INFECTION AND IMMUNITY, Sept. 2005, p. 6187–6190 Vol. 73, No. 9
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.9.6187–6190.2005

Characterization of FimH Adhesins Expressed by *Salmonella enterica* Serovar Gallinarum Biovars Gallinarum and Pullorum: Reconstitution of Mannose-Binding Properties by Single Amino Acid Substitution

Dagmara Kisiela,[1] Anna Sapeta,[1] Maciej Kuczkowski,[2] Tadeusz Stefaniak,[3] Alina Wieliczko,[2] and Maciej Ugorski[1,4]*

Department of Biochemistry, Pharmacology, and Toxicology,[1] Department of Epizootiology and Veterinary Administration with Clinic,[2] and Department and Clinic of Obstetrics, Ruminant Diseases, and Animal Health,[3] Faculty of Veterinary Medicine, Agricultural University of Wroclaw, 50-375 Wroclaw, Poland, and Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland[4]

Received 22 February 2005/Returned for modification 4 April 2005/Accepted 6 May 2005

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-

* Corresponding author. Mailing address: Department of Biochemistry, Pharmacology, and Toxicology, Faculty of Veterinary Medicine, Agricultural University of Wroclaw, Norwida 31, 50-375 Wroclaw, Poland. Phone: 48 71 320 52 09. Fax: 48 71 328 15 67. E-mail: ugorski@ozi.ar.wroc.pl.

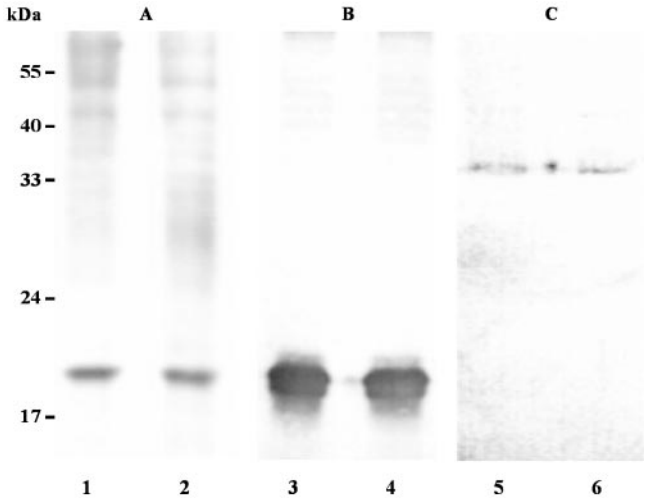

FIG. 1. Immunodetection of FimA and FimH proteins of biovar Gallinarum (lanes 1, 3, and 5) and biovar Pullorum (lanes 2, 4, and 6). Type 1 fimbriae (10 μg), purified according to the method of Müller et al. (11), were dissolved in Laemmli sample buffer supplemented with glycine at pH 2.2 and subjected to SDS-PAGE under reducing conditions in 12% gel and electrophoretically transferred to nitrocellulose. The positions of the molecular mass standards (in kDa) are shown on the left. (A) SDS-PAGE gel stained with Coomassie brilliant blue; (B and C) Western blots stained with polyclonal chicken anti-FimA (B) and polyclonal rabbit anti-FimH (C) antibodies.

