FimH alleles direct preferential binding of *Salmonella* to distinct mammalian cells or to avian cells

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This study aimed to determine whether allelic variants of the FimH adhesin from *Salmonella enterica* confer differential bacterial binding to different types of mammalian cells [murine bone marrow-derived dendritic cells (DCs) and HEp-2 cells] and chicken leukocytes. Although the type 1 fimbriated *S. enterica* serovar Typhimurium strains AJB3 (SR-11 derivative) and SL1344 both aggregated yeast cells, only the former bound efficiently to DCs and HEp-2 cells. Type 1 fimbriae-mediated binding to DCs having previously been shown to require the FimH adhesin and to be inhibited by mannose, FimH sequences from strains SL1344 and AJB3 were compared and found to differ by only one residue, asparagine 158 in SL1344 being replaced by a tyrosine in AJB3. The importance of residue 158 for FimH-mediated binding was further confirmed in recombinant *Escherichia coli* expressing *S. enterica* type 1 fimbriae with a variety of substitutions engineered at this position. Additional studies with the ‘non-adhesive’ FimH of a type 2 fimbriated *S. enterica* serovar Gallinarum showed that this FimH did not mediate bacterial binding to murine DCs or HEp-2 cells. However, the type 2 FimH significantly improved bacterial adhesion to chicken leukocytes, in comparison to the type 1 FimH of strain AJB3, attributing for the first time a function to the type 2 fimbriae of *S. enterica*. Consequently, our data show that allelic variation of the *S. enterica* FimH adhesin directs not only host-cell-specific recognition, but also distinctive binding to mammalian or avian receptors. It is most relevant that this allele-specific binding profile parallels the host specificity of the respective FimH-expressing pathogen.

**INTRODUCTION**

*Salmonella enterica* are Gram-negative facultative intracellular *Enterobacteriaceae* that cause a variety of diseases such as bacteraemia, enteric fever and enterocolitis in a broad range of hosts, including humans (Williams, 1980). The earliest step in *Salmonella* pathogenesis is its attachment to the intestinal mucosa, which is typically mediated by fimbriae that target particular cell types (Bäumler et al., 1996a, b, c). *S. enterica*, like most members of the family *Enterobacteriaceae*, express type 1 fimbriae, which are defined by their capacity to bind mannose residues on soluble molecules or on eukaryotic cells such as yeast cells or guinea pig erythrocytes (Duguid & Anderson, 1967). The type 1 fimbriae of *S. enterica* serovar Typhimurium direct bacterial adhesion and subsequent invasion of epithelial cell lines, including HEp-2 and HeLa cells (Bäumler et al., 1996b; Ernst et al., 1990; Tavendale et al., 1983) and murine dendritic cells (DCs) in vitro (Guo et al., 2007). In vivo, the type 1 fimbriae participate in bacterial virulence by their interaction with enterocytes and promotion of intestinal colonization (Althouse et al., 2003; Duguid et al., 1976; Ewen et al., 1997; van der Velden et al., 1998).

The avian-adapted strains of *S. enterica* serovar Gallinarum encode type 2 fimbriae. These fimbriae are closely related to the type 1 fimbriae of serovar Typhimurium at both the genetic and immunological level. The type 2 fimbriae are
considered to be non-adhesive type 1 fimbriae, since they exhibit neither mannose-sensitive haemagglutination (Crichton et al., 1989), nor any reported binding property. However, whether the type 2 fimbriae definitely do not bind to any mammalian or eukaryotic cells is not known. Replacing one amino acid of biovar Gallinarum FimH with threonine transformed the type 2 to type 1 fimbriae by enabling mannose-sensitive binding (Kisiela et al., 2005). S. enterica serovar Gallinarum that expressed serovar enterica Typhimurium type 1 fimbriae also exhibited increased invasiveness for mammalian cells (Wilson et al., 2000).

Type 1 fimbriae are encoded by the fim gene cluster and assembled by a chaperone-usher system of protein export and assembly. The fimbrial shaft is mainly composed of the major subunit FimA (Hultgren et al., 1991). Although E. coli, S. enterica and other enterobacterial genera have type 1 fimbriae that carry an adhesive mannose-sensitive subunit designated FimH, there is significant heterogeneity among the FimH proteins of different genera (Hancox et al., 1997; Thankavel et al., 1999). It has also been shown that amino acid replacements, resulting from single nucleotide polymorphisms (SNPs) throughout the fimH gene of E. coli or S. enterica, conferred different binding phenotypes (Boddicker et al., 2002; Kisiela et al., 2006; Weissman et al., 2003). Specific amino acid substitutions in the E. coli FimH have been related to variable binding to soluble or membrane-associated mannosylated proteins on epithelial cells (Schembri et al., 2000; Sokurenko et al., 1992, 1994, 1995, 1997) or macrophages (Harris et al., 2001), and even to non-mannosylated proteins (Pouttu et al., 1999; Sokurenko et al., 1992, 1998). FimH alleles of S. enterica serovar Typhimurium also determine the level of bacterial adherence to various substrates or cells. For example, single amino acid substitutions in FimH of the low-binding serovar Typhimurium SL1344 produced a significant improvement in HEp-2 cell adhesion, biofilm formation and host colonization (Boddicker et al., 2002). Comparable FimH sequence alterations probably explain the contradictory results in the literature for S. enterica binding to HEp-2 cells (Bäumler et al., 1996c; Boddicker et al., 2002; Ernst et al., 1990; Tavendale et al., 1983).

