

## Roles of the Extraintestinal Pathogenic *Escherichia coli* ZnuACB and ZupT Zinc Transporters during Urinary Tract Infection<sup>∇</sup>

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**Roles of the ZnuACB and ZupT transporters were assessed in *Escherichia coli* K-12 and uropathogenic *E. coli* (UPEC) CFT073. K-12 and CFT073  $\Delta znu$   $\Delta zupT$  mutants demonstrated decreased  $^{65}\text{Zn}^{2+}$  uptake and growth in minimal medium. CFT073 $\Delta znu$  demonstrated an intermediate decrease of  $^{65}\text{Zn}^{2+}$  uptake and growth in minimal medium, whereas the CFT073 $\Delta zupT$  mutant grew as well as CFT073 and exhibited a less marked decrease in  $^{65}\text{Zn}^{2+}$  uptake. CFT073 mutants grew as well as the wild type in human urine. In competitive infections in CBA/J mice, the  $\Delta zupT$  mutant demonstrated no disadvantage during urinary tract infection. In contrast, the UPEC  $\Delta znu$  and  $\Delta znu$   $\Delta zupT$  strains demonstrated significantly reduced numbers in the bladders (mean 4.4- and 30-fold reductions, respectively) and kidneys (mean 41- and 48-fold reductions, respectively). In addition, in single-strain infection experiments, the  $\Delta znu$  and  $\Delta znu$   $\Delta zupT$  mutants were reduced in the kidneys ( $P = 0.0012$  and  $P < 0.0001$ , respectively). Complementation of the CFT073  $\Delta znu$   $\Delta zupT$  mutant with the *znuACB* genes restored growth in Zn-deficient medium and bacterial numbers in the bladder and kidneys. The loss of the zinc transport systems decreased both motility and resistance to hydrogen peroxide, which could be restored by supplementation with zinc. Overall, the results indicate that Znu and ZupT are required for growth in zinc limited-conditions, that Znu is the predominant zinc transporter, and that the loss of Znu and ZupT has a cumulative effect on fitness during UTI, which may in part be due to reduced resistance to oxidative stress and motility.**

*Escherichia coli* is a versatile bacterial species comprised of innocuous and pathogenic strains. Pathogenic *E. coli* cause intestinal or extraintestinal infections in humans and other animals (28). Extraintestinal pathogenic *E. coli* (ExPEC) cause an array of diseases, including urinary tract infections (UTIs), neonatal meningitis, and septicemia. ExPEC associated with UTI is termed uropathogenic *E. coli* (UPEC). Compared to *E. coli* K-12 and other commensal strains, ExPEC strains encode in their genomes a greater variety of characterized, as well as putative, metal transport systems (11, 54). The importance of pathogen-specific iron transport systems for ExPEC has been established (20, 21, 26). However, the importance of other metal transport systems in ExPEC is less well characterized, and in particular nothing has been reported on the potential implication of the zinc transporters for the virulence of ExPEC.

Zinc ( $\text{Zn}^{2+}$ ) is an essential micronutrient in all living cells, since this transition metal is a component of numerous metalloproteins and serves as an enzymatic cofactor or a structural element (5). In mammals,  $\text{Zn}^{2+}$  has an important immunomodulatory function and is critical for innate and acquired immunity (45). After exposure to lipopolysaccharide (LPS), zinc levels are decreased in the serum, and zinc is accumulated in the liver (38). In addition, during bacterial infection, the host protein calprotectin is released by neutrophils and may reduce bacterial numbers by restricting the availability of metals, including zinc and manganese (16, 39, 50). Also, although zinc concentrations are estimated to be in the millimolar range

within host cells, available zinc may be mostly inaccessible to bacterial pathogens (3) and/or dramatically reduced after activation of innate immune defenses (45).

In bacteria, the uptake of zinc is mediated by two major types of transporters: ZnuACB, which belongs to the cluster C9 family of (TroA-like) ATP-binding cassette (ABC) transporters (15, 27), and ZupT, which is a member of the ZIP (for ZRT/IRT-like protein) family of transporters that are also present in eukaryotes (27). Thus far, the Znu transport system has been shown to be important for virulence in a number of bacterial pathogens, including *Salmonella enterica*, *Brucella abortus*, and *Haemophilus ducreyi* (3, 12, 35, 56). Currently, the roles of the Znu and ZupT transporters for ExPEC virulence have not been investigated.

The genomes of all *E. coli* strains that have been sequenced thus far contain genes encoding the two described zinc transporters: ZupT and ZnuACB. In addition to the transport of zinc, ZupT can also mediate the uptake of  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mn}^{2+}$  (24). The exact mechanism by which ZupT mediates metal uptake is currently unknown, although it may involve a chemiosmotic transmembrane gradient (24). Zinc uptake mediated by the Znu system requires hydrolysis of ATP by ZnuC to transport  $\text{Zn}^{2+}$  captured by the periplasmic binding protein ZnuA through the pore in the cytoplasmic membrane formed by a ZnuB dimer (36, 44). In ExPEC strains, the SitABCD transport system represents an additional ABC transporter belonging to the C9 (TroA-like) cluster (46). Sit transporters have thus far been characterized as manganese and iron transporters (8, 29, 47), although zinc has been shown to be an effective competitive inhibitor of manganese or iron uptake by SitABCD (8, 29). The SitA periplasmic binding protein also shares similarities to the TroA periplasmic binding protein of *Treponema pallidum*, which has been shown to bind both zinc

