
Dagmara Kisiela, Anna Sapeta, Maciej Kuczkowski, Tadeusz Stefaniak, Alina Wieliczko, and Maciej Ugorski

Department of Biochemistry, Pharmacology, and Toxicology, Faculty of Veterinary Medicine, Agricultural University of Wrocław, 50-375 Wrocław, Poland

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Recombinant FimH adhesins of type 1 fimbriae from *Salmonella enterica* serovar Gallinarum biovars Gallinarum and Pullorum, in contrast to those of *Salmonella enterica* serovar Typhimurium, did not bind to high-mannose oligosaccharides or to human colon carcinoma HT-29 cells. However, mutated FimH proteins from biovar Gallinarum and biovar Pullorum, in which the isoleucine at position 78 was replaced by the threonine found in *S. enterica* serovar Typhimurium, bound well to glycoproteins carrying high-mannose oligosaccharides and colon carcinoma cells. The loss of sugar-binding properties by biovar Gallinarum and biovar Pullorum FimH adhesins, which are a part of the type 1 fimbriae, is most probably the result of a single T78I mutation, as was proven by site-directed mutagenesis of FimH proteins.

*Salmonella* spp. are closely related bacteria that can cause disease in many animals, from reptiles to mammals and birds (15). Some of these pathogens are host specific, e.g., *Salmonella enterica* serovar Gallinarum biovar Gallinarum and *Salmonella enterica* serovar Gallinarum biovar Pullorum, which infect only poultry and aquatic birds (1). The biovar Gallinarum is responsible for fowl typhoid, and the biovar Pullorum causes pullorum disease in chickens.

It has been shown that, in contrast to most *Salmonella* serovars, *S. enterica* serovar Gallinarum biovar Gallinarum and *S. enterica* serovar Gallinarum biovar Pullorum do not express the hemagglutinating, mannose-sensitive type 1 fimbriae; however, they produce morphologically similar filamentous organelles which were named type 2 fimbriae (5, 13). The type 1 fimbriae of the genus *Salmonella* are composed primarily of FimA protein subunits (9, 12). However, for direct binding to oligomannosidic structures, another protein, called FimH adhesin and located at the distal end of the fimbrial shaft, is responsible (7, 10, 17). An electron microscope study, using antibodies directed against type 1 fimbriae, and Southern blotting with gene probes for type 1 fimbriae confirmed that type 2 fimbriae are, in fact, type 1 fimbriae (3). Recent cloning and sequencing of *fimH* genes from serovar Typhimurium and biovar Pullorum (6) strongly supported the view that the biovars Gallinarum and Pullorum are able to produce type 1 fimbriae which have lost their functional activity. However, neither the abilities of biovar Gallinarum and biovar Pullorum to produce FimH adhesins nor the consequences of the amino acid substitutions on the adhesive properties of these proteins were ever studied carefully; therefore, the present study was undertaken to address these problems.

The presence of FimH and FimA proteins was shown in type 1 fimbriae of biovar Gallinarum and biovar Pullorum purified by the method of Müller et al. (11). FimH and FimA proteins of biovar Gallinarum and biovar Pullorum type 1 fimbriae were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using the obtained anti-FimH rabbit and anti-FimA chicken polyclonal antibodies (Fig. 1). Because of the high homology in the amino acid sequences of FimH (GenBank accession numbers AAN64295, AAA75420, AAR83178, and AAR83177) and FimA (4, 14) among different *Salmonella* serovars/biovars, heterologous antisera raised against the respective proteins from *Salmonella enterica* serovar Typhimurium (FimH) and *Salmonella enterica* serovar Enteritidis (FimA) were used to identify these proteins from both avian-adopted biovars. Antiserum against FimH proteins was obtained by immunization of rabbits with *S. enterica* serovar Typhimurium recombinant FimH proteins purified on Ni-nitrilotriacetic acid (Ni-NTA) resin (see below), and the immunoglobulin G (IgG) fraction was then purified on protein A Sepharose (Amersham Pharmacia Biotech). Antiserum against FimA proteins was obtained by immunization of hens with recombinant FimA proteins purified on the same Ni-NTA resin (8). In addition, the presence of both fimbrial proteins on the surface of biovar Gallinarum and biovar Pullorum cells was shown by enzyme-linked immunosorbent assay (ELISA) with the same antibodies (data not shown). Bound anti-FimH and anti-FimA antibodies were detected by using alkaline phosphatase-conjugated goat anti-rab-
 amounts of the periplasm (17). However, it was possible to produce large amounts of the murium FimH protein was highly unstable in the bacterial biovars Gallinarum and Pullorum. Recombinant FimH proteins of serovar Typhimurium as well as recombinant protein (16). Using a similar approach, we obtained fimH genes from biovar Gallinarum, biovar Pullorum, and their chaperone proteins are very sensitive to periplasmic proteases, and recombinant Salmonella enterica serovar Typhimurium FimH protein was highly unstable in the bacterial periplasm (17). However, it was possible to produce large amounts of the E. coli FimH receptor binding domain as recombinant protein (16). Using a similar approach, we obtained recombinant FimH proteins of serovar Typhimurium as well as biovars Gallinarum and Pullorum. E. coli DH5α cells were transformed with the expression vector pTrcHis2b (Invitrogen) containing cloned fimH genes from biovar Gallinarum (G.fimH/pTrcHis2b), biovar Pullorum (P.fimH/pTrcHis2b), and S. enterica serovar Typhimurium (T.fimH/pTrcHis2b). The fimH genes from biovar Gallinarum, biovar Pullorum, and S. enterica serovar Typhimurium were cloned by amplification of the genomic DNA sequences with PCR by use of the primers 5′-fim (5′-CGCGGATCCTGAAGAAAATATCAGCTC-3′) and 3′-fim (5′-GCGTCTAGACATCTAATCGACTGCG-3′) based on the published sequence of S. enterica serovar Typhimurium (GenBank accession no. L19338). The PCR primers contained additional sequences corresponding to the BamHI restriction site in the primer 5′-fim and to XbaI in 3′-fim. The fimH genes were amplified as follows: 25 cycles of denaturation (94°C for 1 min), annealing (54°C for 1 min), and elongation (72°C for 1 min). The resulting PCR products were digested with the respective enzymes and ligated to the corresponding sites of pTrcHis2b plasmid. FimH proteins expressed in E. coli were purified on Ni-NTA affinity resin because of the presence of a six-His tag in the C terminus of their polypeptide chain. Coomassie brilliant blue staining of SDS-PAGE-separated proteins revealed the presence of a major band with an apparent molecular mass of about 36 kDa, corresponding to FimH monomer (Fig. 2A). By use of monoclonal antibody 9E10.2 (no. CRL-1792; American Type Culture Collection) directed against the c-myc epitope, which is a part of the recombinant protein, and secondary alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Dako), the identities of these proteins as FimH adhesins were confirmed (Fig. 2B).

