Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection

Sheryl S. Justice*,†, David A. Hunstad**, Patrick C. Seed***, and Scott J. Hultgren*

Departments of *Molecular Microbiology and †Pediatrics, Washington University School of Medicine, St. Louis, MO 63110

Edited by M. J. Osborn, University of Connecticut Health Center, Farmington, CT, and approved November 8, 2006 (received for review July 26, 2006)

To establish disease, an infecting organism must overcome a vast array of host defenses. During cystitis, uropathogenic *Escherichia coli* (UPEC) subvert innate defenses by invading superficial umbrella cells and rapidly increasing in numbers to form intracellular bacterial communities (IBCs). In the late stages of the IBC pathway, filamentous and bacillary UPEC detach from the biofilm-like IBC, fluxing out of this safe haven to colonize the surrounding epithelium and initiate subsequent generations of IBCs, and eventually they establish a quiescent intracellular reservoir. Filamentous UPEC are not observed during acute infection in mice lacking functional Toll-like receptor 4 (TLR4), suggesting that the filamentous phenotype arises in response to host innate immunity. We investigated SulA, a cell division inhibitor associated with the SOS response, to gain insight into the role of filamentous UPEC in pathogenesis. A transcriptional reporter from *P. aeruginosa* revealed spatial and temporal differences in expression within IBCs, and it was active in the majority of filamentous UPEC. Although UTI89 and UTI89 ΔsulA both formed first-generation IBCs equally well, UTI89 ΔsulA was sharply attenuated in formation of second-generation IBCs and establishment of the quiescent intracellular reservoir. The virulence of UTI89 ΔsulA was restored in TLR4-deficient mice, suggesting that filamentation facilitates the transition to additional rounds of IBC formation by subverting innate immune responses. These findings demonstrate that transient SulA-mediated inhibition of cell division is essential for UPEC virulence in the murine model of cystitis.

bacterial filamentation | SOS response | pathogenesis

The interaction between bacterium and host ultimately results in symbiosis, disease, and/or clearance. The innate immune response comprises a myriad of biochemical (e.g., oxidizing agents), cytological (e.g., phagocytic cells), and biomechanical (e.g., exfoliation of epithelial cells) tactics that prevent colonization and eradicate infectious microorganisms. Polymorphonuclear leukocytes (PMNs; neutrophils) are phagocytic cells that represent a first line of defense, and they are recruited from the bloodstream directly to the site of invading bacteria. The primary role for PMNs is the neutralization of invading organisms by using degradative enzymes, reactive oxygen species, and antimicrobial peptides. Phagocytosis of bacteria results in the stimulation of apoptosis within the PMN, modulating inflammation and reducing collateral destruction of host tissues (1). Productive pathogens use multiple mechanisms to evade and/or cope with the stresses presented by these host defenses. Urinary tract infections (UTIs) are an attractive model to study the molecular details of this delicate balance between host and pathogen because the bladder employs diverse tactics to combat infectious organisms. Uropathogenic *Escherichia coli* (UPEC) have adapted multiple mechanisms to evade these host defenses.

UTIs are among the most common infections in the United States. Each year, at least 4 million women seek treatment for UTIs, and ~$1.6 billion will be spent in the diagnosis and treatment of UTIs (2). UPEC are the cause of 70–90% of all community-acquired UTIs (2). Clinicians are frustrated by the rise in antibiotic resistance among organisms that cause UTIs and by frequent recurrences in healthy adult females after an initial UTI. Many women use antibiotics daily to reduce the risk of recurrence only to suffer recrudescence when this prophylactic regimen is discontinued. The use of *in vitro* and *in vivo* models has allowed the description of events that delineate the acute and chronic stages of UTI. Mannosylated uroplakin proteins coating the surface of the superficial umbrella cells of the bladder serve as the receptors for binding and invasion of UPEC by means of the FimH adhesin at the tips of type 1 pili (3, 4). During murine cystitis, intracellular bacterial communities (IBCs) appear initially as rod-shaped, loosely organized colonies with a doubling time of 30 min (5). Between 6 and 8 h postinfection, the IBC matures into a tightly packed, highly organized cluster of coccoid bacteria with a doubling time greater than 45 min. Approximately 16 h postinfection, the bacteria on the outer surface of the IBC regain a rod shape, become motile, detach from the IBC, and eventually they escape from the superficial umbrella cell (5), entering into the bladder lumen to attach to a naive epithelial cell or to leave the host through micturition. During this escape (fluxing), filamentous bacteria (up to 70 μm in length) are observed on the luminal surface (5, 6). In the C3H mouse background, this developmental cascade occurs in two cycles, with the second generation displaying much slower kinetics than the first (5). Exfoliation of the superficial umbrella cells occurs at late stages of the acute infection (6) in an effort to eliminate the bacterial burden. After superficial umbrella cell exfoliation, UPEC establish a chronic, quiescent intracellular reservoir (QIR) within the bladder epithelium (5, 7). Shortly after exfoliation, the urine is sterile on most days, but the bladder tissue maintains a bacterial burden for weeks to months after acute cystitis (7, 8). This QIR resists treatment with oral antibiotics; it is apparently undetected by host immunity, and it can be the source of a recurrent infection (7).

