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New Aspects of RpoE in Uropathogenic *Proteus mirabilis*

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Proteus mirabilis **is a common human pathogen causing recurrent or persistent urinary tract infections (UTIs). The underlying mechanisms for *P. mirabilis* to establish UTIs are not fully elucidated. In this study, we showed that loss of the sigma factor E (RpoE), mediating extracytoplasmic stress responses, decreased fimbria expression, survival in macrophages, cell invasion, and colonization in mice but increased the interleukin-8 (IL-8) expression of urothelial cells and swarming motility. This is the first study to demonstrate that RpoE modulated expression of MR/P fimbriae by regulating *mrpI*, a gene encoding a recombinase controlling the orientation of MR/P fimbria promoter. By real-time reverse transcription-PCR, we found that the IL-8 mRNA amount of urothelial cells was induced significantly by lipopolysaccharides extracted from *rpoE* mutant but not from the wild type. These RpoE-associated virulence factors should be coordinately expressed to enhance the fitness of *P. mirabilis* in the host, including the avoidance of immune attacks. Accordingly, *rpoE* mutant-infected mice displayed more immune cell infiltration in bladders and kidneys during early stages of infection, and the *rpoE* mutant had a dramatically impaired ability of colonization. Moreover, it is noteworthy that urea (the major component in urine) and polymyxin B (a cationic antimicrobial peptide) can induce expression of *rpoE* by the reporter assay, suggesting that RpoE might be activated in the urinary tract. Altogether, our results indicate that RpoE is important in sensing environmental cues of the urinary tract and subsequently triggering the expression of virulence factors, which are associated with the fitness of *P. mirabilis*, to build up a UTI.**

Proteus mirabilis, a common uropathogen, is an infectious agent of pyelonephritis and catheter-associated urinary tract infections (UTIs) (1, 2). *P. mirabilis* has many virulence factors that contribute to UTIs (1–3). These factors include fimbria-mediated adherence to host urothelial cells and the catheters (2, 4), flagella mediated motility (swarming and swimming) (1, 5), hemolysin (6), and invasion of host tissues and immune evasion (1, 7). Flagella-dependent swarm cell formation contributes to establishing infections by migrating along the catheter (5). Hemolysin is also thought to facilitate bacterial spread within the kidney and the development of pyelonephritis by damaging host tissues (6). Bacteria must successfully evade immune responses to persist within the host. *P. mirabilis* uses several strategies to avoid immune attacks in the urinary tract. One is to vary the antigenic structures, such as flagellin by flagellar gene rearrangement (8), and fimbriae by fimbrial gene diversity or phase variation to prevent antibody recognition (1, 3, 9). Other immunoavoidance factors for *P. mirabilis* include capsules (2), IgA proteases (ZapA) (10), and lipopolysaccharides (LPS) (1, 2). Capsules are effective at hiding many bacterial surfaces and preventing opsonization (2). *P. mirabilis* is an antigenically heterogeneous species due to structural differences of LPS (2). Modified LPS promotes bacterial survival by increasing resistance to cationic antimicrobial peptides and by altering host recognition by Toll-like receptors (TLRs) (11). Moreover, capability of invading urothelial cells to survive intracellularly probably represents another mechanism for immune evasion and persistence (1, 3, 7).

Many studies have reported that the presence of mannose-resistant *Proteus*-like (MR/P) fimbriae of *P. mirabilis* is important in UTIs (12, 13). MR/P fimbriae facilitate colonization of the urinary tract, and deficiency of the MR/P fimbriae decreased bacterial loads in the mouse model of UTIs (14, 15). The *mrp* gene cluster contains two transcripts: *mrpABCDEFGHJ* (designated the *mrp* operon) and *mrpI* (12). The promoter for the *mrp* operon, which contains all of the genes required for MR/P fimbrial biogenesis, resides on a 251-bp invertible element (IE) (12). The gene *mrpI*, transcribed divergently from the *mrp* operon and independent of the *mrp* promoter, encodes a recombinase capable of switching the IE from either "ON" to "OFF" or from "OFF" to "ON" to control MR/P fimbria expression (12).

Less is known about the host response to uropathogenic *P. mirabilis* but aspects of the host defense might be similar to uropathogenic *E. coli* (UPEC) (1–3). Urothelial cells secreted soluble mediators such as soluble IgA, lactoferrin, and bactericidal antimicrobial peptides to inhibit attachment of UPEC (16). Microbes that overwhelm these early defenses contact urothelia and activate an innate inflammatory response through TLRs (17). The inflammatory response consists of three principal steps: (i) urothelial cell activation and the production of distinct inflammatory cytokines, (ii) immune cell recruitment to the infectious site, and (iii) local destruction and elimination of the invading bacteria (16, 18).

The bacterial envelope maintains cell homeostasis and is the

Received 19 June 2014 **Returned for modification** 24 July 2014
Accepted 19 December 2014

Accepted manuscript posted online 29 December 2014

Citation Liu M-C, Kuo K-T, Chien H-F, Tsai Y-L, Liaw S-J. 2015. New aspects of RpoE in uropathogenic *Proteus mirabilis*. Infect Immun 83:966–977. doi:10.1128/IAI.02232-14.

Editor: B. A. McCormick

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.02232-14.

doi:10.1128/IAI.02232-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype[a]	Source or reference
Strains		
P. mirabilis		
Wild type	Wild-type (N2); Tet^r	Clinical isolate
rseA mutant	Wild type derivative; *rseA* knockout mutant; Km^r	This study
rpoE mutant	Wild typederivative; *rpoE* knockout mutant; Km^r	This study
rseAc	*rseA* mutant strain containing pGEM-T Easy-*rseA*; RseA-complemented strain; Amp^r	This study
rpoEc	*rpoE* mutant strain containing pGEM-T Easy-*rpoE*; RpoE-complemented strain; Amp^r	This study
E. coli		
DH5α	*fhuA2 lac*Δ*U169 phoA glnV44* W80′ *lacZ* ΔM15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
S17-1 λ*pir*	λ*pir* lysogen of S17-1 [*thi pro hsdR2 hsdM*$^+$ *recA* RP4 2-Tc::Mu-Km::Tn *7*(Tp^r Sm^r)]; permissive host able to transfer suicide plasmids requiring the Pir protein by conjugation to recipient cells	24
Plasmids		
pGEM-T Easy	High-copy TA cloning vector; Amp^r	Promega
pUT/mini-Tn*5*-Km	Suicide plasmid requiring the Pir protein for replication and containing a mini-Tn*5* cassette containing the Km^r gene	24
pACYC184	Low-copy cloning vector, P15A replicon; Cm^r Tet^r	24
pGEM-T Easy-*rseA*	pGEM-T Easy containing intact *rseA* sequence, including its ribosome binding site (RBS); Amp^r	This study
pGEM-T Easy-*rpoE*	pGEM-T Easy containing intact *rpoE* sequence, including its ribosome binding site (RBS); Amp^r	This study
pACYC184-*rpoE- xylE*	*rpoE* reporter plasmid, pACYC184 containing intact *rpoE* promoter sequence before *xylE*; Cm^r	24

[a] Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Sm^r,streptomycin resistance; Km^r, kanamycin resistance; Tp^r, trimethoprim resistance.

site for crucial processes, such as metabolic energy transduction, the transport of nutrients and wastes, signal transduction, and cell-cell communication (19). RpoE, an alternative sigma factor, is essential for the maintenance of cell envelope integrity in Gram-negative bacteria (20–22). In this regard, *rpoE* gene is important in pathogenesis and stress survival in many Gram-negative bacteria (20–22), but the function of RpoE in uropathogenic *P. mirabilis* is still not known. RseA, belonging to the *rpoE* operon, is an anti-RpoE factor under nonstressed conditions. The release of RpoE from RseA binding allows it to combine core RNA polymerase to transcribe RpoE-dependent genes (20). In the present study, we characterized the roles of *P. mirabilis* RpoE in virulence by *in vitro* and *in vivo* assays. This is the first report to show that *P. mirabilis* RpoE affected multiple traits, including swarming motility, hemolysin activity, bacteria-mediated cytotoxicity, fimbria production, survival in macrophage, invasion ability, induction of cytokine expression, and colonization in mice. It is worth noting that *P. mirabilis* RpoE could not only regulate expression of MR/P fimbriae through *mrpI* but also modulate host immune responses. We noticed that fimbria expression, survival in macrophages, invasion ability, and colonization in mice were decreased in the *rpoE* mutant and that the induction of interleukin-8 (IL-8) by *rpoE* mutant was higher relative to the wild type. In addition, we found that RpoE was activated by urea, a component in urine. Altogether, RpoE of *P. mirabilis* could play important roles in establishing UTIs via modulating the appropriate production of virulence factors, including those associated with host immune responses. To our knowledge, this is the first report describing the roles of RpoE in *P. mirabilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium. The LSW$^-$ agar (LB agar plates with glycerol to prevent swarming) was used to prevent the swarming motility when needed (23).

