A Rare Opportunist, *Morganella morganii*, Decreases Severity of Polymicrobial Catheter-Associated Urinary Tract Infection

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ABSTRACT Catheter-associated urinary tract infections (CAUTIs) are common hospital-acquired infections and frequently polymicrobial, which complicates effective treatment. However, few studies experimentally address the consequences of polymicrobial interactions within the urinary tract, and the clinical significance of polymicrobial bacteriuria is not fully understood. *Proteus mirabilis* is one of the most common causes of monomicrobial and polymicrobial CAUTI and frequently cocolonizes with *Enterococcus faecalis*, *Escherichia coli*, *Providencia stuartii*, and *Morganella morganii*. *P. mirabilis* infections are particularly challenging due to its potent urease enzyme, which facilitates formation of struvite crystals, catheter encrustation, blockage, and formation of urinary stones. We previously determined that interactions between *P. mirabilis* and other uropathogens can enhance *P. mirabilis* urease activity, resulting in greater disease severity during experimental polymicrobial infection. Our present work reveals that *M. morganii* acts on *P. mirabilis* in a contact-independent manner to decrease urease activity. Furthermore, *M. morganii* actively prevents urease enhancement by *E. faecalis*, *P. stuartii*, and *E. coli*. Importantly, these interactions translate to modulation of disease severity during experimental CAUTI, predominantly through a urease-dependent mechanism. Thus, products secreted by multiple bacterial species in the milieu of the catheterized urinary tract can directly impact prognosis.

KEYWORDS UTI, pyelonephritis, bacterial infections, medical devices, catheter, CAUTI, *Proteus mirabilis*, bacteremia, urolithiasis, urease, *Enterococcus faecalis*, *Escherichia coli*, *Morganella morganii*, urinary tract infection, polymicrobial

Urinary catheters are common in health care settings, estimated to be utilized in ~60% of critically ill patients, 20% of surgical unit patients, and 10% of nursing homes residents (1–3). The vast majority of individuals with an indwelling urinary catheter will experience bacteriuria and may progress to catheter-associated urinary tract infection (CAUTI) (1). CAUTI is the most common health care-associated condition in the United States and carries an estimated economic burden approaching \$1.7 billion annually (4).

Bacteriuria in catheterized individuals rapidly becomes polymicrobial, involving combinations of *Proteus mirabilis*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Providencia stuartii*, *Morganella morganii*, *Citrobacter* species, *Staphylococcus* species, and *Streptococcus* species (1, 2, 5). It is estimated that 31 to 86% of CAUTIs are also polymicrobial (6–14); however, urine specimens are often suspected of harboring periurethral or vaginal microbiota if multiple colony types are observed, particularly if Gram-positive organisms are present, and are therefore often dismissed as “contamination” (15, 16). Thus, the clinical significance of polymicrobial bacteriuria is not fully understood.

There are numerous experimental and clinical examples of polymicrobial coloniza-

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tion causing more severe disease than infection with a single bacterial species (monomicrobial), including periodontitis, abdominal abscesses, actinomycosis, chronic wounds, otitis media, pneumonia, cystic fibrosis, inflammatory bowel disease, uncomplicated urinary tract infection, and CAUTI (17–20). In many cases, polymicrobial interactions promote an increased level of colonization for one or both of the bacterial species. It is also possible for a species that is generally perceived as a commensal organism to become an “accessory pathogen” by supporting or enhancing the virulence of a more traditional pathogen (21). In contrast to these disease-promoting interactions, other polymicrobial interactions attenuate disease severity or exclude colonization by traditional pathogens (21). Considering the prevalence of polymicrobial bacteriuria and CAUTI in catheterized individuals, exploration of polymicrobial interactions and their impact on disease severity is necessary.

Our recent analysis of 182 clinical CAUTIs from a 3-year study at 12 nursing homes identified *P. mirabilis* as the most common cause of CAUTI (26% of cases), followed by *Enterococcus* species (21%) and *Escherichia coli* (20%) (22). Of the 182 CAUTIs, 31% were polymicrobial, and the most common bacterial combination was *P. mirabilis* with *E. faecalis*, which is in agreement with prior work regarding the composition of polymicrobial CAUTI (1, 5, 12–14, 22, 23). Notably, *E. faecalis*, *M. morganii*, and *P. stuartii* were significantly more prevalent during polymicrobial CAUTI than monomicrobial infection, and each of these bacterial species has been reported to cocolonize catheters with *P. mirabilis* (1, 5, 12–14, 22, 24). These five species therefore represent important constituents of the polymicrobial CAUTI environment.

P. mirabilis poses a significant challenge for effective CAUTI treatment as *Proteus* isolates are intrinsically drug resistant, exhibiting high tolerance to tetracycline and polymyxin, and clinical isolates are often resistant to aminoglycosides and fluoroquinolones (25). Furthermore, there are increasing reports of isolates producing extended-spectrum β -lactamases and carbapenemases (25–29), which threatens the utility of last-resort antibiotics and increases the mortality rate for *P. mirabilis* infection (30–32). *P. mirabilis* also acts as a “hub” species in catheterized nursing home residents, promoting colonization by additional multidrug resistance organisms (33) and providing protection from antibiotic treatment (34). Furthermore, *P. mirabilis* produces a potent urease enzyme that hydrolyzes the urea in urine to carbon dioxide and ammonia, thereby increasing urine pH and facilitating the precipitation of polyvalent ions and resulting in struvite crystals, catheter encrustation, blockage, and formation of urinary stones (urolithiasis) (35–37). Urolithiasis in humans and animal models of infection can elicit bladder obstruction and renal damage (35, 38, 39), which facilitate sepsis and bacteremia. Indeed, *P. mirabilis* is the causative agent in 13 to 21% of bacteremias experienced by nursing home residents, the majority of which are secondary to CAUTI (9, 40–45). However, there are examples of catheterized patients with prolonged colonization by *P. mirabilis* who do not experience catheter blockage or urolithiasis (12, 46), indicating that the magnitude of struvite crystal formation is likely affected by other factors within the urinary tract environment.

In our prior investigations of *P. mirabilis* urolithiasis and pathogenicity, we determined that numerous common urinary tract colonizers are capable of enhancing *P. mirabilis* urease activity during coculture in urine *in vitro* (47). *E. faecalis*, *E. coli*, and *P. stuartii* were the most potent enhancers of *P. mirabilis* urease activity, and experimental coinfection of *P. mirabilis* with *P. stuartii* in a murine model of complicated UTI resulted in higher urinary pH, a greater incidence of urinary stones, and increased disease severity, all of which were dependent on the presence of a functional urease operon in *P. mirabilis* (47, 48). Thus, the concomitant presence of certain urease-enhancing organisms may correspond to likelihood of developing struvite crystals and urolithiasis during *P. mirabilis* CAUTI. In contrast, *M. morganii* was the only species tested that did not enhance *P. mirabilis* urease *in vitro*, and may have even dampened activity (47), suggesting that this species may be able to decrease risk of struvite formation during polymicrobial colonization and therefore possibly reduce disease severity.

