Trade-Off between Iron Uptake and Protection against Oxidative Stress: Deletion of *cueO* Promotes Uropathogenic *Escherichia coli* Virulence in a Mouse Model of Urinary Tract Infection[▽]

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The periplasmic multicopper oxidase (CueO) is involved in copper homeostasis and protection against oxidative stress. Here, we show that the deletion of *cueO* in uropathogenic *Escherichia coli* increases its colonization of the urinary tract despite its increased sensitivity to hydrogen peroxide. The *cueO* deletion mutant accumulated iron with increased efficiency compared to its parent strain; this may account for its advantage in the iron-limited environment of the urinary tract.

Urinary tract infections (UTI) are among the most common infectious diseases of humans and a major cause of morbidity. It is estimated that 40 to 50% of healthy adult women have experienced at least one UTI episode in their lifetime (6). Uropathogenic *Escherichia coli* (UPEC) is the cause of the majority (>80%) of UTI in humans. UPEC isolates exhibit a high degree of genetic diversity due to the possession of specialized virulence genes located on pathogenicity islands (21). Although no single virulence factor is uniquely definitive of UPEC, its ability to cause symptomatic UTI is enhanced by adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., hemolysin) (16, 38). Another important virulence property of UPEC is its ability to sequester iron. The concentration of soluble iron is very low in urine and represents an important growth-limiting factor for bacteria. UPEC possesses multiple mechanisms to acquire iron, including the production of siderophores, such as aerobactin and enterobactin (and the glycosylated enterobactin derivative, salmochelin), and the direct utilization of host iron compounds (particularly heme or hemoglobin) (1, 3, 10, 23, 25, 26). UPEC mutants deleted in these processes display reduced virulence in the mouse urinary tract (32).

The global oxidative stress response regulator OxyR is required for virulence in a mouse model of UTI (14). This indicates that UPEC responds to oxidative stress during infection, consistent with evidence that the attachment of UPEC to the uroepithelium leads to neutrophil recruitment (11, 14). OxyR also acts in concert with Dam methyltransferase to regulate the expression of the antigen 43-encoding *flu* gene (9, 12, 28). Antigen 43 is an autotransporter protein that promotes aggregation, biofilm formation, and long-term persistence of UPEC in the urinary bladder (15, 36).

The *cueO* gene encodes a periplasmic multicopper oxidase which is known to be involved in copper homeostasis and protection against oxidative stress. CueO possesses ferroxidase ($Fe^{2+} \rightarrow Fe^{3+}$), cuprous oxidase ($Cu^{+} \rightarrow Cu^{2+}$), and polyphenol oxidase (oxidation of phenolic compounds, including enterobactin) activities (8, 24, 30). Both Cu^{+} and Fe^{2+} generate toxic hydroxyl radicals via the Fenton reaction (Fe^{2+} or Cu^{+} + $H_2O_2 \rightarrow Fe^{3+}$ or Cu^{2+} + OH^{-} + $OH^{·}$). Oxidation of enterobactin by CueO prevents Cu^{2+} reduction by the reactive catechol groups on enterobactin and has been proposed to form a 2-carboxymuconate derivative in the periplasm that may sequester both copper and iron ions (8) and thereby protect bacteria against metal ion-promoted oxidative stress. In view of the role of CueO in *E. coli* K-12, we were interested in determining whether it contributes to the pathogenesis of the prototypical UPEC strain CFT073 (19, 37).

CueO is required for copper resistance in UPEC CFT073. The deletion of *cueO* renders *E. coli* K-12 sensitive to $CuSO_4$, a phenotype that is enhanced under conditions of iron limitation in which high concentrations of enterobactin are produced. To assess the role of CueO from *E. coli* CFT073 in copper tolerance, a *cueO* deletion strain (CFT073*cueO*) was constructed by λ red-mediated homologous recombination, as previously described (5). Copper sensitivity was assessed by growing CFT073 and CFT073*cueO* on Tris-buffered mineral salts agar supplemented with 0.2% glycerol and 0.3% CAS amino acids in the presence of filter discs impregnated with 5 μl of 1 M $CuSO_4$. In this assay, CFT073 was resistant to copper and produced a distinct brown pigment in the region of growth at the periphery of the clearing zone (Fig. 1). In contrast, CFT073*cueO* was highly sensitive to copper. The copper resistance phenotype of CFT073*cueO* could be restored by complementation with a plasmid containing the *cueO* gene (pCueO) (Fig. 1).