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s) <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>E. coli</i> K-12		
DH5 $\alpha$	F <sup>-</sup> $\lambda$ <sup>-</sup> 80 ( <i>lacZYA-argF</i> ) <i>endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i> ; Nal <sup>r</sup>	Bethesda Laboratories
BW25113	F <sup>-</sup> $\lambda$ <sup>-</sup> ( <i>lacI<sup>s</sup> rrmBT14 lacZWI16 hsdR514 araBADAH33 rhaBADLD78</i> )	17
JW5831-1	BW25113 $\Delta$ <i>znuA::kan</i> ; Km <sup>r</sup>	7
MG1655	F <sup>-</sup> $\lambda$ <sup>-</sup> <i>ilvG rfb-50 rph-1</i>	9
QT1369	MG1655 $\Delta$ <i>znu::kan</i> ; Km <sup>r</sup>	This study
QT1370	MG1655 $\Delta$ <i>znu::FRT</i>	This study
QT1435	MG1655 $\Delta$ <i>znu::FRT</i> $\Delta$ <i>zupT::kan</i> ; Km <sup>r</sup>	This study
QT1460	QT1435(pACYC184); Km <sup>r</sup> Cm <sup>r</sup> Tc <sup>r</sup>	This study
QT1461	QT1435(pIJ28-pACYC184:: <i>sitABCD</i> ); Km <sup>r</sup> Cm <sup>r</sup>	This study
QT1462	QT1435(pIJ156-pACYC184:: <i>znuACB</i> ); Km <sup>r</sup> Cm <sup>r</sup>	This study
QT1463	QT1435(pIJ202-pACYC184:: <i>zupT</i> ); Km <sup>r</sup> Cm <sup>r</sup>	This study
QT1553	MG1655 $\Delta$ <i>zupT::kan</i> ; Km <sup>r</sup>	
<b>ExPEC</b>		
CFT073	UPEC wild-type pyelonephritis strain	43, 54
QT908	CFT073 $\Delta$ <i>sit::tetAR</i> ; Tc <sup>r</sup>	This study
QT1081	CFT073 $\Delta$ <i>lacZYA</i>	This study
QT1239	APEC $\chi$ 7122 $\Delta$ <i>sit::tetAR</i> $\Delta$ <i>mntH::kan</i> ; Km <sup>r</sup> Tc <sup>r</sup>	47
QT1377	CFT073 $\Delta$ <i>znu::kan</i> ; Km <sup>r</sup>	This study
QT1378	CFT073 $\Delta$ <i>sit::tetAR</i> $\Delta$ <i>znu::kan</i> ; Km <sup>r</sup> Tc <sup>r</sup>	This study
QT1434	CFT073 $\Delta$ <i>znu::FRT</i>	This study
QT1554	CFT073 $\Delta$ <i>zupT::kan</i> ; Km <sup>r</sup>	This study
QT1555	CFT073 $\Delta$ <i>znu::FRT</i> $\Delta$ <i>zupT::kan</i> ; Km <sup>r</sup>	This study
QT1634	CFT073 $\Delta$ <i>znu::FRT</i> $\Delta$ <i>zupT::kan</i> $\Delta$ <i>sit::tetAR</i> ; Km <sup>r</sup> Tc <sup>r</sup>	This study
QT1640	CFT073 $\Delta$ <i>znu::FRT</i> $\Delta$ <i>sit::tetAR</i> ; Tc <sup>r</sup>	This study
QT1932	QT1555(pIJ254)(pGP704:: <i>znuACB</i> ); Km <sup>r</sup> Ap <sup>r</sup>	This study
<b>Plasmids</b>		
pACYC184	p15A replicon cloning vector; Cm <sup>r</sup> Tc <sup>r</sup>	14
pCP20	FLP helper plasmid Ts replicon; Ap <sup>r</sup> Cm <sup>r</sup>	17
pGP704	<i>oriR6K mobRP4</i> ; Ap <sup>r</sup>	42
pIJ28	<i>sitABCD</i> region of $\chi$ 7122 cloned into HindIII site of pACYC184 plasmid vector; Cm <sup>r</sup>	47
pIJ156	<i>znuACB</i> region of MG1655 cloned into HindIII site of pACYC184 plasmid vector; Cm <sup>r</sup>	This study
pIJ202	<i>zupT</i> operon of MG1655 cloned into HindIII site of pACYC184 plasmid vector; Cm <sup>r</sup>	This study
pIJ254	<i>znuACB</i> region cloned from pIJ156 into XbaI and EcoRV sites of pGP704	This study
pKD13	Template plasmid for the amplification of the <i>kan</i> cassette bordered by <i>FRT</i> sites	17
pKD46	$\lambda$ -Red recombinase plasmid Ts replicon; Ap <sup>r</sup>	17

<sup>a</sup> Cm<sup>r</sup>, chloramphenicol resistance; Nal<sup>r</sup>, nalidixic acid; Tc<sup>r</sup>, tetracycline resistance; Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance.

and manganese with essentially equal affinities (19). It remains to be determined whether in addition to Znu and ZupT transporters, SitABCD or any other undefined transport systems may contribute to zinc transport or the virulence of ExPEC.

In the present study, the roles of the Znu, ZupT, and SitABCD transport systems for zinc uptake and growth in zinc-restricted medium were compared in an *E. coli* K-12  $\Delta$ *znu*  $\Delta$ *zupT* mutant. In addition, the roles of the Znu and ZupT transporters for growth of ExPEC strain CFT073 in zinc-restricted conditions and for colonization in the murine ascending UTI model were assessed.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in the present study are listed in Table 1. Strains were routinely cultured overnight in Luria-Bertani (LB) broth or on LB agar (15 g/liter agar) at 37°C and stored in 25% glycerol-LB broth at -80°C. Antibiotics were added as required at the following concentrations: kanamycin, 30  $\mu$ g/ml; ampicillin, 100 to 200  $\mu$ g/ml; chloramphenicol, 30  $\mu$ g/ml; and tetracycline, 10  $\mu$ g/ml.

**PCR and gene cloning.** The primers used for the PCRs are presented in Table 2. PCR was used to amplify *znuACB* and *zupT* genes from *E. coli* K-12 strain MG1655. PCR was also used to create and confirm all of the mutant genotypes throughout the present study. The templates used for PCR amplification were either crude lysates of bacterial strains or diluted plasmid DNA. New England Biolabs *Taq* DNA polymerase was used for routine PCR amplifications (confirmation of mutant alleles), and the high-fidelity Herculase DNA polymerase (Stratagene) was used for amplification of cloned fragments. A 5- $\mu$ l portion of each bacterial cell lysate or a 1- $\mu$ l aliquot of plasmid DNA was added to a PCR mixture to achieve a final volume of 25  $\mu$ l containing 6.25 pmol of each primer, 5 nmol of each deoxynucleoside triphosphate, and 0.5 U of either *Taq* polymerase or Herculase in 1 $\times$  buffer. The PCR conditions were as follows: 95°C for 1 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and with a final extension period of 72°C for 1 min. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Plasmid DNA was purified by using Qiagen purification kits according to the supplier's protocol.

Plasmid pIJ156, containing the *znuACB* genes, was obtained by ligating a HindIII-digested fragment obtained by PCR using primers CMD637 and CMD638 (Table 2) into the HindIII site of pACYC184. Similarly, pIJ202 containing *zupT*, was obtained by ligating a HindIII-digested PCR product obtained using the primers CMD717 and CMD718 into HindIII site of pACYC184. The cloned inserts of plasmids pIJ156 and pIJ202 were sequenced and confirmed to be identical to MG1655 genomic sequences of the *znuACB* and *zupT* genes,

TABLE 2. Primers used in this study

Primer	Characteristic(s)	Sequence	$T_m$ (°C)
CMD23	SitA-SitD region amplification (used with CMD42)	CGCAGGGGGCACAACCTGAT	54
CMD42		CTGTGCGCTGCTGTCGGTC	54
CMD264	Amplification of <i>znu::kan</i> from JWK5831_1 (used with CMD265)	AAAACGCCACAATCCAGTTC	54
CMD265		CGGCAGTACGGTCATAGGTT	56
CMD637	Contains a HindIII restriction site; amplification of <i>znuACB</i> region of MG1655 for cloning (used with CMD638)	GACAAGCTTGGCCAGAGTAAGAACGG	56
CMD638	Contains a HindIII restriction site	CGAAAGCTTCGTGGAATCACTTTGGC	56
CMD715	Construction of <i>zupT::kan</i> using pKD13 template plasmid (used with CMD716)	TTCCCGCCGTTTGTAGCAGCACTAAACTG AATCCCATCACTGACATTCCAATTCCGG GGATCCGTCGACC	68
CMD716		CTCTCATTCTGACCATACTGGCGGGGGCA GCCACGTTTATTGGCGCGTTTTGTAGGC TGGAGCTGCTTCG	68
CMD717	Contains a HindIII restriction site; amplification of <i>zupT</i> operon of MG1655 for cloning (used with CMD718)	GTTAAGCTTACGATCTGCCTGAAGGTGAA	54
CMD718	Contains a HindIII restriction site	GAAGAAGCTTATGCGCTGGACAACCTTCTG	54

respectively. The suicide plasmid used for single-copy complementation of *znuACB* genes, pIJ254, was constructed by digestion of plasmid pIJ156 with XbaI and EcoRV and ligation of the appropriate fragment into the same restriction sites of plasmid pGP704.

