Virulent *Shigella flexneri* Affects Secretion, Expression, and Glycosylation of Gel-Forming Mucins in Mucus-Producing Cells

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Mucin glycoproteins are secreted in large amounts by the intestinal epithelium and constitute an efficient component of innate immune defenses to promote homeostasis and protect against enteric pathogens. In this study, our objective was to investigate how the bacterial enteropathogen *Shigella flexneri*, which causes bacillary dysentery, copes with the mucin defense barrier. We report that upon *in vitro* infection of mucin-producing polarized human intestinal epithelial cells, virulent *S. flexneri* manipulates the secretion of gel-forming mucins. This phenomenon, which is triggered only by virulent strains, results in accumulation of mucins at the cell apical surface, leading to the appearance of a gel-like structure that favors access of bacteria to the cell surface and the subsequent invasion process. We identify MUC5AC, a gel-forming mucin, as a component of this structure. Formation of this gel does not depend on modifications of electrolyte concentrations, induction of trefoil factor expression, endoplasmic reticulum stress, or response to unfolded proteins. In addition, transcriptional and biochemical analyses of infected cells reveal modulations of mucin gene expression and modifications of mucin glycosylation patterns, both of which are induced by virulent bacteria in a type III secretion system-dependent manner. Thus, *S. flexneri* has developed a dedicated strategy to alter the mucus barrier by targeting key elements of the gel-forming capacity of mucins: gene transcription, protein glycosylation, and secretion.

The gut mucosal surface is unique among epithelia in that it is in permanent contact with a dense and diverse microbiota, including symbionts and, accidentally, pathogens. Mucosal tissues encompass distinct cell types, each of which employs mechanisms of defense that prevent bacterial invasion and translocation across the epithelial barrier. Epithelial cells, both constitutively and inducibly in response to microbes, secrete many antimicrobial molecules into the intestinal fluid, including antimicrobial peptides and immunoglobulins embedded in a gel constituted by secreted mucins (1). Together, these molecules form a protective barrier against intrusive microorganisms.

Mucins are glycoproteins produced and secreted by goblet cells. They contain a dense array of O-linked carbohydrates representing over 70% of their mass. They can assemble into a viscous gel-like layer that coats the epithelium (2, 3). *In vivo*, the mucus layer is composed of two distinct strata and protects epithelial cells against microbial injury but also mechanical, enzymatic, and chemical injuries (4). The outer layer, of softer consistency, is continually removed by intestinal peristaltic movements. It is colonized with bacteria, whereas the inner layer is resistant to bacterial intrusion, establishing a sort of bacterial no-man's land (5). The mucus layer provides a matrix for retention of antimicrobial molecules, including defensins and cathelicidins, produced by epithelial cells throughout the epithelial lining and secreted in the mucosal environment (6).

The energy invested by mucosal tissues in the production of mucins at the basal level and in response to infection reflects the importance of these glycoproteins. Mucins can be divided into three distinct subfamilies: cell surface mucins (MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20), nonoligomerizing gel-forming mucins (MUC7), and oligomerizing gel-forming mucins (MUC2, MUC5AC,

MUC5B, MUC6, and MUC19) (7). Gel-forming mucins are the major constituent of mucus, to which they confer its viscoelastic properties. These mucins contain N- and C-terminal domains that are both involved in homo-oligomerization (8). Oligomerization is likely to produce either extended filamentous structures or web-like structures able to occupy a large volume at the mucosal surface (9–12).

Gel-forming mucins show constitutive and inducible gene expression in epithelial cells. Their expression can be upregulated by proinflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-6, gamma interferon, and tumor necrosis factor alpha (13–18). Neutrophil elastase and microbial products can also stimulate the transcriptional expression of gel-forming mucins by epithelial cells (17, 19–21). The response to these proinflammatory signals provides a link between mucins, innate mucosal immunity, and mucosal inflammatory responses.

The mucin glycosylation pattern can be altered in response to mucosal inflammation and infection, thereby creating an environment unfavorable to pathogens. The oligosaccharides on gel-forming mucins are clustered into heavily glycosylated domains

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separated by shorter nonglycosylated regions (22). The O-linked glycans typically contain 1 to 20 residues, organized as both linear and branched structures. In addition to the O-linked glycans, mucins contain a smaller number of N-linked oligosaccharides, which have been implicated in folding or oligomerization (23). Changes in mucin glycosylation are a component of innate immune responses to mucosal infection.

The best evidence for the importance of the mucus barrier toward infection is the wide range of strategies used by pathogenic bacteria to escape or subvert it. Motility is important for mucosal pathogens to break through the physical mucus barrier, as highlighted by the fact that a vast proportion of pathogens are flagellated (24). In conjunction with motility, degradative enzymes, such as mucinases, N-acetyl neuraminidases, sialate O-acetyl-esterases, sialidases, and glycosulfatases, are produced by a broad range of bacterial pathogens to destabilize the mucus layers and remove mucin decoy carbohydrates for adhesins (25, 26).

Shigella is a Gram-negative enteroinvasive bacterium that causes bacillary dysentery. This pathogen is poorly efficient at invading intestinal epithelial cells through their apical surface and needs to translocate through the intestinal epithelial lining for the development of infection (27). *Shigella* translocates through M cells of the follicle-associated epithelium that covers the lymphoid nodules associated with the colonic mucosal surface. In subepithelial tissues, *Shigella* induces apoptosis of resident macrophages, allowing bacterial escape into the mucosa and efficient basolateral entry into epithelial cells, followed by cell-to-cell spread and intracellular replication (28–30). Subsequent inflammation disrupts the cohesion of the epithelial barrier, favoring further invasion of luminal bacteria and propagation of infection (31).

Regardless of its mechanisms of invasion of the intestinal mucosa, *S. flexneri* needs to gain access to the epithelial surface. In the absence of expression of flagella and recognized adhesins, it is fully confronted with the surface innate defense systems, particularly the mucus layer and the associated antimicrobial molecules, which it is poised to subvert. In this study, we investigated how *S. flexneri* deals with the mucus barrier, especially whether invasive bacteria modulate the secretion, expression, and glycosylation of mucins during the infectious process established upon contact with mucus-producing epithelial cells. Using an *in vitro* model, we reveal that *S. flexneri* interferes with the extracellular secretion of gel-forming mucins by promoting their trapping and accumulation at the surface of infected cells. We show that this phenomenon favors access of virulent bacteria to the apical cell surface and the following invasion process. We prove that bacteria actively modulate mucin biosynthesis by dampening expression of several of their genes. We also demonstrate that *S. flexneri* reshapes mucin structures by remodeling their glycosylation pattern. This work highlights a novel strategy developed by *S. flexneri* to subvert the mucin-based mechanisms of mucosal defense.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Shigella flexneri* serotype 5a strains were isolated on Congo red agar plates. The virulent wild-type strain M90T (32) and its isogenic derivative, the avirulent *mxlD* mutant (impaired for the MxiD protein, a component of the type III secretion system [TTSS] required for its functionality) (33), were used as previously described. Each strain expresses the AfaE adhesin (34) to favor contact of the bacteria with mucin-secreting HT29-MTX cells. For infection experiments, strains were cultured overnight in BTCS medium (Difco) at 37°C with shaking. Subcultures were set up for 2 h to reach the exponential

phase and spun gently at 2,000 rpm for 10 min. Bacteria were resuspended in Dulbecco modified Eagle tissue culture medium (DMEM; Invitrogen) and used for time course infection experiments.