Construction of mutants and complementation. Sequences flanking the *rpoE* gene were amplified by PCR using the primer pairs rpoE-upF/rpoE-upR and rpoE-downF/rpoE-downR (see Table S1 in the supplemental material), respectively, and cloned into pGEM-T Easy (Promega) to generate prpoEKO-up and prpoEKO-dn. The prpoEKO-up was digested with SalI/XbaI, and the *rpoE* upstream sequence containing fragment was ligated to the SalI/XbaI-digested prpoEKO-dn to produce the prpoEKOupdn plasmid, which contains both upstream and downstream sequences of *rpoE*. A kanamycin resistance (Km^r) cassette was inserted in the XbaI-digested prpoEKOupdn plasmid to generate the prpoEKOupdnKm, a plasmid containing the Km^r cassette-disrupted combined upstream and downstream sequence of *rpoE*. The fragment containing the Km^r cassette-disrupted combined upstream and downstream sequence of *rpoE* was cleaved by SalI/SphI from prpoEKOupdnKm and ligated into SalI/SphI-cleaved pUT/mini-Tn*5*(Km) to generate pUTrpoEKO. Gene inactivation mutagenesis by homologous recombination and confirmation of mutants with double-crossover events were performed as described previously (23). The *rseA* mutant was obtained in the same way using the primer pairs rseA-upF/rseA-upR and rseA-downF/rseA-downR.

For complementation of *rpoE* mutant, the fragments containing full-length *rpoE* and *rseA* were amplified by PCR by using primer pairs rpoEcom-F/rpoEcom-R and rseAcom-F/rseAcom-R and cloned into pGEM-T Easy (Promega), respectively, to generate the plasmids prpoE-com and prseA-com. prpoE-com and prseA-com were transformed into the *rpoE* and *rseA* knockout mutants, respectively, to generate the RpoE-complemented and RseA-complemented strains.

Reporter assay. The *rpoE-xylE* reporter plasmid (24)-transformed wild-type and mutant strains were grown overnight in LB broth with 20 μg of chloramphenicol/ml and diluted 100-fold in the same medium, and then the XylE activity was monitored at 3, 5, and 7 h and overnight after incubation. For testing signals for *rpoE* expression, the wild type and mutants containing the *rpoE-xylE* reporter plasmid were grown overnight in LB broth, diluted 100-fold, cultured to an optical density at 600 nm (OD_{600}) of 0.5 at 37°C, and exposed to 20 μg of polymyxin B (PB)/ml or 0.5 M urea for 2 h. The XylE activity was then measured as described previously (24).

Swarming assay. The swarming migration assays was performed as described previously (24, 25). Briefly, overnight bacterial cultures (5 μl)

were inoculated centrally onto the surface of dry LB swarming plates containing 2% (wt/vol) agar, which were then incubated at 37°C. The swarming migration distance was measured by monitoring the swarm fronts of the bacterial cells at 1-h intervals.

Measurement of the hemolysin activity. The overnight bacterial cultures (120 μl) were inoculated onto the surface of dry LB swarming plates, which were then incubated at 37°C. The hemolysin activity was determined at 5 and 7 h after inoculation, as described previously (24, 25).

Cytotoxicity assay. Cells (5×10^3 cells/well) of the human urothelial cell line NTUB1, which was originally derived from a urinary bladder carcinoma (23), were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) under 5.0% CO_2 for 2 days using 96-well culture plates. Overnight bacterial cultures (120 μl) were inoculated onto the surface of dry LB swarming plates, and bacteria were collected and washed with phosphate-buffered saline (PBS) at 5 h after incubation at 37°C. The bacterial number were adjusted to 10^6 CFU/ml by RPMI 1640, 200 μl of *P. mirabilis* strains were applied to each well at a multiplicity of infection (MOI) of 20, and the plates were incubated at 37°C for 2 h. The culture supernatants were collected, and the lactate dehydrogenase (LDH) release was quantified using a CytoTox 96 nonradioactive cytotoxicity kit (Promega) according to the manufacturer's instructions. Cytotoxicity was determined by calculating the percentage of LDH release relative to the maximum LDH release from uninfected NTUB1 lysed with Triton X-100.

IE assay. The expression of *P. mirabilis* MR/P fimbriae, encoded by *mrp* operon, depends on the promoter orientation (9, 12). The IE "on" means the promoter direction is for *mrp* operon expression. Genomic DNA from overnight and exponential cultures (OD_{600} of 0.5) of various *P. mirabilis* strains were prepared for amplification of the invertible element (IE) element using mrpP1 and mrpP2 primers (see Table S1 in the supplemental material). The PCR product was digested with AflII and resolved on a 2% agarose gel.

Real-time RT-PCR. To study the effect of the RpoE gene on the mRNA levels of *mrpA*, *mrpJ*, *mrpI*, *flhDC*, and *fliC1*, total RNA of the wild type, the *rpoE* mutant, and the *rseA* mutant from overnight LB cultures and cells grown on agar plates for 5 h after seeding was extracted, and real-time reverse transcription-PCR (RT-PCR) was performed as described previously (24). The levels of mRNA were normalized against *gyrB* mRNA.

Expression and purification of the MrpA-His_6 fusion protein and preparation of mouse antiserum against MrpA. The mouse polyclonal antiserum against MrpA was obtained as described previously (26). A 528-bp BamHI/XhoI fragment containing *mrpA* was cloned into the BamHI/XhoI sites of pET32a (Novagen) to construct pET-MrpA. MrpA was expressed as a fusion protein tagged with a His_6 tail from pET-MrpA upon induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The MrpA-His_6 fusion protein was purified using Ni^{2+}-NTA resin (Invitrogen) according to the protocol provided by the manufacturer. The purified MrpA-His_6 fusion protein was desalted on a Ultracel-10K centrifugal filter device (Millipore) buffered with PBS. The protein sample was quantified by a Bio-Rad protein assay. The predicted 40-kDa MrpA-His_6 protein was resolved on an SDS–12.5% polyacrylamide gel and subsequently excised from the gel. To raise antiserum against MrpA, the excised gel containing MrpA was emulsified in Freund complete adjuvant and subcutaneously injected into BALB/c mouse (50 μg of protein/mouse). At 2 and 4 week after the primary immunization, each mouse was given a booster injection of 50 μg of protein emulsified in Freund incomplete adjuvant. Serum was collected 2 weeks after the final booster. The specificity of the antiserum was established using *P. mirabilis hfq* mutant (whose *mrp* operon is OFF) as the negative control, and the correct size of MrpA was predicted to be ~18 kDa.

Western blot analysis. Overnight LB broth cultures (15 ml) of various *P. mirabilis* strains were washed, and the pellets were collected and resuspended in PBS (1 ml). Total proteins of bacteria were extracted by sonication, quantified by Bio-Rad protein assay, and adjusted to the same concentration. The protein samples were denatured in the sample buffer (100°C, 5 min), electrophoresed on an SDS–12.5% polyacrylamide gel, and then transferred to a Amersham Hybond-P membrane (GE Healthcare). The blot was incubated with mouse polyclonal antiserum against MrpA described above, followed by sheep anti-mouse immunoglobulin G-horseradish peroxidase conjugate (GE Healthcare), and then developed using enhanced chemiluminescence detection reagents (Perkin-Elmer).

Macrophage infection assay. The assay was performed as described previously (27), with some modifications. Briefly, 12-well plates were seeded with 10^5 cells (per well) of THP-1 cells, and THP-1 cells were differentiated by using 50 ng of phorbol myristate acetate/ml in RPMI 1640 with 10% FCS under 5.0% CO_2 for 24 h. The overnight cultures of *P. mirabilis* strains were applied to each well at an MOI of 10 (10^6 CFU/well). Bacteria were brought into contact with macrophages by centrifugation, followed by incubation for 30 min at 37°C. After infection, the cells were washed with PBS and incubated for 1 h in RPMI 1640 with 250 μg of streptomycin/ml to kill extracellular bacteria. Immediately, cells in some wells were lysed by 1% Triton X-100 to determine the CFU of intracellular bacterial cells at t_0; others were incubated in medium containing 250 μg of streptomycin/ml for an additional 1 and 4 h to obtain the respective CFU counts.

Cell invasion assay. The wild-type *P. mirabilis* and its mutants were cultured in LB broth at 37°C overnight before the cell invasion assay was performed according to the protocol of Jiang et al. (28). Briefly, human urothelial NTUB1 cells were grown and then infected with a bacterial suspension containing 10^7 cells (MOI = 10) for 1 h. Urothelial cells were then washed and incubated at 37°C in 1 ml of RPMI 1640 medium containing streptomycin for another 1 h. The cells were washed again with PBS and then lysed using 1 ml of lysis solution. Cell lysates were diluted serially and viable bacteria were counted by plating on LSW^- agar plates. Cell invasion ability was determined as the percentage of viable bacteria that survived the streptomycin treatment versus the total inoculum, and the relative invasion was expressed.

