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ZapA, a Virulence Factor in a Rat Model of *Proteus mirabilis*-Induced Acute and Chronic Prostatitis▽

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Our knowledge of pathogenesis has benefited from a better understanding of the roles of specific virulence factors in disease. To determine the role of the virulence factor ZapA, a 54-kDa metalloproteinase of *Proteus mirabilis*, in prostatitis, rats were infected with either wild-type (WT) *P. mirabilis* or its isogenic ZapA$^-$ mutant KW360. The WT produced both acute and chronic prostatitis showing the typical histological progressions that are the hallmarks of these diseases. Infection with the ZapA$^-$ mutant, however, resulted in reduced levels of acute prostatitis, as determined from lower levels of tissue damage, bacterial colonization, and inflammation. Further, the ZapA$^-$ mutant failed to establish a chronic infection, in that bacteria were cleared from the prostate, inflammation was resolved, and tissue was seen to be healing. Clearance from the prostate was not the result of a reduced capacity of the ZapA$^-$ mutant to form biofilms in vitro. These finding clearly define ZapA as an important virulence factor in both acute and chronic bacterial prostatitis.

Proteus mirabilis can be an important infectious agent of the urinary tract that can lead to pyelonephritis or renal calculus, especially in those who are catheterized or who have anomalies in their urinary tract (16, 24, 31). ZapA, which is an extracellular metalloprotease, is one of many well-characterized virulence factors important in urinary tract infections caused by *P. mirabilis* (3, 16, 26, 27). ZapA is a broad-spectrum protease which degrades a number of substrates, including immunoglobulin A (IgA) and defensins of the host's immune system (26, 27). Swarmer cell formation, which is important in the pathogenesis of this organism, results in the upregulation of many *P. mirabilis* virulence factors, including ZapA (1, 27). This appears to be influenced by the ability of *P. mirabilis* to sense surfaces (4), and, not surprisingly, swarmer cell formation is also seen in biofilm formation in artificial urine (12), which may explain the link of pathogenesis to catheterization and anomalies in the urinary tract, where biofilm formation will be encouraged.

Prostatitis is the leading cause for men under 50 years of age to seek treatment from a urologist. The etiology of this disease remains confusing, as bacteria are isolated from only 5 to 10% of clinical cases; however, this is believed to represent a gross underestimation of infection (18, 20). Bacterial prostatitis occurs both as an acute febrile disease that as often responsive to antibiotic treatment and as a chronic infection that is too often nonresponsive to antibiotic treatment and is believed to involve bacterial biofilm formation. Bacterial prostatitis is believed to be the leading cause of recurrent urinary tract infections in men (18, 20). Our lab has established models of bacterial and nonbacterial prostatitis in rats (7, 13, 19) and has demonstrated biofilm formation at the mucosal surface of the prostatic acini in this model (5).

It has been well established that distinct virulence factors are important in both upper and lower urinary tract infections and that defined uropathogenic *Escherichia coli* exists (14); however, much less is known about the role of virulence factors in acute and chronic prostatitis. We have previously demonstrated a role of cytotoxic necrotizing factor type 1 (CNF1) as a virulence factor in *E. coli*-induced prostatitis (22). In the present study we have characterized the role played by ZapA as a virulence factor in both acute and chronic prostatitis by comparing infections by wild-type (WT) *P. mirabilis* and its isogenic ZapA$^-$ mutant in our rat prostatitis model.

MATERIALS AND METHODS

Bacteria, media, and culture conditions. *Proteus mirabilis* BB2000 and its isogenic ZapA-deficient mutant KW360 have previously been well characterized (26). Both strains were stored at −70°C in Microbank vials (Pro-Lab Diagnostics, Toronto, Ontario, Canada) according to the manufacturer's instructions. Second subcultures grown on LB agar were used to establish a culture of 1×10^8/cells per ml in saline, as determined by McFarland standards, to be used as an inoculum for the animal model. Bacterial numbers were later reconfirmed by plate counts.