Deletion of *cueO* promotes colonization of the mouse bladder and shedding in urine. An established mouse model of UTI was employed to examine the role of *cueO* in UPEC virulence (36). Briefly, female C57BL/6 mice (8 to 10 weeks old) were catheterized using a sterile Teflon catheter by inserting the device directly into the bladder through the urethra. An inoculum of 25 μl, containing 5×10^8 CFU of CFT073 or

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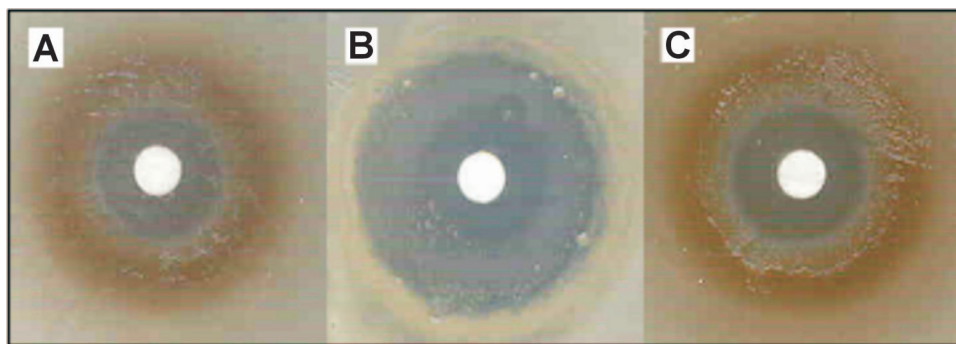
FIG. 1. Copper sensitivities of CFT073 (A), CFT073*cueO* (B), and CFT073*cueO*(pCueO) (C). Overnight cultures were diluted 1/10,000 and spread plated onto solidified Tris-buffered mineral salts media. Discs containing 5 μl of 1 M CuSO$_4$ were placed on plates and incubated overnight. CFT073*cueO* displayed increased sensitivity to copper.

CFT073*cueO* in phosphate-buffered saline (PBS), was instilled directly into the bladder by using a 1-ml tuberculin syringe attached to the catheter. Mice were euthanized at 18 h after challenge by cervical dislocation; bladders and kidneys were then excised aseptically, weighed, and homogenized in PBS for colony counts. Urine samples were also collected from each mouse prior to euthanasia for quantitative colony counts. Compared to CFT073, CFT073*cueO* colonized the mouse bladder in significantly higher numbers in this infection model (Fig. 2A). This also correlated with increased shedding of CFT073*cueO* in urine compared to that of CFT073 (Fig. 2B). No colonization of the kidneys was observed for CFT073 or for CFT073*cueO*; this is consistent with previous data from our laboratory using C57BL/6 mice (36).

Deletion of *cueO* does not affect type 1 fimbria expression, adhesive capacity, or growth in urine. Expression of type 1 fimbriae significantly enhances the attachment of UPEC to uroepithelial cells and the subsequent colonization of the mouse bladder (2, 4, 20). We compared the levels of type 1 fimbria expression in CFT073 and in CFT073*cueO* by using a combination of standard techniques. First, the abilities of CFT073 and CFT073*cueO* to cause mannose-sensitive agglu-

tination of yeast cells were examined. There was no difference in the agglutination titers for the two strains (data not shown). Second, the amounts of FimA produced by the two strains were compared by Western blot analysis using a polyclonal serum raised against purified type 1 fimbriae. There was no difference in the amounts of FimA produced by the two strains (Fig. 3A). Third, a PCR-based assay was employed to determine the orientation of the phase-variable *fimA* promoter (7, 29). There was no difference in the amounts of "on" and "off" fragments amplified from both strains (Fig. 3B). Finally, we compared the abilities of CFT073 and CFT073*cueO* to adhere to HeLa epithelial cells as previously described (35). In these assays, CFT073 and CFT073*cueO* displayed equivalent adherence levels (Fig. 3C). Taken together, the data suggest that the expression levels of type 1 fimbriae were the same in CFT073 and CFT073*cueO*.

In *E. coli* K-12, deletion of *cueO* results in increased aggregation, and this phenotype correlates with the enhanced ex-
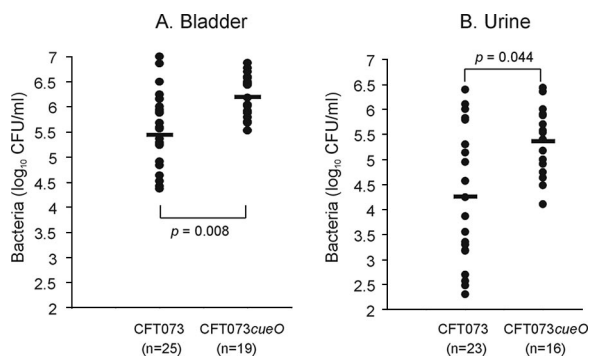