Like *P. mirabilis*, *M. morganii* is a urease-positive organism. However, it produces a

urease enzyme that is distinct from that of *P. mirabilis* (49), and the urine pH change mediated by *M. morganii* urease activity rarely results in development of struvite crystals or catheter blockage (24, 50–52). Interestingly, *M. morganii* is more commonly isolated from unobstructed catheters than blocked catheters in patients catheterized long-term (12), and precolonization of a catheter by *M. morganii* can actually reduce blockage and encrustation by *P. mirabilis* *in vitro* (24), albeit transiently. Taken together, these data support the hypothesis that *M. morganii* may be able to antagonize *P. mirabilis* urease activity and reduce the incidence of struvite crystal formation and urolithiasis during coculture.

In this study, we tested the urease-modulatory capability of *M. morganii* and its impact on disease severity during dual-species and triple-species combinations. We show that *M. morganii* secretes products that decrease *P. mirabilis* urease activity in a contact-independent manner and that the urease-dampening capability of these secreted products can prevent urease enhancement by *E. faecalis*, *P. stuartii*, and *E. coli*. Using a murine model of polymicrobial CAUTI, we further demonstrate that coinfection of *P. mirabilis* with *E. faecalis* dramatically increases disease severity, predominantly due to enhancement of *P. mirabilis* urease activity, and that the addition of *M. morganii* to this polymicrobial combination mitigates disease severity. *M. morganii* was also capable of mitigating cytotoxicity *in vitro*. Thus, identification of the *M. morganii* secreted products that reduce *P. mirabilis* pathogenicity may uncover a novel therapeutic strategy to prevent catheter encrustation, urolithiasis, tissue damage, and secondary bacteremia resulting from *P. mirabilis* catheter colonization.

RESULTS

Uropathogens exhibit comparable competitive fitness during coculture in dilute human urine. To determine whether *P. mirabilis* exhibits cooperative or competitive behavior with *E. faecalis*, *E. coli*, or *M. morganii*, each bacterial species was cultured independently or cocultured in dilute human urine and bacterial viability was assessed by differential plating at hourly time points until the cultures achieved stationary phase (Fig. 1). The viability of *P. mirabilis* was not significantly impacted by coculture with any of the other species (Fig. 1A), and similar results were observed for *E. faecalis* (Fig. 1B) and *M. morganii* (Fig. 1C), indicating that these species do not exhibit competitive behavior during coculture *in vitro*. In contrast, *E. coli* viability was unaffected by *M. morganii* but dramatically reduced during coculture with *P. mirabilis* (Fig. 1D). The reduction in viability appears to be due to urease-dependent production of ammonia, since *E. coli* viability did not decrease during coculture with a *P. mirabilis* urease mutant (Δure). A similar urease-dependent impact on viability was previously observed for *P. stuartii* during coculture with *P. mirabilis* (48). Thus, *E. coli* is sensitive to the increase in urine pH that occurs due to *P. mirabilis* urease activity, but these species do not appear to exhibit any other competition *in vitro*.

***M. morganii* prevents enhancement of *P. mirabilis* urease activity by other bacterial species in a contact-independent manner.** We previously demonstrated that numerous uropathogens, including *E. faecalis*, *P. stuartii*, and *E. coli*, can enhance *P. mirabilis* urease activity during coculture in urine while *M. morganii* appears to dampen activity (47). We therefore sought to determine whether urease modulation by these species requires direct cell-cell contact or whether *P. mirabilis* may be responding to factors present in cell-free supernatants. An example of the experimental approach is shown in Fig. 2A. Briefly, each bacterial species of interest was incubated in filter-sterilized human urine for 90 min, centrifuged to pellet, and filter sterilized. Cell-free supernatants were also generated from cocultures of *M. morganii* with each of the other bacterial species for comparison to 50:50 mixtures of the monomicrobial culture supernatants. In addition, cell-free supernatant was generated from an isogenic urease mutant of *P. mirabilis* HI4320 (Δure) for use as a control for the potential impact of nutrient and cofactor depletion on total urease activity. Wild-type *P. mirabilis* HI4320 was then incubated in each of the cell-free supernatants, and the urease activity was measured over a 120-min time course via a colorimetric assay (Fig. 2B and C). The

FIG 1 Growth of urinary tract isolates in human urine. *P. mirabilis* HI4320 (Pm), *E. faecalis* 3143 (Ef), *M. morganii* TA43 (Mm), and *E. coli* CFT073 (Ec) were cultured in rich laboratory media overnight, diluted 1:100 into filter-sterilized human urine, and incubated at 37°C with aeration for 6 h for hourly assessment of growth and viability by determination of CFU. (A) CFU of *P. mirabilis* from monomicrobial and polymicrobial cultures. (B) CFU of *E. faecalis* from monomicrobial and polymicrobial cultures. (C) CFU of *M. morganii* from monomicrobial and polymicrobial cultures. (D) CFU of *E. coli* from monomicrobial and polymicrobial cultures, including during incubation with *P. mirabilis* Δure. Error bars represent means ± the standard deviations (SD) for three independent experiments. Data were log₁₀ transformed for statistical analyses, and significance was assessed by two-way ANOVA with a Dunnett's multiple-comparison test relative to the monomicrobial cultures. ***, $P < 0.001$.

urease activity was also quantified as the mean change in optical density (OD) per minute for the linear portion of the activity curve (Fig. 2D).

P. mirabilis urease activity was significantly increased during incubation in cell-free spent urine supernatants from *E. faecalis*, *P. stuartii*, and *E. coli* compared to the level of activity for incubation in supernatant from the isogenic Δure mutant, and the activity was significantly reduced during incubation in supernatant from *M. morganii* (Fig. 2B and D). Therefore, modulation of *P. mirabilis* urease activity by each of these species is mediated by a soluble factor present in spent media and independent of direct cell-cell contact. To determine whether either urease-modulatory activity is dominant, we next assessed urease activity in supernatants generated from cocultures of each species with *M. morganii* compared to 50:50 mixtures of the monomicrobial supernatants. The urease activity curves for *E. faecalis* and *M. morganii* are shown in Fig. 2C, and the other coculture urease activity curves are shown in Fig. S1 in the supplemental material. Strikingly, all of the 50:50 mixtures resulted in an intermediate level of urease activity, while all of the coculture supernatants dampened urease activity to a similar level as observed for the *M. morganii* monomicrobial supernatant (Fig. 2C and D). Taken together, these results indicate either that *M. morganii* is able to prevent production of the enhancing signal by each of the other bacterial species or that it inactivates, sequesters, or depletes the enhancing signal from the culture supernatant. In either case, *M. morganii* actively prevents enhancement of *P. mirabilis* urease activity by other bacterial species, in addition to directly dampening *P. mirabilis* urease activity.