UPEC have developed specific strategies to bypass or overcome host defenses. The bladder epithelium produces cytokines, primarily through stimulation of host Toll-like receptor 4 (TLR4) by bacterial LPS, that recruit PMNs to assist in the elimination of the bacteria. UPEC actively suppress the production of cytokines produced by bladder epithelial cells (9). In addition, the intracellular niche provides a safe haven for UPEC evasion from infiltrating PMNs (5). Finally, our recent data suggest that bacterial filamentation might confer resistance to killing by PMNs (5).

Author contributions: S.S.J. and D.A.H. designed research; S.S.J. and D.A.H. performed research; P.C.S. contributed new reagents/analytic tools; S.S.J., D.A.H., and S.J.H. analyzed data; and S.S.J., D.A.H., and S.J.H. wrote the paper. The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: IBC, intracellular bacterial community; MMC, mitomycin C; PMN, polymorphonuclear leukocyte (neutrophil); QIR, quiescent intracellular reservoir; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

*To whom correspondence should be addressed at the present address: Center for Microbial Pathogenesis, Columbus Children’s Research Institute, 700 Children’s Drive, W532, Columbus, OH 43205. E-mail: justices@ccri.net.

†Present address: Department of Pediatrics, Duke University, Durham, NC 27710.

‡Present address: Department of Pediatrics, Duke University, Durham, NC 27710.

§Present address: Department of Pediatrics, Duke University, Durham, NC 27710.

© 2006 by The National Academy of Sciences of the USA

19884-19889 | PNAS | December 26, 2006 | vol. 103 | no. 52 www.pnas.org/cgi/doi/10.1073/pnas.0606329104
Bacteria have developed multiple strategies to cope with environmental stresses. DNA damage can result from certain environmental stresses or insults (e.g., UV light, oxidative conditions). The SOS response is one such strategy that is designed to inhibit cell division while damaged DNA is repaired, thereby preventing transmission of deleterious mutations to daughter cells (for review, see ref. 10). On a molecular level, mutations resulting in mismatched base pairs produce regions of single-stranded DNA. RecA, the major bacterial recombinase, binds single-stranded regions, and it is activated in the presence of nucleotide triphosphates. Activated RecA stimulates the autoproteolysis of the SOS transcriptional repressor, LexA. LexA represses the transcription of at least 17 genes that are involved in DNA repair, and it has recently been implicated in the regulation of >30 unlinked genes (11). The LexA regulon includes a cell division inhibitor, SulA, to prevent transmission of mutant DNA to new daughter cells. SulA specifically inhibits FtsZ polymerization (12, 13), preventing FtsZ ring formation (14–16) and thereby blocking septation. Once DNA repair is complete, LexA repression of the SOS genes is restored. In addition, Lon, the general cytoplasmic protease, degrades SulA, restoring cell division capacity.

UPEC filamentation may serve as an additional mechanism to evade host innate immune responses. The current study directly tested a role for SulA-mediated cell division inhibition in the pathogenic lifestyle of UPEC. Inactivation of sulA in UPEC sharply attenuated virulence in the murine cystitis model, but only when the TLR4-mediated response was intact, demonstrating that filamentation is a critical component of the bacterial arsenal against the innate immune response. Induction of the SOS response in vitro was not sufficient to protect UPEC from PMN phagocytosis, suggesting that factors in addition to filamentous morphology are required for evasion of innate immunity.