### Table 1. Strains of S. enterica serovar Typhimurium and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Parental strain and relevant properties</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
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<tr>
<td>AJB3</td>
<td>S. enterica serovar Typhimurium p4252 (Nal&lt;sup&gt;R&lt;/sup&gt;) derived from SR-11 (highly virulent in mice)</td>
<td>Bäumler et al. (1996a), Bäumler et al. (1996c), Schneider &amp; Zinder (1956)</td>
</tr>
<tr>
<td>LB5010</td>
<td>S. enterica serovar Typhimurium (LT-2 derivative)</td>
<td>Bullas &amp; Ryu (1983)</td>
</tr>
<tr>
<td>LBH4</td>
<td>S. enterica serovar Typhimurium LB5010 fimH:: Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Hancox et al. (1997)</td>
</tr>
<tr>
<td>AZb57</td>
<td>S. enterica serovar Gallinarum biovar Gallinarum</td>
<td>ATCC 700623</td>
</tr>
<tr>
<td>AAEC189</td>
<td>E. coli MM294 Δlac recA endA Δfim (r&lt;sup&gt;+&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Blomfeld et al. (1991)</td>
</tr>
<tr>
<td>AZb62</td>
<td>AAEC189(pISF101)</td>
<td>This study</td>
</tr>
<tr>
<td>AZb74</td>
<td>AAEC189(pAZ37)</td>
<td>This study</td>
</tr>
<tr>
<td>AZb84</td>
<td>AAEC189(pAZ37)(pAZ30)</td>
<td>This study</td>
</tr>
<tr>
<td>AZb85</td>
<td>AAEC189(pAZ37)(pAZ31)</td>
<td>This study</td>
</tr>
<tr>
<td>AZb86</td>
<td>AAEC189(pAZ37)(pAZ32)</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pMal-2c</td>
<td>Expression vector (P&lt;sub&gt;lac&lt;/sub&gt; lac&lt;sup&gt;β&lt;/sup&gt;) for MBP (malE) fusions, ColE1 ori, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pISF101</td>
<td>pACYC184 fimAICDHZYWU (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>GenBank accession L19338</td>
</tr>
<tr>
<td>pISF204</td>
<td>fimH in Ap&lt;sup&gt;+&lt;/sup&gt; plasmid</td>
<td>Hancox et al. (1997)</td>
</tr>
<tr>
<td>pISF211</td>
<td>pISF204 fimH</td>
<td>Hancox et al. (1997)</td>
</tr>
<tr>
<td>pAZ8</td>
<td>GPMut3.1 gene in pLG339 (Tet&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>Guo et al. (2007)</td>
</tr>
<tr>
<td>pAZ22</td>
<td>fimH of AZb29 in pMal-2C (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pAZ30</td>
<td>fimH of AJB3 in pMal-2C (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<td>pAZ21</td>
<td>fimH of SL1344 in pMal-2C (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<td>pAZ32</td>
<td>fimH of AZb57 in pMal-2C (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pAZ37</td>
<td>pISF101 fimH (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
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<td>pAZ30 (Y158I)</td>
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<td>p158-5</td>
<td>pAZ30 (Y158A)</td>
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Attachment of serovar Typhimurium to the intestinal mucosa, and especially to the Peyer’s patches, is critical for 
S. enterica infection (Giannella, 1979; Jones et al., 1994). 
DCs, which are widespread in the lamina propria of the gut 
and in Peyer’s patches, can also capture 
S. enterica that cross M cells or that are directly on the mucosal surface by 
dendrites that extend between the intestinal epithelial cells 
(Hopkins et al., 2000; Niedergang et al., 2000; Rescigno 
et al., 2001). These DCs, by processing and presenting 
S. enterica antigens, are active participants in the induction of 
the adaptive immune response (Kelsall & Rescigno, 2004; 
Wick, 2003). In a previous study, we determined that the 
interaction of type 1 fimbriae and FimH with DCs resulted in 
efficient binding and uptake of 
S. enterica and epithelial cells. We present evidence that specific 
acidoic residues of FimH determine differential 
host specificity for receptors on mammalian or avian cells.