Cytokine expression of NTUB1 cells. Determination of the relative levels of selected human cytokines and chemokines was performed using Human Cytokine Array Panel A (R&D Systems, USA) according to the manufacturer's instructions. In brief, the NTUB1 cells were grown in the 12-well plate (10^6 cells/well) and incubated with overnight bacterial cultures in RPMI 1640 (10^7 cells/well) at an MOI of 10 for 3 h at 37°C. The culture supernatant and NTUB1 cells were then subjected to analyses of the cytokine array and real-time RT-PCR, respectively. A mixture of NTUB1 cell culture supernatant and the detection antibody was added to the membrane spotted with capture antibodies. After incubation, the membrane was washed, and the chemiluminescence produced by the sample/antibody hybrid on the membrane was detected after the addition of streptavidin-labeled horseradish peroxidase. NTUB1 cells were washed, the total RNA was extracted, and real-time RT-PCR was performed as described previously (24, 29) using the primers listed in Table S1 in the supplemental material to examine the mRNA expression of IL-8, MIF, PAI-1, and CXCL1 against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. To study the effect of LPS on the expression of IL-8 mRNA, LPS (0.1, 1, and 5 μg/ml) extracted from *P. mirabilis* strains as described previously (24, 28) was applied to wells with NTUB1 cells, the plates were incubated for 3 h, and the total RNA was isolated for real-time RT-PCR.

Infection of mice. The C57BL/6 mouse model of UTIs was used as described previously (29). Briefly, 6-week-old female mice were injected transurethrally with a 50-μl overnight culture suspension of *P. mirabilis* strains at a dose of 10^7 CFU per mouse. On days 3 and 6 after injection, the mice were sacrificed, and bladder and kidney samples were collected, weighed, suspended in 0.5 ml of PBS, and then homogenized to determine the viable bacterial count by plating on LSW^- agar plates. All animal experiments were performed in strict accordance to the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Laboratory Animal Center (Taiwan), and the protocol was ap-

proved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Hematoxylin and eosin staining. One and six days after the transurethral inoculation of PBS, the wild type, the *rseA* mutant, and the *rpoE* mutant, respectively, C57BL/6 mice (as described above) were sacrificed, and for each mouse, bladder and the kidney samples were collected, preserved in 10% formalin (pH 7.2), embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. The extent of renal pathology was scored as follows (30): 0, no inflammation; 1, polymorphonuclear neutrophils (PMNs) confined to the peripelvic region; 2, PMN clusters detectable in the papilla or peripelvic cortex; and 3, widespread extension of PMNs into the cortex or outer medulla. The severity of histological modifications of each bladder was scored as follows (30): 0, no histological modifications; 1, occasional submucosal immune cell infiltrates; 2, widespread submucosal immune cell infiltration with minimal spread to the muscularis or epithelium; and 3, widespread inflammation with dense perivascular cuffs, transmural distribution, and intraepithelial inflammatory cells. The investigator was blind to the identity of the bacterial strain.

Nucleotide sequence accession numbers. The nucleotide sequences of *P. mirabilis* intergenic region between *mrpA* and *mrpI* and the region containing *rpoE* and *rseA* genes have been deposited in GenBank under accession numbers KJ814628 and KJ814629, respectively.

RESULTS

Identification and characterization of the *P. mirabilis rpoE* operon. To investigate the roles of *rpoE* in *P. mirabilis*, we first searched in the released genome of *P. mirabilis* HI4320 (accession no. AM942759) and found that the *rpoE* operon of *P. mirabilis*, like that of *E. coli*, consists of *rpoE*, *rseA*, *rseB*, and *rseC* genes. Using primers annealing to conserved sequences, we cloned and sequenced the fragment containing *rpoE*, *rseA*, and the region upstream of *rpoE* in *P. mirabilis* N2. The nucleotide sequences of *rpoE* and *rseA* were both found to be 99% identical to those of *P. mirabilis* HI4320. The amino acid sequences of RpoE and RseA were 87 and 57%, respectively, identical to UPEC strain CFT073. Analysis of the upstream sequence of the *rpoE* operon revealed a putative binding site for RpoE, 5′-AACttcattctattttaatgcTCtaA-3′, based on the conserved binding sequence (5′-AACtt-N_{16}-TCnaA-3′) (20). Capital and lowercase letters represent the relative frequencies of each base at that position; the sequences in >80% are in capital letters. Knowing RseA is an anti-RpoE factor and RpoE is autoregulated (20), we hypothesized that mutation in *rseA* might cause RpoE overexpression in *P. mirabilis*. Therefore, the *rpoE* and *rseA* mutants were constructed, and a reporter assay was performed in the wild type, the *rseA* mutant, and the *rpoE* mutant containing the *rpoE-xylE* plasmid. As shown in Fig. 1, the promoter activity of *rpoE* (XylE activity) was decreased in the *rpoE* mutant but increased in the *rseA* mutant compared to the wild type at 3, 5, and 7 h and overnight after incubation. The XylE activities of both the wild type and the *rseA* mutant, but not the *rpoE* mutant, exhibited a growth-phase-dependent pattern, which was most pronounced during the stationary phase. Thus, we concluded RpoE was positively autoregulated and that *rseA* mutant was an *rpoE* overexpression strain of *P. mirabilis*.

***P. mirabilis* RpoE regulated swarming motility, hemolysin activity, and cytotoxicity to urothelial cells.** The swarming phenomenon is a metabolically complicated process, so any factors that affect this phenomenon would likely affect the fitness of the organism (1, 2). We thus tested the swarming behavior of wild-type and mutant strains and found that the swarming distance was

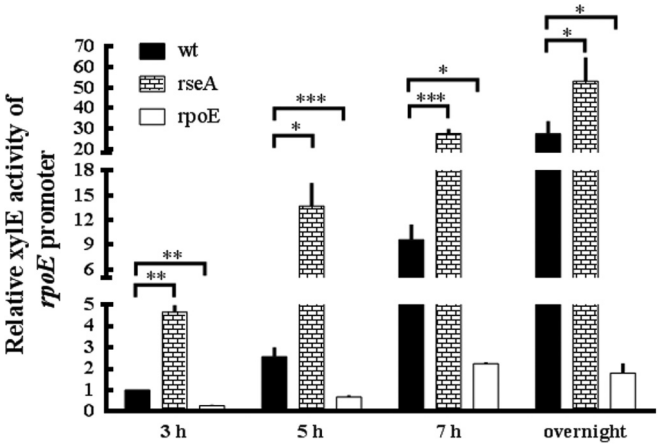

FIG 1 Promoter activity of *rpoE* in wild-type *P. mirabilis*, *rseA* mutant, and *rpoE* mutant strains. The activities of XylE in the *rpoE-xylE* reporter plasmid-transformed wild-type, *rseA* mutant, and *rpoE* mutant strains were determined by reporter assay at 3, 5, and 7 h and overnight after incubation. The XylE activity of the wild type at 3 h was set at 1, and other data are presented relative to this value. The data are averages and standard deviations of three independent experiments. Significant differences were determined by using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant.

increased for the *rpoE* mutant but decreased for the *rseA* mutant at 4, 5, 6, 7, and 8 h after inoculation onto the swarming plate (Fig. 2A). Both complemented strains (designated rseAc and rpoEc) restored almost wild-type swarming. Previous studies have shown that multiple virulence factors are coordinately expressed during *P. mirabilis* swarming migration (31, 32). *P. mirabilis* hemolysin was a pore-forming cytotoxin, contributing to the damage of human renal cells (6). We then tested the hemolysin activity. As shown in Fig. 2B, the hemolysin activity was decreased in the *rseA* mutant but increased in the *rpoE* mutant compared to the wild-type strain after growing on swarming plates for 5 h. At 7 h after inoculation, the *rpoE* mutant exhibited significant hemolysin activity in contrast to other strains. Accordingly, we found that the *rpoE* mutant differentiated into more swarmer cells at 7 h after incubation than did the other strains (data not shown). These data indicated that RpoE could inhibit swarming motility, swarm cell differentiation, and swarming-related hemolysin activity. Knowing *P. mirabilis* RpoE inhibited hemolysin activity, we hypothesized that RpoE might influence cytotoxicity. To identify bacterium-induced cytotoxicity, the wild-type and mutant (*rseA* and *rpoE*) strains were cocultured with NTUB1 cells, respectively, and the LDH released was measured. As shown in Fig. 2C, the *rpoE* and *rseA* mutants displayed higher and lower cytotoxicities, respectively, relative to the wild type. Thus, mutation of *rpoE* increased the cytotoxicity of *P. mirabilis*.