Results

SulA Mediates Filamentation in Vivo. Bacterial filamentation has been proposed as a mechanism of evasion of the innate immune response in murine cystitis (5). In addition, filamentation may play a role in human cystitis because filamentous UPEC are observed in the urine of UTI patients (D. Rosen and S.J.H., unpublished data). To assess directly a role for filamentation in cystitis, the bacterial system responsible for filamentation was investigated. Because PMNs use oxidative molecules to eradicate bacterial pathogens, the oxidative environment of the host inflammatory response could lead to mutation of bacterial DNA. DNA repair would be accomplished by induction of the SOS response, ultimately leading to bacterial filamentation caused by the cell division inhibitor, SulA (17). Therefore, a kanamycin cassette disruption of sulA was transduced into our prototypic cystitis UPEC isolate, UTI89. Cystitis was induced in 7- to 9-week-old female C3H/HeN mice by transurethral inoculation of 10^7 cfu of statically grown UTI89 or UTI89 ΔsulA. The ability of the sulA mutant to proceed through all stages of the developmental pathway was evaluated by fluorescence microscopy or by recovery of cfu from the bladder. UTI89 and UTI89 ΔsulA were transformed with pCOMGFP (18) for ease of visualization of intracellular bacteria by fluorescence microscopy. During the first 16 h postinfection, the size and morphology of IBCs were indistinguishable between UTI89 and UTI89 ΔsulA (Fig. 1a and b), suggesting that UTI89 ΔsulA is competent for first-generation IBC formation. Consistent with these microscopic results, the total numbers of bacteria recovered at 6 and 16 h from the bladders infected with UTI89 ΔsulA were indistinguishable from bladders infected with UTI89 (Fig. 2a). However, at 16 h, when filamentous UTI89 is readily observed in infected bladders (Fig. 1c), UTI89 ΔsulA appeared only in bacillary form, both intracellularly and on the luminal surface. To characterize more fully the absence of filamentation in UTI89 ΔsulA, bladders coinfected with equivalent numbers of UTI89 and UTI89 ΔsulA/pCOMGFP were studied by fluorescence microscopy at 16 h postinfection. Wild-type UTI89 displayed red fluorescence caused by the counterstaining of whole mounts.
bacterial load was much lower than in wild-type infection at this time point (Fig. 2a). After the second generation of IBC formation, UPEC enter into a QIR (5, 7, 19). Bacteria were not recovered from any of the bladders infected with UTI89 Δ.sulA at 2 weeks postinfection, whereas bladders infected with UTI89 all contained viable cfu, reflecting the presence of a QIR (Fig. 2a) (10^1 to 10^2 cfu per bladder) as observed in our earlier studies (7).

The inability of the sulA mutant to initiate effectively second-generation IBC formation suggests that SulA-mediated bacterial filamentation is important for the transition between generations of IBC formation. Moreover, the transition into a QIR is also preceded by the emergence of a large number of filamentous bacteria on the luminal surface (5). Thus, the pathogenesis of murine cystitis hinges on SulA-mediated filamentation, a phenotype that might underlie reentry into naïve superficial umbrella cells or confer resistance to host immune responses, including PMN engulfment and killing.

**SulA Is Not Required in the Absence of Functional TLR4.** PMN influx into the bladder during UTI is greatly impaired in the C3H/HeJ mouse strain (20) because of a mutation in the cytoplasmic tail of TLR4, blocking transduction of LPS-initiated signals (21, 22). Our previous studies indicated that filamentous UTI89 are not observed during the acute infection in the C3H/HeJ mouse strain (5). Therefore, we hypothesized that inactivation of sulA would have no effect on the course of cystitis in the C3H/HeJ mouse. UTI89 Δ.sulA persisted to the same extent as UTI89 after inoculation into the bladders of C3H/HeJ mice (Fig. 2b) from 6 h to 2 weeks. IBCs formed by UTI89 Δ.sulA in C3H/HeJ bladders were similar in number and architecture at 24 h postinfection to those observed in C3H/HeJ bladders infected with UTI89 (Fig. 1f). Moreover, the ability of UTI89 Δ.sulA to establish a QIR was restored in the Tlr4^−/− mouse strain (Fig. 2b), suggesting that the requirement for SulA-mediated filamentation during IBC maturation and establishment of cystitis relates to evasion of host immunity.