***M. morganii* prevents enhancement of *P. mirabilis* cytotoxicity by other bacterial species.** Our prior studies with *P. mirabilis* and *P. stuartii* demonstrated that coculture increased cytotoxicity to HEK293 cells *in vitro* compared to monomicrobial cultures and that this process was not dependent upon urease activity from either species (47). We therefore

FIG 2 Urease activity of *P. mirabilis* during incubation in cell-free urine supernatants. (A) An example of the experimental approach is displayed. Cell-free urine supernatants were generated from monomicrobial cultures of strains of interest (*P. mirabilis* Δure , *E. faecalis* 3143, *E. coli* CFT073, and *M. morganii* TA43) by 90-min incubation in filter-sterilized human urine. Supernatants were also generated from cocultures of *M. morganii* with each of the other bacterial species. *P. mirabilis* HI4320 was then incubated in each of the cell-free urine supernatants supplemented with excess urea, and urease activity was monitored at 60-s intervals over 120 min. (B) *P. mirabilis* urease activity in each of the monomicrobial supernatants (sup). (C) *P. mirabilis* urease activity during incubation in *E. faecalis* and *M. morganii* coculture supernatants compared to a 50:50 mixture of monomicrobial supernatants. Dashed lines indicate the OD₅₆₂ values of wells containing urine and urea in the absence of bacteria to control for pH change due to urea breakdown; error bars represent means $\pm$ the SD from a representative experiment with three to six replicates. (D) Urease activity is expressed as the mean change in OD per minute (mOD/min) for the linear portion of the urease activity curves. The dashed lines indicate the average urease activity for *P. mirabilis* during incubation in *P. mirabilis* Δure supernatant and the background level of pH change due to urea breakdown in the negative-control wells. Error bars represent means $\pm$ the SD from three independent experiments, with at least three replicates each. ns, not significant ($P > 0.05$); ***, $P < 0.001$ (as determined by Student *t* test).

utilized the *in vitro* cytotoxicity assay to determine whether other bacterial species are capable of enhancing *P. mirabilis* cytotoxicity in a urease-independent manner and whether *M. morganii* can prevent enhancement. An example of the experimental approach is shown in Fig. 3A. Briefly, each bacterial species of interest was incubated in filter-sterilized human urine for 90 min, washed twice, and overlaid onto a monolayer of HEK293 cells. After a 4-h incubation, cytotoxicity was assessed by measuring the lactate dehydrogenase (LDH) activity in the cell supernatants. For each polymicrobial condition, a predicted level of cytotoxicity (*P*) was calculated based on the proportion of the bacterial species in the culture and their relative cytotoxicity during monomicrobial culture. The level of cytotoxicity was then expressed relative to that of wild-type *P. mirabilis*, for ease of comparison between conditions.

Cytotoxicity was first compared for wild-type *P. mirabilis*, the isogenic urease mutant (*P. mirabilis* Δure), and *M. morganii* (Fig. 3B). As expected, incubation with the urease mutant resulted in a similar level of cytotoxicity to HEK293 cells as wild-type *P. mirabilis*, confirming that urease does not contribute to cytotoxicity under these conditions. The urease mutant was therefore utilized for all further cytotoxicity experiments. Interestingly, *M. morganii* monomicrobial cultures exhibited a level of cytotoxicity similar to

FIG 3 Urease-independent cytotoxicity of monomicrobial and polymicrobial cultures. Cytotoxicity to HEK293 cells was assessed by LDH release during a 4-h incubation with various single-species, dual-species, and triple-species combinations as indicated. (A) An example of the experimental approach is displayed. Bacteria were cultured in rich laboratory media to mid-log phase, centrifuged to pellet, resuspended in filter-sterilized human urine, and incubated for 90 min at 37°C with aeration prior to infection of HEK293 cells. (B to E) The level of HEK293 lysis under each treatment condition is expressed relative to the level of treatment with *P. mirabilis* Δ ure (dashed line), and the dotted line indicates background cytotoxicity during treatment with sterile urine. For each dual-species and triple-species combination, a predicted (P) level of cytotoxicity was calculated based on the proportion of each bacterial species in the mixture and the cytotoxicity of their respective monomicrobial cultures. For each panel, the predicted value refers to the same combination of organisms present in the coculture bar immediately to the right. (B) Relative cytotoxicity of *P. mirabilis* HI4320, *P. mirabilis* Δ ure, *M. morganii*, and a dual-species culture of *P. mirabilis* Δ ure with *M. morganii*. (C) Relative cytotoxicity of *P. mirabilis* Δ ure, *E. faecalis*, a dual-species culture of *P. mirabilis* Δ ure with *E. faecalis*, and a triple-species culture of *P. mirabilis* Δ ure with *E. faecalis* and *M. morganii*. (D) Relative cytotoxicity of *P. mirabilis* Δ ure, *P. stuartii* Δ ure, a dual-species culture of *P. mirabilis* Δ ure with *P. stuartii* Δ ure, and a triple-species culture of *P. mirabilis* Δ ure with *P. stuartii* Δ ure and *M. morganii*. (E) Relative cytotoxicity of *P. mirabilis* Δ ure, *E. coli*, a dual-species culture of *P. mirabilis* Δ ure with *E. coli*, and a triple-species culture of *P. mirabilis* Δ ure with *E. coli* and *M. morganii*. Statistical significance was assessed by nonparametric Mann-Whitney test for indicated comparisons. ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

that of *P. mirabilis* Δ ure, and coculture had no detectable impact on total cytotoxicity (Fig. 3B).

E. faecalis monomicrobial cultures also exhibited comparable cytotoxicity as *P. mirabilis* Δ ure, but coculture of *P. mirabilis* Δ ure with *E. faecalis* significantly increased cytotoxicity over the level observed for the monomicrobial cultures, as well as the predicted value for the dual-species culture (Fig. 3C). Thus, the interaction between these species appears to enhance production of other virulence factors in addition to increasing urease activity. However, the addition of *M. morganii* dampened cytotoxicity back to a level similar to that observed for each respective monomicrobial culture (Fig. 3C). Taken together, these results indicate that coculture of *P. mirabilis* with *E. faecalis* increases cytotoxicity in a urease-independent manner, and *M. morganii* dampens this enhancement.

