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 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 (Fe2⁺ → Fe3⁺), cuprous oxidase (Cu⁺ → Cu²⁺), and polyphenol oxidase (oxidation of phenolic compounds, including enterobactin) activities (8, 24, 30). Both Cu⁺ and Fe²⁺ generate toxic hydroxyl radicals via the Fenton reaction (Fe²⁺ or Cu⁺ + H₂O₂ → Fe³⁺ or Cu²⁺ + OH⁻ + OH⁻). Oxidation of enterobactin by CueO prevents Cu²⁺ reduction by the reactive cationic copper on enterobactin and has been proposed to form a 2-carboxymuconate derivative in the periplasm that may sequester both copper and iron oxides (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 CuSO₄, 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 (CFT073cueO) was constructed by λ red-mediated homologous recombination, as previously described (5). Copper sensitivity was assessed by growing CFT073 and CFT073cueO 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 CuSO₄. 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, CFT073cueO was highly sensitive to copper. The copper resistance phenotype of CFT073cueO 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 × 10⁸ CFU of CFT073 or...
CFT073cueO 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, CFT073cueO colonized the mouse bladder in significantly higher numbers in this infection model (Fig. 2A). This also correlated with increased shedding of CFT073cueO in urine compared to that of CFT073 (Fig. 2B).

No colonization of the kidneys was observed for CFT073 or for CFT073cueO; 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 CFT073cueO by using a combination of standard techniques. First, the abilities of CFT073 and CFT073cueO to cause mannose-sensitive agglutination 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 CFT073cueO to adhere to HeLa epithelial cells as previously described (35). In these assays, CFT073 and CFT073cueO 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 CFT073cueO.

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

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**FIG. 1.** Copper sensitivities of CFT073 (A), CFT073cueO (B), and CFT073cueO(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 CuSO4 were placed on plates and incubated overnight.

CFT073cueO displayed increased sensitivity to copper.

**FIG. 2.** CFT073 and CFT073cueO 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 CFT073cueO (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 CFT073cueO. Lane 1, HinfI-digested CFT073 *P*<sub>fim</sub> PCR product; lane 2, HinfI-digested CFT073cueO *P*<sub>fim</sub> 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 CFT073cueO adherence to HeLa cell monolayers. Bacteria (5 × 10<sup>6</sup> 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.
performed 55Fe2

The abilities of CFT073 and CFT073

enhanced uptake could provide a growth advantage. To compare

ditions of oxidative stress, iron is a limiting nutrient in urine, and

between the two strains (data not shown).

ously described (8, 26). No significant difference was observed

expression of genes encoding antigen 43 and curli (33). CFT073

and CFT073cueO produced equal levels of antigen 43, as de-
termined by Western blot analysis (data not shown). We also

examined the growth of CFT073 and CFT073cueO and their

condordant production of catechols in human urine as previ-

erously described (8, 26). No significant difference was observed

between the two strains (data not shown).

CueO is required for hydrogen peroxide resistance. The gene-

eration of hydroxyl radicals from hydrogen peroxide and iron

(the Fenton reaction) is thought to be the primary bacte-

ricial 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

CFT073cueO, we tested whether this strain displayed increased

susceptibility to hydrogen peroxide stress by using an estab-

lished protocol (34). Despite its hypercolonization phenotype,

CFT073cueO was significantly more sensitive to hydrogen per-

oxide challenge than CFT073 (Fig. 4). CFT073cueO 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 CFT073cueO

is that this strain can take up iron with increased efficiency. Thus,

while excessive iron accumulation may be detrimental under con-

ditions 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 CFT073cueO to take up iron, we

performed 55Fe2+ uptake experiments as previously described

(18). In these assays, CFT073cueO accumulated 5.5-fold more

55Fe2+ than did CFT073 (Fig. 5). The introduction of plasmid

pCueO into CFT073cueO restored the level of 55Fe2+ accumu-

lated 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 coloni-
zation observed for CFT073cueO in the mouse bladder was also

reflected in colonization of other sites, we tested CFT073

and CFT073cueO in a mouse systemic infection model. Mice

were infected with 2 × 107 cells of CFT073 (n = 15) or

CFT073cueO (n = 15) by a subcutaneous injection into the

abdomen and monitored to assess the clinical effects of infec-
tion, 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 abil-

ties 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 (Km of 1.5 μM) and

that oxidation of the enterobactin precursor 2,3-dihydroxyben-

zoic 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 CFT073cueO strain displayed enhanced blad-

der colonization in comparison to CFT073, this did not trans-

late into increased growth in human urine. Recent transcrip-

tional profiling of UPEC during the formation of intracellular

bacterial communities within epithelial cells revealed that the

intracellular environment is iron limited and aerobic (23). In-

creased iron acquisition from damaged epithelial cells could

explain the enhanced colonization of CFT073cueO 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 cue 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 CFT073cueO would be diminished in inflamed tissues in which reactive oxygen species cause oxidative stress. This is consistent with our observations that the CFT073cueO mutant is not attenuated in a systemic infection model.

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