**Construction of mutant strains.** All mutants were made by using one-step PCR fragment-mediated  $\lambda$ -Red recombination mutagenesis (17). The *znuA::kan* allele was amplified directly from strain JW5831-1 ( $\Delta znuA::kan$ ) (7) using the primers CM264 and CMD265 (Table 2) to generate  $\Delta znuA::kan$  derivatives of K-12 strain MG1655 (QT1369) and UPEC strain CFT073 (QT1377) by recombination-mediated allelic exchange using plasmid pKD46 (17). The kanamycin cassettes, which are flanked by *FRT* sites, were lost in these strains through FLP recombinase-mediated excision using plasmid pCP20, generating the strains QT1370 (MG1655  $\Delta znuA::FRT$ ) and QT1434 (CFT073  $\Delta znuA::FRT$ ). The *zupT::kan* allele was obtained using primers CMD715 and CMD716 and specific amplification of the allele from the template plasmid pKD13. This allele was introduced into strains MG1655 and CFT073 as described above, generating strains QT1553 (MG1655  $\Delta zupT::kan$ ) and QT1554 (CFT073  $\Delta zupT::kan$ ). Similarly, the  $\Delta zupT::kan$  allele was also introduced to the  $\Delta znuA::FRT$  mutants to generate the  $\Delta znuA::FRT \Delta zupT::kan$  double mutant strains of MG1655 (QT1435) and CFT073 (QT1555). A  $\Delta sit::tetAR$  allele amplified from APEC QT205 (46) with the primers CMD23 and CMD42 was used to generate strains QT1640 (CFT073  $\Delta znuA::FRT \Delta sit::tet$ ) and QT1634 (CFT073  $\Delta znuA::FRT \Delta zupT::kan \Delta sit::tet$ ). Strain QT1932, the UPEC *znuACB* complemented  $\Delta znuA::FRT \Delta zupT::kan$  derivative of CFT073, was obtained by conjugation of suicide plasmid pIJ254 into strain QT1555 by using previously described methods (46). UPEC strain QT1081 (CFT073  $\Delta lacZYA$ ) was generated as previously described for strain  $\chi$ 7122 (40).

**Growth in minimal medium and ion rescue.** All media used for growth assays were made using the purest available water processed with the Milli-Q water purification system (Millipore) and were prepared in polypropylene labware rinsed three times with Milli-Q water. M9-glucose medium and M9-glucose agar (using 1.5% Noble agar from Sigma) were used as a defined minimal metal-limited medium to assess the growth of strains. For growth in a complex minimal medium, DT-glucose (diluent tryptone) was used as described previously, except that it was supplemented with a 200  $\mu$ M concentration of the metal-chelator EGTA (47). The EGTA affinity constants for  $Mn^{2+}$ ,  $Fe^{3+}$ , and  $Zn^{2+}$ , taken from the NIST critically selected stability constants of metal complexes database are as follows:  $\log K = 12.2$  ( $Mn^{2+}$ ),  $\log K = 20$  ( $Fe^{3+}$ ), and  $\log K = 12.6$  ( $Zn^{2+}$ ). The growth of *E. coli* strains was assessed in DT-EGTA or M9-glucose broth. Briefly, after growth in rich LB medium, the strains were precultured in the appropriate minimal medium overnight. The following day, the strains were adjusted to an optical density at 600 nm ( $OD_{600}$ ) of 0.02. Growth as measured at  $OD_{600}$  was monitored every 2 h. All strains were tested in triplicate. Metal supplements (5  $\mu$ M concentrations of  $ZnCl_2$ ,  $MnCl_2$ , or  $FeCl_2$ ) were added to determine the effects of ion rescue on growth of strains. Human urine, pooled from three healthy women and sterile filtered, was also used in the present study to char-

acterize growth of strain CFT073 and its mutant derivatives. LB broth was used as the metal-replete rich medium.

**Isotope uptake.** Zinc isotope ( $^{65}Zn^{2+}$ ) uptake was determined in the *E. coli* K-12 zinc transport-deficient ( $\Delta znuA::FRT \Delta zupT::kan$ ) strain derivative of MG1655 (QT1435) transformed with the plasmids pIJ156 (*znuACB*), pIJ202 (*zupT*), pIJ28 (*sitABCD*), or pACYC184 (vector control) and the mutant derivatives of UPEC strain CFT073 lacking functional Znu, ZupT, and/or Sit transporters. All strains were grown in DT broth overnight. The following day, the strains were suspended in DT-EGTA (200  $\mu$ M) for 2 h at 37°C with moderate shaking. The strains were washed three times with DT-EGTA (500  $\mu$ M) and adjusted to an  $OD_{600}$  of 0.5 (corresponding to  $10^8$  cells/ml). Zinc isotope  $^{65}Zn^{2+}$  was added at a concentration of 20 nM. The strains were left in the presence of the isotope for 15 min at 37°C. Then, 1-ml samples of the strain and/or isotope suspension in DT-EGTA were distributed in 2-ml Eppendorf tubes. Triplicates of each strain were prepared, and samples were washed three times with cold DT-EGTA (1 mM) for 2 min and resuspended in Wallac Optiphase scintillation buffer. Scintillations counts were read on the channels 0 to 748 of an LS1701 scintillation counter (Beckman). Samples from the zinc-transport deficient strain without isotope were used as a blank to determine the value of the background that was subsequently subtracted. A triplicate of samples containing 100  $\mu$ l of 20 nM  $^{65}Zn^{2+}$  DT-EGTA without bacterial cells was used as a standard to convert the value of the uptake from counts per minute to pmol/ $10^8$  cells.

**Oxidative stress resistance.** Oxidative stress resistance against hydrogen peroxide (30% [vol/vol]) using a disk diffusion assay was done using strain CFT073 and its mutant derivatives as previously described (46).

**Motility assay.** Motility assays were done using strain CFT073 and its mutant derivatives as previously published (32), except that all of the strains screened for motility were washed three times with M9 medium prior to inoculation on motility agar plates. The motility area was measured after 18 h of growth at 37°C.

**Experimental UTI in CBA/J mice.** A murine ascending UTI model was used in which a virulent  $\Delta lacZYA$  derivative of UPEC CFT073 (strain QT1081) was coinfecting with the different metal transporter mutants. Six-week-old CBA/J female mice were coinfecting with 25  $\mu$ l ( $10^9$  CFU) of a mixed culture containing nearly equal amounts of two test strains through a catheter inserted in the urethra. The infected animals were euthanized 48 h postinfection, and the bacterial counts of the bladders and kidneys were determined on MacConkey agar plates, which allowed for direct comparison of the virulent  $\Delta lac$  CFT073 derivative (white colonies) and the metal transport mutants (red colonies). Competitive indexes (CI; calculated as the mutant CFU/ $\Delta lac$  CFT073) were determined for each sample and normalized for the input ratio of the inoculum. The log CI values were used for graphical representation, with negative log CI values indicating a decreased capacity of the transport mutant to compete with the virulent UPEC CFT073 derivative. In the single-strain experimental UTI model, individual groups of CBA/J mice were infected as described above but with pure cultures of each strain, and 48 h postinfection bacterial counts were determined from the bladders and kidneys.