Similarly, coculture of *P. mirabilis* Δ ure with a *P. stuartii* urease mutant (*P. stuartii* Δ ure) increased cytotoxicity above the level observed for either monomicrobial culture, as well as the predicted coculture level, which is consistent with our previous work (47). For this coculture condition, *M. morganii* was capable of dampening enhancement back down to the level observed for *P. mirabilis* Δ ure alone, but not quite to the predicted level of activity (Fig. 3D). In contrast, coculture of *P. mirabilis* Δ ure with *E. coli* resulted in an intermediate level of cytotoxicity that was significantly greater than that of *E. coli* alone, as well as the predicted value, but not greater than that of *P. mirabilis* Δ ure alone (Fig. 3E). However, the addition of *M. morganii* had no discernible impact on cytotoxicity for this combination (Fig. 3E). In summary, coculture of *P. mirabilis* with *E. faecalis*, *P. stuartii*, or *E. coli* increases cytotoxicity to various degrees through a urease-independent mechanism, and *M. morganii* is capable of preventing increased cytotoxicity from *E. faecalis* and *P. stuartii*, but not *E. coli*.

FIG 4 Experimental schematic for murine CAUTI. (A) Overview of experimental approach for murine CAUTI. Female CBA/J mice were purchased from Jackson Laboratories at 5 to 7 weeks of age, allowed to acclimate to the vivarium for 1 week, and infected at 6 to 8 weeks of age by transurethral instillation of $50 \mu\text{l}$ of a bacterial inoculum over 25 s. At the time of inoculation, a 4-mm segment of sterile silicone catheter tubing was inserted into the bladder. All mice were weighed daily in the morning to monitor weight loss. After 96 h, urine was collected from all mice for determination of bacterial burden, mice were euthanized, and urolithiasis was assessed by the presence of macroscopic kidney stones. Images 1 and 2 display infected kidneys lacking stones, while images 3 and 4 demonstrate the presence of severe macroscopic kidney stones. Bladders, kidneys, and spleens were also removed for determination of bacterial burden. (B) Overview of bacterial inoculations to establish monomicrobial and polymicrobial infections. All mice were inoculated with 1×10^5 CFU of bacteria: *Proteus mirabilis* HI4320 (Pm, blue), *Escherichia coli* CFT073 (Ec, orange), *Enterococcus faecalis* 3143 (Ef, green), and *Morganella morganii* TA43 (Mm, light blue).

***M. morganii* dampens disease severity during polymicrobial CAUTI.** Our prior studies demonstrated that coinfection of *P. mirabilis* and *P. stuartii* increased disease severity compared to monomicrobial infection in a murine model of UTI and CAUTI. Specifically, enhancement of *P. mirabilis* urease activity by *P. stuartii* resulted in a greater incidence of urolithiasis and bacteremia (47, 48). We therefore hypothesized that coinfection of *P. mirabilis* with *E. faecalis* and *E. coli* may similarly increase disease severity and that *M. morganii* may be capable of preventing progression to severe disease during polymicrobial infection.

To test this hypothesis, we utilized our robust animal model of CAUTI via the experimental approach detailed in Fig. 4. Briefly, female CBA/J mice were transurethrally inoculated with 10^5 CFU of each species alone or dual-species combinations, as follows: (i) *P. mirabilis* with *E. coli*, (ii) *P. mirabilis* with *E. faecalis*, (iii) *P. mirabilis* with *M. morganii*, and (iv) *M. morganii* with *E. faecalis*. A triple-species infection was also conducted for *P. mirabilis* with *E. faecalis* and *M. morganii*, since cocultures involving *E. faecalis* consistently presented the most severe phenotypes *in vitro*. For each mouse, a 4-mm segment of sterile silicone catheter tubing was inserted into the bladder at the time of inoculation to recapitulate CAUTI, as previously reported (47). Weight loss was monitored daily (see Fig. S2 in the supplemental material), and mice were euthanized at 96 h postinoculation (hpi) to quantify bacterial burden (Fig. 5 and Fig. S3) and the incidence of bacteremia and macroscopic kidney stones (Table 1). For each infection group, a subset of mice had the entire bladder and both kidneys sectioned and paraffin embedded for assessment of histopathology rather than quantification of bacterial burden (Fig. 5F to M and Fig. S4).

All mice exhibited weight loss during the first 24 hpi, regardless of infection group, and the majority of mice failed to recover weight during the study. The only group of mice that

FIG 5 Urinary tract bacterial burden and tissue histopathology during monomicrobial and polymicrobial CAUTI. The log₁₀ CFU recovered from the urine, bladder, kidneys, and spleen per gram of tissue or milliliter of urine are shown for monomicrobial infections (A), as are the total CFU recovered from polymicrobial infections (B). Each symbol represents the CFU recovered from a single mouse, gray bars indicate the median, and dashed lines indicate the limit of detection (200 CFU). The number of mice in each infection group is provided in Table 1. For statistical analysis, all CFU data were log₁₀ transformed, and significance was assessed by two-way ANOVA with Tukey's multiple-comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C to M) Bladder and kidney sections from mice inoculated with PBS ($n = 2$), *P. mirabilis* ($n = 5$), *P. mirabilis*-*M. morganii* ($n = 6$), *P. mirabilis*-*E. faecalis* ($n = 8$), or *P. mirabilis*-*E. faecalis*-*M. morganii* ($n = 5$) were paraffin embedded, sectioned, and stained with hematoxylin-eosin to assess tissue damage and inflammation using the scoring rubric provided in Table S1. Bladder scores (C), kidney scores (D), and combined scores for bladder and kidney (E) from each individual mouse are shown. *, $P < 0.05$ (by nonparametric Mann-Whitney test). (F to M) Representative bladder images from select infection groups, including both a low-magnification (2× objective) scanning view (F, H, J, and L) and a higher-magnification detail view (G, I, K, and M). (F and G) PBS/mock-infected mice, normal healthy bladder. (H) *P. mirabilis* monomicrobial infection with severe edema (arrow) and multifocal vasculitis. (I) Detail of vasculitis (arrows) and widespread neutrophilic inflammation. (J) *P. mirabilis*-*E. faecalis* coinfection showing severe edema, neutrophilic inflammation, and ulceration with embedded calculus (arrow). (K) Detail of calculus (arrow) and surrounding inflammation and necrosis. (L) *P. mirabilis*-*E. faecalis*-*M. morganii* coinfection showing intact mucosa and moderate edema (arrow). (M) Detail of intact mucosa.

exhibited recovery from the initial weight loss were those that received an *E. coli* monomicrobial inoculum (Fig. S2A, C, and H). This is likely because monomicrobial inoculation with 1×10^5 CFU of *E. coli* resulted in the lowest bacterial burden of all of the infection groups (Fig. 5A), as well as the lowest overall infection severity (Table 1), followed by the *E. faecalis* monomicrobial infection. No other significant differences in weight loss were observed.