FIG. 2. CFT073 and CFT073*cueO* colonization of C57BL/6 mice bladders (A) and shedding in urine (B). Data for individual mice are expressed as the total number of CFU per 0.1 g of bladder tissue or as the total number of CFU per ml urine. The number of mice used for each experiment (n) is indicated. The data represent a compilation of the results for three individual experiments. Statistical analysis was performed using independent sample *t* test within the SPSS v9.0.2 software package.
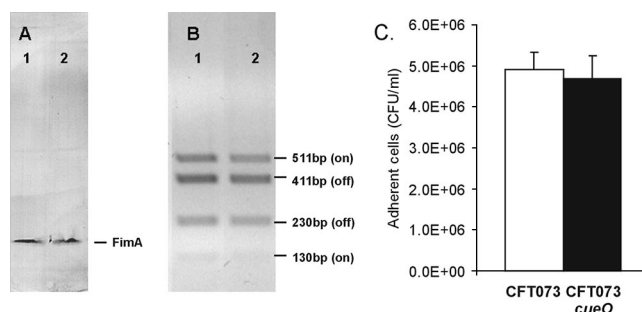

FIG. 3. Expression of type 1 fimbriae and adherence to HeLa cell monolayers. (A) Western blot analysis of whole-cell lysates prepared from CFT073 (lane 1) and CFT073*cueO* (lane 2) using a type 1 fimbria-specific antibody. The FimA major subunit protein of type 1 fimbriae was detected in equivalent amounts in both strains. (B) *fim* promoter orientation in CFT073 and CFT073*cueO*. Lane 1, HinfI-digested CFT073 P$_{fim}$ PCR product; lane 2, HinfI-digested CFT073*cueO* P$_{fim}$ PCR product. Bands at 511 bp and 130 bp indicate the relative proportions of the *fim* promoter in the "on" orientation, and bands at 411 bp and 230 bp indicate the relative proportions of the *fim* promoter in the "off" orientation. (C) CFT073 and CFT073*cueO* adherence to HeLa cell monolayers. Bacteria (5 × 10^6 CFU) were incubated on confluent HeLa cell monolayers for 2 h. Monolayers were washed three times with PBS, and adherent cells were recovered. Error bars represent standard errors.
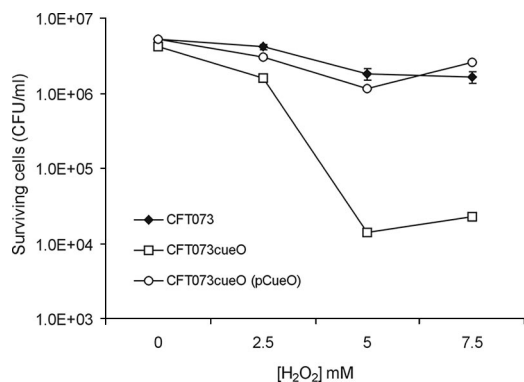
FIG. 4. Sensitivities of CFT073, CFT073*cueO*, and CFT073*cueO* (pCueO) to H_2O_2. Approximately 1×10^6 cells for each strain were harvested in mid-exponential growth phase and incubated for 1 h in the presence of 0, 2.5, 5.0, and 7.5 mM H_2O_2. The reaction was stopped by serial dilution of cells into PBS containing 1,000 U/ml catalase. The number of viable cells remaining after H_2O_2 treatment was determined by direct colony counts. Error bars represent the standard errors from the results for triplicate assays.

FIG. 5. Iron uptake by CFT073, CFT073*cueO*, and CFT073*cueO* (pCueO). Strains were incubated in the presence of 100 μM $^{55}Fe^{2+}$. The rate of ferrous iron uptake was determined by removing samples at 30, 60, 90, 120, 180, and 300 s and monitoring β emissions by using a Beckman LS3801 scintillation counter. $^{55}Fe^{2+}$ uptake rates were normalized to CFU/ml. Data are presented as the mean $^{55}Fe^{2+}$ uptake rates per CFU (±standard error) and represent the averages of the results for three independent experiments.

pression of genes encoding antigen 43 and curli (33). CFT073 and CFT073*cueO* produced equal levels of antigen 43, as determined by Western blot analysis (data not shown). We also examined the growth of CFT073 and CFT073*cueO* and their concordant production of catechols in human urine as previously described (8, 26). No significant difference was observed between the two strains (data not shown).