Infection of polarized and differentiated cell monolayers. HT29-MTX human intestinal epithelial cells (35) derived from a colonic carcinoma were cultured in 6- or 12-well plates (1.5×10^7 cells/well or 0.75×10^7 cells/well, respectively) with DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 1% nonessential amino acids (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in 10% CO₂. Cells were grown for 3 weeks to reach full differentiation and polarization. Culture medium was changed 2 times per week. Infections were performed in triplicate in DMEM without serum at a multiplicity of infection (MOI) of 10 bacteria per cell and 37°C in 10% CO₂ for the times indicated below.

Histochemical AB staining. HT29-MTX cell monolayers were grown on glass coverslips in 12-well plates. After the infection experiments, cells were fixed with Carnoy's reagent (60% ethanol, 30% acetic acid, 10% chloroform) at room temperature and washed with distilled water. Then, detection of the acid mucopolysaccharides harbored by mucin proteins was performed by addition of 1% alcian blue (AB) 8GX (Sigma-Aldrich) solution (in 1% acetic acid) to each well for 20 min, followed by a washing step with distilled water. Finally, cell monolayers were dehydrated through sequential incubations in 90% ethanol, 100% ethanol, and 100% xylene. Coverslips were mounted using Eukitt medium (Sigma-Aldrich) and dried overnight at room temperature before microscope examination.

Immunofluorescence experiments. Cell monolayers were fixed with Carnoy's reagent, and detection of proteins was performed as follows. Cell monolayers were permeabilized for 10 min using permeabilizing buffer (1× phosphate-buffered saline [PBS], 0.1% Triton), followed by an incubation step in saturation buffer (1× PBS, 3% bovine serum albumin [BSA], 5% FCS) for 1 h at room temperature. Cell monolayers were incubated for 1 h at room temperature with the primary polyclonal antibody to human MUC5AC (sc-20118; Santa Cruz Biotechnology). Cell monolayers were washed 2 times with saturation buffer, incubated for 45 min at room temperature with the secondary antibody coupled to Alexa Fluor 488 (Invitrogen) and rhodamine phalloidin (Sigma-Aldrich), washed 2 times with saturation buffer, and finally, incubated for a few seconds with DAPI (4',6-diamidino-2-phenylindole) solution (1× PBS) (Invitrogen). Coverslips were mounted using 1% DABCO mounting medium (80% glycerol) (Sigma-Aldrich), sealed, and dried overnight before examination.

Infection of rabbit ligated ileal loops. The rabbit ligated ileal loop model protocol was approved by the French Comité Régional d'Éthique pour l'Expérimentation Animale, Paris, France (reference 20070004). Two male New Zealand White rabbits weighing 2.5 kg (Charles River) were used for 360 min of infection. Before the infection protocol, the animals were pretreated for 7 days with 2.8 g/liter of sodium sulfadimethoxine salt (Mucoxid; CEVA Santé Animale) in drinking water to minimize infection with coccidian parasites. Two independent experiments, each of which used one rabbit, were performed. Rabbits were fasted 24 h before infection, sedated intravenously with 0.05 ml/kg of body weight 0.5% acepromazine (Calmivet; Vétquinol), and anesthetized by the same route with 0.2 ml/kg of 10% ketamine HCl (Imalgene 1000; Merial). Prior to laparotomy, 2 ml 2,1% lidocaine (Xylovet; CEVA Santé Animale) was injected intradermally in the abdomen along the site of the incision. The small intestine was exteriorized, and loops of 5-cm segments of ileum were ligated. Each loop was injected with 0.5 ml of bacterial suspension (10^9 bacteria/ml) or the saline control. Each condition was performed in duplicate within an animal. Loops were returned into the abdominal cavity, the abdomen was closed, and the animals were returned to their cage for 360 min. Animals were sacrificed by intravenous injection of 120 mg/kg sodium pentobarbital (Vétquinol). Loops were dissected, fixed with Carnoy's reagent, and processed for histology.

Histological AB/periodic acid-Schiff stainings. Tissue sections of 7 μ m were prepared on slides from rabbit loops conserved in paraffin. Sections were deparaffinized, rehydrated, stained with 1% AB 8GX (Sigma-Aldrich) solution (in 1% acetic acid) for 5 min, washed with tap water, treated with periodic acid (Merck) for 10 min, washed with tap water, stained with the Schiff reagent (Merck) for 15 min, washed with tap water, and sequentially dehydrated with ethanol and xylene. Slides were mounted using Eukitt medium (Sigma-Aldrich) and dried overnight at room temperature before examination under a microscope.

Gel-forming mucin separation on polyacrylamide gels and detection. Supernatant fractions from infected cell monolayers were centrifuged at $13,000 \times g$ for 30 min at 4°C. Pellets were resuspended in loading buffer (6 g SDS plus 20 mg bromophenol blue in 25% Tris, 20% glycerol, 55% H₂O) and run in 4 to 15% SDS-polyacrylamide precast gels (Bio-Rad). Gels were stained using 0.25% AB 8GX solution (45.5% ethanol, 9% acetic acid) overnight and destained using stop solution (45.5% ethanol, 9% acetic acid) (4).

MUC5AC mucin quantification using ELISA. MUC5AC protein expression was measured using enzyme-linked immunosorbent assay (ELISA). Cell supernatants were diluted in 4 M guanidine chloride solution (diluted by volume). A volume of 100 μ l of each sample was incubated in a 96-well plate at 4°C overnight. Plates were washed three times with PBS and blocked with protein-free blocking buffer (Thermo Fisher Scientific) for 2 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μ l of a mouse monoclonal MUC5AC antibody (45M1 diluted 1/200; a gift from J. Bara) diluted in PBS containing 0.05% Tween 20. After 1 h, the wells were washed three times with PBS and incubated with 100 μ l of horseradish peroxidase–goat anti-mouse IgG conjugate (diluted 1/3,000; Invitrogen). After 1 h, the plates were washed three times with PBS. The color reaction was developed with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] solution: 22 mg ABTS (Sigma-Aldrich) diluted in 100 ml of citrate buffer (50 mM sodium citrate, 0.05% H₂O₂, pH 4.0). The optical densities at 450 nm were measured on an Elx800 microplate reader (Biotek Instrument).

