RpoS Contributes to Phagocyte Oxidase-Mediated Stress Resistance during Urinary Tract Infection by *Escherichia coli* CFT073

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**ABSTRACT** Uropathogenic *Escherichia coli* (UPEC) is the most common causative agent of community-acquired urinary tract infection (UTI). In order to cause UTI, UPEC must endure stresses ranging from nutrient limitation to host immune components. RpoS (σ70), the general stress response sigma factor, directs gene expression under a variety of inhibitory conditions. Our study of rpoS in UPEC strain CFT073 began after we discovered an rpoS-frameshift mutation in one of our laboratory stocks of wild-type CFT073. We demonstrate that an rpoS-deletion mutation in CFT073 leads to a colonization defect during UTI of CBA/J mice at 48 hours postinfection (hpi). There is no difference between the growth rates of CFT073 and CFT073 rpoS in urine. This indicates that rpoS is needed for replication and survival in the host rather than being needed to address limitations imposed by urine nutrients. Consistent with previous observations in *E. coli* K-12, CFT073 rpoS is more sensitive to oxidative stress than the wild type. We demonstrate that peroxide levels are elevated in voided urine from CFT073-infected mice compared to urine from mock-infected mice, which supports the notion that oxidative stress is generated by the host in response to UPEC. In mice that lack phagocyte oxidase, the enzyme complex expressed by phagocytes that produces superoxide, the competitive defect of CFT073 rpoS in bladder colonization is lost. These results demonstrate that σ70 is important for UPEC survival under conditions of phagocyte oxidase-generated stress during UTI. Though σ70 affects the pathogenesis of other bacterial species, this is the first work that directly implicates σ70 as important for UPEC pathogenesis.

**IMPORTANCE** UPEC must cope with a variety of stressful conditions in the urinary tract during infection. RpoS (σ70), the general stress response sigma factor, is known to direct the expression of many genes under a variety of stressful conditions in laboratory-adapted *E. coli* K-12. Here, we show that σ70 is needed by the model UPEC strain CFT073 to cope with oxidative stress provided by phagocytes during infection. These findings represent the first report that implicates σ70 in the fitness of UPEC during infection and support the idea of the need for a better understanding of the effects of this global regulator of gene expression during UTI.

**RESEARCH ARTICLE**

Utirinary tract infections (UTIs) are among the most common human bacterial infections. UTIs cause significant discomfort, malaise, and lethargy, frequently require antibiotic treatment, and can become life threatening. It is estimated that 150 million cases of UTI occur per year, resulting in global health care costs totaling over 6 billion U.S. dollars (1). Forty percent of women will have a UTI in their lifetimes, and 25% of this population will suffer recurrent UTIs, with subsequent infections usually occurring within 6 to 12 months of the previous occurrence (2, 3). Additionally, 12% of men, with a large proportion of those being elderly, will experience a UTI (2, 3). Though many microbes are known to cause UTI (4), *Escherichia coli* is the most common causative agent, accounting for 70 to 95% of all reported cases (1). Uropathogenic *E. coli* (UPEC) strains are residents of the gut microbiota and can gain access to the urinary tract through an ascending route (4). This route of infection involves the colonization of the periurethral area and ascension through the urethra to the bladder, where the bacteria cause cystitis. If cystitis is left untreated, the bacteria can ascend into the ureters and kidneys, where they cause pyelonephritis.

Neutrophils have been shown to be critical for the clearance of UPEC during infection (5). Neutrophils produce many antimicrobial factors, a primary component being reactive oxygen species (ROS) (6). These ROS originate from superoxide (O2−), which is produced by phagocyte oxidase, a multisubunit enzyme complex expressed by phagocytic cells (7–9). ROS have pleiotropic effects on bacterial cells, where they react with thiols, lipids, metal centers, nucleic acids, and tyrosine residues (8, 10, 11). Indeed, *E. coli* is capable of coping with oxidative stress by a variety of mechanisms, and it is thought that resistance to ROS may be important for UPEC pathogenesis (12).

Transcription of genes in *E. coli* is catalyzed by the RNA polymerase holoenzyme, which is composed of core RNA polymerase (α2ββ′ω) and a dissociable sigma factor (σ). *E. coli* has one housekeeping sigma factor (σ70) and six other “alternative” σ factors (13). In response to stimuli, each of these σ factors facilitates tran-
scription from a variety of different promoters when bound to core polymerase. The best studied of the alternative sigma factors, σ^S, positively regulates ~500 genes involved in stationary-phase survival and in resistance to a variety of stresses, including oxidative stress in E. coli K-12 (13–17). Insight into the role of σ^S in uropathogenesis is lacking, despite an appreciated role in the pathogenesis of other bacterial species and epidemiological data suggesting that a loss of rpoS function may be detrimental to E. coli during human extraintestinal pathogenic E. coli (ExPEC) infections (18, 19).