Biofilm growth. Growth of *P. mirabilis* BB22000 and KW360 as biofilms was carried out using the Calgary biofilm device (Innovotech Inc., Edmonton, Canada) according to our published procedures (6). Simply, bacteria were brought to a McFarland standard of 1 in LB broth and diluted 1/20 in LB before being added to the MBEC P & G plate for growth. At each time point, three pegs were removed from the lid, sonicated, and plated to determine the biofilm growth curve of each isolate.

Prostatitis model. The rat model of prostatitis described previously (5, 7, 13, 19) was employed in this study. Briefly, male Sprague-Dawley rats of approximately 300 g were obtained from the Life and Environmental Sciences Animal Resource Center of the University of Calgary. The rats were maintained in polycarbonate box cages in corncob bedding and housed at 20 ± 2°C and 40% ± 10% relative humidity with 12 h of daily illumination. Animals were provided rat chow and water ad libitum. Rats were anesthetized with 4% halothane prior to transurethral catheterization with a lubricated sterile PE10 polyethylene feeding

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tube. A volume of 0.2 ml of bacterial suspension, prepared as described above, was administered through the catheter to the base of the prostate. *P. mirabilis* strains BB2000 and KW360 were each instilled into six rats per experimental group, with three independent experiments (for both acute and chronic infections, $n = 18$ for ZapA$^-$ and WT infections). For the acute-infection model, animals were asphyxiated with CO_2 on day 2 following infection; prostates from chronically infected animals were collected on day 8 postinfection. Protstates from one set of animals were later collected on day 12 postinfection to follow the reduction in bacterial counts (data not shown). Approval for the studies was granted by The Life and Environmental Sciences Animal Care Committee in accordance with guidelines of the Canadian Council of Animal Care.

Tissue preparation. The ventral prostate was sterilely removed from the animal, photographed, and scored by a trained, blinded observer for gross morphology, with scoring for edema, hyperemia, and congestion (scored from 0 [no change from normal] to 3 [most severe] for each component, giving a total possible score of 9). The tissue was divided into four pieces; one was homogenized in sterile saline to determine bacterial counts/g tissue, one was immediately frozen and stored at −70°C for later cytokine analysis, one was fixed in 10% neutral buffered formalin for histological processing, and one was stored on ice for immediate myeloperoxidase (MPO) determinations.

Histological sections. Tissue fixed as described above was embedded in paraffin for sectioning and staining with standard hematoxylin and eosin protocols. Sections were scored for edema, neutrophil infiltration in the lumen, neutrophil infiltration in interstitial spaces, and integrity of epithelium, with each criterion assigned a value from 0 (no change from normal) to 3 (most severe). These scores were totaled to give a final histology of score from 0 to 9.

MPO assay. Prostate tissue was homogenized in hexadecyltrimethyl ammonium bromide buffer and then sonicated. The homogenates were centrifuged, the supernatants were mixed with *o*-dianisidine in phosphate buffer and assayed at 450 nm, and the activity per mg protein was recorded (17).

Cytokine assays. Tissue to be screened for cytokine levels was homogenized in phosphate-buffered saline (PBS) containing 1 μg/ml each of the protease inhibitors leupeptin, pepstatin A, and aprotinin as previously reported (8). The homogenates were centrifuged at 1,800 × *g* for 10 min and supernatants used for assays. Assays were performed using commercial kits for rat interleukin-1β (IL-1β) (R&D Systems) and rat Gro/CINC-1 (Amersham) according to the manufacturer's instructions.

Statistical analysis. Group data are expressed as mean values ± standard errors of the means (SEM). Figures and statistical analyses were compiled using GraphPad Prism v3.00 software (GraphPad Software). Morphological and histological scores were analyzed by the nonparametric Kruskal-Wallis analysis of variance or ranks test and Dunn's multiple-comparison test of groups. The results of enzyme-linked immunosorbent assays and MPO assays from in vivo experiments were $\log_{10}$ transformed and analyzed by one-way analysis of variance and Tukey's multiple-comparison test. *P* values of <0.05 were considered statistically significant.