CueO is required for hydrogen peroxide resistance. The generation of hydroxyl radicals from hydrogen peroxide and iron (the Fenton reaction) is thought to be the primary bactericidal activity of hydrogen peroxide as, in the presence of iron chelators, hydrogen peroxide toxicity is greatly reduced (13). CueO has previously been shown to protect *Salmonella enterica* serovar Typhimurium from peroxide stress (17). Given the significant increase in the colonization of the mouse bladder by CFT073*cueO*, we tested whether this strain displayed increased susceptibility to hydrogen peroxide stress by using an established protocol (34). Despite its hypercolonization phenotype, CFT073*cueO* was significantly more sensitive to hydrogen peroxide challenge than CFT073 (Fig. 4). CFT073*cueO* resistance to hydrogen peroxide could be restored by complementation with plasmid pCueO (Fig. 4).

Deletion of *cueO* increases ferrous iron uptake. A possible explanation for the hypercolonization phenotype of CFT073*cueO* is that this strain can take up iron with increased efficiency. Thus, while excessive iron accumulation may be detrimental under conditions of oxidative stress, iron is a limiting nutrient in urine, and enhanced uptake could provide a growth advantage. To compare the abilities of CFT073 and CFT073*cueO* to take up iron, we performed $^{55}Fe^{2+}$ uptake experiments as previously described (18). In these assays, CFT073*cueO* accumulated 5.5-fold more $^{55}Fe^{2+}$ than did CFT073 (Fig. 5). The introduction of plasmid pCueO into CFT073*cueO* restored the level of $^{55}Fe^{2+}$ accumulated to approximately wild-type levels (Fig. 5).

Deletion of *cueO* does not confer a growth advantage in a systemic infection model. To examine if the increased colonization observed for CFT073*cueO* in the mouse bladder was also reflected in colonization of other sites, we tested CFT073
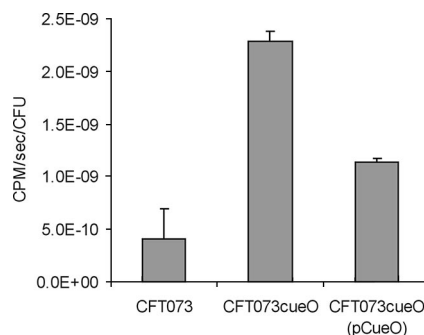
and CFT073*cueO* in a mouse systemic infection model. Mice were infected with 2×10^7 cells of CFT073 ($n = 15$) or CFT073*cueO* ($n = 15$) by a subcutaneous injection into the abdomen and monitored to assess the clinical effects of infection, as previously described (22). There was no difference in the virulence levels of the two strains (the time taken to kill the mice was approximately 24 h for both strains). Bacterial colony counts were also performed from the liver, spleen, and kidneys of each mouse; there was no significant difference in the abilities of the strains to colonize these organs.

Conclusions. The results presented herein show that while deletion of *cueO* in *E. coli* CFT073 renders the cell sensitive to hydrogen peroxide stress, it also promotes increased uptake of iron. Under the iron-limited conditions encountered in the urinary tract (25, 27, 31, 32), this may represent a competitive advantage, though the consequence may be that it leaves the cell vulnerable to iron-promoted oxidative stress. Recent work by Grass and coworkers (8) has shown that CueO in *E. coli* K-12 has a high affinity for Fe-enterobactin (K_m of 1.5 μM) and that oxidation of the enterobactin precursor 2,3-dihydroxybenzoic acid leads to formation of a polymer capable of chelating copper and iron in the periplasm (8). Production of an iron-chelating polymer in the periplasm of CFT073 under iron-limited conditions may account for the restricted iron uptake observed in this strain; loss of this biological function in the *cueO* mutant may lead to increased uptake of ferrous iron.

Although the CFT073*cueO* strain displayed enhanced bladder colonization in comparison to CFT073, this did not translate into increased growth in human urine. Recent transcriptional profiling of UPEC during the formation of intracellular bacterial communities within epithelial cells revealed that the intracellular environment is iron limited and aerobic (23). Increased iron acquisition from damaged epithelial cells could explain the enhanced colonization of CFT073*cueO* in the mouse bladder. Our results lead us to conclude that CueO is critical for iron and copper homeostasis in UPEC and that it has a key role in maintaining a tightly controlled flux of iron into the cell to avoid oxidative stress. The attachment of UPEC to the uroepithelium induces neutrophil recruitment with a

concomitant oxidative burst that would expose UPEC to reactive oxygen species (11). In view of this, the superior colonization of the bladder by the *cueO* mutant is surprising. However, we note that we employed a short-term infection model, and thus, it is possible that the long-term fitness of CFT073*cueO* would be diminished in inflamed tissues in which reactive oxygen species cause oxidative stress. This is consistent with our observations that the CFT073*cueO* mutant is not attenuated in a systemic infection model.

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