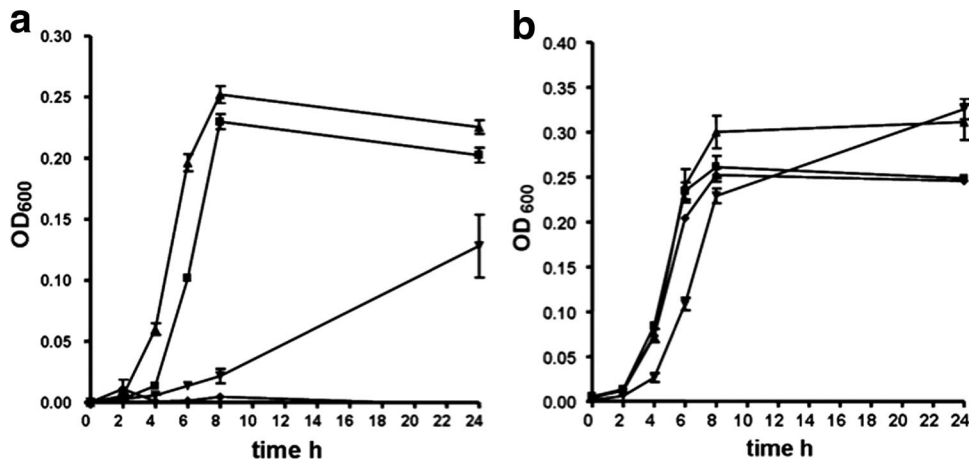


FIG. 1. Growth of the *E. coli* K-12  $\Delta znu \Delta zupT$  mutant QT1435 containing plasmid-encoded metal transporters in DT-EGTA medium (a) or DT-EGTA with 5  $\mu\text{M}$  Zn<sup>2+</sup> (b). Symbols: ◆, QT1435(pACYC184) (vector control); ▲, QT1435(pIJ202) *zupT*; ■, QT1435(pIJ156) *znuACB*; ▼, QT1435(pIJ28) *sitABCD*.

**Statistical analyses.** All data were analyzed by using GraphPad Prism 4 software. A Wilcoxon signed-rank test (two-tailed  $P \leq 0.05$ ) was used to determine statistical significance in the log CI values between groups in the competitive-infection experiments. A Mann-Whitney test was used to determine significant differences between groups in single-strain infection experiments.

## RESULTS

**Individual contribution of metal transport systems to the growth of *E. coli* zinc transport-deficient mutants.** To investigate the individual roles of the Znu, ZupT, and SitABCD transport systems for growth in medium with limited zinc availability (DT-EGTA), genes encoding these systems on plasmid pACYC184 were introduced into *E. coli* K-12 MG1655 derivative strain QT1435, which lacks the known zinc transporters ZnuACB and ZupT. Strain QT1435 containing the

pACYC184 vector alone failed to grow in DT-EGTA (Fig. 1a). In contrast, strain QT1435 complemented with *znuACB* (pIJ156) or *zupT* (pIJ202) regained growth. By comparison, introduction of the *sitABCD* genes (pIJ28), encoding a manganese/iron transport system, conferred a limited and late increase in growth (Fig. 1a). All mutant strains grew well in DT-EGTA medium supplemented with 5  $\mu\text{M}$  ZnCl<sub>2</sub>, suggesting that the growth defect of the *znu/zupT* mutant was due to the lack of available Zn<sup>2+</sup> (Fig. 1b).

The CFT073  $\Delta znu$  mutant also exhibited a marked lag and decreased growth in minimal medium (Fig. 2a). Loss of the *sit* genes from the  $\Delta znu$  strain had no discernible effect on growth (Fig. 2a). The  $\Delta zupT$  mutant (QT1554) demonstrated no growth defect compared to the wild-type parent strain,

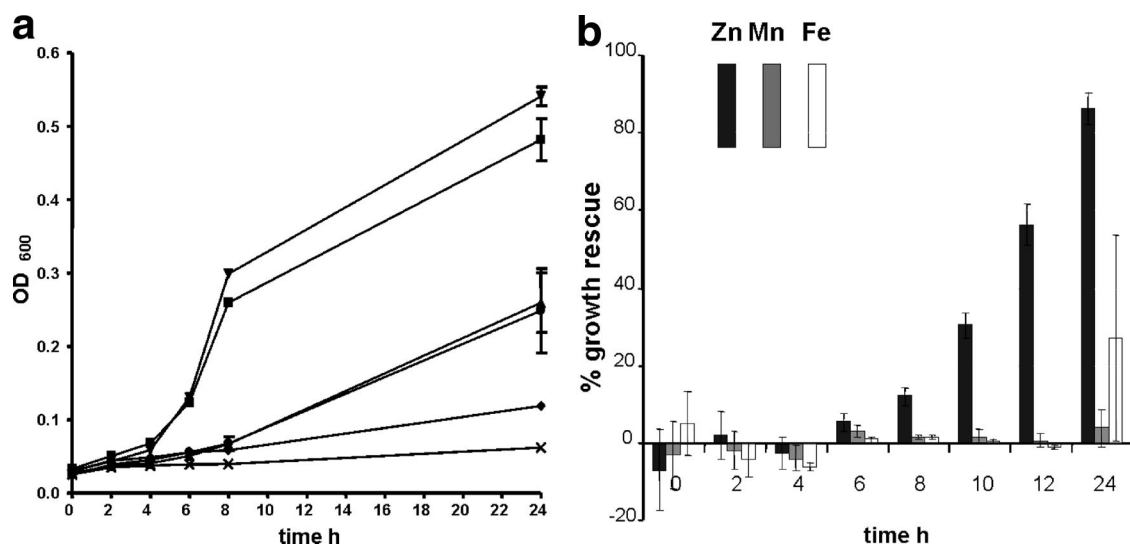


FIG. 2. (a) Growth of UPEC strain CFT073 and metal transport-defective mutant derivatives in M9-glucose medium. Symbols: ■, CFT073 wild type; ▲,  $\Delta znu$  mutant (QT1434); ▼,  $\Delta zupT$  mutant (QT1554); ◆,  $\Delta znu \Delta zupT$  mutant (QT1555); ●,  $\Delta znu \Delta sitABCD$  mutant (QT1640); ×,  $\Delta znu \Delta zupT \Delta sitABCD$  mutant (QT1634). (b) Growth rescue of the UPEC  $\Delta znu \Delta zupT$  strain QT1555 in M9-glucose medium supplemented with 5  $\mu\text{M}$  Zn<sup>2+</sup> (■), Mn<sup>2+</sup> (▣), or Fe<sup>3+</sup> (□). Growth of the strain is presented as the percent growth compared to the wild-type strain CFT073 based on the formula:  $[(\text{OD}_{600} \text{ of the mutant in metal-enriched medium}) - (\text{OD}_{600} \text{ of the mutant})]/(\text{OD}_{600} \text{ of the wild-type strain}) \times 100$ .