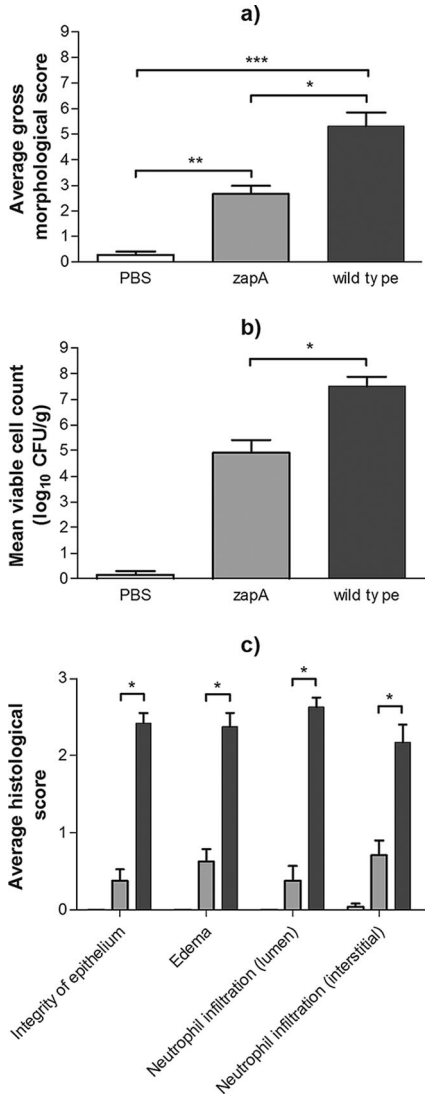

FIG. 1. Severity of acute prostatitis. *P. mirabilis* strains BB2000 (WT) and KW360 (isogenic ZapA$^-$ mutant) were compared for gross morphological scores, bacterial numbers in prostatic tissue, and tissue histology scores. (a) Gross morphological scores for day 2 prostates instilled with saline (PBS), the ZapA$^-$ mutant, or WT *P. mirabilis* as described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (b) Bacterial numbers in prostates instilled with sterile saline, the ZapA$^-$ mutant, or WT *P. mirabilis*. *, $P < 0.05$. (c) Histological scores for prostates instilled with sterile saline (open boxes), the ZapA$^-$ mutant (stippled boxes), or WT *P. mirabilis* (filled boxes). Error bars indicate SEM.

RESULTS

Role of ZapA in acute infection. Our rat model of prostatitis is initiated as an ascending infection, as is believed to be the case in human disease, and follows a histological progression over time that mirrors the histological progression seen in the human disease (reviewed in reference 5). On the basis of this histological time line, day 2 postinfection was defined as typical of an acute infection. Gross morphology, bacterial numbers, and tissue histology all supported the conclusion that infection with the WT BB2000 was more severe than infection with its isogenic ZapA$^-$ mutant KW360. Gross morphology scores (Fig. 1a), which included values for edema, hyperemia, and congestion, for prostates infected with the WT (5.5/9) were greater than those for ZapA$^-$ mutant-infected prostates (3.0/9), and both scores were significantly higher than those seen for saline-administered controls (0.4/9; $P < 0.01$ and $P < 0.001$ for mutant and WT, respectively). Numbers of WT bacteria in the prostate were 5×10^7/g of tissue, while the ZapA$^-$ cell numbers were significantly lower, at only 3×10^5/g (Fig. 1b) ($P < 0.05$). Tissue histology scores for control, WT-infected and ZapA$^-$ mutant-infected prostates (Fig. 1c and 2a, b, and c) again clearly showed a difference in the disease process. The histological scoring of these sections (Fig. 1c) ($P < 0.05$) showed a significant difference in the metrics used to measure the degree of pathology seen between WT and ZapA$^-$ infections, supporting the importance of ZapA as a virulence factor in prostatitis. For example, WT-infected prostates showed high levels of edema, neutrophil infiltration into both stromal and glandular tissue, and the breakdown of the membranes of the glandular structure (Fig. 2b), whereas prostates taken from ZapA$^-$ mutant-infected animals showed lower levels of edema