**UPEC Virulence Requires Recovery from Filamentation.** Previous studies indicated that cell division capacity was restored in filamentous UPEC during UTI (5). To determine whether restoration of cell division within the filamentous population is essential for uropathogenesis, lon was inactivated in UTI89 to prevent recovery from SulA-mediated filamentation. The cytoplasmic protease, Lon, degrades SulA to restore FtsZ polymerization and septation after repair of damaged DNA (23). UTI89 Δ.lon displayed a defect in cfu per bladder of 5 orders of magnitude in C3H/HeN mice at 48 h (Fig. 2a), but virulence of UTI89 Δ.lon was restored in the C3H/HeJ mice (Fig. 2b), similar to that observed with UTI89 Δ.sulA infection.

When filamentous UTI89 on the bladder surface is visualized by SEM, the majority of filaments exhibit a smooth surface (5). This observation is consistent with previous studies reporting a smooth surface for *E. coli* that have undergone filamentation mediated by SulA (5, 6). However, occasionally, a partial septum is observed on the surface of filamentous UPEC by SEM. Luktenhaus and colleagues (24) have previously demonstrated that disassembly of preformed septation complexes results in an aborted septum that cannot be used in further septation events. Thus, the majority of daughter cells arising after Lon-mediated restoration of cell division would display the normal cell length that we observed. SulA-mediated division inhibition has been described as a transient process to prevent septation temporarily during the repair of DNA damage (25). Our data suggest that the filamentation that occurs during pathogenesis must also be a transient process and that septation must be restored for continued progression through the IBC cascade during acute infection.

![Figure 2](image-url)
sulA Is Expressed in Vivo. To demonstrate further that SulA is involved in UPEC pathogenesis, the in vivo transcriptional profile of sulA was evaluated. To observe directly expression of sulA during UTI pathogenesis in vivo, the sulA promoter was used to drive expression of the gene encoding GFP from a single copy integrated at the attB site. In vitro, UTI89 attB::P_sulA-gfp exhibited strong fluorescence only on exposure to SOS-inducing agents, e.g., mitomycin C (MMC; data not shown). Expression of sulA was readily observed within a minority of the individual bacteria in early IBCs in C3H/HeN mice infected with UTI89 attB::P_sulA-gfp (Fig. 3 a and b). The number of bacteria within an IBC that expressed sulA varied among IBCs depending on their size and maturation state. Approximately 60% of the >800 IBCs observed within 10 murine bladders infected with UTI89 attB::P_sulA-gfp exhibited differential sulA expression, both temporally and spatially, by confocal microscopy. Fluorescence emitted from GFP was also observed in the majority of filamentous E. coli (Fig. 3 c and d) analyzed in 15 murine bladders infected with UTI89 attB::P_sulA-gfp, consistent with the hypothesis that expression from P_sulA leads to filamentation of UPEC during cystitis. Infection of C3H/HeJ (Thr4++) mice with UTI89 attB::P_sulA-gfp did not lead to detectable expression of sulA within >600 IBCs analyzed (Fig. 3 e and f). Thus, TLR4-dependent innate responses lead to induction of sulA expression, likely reflecting induction of the SOS response caused by host production of DNA-damaging (e.g., oxidative) agents.

SulA Overproduction Does Not Protect from PMNs in Vitro. Our data suggest that SulA is critical in uropathogenesis, but they do not directly address the function of SulA in subverting innate defenses in vivo. Previous studies have suggested that filamentous UTI89 evades PMN killing (5). Current methods do not permit isolation of sufficient numbers of filamentous bacteria from the urine and/or bladders to assess directly the ability of in vivo-produced filamentous UPEC to survive PMN phagocytosis ex vivo. To circumvent this problem in vitro, UTI89 was transformed with a plasmid (pGC165) that encodes sulA under control of P_lac. Filamentous and bacillary UTI89 were opsonized with 50% normal human serum and challenged with purified human PMNs. There was no apparent difference in the ability of the PMNs to engulf and kill the filamentous and bacillary forms of UTI89 (Fig. 4 a and b), suggesting that filamentation per se is not sufficient for protection from PMNs. The observation that size alone is not sufficient to protect the bacteria from engulfment is not surprising, considering that PMNs engulf the much larger hyphal form of Candida albicans (26). The ability of PMNs to engulf the SulA-induced filaments in vitro suggests that there are additional factors coregulated with the filamentous response, needed to subvert PMNs. Such factors might represent other components of the SOS regulon, or they may be produced independent of the SOS response. Alternatively, the extended length might provide a more secure attachment to the bladder epithelium, with increased number of adhesins over a greater surface area that would make removal by PMNs more difficult.