Regulation of MR/P fimbria expression by RpoE and the effect of RpoE-regulated *mrpJ* on expression of *flhDC* and *fliC1*. *P. mirabilis* MR/P fimbriae have been shown to be important in UTIs (12–15). We investigated whether RpoE affects expression of MR/P fimbriae in DNA, mRNA, and protein levels. The IE assay using exponential-phase and overnight LB broth cultures was performed to determine the ON (fragments of 336 and 407 bp) or OFF (fragments of 204 and 539 bp) position of the IE. The IE was located on an intergenic region between *mrpA* and *mrpI* (Fig. 3A). Both ON and OFF orientations were found in stationary-phase

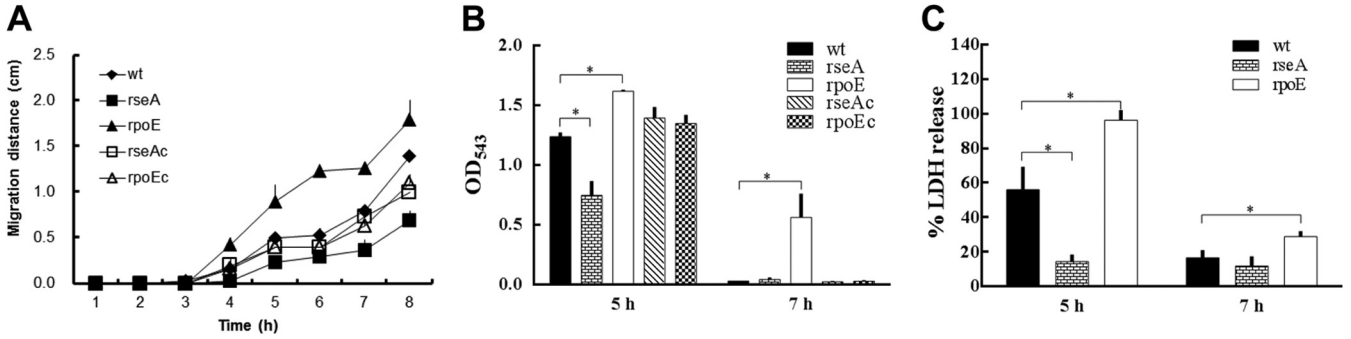

FIG 2 Effect of *P. mirabilis* RpoE on swarming, hemolysin activity, and cytotoxicity. (A) Swarming migration of the wild-type, *rseA* (rseA) and *rpoE* (rpoE), and complemented *rseA* (rseAc) and *rpoE* (rpoEc) strains. Aliquots (5 μl) of overnight culture were inoculated centrally onto LB swarming plates. The plates were incubated at 37°C, and the migration distance was measured hourly after inoculation. (B) Hemolysin activities of the wild-type, *rseA* and *rpoE* mutants, and complemented *rseA* and *rpoE* strains. The hemolysin activity was determined by measuring the OD_{543} at 5 and 7 h after seeding the bacteria on LB swarming plates. (C) Cytotoxicity of wild-type, *rseA* mutant, and *rpoE* mutant strains to NTUB1 cells. Bacterial strains after seeding onto LB swarming plates for 5 and 7 h were cocultured with NTUB1 cells. After 2 h, the cytotoxicity was determined by quantifying the percentage of LDH release relative to the maximum LDH release from uninfected NTUB1 lysed with Triton X-100. All data are the averages, and standard deviations of three independent experiments are given. Significant differences were determined by using the Student *t* test (*, $P < 0.05$).

cultures of the wild type, the *rseA* mutant, and the *rpoE* mutant, with less "ON" in the *rpoE* mutant (Fig. 3B). Exponential-phase cultures of all strains appeared to favor the OFF position compared to the stationary phase and almost no "ON" in the *rpoE* mutant. Based on ImageJ software quantification, the relative proportions of DNA in the ON phase of stationary wild-type, *rseA* mutant, and *rpoE* mutant strains were 34, 34, and 10.5%, respectively, while those of exponential-phase cells were 0.5, 3.5, and

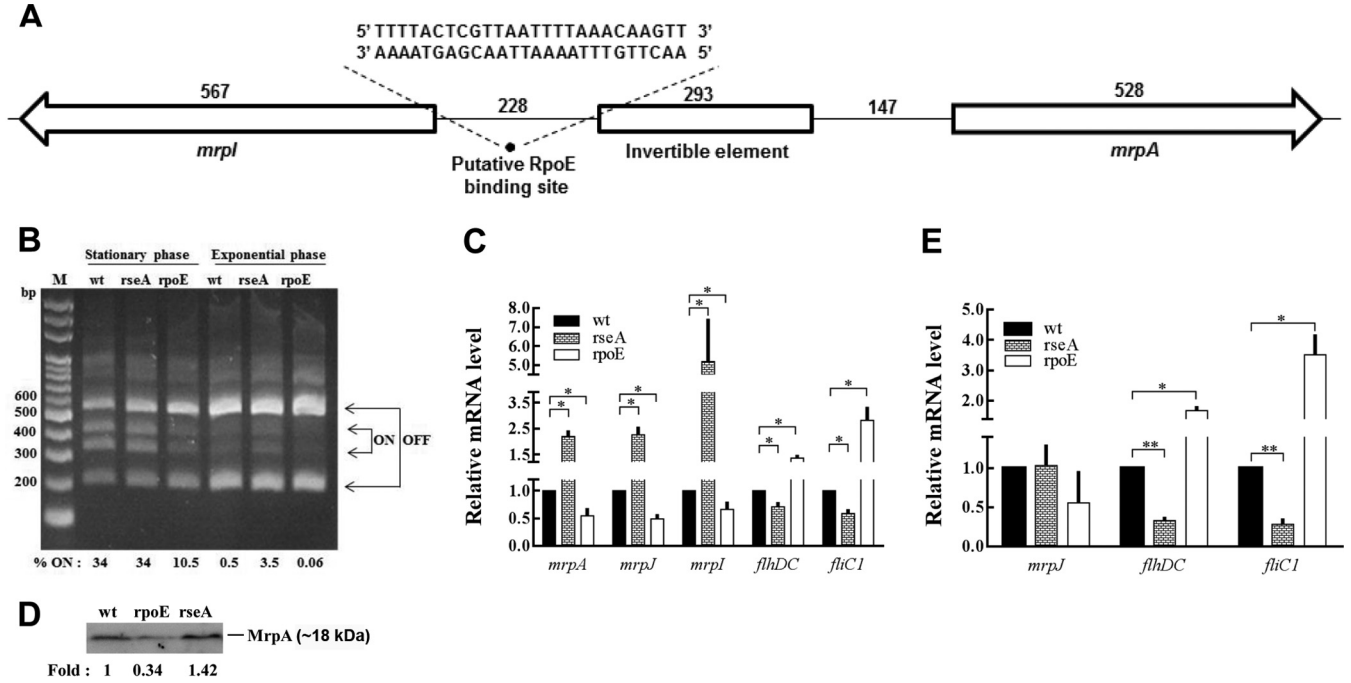

FIG 3 Regulation of *mrp* operon, *mrpI*, *flhDC*, and *fliC1* by *P. mirabilis* RpoE. (A) Schematic drawing of the intergenic region between *mrpA* and *mrpI*. The sequence of the putative RpoE binding site upstream of *mrpI* is "5′-AACttgtttaaaattaacgagTaaaA-3". The numbers indicate the sizes (in bp) of each DNA fragment. (B) Promoter orientation of *mrp* operon in wild-type, *rseA* mutant, and *rpoE* mutant strains determined by the IE assay. The assay was performed as described in Materials and Methods using overnight and exponential cultures. "ON" means the direction of *mrp* promoter is for *mrp* operon expression. The "% ON" is indicated by the band intensity of the ON band compared to those of the ON and OFF bands. M, marker. (C) Expression of *mrpA*, *mrpJ*, *mrpI*, *flhDC*, and *fliC1* in wild-type, *rseA* mutant, and *rpoE* mutant strains cultured in LB broth. Total RNA was extracted from overnight cultures of wild-type, *rseA* mutant, and *rpoE* mutant strains, and the mRNA amounts of *mrpA*, *mrpJ*, *mrpI*, *flhDC*, and *fliC1* were quantified by real-time RT-PCR. (D) Effect of RpoE on the protein level of MrpA. The total proteins were extracted from overnight cultures of wild-type, *rseA* mutant, and *rpoE* mutant strains, and Western blot analysis with antiserum against MrpA was performed as described in Materials and Methods. The fold change of the mutants is given relative to the band intensity of the wild type. (E) Expression of *mrpJ*, *flhDC*, and *fliC1* in wild-type, *rseA* mutant, and *rpoE* mutant strains cultured on LB agar plates. Total RNA was extracted from bacterial cells grown on LB agar plates at 5 h after seeding, and the mRNA amounts of *mrpJ*, *flhDC*, and *fliC1* were quantified by real-time RT-PCR. In panels B and D, the representative result from three independent experiments is shown. In panels C and E, the value obtained for the wild-type cells was set at 1, and all other data were expressed relative to this value after being normalized to a housekeeping gene (*gyrB*). The data are the averages and standard deviations of three independent experiments, and significant differences were determined using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$). wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant.