METHODS

Bacterial strains and plasmid constructs. The strains and 
plasmids used in this study are detailed in Table 1. The 
S. enterica strains were derivatives of serovars Typhimurium and Gallinarum. 
The E. coli strains lacked the type 1 fimbrial genes of 
E. coli. Bacteria were routinely grown in LB broth at 37 °C for 48 h under static 
conditions to enrich for 
S. enterica type 1 fimbriated bacteria. All the 
transformants were prepared by electroporation and cultured in LB broth supplemented with the appropriate antibiotics, as described 
previously (Guo et al., 2007). Expression of the type 1 fimbriae and 
mannose-specific recognition by FimH were determined by 
slide agglutination with anti-type 1 fimbriae antibodies (seroagglutination) 
and by yeast cell (Saccharomyces cerevisiae) aggregation, respectively. 
Bacterial counts (c.f.u) were estimated by the determination of optical densities (OD600), using standard methods. The fimH mutant 
was constructed by deleting a Stul fragment of 33 bp that is internal 
to the fimH gene of pSF101, resulting in plasmid pAZ37. Full-length 
fimH genes were amplified by PCR using the Expand High Fidelity 
polymerases (Roche) with optimized conditions (Oligo software, MBL Inc.), various 
S. enterica genomes as templates, and primers matching the 
fimH sequence in pSF101 (accession no. L19338) with 
the incorporation of restriction sites. The forward and reverse primers were 
5’-GGATCCATATGAAATATATCTACGGCTATT-3’ 
(underlined: Ndel restriction site) and 5’-GGGATCCTTAATCATACTCATGCTAGATA-3’ 
(underlined: BamHI restriction site). The amplified fimH genes of 
S. enterica AJ3, SL1344 and AZb57 were cloned into pMal-2c, substituting 
malle for fimH, to obtain plasmids 
pAZ20, pAZ31 and pAZ32, respectively. The corresponding fimH 
genes were sequenced and new data were deposited in GenBank 
(accession numbers EU445777 for strain AJ3; fimH in pAZ30 and 
EU445778 for strain AZB57 fimH in pAZ32; strain SL1344 fimH in 
pAZ31 was the same as that determined by the Sanger Institute, http://www.sanger.ac.uk/Projects/Salmonella/). 
The codon of tyrosine 
residue 158 in AJ3 FimH from plasmid pAZ30 was mutated by 
random substitutions of the three corresponding nucleotides. 
For this, two primers containing random nucleotides at the desired sites 
(primers 5’-CGGAGGAGATCTCCAGTCAGGTCATATAAGGG-3’ 
and 5’-GATACGCCCTTATTATNNNATGTTAGCAGACC-3’; 
N corresponds to A, T, G or C) were used with the QuikChange site-
directed mutagenesis kit (Stratagene) according to the manufacturer’s 
instructions. Briefly, PCR was performed with the high-fidelity Pfu 
Turbo DNA polymerase and the amplified product was treated with 
DpnI to digest the methylated and hemimethylated parental DNA 
template before preparing a bacterial host. The complete fimH 
sequences of all the various constructs were checked with flanking 
primers (upper primer 5’-GAGCTTGGACAAATATCATCGG-3’ 
and lower primer 5’-CCGGCTCTTCGCTATTACG-3’) by DNA 
sequencing (Cell Center, University of Pennsylvania, Philadelphia, 
PA, or Shanghai Sangon Biological Engineering Technology 
& Services Co., Shanghai, China). All the sequence alignments were 
carried out with the MEGALIGN program (Lasergene, DNASTAR).

Preparation of type 1 fimbriae and specific antibodies. The 
S. enterica serovar Typhimurium type 1 fimbriae were isolated from 
recombinant E. coli strains AZh62 (fimH++) and AZh74 (fimH-) and 
purified as described elsewhere (Dodd & Eisenstein, 1982). The 
fimbriae were kept in distilled water at 4 °C. The purity of the 
fimbrial preparation was confirmed by SDS-PAGE, after disassembling 
the fimbriae by acidification (pH 2.0) and 100 °C treatment for 
2–5 min. The protein concentration was measured as described 
previously (Markwell et al., 1978). The type 1 fimbriae were used as 
immunogens to prepare specific antibodies in rabbits (Cocalico 
Biologicals). The antisera obtained (ELISA titres>10000) agglutinated 
type 1 fimbriated S. enterica or recombinant E. coli that express the 
S. enterica type 1 fimbriae, but not non-fimbriated bacteria. 
Consistent with other studies (Crichton et al., 1989), the antibodies 
also reacted with the type 2 fimbriae of S. enterica serovar Gallinarum.

Preparation of DCs, chicken leukocytes and HEP-2 cells. DCs 
were prepared essentially as described previously (Guo et al., 2007; 
Lutz et al., 1999). Briefly, 6- to 7-week-old BALB/c mice were purchased from 
Charles River Laboratory and housed temporarily in 
filter-top cages in an air-conditioned animal facility at the University of 
Pennsylvania School of Veterinary Medicine. Mice were sacrificed 
and the bone marrow from femurs was flushed out from the 
shafts and seeded into Petri dishes with complete RPMI 1640 media 
(Glutamax, Gibco) containing 20 ng ml-1 murine recombinant 
granulocyte/macrophage colony-stimulating factor (rGM-CSF; Pepro 
Tech), 10 % fetal calf serum (Gibco), 1 mM sodium pyruvate, 50 mM 
β-mercaptoethanol, 100 U penicillin-streptomycin and 100 mg 
gentamicin sulphate ml-1. The cells were incubated in a humidified 
incubator at 37 °C and 5 % CO2 for 10 days, with medium changes 
every 3 days. The non-adherent cells were harvested and the cells were 
tested by flow cytometry for the expression of various surface markers 
to ensure cell batch consistency, immature DCs being characterized 
by high-level expression of CD11c, middle-level expression of MHC II 
and low-level or no expression of CD80, CD86 and CD40 (Guo et al., 
2007). Peripheral blood leukocytes from chickens were isolated from 
whole blood. The birds were native female Cochin-China chickens 
(~2 kg) from Huazhong Agricultural University. Briefly, heparinized (15 U ml-1) 
blood was added to an equal volume of 0.83 % NH4Cl to 
deplete red cells for 5 min at room temperature. The leukocytes 
were harvested and washed with PBS several times by centrifugation 
(360 g, 10 min) until the pellet became white. The cells were 
resuspended in complete RPMI 1640 medium (Glutamax, Gibco). 
HEP-2 cells (China Center for Type Culture Collection) were cultured in 
 Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supple-
mented with 10 % fetal calf serum and 100 U penicillin-streptomycin. 
HEP-2 cells were collected by trypsinization and suspension in RPMI 1640 medium.