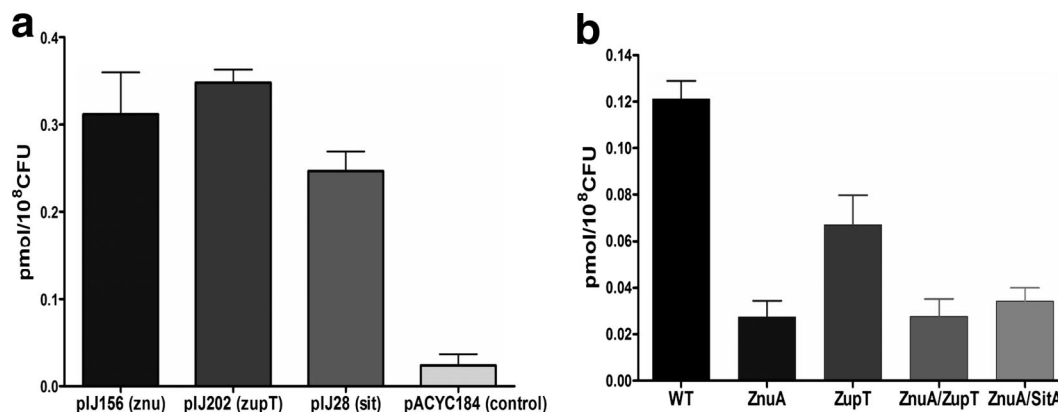


FIG. 3. Uptake of  $^{65}\text{Zn}^{2+}$  by *E. coli* K-12  $\Delta znu \Delta zupT$  mutant QT1435 containing plasmid-encoded metal transporters or the control vector (a) or UPEC strain CFT073 and mutant derivatives (b). WT, wild-type CFT073; ZnuA,  $\Delta znu$  strain QT1434; ZupT,  $\Delta zupT$  strain QT1554; ZnuA/ZupT,  $\Delta znu \Delta zupT$  strain QT1555; ZnuA/SitA,  $\Delta znu \Delta sitABCD$  strain QT1640.

whereas the  $\Delta znu \Delta zupT$  mutant (QT1555) grew very poorly, and additional loss of *sitABCD* completely impeded growth of strain QT1634 (Fig. 2a). Supplementation with 5  $\mu\text{M}$   $\text{Zn}^{2+}$  restored growth of the CFT073  $\Delta znu \Delta zupT$  mutant (86% growth of the wild type at 24 h), whereas addition of  $\text{Fe}^{2+}$  only slightly increased growth (27% compared to the wild type at 24 h of incubation), and the addition of  $\text{Mn}^{2+}$  had no appreciable effect (Fig. 2b). These results confirmed that the lack of  $\text{Zn}^{2+}$  in minimal medium was likely responsible for the growth-deficient phenotypes observed for the  $\Delta znu$  and  $\Delta znu \Delta zupT$  mutant derivatives of CFT073.

**Roles of Znu, ZupT, and Sit metal transport systems for zinc transport.** We investigated the capacity of the Znu, ZupT, and Sit systems to transport zinc by comparing  $^{65}\text{Zn}^{2+}$  uptake in the *E. coli* K-12  $\Delta znu \Delta zupT$  strain QT1435, complemented with plasmids encoding the Znu or ZupT transporters and in mutants of UPEC strain CFT073 (Fig. 3). Strain QT1435 complemented with the vector control demonstrated only a background level of  $^{65}\text{Zn}^{2+}$  uptake. In contrast, complementation with plasmids encoding either Znu (pIJ156), or ZupT (pIJ202) systems conferred increased uptake of  $^{65}\text{Zn}^{2+}$ . Further, introduction of *sit* genes (pIJ28 plasmid) increased  $^{65}\text{Zn}^{2+}$  accumulation, albeit at lower levels than the Znu- or ZupT-encoding plasmids (Fig. 3a). In uptake studies with UPEC strain CFT073 and mutant derivatives, loss of the Znu system resulted in the greatest decrease in  $^{65}\text{Zn}^{2+}$  accumulation. The decrease in  $^{65}\text{Zn}^{2+}$  uptake observed was similar for the  $\Delta znu$  (QT1434),  $\Delta znu \Delta zupT$  (QT1555) and  $\Delta znu \Delta sitABCD$  (QT1640) strains, whereas loss of the ZupT system had a less marked effect on  $^{65}\text{Zn}^{2+}$  uptake (Fig. 3b). These results indicate that, despite its ability to acquire  $^{65}\text{Zn}^{2+}$  when expressed from a medium-copy-number plasmid (pIJ202), ZupT is a less efficient zinc transporter than the ZnuACB transporter when expressed at wild-type levels in UPEC strain CFT073. Finally, the loss of *sit* in the *znu* mutant strain did not result in a cumulative reduction in  $^{65}\text{Zn}^{2+}$  acquisition, suggesting that despite its ability to accumulate  $^{65}\text{Zn}^{2+}$  when expressed from a medium-copy-number plasmid (pIJ28), SitABCD does not contribute significantly to zinc acquisition by UPEC strain CFT073.

**Contribution of the Znu and ZupT transporters in the murine UTI model.** UPEC strain CFT073 zinc transporter mutants were tested in a murine coinfection model using virulent  $\Delta lacZYA$  CFT073 strain QT1081 as the competitor strain (Fig. 4). In this infection model, bacterial numbers of the  $\Delta zupT$  mutant in the bladders and kidneys were similar to the virulent competitor strain, indicating that *zupT* alone does not contribute significantly to colonization and survival during UTI. The  $\Delta znu$  mutant strain QT1434 was significantly attenuated and, compared to the virulent competitor strain, demonstrated a mean 4.4-fold reduction in bladders ( $P = 0.0005$ ) and a mean 44-fold reduction in kidneys ( $P < 0.0001$ ). Further, the  $\Delta znu \Delta zupT$  strain (QT1555) was even more attenuated and demonstrated a mean 30-fold decrease in bladders ( $P = 0.0001$ ) and a mean 48-fold decrease in kidneys ( $P < 0.0001$ ). Reintroduction of the *znuACB* genes into the  $\Delta znu \Delta zupT$  strain, QT1932, resulted in complementation as demonstrated by a significant increase of the CI in the bladder ( $P = 0.001$ ) and a complete regain in the kidneys ( $P < 0.0001$ ) compared to the attenuated  $\Delta znu \Delta zupT$  mutant QT1555.

In the single-strain infection model, the  $\Delta znu$  and  $\Delta znu \Delta zupT$  mutant strains showed a significant decrease in bacterial numbers in the kidneys ( $P = 0.0012$  and  $P < 0.0001$ , respectively) but were present in the bladder at levels similar to the virulent CFT073  $\Delta lac$  strain (Fig. 5). Thus, taken together, these results indicate that zinc transport systems impart a competitive advantage to ExPEC strain CFT073 during UTI in the murine model and that the ZnuACB system plays a predominant role, whereas ZupT appears to be a zinc transporter of secondary importance.

Since the zinc transporter mutants demonstrated a growth defect in zinc-limited medium (Fig. 1 and 2), we investigated whether the UPEC zinc transport mutants were less able to grow in human urine. Loss of either or both of the Znu and ZupT systems in strain CFT073 did not effect growth of the strains in human urine (data not shown). Moreover, we performed competition assays between the zinc transport-deficient mutants and the CFT073  $\Delta lac$  derivative in human urine in vitro. During competitive growth in human urine, the zinc transport mutants remained at levels similar to the CFT073  $\Delta lac$  derivative, and after 24 h the