Shigella flexneri LPS immunostainings. HT29-MTX cell monolayers were grown on glass coverslips in 12-well plates. After infection with *S. flexneri*, cells and bacteria were fixed with Carnoy's reagent and washed with PBS. Coverslips were treated with 1% BSA (in PBS) for 1 h, incubated with a polyclonal antibody to *S. flexneri* serotype 5a lipopolysaccharide (LPS) for 1 h 30 min, washed with PBS, incubated with a peroxidase-conjugated secondary antibody (Dako) for 1 h 30 min, and washed with PBS. Detection was accomplished by addition of the liquid diaminobenzidine substrate-chromogen system (Dako). Then, the coverslips were washed with distilled water, treated with 1% AB 8GX (Sigma-Aldrich) solution (in 1% acid acetic) for 20 min, and washed with distilled water. Coverslips were mounted using aqueous permanent solution (Dako) and sealed before examination under a microscope.

Plaque assay experiments. The gel-forming mucin-producing HT29-MTX cell line (mucus positive) and its parental HT29 cell line, cells which do not secrete gel-forming mucins, were cultured in 6-well plates (1.5×10^7 cells/well) for 3 weeks to reach full differentiation and polarization. Infections were performed at an MOI of 10 bacteria per cell at 37°C in 10% CO₂ for 270 min. Cell monolayers were washed with DMEM (Invitrogen) and covered with an agar layer prepared with DMEM (Invitrogen) supplemented with 10% inactivated FCS (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.5% agar, and 50 μ g/ml gentamicin. Plates were incubated at 37°C in 10% CO₂ and examined daily for plaque formation. Plaques were visualized after cell fixation with ethanol and cell staining with 1/20-diluted Giemsa solution (Merck).

mRNA isolation and quantitative real-time PCR. mRNA was isolated using an RNeasy minikit (Qiagen). Reverse transcription (RT)-PCRs were performed on 4- μ g RNA samples using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)₁₅ primers (Promega), as recommended by the suppliers. Gene-specific primers were designed and purchased from Sigma: for MUC1, GTGGTGGTACAATTGACTCTGG and

GTATATCGAGAGGCTGCTTCC; for MUC2, CTCCTCTGTGCTTA TCTGCTG and TGACGAAATAACAGGTGTCTCC; for MUC3A, CTGC AACTACCAGCACTTCTTC and TATAGTTCTCGACAGGGTGTG; for MUC4, AGGCTACCTCAAGACTCACCTC and TCATTCTCCTTGA AGAATCCTG; for MUC5AC, ACGCTCCATCAGTAGAGAAAGAC and TGAGATTTCAGATCTGTCTC; for MUC5B, CCACTTCTCTACTC CCTGCTC and TCGGTCGGTCTTATTGTAGATG; for MUC12, CTA CGTTGGTTACCAAGTCTTG and TCATACCTAAAGTGGCGTTGAG; for MUC13, GACATAATCACCGCTTCATCTC and TGATTGATTGTC TTCTGTGGTG; for MUC15, CTGGGTGTCTCATTGCTTACTC and GAACTGGTTCATTTCTGTCTG; for TFF1, CATGGAGAACAAGGT GATCTG and GCCACTGTACAGTCTCTGTG; for TFF2, GTCTCAGA CCGAAGAACTGTG and TCAAAGATGAAGTTGGAGAAGC; for TFF3, CTGTCTGGGAGCTTGACAAAG and TCATTTATGCACCGTT GTTTG; for HSP5A, CCCAATACAGCCATTAAAGATG and AGTGAG AGCCTTTGAGGTAAGG; and for HSP90B1, AGTTTGAACATTGACC CTGATG and TTGCTCTGTGTCTTCTGTTGTG. Quantitative RT-PCRs (qRT-PCRs) were carried out in a 20- μ l volume containing 8 μ l of cDNA (diluted 1/100), 2 μ l of primers (0.2 μ M each), and 10 μ l of Power SYBR green mix (Applied Biosystems). Reactions were run on an ABI 7900HT instrument (Applied Biosystems) under conditions with the universal thermal cycling parameters recommended by the manufacturer. Each reaction was run in duplicate on the same plate. Relative quantification of gene expression was performed using the comparative threshold cycle method. Results were normalized using the human β_2 -microglobulin (B2M) housekeeping gene.

PCR-based assay for XBP1 mRNA splicing. mRNA was isolated using the RNeasy minikit (Qiagen). cDNAs were synthesized from 4 μ g of each sample and used as the template for PCRs using XBP1 primers flanking the splice site on the transcript (AAACAGAGTAGCAGCTCAGACTGC and TCCTTCTGGGTAGACCTCTGGGA). After amplifications (denaturation at 95°C for 10 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s for 35 cycles), the unspliced XBP1 cDNA gave a 480-bp product and the spliced XBP1 cDNA gave a 454-bp product (36).

Electrolyte dosage. Electrolyte concentrations from cell supernatant fractions were measured using a Dade Behring Dimension Xpand chemistry analyzer (Siemens), as recommended by the supplier, thanks to the Pasteur Institute medical analysis center.

Mucin glycosylation analysis by MALDI-TOF mass spectrometry. Cell supernatant fractions were collected and diluted (by volume) in 4 M guanidine chloride solution containing 5 mM EDTA, 10 mM benzamidine, 5 mM *N*-ethylmaleimide, 0.1 mg/ml soy bean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, as previously published (37). CsCl was added to an initial density of 1.4 g/ml, and mucins were purified by isopycnic density gradient centrifugation (58,000 rpm at 15°C for 72 h; 70.1 Ti rotor, Beckman Coulter LE80K ultracentrifuge). The mucin-containing fractions were pooled, dialyzed into water, and lyophilized. Mucins were further submitted to β elimination under reductive conditions (0.1 M NaOH, 1 M KBH₄, 24 h at 45°C). Permethylated oligosaccharide alditols was carried out with the sodium hydroxide procedure described by Ciucanu and Kerek (38). After derivatization, the reaction products were dissolved in 200 μ l of methanol and further purified on a C₁₈ Sep-Pak column (Waters). Permethylated oligosaccharides were analyzed by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry (MS) in positive ion reflective mode as $[M + Na]^+$. Quantification through the relative percentage of each oligosaccharide was calculated on the basis of integration of peaks on MS spectra.

Statistical analysis. Data are expressed as the mean $\pm$ standard deviation (SD) of three separate experiments. Statistical significance was assessed by Student's *t* test.

RESULTS

Virulent *S. flexneri* compromises the extracellular secretion of gel-forming mucins in mucus-producing cells. To investigate

the dynamics of the extracellular release of gel-forming mucins in cells infected with *S. flexneri*, we used a previously established *in vitro* model of infection based on polarized and fully differentiated HT29-MTX human colonic epithelial cells, which produce and secrete mucins (35). Monolayers of these cells mimic an epithelial lining, allowing us to study the relationship between *Shigella* and gel-forming mucins during the infectious process. Infections were carried out on cell monolayers with the virulent wild-type strain M90T and its isogenic derivative, an avirulent *mxiD* mutant impaired for the assembly of a functional TTSS. Each strain expressed the AfaE adhesin to favor contact of the bacteria with mucin-secreting HT29-MTX cells (34). Time course experiments were performed and stopped at 30, 90, 180, and 270 min by addition of Carnoy's fixative buffer. The extracellular secretion of gel-forming mucins was characterized by analyzing the cytoplasmic content of these glycosylated proteins, using AB staining (39).

