via the same linker as Le$^a$ (-O(CH$_2$)$_3$NH(O)(CH$_2$)$_3$NHC(O)(CH$_2$)$_3$NHC(O)$_2$-). Although binding of plasmid-encoded fimbriae to the remaining sugars scored below the cutoff value, it should be mentioned that the biotinylated carbohydrate ligand producing the greatest average S/N ratio was tri-Lewis X-biotin (average S/N ratio=1.65) (Figure 3). Results from the glycanarray experiments thus suggested that Le$^a$ is a carbohydrate ligand specifically bound by plasmid-encoded fimbriae.

**Confirmation of glycanarray results** – We next wanted to confirm data from our glycomics screen by performing more detailed binding studies for those interactions that scored positive in the glycanarray. Increasing concentrations of plasmid-encoded fimbriae were added to 96-well plates coated with Le$^a$-polyacrylamide (PAA)-biotin and binding was detected with anti-PefA serum and goat anti-rabbit alkaline phosphatase conjugate (Figure 4A). Binding of plasmid-encoded fimbriae to Le$^a$-PAA-biotin-coated wells was concentration dependent and saturation was reached with a fimbrial preparation containing 2 µg of protein per liter. Half maximum binding was observed at concentrations between 0.1 and 0.01 µg/l of plasmid-encoded fimbriae. No binding of plasmid-encoded fimbriae to wells coated with α-D-Mannose-PAA-biotin or HOCH$_2$(HOCH)$_3$C$_2$NH-PAA-biotin was observed. These data further supported the idea that plasmid-encoded fimbriae specifically bind the carbohydrate ligand Le$^a$, thus conforming results from the glycanarray analysis.

To determine the specificity of Le$^a$-binding by plasmid-encoded fimbriae, we tested the ability of Le$^a$-PAA to inhibit binding of plasmid-encoded fimbriae to Le$^a$-PAA-biotin coated wells in a solid phase binding assay (Figure 4B). Plasmid-encoded fimbriae (2µg/l) were incubated in wells coated with Le$^a$-PAA-biotin in the presence of increasing concentrations of Le$^a$-PAA. Soluble Le$^a$-PAA blocked binding of plasmid-encoded fimbriae to Le$^a$-PAA-biotin coated wells in a concentration dependent fashion. Inhibition was observed at Le$^a$-PAA concentrations of >2µg/l (which corresponds to concentrations of Le$^a$-polyacrylamid repeat units of >1.8nM). In contrast, no inhibition of binding was observed when plasmid-encoded fimbriae (2µg/l) were incubated in wells coated with Le$^a$-PAA-biotin in the presence of increasing concentrations of α-D-mannose-PAA. Collectively, solid based binding studies supported the idea that plasmid-encoded fimbriae specifically bind the Le$^a$ blood group antigen, thereby validating results from our glycanarray analysis. These data demonstrate that glycanarrays can be an effective tool in determining the carbohydrate binding specificity of intact bacterial fimbrial filaments.
DISCUSSION

The human blood group antigens are defined by the presence of glycosphingolipids on the surface of erythrocytes, which carry characteristic terminal ends in their saccharide chains. In addition to their presence on erythrocytes, blood group antigens are abundant in epithelia of skin and mucosal surfaces (histo-blood group antigens), where they are present at the terminal ends of saccharide chains of surface localized glycoproteins or glycosphingolipids (21). Thus the heterogeneity of blood group antigens within the human population reflects the heterogeneity of glycans that cover the surface of the intestinal tract. Pathogenic microbes colonizing the intestinal tract can bind to blood group antigens (22) and it has been speculated that their role as ligands for microbial adhesins represents one of the driving forces for evolving the structural heterogeneity found in the glycans that cover the surface of the digestive tract. In other words, the human blood group polymorphism may have developed in part through the selective pressures exerted by pathogenic microorganisms (23). At the same time, the heterogeneity of glycans found in a particular host species may have selected for the presence of a diverse array of different adhesins in microbes colonizing its intestinal tract. For example, the S. Typhimurium genome contains 12 operons encoding chaperone/usher-type fimbriae (11,24) and it is tempting to speculate that the presence of this large repertoire of adhesins may in part be explained by the necessity for the pathogen to deal with the heterogeneity of carbohydrate receptors encountered either during its transmission between different individuals within a host species and/or during its transmission between different host species.