Despite differences in the colonization density achieved by each species during monomicrobial infection (Fig. 5A), there were no significant differences in total bacterial

TABLE 1 CAUTI disease severity^a

	Number of mice	Bacteremia ^A	<i>P</i> value	Urolithiasis ^B	<i>P</i> value
<i>Monomicrobial Infections</i>					
<i>Ec</i>	10	20%	.001*	0%	---
<i>Ef</i>	15	47%	.040*	0%	---
<i>Mm</i>	15	62%	.222	19%	.666
<i>Pm</i>	27	78%	---	26%	---
<i>Polymicrobial Infections</i>					
<i>Pm+Ec</i>	17	82%	.714	29%	.800
<i>Pm+Ef</i>	25	96%	.054	56%	.027*
<i>Pm+Ef+Mm</i>	21	71%	.614	24%	.867
<i>Pm+Mm</i>	26	73%	.691	15%	.344
<i>Ef+Mm</i>	10	70%	.624	10%	.296
<i>Urease-Independent Infections</i>					
<i>Pm Δure</i>	10	40%	.001*	0%	---
<i>Δure+Ec</i>	10	40%	.999	0%	---
<i>Δure+Ef</i>	12	58%	.392	0%	---
<i>Δure+Mm</i>	8	37%	.914	12%	---
<i>Δure+Ef+Mm</i>	12	33%	.746	8%	---

^aAbbreviations: *Ec*, *E. coli*; *Ef*, *E. faecalis*; *Mm*, *M. morganii*; *Pm*, *P. mirabilis*. (Superscript A) Bacteremia was assessed by spleen CFU above the limit of detection. (Superscript B) Urolithiasis was assessed by the presence of macroscopic kidney stones, as shown in Fig. 4. Severity is expressed relative to *P. mirabilis* (white), with more severe being indicated by orange and less severe being indicated by blue. *P* values were determined by chi-square analysis versus *P. mirabilis* monomicrobial infection for all groups except for the urease-independent infections, which were compared to *P. mirabilis Δure* infections.

burden within the urinary tract during any of the polymicrobial infections (Fig. 5B). The CFU recovered for each individual bacterial species from the polymicrobial infections are shown across infection groups in Fig. S3A to D and on a per-mouse basis in Fig. S3E to I. *P. mirabilis* and *E. faecalis* CFU counts were stable across all infection types (Fig. S3A and B), *M. morganii* CFU were reduced in the presence of *P. mirabilis* but unaffected by *E. faecalis* (Fig. S3C), and *E. coli* CFU were augmented by *P. mirabilis* (Fig. S3D). Despite these shifts in bacterial burden for each species, the total combined CFU achieved within the urinary tract were consistent across all of the polymicrobial infections (Fig. 5B).

The similarities in total bacterial burden across infection groups are particularly noteworthy considering that there were dramatic differences in disease severity between infection groups (Table 1). *P. mirabilis* exhibited the greatest disease severity of the monomicrobial infections, with 78% of mice exhibiting bacteremia and 26% urolithiasis. Coinfection of *P. mirabilis* with *E. coli* did not significantly impact the incidence of bacteremia or urolithiasis compared to *P. mirabilis* monomicrobial infection, despite the ability of this combination to increase urease activity and cytotoxicity *in vitro*. In contrast, coinfection of *P. mirabilis* with *E. faecalis* resulted in the most severe disease presentation, since 96% of these mice exhibited bacteremia (an increase of 18% relative to *P. mirabilis* monomicrobial infection, *P* = 0.054) and 56% exhibited urolithiasis (an increase of 30% relative to *P. mirabilis* monomicrobial infection, *P* = 0.027). This coinfection combination also resulted in the greatest incidence of maximum histopathology scores for tissue damage and inflammation in the bladder (Fig. 5C) and the highest combined scores for bladder and kidney damage (Fig. 5E). Representative bladder sections are displayed in Fig. 5F to M, and representative kidney sections are displayed in Fig. S4.

Remarkably, the addition of *M. morganii* to the *P. mirabilis* and *E. faecalis* coinfection dramatically reduced infection severity to a level that was indistinguishable from that seen in mice infected with *P. mirabilis* alone or from mice coinfecting with *P. mirabilis* and *M. morganii* (Table 1). A total of 71% of mice from the triple-species infection

FIG 6 Urinary tract bacterial burden during monomicrobial and polymicrobial infection with *P. mirabilis* Δure . The $\log_{10}$ CFU values recovered from the urine, bladder, kidneys, and spleen per gram of tissue or milliliter of urine are displayed for *P. mirabilis* Δure monomicrobial infection, as are the total CFU recovered from polymicrobial infections. Each symbol represents the CFU recovered from a single mouse, gray bars indicate the median, and dashed lines indicate the limit of detection (200 CFU). The number of mice in each infection group is provided in Table 1. All CFU data were $\log_{10}$ transformed, and no significant differences were identified by two-way ANOVA with Tukey's multiple-comparison test.

exhibited bacteremia (-25% compared to *P. mirabilis*-*E. faecalis* coinfection, $P = 0.0208$) and 24% exhibited urolithiasis (-32% compared to *P. mirabilis*-*E. faecalis* coinfection, $P = 0.0272$). The decrease in disease severity was also clearly evident by histopathology, since the triple-species infection resulted in significantly reduced bladder scores and combined scores compared to the *P. mirabilis*-*E. faecalis* coinfection (Fig. 5C and E). Thus, the ability of *M. morganii* to dampen enhancement of urease activity and cytotoxicity *in vitro* does indeed translate to a reduction in infection severity *in vivo*. Taken together, these data indicate that disease severity during polymicrobial CAUTI is dependent on the effects of the specific coinfecting organisms, rather than bacterial burden alone, and that an organism such as *M. morganii* can reduce infection severity even when present at a low density.

Modulation of polymicrobial infection severity is predominantly mediated through a urease-dependent mechanism. Our prior studies of *P. mirabilis* with *P. stuartii* demonstrated that increased disease severity during coinfection of these species was dependent on the presence of a functional urease operon in *P. mirabilis* and enhancement of urease activity (47, 48). We therefore tested the hypothesis that *P. mirabilis* urease activity is required for modulation of disease severity during polymicrobial infection. Mice were inoculated as described above, only using the isogenic urease mutant of *P. mirabilis* HI4320 (Δure). Fig. S5 shows the percent weight change. Consistent with the prior infections, all mice exhibited weight loss during the first 24 hpi, regardless of infection group. However, as expected, the majority of mice inoculated with the Δure mutant exhibited decreased disease severity compared to infection with the wild-type strain, as indicated by a trend toward weight gain from 72 to 96 hpi.