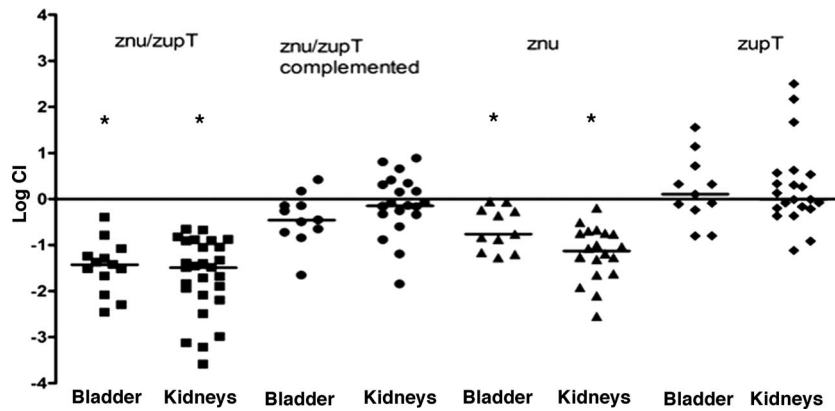


FIG. 4. Comparative colonization of bladders and kidneys of CBA/J mice coinfecting with UPEC CFT073 derivatives defective in zinc transporter systems and virulent CFT073  $\Delta lac$  derivative strain QT1081. A competitive coinfection model was used in which a mixture of QT1081 and different metal transporter mutants were inoculated simultaneously. At 48 h postinfection, tissues were sampled, and results are presented as the  $\log_{10}$  CI. The CI represents the ratio of numbers of the two test strains (mutant/ $\Delta lac$  virulent strain QT1081) from the tissues sampled (the output ratio) normalized for the initial numbers of each strain in the inoculum (input). Negative CI values indicate a decreased capacity for the mutant to compete with the virulent test strain QT1081. Horizontal bars indicate the median  $\log_{10}$  CI values. Each data point represents a sample from an individual mouse. Both kidneys were sampled separately. Strains tested: strain QT1434 ( $\Delta znu$ ), strain QT1554 ( $\Delta zupT$ ), strain QT1555 ( $\Delta znu \Delta zupT$ ), and strain QT1932, the  $\Delta znu \Delta zupT$  mutant complemented with *znuACB*. Statistically significant decreases in CI values are indicated by asterisks (\*,  $P < 0.001$ ) as determined by the Wilcoxon matched-pair test.

CI values were 0.92 for the  $\Delta znu$  (QT1434) strain and 0.78 for the  $\Delta znu \Delta zupT$  (QT1555) strain. These results suggest that the levels of zinc present in urine from healthy individuals were adequate for growth of the strains in vitro.

**Zinc transporters contribute to resistance to  $H_2O_2$ .** Since other metal transporters contribute to bacterial resistance to oxidative stress mediated by exogenous  $H_2O_2$  (10, 47), we assessed whether the loss of zinc transporters increased susceptibility of strain CFT073 to  $H_2O_2$  (Fig. 6). The  $\Delta znu$  mutant strain (QT1434) was more sensitive to  $H_2O_2$ , whereas the  $\Delta zupT$  strain QT1554 was as resistant as the wild-type strain (Fig. 6a). Without zinc supplementation, the  $\Delta znu \Delta zupT$  strain (QT1555) was unable to grow on M9-glucose plates. However, when QT1555 was complemented with the *znuACB* genes (strain QT1932), both growth and resistance to  $H_2O_2$  were restored (Fig. 6a). Supplementation with 5  $\mu M$  zinc re-

stored the growth of all strains and was sufficient to restore resistance of the  $\Delta znu$  strain QT1434 (Fig. 6b). In contrast, although the  $\Delta znu \Delta zupT$  strain (QT1555) regained the capacity to grow when supplemented with 5  $\mu M$  zinc, it was even more sensitive to  $H_2O_2$  than the  $\Delta znu$  strain QT1434 without zinc supplementation. However, supplementation with 10  $\mu M$   $Zn^{2+}$  resulted in a regain in resistance of strain QT1555 to  $H_2O_2$  (Fig. 6b). Supplementation with zinc had no effect on the resistance of the wild-type strain or the  $H_2O_2$ -sensitive  $\Delta sit \Delta mntH$  control strain, which is defective for the transport of manganese. Therefore, supplementation with zinc alone did not have a nonspecific effect of increased resistance to  $H_2O_2$ .

**Zinc transport mutants demonstrate decreased motility.** Because genes required for the production of flagella and motility are affected by zinc availability (34, 49) and since motility contributes to colonization and persistence in the uri-

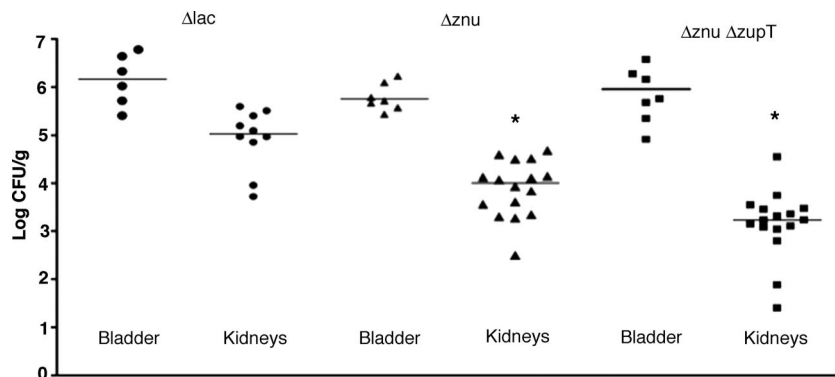


FIG. 5. Colonization of bladders and kidneys of CBA/J mice infected with virulent UPEC CFT073 derivative strain QT1081 ( $\Delta lac$ ) and transport mutant CFT073 derivatives in the single-strain infection model. The data are presented as the  $\log$  CFU/gram of tissue. Each data point represents a tissue sample from an individual infected mouse 48 h postinfection. Both kidneys were sampled separately. Strains tested were the CFT073  $\Delta lacZYA$  strain QT1081 ( $\Delta lac$ ), the QT1434  $\Delta znu$  strain ( $\Delta znu$ ), and the QT1555  $\Delta znu \Delta zupT$  strain ( $\Delta znu \Delta zupT$ ). Horizontal bars represent the median values of each group. Samples that were significantly decreased compared to the virulent  $\Delta lac$  strain are indicated with asterisks (\*,  $P < 0.005$ ).  $P$  values of comparative differences were determined by the Mann-Whitney test.