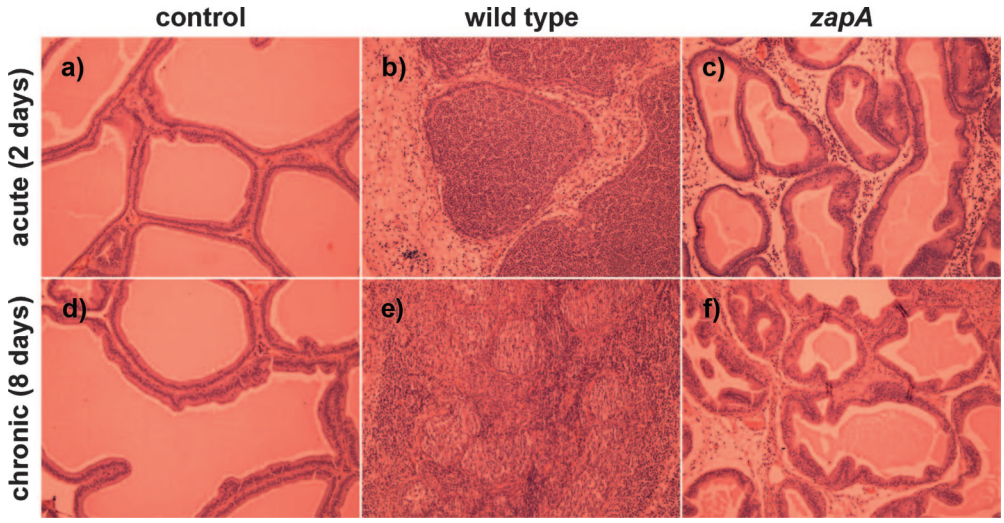

FIG. 2. Tissue histology of acute and chronic *P. mirabilis* prostate infections. (a to c) Sections stained as described in Materials and Methods, showing the typical appearance of saline-treated controls (a) and WT (b)- and ZapA$^-$ mutant (c)-infected prostate tissue in acute infection. (d to f) Histology of chronic infection for saline controls (d), WT infection (e), and ZapA$^-$ mutant infection (f).

and interstitial neutrophils, with very few immune cells in the glandular component of the tissues and little loss of membrane integrity (Fig. 2c); however, these changes seen in ZapA$^-$ infection were still significantly different from the case for the control animals given saline (Fig. 2A).

ZapA and the host response to acute infection. The differences seen in the levels of pathogenesis between WT *P. mirabilis* and its isogenic ZapA$^-$ mutant were also reflected in the difference in the level of immune response seen in the acute state of infection. MPO activity, commonly used to quantify neutrophil involvement in inflammation, was seen to be fourfold lower in the ZapA$^-$ compared to WT infections and was not significantly above background levels seen in saline-injected animals; however, MPO levels in WT-infected animals were significantly higher than those in control and ZapA$^-$ mutant infected animals (Fig. 3a) ($P < 0.05$). Levels of the proinflammatory cytokine IL-1β and the chemokine Gro/CINC-1, the rat analogue for human IL-8, were both measured using commercial assay kits as markers to compare host response to infection by the WT and the ZapA$^-$ mutant. As with MPO levels, the IL-1β and Gro/CINC-1 responses were significantly higher in WT infections than in those mediated by the ZapA$^-$ mutant ($P < 0.05$ for IL-1β and $P > 0.001$ for Gro/CINC-1); however, levels in ZapA$^-$ mutant-infected animals were significantly higher than those in the saline-injected control animals for Gro/CINC-1 ($P < 0.05$) but not for IL-1β (Fig. 3b and c). These findings therefore suggest that while the host response to infection by *P. mirabilis* lacking ZapA is much reduced from that seen in WT infections, the response is still significant.