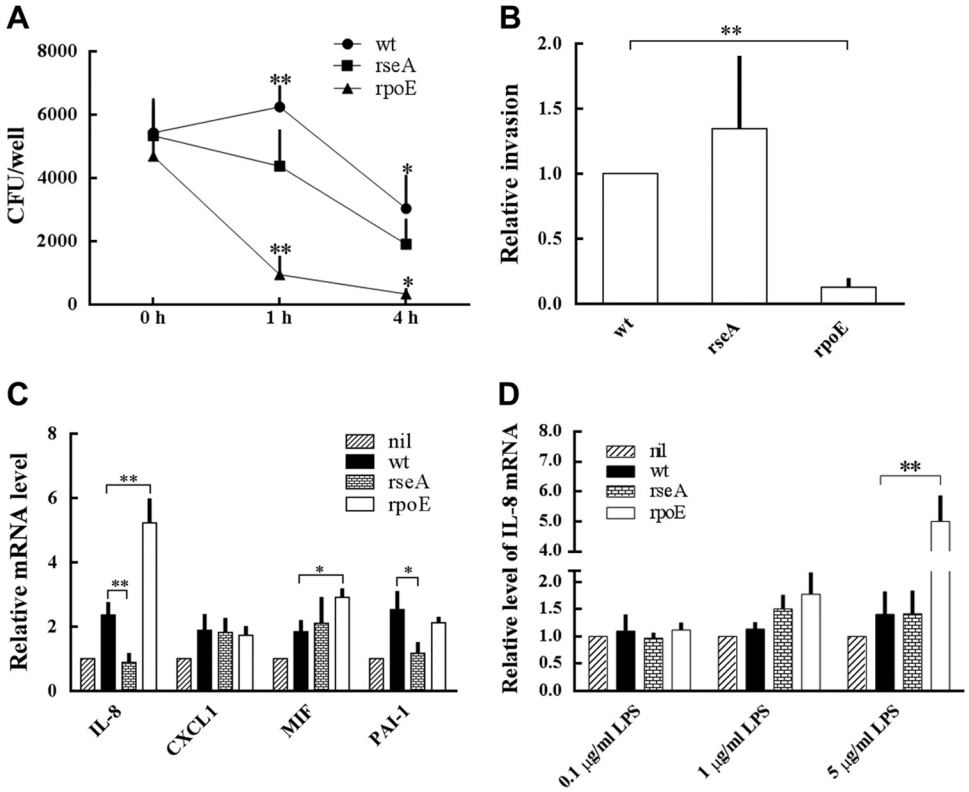

FIG 4 Effect of *P. mirabilis* RpoE on survival in macrophages, cell invasion, and cytokine expression. (A) Survival of the wild-type, *rseA* mutant, and *rpoE* mutant strains in macrophages. THP-1 cells were infected with bacteria for 30 min at an MOI of 10, and the survival of the intracellular bacteria was determined by the streptomycin protection assay at 0, 1, and 4 h. Asterisks indicate significant differences between wild-type and *rpoE* mutant strains at 1 and 4 h (*, $P < 0.05$; **, $P < 0.01$). (B) Invasion abilities of wild-type, *rseA* mutant, and *rpoE* mutant strains. The ability to invade NTUB1 cells was determined as described in Materials and Methods. The ability of the wild type was set at 1, and other data are presnted relative to this value. **, Significant difference between wild-type and *rpoE* mutant strains ($P < 0.01$). (C) Expression of IL-8, CXCL1, MIF, and PAI-1 in NTUB1 cells treated with wild-type, *rseA* mutant, or *rpoE* mutant strains. IL-8, CXCL1, MIF, and PAI-1 mRNA levels of NTUB1 cells after challenging with the bacteria were determined by real-time RT-PCR. (D) IL-8 expression of NTUB1 cells treated with LPS extracted from the wild-type, *rseA* mutant, or *rpoE* mutant strains. LPS at 0.1, 1, or 5 μg/ml was coincubated with NTUB1 cells, and the mRNA amounts of IL-8 were measured 3 h after incubation by real-time RT-PCR. In panels C and D, the value of NTUB1 cells without treatment (nil) was set at 1, and other data are given relative to this value. The data are averages and standard deviations of three independent experiments, and significant difference were determined by using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$). wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant.

0.06%, respectively. Real-time RT-PCR and Western blot analysis were used to further confirm the results of the invertible element assay. Accordingly, the *mrpA* and *mrpJ* mRNA levels of the *rpoE* mutant were lower, but those of the *rseA* mutant were significantly higher than the that of the wild type (Fig. 3C). Figure 3D indicates that the MrpA protein level of *rpoE* mutant was also lower than that of the wild type and *rseA* mutant. These results indicated that RpoE regulated the expression of the *mrp* operon. Therefore, we searched the consensus sequence of RpoE binding site in the intergenic region between *mrpA* and *mrpI* and found the putative binding sequence, 5′-AACttgtttaaaattaacgagTaaaA-3′, only upstream of *mrpI* (Fig. 3A). It has been shown that switching to the ON orientation of IE occurred to a greater extent when expression of MrpI was induced by IPTG (9). To demonstrate whether the expression of *mrpI* gene is regulated by RpoE, which further influences the ON/OFF of the *mrp* operon, we tested for the expression of *mrpI* in wild-type, *rseA* mutant, and *rpoE* mutant strains. As shown in Fig. 3C, the mRNA amount of *mrpI* was decreased in the *rpoE* mutant but increased in the *rseA* mutant. In summary, the data revealed *P. mirabilis* RpoE regulated *mrpI* expression and suggested that RpoE affected *mrp* expression through regulating *mrpI*. In addition, we found that excess MrpI does not contribute more to turn on the *mrp* operon, based on the observation that the ON percentages of wild-type and *rseA* mutant were almost the same (Fig. 3B and C).

Given that the *P. mirabilis mrp* operon is off in agar-grown cells (9, 12), MrpJ is a repressor of *flhDC* (encoding the flagellar transcriptional regulator) (26, 33), and RpoE regulated the expression of the *mrp* operon (Fig. 3B and D), we compared the mRNA expression of *mrpJ*, *flhDC*, and *fliC1* (encoding flagellin) in wild-type, *rseA* mutant, and *rpoE* mutant strains cultured in LB broth and on plates to clarify the effect of *rpoE*-mediated *mrpJ* expression on the expression of *flhDC* and *fliC1*. We found that *mrpJ* was positively regulated by RpoE in broth cultures but that alterations of RpoE had no effect on the expression of *mrpJ* in plate cultures, a phase-off condition for *mrp* (Fig. 3C and E). In addition, *flhDC* and *fliC1*mRNA levels were decreased in the *rseA* mutant and increased in the *rpoE* mutant from both broth and plate cultures (Fig. 3C and E). In this regard, we found that the *rseA* mutant displayed a significant reduction in swimming (see Fig. S1 in the supplemental material) and swarming (Fig. 2A) compared to the wild type. These results indicate that RpoE-mediated repression of

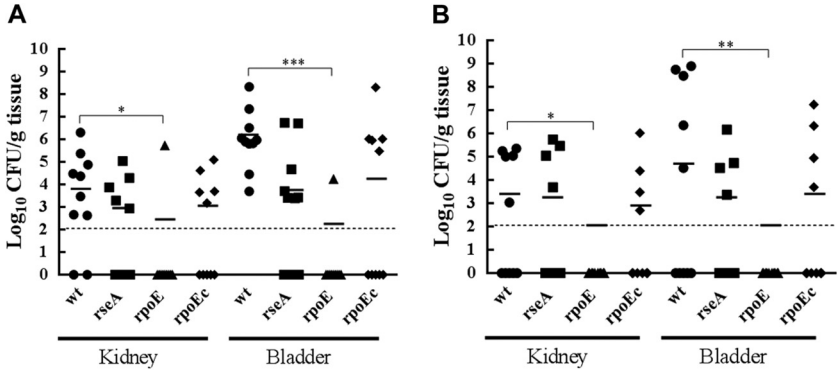

FIG 5 Colonization of wild-type *P. mirabilis*, *rseA* mutant, *rpoE* mutant strains and the RpoE-complemented strain in mice. C57BL/6 mice (ten mice per group) were inoculated transurethrally with overnight cultures of bacterial strains at a dose of 10^7 CFU per mouse. Bacterial loads were determined in the bladder and kidneys on day 3 (A) and day 6 (B) after inoculation. Horizontal bars indicate median values for each group, and the dotted horizontal line indicates the limit of detection (100 CFU). Circles, squares, triangles, and diamonds represent the wild type, the *rseA* mutant, the *rpoE* mutant, and the RpoE-complemented strain retrieved from mice, respectively. Significant differences were determined observed by using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant; rpoEc, RpoE-complemented strain.

swarming and the expression of *flhDC* or *fliC1* in cells from plate cultures is not MrpJ dependent but that MrpJ contributes to the RpoE-mediated reduction of swimming motility and *fliC1* expression in cells from broth cultures.

