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## Contribution of the SitABCD, MntH, and FeoB Metal Transporters to the Virulence of Avian Pathogenic *Escherichia coli* O78 Strain $\chi$ 7122<sup>∇</sup>

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The roles of SitABCD, MntH, and FeoB metal transporters in the virulence of avian pathogenic *Escherichia coli* (APEC) O78 strain  $\chi$ 7122 were assessed using isogenic mutants in chicken infection models. In a single-strain infection model, compared to  $\chi$ 7122, the  $\Delta$ sit strain demonstrated reduced colonization of the lungs, liver, and spleen. Complementation of the  $\Delta$ sit strain restored virulence. In a coinfection model, compared to the virulent APEC strain, the  $\Delta$ sit strain demonstrated mean 50-fold, 126-fold, and 25-fold decreases in colonization of the lungs, liver, and spleen, respectively. A  $\Delta$ mntH  $\Delta$ sit strain was further attenuated, demonstrating reduced persistence in blood and mean 1,400-fold, 954-fold, and 83-fold reduced colonization in the lungs, liver, and spleen, respectively. In coinfections, the  $\Delta$ feoB  $\Delta$ sit strain demonstrated reduced persistence in blood but increased colonization of the liver. The  $\Delta$ mntH,  $\Delta$ feoB, and  $\Delta$ feoB  $\Delta$ mntH strains were as virulent as the wild type in either of the infection models. Strains were also tested for sensitivity to oxidative stress-generating agents. The  $\Delta$ mntH  $\Delta$ sit strain was the most sensitive strain and was significantly more sensitive than the other strains to hydrogen peroxide, plumbagin, and paraquat. sit sequences were highly associated with APEC and human extraintestinal pathogenic *E. coli* compared to commensal isolates and diarrheagenic *E. coli*. Comparative genomic analyses also demonstrated that sit sequences are carried on conjugative plasmids or associated with phage elements and were likely acquired by distinct genetic events among pathogenic *E. coli* and *Shigella* sp. strains. Overall, the results demonstrate that SitABCD contributes to virulence and, together with MntH, to increased resistance to oxidative stress.

The *Salmonella* iron transporter, SitABCD, was first identified in *Salmonella enterica* serovar Typhimurium as a homolog of the *Yersinia pestis* YfeABCD transporter (68) and is a member of the periplasmic binding protein-dependent ATP-binding-cassette (ABC) family of metal transporters (12). In *Y. pestis* and *S. enterica* serovar Typhimurium, inactivation of the genes encoding the Yfe and Sit transporters, respectively, resulted in decreased virulence in murine experimental infection models (3, 25). SitABCD homologs are also present in other pathogenic enterobacteria, including *Shigella* spp. (26, 55, 66), uropathogenic *Escherichia coli* (UPEC) (6, 11, 67), and avian pathogenic *E. coli* (APEC) (28, 50, 56, 59). The genes encoding the Sit system are located on either genomic islands or large plasmids, and some strains carry more than one copy of the sit system (27, 28, 56, 59).

Previously, SitABCD from APEC strain  $\chi$ 7122 was shown to transport iron and manganese, and in the absence of the endogenous manganese transporter MntH, SitABCD contributed to protection of APEC and *E. coli* K-12 against hydrogen peroxide (56). SitABCD from *S. enterica* serovar Typhimurium similarly mediated transport of manganese and iron and, in combination with MntH, contributed to resistance to hydrogen

peroxide (4). Furthermore, the loss of Sit in combination with the loss of either MntH or the Feo ferrous iron transporter resulted in greater attenuation of *Salmonella* virulence in susceptible mice (4). A *Shigella flexneri* 2a sit mutant was less able to grow in iron-restricted medium (38, 55). However, loss of Sit had no appreciable effect on intracellular multiplication or cell-to-cell spread in epithelial cells or on virulence in the guinea pig keratoconjunctivitis model (38, 55). In contrast, the cumulative loss of Sit and iron transporters such as Feo or the aerobactin siderophore system resulted in a reduced capacity to form plaques on epithelial cells (55). In addition, the cumulative loss of Sit and MntH resulted in a reduced capacity of *Shigella flexneri* 2a to survive in macrophage cell lines (53).

Divalent ion transporters such as SitABCD, MntH, and Feo may contribute to the virulence of APEC and other bacterial pathogens by facilitating acquisition of iron and/or manganese. Both manganese and iron are essential cofactors for enzymes required for metabolic processes and protection against oxidative stress (1, 23, 33). Thus far, the role of the SitABCD transporter, alone or in combination with other divalent metal transporters, such as MntH and Feo, in the virulence of pathogenic *E. coli* has not been investigated. A sitABCD mutant derivative of APEC strain  $\chi$ 7122 (O78:K80:H9) caused lesions in chickens similar to those induced by the parental strain and was also as resistant to hydrogen peroxide as the wild-type parent, suggesting that other metal transporters were possibly compensating for the absence of the Sit system (56). However, a sitB signature-tagged transposon mutant of APEC strain