In most of the noninfected cells, we observed the presence of mucins in the cytoplasm, as revealed by strong AB staining (Fig. 1A). Only a few clusters of cells appeared to be defective for mucin production. Infection of cells with the *mxiD* mutant was followed by the time-dependent release of the cytoplasmic mucin content, as shown by the weak AB staining observed at the last point of 270 min after infection (Fig. 1C) compared with the level of staining of noninfected cells (Fig. 1A). Strikingly, in cells infected with the wild-type strain M90T, we observed the time-dependent appearance of an extracellular gel-like structure covering the surface of cell monolayers concomitantly with the secretion of the cytoplasmic mucin content (Fig. 1B). The association of the strong AB staining of this gel revealed a web-like structure, and the weak AB staining of the cell cytoplasm indicated that gel-forming mucins were components of the cell surface-associated structure. Upon extended infection, the *mxiD* mutant failed to form the surface structure.

To identify which mucins constituted the gel-like structure typically observed at the surface of cell monolayers infected with the wild-type strain M90T, we next performed immunofluorescence staining experiments using specific antibodies directed against the MUC2, MUC5AC, and MUC5B gel-forming mucins. Among them, the MUC5AC antibody provided a strong signal in the cytoplasmic granules of noninfected cell monolayers, as revealed by epifluorescence (Fig. 1D) and confocal (Fig. 1G) microscopy. Infection experiments with the *mxiD* mutant were followed by a time-dependent disappearance of this signal from the cytoplasm (Fig. 1F and I), which did not occur in noninfected cells (Fig. 1D and G), demonstrating the extracellular release of MUC5AC in the supernatant fraction. Interestingly, disappearance of the signal in the cell cytoplasm was concomitant with the time-dependent appearance of a web-like signal organized at the surface of cells infected with the wild-type strain M90T (Fig. 1E and H), showing the secretion of MUC5AC from the cytoplasm and its trapping at the cell surface. Similar results were observed with the MUC2 and MUC5B antibodies, but to a lesser extent (unpublished data).

To examine the occurrence of the mucin web-like structure *in vivo*, we finally used the established rabbit ligated ileal loop model of shigellosis and inspected the process of mucin secretion from goblet cells at the 360-min time point after *S. flexneri* infection. In noninfected tissues, we observed goblet cells with a full mucin cytoplasmic content, as revealed by strong AB staining, and the release of little mucin into the lumen (Fig. 1J). Infection with the

mxiD mutant was associated with the gushing of mucins from goblet cell cytoplasm into the lumen, far from the epithelial surface (Fig. 1L), which did not occur in noninfected tissues (Fig. 1J). Interestingly, in tissues infected with the wild-type strain M90T, a lattice-like gel comparable to the web-like structure observed *in vitro* was detected in a patchy way, depending upon the areas of significant epithelial invasion (Fig. 1K). This mucin layout was never observed in *mxiD* mutant-infected tissues.

Together, these observations demonstrate that infection of cells with both strains of *S. flexneri* promotes the extracellular release of gel-forming mucins, including MUC5AC, but also revealed the ability of the wild-type strain M90T to interfere with this process by promoting the appearance of a gel-like structure coating the surface of infected cells.

Gel-forming mucins are trapped and accumulated at the surface of cells upon infection with virulent *S. flexneri*. To further characterize the gel-like structure observed at the surface of cell monolayers infected with the wild-type strain M90T of *S. flexneri* but not the surface of monolayers infected with the *mxiD* mutant, we measured parameters reflecting the properties of this structure, such as its volume and mucin content, at 30, 90, 180, and 270 min postinfection.

The release of granules containing stored gel-forming mucins from mucus-producing cells occurs through the exocytosis pathway and results in a 100- to 1,000-fold expansion in mucin volume upon hydration (40, 41). To analyze this process upon *S. flexneri* infection, we carefully collected supernatant fractions from cells infected with the wild-type strain M90T or the *mxiD* mutant and measured their volume. Then, we deduced the volume of their respective cell fractions, using the total volume of noninfected cells plus their supernatant as a reference. In noninfected cells, the volumes associated with supernatant and cell fractions were constant throughout the experiment and represented 95% and 5% of the total volumes, respectively (unpublished data). Similar values were obtained for both fractions from cells infected with the *mxiD* mutant (Fig. 2A). Strikingly, measures determined from cells infected with the wild-type strain M90T revealed a time-dependent decrease of the volume associated with the supernatant fraction, to the benefit of the one associated with the cell fraction (Fig. 2A). Quantitatively, the cell fraction volume increased from 5% to 15% during infection with M90T. Because the gel typically observed at the surface of cells infected with the wild-type strain M90T was associated with the cell fraction, these observations reflect expansion of the volume of this structure.

To determine the content of gel-forming mucins in supernatant fractions upon *S. flexneri* infection, samples were collected from cell monolayers infected with the wild-type strain M90T or the *mxiD* mutant at the different time points of the experiments, loaded, run in a 4 to 15% gradient polyacrylamide gel allowing the migration of mucins, and stained by AB to detect glycosylated proteins. At 30 min after infection, the supernatant fractions contained the same amount of gel-forming mucins whether the cells were infected with the wild-type strain M90T or the *mxiD* mutant, as revealed on the gel by the smears of the proteins stained by AB (Fig. 2B) and the dosage of MUC5AC determined by ELISA (Fig. 2C). In contrast, we detected significant variations of the mucin content in supernatant fractions collected 270 min after infection with the two *S. flexneri* strains (Fig. 2B). Qualitatively, smears of proteins contained in the supernatant fractions appeared much more intense upon infection of cells with the *mxiD* mutant than