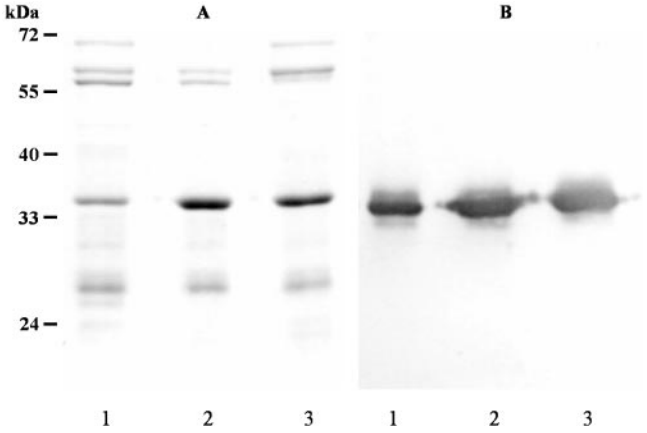

FIG. 2. Coomassie brilliant blue-stained SDS-PAGE (A) and immunostaining (B) of recombinant FimH adhesins from biovar Gallinarum (lanes 1), biovar Pullorum (lanes 2), and *Salmonella enterica* serovar Typhimurium (lanes 3). FimH proteins (10 μg), purified on Ni-NTA resin, were subjected to SDS-PAGE under reducing conditions in 10% gel. The migration positions of protein standards (in kDa) are indicated on the left.

bit IgG (Dako) and alkaline phosphatase-conjugated rabbit anti-chicken IgG (Chemicon), respectively.

Information on the structure-function relationship of FimH molecules is still quite limited, except perhaps for *Escherichia coli*, mostly because purification of type 1 fimbrial adhesins is extremely complicated (16). In addition, adhesins devoid of 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 Galliarum, 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′-CGCGGATCCAATGAAAATATACTCAGC-3′) and 3′-fim (5′-GCGTCTAGAGCATCATAATCGACTCG-3′) based on the published sequence of *S. enterica* serovar Typhimurium (GenBank accession no. L19338). The PCR primers contained additional sequences corresponding to a 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-

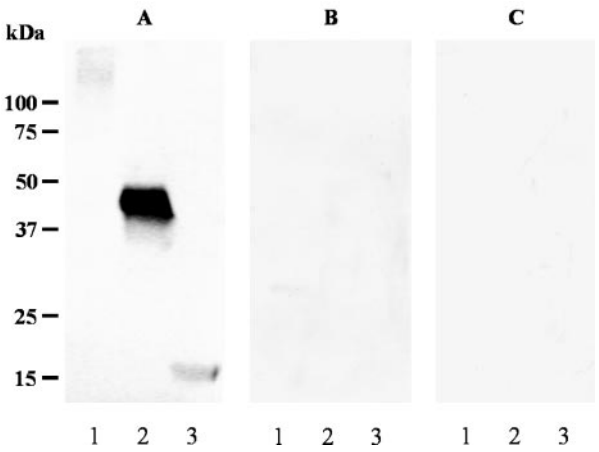

FIG. 3. Western blot analysis of FimH binding to glycoproteins carrying high-mannose-type oligosaccharide chains. Binding of the recombinant FimH adhesins from *Salmonella enterica* serovar Typhimurium (A), biovar Gallinarum (B), and biovar Pullorum (C) to mannan-BSA (lanes 1), HRP (lanes 2), and RNase B (lanes 3). Individual glycoproteins (10 μg) were subjected to SDS-PAGE under reducing conditions in 10% gel and electrophoretically transferred to nitrocellulose. The positions of the molecular mass standards (in kDa) are shown on the left.