Here, we show that σ^S allows UPEC strain CFT073 to cope with phagocyte-mediated oxidative stress during urinary tract infection. These data support the hypothesis that σ^S is needed for efficient bladder colonization and that σ^S-regulated genes play a significant role during UTI in tolerating oxidative stress provided by the host innate immune response.

RESULTS

CFT073 rpoS is attenuated for colonization relative to wild type in CBA/J mouse bladders at 48 hpi. To assess if rpoS is important for E. coli CFT073 during urinary tract infection, we utilized the murine model of UTI. When coinoculated with wild type into CBA/J mice, CFT073 rpoS was recovered from bladder homogenates at approximately 50-fold-lower levels at 48 hours postinfection (hpi) (Fig. 1A). This competitive disadvantage was lost when a functional copy of rpoS was reintroduced into its native location in the rpoS mutant by φEB49-mediated allelic transductions (Fig. 1A).

To further investigate the defect shown by a CFT073 rpoS mutant during experimental UTI, we infected CBA/J mice with single strains, either wild type CFT073 or the rpoS mutant. Animals were sacrificed at 48 hpi, and bacterial burdens were assessed. The rpoS mutant was recovered at significantly lower levels than wild type at 48 hpi (Fig. 1B). We assessed several phenotypes known to influence colonization in the murine UTI model. There was no statistically significant difference between mannose-sensitive guinea pig erythrocyte agglutination results nor was there a difference in motility on Adler motility agar (data not shown). Furthermore, the growth kinetics of these two strains in human urine were identical (data not shown).

CFT073 rpoS is more sensitive to oxidative stress than wild type. It was previously demonstrated in E. coli K-12 that many σ^S-regulated genes are involved in detoxifying ROS or repairing damage caused by ROS. To show that rpoS contributes to oxidative stress resistance in CFT073, we utilized a hydrogen peroxide killing assay (modified from reference 20). CFT073 rpoS is killed more readily than wild type by treatment with 5mM hydrogen peroxide (Fig. 2).

Mice infected with CFT073 or CFT073 rpoS have elevated levels of peroxide in urine. To demonstrate that the infected bladder environment provides oxidative stress to bacteria, we measured the levels of H$_2$O$_2$, a relatively stable byproduct of superoxide, in infected CBA/J mouse urine at 24 hpi and 48 hpi. Urine was collected from anesthetized mice that were infected with either strain and subjected to a Xylenol Orange-based Pierce quantitative peroxide assay (n = 12 each). The fold changes in urine peroxide levels were calculated relative to phosphate-buffered saline (PBS) mock-infected control levels. CFT073 rpoS-infected animals did not have increased levels of H$_2$O$_2$ in their urine relative to wild-type-infected animals at either of these time points. From 24 hpi to 48 hpi, however, the concentration of peroxide in all infected animals increased significantly (Fig. 3).

The competitive defect shown by CFT073 rpoS in bladders at 48 hpi is diminished in mice that lack functional phagocyte oxidase. During infection, inflammatory cells produce a variety of antimicrobial factors. A primary antimicrobial agent in this repertoire is superoxide, which reacts with other molecules to form ROS. The primary source of bactericidal ROS during infection is provided by phagocyte oxidase. To assess the contribution of
phagocyte oxidase to the colonization defect of CFT073 rpoS, we utilized C57BL/6 mice and a congenic strain lacking the p47 subunit of this enzyme complex. p47phox knockout mice lack functional phagocyte oxidase (21, 22). As seen in Fig. 4, the competitive disadvantage seen in C57BL/6 mice was significantly diminished in the mice lacking functional phagocyte oxidase.

**DISCUSSION**

Here we present data showing that rpoS is needed for efficient UTI by UPEC. These studies began after we discovered that the sequenced CFT073 strain (23) has a 5-bp duplication in rpoS, which results in the production of a nonfunctional, truncated σS protein. The wild-type strain used in the present study was obtained from the original CFT073 clinical isolate (Table 1). The nucleotide sequence of the CFT073 wild-type rpoS allele has been reported previously (24) and can be found in GenBank (accession no. AF270497).