Role of ZapA in chronic infection. The time point for chronic infection is again based on the histological comparison between the rat model and the disease progression as seen in humans. Gross morphology scores for prostates collected on day 8 postinfection can be seen in Fig. 4a. Based on the same criteria used in the evaluation of the acute infections, both WT- and ZapA$^-$ mutant-infected prostates showed significant

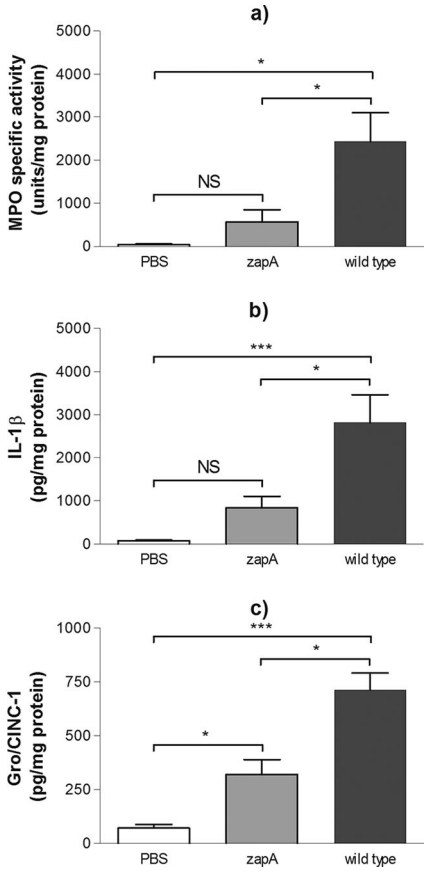

FIG. 3. Host response to acute infection with *P. mirabilis* (saline, $n = 9$; ZapA$^-$ and WT, $n = 18$). (a) MPO levels in prostates from rats instilled with saline (PBS), the ZapA$^-$ mutant, or the WT. *, $P < 0.05$; NS, not statistically different. (b) IL-1β levels in prostates treated as for panel a. *, $P < 0.05$; ***, $P < 0.001$; NS, not statistically different. (c) Gro/CINC-1 levels in prostates treated as for panel a. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate SEM.