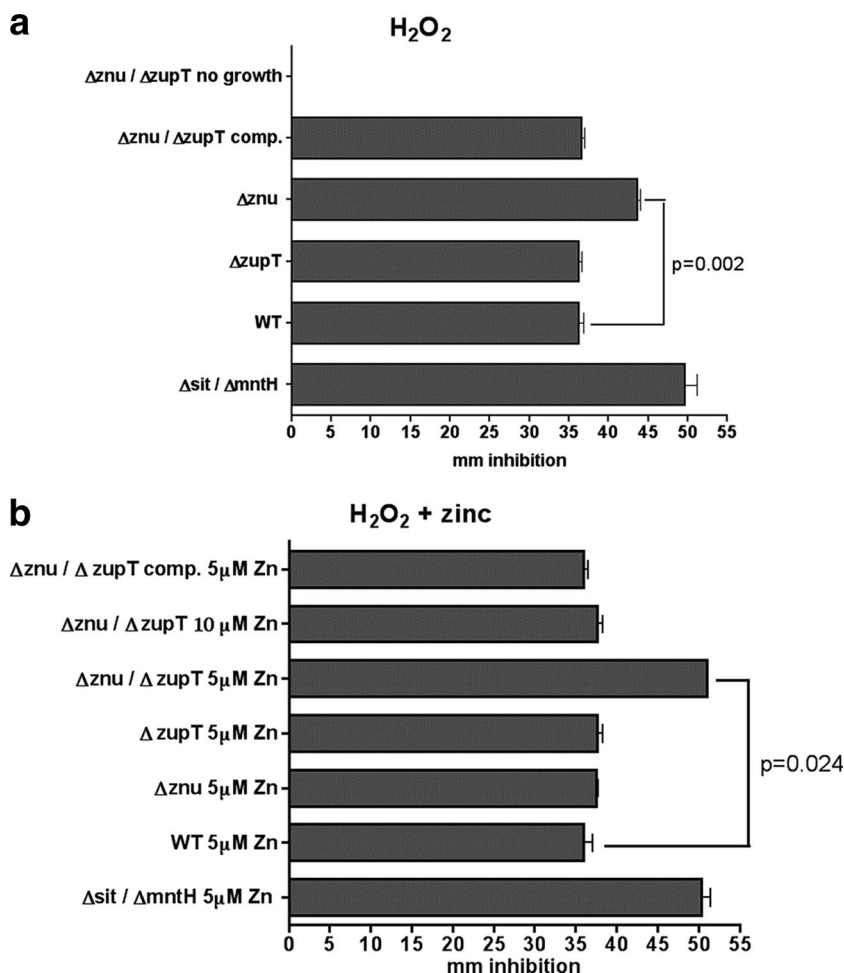


FIG. 6. Sensitivity to  $H_2O_2$ . UPEC strain CFT073 and derivatives were seeded onto M9-glucose agar plates without zinc supplementation (a) or with zinc supplementation (b) and subjected to  $H_2O_2$  placed on sterile filter disks. Differences in resistance were assessed from the diameter of the inhibition zone of a culture after overnight growth. Samples were done in triplicate, and bars represent the means  $\pm$  the standard deviation. Strains used were wild-type CFT073 (WT), strain QT1434 ( $\Delta znu$ ), strain QT1554 ( $\Delta zupT$ ), strain QT1555 ( $\Delta znu \Delta zupT$ ), and the QT1555 *znuACB*-complemented strain, QT1932. Avian pathogenic *E. coli* strain QT1239 ( $\Delta sitABCD \Delta mntH$ ), which is sensitive to  $H_2O_2$ , was used as an internal control. Significant differences between the WT parent and isogenic zinc transporter mutants are indicated by *P* values to the right of the figures.

nary tract (32), we investigated whether the loss of zinc transporters affected motility. No difference in motility was observed for the  $\Delta znu$  or  $\Delta zupT$  mutants. However, the  $\Delta znu \Delta zupT$  strain QT1555 was less motile than the UPEC strain CFT073. The motility of this mutant was partially restored by supplementation with zinc (Fig. 7), and the *znu* complemented mutant strain QT1932 regained motility to wild-type levels.

## DISCUSSION

In the present study, we determined the roles of the Znu and ZupT zinc transporters for growth and  $^{65}Zn^{2+}$  uptake by *E. coli* K-12 and UPEC strain CFT073, and the contribution of these transporters to UTI by strain CFT073 in mice. Our results indicate that the Znu and ZupT transporters are required for growth in medium where zinc is limited.  $Zn^{2+}$  rescued the growth of the zinc transporter-deficient *E. coli* strains in minimal medium, while  $Mn^{2+}$  and  $Fe^{2+}$  failed to do so, indicating

that zinc deficiency in the absence of these transporters was a limiting factor for growth (Fig. 1 and 2).

When expressed from a medium-copy plasmid, *znuACB* or *zupT* genes complemented the growth defect of a  $\Delta znu \Delta zupT$  *E. coli* K-12 mutant strain, whereas the *SitABCD* transporter only provided a slight gain in growth (Fig. 1). The ZnuACB transporter was shown to be the predominant Zn transporter in UPEC strain CFT073 in growth and uptake assays, and in a  $\Delta znu$  background a cumulative loss of *zupT* or *sitABCD* did not reduce  $^{65}Zn^{2+}$  uptake any further (Fig. 3). ZnuACB belongs to the same family of transporters as *SitABCD*, the C9 cluster of ABC manganese and zinc permeases (15), whereas ZupT belongs to the ZRT IRT-like proteins (ZIP) family of transporters (25). While ZnuACB uses ATP hydrolysis for transport, ZupT is thought to require a chemo-osmotic gradient. ZnuACB functions as a  $Zn^{2+}$ -specific transporter, whereas ZupT has also been shown to mediate the uptake of  $Mn^{2+}$  and  $Fe^{2+}$  (24, 57). The transport specificity and higher affinity of

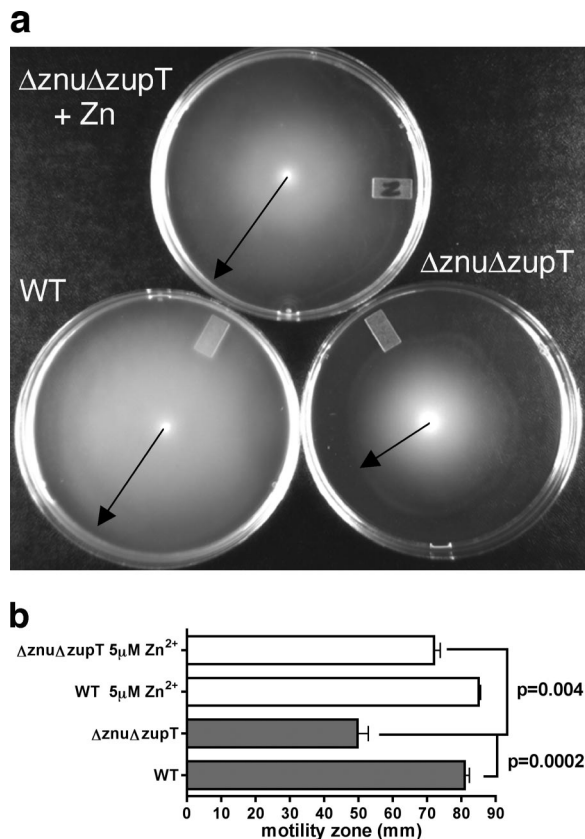


FIG. 7. Loss of zinc transporters affects the swimming motility. Uropathogenic strain CFT073 and derivatives were tested after 18 h growth at 37°C on tryptone motility agar plates (44). (a) Image showing motility distances of the  $\Delta znu \Delta zupT$  strain QT1555 ( $znu/zupT$ ) with or without 5  $\mu M$   $Zn^{2+}$  supplementation compared to the CFT073 wild-type strain (WT). (b) Bar graph indicating distances of motility zones. Statistically different motility between the WT parent and isogenic QT1555 ( $\Delta znu \Delta zupT$ ) strain or QT1555 with or without  $Zn^{2+}$  are indicated by *P* values to the right of the figure. Other mutants—strain QT1434 ( $\Delta znu$ ), strain QT1554 ( $\Delta zupT$ ), and the QT1555  $znuACB$ -complemented strain, QT1932—were as motile as the WT parent with or without zinc supplementation (data not shown).

ZnuACB could explain why the loss of ZnuACB had greater impact on the growth of CFT073 than loss of the ZupT transporter. The higher  $Zn^{2+}$  affinity of ZnuACB is probably due to the ZnuA periplasmic ligand binding protein, which contains a metal-binding histidine-rich loop protruding from the vicinity of the protein in addition to the high-affinity  $Zn^{2+}$ -binding pocket located inside the protein (13, 57).