This report represents the first use of φEB49, the uropathogenic E. coli transducing phage (25), to conduct allelic repair of the rpoS null allele. Allelic repair involves the cotransduction of a functional copy of an allele of interest with a closely linked antibiotic resistance marker. Because the concentration of σS within cells and the subsequent influence on σS-dependent genes are regulated at several different levels (17), allelic repair was used rather than more “traditional” methods of genetic complementation (i.e., single-copy complementation in cis or low-copy-number vector complementation in trans) to maintain wild-type regulation of σS levels. The CFT073 rpoS::φEB49 repaired strain shown in Fig. 1, as well as other CFT073 rpoS::φEB49 transductants tested (n = 10), were shown to have growth kinetics in Luria-Bertani (LB) broth identical to those of wild-type CFT073. Furthermore, transductants that incorporated the kanamycin (Kan) cassette but not the functional rpoS allele (n = 3) and CFT073 rpoS exhibited a

**FIG 2** CFT073 rpoS is more sensitive to oxidative stress in vitro than wild type. CFT073 and CFT073 rpoS were grown overnight in LB medium and diluted 1:1,000 into PBS–5 mM H2O2 or an equivalent volume of distilled water (dH2O). Cells were then incubated at 37°C and were enumerated every 5 min by plating onto LB agar. Data from four independent experiments are represented as mean percent survival ± standard error of the mean (SEM) relative to bacterial counts at t = 0.

**FIG 3** Levels of peroxide in urine are elevated in urine from infected CBA/J mice. CBA/J mice were infected with either CFT073 or CFT073 rpoS (n = 12 each). At 24 and 48 hpi, urine was collected from these animals and H2O2 concentrations were measured using a Xylenol Orange-based quantitative peroxide assay kit (Pierce). Data are represented as mean fold change in [H2O2] ± SEM relative to PBS mock-infected control animals. Mean fold changes in [H2O2] did not differ between mice infected with CFT073 and mice infected with CFT073 rpoS at 24 or 48 hpi, but there was a significant change in [H2O2] in infected animals from 24 to 48 hpi, as assessed by an unpaired t test and a paired t test, respectively.

**FIG 4** The competitive defect of CFT073 rpoS during UTI is diminished in mice that lack functional phagocyte oxidase. C57BL/6 mice (n = 16) and age-matched congenic mice lacking phagocyte oxidase [p47phox (−/−)] (n = 16) were infected with a 1:1 ratio of CFT073 rpoS and wild-type strains. Animals were infected and sacrificed at 48 hpi, and bacteria were enumerated as described in the Fig. 2 legend. Lines are drawn at the geometric mean relative competitive index (RCI). Statistical significance was assessed by the Mann-Whitney U test.

| Strain name | Genotype | Source or reference |
|-------------|----------|---------------------|
| WAM 4505    | *Escherichia coli* CFT073 | Original clinical isolate |
| WAM 4519    | CFT073 rpoS | Our laboratory |
| WAM 4520    | CFT073 lacZYA | Our laboratory |
| WAM 4530    | CFT073 rpoS* Kan | Our laboratory |
| WAM 4560    | CFT073 rpoS::φEB49 Kan | Our laboratory |

φEB49 Generalized transducing phage 25
small decrease in the final optical density at 600 nm (OD$_{600}$) in LB broth in late stationary phase (data not shown).

In *E. coli* K-12, many $\sigma^{d}$-regulated genes are involved in mitigating the toxic effects of reactive oxygen species by a variety of mechanisms. Although these genes (*dps*, *katE*, *sodC*, *xthA*, and *otsLB*, among others) are shared between K-12 and CFT073, their expression during infection and their degree of dependence on $\sigma^{d}$ in CFT073 are not known. Regardless, we show that CFT073 $rpoS$ is killed more readily than wild type by treatment with hydrogen peroxide (Fig. 2), indicating that one or more mechanisms of oxidative stress resistance are regulated by $\sigma^{d}$ in CFT073. The extent of the $\sigma^{d}$ regulon in CFT073 during experimental UTI is a subject of ongoing investigation in our laboratory. A role of $\sigma^{d}$-regulated genes in resistance to oxidative and pH stress in the model UPEC strain UTI89 has been suggested (26). Our observations provide further evidence that $\sigma^{d}$ regulates the expression of oxidative stress resistance genes in UPEC.

Interestingly, there was a significant increase in the concentration of peroxide in urine from 24 hpi to 48 hpi in all infected animals (Fig. 2). This increase in peroxide concentration correlates with a significant change in the bladder relative competitive index (RCI) but not in total bacterial loads in animals coinfected with the wild-type and *rpoS* strains (data not shown). These observations support the notion that CFT073 $rpoS$ is more sensitive than wild type to oxidative stress in *in vivo*. Indeed, when the source of phagocyte-mediated oxidative stress was removed, CFT073 $rpoS$ no longer showed a pronounced colonization defect (Fig. 4).