Above considerations suggest that some of the 12 chaperone/usher-type fimbrial operons present in the S. Typhimurium genome may encode adhesins that bind human blood group antigens. Our finding that plasmid-encoded fimbriae bind the Le^a blood group antigen represents the first evidence supporting this concept for the human pathogen S. Typhimurium. The Le^a antigen is defined by the presence of a terminal Galβ1-4(Fucα1-3)GlcNAc-R moiety on saccharide chains of glycoproteins or glycosphingolipids. In the human intestine, the Le^a histo-blood group antigen is expressed mainly by crypt epithelial cells (21). Studies in bovine ligated ileal loops show that S. Typhimurium initiates infection by invading epithelial cells at the tips of absorptive villi and in the follicle associated epithelium of Peyer’s patches (25,26). However, these surfaces rapidly become unavailable because the ensuing inflammatory reaction leads to necrosis of the uppermost mucosa, while the crypt epithelium remains intact (27). Abundant crypt abscesses are commonly found in S. Typhimurium patients (28,29), thus raising the possibility that the pathogen may bind to human crypt epithelium at later stages of infection. Our finding that S. Typhimurium possesses an adhesin that binds a crypt-specific histo-blood group antigen may thus be relevant to the pathogenesis of human infections.

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FOOTNOTES

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Abbreviations: BSA, bovine serum albumin; cfu, colony forming units; FITC, fluorescein isothiocyanate; IPTG, isopropyl-β-D-thiogalactopyranoside; Le³, Galβ1-4(Fucα1-3)GlcNAc-R; PAA, poly-acrylamide; SDS- PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Figure 1: Expression of *S. Typhimurium* plasmid-encoded fimbriae by *E. coli* strain ORN172(pFB11). (A-B) Expression of plasmid-encoded fimbriae by strain ORN172(pFB11) was detected by flow cytometry, using the DNA stain propidium iodide for gating bacteria (A), followed by detection of PefA expression using rabbit anti-PefA serum and goat anti-rabbit IgG FITC conjugate (B). A non-fimbriate *E. coli* strain (ORN172) is shown as a negative control. (C-F) Electron microscopic examination of *E. coli* strain ORN172(pFB11) expressing plasmid-encoded fimbriae. Fimbriae were visualized in ORN172(pFB11) by negative staining (C) or by immunoelectron microscopy with anti-PefA serum (D) or anti-PefD serum (F). A negative control (ORN172) for immunoelectron microscopy with anti-PefA serum is shown in (E). Binding of antiserum was detected using goat anti-rabbit 10nm gold.

Figure 2: Characterization of preparations of plasmid-encoded fimbriae. (A) Fimbrial filaments visualized by electron microscopy. (B) Western blot of purified plasmid-encoded fimbriae using anti-PefA serum (right lane). Molecular masses of standard proteins are indicated on the left. (C) Analysis of purified plasmid encoded fimbriae (right lane) by SDS-PAGE and coomassie staining. The left lane contains standard proteins (10, 15, 20, 25, 37, 50, 75, 100, 150 and 200kDa). (D) Position of amino acid sequences (bold font) determined by N-terminal sequencing of the indicated protein bands (arrows) in the PefA primary structure are shown.

Figure 3: Binding of purified plasmid-encoded fimbriae to glycanarrays containing wells coated with different biotinylated carbohydrates (x-axes). A dashed line indicates the two-fold increase in the average S/N ratio, which was used as an arbitrary cutoff for scoring binding to biotinylated carbohydrate. The identities of carbohydrates producing the greatest average S/N ratio are indicated.