Bacterial binding assays. Bacteria were grown as described above, 
washed three times in cold PBS and co-incubated with the various 
cells. Bacterial binding to DCs was undertaken at 4 °C for 30 min at 
an m.o.i. of 50 with gentle end-to-end agitation. The unbound 
bacteria were removed from cells by extensive washing with PBS.
containing 2% fetal calf serum. Bacterial binding to DCs was examined by flow cytometry, essentially as described previously (Guo et al., 2007). Specifically, cells were preincubated with anti-mouse CD16/CD32 (FcIII/II receptor) Fc block (mAb 2.4G2; Pharmingen, BD Biosciences) and then labelled with R-phcoeytherin (PE)-mAb to CD11c. The S. enterica cells were labelled green either by carrying plasmid GFPmut3.1 (Guo et al., 2007) or by staining with anti-type 1 fimbriae antibodies followed by Alexa Fluor 488- or FITC-labelled goat anti-rabbit IgG (H+L) (Invitrogen, Molecular Probes). E. coli strains that carried different plasmids were probed with rabbit polyclonal antibody to E. coli (Accurate Chemical & Scientific Corp.) and the FITC-labelled goat anti-rabbit IgGs. Isotype controls were Alexa Fluor 488-labelled non-specific antibodies. For the binding-inhibition assays, incubation of the bacteria with DCs was done in the presence of 100 mM methyl α-D-mannoside. For fimbrial binding, diluted purified fimbiae were added to the DCs instead of bacteria and labelled with the anti-type 1 fimbiae antibodies, as described above. All the incubations with fluorescent-labelled antibodies were carried out for 30 min in the dark at 4°C. The washed DCs were suspended in 300–500 µl cold RPMI 1640 for two-colour flow cytometry analysis (FACScalibur cytometer with the CellQuest pro software, BD Biosciences). Bacterial binding to DCs was further evaluated by confocal laser scanning microscopy (Zeiss LSM 510), using CD11c-PE-labelled DCs and Alexa-Fluor-labelled E. coli, as described above. All the experiments were repeated independently at least three times. Bacterial binding to HEp-2 cells was studied by flow cytometry after adding the bacteria to the cells in RPMI 1640 at an m.o.i. of 50 and incubating for 20 min at 37°C under gentle agitation. After extensive washing of the cells, bound bacteria were stained with rabbit antiserum to S. enterica serovar Typhimurium O-antigens (Accurate Chemical & Scientific Corp.) and Alexa Fluor 488-labelled goat antibody to rabbit IgG (Pierce Biotechnology). Fluorescent-labelled cells were detected by flow cytometry as described above for the DCs. Bacterial binding to chicken leukocytes was evaluated in RPMI 1640 medium after adding the bacteria to the leukocytes at an m.o.i. of 10, in the presence or absence of 100 mM methyl α-D-mannoside, and incubating for 5–10 min at 4°C. The unbound bacteria were removed by five washes with PBS and the cells were suspended in 100 µl PBS, deposited on slides, air-dried, fixed with methanol and Giemsa-stained. The number of leukocytes associated with none, one, or more bacteria in 10 fields per slide (namely for approximately 120 cells on average for each sample) were recorded. Binding was evaluated both as the mean number of leukocyte-associated bacteria (number of bacteria per leukocyte divided by the total number of leukocytes counted) and as the percentage of bacteria-associated leukocytes (number of leukocytes with bound bacteria divided by the total number of leukocytes counted) × 100).

Statistical analysis. Groups of data were compared with the unpaired Student’s t-test or the chi-squared test (percentage binding). Statistical significant differences between groups were determined by probability (P) values of less than 0.05.

RESULTS

Differential binding to DCs by various S. enterica serovar Typhimurium strains

We have previously shown that the FimH adhesin of the type 1 fimbiae from S. enterica serovar Typhimurium AJB3 mediates efficient bacterial binding to murine DCs (Guo et al., 2007). Since others had demonstrated differential binding of diverse FimH alleles to epithelial cells, we wondered whether differential binding would also occur with DCs, a professional phagocytic cell with a potentially broader repertoire of receptors than epithelial cells. For this we compared DC association with type 1 fimbrated S. enterica serovar Typhimurium AJB3 and SL1344, the latter strain being unable to bind significantly to HEp-2 cells (Boddicker et al., 2002). The two strains were well fimbrated, as determined by seroagglutination, and aggregated yeast cells, a general property of type 1 fimbrated bacteria. S. enterica binding to murine DCs was analysed by flow cytometry using GFP-expressing bacteria and DCs defined as CD11c+-gated cells. Fig. 1 shows that most of the DCs (93%) were associated with S. enterica AJB3. In contrast, S. enterica SL1344 showed no relevant interaction with the DCs. This result indicated that not all type 1 fimbrated S. enterica interact with DCs, despite their capacity to bind to yeast cells, highlighting the existence of different types of FimH receptors.