Our findings that $rpoS$ is needed during UTI conflict with previously reported results from Culham et al. (24), where an assessment contrasts with the well-established stationary-phase induction of $\sigma^{d}$ levels in laboratory-adapted *E. coli* strains (17). We demonstrate that $rpoS$ is needed to cope with phagocyte oxidase-mediated stress during infection. However, we cannot discount the possibility that this global regulator may be important for directing the expression of other genes important during UTI. With a better understanding of $\sigma^{d}$-regulated genes and the regulation of cellular $\sigma^{d}$ levels in UPEC, we expect to garner a greater understanding of how UPEC causes UTIs.

**MATERIALS AND METHODS**

**Strains.** Strains used in this study are listed in Table 1. In-frame deletion mutants of *E. coli* CFT073 were constructed using the lambda Red mutagenesis protocol (39), which was modified to incorporate a generalized transduction step using dEB49 prior to pCP20-mediated antibiotic resistance cassette removal (25). Allelic repair of the *rpoS* null allele was carried out by dEB49-mediated cotransduction of a functional *rpoS* allele linked to a kanamycin resistance cassette (see Fig. S1 in the supplemental material).

**Media and bacterial growth conditions.** All strains were grown in Luria-Bertani (LB) broth, on LB agar, or on filter-sterilized human urine samples. Urine was collected and pooled from healthy human volunteers (*n = 2*) with no recent history of antibiotic use. Inocula for murine model UTI were prepared as described previously (38). For *in vitro* growth analysis, bacteria were grown overnight at 37°C with aeration, washed twice in phosphate-buffered saline (PBS), and added to fresh growth medium to reach an OD$_{400}$ = 0.01. Bacterial growth was measured by OD$_{400}$ readings over time in a Synergy HT plate reader (Bio-Tek). Antibiotic selection (kanamycin [*50 μg/ml*] and chloramphenicol [20 μg/ml]) was used when applicable.

**Murine model UTI.** Six-to-nine-week-old mice were used for all experiments described here. CBA/J mice were purchased from Harlan Laboratories (Indianapolis, IN). C57/BL6 mice and age-matched, congenic *p47-phox/−/−* mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were inoculated with either a single strain (single infection) or equal numbers of two strains (competitive infection) as described previously (38) and sacrificed at 48 hours postinfection (hpi). Bladders were homogenized in PBS–0.0025% Triton X-100, and 10-fold serial dilutions in PBS were plated onto MacConkey’s agar containing lactose. In competitive infections, CFT073 *lacZYA* was used to facilitate enumeration of wild type/mutant ratios. It has been shown previously that CFT073 *lacZYA* colonizes the murine urinary tract as well as wild-type CFT073 (37).
Competitive infection data are reported as a relative competitive index (RCI), which is defined as the ratio of mutant to wild type recovered normalized to the ratio of mutant to wild type in the inoculum.

**Sensitivity to hydrogen peroxide.** Bacteria were grown overnight at 37°C in LB broth with aeration and diluted 1:1,000 in PBS. Diluted cultures were incubated at 37°C, and hydrogen peroxide (Fisher Scientific) was added to reach a final concentration of 5 mM (20). After the addition of peroxide, samples of cells were plated at various time points for viable counts on LB agar and compared to samples where hydrogen peroxide was not added.

**Fold change in levels of peroxides in infected urine samples.** A Pierce quantitative peroxide colorimetric assay kit (Thermo Scientific) was used according to the manufacturer’s protocol, and a Synergy HT plate reader (Bio-Tek) was used to measure peroxide concentration in infected and PBS mock-infected mouse urine samples.

**Assays for motility and expression of type 1 pili.** The motility of single-strain inocula containing CFT073 or CFT073 rpoS was assessed on Adler motility agar as described previously (40). The diameter of the growth area was measured after 22 h of incubation at room temperature. Semiquantitative mannose-sensitive guinea pig erythrocyte agglutination was carried out to assess the presence of type 1 pili as described previously (37). For each assay, the OD₅₀₀ of the inocula was normalized by dilution in PBS where necessary and viable counts on LB agar were used to confirm that there was no significant difference in numbers of bacterial cells used in the assays.

**Statistical analyses.** All statistical analyses were carried out using Prism 4.0 (GraphPad, Inc.). Statistical significance was determined by the U test for log distributed data and was determined by paired or unpaired t tests for normally distributed data where applicable. P values ≤ 0.05 represent statistically significant differences between data sets.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00023-13/-/DCSupplemental.

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