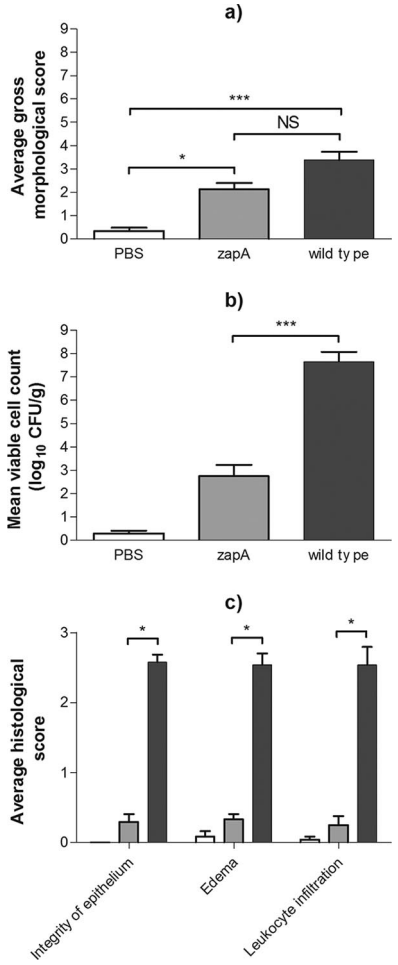

FIG. 4. Severity scores for chronic prostatitis (day 8 postinfection) seen in saline controls, ZapA$^-$ mutant and WT infections. (a) Gross morphology scores for day 8 prostates instilled with sterile saline (PBS), the ZapA$^-$ mutant, or the WT. *, $P < 0.05$; ***, $P < 0.001$; NS, not statistically different. (b) Bacterial numbers in prostates (day 8 postinfection) instilled with sterile saline, the ZapA$^-$ mutant, or the WT. ***, $P < 0.001$. (c) Histology scores of day 8 prostates instilled with sterile saline (open boxes), the ZapA$^-$ mutant (stippled boxes), or the WT (filled boxes). *, $P < 0.05$. Error bars indicate SEM.

differences from those of saline-injected control animals. Both scores were much reduced compared to those at the acute stage of the disease, as the prostate begins to atrophy and shrink in chronic infection. The scores were no longer significantly different between WT- and ZapA$^-$ mutant-infected prostates; however, the similar scores may not reflect similar states of infection, as can be seen looking at bacterial numbers derived from infected prostates. WT-infected prostates had bacterial numbers similar to those seen at day 2 postinfection, i.e., approximately 6×10^7 CFU/g of tissue, while in the ZapA$^-$ mutant-infected rats the bacterial numbers had decreased to only 1×10^2 CFU/g of tissue (Fig. 4b) ($P < 0.001$). This would suggest that the ZapA$^-$ isogenic mutant was not able to maintain itself in the prostate to produce a chronic infection as had the WT *P. mirabilis*. This contention is supported by the histological data (Fig. 4c) ($P < 0.05$), where scores for loss of membrane integrity, edema, and leukocyte infiltration were significantly higher for WT infection than for infection with the ZapA$^-$ mutant, where the scores had fallen to mean levels not significantly different from those for controls. Again, for example, WT sections (Fig. 2e) demonstrate the typical loss of tissue architecture seen in chronic infections in both rats (7, 13, 19) and humans (5), while the sections derived from the ZapA$^-$ mutant-infected rats appear to have essentially healed and only light signs of edema and few inflammatory cells can be seen in even the sections with the highest scores (Fig. 2f). To determine if the progression to healing continued, rats were also examined on day 12 postinfection, where prostates taken from ZapA$^-$ mutant-infected rats were seen to have no significant changes from those from saline-injected animals, while WT-infected animals continued to show loss of tissue architecture (data not shown).

ZapA and the host response to chronic infection. As seen with the acute phase of infection, the level of immune response was related to the degree of pathogenesis, and therefore the response to the WT chronic infection was high and that to the ZapA$^-$ infection was diminishing as the infection cleared. MPO levels were lower in the chronically WT-infected prostate than in the acute WT infection; however, the values were significantly higher than the MPO levels seen in chronically ZapA$^-$ mutant-infected tissue, where levels had returned to control levels (Fig. 5a) ($P < 0.01$). Similarly, values for both IL-1β and Gro/CINC-1 were significantly elevated in the WT-infected prostates compared to in ZapA$^-$ mutant-infected prostates ($P < 0.05$), but levels did not significantly differ between the ZapA$^-$ mutant-infected and control tissues (Fig. 5b and c). It should be noted that IL-1β levels had decreased in WT infections from day 2 to day 8, as would be expected over time; however, Gro/CINC-1 levels had gone up with increased cellular infiltration. This would again support the hypothesis that the ZapA$^-$ mutant was being cleared from the prostate and that the inflammation had subsided in response.

ZapA and biofilm formation. The chronic prostatitis initiated by WT *P. mirabilis* is in stark contract to the clearance of the ZapA$^-$ isogenic mutant and the corresponding healing which followed. To determine if differences in biofilm development between the WT and the ZapA$^-$ isogenic mutant could account for the differences in bacterial clearance, biofilm formation was followed using the Calgary biofilm device (6). No difference in biofilm formation was seen over 24 h of biofilm growth of the WT and the ZapA$^-$ mutant isolates (Fig. 6).