Strain-specific FimH determines differential bacterial binding to DCs

Four lines of evidence obtained previously indicated that the FimH adhesin of S. enterica serotype Typhimurium determines bacterial binding to DCs (Guo et al., 2007): (i) only strain AJB3, but not its isogenic fim mutant, bound to...
DCs; (ii) *S. enterica* serovar Typhimurium strain LB1510 bound to DCs, whereas the isogenic *fimH* mutant bound to DCs only after complementation with *fimH*; (iii) AJB3 binding to DCs was mannose-dependent; (iv) recombinant *E. coli* expressing the AJB3 type 1 fimbriae bound to DCs only when the mannose-specific adhesin FimH was produced. Considering the observed differential binding of various strains of *S. enterica* to DCs in the current study, it was likely that FimH sequence variation was responsible for bacterial adhesion to DCs. To evaluate this possibility, *fimH* genes of *S. enterica* serovar Typhimurium AJB3 and SL1344 were sequenced and the predicted FimH sequences were aligned with available sequences from *S. enterica* serovar Typhimurium LT2 (GenBank accession no. NC_003197), LB5010 (on pISF101, GenBank accession no. L19338) and SL1344 (NCTC 13347, http://www.sanger.ac.uk/Projects/Salmonella/) (Table 2). The FimH sequence of SL1344 corresponded to the published one of strain LT2. Although strain LB5010 was reported in the literature to derive from strain LT2 (Bullas & Ryu, 1983), the deduced FimH sequence of strain LB5010 (GenBank, protein accession no. AAA75420) corresponded to our sequencing results, but differed from the FimH sequence of strain LT2 (GenBank, protein accession no. P37925). When compared to the FimH of SL1344, either one novel substitution at position 158 (tyrosine instead of an asparagine, N158Y) in FimH from AJB3 or two substitutions (G61A and F118S) in FimH from LB5010 (pISF101) correlated with DC-recognizing FimH. The DC-binding profiles of LB5010 and SL1344 corresponded to the previously described binding profiles of these two strains for HEP-2 cells (Boddicker et al., 2002). To confirm that the differential binding of these strains was due to their FimH sequence, we examined DC recognition by their FimH adhesins in recombinant *E. coli* using a complementation assay. Plasmid pAZ37, which directs the expression of all the *S. enterica* *fim* genes, with the exception of a functional *fimH* gene, was engineered. *E. coli* AZb74, which lacks type 1 fimbrial genes but expresses *S. enterica* type 1 fimbriae from pAZ37, as determined by seroagglutination, did not aggregate yeast cells or bind to DCs (Fig. 2, mock-infected DCs and AZb74). In contrast, when strain AZb74 was complemented with pAZ30, which carries the *fimH* gene of AJB3, the resulting recombinant *E. coli* adhered to DCs (Fig. 2, AZb84), confirming that FimH is the determinant protein for DC binding. The relatively low level of AZb84 association with DCs (31%) can be attributed to some binding inhibition mediated by the non-functional FimH from AZb74 (expressed by pAZ37), which competes with the functional FimH from AJB3 (expressed by pAZ30) for incorporation into the export and/or assembly apparatus of the type 1 fimbriae. In comparison, 58.1% of the DCs demonstrated bound AZb62 (not shown), which expresses only the adhesive FimH of *S. enterica* LB5010, as determined previously (Guo et al., 2007). In contrast to the *fimH* genes of AJB3 or

Table 2. Amino acid changes in FimH sequences of *S. enterica* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Position of deduced amino acids*</th>
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<tr>
<td></td>
<td>61</td>
</tr>
<tr>
<td>SL1344†</td>
<td>G</td>
</tr>
<tr>
<td>AJB3</td>
<td>G</td>
</tr>
<tr>
<td>AZb62 (pISF101)</td>
<td>A</td>
</tr>
<tr>
<td>AZb57</td>
<td>G</td>
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</table>

*The exported FimH molecules comprise 335 amino acids.
†This sequence corresponds that published for strain LT2 (GenBank accession no. P37925).