Role of *P. mirabilis* RpoE in survival in macrophages, invasion ability, and induction of cytokine expression. To understand whether RpoE is involved in the innate response of macrophages (34) to eliminate *P. mirabilis*, we challenged THP-1 cells with wild-type and mutants, killed external bacteria with streptomycin, and assessed the survival of internalized *P. mirabilis*. We found that there was no significant difference between the intramacrophage survival of wild-type and mutant cells immediately after streptomycin treatment. However, the CFU counts of intramacrophage *rpoE* mutant were reduced significantly after further incubation for 1 and 4 h compared to the wild type (Fig. 4A), indicating that the loss of *rpoE* impaired the survival of *P. mirabilis* in macrophages. The *rseA* mutant exhibited a survival pattern similar to that of the wild type.

Given that UPEC evaded the host defense mechanism by invading into the epithelium (2, 18, 35) and *P. mirabilis* also can invade into urothelial cells (7, 28), we then assessed the role of RpoE in invasion into NTUB1 cells. As shown in Fig. 4B, the *rpoE* mutant was significantly less able to invade cells than was the wild type.

The production of cytokines and chemokines from epithelial cells initiates the innate immune responses in UTIs, and the immune cells such as neutrophils would be attracted to the infection site to eliminate the pathogens (16). Therefore, we next examined the role of RpoE in inducing the expression of cytokines and chemokines. To investigate a variety of cytokines and chemokines produced by NTUB1 cells after coculture with *P. mirabilis* wild-type or *rpoE* mutant strains, a human cytokine array was used. We found that spots of IL-8, CXCL1, MIF, and PAI-1 appeared more intense from supernatants of NTUB1 cells treated with the *rpoE* mutant (data not shown). To confirm the expression of IL-8, CXCL1, MIF, and PAI-1, real-time RT-PCR was performed using NTUB1 cells treated with various *P. mirabilis* strains. Both wild-type and *rpoE* mutant strains can induce IL-8 mRNA, though significantly more was induced by the *rpoE* mutant than by the wild type (Fig. 4C). *rseA* mutant (RpoE overexpression)-treated cells expressed IL-8 mRNA similarly to the untreated control (Fig. 4C). In addition, the *rpoE* mutant induced a significantly higher level of MIF mRNA than did the wild type, and the *rseA* mutant inhibited the mRNA level of PAI-1 (a cytokine supporting IL-8-mediated neutrophil migration) relative to the wild type (Fig. 4C). It has been reported that the innate immune response relies on recognition of pathogen-associated molecular patterns (PAMPs) (17). LPS is a prominent feature of Gram-negative bacteria, being one of the most potent PAMPs known and responsible for the inflammatory response (17). Moreover, RpoE regulates biogenesis and the modification of LPS in Gram-negative bacteria (20, 36), so we hypothesized that RpoE-regulated LPS might be one of the bacterial components to affect the IL-8 expression of NTUB1 cells. To this end, we treated NTUB1 cells with LPS (0.1, 1, and 5 μg/ml) extracted from wild-type, *rseA* mutant, and *rpoE* mutant strains, respectively, and the IL-8 mRNA level was determined. As shown in Fig. 4D, the IL-8 mRNA amount of NTUB1 cells was induced ~5-fold by 5 μg of LPS/ml from the *rpoE* mutant only. Altogether, RpoE-mediated LPS changes could explain the difference of IL-8 expression induced by wild-type *P. mirabilis* and the *rpoE* mutant.

Colonization of the urinary tract was attenuated in the *P. mirabilis rpoE* mutant. Knowing that *P. mirabilis* RpoE controlled the expression of diverse virulence factors mentioned above, including swarming, hemolysin, fimbriae, invasion, and modulating the host immune responses, we established the C57BL/6 mouse model for UTIs (29) to determine the effect of inactivation of either *rpoE* or *rseA* on the colonization ability of *P. mirabilis*. As shown in Fig. 5, the *rpoE* mutant had a severely impaired ability to colonize within the bladders and kidneys relative to the wild type. The colonization of the RpoE-complemented strain showed no significant difference from the wild type, and the *rseA* mutant did so except on day 3 from bladder samples. A significant difference in the bacterial load was observed between the wild type and the *rpoE* mutant on days 3 and 6 after transurethral inoculation. This result indicated RpoE is required for the colonization and survival of *P. mirabilis* in the mouse urinary tract.

Histopathologic evaluation of mouse bladders and kidneys. Knowing that the loss of *rpoE* led to higher mRNA levels of IL-8 and MIF (Fig. 4C), which could attract the immune cells to con-

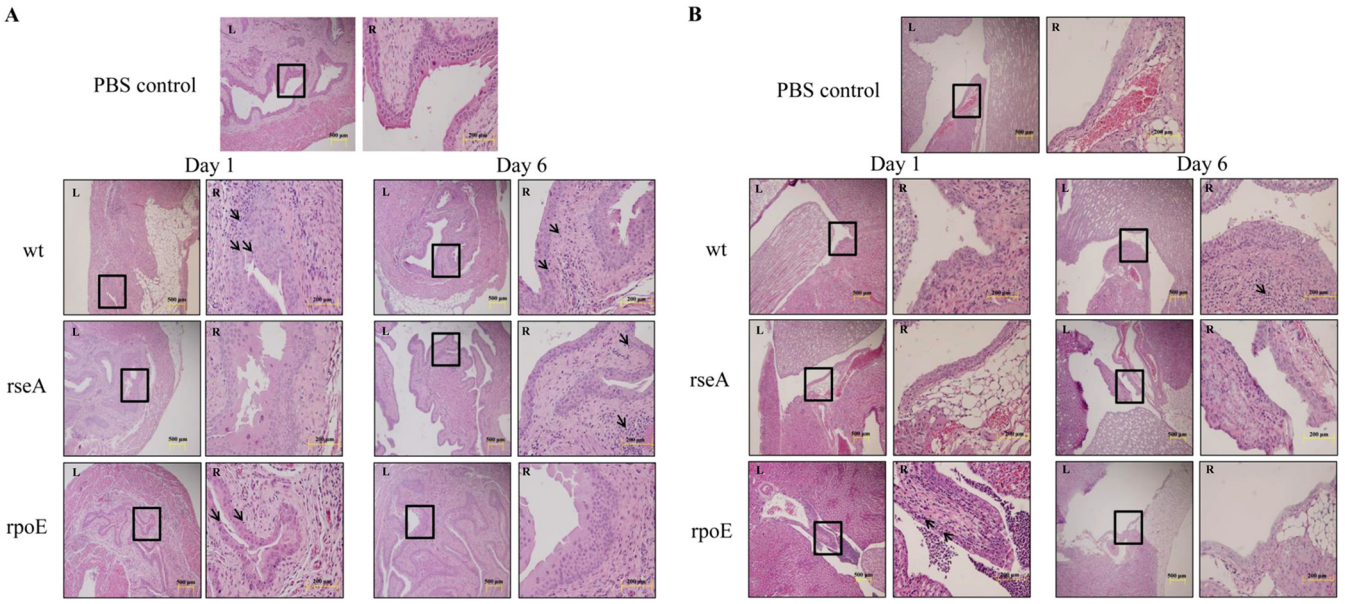

FIG 6 Histological images of mice infected with wild-type *P. mirabilis*, *rseA* mutant, or *rpoE* mutant strains. C57BL/6 mice were infected with wild-type, *rseA* mutant, or *rpoE* mutant strains as in Fig. 5, and mice were sacrificed on day 1 and day 6 postinfection. Bladder (A) and kidney (B) samples were collected, preserved in formalin, embedded, sectioned, stained, and observed microscopically. The amplified image of the rectangle area of each left panel (×100) is shown on the right at ×400. The site of immune cell infiltration is indicated by arrows. wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant.

fine the bacterial loads *in vivo*, we also evaluated the capacity of the wild type and mutants to induce immune cell infiltration in mouse bladders and kidneys. The histopathology of the bladder and kidneys was evaluated according to the criteria described by Alamuri et al. (30) and was expressed as a semiquantitative score. On day 1 postinoculation, light microscopic evaluation revealed a significant increase of immune cell infiltrates in bladders of the wild type- or *rpoE*-treated mice compared to the PBS control, while that of the *rseA* mutant was similar to the PBS control (Fig. 6A). Only the kidneys of *rpoE*-treated mice exhibited widespread extension of immune cells into the cortex or outer medulla, scored as 3, at 1 day after inoculation (Fig. 6B and Table 2). On day 6 postinoculation, colonization of the bladders by the wild type or *rseA* mutant was accompanied by occasional submucosal inflammatory cell infiltrates (scored as 1), and those of the *rpoE* mutant showed almost no sign of inflammatory cell infiltration (Fig. 6A and Table 2). At 6 days after inoculation, the kidneys of *rseA* or *rpoE* mutant-inoculated mice displayed almost no sign of immune cell infiltration, in contrast to those of the wild type, with PMN clusters detectable in the papilla or the peripelvic cortex (Fig. 6B and Table 2). Altogether, the *rpoE* mutant could recruit immune cells in the bladder and kidneys during the early stage of infection (Fig. 6). The wild type induced immune cell infiltration in the bladder but not kidneys on day 1 after inoculation and in both the bladder and the kidneys on day 6 (Fig. 6). Interestingly, the *rseA* mutant could not recruit immune cells in the kidneys (Fig. 6B).