FIG 1 Secretion of mucins is altered upon infection with virulent *S. flexneri*. (A to C) AB staining of mucins produced by HT29-MTX cell monolayers after 270 min of infection with the wild-type *S. flexneri* strain M90T (B) or the *mxuD* mutant (C) compared to mucins produced by noninfected cells (A). Arrows, areas of cells with a poor cytoplasmic content of mucins in monolayers infected with wild-type M90T or *mxuD* mutant bacteria; arrowheads, constitution of gel-forming mucins in a gel-like structure at the surface of cell monolayers, observed only upon infection with the wild-type *S. flexneri* strain M90T. Experiments were performed in 12-well plates (0.75×10^7 cells/well) at an MOI of 10 bacteria per cell. Results are representative of five independent experiments. (D to I) Detection by immunofluorescence experiments and epifluorescence (D to F) or confocal (G to I) microscopy analysis of the MUC5AC gel-forming mucin in HT29-MTX cell monolayers after 270 min of infection with the wild-type *S. flexneri* strain M90T (E, H) or the *mxuD* mutant (F, I) and in noninfected cells (D, G). Arrows, extracellular release of cytoplasmic granules containing the MUC5AC gel-forming mucin, typically observed in cell monolayers infected with M90T and *mxuD* bacteria; arrowheads, trapping of MUC5AC at the cell surface after its extracellular secretion from granules and its constitution in a web-like structure, observed only upon infection with the wild-type *S. flexneri* strain M90T. Experiments were performed in 12-well plates (0.75×10^7 cells/well) at an MOI of 10 bacteria per cell. MUC5AC is in green, actin is in red, and nuclei are in blue. Results are representative of four independent experiments. (J to L) AB and periodic acid-Schiff (PAS) staining of ligated ileal loop sections from rabbits infected for 360 min with the wild-type *S. flexneri* strain M90T (K) or the *mxuD* mutant (L) and noninfected animals (J). Arrows, goblet cells from tissue with their full (J) or empty (K, L) content of mucins; arrowheads, a lattice-like structure at the surface of tissue (K), observed only upon infection with the wild-type *S. flexneri* strain M90T. Results are for two independent loops from two independent infected rabbits. Magnifications, $\times 100$.

infection of cells with the wild-type strain M90T. In agreement, the dosages of MUC5AC in the supernatant fractions of infected cells revealed a concentration reaching 7 mg/ml for cells infected with the *mxuD* mutant, in contrast to a concentration of 2 mg/ml for cells infected with the wild-type strain M90T, as measured by

ELISA at 270 min after infection (Fig. 2C). The observation that MUC5AC is secreted by M90T-infected cells (Fig. 1E and H) but is detected in only small amounts in the supernatant (Fig. 2C) shows the trapping and accumulation of gel-forming mucins at the surface of cell monolayers.

FIG 2 Virulent *S. flexneri* infection leads to the trapping of mucins at the surface of cells. (A) Effect of appearance of the M90T-dependent gel-like structure at the surface of HT29-MTX cell monolayers on the volume of supernatant and cell fractions. Volumes were measured in 6-well plates (1.5×10^7 cells/well) at 30 and 270 min after infection at an MOI of 10 bacteria per cell. Values are given in percentages and represent variations of volumes associated with the supernatant fractions and the respective cell fractions. Black bars, volume of cell fractions; white bars, volume of supernatant fractions. Results are representative of three independent experiments. Error bars represent the SD. *, $P < 0.01$ for infections with the M90T strain compared with the *mxid* mutant. (B) Migration of supernatant fractions from infected cell monolayers on an acrylamide gradient gel. Supernatant fractions of cell monolayers infected with wild-type and mutant *S. flexneri* strains were collected at 30 and 270 min after infection, loaded and run on an SDS-PAGE 4 to 15% polyacrylamide gradient gel, and stained with AB to detect the smear-type migration of extracellular gel-forming mucins. Results are representative of three independent experiments. (C) ELISA measurement of the amount of the MUC5AC protein in supernatant fractions from infected cell monolayers. Supernatant fractions of cell monolayers infected with wild-type and mutant *S. flexneri* strains were collected at 30 and 270 min after infection and used for measurement of the extracellular MUC5AC content. Values are presented as the ratio of MUC5AC proteins detected in supernatant fractions from infected cells to MUC5AC proteins in supernatant fractions from noninfected cells. Black bars, wild-type strain M90T; white bars, *mxid* mutant strain. Results are representative of three independent experiments. Error bars represent the SD. *, $P < 0.01$ for infections with M90T compared with the *mxid* mutant.

Collectively, these results highlight the time-dependent secretion of gel-forming mucins from the cytoplasm to the supernatant of cells infected with the *mxid* mutant and a manipulation of this process by the wild-type strain M90T leading to the appearance of a gel-like structure promoted by the trapping and accumulation of gel-forming mucins at the surface of cells.

Trapping of gel-forming mucins favors access of virulent *S. flexneri* to the cell surface and the subsequent invasion process. To study the overall relevance of the trapping and accumulation of gel-forming mucins at the apical cell surface upon *S. flexneri* infection, we investigated the presence of bacteria at the surface of

cells during appearance of the gel-like structure and evaluated the impact of this process on the outcome of the infection.

To label bacteria at the surface of cells upon infection with the wild-type *S. flexneri* strain M90T or the *mxid* mutant, we performed *S. flexneri* LPS immunostaining of the infected HT29-MTX cell monolayers at different time points (Fig. 3A). At 30 min after infection, both strains were observed at the cell surface at similar amounts, as revealed by the red dots uniformly distributed throughout the surface (Fig. 3A). In contrast, we saw significant variations between the two strains at 270 min after infection. Whereas we could detect only a few bacteria of the *mxid* mutant at

FIG 3 Trapping of mucins favors access of virulent *S. flexneri* to the cell surface and subsequent invasion. (A) Anti-*S. flexneri* LPS immunostaining of bacteria (red) and AB staining of mucins (blue) at 30 and 270 min after infection of HT29-MTX cell monolayers with the wild-type *S. flexneri* strain M90T or the *mxid* mutant. Arrowheads, bacteria present at the cell surface. Experiments were performed in 12-well plates (0.75×10^7 cells/well) at an MOI of 10 bacteria per cell. Results are representative of two independent experiments. (B) Plaque assays on gel-forming mucin-producing HT29-MTX cells (mucus positive) or gel-forming mucin-nonproducing HT29 cells (mucus negative) after 270 min of infection with the wild-type *S. flexneri* strain M90T or the *mxid* mutant. Experiments were performed in 6-well plates (1.5×10^7 cells/well) at an MOI of 10 bacteria per cell. Results are representative of three independent experiments.

the surface of cells, we spotted more wild-type M90T bacteria covering cell monolayers and entangled in the AB-stained gel-like structure (Fig. 3A).

To correlate the trapping and accumulation of gel-forming mucins at the cell surface to the efficacy of the *S. flexneri* invasion process, we carried out plaque assay experiments using fully differentiated monolayers of either HT29-MTX gel-forming mucin-producing cells (mucus positive) or cells of the parental HT29 cell line, which do not produce gel-forming mucins (mucus negative). Experiments were performed in parallel on the two cell lines with the virulent wild-type strain M90T or the *mxiD* mutant. Infections were stopped at 270 min after infection, and plaque formation was examined on the day after (Fig. 3B). Infection of HT29-MTX (mucus-positive) cells with the wild-type strain M90T was followed by the appearance of large and numerous plaques uniformly covering the whole monolayer (Fig. 3B). Strikingly, in HT29 (mucus-negative) cells infected with the same *S. flexneri* strain, we observed plaques with smaller sizes, and these plaques were mostly distributed at the periphery of the monolayers (Fig. 3B). As a control, infection of the two cell lines with the *mxiD* mutant impaired for a functional TTSS was not followed by plaque formation (Fig. 3B).