Fig. 2. Binding of isogenic recombinant *E. coli* to DCs. The *E. coli* expressed the type 1 fimbriae of *S. enterica* serovar Typhimurium with various *fimH* alleles. Flow cytometry was done of DCs alone (mock infection) or interacting with fimbriated *E. coli* AZb74 that were expressing the mutated and non-adhesive *Salmonella* FimH from pAZ37 alone or complemented with the pAZ30 *fimH* from *S. enterica* serovar Typhimurium AJB3 (AZb84), with the pAZ31 *fimH* from serovar Typhimurium SL1344 (AZb85), or with the pAZ32 *fimH* from serovar Gallinarum (AZb86). All the bacteria were co-incubated with DCs at 4 °C for 30 min at an m.o.i. of 50. DCs were gated as PE-labelled CD11c+ cells, and the bacteria were detected with FITC-labelled antibody to *E. coli*. The numbers in the upper right squares of each dot-plot represent the percentage of DCs associated with bacteria. The data are from one experiment and represent reproducible data from three experiments.
LB5010, the fimH genes from strain SL1344 (Fig. 2, AZb85) did not complement AZb74 for DC binding. That the FimH of strains LB5010 and AJB3, unlike the FimH of strain SL1344, directed efficient bacterial binding to DCs was confirmed by confocal microscopy (data not shown). To ensure that potential slight variations in bacterial fimbriation between the recombinant E. coli did not affect the interpretation of the results, the effect of FimH on fimbriae-mediated binding was evaluated with purified fimbriae. After incubation of the DCs with various concentrations of type 1 fimbriae from E. coli AZb62 and AZb74, only the fimbriae from AZb62 bound to the DCs efficiently, as determined by flow cytometry and fluorescence microscopy (data not shown), further confirming that bacterial association with DCs was FimH allele-dependent. Thus, our results indicated that different point mutations in the primary sequence of FimH can achieve comparable binding specificities.

**Effects of FimH residue 158 substitutions on HEp-2 and DC binding**

Since the type 1 fimbriae of S. enterica AJB3 have previously been shown to mediate bacterial binding to epithelial cells (Baumler et al., 1996c), we determined whether FimH residue 158, which determined AJB3 binding to DCs, was also involved in the recognition of epithelial cells. To test this hypothesis, we evaluated bacterial association with HEp-2 cells by flow cytometry. We first confirmed that, as shown previously (Boddicker et al., 2002), the FimH from strain LB5010 mediated efficient bacterial binding to HEp-2 cells, 97.6 ± 0.7 % of the HEp-2 cells being associated with bacteria, as determined by flow cytometry. Second, we used a complementation system to study a collection of engineered amino acid substitutions together, these results show that FimH alleles varying at position 158 have differential bacterial cell-binding specificities, potentially modulating the virulence properties of the FimH-expressing Salmonella.

**Table 3.** Fimbriation, yeast cell aggregation and adhesion to HEp-2 cells and DCs by S. enterica serovar Typhimurium expressing various FimH alleles

<table>
<thead>
<tr>
<th>Strain:</th>
<th>LB5010</th>
<th></th>
<th>LBH4 (fimH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid:</td>
<td></td>
<td>pAZ30</td>
<td></td>
</tr>
<tr>
<td>FimH from:</td>
<td></td>
<td>p158-1</td>
<td>p158-2</td>
</tr>
<tr>
<td>FimH residue 158</td>
<td>N</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Fimbriation*</td>
<td>+ + +</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>Yeast aggregation</td>
<td>+ + +</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>% Bacteria-associated HEp-2 cells</td>
<td>95.1 ± 4.9</td>
<td>12.9 ± 5.6</td>
<td>40.0 ± 1.4</td>
</tr>
<tr>
<td>% Bacteria-associated DCs</td>
<td>94.1 ± 3.5</td>
<td>17.6 ± 4.5</td>
<td>93.9 ± 1.3</td>
</tr>
<tr>
<td>% Bacteria associated-DCs in 100 mM methyl α-D-mannoside</td>
<td>14.5 ± 5.5</td>
<td>16.5 ± 5.8</td>
<td>27.6 ± 3.6</td>
</tr>
</tbody>
</table>

*Fimbrial expression was detected by seroagglutination.*

The substitutions of tyrosine 158 from AJB3 FimH with phenylalanine (Y158F), histidine (Y158H) or leucine (Y158L) were all capable of complementing strain LBH4 for fimbriation, yeast cell aggregation and adhesion to HEp-2 cells, but to various extents (Table 3). Interestingly, FimH substitutions Y158F and Y158H directed significantly better bacterial binding to the HEp-2 cells than the parental non-mutated FimH (P < 0.05). Although binding mediated by the FimH substitution Y158L appeared comparable to that of the non-mutated FimH proteins, this substitution affected fimbriation and yeast aggregation, suggesting that the mutation interfered with fimbrial biogenesis, negating a potential improvement in adhesive properties. The Y158I and Y158A substitutions in FimH abrogated fimbriation, and thus, not surprisingly, bacterial association with yeast and HEp-2 cells. The effects of FimH residue 158 substitutions on DC association paralleled those obtained with the HEp-2 cells (Table 3). All well-fimbriated bacteria bound efficiently to DCs, while the non-fimbriated ones were poorly adhesive (e.g. LBH4 versus LB5010; P < 0.001). Interestingly, the AJB3 FimH (pAZ30) mediated better adhesion to DCs than to HEp-2 cells, indicating a more differentiated cell specificity than the FimH of the mutated strains (p158-1 and p158-2). Binding of all the fimbriated bacteria was significantly inhibited by methyl α-D-mannoside (P < 0.001) (Table 3), indicating that the various adhesive FimH alleles were all involved in recognizing mannose residues on DCs. Taken together, these results show that FimH alleles varying at position 158 have differential bacterial cell-binding specificities, potentially modulating the virulence properties of the FimH-expressing Salmonella.