The binding properties of FimH proteins from biovar Gallinarum and biovar Pullorum were analyzed by use of two glycoproteins with different oligomannosidic structures, RNase B and horseradish peroxidase (HRP), both from Sigma, and synthetic neoglycoprotein, containing derivatives of Saccharomyces cerevisiae mannan conjugated to bovine serum albumin (mannan-BSA), made according to the method of Boratyński and Roy (2). As expected, both recombinant adhesins did not bind, in contrast to S. enterica serovar Typhimurium FimH, to any of the glycoproteins carrying high-mannose structures, as found by Western blotting (Fig. 3). In addition, by use of cellular ELISA, it was shown that FimH adhesins did not bind to human HT-29 colon carcinoma cells (Fig. 5). To detect FimH proteins, rabbit anti-FimH IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako) were used.

Taken together, our results support an earlier proposition by Hancox et al. (6) that small differences in the amino acid sequences of FimH proteins are most probably responsible for the lack of mannose-binding activities of type 1 fimbriae from biovar Gallinarum and biovar Pullorum. A comparison of amino acid sequences of active FimH adhesins from S. enterica serovar Typhimurium (17), Salmonella enterica serovar Typhi (12), and S. enterica serovar Enteritidis (unpublished data) revealed that both inactive adhesins from biovar Gallinarum and biovar Pullorum are characterized by the presence of iso-
leucine at position 78 instead of the threonine found in active FimH adhesins in all other analyzed Salmonella serovars, and it is the only consistent amino acid substitution differentiating active and inactive proteins. This finding raised the possibility that the T78I mutation could be responsible for the loss of their binding properties. To address this hypothesis, fimH gene mutations with the I78T mutation were constructed. The mutagenesis of biovar Gallinarum and biovar Pullorum fimH was performed by using a PCR-based method. The mutation of interest was generated by two separate PCRs using two pairs of primers. The first PCR was carried out with the original 5'-fim primer for the fimH gene (5'-CGCGGATCCATGAAAAT TAGACCATCATAATCGACTCG-3') and a 3' primer containing the desired I78T mutation (5'-GGACAACGGTAAATTATACCTAGATACTCAGC-3') and also a plasmid (P.fimH/pTreHis2b or G.fimH/pTreHis2b) as a template. The second PCR was performed with the original 3'-fim primer for the fimH gene (5'-GCCGGATCCCAATGAAAAT TAGACCATCATAATCGACTCG-3') and a 5' primer carrying the mutation (5'-GGACAACGGTAAATTATACCTAGATACTCAGC-3') and also a template as described before. The resulting DNA fragments were used as a template for the third PCR with the original primers for the fimH gene. In each case, amplification was performed as follows: 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C (25 cycles). The mutations introduced into the fimH genes were confirmed by DNA sequencing. Mutated fimH genes were cloned into pTrcHis2b plasmid and expressed in E. coli DH5α as described for wild-type FimH adhesins. The construction of FimH proteins in which isoleucine was replaced by threonine fully confirmed our hypothesis. That the T78I mutation could be responsible for the loss of adhesive properties by type 1 fimbriae from biovar Gallinarum and biovar Pullorum. At this point, it is difficult to hypothesize what the biological consequences of such a mutation are, e.g., the effect on the host specificities of these avian-adopted biovars. Recently, it was shown that biovar Gallinarum and biovar Pullorum expressing S. enterica serovar Typhimurium strain LT2 type 1 fimbriae adhered 10 to 20 times better to human epithelial HEp-2 cells and were characterized by 20- to 60-fold increased invasiveness (18). In addition, a 32% increase in the binding of serially diluted FimH adhesins (starting from 100 μg/ml) to a monolayer of human colon cancer HT-29 cells growing in 96-well plates. ▲, FimH from S. enterica serovar Typhimurium; ■, native FimH from biovar Gallinarum; □, mutated FimH from biovar Gallinarum; ○, native FimH from biovar Pullorum; △, mutated FimH from biovar Pullorum.
number of M-cell ruffles was observed in a murine-ligated ileal loop model.

In summary, we have shown that biovar Gallinarum and biovar Pullorum produce FimH adhesins, which are a part of the type 1/type 2 fimbriae expressed by these biovars. The loss of sugar-binding properties by these proteins is the result of a single T78I mutation, as was proven by site-directed mutagenesis.

**Nucleotide sequence accession numbers.** The sequences for the FimH protein reported in this study have been submitted to GenBank under accession numbers AAR83178, AAR83177, and AAN64295.

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