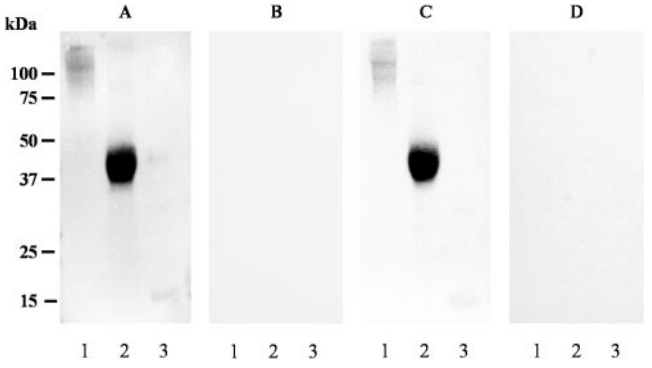

FIG. 4. Western blot analysis of mutant FimH adhesins binding to glycoproteins carrying high-mannose-type oligosaccharide chains. Binding of the mutant FimH proteins from biovars Gallinarum and Pullorum in the absence (A and C) and presence (B and D) of 0.2 M D-mannose to mannan-BSA (lanes 1), HRP (lanes 2), and RNase B (lanes 3). The mutants, constructed by site-directed mutagenesis, are identical to wild-type adhesins except at the position corresponding to the amino acid at position 78, where isoleucine was replaced by threonine (I78T). Individual glycoproteins (10 μg) were subjected to SDS-PAGE under reducing conditions in 10% gel and electrophoretically transferred to nitrocellulose. The positions of the molecular mass standards (in kDa) are shown on the left.

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 mutants 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′-CGCGGATCCAATGAAAAT ATACTCAGC-3′) and a 3′ primer containing the desired I78T mutation (5′-GCTTCGGTAGGTATAATTTACCGTT GTCC-3′) and also a plasmid (P.fimH/pTrcHis2b or G.fimH/ pTrcHis2b) as a template. The second PCR was performed with the original 3′-fim primer for the *fimH* gene (5′-GCGTC TAGAGCATCATAATCGACTCG-3′) and a 5′ primer carrying the mutation (5′-GGACAACGGTAAATTATACCTACC GAAGC-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. Both mutants bound well to glycoproteins carrying high-mannose oligosaccharides (Fig. 4) as well as colon carcinoma cells (Fig. 5) as shown by Western blotting and ELISA with rabbit polyclonal antibodies directed against FimH and alkaline phosphatase-conjugated goat anti-rabbit IgG. Because the only common difference in amino acid sequence between active and inactive FimH adhesins is the presence of isoleucine instead of threonine at position 78, our data strongly suggest that just one amino acid difference is fully 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

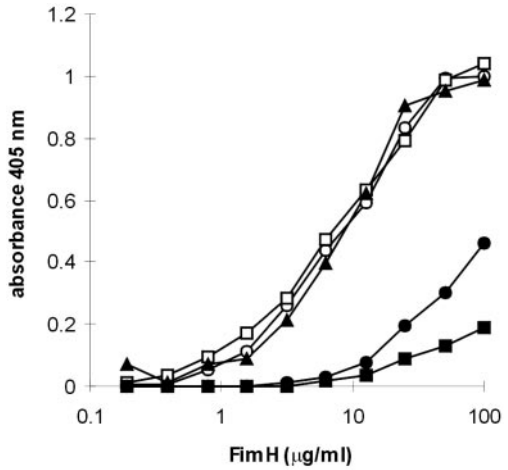

FIG. 5. Binding of serially diluted FimH adhesins (starting from 100 μg/ml) to a monolayer of human colon cancer HT-29 cells growing in 96-well plates. ▲, FimH from *S. enterica* serovar Typhimurium; ■, native FimH from biovar Gallinarum; □, mutated FimH from biovar Gallinarum; ●, native FimH from biovar 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.

This work was supported by grant no. 3PO6K 003 22 of the State Committee for Scientific Research (KBN), Warsaw, Poland.