Figure 4: Binding of purified plasmid-encoded fimbriae to Leα. (A) ELISA plates were coated with equal amounts of biotinylated Leα (Leα-PAA-biotin, closed circles) or control carbohydrates (Mannose-PAA-biotin, open circles, or HOCH2(HOCH)2CH2NH-PAA-biotin, open squares). Binding of increasing amounts of purified plasmid-encoded fimbriae to wells was detected with rabbit anti-PefA serum and goat anti-rabbit alkaline phosphatase conjugate. Data are shown as averages ± standard error from three independent experiments. (B) Binding of purified plasmid-encoded fimbriae (100 µl/well of a preparation containing 2 µg protein/l) to Leα-PAA-biotin-coated wells in the presence of increasing concentrations of the inhibitors Leα-PAA or D-mannose-PAA. Data are shown as averages ± standard error from three independent experiments.

Table 1: Carbohydrates present on the glycanarray.

| No.* | Glycanb | Trivial Name                          |
|------|---------|--------------------------------------|
| 1    | -O(CH2)2NH-CO(CH2)2NH-Biotin. | aminogluitol                          |
| 2    | α-β-Glc#SP2.BT                  | α-D-glucose                           |
| 3    | β-β-Glc#SP2.BT                  | β-D-glucose                           |
| 4    | α-β-Gal#SP2.BT                  | α-D-galactose                         |
| 5    | β-β-Gal#SP2.BT                  | β-D-galactose                         |
| 6    | 3-O-Su-Galβ#SP2.BT              | β-D-galactose-3-sulfate               |
| 7    | β-GlcNAc#SP2.BT                 | β-N-acetyl-D-glucosamine             |
| 8    | α-β-GalNAc#SP2.BT               | α-N-acetyl-D-galactosamine (T1)      |
| 9    | β-GalNAc#SP2.BT                 | β-N-acetyl-D-galactosamine           |
| 10   | α-D-Man#SP2.BT                  | α-D-mannose                           |
| Position of saccharide on glycanarray |
|--------------------------------------|
| Abbreviations: SP1.BT, 'O(CH$_2$)$_2$NH(O)(CH$_2$)$_2$NHC(O)(CH$_2$)$_2$NHC(O)-biotin; SP2.BT, 'O(CH$_2$)$_3$NH-CO(CH$_2$)$_3$NH-biotin; SP4.BT, 'O(CH$_2$)$_3$NH-CO(CH$_2$)$_3$NH-CO(CH$_2$)$_3$NH-biotin. |
Figure 1

A

[Histograms showing Propidium iodide fluorescence intensity for ORN172 and ORN172(pFB11).]

B

[Scatter plots showing Side scatter for PefA (FITC fluorescence intensity) for ORN172 and ORN172(pFB11).]

C

[Images showing Negative staining and ORN172(pFB11), ORN172, and α-PefD immunogold.]
Figure 2

Electron microscopy

Western blot

SDS-PAGE

PefA primary structure:

MKKSIIASIIALGVLLGTAHA **ANEVTF** LGSVSATTCDL TTSVNGAAQPNQVVLGTVQANQPGNFVDFAMKPVD

**PNAQGC** ANLAAKTATVSWASAALDGEFGATSGTA TDAKVLVESVNSKNPAGVANANASTVFEGAKLTDDG LQFKAKLGATEGDFKSVASFAVAYK
Average S/N ratio

Position of carbohydrate on glycanarray

Le^X

Tri-Le^X

Figure 3
Plasmid-encoded fimbriae (g/l)

- Le-x-PAA-biotin
- Mannose-PAA-biotin
- HOCH₂(HOCH)₄CH₂NH-PAA-biotin

Concentration of inhibitor (g/l):  
- Le-x-PAA
- Mannose-PAA

Figure 4
Binding specificity of Salmonella plasmid-encoded fimbriae assessed by glycomics
Daniela Chessa, Caleb W. Dorsey, Maria G. Winter and Andreas J. Baumler

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