**FimH, and not the fimbrial shaft, determines DC binding**

The type 2 fimbriae of S. enterica serovar Gallinarum were originally differentiated from the type 1 fimbriae by their lack of binding properties for red blood cells. Later studies showed that these two types of fimbriae were genetically
The FimH of the avian-adapted S. enterica serovar Gallinarum recognizes chicken leukocytes

The type 1 FimH adhesins are expressed by S. enterica serovars that can be isolated from mammalian and avian hosts. In contrast, the type 2 FimH is expressed exclusively by the avian-adapted S. enterica serovar Gallinarum. Thus, we wondered whether the ‘non-adhesive’ type 2 FimH proteins are avian-specific adhesins. Since adhesive FimH mediate S. enterica binding to a variety of mammalian cells, including leukocytes (Ohman et al., 1985), the potential existence of avian receptors for the ‘non-adhesive’ FimH was evaluated with chicken peripheral blood leukocytes. We first determined the binding properties of S. enterica serovar Typhimurium AJB3 and avian Gallinarum AZB57 (biovar Gallinarum ATCC 700623). Both strains bound to chicken leukocytes (Fig. 4A), the latter in greater numbers than the former (P<0.05), although adhesion by the former strain was better inhibited by methyl α-D-mannoside [65 % (P<0.001) versus 34 % (P=0.005)], suggesting that the latter strain possesses a mannose-resistant adhesin that recognizes chicken leukocytes. Whether the binding property of S. enterica serovar Gallinarum was linked to the type 2 fimbrial adhesin FimH was determined with recombinant E. coli AZB74 expressing only the non-adhesive S. enterica type 1 fimbriae from pAZ37 (fimH mutant) or complemented with the fimH genes from S. enterica serovar Typhimurium AJB3 (pAZ30) or Gallinarum AZB57 (pAZ32). As expected, all three strains were well fimbriated (seroagglutination +++) and only E. coli expressing the AJB3 fimH aggregated yeast cells. Although the observed leukocyte binding of the recombinant strain expressing the AJB3 FimH was a novel finding (Fig. 4A), the most interesting result was obtained with the recombinant strain expressing the serovar Gallinarum FimH, which bound significantly better to chicken leukocytes than the control strain that did not express a functional FimH (P<0.001) (Fig. 4A). Whereas methyl α-D-mannoside inhibited AJB3 FimH-mediated bacterial binding, this inhibitor’s effect on...
serovar Gallinarum FimH-directed adhesion was less significant [82% (P<0.001) versus 31% (P<0.01) inhibition, 12% inhibition being considered non-specific, as determined with the non-adhesive fimbriated fimH mutant E. coli pAZ37]. Consistent inhibition profiles were obtained by comparing numbers of bacteria-associated leukocytes (65% versus 24% inhibition, with 14% non-specific inhibition) (Fig. 4B). To summarize, these new results indicated that the type 2 FimH molecule of S. enterica serovar Gallinarum is an adhesin that mediates bacterial binding to chicken leukocytes and that mannose is not an efficient inhibitor of this adhesion. Since the type 2 fimbriae have never been observed to bind to mannosylated molecules, it is suggested that the methyl \(\alpha\)-D-mannoside-mediated inhibition of the type 2 FimH was more likely due to its methyl than its mannose moiety, and that the type 2 FimH recognizes a chicken-specific non-mannosylated residue that remains to be determined.

**DISCUSSION**

DCs are one of the target cells for S. enterica (Hopkins et al., 2000; Niedergang et al., 2000; Rescigno et al., 2001). We previously showed that the S. enterica type 1 fimbriae of strain AJB3 mediated bacterial binding to murine DCs (Guo et al., 2007). The FimH adhesion of AJB3 was found to be essential for binding, and a mannose-inhibited interaction pointed to the involvement of one or more mannosylated receptor(s) on DCs. Here we observed that not all type 1 fimbriae from S. enterica serovar Typhimurium strains interacted significantly with DCs. Type 1 fimbriated S. enterica, E. coli engineered to express intact S. enterica type 1 fimbriae, or purified type 1 fimbriae themselves required an intact FimH to bind efficiently to the DCs. More importantly, the bacteria had to express certain specific FimH alleles to adhere to DCs. For example, unlike the FimH of strain AJB3, the FimH of strain SL1344 (which is identical to the FimH of strain LT2) mediated bacterial binding to yeast cells, but not to DCs. A single amino acid substitution in the SL1344 FimH, replacing asparagine 158 by tyrosine, was responsible for the observed gain of function of the AJB3 FimH. Binding to DCs was also detected with strain LB5010, which is characterized by two different amino acid changes in FimH (G61A, F118S). This finding was consistent with a previous investigation on S. enterica adhesion to the epithelial cell line HEp-2, which showed that each mutation additively improved binding (Boddicker et al., 2002). As determined in the current study, the FimH of strain AJB3 also conferred the adhesion of type 1 fimbriated bacteria to HEp-2 cells, which indicates that a variety of unique individual mutations in FimH (G61A and F118S for LB5010 or N158Y for AJB3) can achieve a convergent adhesive phenotype. The data also suggest the presence of similar types of mannosylated receptor moieties on murine DCs and HEp-2 cells, although this remains to be demonstrated. Having found that residue 158 of FimH