DISCUSSION

In this study, using a rat prostatitis model, we have for the first time verified that the metalloprotease ZapA of *P. mirabilis* is a virulence factor in the urinary tract. ZapA was proposed as a virulence factor due to its substrate specificity for IgA and antimicrobial peptides (27), as well as for its expression during swarmer cell development (26), the morphotype believed to be associated with virulence of *P. mirabilis* in the urogenital tract (16). Here we have directly demonstrated the importance of ZapA in both the acute and chronic stages of prostatitis caused by *P. mirabilis*.

The ZapA$^-$ isogenic mutant (KW360) showed reduced gross morphology scores, lower bacterial numbers, and less severe histological changes (Fig. 1a to c and 2) than the WT

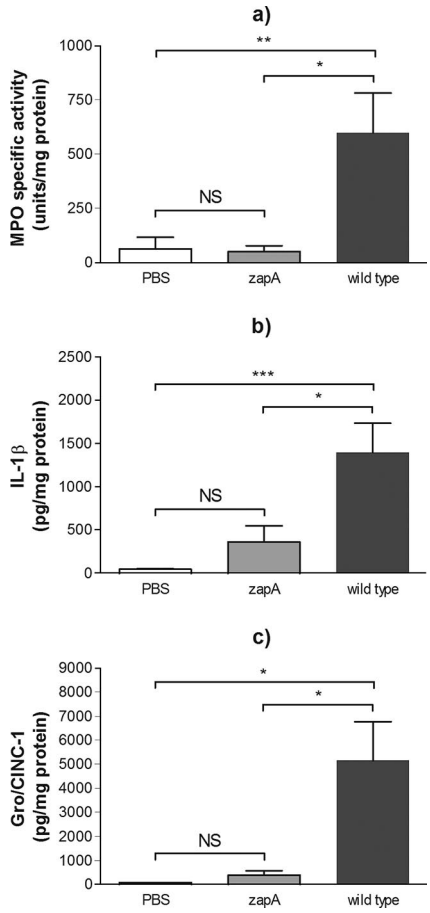

FIG. 5. Host response to chronic infection (day 8 postinfection). (a) MPO values derived from day 8 prostates instilled with sterile saline (PBS), the ZapA$^-$ mutant, or the WT. *, $P < 0.05$; **, $P < 0.01$; NS, not statistically different. (b) IL-1β levels in prostates treated as for panel a. *, $P < 0.05$; ***, $P < 0.001$; NS, not statistically different. (c) Gro/CINC-1 levels in prostates treated as for panel a. *, $P < 0.05$; NS, not statistically different. Error bars indicate SEM.

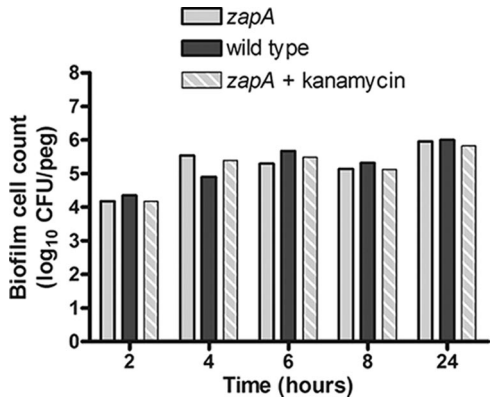

FIG. 6. Biofilm growth curves for the WT and the ZapA$^-$ mutant (with and without antibiotic selection) on the Calgary biofilm device. The data presented are from triplicate samples from a single experiment; similar data were obtained in three independent experiments.