REFERENCES

1. **Barrow, P. A., M. B. Huggins, and M. A. Lovell.** 1994. Host specificity of *Salmonella* infection in chickens and mice is expressed in vivo primarily at the level of the reticuloendothelial system. Infect. Immun. **62:**4602–4610.
2. **Boratyński, J., and R. Roy.** 1998. High temperature conjugation of proteins with carbohydrates. Glycoconj. J. **15:**131–138.
3. **Crichton, P. B., D. E. Yakubu, D. C. Old, and S. Clegg.** 1989. Immunological and genetical relatedness of type-1 and type-2 fimbriae in salmonellas of serotypes Gallinarum, Pullorum and Typhimurium. J. Appl. Bacteriol. **67:** 283–291.
4. **Doran, J. L., S. K. Collinson, C. M. Kay, P. A. Banser, J. Burian, C. K. Munro, S. H. Lee, J. M. Somers, E. C. Todd, and W. W. Kay.** 1994. fimA and tctC based DNA diagnostics for *Salmonella*. Mol. Cell. Probes **8:**291–310.
5. **Duguid, J. P., E. S. Anderson, and I. Campbell.** 1966. Fimbriae and adhesive properties in *Salmonellae*. J. Pathol. Bacteriol. **92:**107–138.
6. **Hancox, L. S., Y. Kuang-Sheng, and S. Clegg.** 1998. Construction and characterization of type 1 non-fimbriate and non-adhesive mutants of *Salmonella* Typhimurium. FEMS Immunol. Med. Microbiol. **19:**289–296.
7. **Jones, C. H., J. S. Pinkner, R. Roth, J. Heuser, A. V. Nicholes, S. N. Abraham, and S. J. Hultgren.** 1995. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. Proc. Natl. Acad. Sci. USA **92:**2081–2085.
8. **Kisiela, D., M. Kuczkowski, A. Wieliczko, I. Sambor, M. Mazurkiewicz, and M. Ugorski.** 2003. Comparison of SefA, FimA and AgfA fimbrial proteins of *Salmonella* Enteritidis in their abilities to elicit humoral immune response in hens. Bull. Vet. Inst. Pulawy **47:**95–105.
9. **Korhonen, T. K., K. Lounatmaa, H. Ranta, and N. Kuusi.** 1980. Characterization of type 1 pili of *Salmonella* Typhimurium LT2. J. Bacteriol. **144:**800–805.
10. **Krogfelt, K. A., H. Bergmans, and P. Klemm.** 1990. Direct evidence that the FimH protein is the adhesin of *Escherichia coli* type 1 fimbriae. Infect. Immun. **58:**1995–1999.
11. **Müller, K.-H., K. Collinson, T. J. Trust, and W. W. Kay.** 1991. Type 1 fimbriae of *Salmonella enteritidis*. J. Bacteriol. **173:**4765–4772.
12. **Muscas, P., G. M. Rossolini, A. Chiesurin, A. Santucci, and G. Satta.** 1994. Purification and characterization of type 1 fimbriae of *Salmonella* Typhi. Microbiol. Immunol. **38:**353–358.
13. **Old, D. C., and S. B. Payne.** 1971. Antigens of the type-2 fimbriae of *Salmonellae*: "cross-reacting material" (CRM) of type-1 fimbriae. J. Med. Microbiol. **4:**215–225.
14. **Rossolini, G. M., P. Muscas, A. Chiesurin, and G. Satta.** 1993. Analysis of the *Salmonella fim* gene cluster: identification of a new gene (*fimI*) encoding a fimbrin-like protein and located downstream from the *fimA* gene. FEMS Microbiol. Lett. **114:**259–265.
15. **Schechter, L. M., and C. A. Lee.** 2000. *Salmonella* invasion of non-phagocytic cells. Subcell. Biochem. **33:**289–320.
16. **Schembri, M. A., H. Hasman, and P. Klemm.** 2000. Expression and purification of the mannose recognition domain of the FimH adhesin. FEMS Microbiol. Lett. **188:**147–151.
17. **Thankavel, K., A. H. Shah, M. S. Cohen, T. Ikeda, R. G. Lorenz, R. Curtiss, and S. N. Abraham.** 1999. Molecular basis for the enterocyte tropism exhibited by *Salmonella* Typhimurium type 1 fimbriae. J. Biol. Chem. **274:**55797–55809.
18. **Wilson, R. L., J. Elthon, S. Clegg, and B. D. Jones.** 2000. *Salmonella enterica* serovars Gallinarum and Pullorum expressing *Salmonella enterica* serovar Typhimurium type 1 fimbriae exhibit increased invasiveness for mammalian cells. Infect. Immun. **68:**4782–4785.

Editor: F. C. Fang