To test whether chemical induction of the SOS response provides protection from PMN phagocytosis in vitro, UTI89 was grown under static conditions with the addition of 300 ng/ml MMC in early log phase at 37°C. PMNs were isolated from both humans and mice as described in ref. 27, and UTI89 was grown in the presence or absence of MMC and opsonized with normal serum or mixed directly with human or mouse PMNs. As visualized by microscopy, both human and mouse PMNs were able to engulf both opsonized (data not shown) and nonopsonized (Fig. 4 c-f) filamentous and bacillary UTI89 readily. From these in vitro data, it appears that a putative bacterial component for protection is not induced by brief exposure to a chemical DNA-damaging agent. Additionally, it is unknown whether the observed induction of sulA in vivo occurs as a component of the SOS response. LexA-dependent induction mediated by DNA damage is the only known mechanism that leads to sulA expression. Recently, microarray analysis of the SOS response in laboratory (K-12) strains of E. coli have demonstrated that additional genes are transcriptionally active on initiation of DNA damage (11). Given the increased size of the UTI89 genome
relative to E. coli K-12 (28), it is likely that additional genes may be present in the SOS regulon within pathogenic organisms. However, the presence of other signals that induce sulA in vivo cannot be eliminated. SOS induction for periods longer than 4 h may result in the expression of additional molecules that may be protective. The putative bacterial component for PMN protection may be produced in response to the same signal that results in $P_{\text{sulA}}$ expression, or it may occur through an alternative signal that occurs coincidently with the signal that induces $P_{\text{sulA}}$ in vivo.

Discussion

The data presented here provide strong evidence that filamentation, mediated by SulA, is a vital component of the UPEC pathogenic cascade within an immunocompetent host. The transition of UPEC from the first to second generation of IBC formation depends on SulA-mediated division inhibition and Lon-mediated restoration of cell division competence. This conclusion is based on the observation of a rapid decline in cfu per bladder 24 h postinfection, which is the time point associated with formation of second-generation IBCs (5). The restoration of virulence of UTI89 ΔsulA in C3H/HeJ mice argues that SulA-mediated filamentation protects UPEC from components of host innate defenses. However, overproduction of SulA or chemical induction of the SOS response was insufficient to confer protection from phagocytosis in vitro. Although sulA is expressed in vivo during cystitis, the mechanism of its induction is unknown. We propose that components of the innate immune response (e.g., oxidative stresses) likely induce the SOS response in vivo but that sulA induction in vivo might instead rely on signals independent of the SOS pathway. Our data suggest that the filamentous phenotype that is observed at the transition between stages of murine cystitis is more than a morphologic one and that filamentation may provide multiple means of protection for UPEC. For example, previous reports have suggested that induction of the SOS response and subsequent SulA-mediated division inhibition protect against antibiotics that act on dividing cells (29). In addition, the potential roles of UPEC filamentation in resistance to killing mediated by neutrophil extracellular traps (30) and by antimicrobial peptides such as cathelicidin (31) warrant further investigation.

In summary, we have demonstrated that in response to innate immunity, UPEC employs filamentation as a means to evade this immune response. Characterization of putative host signals and further understanding of the regulation of sulA will offer insight into the pathogenesis of diverse bacterial infections and will foster the design of novel therapeutic agents.