Figure 6 shows the total CFU recovered from mice inoculated with *P. mirabilis* Δure alone for comparison to each of the polymicrobial infections. The CFU recovered for each individual bacterial species from the polymicrobial infections are shown across infection groups in Fig. S6A to D and on a per-mouse basis in Fig. S6E to H. Similar to infections with wild-type *P. mirabilis*, there were no significant differences in total bacterial burden within the urinary tract between infection groups (Fig. 6). *P. mirabilis* Δure CFU were again largely similar across all infection types (Fig. S6A). Interestingly, *E. faecalis* CFU were significantly decreased in the urine and kidneys when *P. mirabilis* Δure was present (Fig. S6B), which was not observed during infection with the wild-type strain (Fig. S3B), suggesting that *E. faecalis* colonization may be perturbed by *P. mirabilis* in the absence of urease activity. *M. morganii* colonization was again significantly decreased in the urine, bladder, and kidneys when *P. mirabilis* Δure was present, indicating that the impact of *P. mirabilis* on *M. morganii* colonization is urease independent (Fig. S6C). Similarly, *E. coli* colonization was significantly increased when *P.*

mirabilis Δure was present, indicating that urease activity is not required for enhancement of *E. coli* colonization during coinfection (Fig. S6D).

Despite these differences in the colonization density achieved by each individual bacterial species during coinfection, the total bacterial burden within the urinary tract again remained consistent across infection groups (Fig. 6). Compared to monomicrobial infection with wild-type *P. mirabilis*, Δure mutant infections exhibited a significant reduction in the incidence of bacteremia (40% compared to 78%, $P < 0.001$), which is consistent with the role of urease activity and urolithiasis in tissue damage and progression to bacteremia (47, 48). Even more striking was the observation that disease severity remained consistent between monomicrobial infection with *P. mirabilis* Δure and each of the polymicrobial infections, in stark contrast to the results obtained with wild-type *P. mirabilis* (Table 1). Thus, while urease-independent interactions still have the potential to influence disease severity during polymicrobial *P. mirabilis* CAUTI, modulation of urease activity is the main contributor to infection severity in this model system. The mechanism(s) by which *M. morganii* dampens *P. mirabilis* urease activity and prevents enhancement by other bacterial species may therefore elucidate potent inhibitors of infection severity and thus could have clinical utility for CAUTIs involving *P. mirabilis*.

DISCUSSION

It is now widely recognized that microbes generally exist in complex polymicrobial communities rather than living in isolation. There are numerous experimental and clinical examples of polymicrobial interactions increasing disease severity compared to monomicrobial infection (17–20), as well as examples of interactions that attenuate disease severity (21). However, the clinical significance of polymicrobial interactions is less clear in the context of bacteriuria, particularly for individuals with indwelling urinary catheters. In the present study, we show that bacterial species colonizing the catheterized urinary tract can both enhance (*E. faecalis*, *E. coli*, and *P. stuartii*) and dampen (*M. morganii*) disease severity by exerting an effect on *P. mirabilis* and that this process does not require physical contact or close proximity of the bacteria. We further demonstrate that the predominant cause of disease severity is modulation of *P. mirabilis* urease activity, but additional urease-independent factors also contribute to cytotoxicity to host cells.

M. morganii is a common constituent of the intestinal tract and considered to be a rare opportunistic pathogen, mainly causing nosocomial urinary tract infections in elderly catheterized individuals (53). This species has occasionally been identified as a cause of severe UTI-related complications such as sepsis and bacteremia, although it is less common than *P. mirabilis* or *E. coli* and more likely to be present during polymicrobial sepsis than as the sole causative agent of disease (54). Thus, severe disease manifestations, such as entry of *M. morganii* to the bloodstream, are likely facilitated by other bacterial species, particularly within the urinary tract, where *M. morganii* is generally present during polymicrobial infection (6, 55). Patients experiencing *M. morganii* sepsis are also more likely than patients with *E. coli* to have multiple comorbidities and reduced functional status (54), underscoring the opportunistic nature of this organism. It is therefore fascinating that this rare opportunist has such a dramatic impact on the pathogenesis of polymicrobial CAUTI involving *P. mirabilis*. Our results clearly demonstrate that *M. morganii* dampens *P. mirabilis* urease activity and even prevents enhancement by other bacterial species, which is consistent with prior reports of *M. morganii* reducing catheter blockage and encrustation by *P. mirabilis* *in vitro* (24). However, our study further extends these observations to show that *M. morganii* significantly attenuates polymicrobial CAUTI.

Our experimental findings have important clinical implications, particularly for patient populations requiring long-term catheterization. Approximately 50% of individuals catheterized for 28 days or longer experience catheter blockage and/or encrustation from crystalline deposits, the vast majority of which are the result of *P. mirabilis* urease activity (10, 12, 56, 57). These crystalline deposits accumulate on the catheter

FIG 7 Implications of polymicrobial interactions during CAUTI. (A) *P. mirabilis* (blue) monomicrobial colonization. Urease activity from this bacterial species results in crystalline deposits in the bladder (a, bladder inset) and on the catheter (b, catheter inset), promoting bacterial colonization and in some cases bacteremia. (B) Polymicrobial colonization involving *P. mirabilis* and *P. stuartii* (maroon), *E. coli* (orange), or *E. faecalis* (green). Cocolonizing species secrete factors that act on *P. mirabilis* to enhance urease activity, thereby raising urine pH and increasing crystal formation, which increases the risk of catheter encrustation and blockage. Cocolonization with *E. faecalis* also increases the risk of urolithiasis and bacteremia, and *E. coli* colonization is augmented by the presence of *P. mirabilis*. (C) Polymicrobial colonization involving *P. mirabilis* and *M. morganii* (light blue) with *P. stuartii*, *E. coli*, or *E. faecalis*. *M. morganii* secretes factors that act on *P. mirabilis* to dampen urease activity and prevent enhancement by other species, such as *E. faecalis*, thereby reducing the formation of crystals and the risk of catheter encrustation and blockage. The presence of *M. morganii* also decreases risk of urolithiasis and bacteremia during coinfection with *P. mirabilis* and *E. faecalis*. (Catheterized bladder image adapted from reference 78 and from reference 79 with permission of the publisher.)

surface and facilitate biofilm formation (58), a bacterial mode of growth that is associated with immune evasion and antimicrobial resistance (59). Many of the urease-positive organisms that colonize urinary catheters can persist within a catheterized host for months, even after antibiotic treatment and catheter changes (14). Thus, interactions between bacteria within the catheterized bladder and in catheter biofilms have the potential to significantly impact catheter encrustation, tissue damage, urolithiasis, and dissemination to the kidneys and bloodstream.