TABLE 2 Histopathological evaluation of mice inoculated with wild-type *P. mirabilis*, *rseA* mutant, and *rpoE* mutant

Strain	Score[a]			
	Day 1		Day 6	
	Bladder	Kidney	Bladder	Kidney
Wild type	2	0	1	2
rseA mutant	0	0	1	0
rpoE mutant	3	3	0	0
PBS control	0	0	0	0

[a] The scores for histological modifications of the kidney are presented: 0, no inflammation; 1, PMNs confined to the peripelvic region; 2, PMN clusters detectable in the papilla or peripelvic cortex; and 3, widespread extension of PMNs into the cortex or outer medulla. The scores for the histological modifications of each bladder are also presented: 0, no histological modifications; 1, occasional submucosal immune cell infiltrates;2, widespread submucosal immune cell infiltration with minimal spread to the muscularis or epithelium; and 3, widespread inflammation with dense perivascular cuffs, transmural distribution, and intraepithelial inflammatory cells.

Urea and polymyxin B induced promoter activity of the *rpoE* gene in *P. mirabilis*. Based on the fact that *P. mirabilis* RpoE plays such an important role in virulence factor expression and colonization in mouse, it was tempting to determine what the signals activate the expression of RpoE in the urinary tract, an environment containing antimicrobial peptides and a lot of urea. Therefore, the XylE activities of *rpoE-xylE* reporter in the wild type and mutants were determined in the presence of urea or polymyxin B, a cationic antimicrobial peptide. The promoter activity of the *rpoE* gene was induced by 20 μg of PB/ml or 500 mM urea after incubation for 2 h in the wild type but not in the *rseA* and *rpoE* mutants (Fig. 7). The results suggested that *P. mirabilis* RpoE could be activated in the host urinary tract during infection.

DISCUSSION

RpoE is involved in virulence, surface stress responses, and modulation of the inflammatory response during infections (20-22, 37). RpoE of uropathogenic *E. coli* plays roles in motility, biofilm formation, and sensitivity to polymyxin B (38). In the present study, we demonstrated the role of RpoE in the regulation of virulence in uropathogenic *P. mirabilis*. Swarming motility, hemoly-

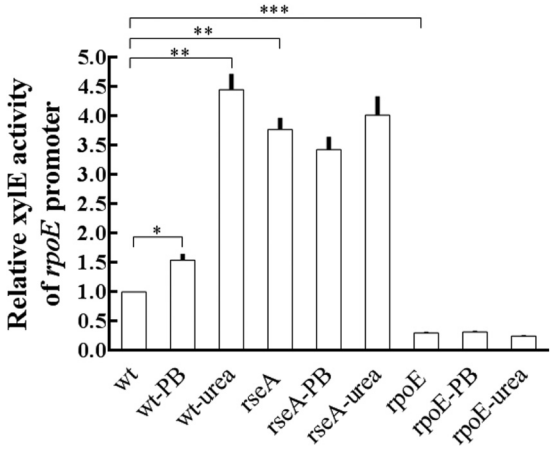

FIG 7 Promoter activity of *P. mirabilis rpoE* induced by urea and PB. The wild-type, *rseA* mutant, and *rpoE* mutant strains containing the *rpoE-xylE* reporter plasmid were grown overnight in LB broth, diluted, cultured to an OD_{600} of 0.5 at 37°C, and exposed to 20 μg of polymyxin B (PB)/ml or 0.5 M urea for 2 h. The XylE activity was measured. The XylE activity of wild-type without treatment was set at 1, and other data are given relative to this value. The data are the averages and standard deviations of three independent experiments. Significant differences were determined by using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). wt, wild type; rseA, *rseA* mutant; rpoE, *rpoE* mutant.

sin activity, and cytotoxicity were increased, but fimbria expression, cell invasion, and survival in macrophages were decreased in the *rpoE* mutant (Fig. 2, 3, and 4). The *rpoE* mutant induced higher mRNA levels of IL-8 and MIF than did the wild type, but RpoE overexpression (in the *rseA* mutant) decreased the mRNA levels of IL-8 and PAI-1 (Fig. 4C). Moreover, the *rpoE* mutation significantly impaired the strain's ability to colonize mice (Fig. 5).

Many observations have indicated that MR/P fimbriae are an important virulence factor for uropathogenic *P. mirabilis*. In a murine model, UTIs with *P. mirabilis* elicited a strong immune response to MrpA, indicating that MR/P fimbriae were expressed *in vivo* (39). Increased expression of MR/P fimbriae correlates with higher levels of colonization in the mouse bladder (14, 15). Vaccine trials in mice using MR/P fimbriae reduce bacterial burdens after challenge (40). Transcriptome analysis also revealed that genes encoding MR/P fimbriae were highly upregulated *in vivo* (13). The phase variation of MR/P fimbriae, mediated by MrpI, has been suggested as a virulence factor for uropathogenic *P. mirabilis* (12). Given that MrpI switches the IE in both directions, why can expression of *mrp* operon be almost off (on agar plates) or on (during UTIs in mice) (12)? Selection pressures or environmental signaling in the urinary tract may determine the outcome, while the regulation of MrpI expression and epigenetic modification of IE may be involved. This is the first study to demonstrate that RpoE increased fimbria (*mrp* operon) expression by regulating *mrpI*. Several lines of evidence support the notion. First, an RpoE binding site exists upstream of *mrpI* (Fig. 3A). Second, more OFF positions of IE were observed in the *rpoE* mutant than in the wild-type and *rseA* mutant strains (Fig. 3B). Third, RpoE positively regulated the mRNA levels of *mrpI* and two genes belonging to the *mrp* operon, *mrpA* and *mrpJ* (Fig. 3C). Fourth, the *rpoE* mutant had less MrpA protein relative to the wild type (Fig. 3D). On swarming agar plates, the IE of wild-type, *rseA* mutant, and *rpoE* mutant strains was in the OFF position (data not shown), and both ON and OFF appeared on subculturing in broth, being more "ON" in the stationary phase when the RpoE expression was increased (Fig. 3B and Fig. 1). On the contrary, Lane et al. suggested that MrpI preferentially switches the IE from ON to OFF (41). They found that there was more ON in low-O_2 conditions than in atmospheric O_2, a condition wherein *mrpI* expression is higher than under low O_2 (41). It is possible that *mrpI* may be subjected to different regulation in atmospheric-O_2 and low-O_2 conditions. In this regard, an *Escherichia coli* two-component regulator, RcsB, influences the piliation state by controlling transcription of *fimB* and *fimE* recombinases in response to environmental cues (42).

We found that alterations of RpoE did affect expression of *mrpJ* in cells from broth cultures but not those from plate cultures, a phase-off condition for *mrp* (Fig. 3C and E). It is reasonable to infer that the agar surface may be sensed by *P. mirabilis* to swarm, a condition in favor of downregulating fimbriae and thus repressing expression of MrpJ. It is notable that the *flhDC* and *fliC1*mRNA levels were decreased in the *rseA* mutant from both broth and plate cultures (Fig. 3C and E). Moreover, the *rseA* mutant displayed a significant reduction in motility of swimming and swarming (see Fig. S1 in the supplemental material; Fig. 2A). These results suggest that RpoE-mediated swarming repression on agar plates is not MrpJ dependent but that RpoE-regulated MrpJ is involved in the reduction of swimming motility. Overexpression of RpoE (*rseA* mutant) could upregulate MrpI expression; MrpI then switches IE to the ON position to express the *mrp* operon, and then the production of MrpJ inhibits *flhDC* and *fliC1* expression when *P. mirabilis* was subcultured from the LB agar plate into LB broth. The reciprocal regulation of MR/P fimbriae and flagella involving RpoE highlights the role of RpoE in establishing a UTI and the necessity for investigating the dynamics of RpoE expression during infection. Mobley et al. also reported elevated expression of MrpJ in *P. mirabilis* resulted in reduction of FliC1 protein and swimming motility using overnight broth cultures (26). These researchers found the phase ON mutant has a lower level of FlaA than the OFF mutant and the wild type (26). Obviously, other factors and not MrpJ regulated by RpoE account for the repression of swarming and expression of *flhDC* or *fliC1* in cells from plate cultures. It is also possible that *rpoE* mutation may have other effects, except low expression of MrpJ, to result in higher expression of FliC1 than was seen with the wild type in broth cultures. In this view, De Lay et al. found several small RNAs inhibited motility of *E. coli* (43). Among them, we found an ArcZ homologue in *P. mirabilis* and a putative RpoE binding site in its promoter region. The work to characterize the role of *P. mirabilis* ArcZ in motility has been undertaken.