Materials and Methods

Strains and Media. Bacterial strains were grown in Luria broth statically at 37°C for 16 h to facilitate type 1 pilus production (32). UTI89 is a clinical UPEC isolate obtained from a patient with cystitis (8). The sulA:kan insertional mutation (33) was transduced into UTI89 by using P1 transduction (34), and transductants were selected by using 50 μg/ml kanamycin sulfate. Transcriptional activity of sulA in vivo was assessed by introduction of the sulA promoter driving expression of the gene encoding for green fluorescent protein (gfp) in single copy into UTI89. Briefly, the sulA promoter region (~322 to +51 relative to the start ATG) was amplified by PCR from UTI89 genomic DNA by using primers ACTGTATCGATCAACGGTCAGGCTGTA-322 to GAGAACGACGAATCA. The 373-bp product was restricted using primers ACTGTATCGATCAACGGTCAGGCTGTA-322 to ACTACCAAA and ATGAACCCGGGTGCTGTTGAT-GAGAACCAGCAATCA. The 373-bp product was restricted with Clal and Smal, cloned into the same sites of pPSSH10, sequenced for verification, and integrated into the λ attachment site of E. coli MG1655 as described in ref. 35. The fusion was moved into UTI89 by P1 phage transduction. Plasmid pGC165 contains sulA under the control of the Plac promoter (36). To induce the SOS response in vitro, saturated cultures were diluted 1:1,000 and allowed to grow with aeration or statically at 37°C until they reached early log phase (A600 = ~0.3). MMC (Sigma–Aldrich, St. Louis, MO) was added to a final concentration of 300 ng/ml, and growth continued under the same conditions for 2–4 h.

Fig. 4. PMN phagocytosis in vitro. Human and mouse PMNs were incubated with UTI89 under inducing conditions for 20 min, and they were visualized by using phase microscopy (a, b, e, and f) or fluorescent microscopy (c and d). (a) Oposonized, uninduced UTI89 with human PMNs. (b) Oposonized induced UTI89 Plac::sulA (c and d) MMC-induced UTI89 with mouse PMNs. (e) Uninduced UTI89 with human PMNs. (f) MMC-induced UTI89 with human PMNs. (Scale bars: a–d, 30 μm; e and f, 50 μm.)
**Murine Cystitis.** Female C3H/HeN (Harlan, Indianapolis, IN) or C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) mice, 7–9 weeks old, were infected with $10^7$ cfu of *E. coli* transurethrally, in a volume of 50 μl as described in ref. 6. At the times indicated, bladders were aseptically harvested and homogenized for bacterial enumeration; or they were bisected, splayed, and fixed in 3% paraformaldehyde for 1 h for visualization by laser scanning confocal fluorescent microscopy (Zeiss LSM510 Meta; Carl Zeiss, Inc., Thornwood, NY). Fixed bladders were treated with 0.01% Triton X-100 in PBS for 10 min, and then they were counterstained with a red fluorescent monomeric cyanine nucleic acid dye (ToPro3; Molecular Probes, Eugene, OR) for an additional 10 min. Three-dimensional image reconstruction was performed by using Volocity software (Improvision, Lexington, MA).

**PMN Isolation.** Human blood was collected from peripheral veins of healthy donors in accordance with a protocol approved by the Justice et al. PMN Isolation. Female C3H/HeN (Harlan, Indianapolis, IN) or C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) mice, 7–9 weeks old, were infected with $10^7$ cfu of *E. coli* transurethrally, in a volume of 50 μl as described in ref. 6. At the times indicated, bladders were aseptically harvested and homogenized for bacterial enumeration; or they were bisected, splayed, and fixed in 3% paraformaldehyde for 1 h for visualization by laser scanning confocal fluorescent microscopy (Zeiss LSM510 Meta; Carl Zeiss, Inc., Thornwood, NY). Fixed bladders were treated with 0.01% Triton X-100 in PBS for 10 min, and then they were counterstained with a red fluorescent monomeric cyanine nucleic acid dye (ToPro3; Molecular Probes, Eugene, OR) for an additional 10 min. Three-dimensional image reconstruction was performed by using Volocity software (Improvision, Lexington, MA).

**PMN Isolation.** Human blood was collected from peripheral veins of healthy donors in accordance with a protocol approved by the Justice et al. PMN Isolation. Female C3H/HeN (Harlan, Indianapolis, IN) or C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) mice, 7–9 weeks old, were infected with $10^7$ cfu of *E. coli* transurethrally, in a volume of 50 μl as described in ref. 6. At the times indicated, bladders were aseptically harvested and homogenized for bacterial enumeration; or they were bisected, splayed, and fixed in 3% paraformaldehyde for 1 h for visualization by laser scanning confocal fluorescent microscopy (Zeiss LSM510 Meta; Carl Zeiss, Inc., Thornwood, NY). Fixed bladders were treated with 0.01% Triton X-100 in PBS for 10 min, and then they were counterstained with a red fluorescent monomeric cyanine nucleic acid dye (ToPro3; Molecular Probes, Eugene, OR) for an additional 10 min. Three-dimensional image reconstruction was performed by using Volocity software (Improvision, Lexington, MA).