The ExPEC-associated SitABCD transporter was suspected to be a possible zinc transporter, since SitA is a member of the cluster 9 transition metal transporters, such as TroA, which has been shown to efficiently bind zinc and other metals such as manganese and iron (19). As well, uptake of  $Fe^{2+}$  and  $Mn^{2+}$  by the *Salmonella* Sit and of  $Fe^{2+}$  by the orthologous *Yersinia* Yfe transporter was inhibited by  $Zn^{2+}$  at micromolar concentrations (8, 29). Indeed, when expressed from a medium-copy plasmid, SitABCD partially restored growth and also increased accumulation of  $^{65}Zn^{2+}$  in a  $\Delta znu \Delta zupT$  K-12 strain. However, SitABCD-mediated  $^{65}Zn^{2+}$  accumulation did not correlate with an increase in growth in minimal media. It is there-

fore likely that SitA can bind  $Zn^{2+}$  in the periplasm resulting in zinc accumulation, although this binding results in little or no  $Zn^{2+}$  uptake into the cytoplasm. Since *sitABCD* only provided a marginal growth increase to the CFT073  $\Delta znu \Delta zupT$  strain in growth assays (Fig. 2), and since the CFT073  $\Delta znu \Delta zupT$  mutant was no more able to grow on minimal plates than the *E. coli* K-12  $\Delta znu \Delta zupT$  mutant, these results suggest that Sit is unable to compensate for the transport of  $Zn^{2+}$  in the absence of the Znu and ZupT systems. Hence, Znu and ZupT appear to be the only transporters that can provide sufficient  $Zn^{2+}$  uptake for the growth of either *E. coli* K-12 strain MG1655 or UPEC strain CFT073.

Our results demonstrate the importance of zinc transporters for UPEC strain CFT073 during UTI (Fig. 4 and 5). Loss of the Znu transporter attenuated UPEC strain CFT073. Loss of both the Znu and the ZupT systems further decreased bacterial numbers in the urinary tract in the murine coinfection challenge and in the kidneys in the single-strain infection model. It is interesting that in the single-strain infection model, Zn transport mutants were only significantly reduced in kidneys and not in bladders. These results suggest that colonization of, access to, and/or survival in the kidneys is affected in the Zn transport mutants. Known factors that contribute to enhanced colonization of the kidneys include P fimbriae, which mediate mannose-resistant hemagglutination, and increased tissue tropism for the kidneys (28, 33, 41). However, since mannose-resistant hemagglutination titers of the wild-type CFT073 and Zn transport mutants were similar after growth in different conditions, including urine, as well as liquid and solid culture media, it is unlikely that decreased bacterial numbers in the kidney in the Zn transport mutants are due to decreased production of P fimbriae. Decreased bacterial numbers in the kidneys compared to the bladder may be due to increased sensitivity of these strains to oxidative stress. Compared to the bladder, the kidneys may represent a tissue with a higher level of oxidative stress due to increased infiltration of inflammatory mediators and immune cells, as well as oxygen from the circulatory system. In addition, decreased swimming motility in Zn transport mutants could also reduce access to the distal renal tissues of the urinary tract.

The UPEC zinc transport mutants grew well in human urine, which is considered to be a relatively zinc-replete environment, since urinary zinc losses amount to an average of 9  $\mu M$ /day in healthy subjects and vary little when a diet is zinc balanced (31). However, despite the apparently adequate availability of zinc in urine in healthy individuals, zinc availability after infection may be dramatically altered following infection in both extracellular and intracellular host environments (3, 38, 45). A recent study on zinc transport-related virulence phenotypes of *S. enterica* demonstrated that ZnuACB is expressed in intracellular environments, which strongly suggests that  $Zn^{2+}$  availability is limited within such cells (3). UPEC strains have been shown to form intracellular bacterial communities in bladder epithelial cells (4), whereas intracellular zinc has been shown to be bound to metallothioneins and transported to specific cellular vacuoles for storage (37, 48). Thus, zinc could be limited during intracellular-bacterial-community formation. ExPEC also cause septicemia, and in plasma zinc is mainly bound to albumin and to a lesser extent to  $\alpha_2$ -macroglobulin (22). Zinc also could be bound by other immune-induced pro-



teins such as calprotectin released by neutrophils during response to bacterial infection (16, 38). Thus, overall, during UTI as with other extraintestinal infections, zinc availability may be reduced, and bacterial zinc transporters may therefore contribute to increased fitness for colonization of host tissues or cells.

Zinc is second only to iron in abundance inside *E. coli* cells and is the only metal that is a cofactor for enzymes belonging to six different major functional groups (6). Although decreased zinc availability could potentially result in decreased activity of many bacterial enzymes implicated in cellular metabolism, physiology, and growth, we have specifically identified two phenotypes that could contribute to urinary tract virulence that were impaired in the zinc transport mutants: sensitivity to oxidative stress and decreased motility.

The implication of zinc transporters in oxidative stress resistance is a new finding which will require further investigation. Zinc protects against iron-triggered membrane lipid oxidation (30, 58), which may partially explain why Znu- and ZupT-mediated zinc transport may provide resistance to oxidative stress. One explanation for increased sensitivity to hydrogen peroxide may be due to lowered activity or decreased expression of the *E. coli* SodC, Zn/Cu superoxide dismutase. In *E. coli* SodC has been shown to contribute to increased resistance to H<sub>2</sub>O<sub>2</sub> (23, 30), and zinc ions have been shown to be required for stable binding of copper to the SodC catalytic site (2). In *S. enterica* serovar Typhimurium, Ammendola et al. (2) recently reported that zinc limitation and the loss of ZnuA resulted in a decreased level of expression of SodCII, which is the *Salmonella* ortholog of *E. coli* SodC. Hence, increased zinc availability through zinc transporters, particularly the Znu transporter, may provide an important signal for increased SodC expression that may protect against oxidative stress.

Another possible explanation of a role for Zn transport in resistance to oxidative stress is suggested by the fact that the global iron uptake repressor Fur is a zinc metalloprotein (1, 18). Fur expression is under the control of the global oxidative stress resistance regulators OxyR and SoxS; Fur is directly implicated in oxidative stress, and its inactivation results in increased oxidative damage to the cell through the iron-dependent Fenton chemistry reactions (51, 52). In addition, in *E. coli* K-12 *znu* genes have been shown to be induced under iron repletion and repressed under iron depletion (44). Under iron repletion, bacterial cells require the Fur regulator to control iron homeostasis and reduce iron overload and oxidative stress. Therefore, reduced Zn<sup>2+</sup> uptake may possibly compromise Fur function and lead to increased susceptibility to oxidative stress.

Loss of both the Znu and ZupT transporters was required to reduce the motility of UPEC strain CFT073, and this decrease in motility was Zn dependent. FlhC, a subunit of the FlhCD transcriptional regulator of flagellar genes, is a zinc metalloprotein (53), and expression of flagellar genes has been shown to decrease under conditions of zinc restriction (49). The importance of motility for the competitive fitness and establishment of UTI by UPEC strain CFT073 and particularly colonization of the kidneys has been established previously (32, 55). In the present study, using both coinfection and single-strain infection models, colonization of the kidneys was shown to be

reduced in the zinc transport deficient strains, which could at least in part be due to a decrease in motility.

Taken together, our overall findings establish that in *E. coli* Znu plays a predominant role in zinc transport and UTI and that ZupT plays a secondary role. Future investigations will be important to establish the specific mechanisms by which loss of these transporters compromise ExPEC fitness and physiology during extraintestinal infections.

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