The implications of our findings, in this context, are depicted in Fig. 7. When *P. mirabilis* is present in the bladder in the absence of other bacterial species, it is capable of causing stone formation and crystalline catheter biofilms, ascending to the kidneys, and potentially disseminating to the bloodstream (Fig. 7A). When an enhancing species is present, such as *P. stuartii*, *E. coli*, or *E. faecalis*, secreted factors produced by these organisms act on *P. mirabilis* to increase urease activity and cytotoxicity. This leads to an increase in tissue damage, urine pH and crystalline deposits, and catheter encrustation and blockage, in addition to promoting urolithiasis and bacteremia. Bladder cocolonization of *P. mirabilis* with *E. coli* also bolsters *E. coli* CFU, which may potentiate its impact on *P. mirabilis* (Fig. 7B). However, when *M. morganii* is present, it secretes factors that counteract the effects of these other microbes on *P. mirabilis* urease activity and cytotoxicity, thereby reducing crystal formation, catheter encrustation and blockage, urolithiasis, and bacteremia (Fig. 7C). Considering the level of activity present in cell-free urine supernatants of these organisms, it is likely that if any of these modulatory organisms are present in the bladder, they would exert an impact on catheter-resident *P. mirabilis*.

A few other dual-species polymicrobial combinations have been characterized in murine models of UTI or CAUTI, although most studies have focused on the impact of coinfection on colonization rather than disease severity. For instance, *E. faecalis* and *Streptococcus agalactiae* are both capable of promoting *E. coli* colonization by sup-

pressing the host immune response (60, 61). Coinfection of *E. coli* with *P. mirabilis* promotes colonization of both species during uncomplicated UTI through a dampening of the immune response (62), although only *E. coli* received a benefit from cocolonization in our CAUTI model. Coinfection of *P. mirabilis* with *P. stuartii* has no impact on the bacterial burden of either species within the urinary tract, but their interactions actually promote a potent proinflammatory response, greater tissue damage, and an increased incidence of severe disease during both UTI and CAUTI (47, 48). This is likely also the case for coinfection of *P. mirabilis* with *E. faecalis*, given the increase in cytotoxicity that occurs *in vitro* and the incidence of bacteremia in our CAUTI model. An increased likelihood of severe disease has also been observed during coinfection of *P. mirabilis* with *Staphylococcus saprophyticus* (63), as well as for *P. aeruginosa* with *E. faecalis* (64).

In contrast to these infection-promoting interactions, there are limited examples of polymicrobial interactions that attenuate disease severity during UTI or CAUTI. Production of an iron-binding factor by commensal *E. coli* and other *Enterobacteriaceae* antagonizes iron acquisition by *P. aeruginosa*, thereby limiting growth and possibly survival in the catheterized bladder (65). Other commensal *E. coli* strains have also been shown to inhibit growth and catheter colonization by traditional uropathogens *in vitro* (66, 67). Thus, our observation of disease modulation by *M. morganii* is consistent with the idea that commensal or nontraditional pathogens can hinder the establishment of infection and disease severity by other pathogens.

In conclusion, our results indicate that further assessments of the clinical significance of polymicrobial bacteriuria and CAUTI need to consider the exact combination of bacterial species that are present, in addition to Gram-positive versus Gram-negative and bacterial burden within the sample. Such interactions may be difficult to capture from single urine samples if colonization of the disease-modulating organism is impacted during coinfection, as is the case for *M. morganii* and *E. coli*. However, a deeper understanding of the importance of these interactions is necessary to help guide novel treatment strategies. Furthermore, identification of *M. morganii* secreted products that modulate *P. mirabilis* pathogenicity has the potential to lead to new therapeutic treatments for CAUTI. The abundance of urease-dampening and urease-enhancing factors during asymptomatic colonization may also have predictive value for patient outcomes in long-term catheterization.

MATERIALS AND METHODS

Study approval. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University at Buffalo (MIC31107Y), in accordance with the Office of Laboratory Animal Welfare, the U.S. Department of Agriculture, and the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were anesthetized with a weight-appropriate dose (0.1 ml for a mouse weighing 20 g) of ketamine/xylazine (80 to 120 mg/kg ketamine and 5 to 10 mg/kg xylazine) by intraperitoneal injection and euthanized by inhalant anesthetic overdose with vital organ removal.

Bacterial strains and growth conditions. *Proteus mirabilis* HI4320, *Providencia stuartii* BE2467, and *Morganella morganii* TA43 were isolated from the urine of catheterized patients in a chronic care facility in Maryland (12). *Escherichia coli* CFT073 was isolated from a patient hospitalized for acute pyelonephritis (68). *Enterococcus faecalis* 3143 was isolated from the urine of a catheterized nursing home resident in Michigan (47). *P. mirabilis* Δ ure refers to *P. mirabilis* HI4320 lacking urease activity due to an insertional disruption of the *ureF* gene, and *P. stuartii* Δ ure refers to *P. stuartii* BE2467 lacking the portion of its plasmid that carries the urease operon (47, 69, 70). Bacteria were routinely cultured at 37°C with aeration in 5 ml of lysogeny broth (LB) (10 g/liter tryptone, 5 g/liter yeast extract, 0.5 g/liter NaCl) or on LB medium solidified with 1.5% agar. *Enterococcus faecalis* isolates were cultured in brain heart infusion broth (BHI). LB medium was supplemented with 25 μ g/ml kanamycin, 20 μ g/ml chloramphenicol, 25 μ g/ml ampicillin, 2.5 μ g/ml tetracycline, or 100 μ g/ml streptomycin to distinguish between species.

Urine growth curves. Pooled, filter-sterilized human urine from female donors (Cone Bioproducts) was diluted 1:1 with sterile saline to achieve a uniform specific gravity of $\sim$ 1.005. Overnight cultures of bacterial species were diluted 1:100 into 5 ml of urine for monomicrobial growth curves. For cocultures each bacterial species was diluted 1:200 to maintain the same total starting CFU, and for triple cultures each bacterial species was diluted 1:300. Bacterial burden was assessed by plating for CFU. To distinguish between bacterial species, samples were plated as follows: (i) *P. mirabilis*-*E. faecalis* coinfection samples were plated on plain LB medium (total CFU) and BHI with streptomycin (*E. faecalis* CFU), (ii) *P. mirabilis*-*M. morganii* coinfection samples were plated on plain LB medium (total CFU) and LB medium with ampicillin (*M. morganii* CFU), (iii) *P. mirabilis*-*E. coli* coinfection samples were plated on plain LB medium (total CFU)

and LB medium with tetracycline (*P. mirabilis* CFU), (iv) *E. faecalis*-*M. organii* coinfection samples were plated on plain LB medium (total CFU) and BHI with streptomycin (*E. faecalis*), and (v) *P. mirabilis*-*E. faecalis*-*M. organii* triple-infection samples were plated on plain LB (total CFU), LB medium with ampicillin (*M. organii* CFU), and BHI with streptomycin (*E. faecalis* CFU).