Several results of the present study suggested *P. mirabilis* RpoE could modulate immune responses. First, *rpoE* mutant induced higher levels of IL-8 and MIF (both protein and mRNA), and *rseA* mutant decreased IL-8 and PAI-1 mRNA relative to the wild type (Fig. 4C). Second, LPS of the *rpoE* mutant induced high levels of IL-8 mRNA (Fig. 4D). Third, the *rpoE* mutant exhibited lower survival inside macrophages than did the wild type. Fourth, the *rpoE* mutant caused more immune cell infiltration in both bladders and kidneys on day 1 after inoculation (Table 2 and Fig. 6), which corresponds to the increased proinflammatory cytokine expression (Fig. 4C and D). This is the first study to demonstrate that *P. mirabilis* RpoE affected proinflammatory cytokine expression

of urothelial cells. IL-8, MIF, and PAI-1 could all induce the migration of immune cells to the infection site (16, 44–47). IL-8 has been shown to be the main neutrophil attractant in humans and is secreted by both bladder and kidney cell lines in response to uropathogenic *E. coli* (44). Our finding also supported that IL-8 could be important during UTIs caused by *P. mirabilis*.

The intracellular environment of a phagocyte may protect the bacteria during the early stages of infection or until they develop a full complement of virulence factors. It has been indicated that the deficiency of bactericidal activity of macrophages from cystic fibrosis patients against *Pseudomonas aeruginosa* is correlated with the increased susceptibility to bacterial infections in these patients (34). Our results that surviving bacteria of the *rpoE* mutant decreased rapidly inside macrophages indicated that RpoE may improve the fitness of *P. mirabilis* during the early stage of infection.

Bacterial LPS are capable of inducing bladder inflammation characterized by an increase in the release of proinflammatory cytokines and recruitment of the immune cells (17, 48). Many Gram-negative pathogens modify LPS to alter TLR4 responses (11, 49). For example, *Salmonella* PhoP/Q (a two-component system) can sense host environments, regulating genes involved in LPS modification (11). *Salmonella* modified LPS are up to 100-fold less active for TLR4-driven NF-κB activation, and the PhoP-null mutant is immunostimulatory *in vivo* (11, 50). We found that LPS at up to 5 μg/ml from the *rpoE* mutant but not from the wild type induced a significant level of IL-8 mRNA (Fig. 4D), suggesting that *P. mirabilis* RpoE may participate in LPS modification and that the absence of RpoE-mediated LPS modification results in TLR4 activation and increased IL-8 expression. RpoE in *E. coli* has been shown to be involved in LPS modification (20, 36). LPS profile analysis also revealed a difference in LPS between the *P. mirabilis rpoE* mutant and the wild type (data not shown). Moreover, knowing LPS modification influences PB resistance in *P. mirabilis* (23, 24, 28), the finding that the PB MIC for the wild type was higher than that for the *rpoE* mutant (>50,000 versus 12,500 μg/ml) also supports the notion.

Different authors have proposed that the induction of an innate response could prevent the progress of infection and that TLR4-mediated recognition of LPS was thought to activate the host defense against Gram-negative bacterial pathogens (17). For example, intranasal immunization of mice with MR/P fimbriae protects *P. mirabilis* from UTIs (40) and activating TLR4 by LPS impairs colonization of *Bordetella pertussis* (51). Accordingly, we found *rpoE* mutant increased the proinflammatory cytokines and the immune cell recruitment and had an impaired ability of colonization.

P. mirabilis could cause persistent UTIs despite antibiotic treatment and catheter changes (1, 2, 7). Variation of MR/P fimbriae (9) and flagellar antigens (8), secretion of ZapA protease (10), and the ability of cell invasion are known mechanisms that were utilized by *P. mirabilis* to contribute to immune evasion during infection (1, 3). The formation of biofilms and urinary stones is also thought to limit bacterial exposure to antibiotics and antibodies (1, 2, 7). We found that the loss of RpoE did not affect biofilm formation and, by reporter assay, that RpoE did not regulate *zapA* and *ureC* (mutation of the urease gene, *ureC*, prevents stone formation [1]) in *P. mirabilis* (data not shown). This indicates that RpoE might participate in immune evasion and the persistence of *P. mirabilis* during UTIs by facilitating invasion into urothelial cells and modulating the expression of fimbria and flagella and immune responses.

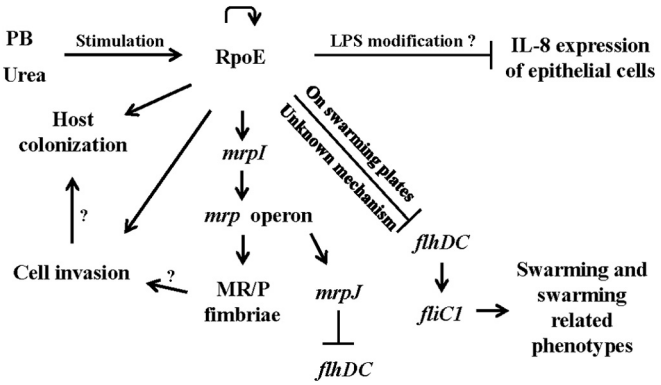

FIG 8 Summary of proposed RpoE roles in *P. mirabilis*. RpoE is autoregulated (curved arrow) and can be activated by urea or antimicrobial peptides in the urinary tract. Activated RpoE upregulates *mrpI* expression, and in turn MrpI controls the production of MR/P fimbriae, which are important for cell invasion and subsequent colonization. RpoE inhibits expression of the host cytokine (IL-8; line with a vertical bar). LPS plays a role in the RpoE-mediated IL-8 expression. In cells from broth cultures, *mrpJ* (one member of the *mrp* operon) contributes to RpoE-mediated *flhDC* repression. In cells from swarming agar plates, the regulation of *flhDC* expression, swarming, and swarming-related phenotypes by RpoE occurs through an MrpJ-independent pathway. A question mark (?) indicates that something was not demonstrated in the present study. PB, polymyxin B.

The ability to perceive changes in different environments and to modify gene activity is important for bacteria. The activation signals of RpoE could be heat/cold shock, oxidative stresses, osmotic stresses, stationary phase, and growth *in vivo* in Gram-negative bacteria (20). In the present study, we demonstrated three signals—PB, urea, and the stationary phase (Fig. 1)—for RpoE activation in *P. mirabilis*. We found that urea, a component of urine, and PB (a cationic antimicrobial peptide) can serve as signals to induce expression of RpoE, reinforcing the role of *P. mirabilis* RpoE in establishing UTIs. In 2011, Pearson et al. detected gene expression of *P. mirabilis* from infected mice by microarray analysis (microarray data accession number GSE25977) (13). These researchers found that genes encoding MR/P fimbriae were upregulated and that those for flagella were downregulated compared to an *in vitro* overnight broth culture. Accordingly, we observed that RpoE positively regulated *mrp* operon (fimbria expression) and negatively regulated *fliC1* (flagellin) in the present study. We suggest that urea could maintain the appropriate level of activated RpoE *in vivo*.

We characterized here the significance of *P. mirabilis* RpoE in building up UTIs (Fig. 8). RpoE could sense the component of urine to regulate multiple virulence factors, including fimbria expression, swarming, cell invasion, survival in macrophages, colonization in mice, and modulation of host immune responses. Given the diverse regulons of RpoE, it is not surprising for such global effects of RpoE. Altogether, RpoE could help *P. mirabilis* adapt to its unique environmental niches for building up a UTI. Our discovery adds RpoE into the complex network of pathogenesis in uropathogenic *P. mirabilis* and deepens our understanding of the factors affecting the infectious process. The information is expected to contribute to the design of new strategies to improve the control of UTIs caused by *P. mirabilis*.

ACKNOWLEDGMENTS

This study was supported by grant NSC-100-2320-B-002-075 from the National Science Council.

We thank Yeong-Shiau Pu (National Taiwan University Hospital) for providing the NTUB1 cell line and Betty A. Wu-Hsieh and Li-Chung Hsu for providing the THP-1 cells and technique support in the macrophage infection assay.

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