Together, these observations prove that the trapping and accumulation of gel-forming mucins at the surface of infected cells assist with the access of virulent *S. flexneri* to the apical cell surface and support the following processes of cell invasion and cell-to-cell spread.

Trapping of gel-forming mucins is not linked either with the variation of electrolyte concentrations or with induction of trefoil factor (TF) expression, ER stress, or UPR. To investigate whether variation of the cell physiological parameters could explain the trapping of gel-forming mucins at the cell surface, as typically observed upon infection with the *S. flexneri* wild-type strain M90T, we analyzed parameters that are known to influence physicochemical properties of the mucus, such as electrolyte concentrations (42–44) or trefoil factor expression (45–49), and, conversely, parameters that are influenced by mucin misfolding, such as activation of endoplasmic reticulum (ER) stress (50, 51) or the unfolded protein response (UPR) (36, 51, 52).

To measure the extracellular electrolyte concentrations upon *S. flexneri* infection, we collected supernatant fractions from cell monolayers infected with the virulent wild-type strain M90T or the avirulent *mxiD* mutant at different time points during experiments and used an automatic chemistry analyzer to monitor the concentrations of the main electrolytes, including sodium (Na^+), potassium (K^+), chloride (Cl^-), carbonate (HCO_3^-), calcium (Ca^{2+}), and magnesium (Mg^{2+}) ions. Among them, potassium and carbonate ion concentrations were significantly modified in supernatants collected from infected cells compared with supernatants collected from noninfected cells; however, these concentrations were found to be statistically equivalent between the M90T and *mxiD* strains (see Fig. S1A in the supplemental material). As a control, noninfected cells were stimulated to release their mucin content by different secretagogues (ATP, carbachol, vasoactive intestinal peptide) with changes of extracellular potassium and carbonate concentrations or pH level. We failed to mimic the trapping of gel-forming mucins at the surface of cells (unpublished data).

To study the expression of trefoil factors in cells infected with *S. flexneri*, we performed a transcriptional analysis of the TFF1,

TFF2, and TFF3 genes in fully differentiated cell monolayers. mRNA was collected at different time points of infection and analyzed by qRT-PCR for expression of the three genes. We detected a basal expression of the TFF1, TFF2, and TFF3 genes in noninfected cells over the observed course of time (unpublished data) and in a comparison found a similar level of transcription of these genes upon infection with both the M90T and *mxiD* strains (see Fig. S1B in the supplemental material). As a control, expression and secretion of the three trefoil factor peptides from cells infected with the two *S. flexneri* strains were followed by immunofluorescence, using specific antibodies, which was found to be comparable for both strains throughout the infection (unpublished data).

To evaluate ER stress in cells upon *S. flexneri* infection, we studied expression of the HSP5A and HSP90B1 genes, encoding heat shock proteins that are induced under ER stress, when these chaperones accumulate with misfolded proteins (51). We carried out a transcriptional analysis of these two genes in cell monolayers infected with the wild-type strain M90T and the *mxiD* mutant at successive time points. We detected a basal level of expression of HSP5A and HSP90B1 genes in noninfected cells (unpublished data) and in a comparison found a similar level of transcription upon infection with both *S. flexneri* strains, even after 270 min, as measured by qRT-PCR (see Fig. S1C in the supplemental material). As a control, expression of the two proteins was tracked by immunofluorescence, using specific antibodies, and was found to be similar under all tested conditions over the observed course of time (unpublished data).

To examine the UPR in cells infected with *S. flexneri*, we analyzed splicing of mRNAs encoding the XBP1 transcription factor, one of the molecular events leading to activation of the UPR (51). We performed RT-PCR experiments on total mRNA extracted from cell monolayers infected with the wild-type strain M90T and the *mxiD* mutant at different time points. We detected a single product of amplification with a similar size of 480 bp under all tested conditions (see Fig. S1D in the supplemental material). This product, corresponding to the unspliced form of the XBP1 mRNA, revealed the absence of activation of the UPR (51).

Together, these results show only weak variations in electrolyte concentrations and the absence of induction of trefoil factor expression, ER stress, and the UPR in cells infected with *S. flexneri*, allowing us to exclude a causative relation between these cell physiological parameters and the M90T-dependent trapping of gel-forming mucins at the cell surface.

Virulent *S. flexneri* modulates expression of genes encoding gel-forming and cell surface mucins in mucus-producing cells.

To examine whether *S. flexneri* altered expression of genes encoding cell surface and gel-forming mucins upon infection, we performed a transcriptional analysis of the MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC12, MUC13, and MUC15 genes in fully differentiated cell monolayers infected with the virulent wild-type strain M90T or the avirulent *mxiD* mutant. mRNA was extracted at 90, 180, and 270 min after infection and analyzed by qRT-PCR for expression of the selected set of genes.

In noninfected cells, we detected a basal transcription of the nine mucin-encoding genes at the different time points (unpublished data). Infection of cells with the *mxiD* mutant was followed by transcriptional induction of the gel-forming mucin MUC5AC gene (2-fold) (Fig. 4A) and the cell surface mucin MUC4 and MUC15 genes (7- and 8-fold, respectively) (Fig. 4B) at the latest time point of 270 min. In contrast, the MUC1, MUC2, MUC3A,

FIG 4 Expression of genes encoding mucins is modulated by virulent *S. flexneri* upon cell infection. Transcriptional expression of genes encoding the gel-forming mucins MUC2, MUC5AC, and MUC5B (A) and the cell surface mucins MUC1, MUC3A, MUC4, MUC12, MUC13, and MUC15 (B) in HT29-MTX cell monolayers throughout the infection. After mRNA extractions and RT reactions, qRT-PCR was performed on each sample at each time point with specific primers to determine the relative expression of genes under each condition, using the comparative threshold cycle method. Values are presented on a linear scale as the ratio of gene expression in infected cells compared to that in noninfected cells. Black squares, wild-type strain M90T; black triangles, *mxiD* mutant strain. Experiments were performed in 6-well plates (1.5×10^7 cells/well) at an MOI of 10 bacteria per cell. Results are representative of three independent experiments. Error bars represent the SD. *, $P < 0.01$ for infections with M90T compared with the *mxiD* mutant.

MUC4, MUC5B, MUC12, and MUC13 genes remained transcribed at their basal level of expression throughout the infection (Fig. 4).

Interestingly, infection with the wild-type strain M90T showed differential transcriptional expression of the MUC4, MUC5AC, and MUC15 genes compared with the level of expression by the *mxiD* mutant strain (Fig. 4). These genes were not induced in cells infected with M90T but were induced in cells infected with the *mxiD* mutant, even after 270 min of infection. Conversely, infection of cells with M90T was transiently followed by a transcriptional induction of the gel-forming mucin MUC2 gene (1.8-fold) at 180 min after infection, which did not occur in cells infected with the *mxiD* mutant (Fig. 4A). In contrast, expression of the MUC1, MUC3A, MUC5B, MUC12, and MUC13 genes was found to be similar upon infection with both *S. flexneri* strains (Fig. 4).