Urease assay. A previously described alkalimetric screen was used to measure urease activity in whole bacterial cells (47). Briefly, bacterial strains were cultured to mid-log phase ($OD_{600} = 0.5$), centrifuged to pellet, and resuspended in filter-sterilized human urine (using a batch pool from deidentified donors, purchased from Cone Bioproducts). Resuspended cultures were incubated individually at 37°C with aeration or mixed in equal proportions to generate cocultures. After 90 min, the cultures were centrifuged to pellet, and supernatants were passaged through a 0.22- μ m-pore-size filter to generate cell-free spent urine supernatants for determination of *P. mirabilis* urease activity. For measurement of urease enhancement in spent urine supernatants, *P. mirabilis* HI4320 was cultured for ~16 h at 37°C with aeration in 5 ml of LB, centrifuged to pellet, and resuspended in 0.9% sterile saline. The urine supernatants were dispensed into replicated wells of a 96-well plate, supplemented with a phenol red and urea mixture to a final concentration of 0.001% (wt/vol) phenol red and 500 mM urea, and inoculated with 20 μ l of the bacterial saline suspension. The OD_{562} was measured every 60 s for 2 h using a Synergy H1 (BioTek). The urease activity was expressed as the mean change in OD per minute (mOD/min) for the linear portion of the activity curve, as calculated by the Gen5 software (BioTek).

Cytotoxicity. Toxicity of bacterial species independently or during coculture was estimated by an LDH release cytotoxicity assay, as previously described (47, 71). HEK293 cells were purchased from the American Type Culture Collection in 2018 and used for cytotoxicity studies after ≤ 3 passages. Briefly, bacteria were cultured in LB medium to mid-log phase ($OD_{600} = 0.5$), centrifuged to pellet, and resuspended in filter-sterilized human urine to induce urease activity. Resuspended cultures were incubated for 60 min at 37°C. Cultures were then pelleted, washed twice, resuspended in Dulbecco modified Eagle medium without supplements or antibiotics to a concentration of 2×10^7 CFU/ml, and added to a monolayer of low-passage-number HEK293 cells at a multiplicity of infection of approximately 100:1. After a 4-h incubation at 37°C and 5% CO_2 , the amount of LDH released into the supernatant was measured using an LDH cytotoxicity assay kit (Cayman Chemical). Treatment with 9% Triton X-100 for the 4-h incubation was used as a positive control for maximum lysis, and treatment with sterile urine alone was used as a negative control. The level of HEK293 lysis under each treatment condition is expressed relative to the level for *P. mirabilis* Δ ure. For all dual-species and triple-species cultures, a predicted (P) level of cytotoxicity was calculated based on the proportion of each bacterial species in the culture (determined by differential plating for CFU) and the relative cytotoxicity of monomicrobial cultures for each species.

Mouse model of CAUTI. Infection studies were carried out as previously described (47). Briefly, bacteria were cultured overnight in LB medium, washed in phosphate-buffered saline (PBS; 0.128 M NaCl, 0.0027 M KCl [pH 7.4]), adjusted to 2×10^8 CFU/ml ($OD_{600} = 0.2$ for *P. mirabilis*, *P. stuartii*, and *M. organii*, and $OD_{600} = 0.4$ for *E. coli* and *E. faecalis*), and diluted 1:100 to achieve an inoculum of 2×10^6 CFU/ml. Female CBA/J mice at 6 to 8 weeks of age (Jackson) were inoculated transurethrally with 50 μ l of 2×10^6 CFU/ml (1×10^5 CFU/mouse) of a single bacterial species (monomicrobial), a 1:1 mixture of two species for coinfections, or a 1:1:1 mixture of *P. mirabilis* with two other species for polymicrobial infections. In all cases, a 4-mm segment of sterile silicone tubing (0.64 mm [outer diameter], 0.30 mm [inner diameter]; Braintree Scientific, Inc.) was carefully advanced into the bladder during inoculation as described elsewhere (47, 72–74). Mice were euthanized 96 hpi, and bladders, kidneys, and spleens were harvested into 5-ml Eppendorf tubes containing 1 ml of PBS. Gross inspection of kidneys was performed to assess macroscopic urolithiasis. Tissues were homogenized using a Bullet Blender 5 Gold (Next Advance) and plated using an EddyJet 2 spiral plater (Neutec Group) for determination of CFU using a ProtoCOL 3 automated colony counter (Synbiosis). To distinguish between bacterial species, samples were plated as follows: (i) *P. mirabilis*-*E. faecalis* coinfection samples were plated on plain LB medium (total CFU) and BHI with streptomycin (*E. faecalis* CFU), (ii) *P. mirabilis*-*M. organii* coinfection samples were plated on plain LB medium (total CFU) and LB medium with ampicillin (*M. organii* CFU), (iii) *P. mirabilis*-*E. coli* coinfection samples were plated on plain LB medium (total CFU) and LB medium with tetracycline (*P. mirabilis* CFU), (iv) *E. faecalis*-*M. organii* coinfection samples were plated on plain LB medium (total CFU) and BHI with streptomycin (*E. faecalis*), and (v) *P. mirabilis*-*E. faecalis*-*M. organii* triple infection samples were plated on plain LB medium (total CFU), LB medium with ampicillin (*M. organii* CFU), and BHI with streptomycin (*E. faecalis* CFU).

Pathological evaluation. A subset of mice from each infection group were selected for histopathological examination rather than determination of bacterial burden. For these mice, bladders were cut longitudinally and each kidney was transversely. Organs were preserved in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were examined microscopically and scored in a blinded fashion by a veterinary pathologist to determine the severity and extent of inflammation and lesions using an adaptation of a previously developed semiquantitative scoring system (47, 71, 75–77). The scoring rubric is provided in Table S1 in the supplemental material.

Statistics. Normalcy was assessed for all data sets by the Shapiro-Wilk and D'Agostino-Pearson normality tests. Significance was assessed using two-way analysis of variance (ANOVA), a nonparametric Mann-Whitney test, an unpaired *t* test, or a chi-square test, as indicated in the figure legends. All *P* values are two tailed at a 95% confidence interval. All analyses were performed using Prism, version 7.03 (GraphPad Software, San Diego, CA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 7.5 MB.

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