(BB2000), indicating that ZapA is important in acute infection. Importantly, the 2-log-unit reduction in bacterial number (Fig. 1b) compared to the WT parental strain suggests that without ZapA, the ability of *P. mirabilis* to survive in the prostate is compromised. In a previous study using this same model, we demonstrated that CNF1 was also an important virulence factor of *E. coli* in acute prostatitis (22), supporting both epidemiological evidence linking CNF1 to upper and lower urinary tract infections (15, 25) and also experimental findings from a model of ascending urinary tract infection in mice (23). While the level of disease in the prostate was again reduced in animals infected with the isogenic CNF1$^-$ mutant, the results differed in that the isogenic mutant was able to persist in the prostate at the same level as the WT and in fact could compete against the WT in dually infected rats (22). With respect to the host response to infection, the ZapA$^-$ mutant induced a reduced level of host immunological response to infection compared to the isogenic parent. Levels of MPO, IL-1β, and Gro/CINC-1, all proinflammatory markers (Fig. 4a to c), were seen to be lower in ZapA$^-$ mutant infections than in WT infections. While the levels of these proinflammatory mediators were also seen to drop in the CNF1$^-$ mutant infections, the changes were not seen to be significantly different (M. D. Lang, A. D. O'Brien, and H. Ceri, unpublished data). Taken together, these data suggest that ZapA affects acute disease and the host response in prostatitis.

Chronic infection involving WT *Proteus mirabilis* followed the expected disease trajectory characteristic of bacterial prostatitis as seen with *E. coli*, both in this model and in humans as judged by tissue integrity (5). In our model the gross morphology scores were reduced as the prostates typically shrunk in size, the bacterial numbers remained at the levels seen in acute disease, and the tissue histology demonstrated the characteristic loss of architecture, as neutrophil infiltration led to the bursting of the acini and the resultant loss of structure and function of the organ (Fig. 4a to c and 2e). The continuous presence of high levels of bacteria and tissue damage also resulted in sustained expression of the proinflammatory markers MPO, IL-1β, and Gro/CINC-1 (Fig. 5). Infection with the isogenic mutant lacking ZapA showed a mark reduction in the chronic state, and there appeared to be progression toward healing as judged by the gross morphology scores, bacterial numbers, and tissue histology (Fig. 4a to c and 2f). To follow through on this observation, six rats were infected with the WT or the ZapA$^-$ mutant and prostates were collected after 12 days, at which time the ZapA$^-$ mutant-infected animals showed only minor signs of infection and clearance of bacteria (data not shown).

While it is well recognized that renal damage in infection can result from bacterial and host factors (11), the mechanisms by which the metalloproteinase ZapA contributes to virulence are not understood. Proteases, even host derived, may directly induce tissue damage, such as, for example, the matrix metalloproteinases in the estradiol-induced prostatitis model (28). As the ZapA$^-$ mutant was more readily cleared from the prostate than the WT, the simplest explanation for the importance of ZapA in virulence may be its specificity for IgA and human β-defensin 1 (hBD1), thereby preventing immune clearance. We have, however, previously shown that high levels of bacterium-specific IgA in the prostate are not protective

against prostatitis (7), and further, it is not known if ZapA is effective against rat defensins, as ZapA is uniquely specific for hBD1 but not hBD2 (3). The inability to clear infection may also be related to biofilm formation (10), which we previously showed to occur in *E. coli*-induced chronic prostatitis (5).

In vitro biofilm formation has been compared previously using the Calgary biofilm device (2, 9, 21), and in this study no difference in biofilm (Fig. 6) or planktonic growth (data not shown) was seen between the WT and the ZapA$^-$ mutant. While this observation does not rule out differences in biofilm formation in the prostate, it does indicate that there is no inherent inability of the ZapA$^-$ mutant to form biofilms.

Uropathogenic *E. coli* strains possess specific virulence factors that distinguish them from commensal flora and that are clearly linked to infection of the urinary tract (14, 29, 30). Our data demonstrate that in *Proteus mirabilis*, ZapA plays a similar role.

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