Collectively, these results indicate that the wild-type strain M90T impairs transcription of specific genes encoding cell surface and gel-forming mucins in cell monolayers in a TTSS-dependent manner, thereby affecting the *de novo* mucin expression.

Virulent *S. flexneri* remodels glycosylation of gel-forming mucins and induces appearance of sulfated oligosaccharides in mucin-producing cells. To analyze whether *S. flexneri* shapes the mucin structure, in addition to modulating mucin expression, we studied the glycosylation pattern of gel-forming mucins upon infection by carrying out a biochemical analysis of the glycans harbored by these proteins. For this purpose, gel-forming mucins trapped and accumulated at the surface of infected cells were gently resuspended in supernatant fractions and collected at successive time points. Then, mucins were purified and glycans were characterized and quantified by MALDI-TOF mass spectrometry (Fig. 5 and Table 1).

Our study identified 12 different glycans present on gel-forming mucins in supernatant fractions from noninfected cells (see Fig. S2 and Table S1 in the supplemental material). Among them, 5 glycans appeared to be strongly dominant, constituting 80% of total oligosaccharides: the TF antigen at m/z 534 (13%), the sialyl TF at m/z 895 (36%), the disialyl TF at m/z 1256 (6.5%), as well as two other glycans at m/z 1344 (7%) and m/z 1705 (19.5%), respec-

FIG 5 Glycosylation of secreted mucin proteins is remodeled by virulent *S. flexneri* upon cell infection. The glycosylation patterns of gel-forming mucin proteins released from cell monolayers infected with *S. flexneri* were analyzed biochemically. Gel-forming mucins trapped at the surface of infected cell monolayers were resuspended in supernatant fractions, collected at successive time points of infection, and used for a quantitative analysis of oligosaccharides by MALDI-TOF mass spectrometry. Values are given in percentages on a logarithmic scale and represent the relative amount of each oligosaccharide calculated on the basis of integration peaks from the mass spectrometry spectra. Black bars, wild-type strain M90T; white bars, *mxiD* mutant strain; *, sulfated oligosaccharides. Results are for three independent pooled samples.

tively. This pattern of oligosaccharides constitutively expressed on gel-forming mucins, as well as their respective proportions, is in agreement with the findings of previous studies carried out on the same cell line (53).

Strikingly, in supernatant fractions from cells infected with the wild-type strain M90T or the *mxiD* mutant, gel-forming mucins were characterized not only by a time-dependent increase in glycan diversity but also, above all, by the appearance of sulfated

oligosaccharides. Qualitatively, we characterized a repertoire of 38 different glycans harbored by gel-forming mucins upon cell infection, including 9 sulfated glycans (*m/z* 663, 867, 1286, 1316, 1419, 1617, 1735, 1765, and 1909) (see Fig. S3 and Table S2 in the supplemental material). Among them, 20 glycans were more abundant on mucin proteins secreted by cells infected with the wild-type strain M90T than on mucin proteins secreted by cells infected with the *mxiD* mutant (Fig. 5 and Table 1), including 6 sulfated glycans (Table 1). Interestingly, some of these oligosaccharides appeared exclusively upon infection with the wild-type strain M90T in a time-dependent manner, such as the glycans at *m/z* 1024, 1419, 1430, 1432, 1518, 1576, 1617, 1765, and 1881, representing more than 5% of total oligosaccharides, as soon as 90 min after infection.

Together, these results demonstrate that the *S. flexneri* wild-type strain M90T induced quick and profound modifications to the glycosylation pattern of mucin proteins secreted by cells upon infection through a TTSS-dependent process, thus qualitatively altering the mucin structure.

DISCUSSION

The gut epithelium forms a continuous lining that acts as a barrier between the outside environment, particularly the gut microbiota, and the host's interior milieu. This barrier protects against colonization, invasion, and systemic dissemination of both symbiotic and pathogenic microorganisms. The epithelium can be seen as three barriers in one: a physical barrier, an innate immune barrier, and an adaptive immune barrier (54). The mutually beneficial relationship between symbionts and the host is a delicate balance that is maintained by appropriate host barrier functions and by specific adaptation of the microorganisms. In order to protect the mucosa, the host produces a thick complex layer of mucus that covers the gastrointestinal tract. The constituents of this mucus layer are rapidly turned over, can respond dynamically to infec-

TABLE 1 Glycans overexpressed on mucins upon virulent *S. flexneri* infection^a

[M + Na] ⁺	Composition
534	1 Hex, GalNAcol
691	1 NeuAc, GalNAcol
779	1 Hex, 1 HexNAC, GalNAcol
867*	1 Hex, 1 HexNAC, 1 SO ₃ , GalNAcol
895	1 Hex, 1 NeuAc, GalNAcol
925	1 Hex, 1 HexNAC, 1 Fuc, GalNAcol
1024	1 Hex, 1 HexNAC, 2 Fuc, GalNAcol
1228	2 Hex, 1 HexNAC, GalNAcol
1256	1 Hex, 2 NeuAc, GalNAcol
1316*	2 Hex, 2 HexNAC, 1 SO ₃ , GalNAcol
1419*	2 Hex, 1 HexNAC, 2 Fuc, 1 SO ₃ , GalNAcol
1430	1 Hex, 1 Fuc, 2 NeuAc, GalNAcol
1432	3 Hex, 2 HexNAC, GalNAcol
1518	2 Hex, 1 HexNAC, 1 Fuc, 1 NeuAc, GalNAcol
1576	2 Hex, 2 HexNAC, 2 Fuc, GalNAcol
1617*	2 HexNAC, 2 Fuc, 1 NeuAc, 1 SO ₃ , GalNAcol
1675	1 Hex, 1 HexNAC, 1 Fuc, 2 NeuAc, GalNAcol
1765*	3 Hex, 3 HexNAC, 1 SO ₃ , GalNAcol
1881	4 Hex, 3 HexNAC, GalNAcol
1909*	2 Hex, 3 HexNAC, 2 Fuc, 1 SO ₃ , GalNAcol

^a Symbols and abbreviations: *, sulfated oligosaccharides; Fuc, fucose; GalNAcol, reduced *N*-acetylglactosamine; Hex, hexose; HexNAC, *N*-acetylhexosamine; NeuAc, *N*-acetyl neuraminic acid; SO₃, sulfate.

tion, and are regulated by underlying innate and adaptive immune mechanisms. Mucus is a complex fluid that is rich in mucin glycoproteins that provide its viscous properties and a broad range of antimicrobial molecules, including antimicrobial peptides and immunoglobulins. The spatial separation of the microbiota and the epithelium by the mucus layer is the best demonstration of the effectiveness of mucus as a defense barrier (4, 55). However, homeostasis of the epithelial barrier can be subverted by cross talk between pathogenic bacteria and mucosal tissues. Pathogens have developed strategies targeting specific steps of mucin dynamics to allow them to interfere with their secretion, expression, or glycosylation in order to reach the underlying epithelium, where they can initiate disease. For example, *Helicobacter pylori* has been shown to alter mucin exocytosis (56) and to affect mucin biosynthesis (57) and the mucin structure (58), thereby representing a strategy by which the bacterium can favorably modulate the mucus defense barrier.