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**Fig. 4.** Type 2 FimH-mediated bacterial adhesion to chicken leukocytes. Bacteria and chicken leukocytes were incubated together for 5–10 min at 4 °C and stained with Giemsa after removing the non-adhesive bacteria by several washing steps. Mean numbers (± se) of leukocyte-associated bacteria (A) and bacteria-associated leukocytes (B) were counted microscopically for approximately 120 leukocytes per sample. The bacteria studied were either S. enterica serovar Typhimurium AJB3, S. enterica serovar Gallinarum AZb57, and E. coli AZb74 expressing only the non-adhesive S. enterica type 1 fimbriae from pAZ37 (fimH\(^{-}\)) or complemented with the fimH genes from S. enterica serovar Typhimurium AJB3 (pAZ30, fimHT) or Gallinarum AZb57 (pAZ32, fimHG). The black and grey columns represent the results obtained in the absence or presence, respectively, of 100 mM methyl \(\alpha\)-D-mannoside. The graphs are a representation of one of three separate, reproducible experiments.
was involved in type 1 fimbriae-mediated binding to host cells, tyrosine 158 in the FimH adhesin of strain AJB3 was selected for substitution mutagenesis. Some mutations improved bacterial adhesion to HEp-2 cells (Y158F, Y158H), whereas others affected fimbriation and maintained (Y158L) or abolished (Y158I, Y158A) binding. Based on the model of fimbrial biogenesis (Hultgren et al., 1991), it is likely that the three mutations that affected fimbriation interfered with the proper conformation of FimH for productive interactions with the periplasmic chaperone protein, the usher protein that channels fimbrial subunits through the outer membrane or the following subunit, resulting in the inhibition of fimbrial growth. In the absence of structural data on the Salmonella FimH protein, it remains to be determined whether residues 158, 61 or 118 participate directly or indirectly in the FimH–receptor interactions.

The type 2 fimbriae of avian-adapted S. enterica serovar Gallinarum strains have not been reported to bind to any host cells or to interact with any host receptor (Boddicker et al., 2002; Wilson et al., 2000). Despite the large repertoire of receptors expressed by murine DCs (van Vliet et al., 2008), by demonstrating the lack of binding to these cells, the current study further confirmed that type 2 fimbriated bacteria do not bind to mammalian cells. This binding deficiency could be reversed, however, by transforming bacteria with a plasmid that directed the expression of the FimH adhesin of serovar Typhimurium AJB3 or LB5010. This successful complementation indicated that the binding deficiency for DCs was uniquely FimH-specific. This result was consistent with a previous mutagenesis study which showed that the substitution of the isoleucine 78 of S. enterica serovar Gallinarum with a threonine altered the binding to high-mannose oligosaccharides and human colon carcinoma HT-29 cells (Kisiela et al., 2005).

As the non-mutated FimH of the avian-adapted S. enterica serovar Gallinarum was apparently unable to mediate bacterial binding to mammalian cells, we wondered whether this protein acted instead as an avian receptor-specific ligand. Although earlier studies had shown that type 2 fimbriated S. enterica were non-haemagglutinating with both mammalian and avian erythrocytes (Duguid et al., 1966), many fimbrial adhesins bind to mammalian host cell receptors that are absent on red blood cells. As S. enterica serovar Gallinarum has been shown to associate with chicken lymphocytes (Monno et al., 1986), we investigated more generally how effectively chicken leukocytes interact with bacteria that express the S. enterica serovar Gallinarum FimH protein. Interestingly, when this protein was expressed in combination with a S. enterica serovar Typhimurium fimbrial shaft, the bacteria bound efficiently to chicken leukocytes, but not to murine DCs. Conversely, when the murine DC-adhesive FimH of strain AJB3 (S. enterica serovar Typhimurium) was expressed with the same fimbrial shaft, binding to chicken leukocytes was significantly less efficient. It remains to be investigated which one(s) of the few amino acids that differ between the two serovars are involved in the FimH binding specificity for chicken leukocytes. Although further studies are needed to identify the avian receptor for the type 2 FimH, our results highlight a link between host adaptation and FimH binding profiles. Moreover, in order to cross host class barriers, allelic variation of the fimH gene has apparently evolved even further than merely adjusting to a variety of mannosylation patterns for mammalian cells.

In conclusion, we have characterized the binding properties of some S. enterica type 1 fimbriae to murine DCs and identified a new fimH allele that encodes an adhesin that binds well to both murine DCs and human epithelial HEp-2 cells. This allele was found in strain AJB3 derived from SR-11, a strain that is highly pathogenic to mice. A comparison of FimH sequences from AJB3 with SL1344 highlighted the importance of amino acid 158 for binding to DCs and HEp-2 cells, as confirmed by substitution mutagenesis. Finally, this study identified for the first time an adhesive property for the FimH protein from the type 2 fimbriae of S. enterica serovar Gallinarum.

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REFERENCES


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binding to mannoses and uroepithelial cells. *J Biol Chem* 272, 17880–17886.


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