Secretion of gel-forming mucins by epithelial cells occurs via both constitutive and inducible pathways (59). The constitutive pathway continuously secretes a sufficient amount of mucins to maintain the mucus layer, whereas the regulated pathway affords a capacity of massive discharge of stored mucins as a response to a range of stimuli, including inflammatory cytokines and a variety of microbial products (13, 15, 17). Mucin release occurs immediately and is accompanied by hydration, resulting in expansion in the volume of the secretory granule content (40, 41). Investigation of secretion of gel-forming mucins in cells infected with *S. flexneri* revealed the ability of the pathogen to manipulate this secretory pathway by promoting the trapping and accumulation of mucins at the surface of cells. Strikingly, this process was triggered only by the virulent strain and resulted in the appearance of a dense and tightly bound gel-like structure covering infected cells and favoring the bacterial invasion process. The MUC5AC mucin was identified as the principal component of this structure. Given the observed changes in mucin gene expression and mucin protein glycosylation patterns after virulent *S. flexneri* infection, it is likely that profound changes in the rheological and functional properties of the mucus occurred. Although these changes remain to be evaluated, they could be at the origin of the formation of this gel. Moreover, proteomic studies have shown that mucus contains a large number of proteins, in addition to mucins (60). For many of these molecules, their function within the mucus remains unclear. Therefore, we can speculate that modification of expression of these additional proteins upon *S. flexneri* infection may influence the biophysical properties of mucus. One common feature of enteric pathogens is the presence of flagella, which allow the bacteria to propel themselves within the viscous environment (61). Since *Shigella* lacks expression of flagella and recognized adhesins, one could hypothesize that induction of changes leading to the formation of this gel-like matrix, combined with the ability to actively suppress the production of antimicrobial peptides (62), increases the persistence of bacteria embedded in the mucus and thereby facilitates easier access to the surface of epithelial cells. Furthermore, it could promote the creation of a favorable niche for extracellular survival and, possibly, propagation.

Expression of both gel-forming and cell surface mucin genes is constitutive and inducible in mucosal epithelial cells. The promoters of mucin genes are not fully characterized yet; however, partial analysis indicates the presence of regulatory boxes associated with proinflammatory pathways for human MUC1, MUC2,

MUC3, MUC4, MUC5AC, and MUC5B genes (63–68). Here, transcriptional analysis of infected cells revealed modulation of mucin gene expression by virulent *S. flexneri* in a TTSS-dependent manner. This subversive mechanism led to the dampening of MUC4, MUC5AC, and MUC15 gene expression. A similar modulation of MUC5AC gene expression has already been found in human cells infected with *H. pylori*, as well as in infected rhesus monkeys, in which mucin glycosylation shows strong similarities to that in humans (69, 70). One could hypothesize that modulation of mucin gene expression could critically determine pathogenesis and the host response, in terms of the structural changes to the nature of mucus, both of which would be to the benefit of the pathogen. Given our current understanding of the proinflammatory signaling pathways targeted by the set of *S. flexneri* effector proteins, the mucin-encoding genes are therefore excellent candidates to be regulated by the pathogen. The TTSS of *S. flexneri* allows injection of effectors that have the capacity to target specific steps of activation of key innate response pathways, such as the NF- κ B pathway in epithelial cells. As soon as bacteria establish contact with epithelial cells, these effectors are expressed and secreted into cells, which, at the same time, undergo strong proinflammatory signaling via the Toll-like receptor pathways. This system enables the bacteria to suppress the expression of genes whose promoter contains NF- κ B motifs, as well as possibly other regulatory sequences.

Besides regulation of their secretion and expression, mucins are regulated in terms of their glycosylation. As suggested earlier, changes in mucin glycosylation can be seen as an attempt by the host to alter the pattern of glycosylation that was unable to prevent infection by a pathogen (71, 72). Infection and the associated inflammation can induce changes in the extensive glycosylation pattern of mucins and sulfation, hence influencing their chemical and physical properties and thereby protecting them from the action of bacterial glycosidases (22). On the other hand, mucin glycosylation can be actively shaped by microbes themselves, with the consequence of changing their mucosal niche to their favor and promoting their colonization (58, 73). How this is achieved is just being unraveled. By taking advantage of mass spectrometry-based technology to analyze and quantify complex oligosaccharides, the study of cells infected with virulent *S. flexneri* revealed time-dependent changes of mucin glycosylation motives, an increase in glycan diversity, and the appearance of sulfated forms. One could hypothesize that such fine-tuned dynamic modifications of the mucin glycosylation pattern, found to be induced by virulent *S. flexneri* in a TTSS-dependent manner, modulate host-bacterium interactions to the benefit of the pathogen. As such, because sulfated glycans are ligands for only a few bacterial species (74), the appearance of sulfated glycans upon an infectious process can be seen as an advantage for the colonization of pathogenic bacteria expressing glycosulfatases by favoring establishment of their own niche to the detriment of species that are not equipped with such degradative enzymes. Interestingly, this could be the strategy used by *S. flexneri*, whose genome sequence reveals the existence of at least three genes encoding putative sulfatases, named *yefM*, *yidJ*, and ORF1680 (75).

The nature of mucus is very flexible through the expression and secretion of different mucins, variations in mucin glycosylation, and the cosecretion of mucin-associated molecules and can be adapted to physiological requirements, including in response to bacterial colonization and infection. The responsiveness to cy-

tokines and other immune regulators provides a link between mucin-based defense, innate mucosal immunity, and mucosal inflammatory responses. Supporting the view that the mucus layer is an effective infection barrier whose protection is augmented by the epithelial production of antimicrobial peptides, it seems logical that pathogens have developed mechanisms to subvert these host defense systems (62). Thus, the main strategies for pathogenic bacteria to successfully thwart the mucus layer are to degrade it or to target key elements of the gel-forming capacity of mucins, such as their secretion, expression, and glycosylation. The last three options appear to be the strategies that *S. flexneri*, along with other pathogens, such as *H. pylori*, has evolved, although the mechanisms employed by these two bacteria have to be further characterized. Finding the molecular mechanisms used by *S. flexneri* to dampen mucin gene transcription and reshape mucin protein structures will lead to a better understanding of the pathways that regulate their expression and glycosylation and will provide another example of the contribution of pathogens and their subversive strategies to decipher mechanisms of host mucosal defense.

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