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s) <sup>a</sup>	Reference or source
<i>E. coli</i> K-12 strains		
BW25113	<i>rnnB3ΔlacZ4787 hsdR514 ΔaraBAD ΔrhaBAD568 rph-1</i>	2
DH5α	F <sup>-</sup> λ <sup>-</sup> φ80 Δ( <i>lacZYA-argF</i> ) <i>endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i>	Invitrogen
JW3372	BW25113 Δ <i>feoB</i> :: <i>kan</i> Km <sup>r</sup>	2
JW3933	BW25113 Δ <i>oxyR</i> :: <i>kan</i> Km <sup>r</sup>	2
JW4023	BW25113 Δ <i>soxS</i> :: <i>kan</i> Km <sup>r</sup>	2
MGN-617	<i>thi thr leu tonA lacY glnV supE ΔasdA4 recA::RP4 2-Tc::Mu [λpir]</i> Km <sup>r</sup>	16
APEC χ7122 and derivatives		
χ7122	Wild-type APEC O78:K80:H9; <i>gyrA</i> Nal <sup>r</sup>	47
QT51	χ7122 Δ <i>lacZYA</i> Nal <sup>r</sup>	40
QT205	χ7122 Δ <i>sitABCD</i> :: <i>tetAR</i> Tc <sup>r</sup>	56
QT770	QT205::pIJ93; <i>sitABCD</i> single-copy integrant	This study
QT877	χ7122 Δ <i>feoB</i> :: <i>kan</i> Nal <sup>r</sup> Km <sup>r</sup>	This study
QT878	χ7122 Δ <i>mntH</i> :: <i>kan</i> Nal <sup>r</sup> Km <sup>r</sup>	56
QT1239	QT205 Δ <i>mntH</i> :: <i>kan</i> Tc <sup>r</sup> Nal <sup>r</sup> Km <sup>r</sup>	56
QT1240	QT205 Δ <i>feoB</i> :: <i>kan</i> Tc <sup>r</sup> Nal <sup>r</sup> Km <sup>r</sup>	This study
QT1517	QT878 Δ <i>mntH</i> ::FRT Nal <sup>r</sup>	
QT1539	QT1517 Δ <i>feoB</i> :: <i>kan</i> Nal <sup>r</sup> Km <sup>r</sup>	This study
Plasmids		
pACYC184	p15A replicon cloning vector; Cm <sup>r</sup> Tc <sup>r</sup>	9
pCP20	FLP helper plasmid; Ts replicon; Ap <sup>r</sup> Cm <sup>r</sup>	14
pGP704	<i>oriR6K mobRP4</i> Ap <sup>r</sup>	45
pIJ28	8-kb HindIII fragment containing <i>sitABCD</i> from χ7122 cloned into pACYC184; Cm <sup>r</sup>	56
pIJ93	<i>sitABCD</i> genes from pIJ28 cloned into pGP704	This study
pKD46	λ red recombinase plasmid; Ts replicon; Ap <sup>r</sup>	14

<sup>a</sup> Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, naladixic acid; Tc, tetracycline; Ts, temperature sensitive.

IMT5155 (O2:K5), which contains two copies of the *sit* genes, was moderately attenuated compared to its wild-type parent in a coinfection competition model in chickens (37), suggesting a role for the Sit transporter in virulence. Herein we investigate the individual role of SitABCD in the virulence of APEC O78:K80 strain χ7122 in single-strain infection and competitive infection models in chickens. In addition, we determined the roles of the MntH and Feo transporters, alone or combined with SitABCD, in the virulence of APEC strain χ7122 in the chicken and in resistance to oxidative stress agents. The distribution of *sit* genes among pathogenic *E. coli* and *E. coli* reference strains was also assessed. Finally, the *sit* sequences available from genomic databases were compared and suggest that *sit* genes were likely inherited through distinct transfer events leading to integration within conjugative plasmids in APEC or on prophage-associated genomic islands among *E. coli* and *Shigella* sp. strains.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. In addition, clinical and commensal fecal isolates from various sources were used to screen for the presence of *sit* sequences. The 72 members of the *Escherichia coli* reference (ECOR) collection represent a diverse population of *E. coli* strains that have been grouped phylogenetically by multilocus enzyme electrophoresis (22). The 297 APEC clinical isolates were described elsewhere (16). APEC strains were previously classified for virulence based on lethality for 1-day-old chicks following subcutaneous inoculation, where LC1 corresponds to the high-lethality class, LC2 corresponds to the low-lethality class, and LC3 corresponds to the nonlethal class (16). Human extraintestinal pathogenic *E. coli* (ExPEC) isolates included 32 strains from urosepsis and other extraintestinal infections from the United States obtained from J. R. Johnson (Veterans Administration Medical Center, Minneapolis, MN). Thirty-two *E. coli* fecal isolates from healthy poultry and 23 diarrheagenic *E. coli* isolates belonging to various pathotypes (12 entero-

toxigenic *E. coli* [ETEC], 6 enteropathogenic *E. coli*, and 5 enterohemorrhagic *E. coli* strains) were kindly provided by J. M. Fairbrother (University of Montreal, Canada). Strains were grouped phylogenetically by either multilocus enzyme electrophoresis or a multiplex PCR method (13). Strains were maintained at -80°C in 25% glycerol following overnight culture in Luria-Bertani (LB) broth (10 g yeast extract, 5 g tryptone, and 10 g NaCl per liter). Strains and clones were routinely grown in LB broth or on LB agar plates (15 g agar per liter) at 37°C. *E. coli* strain DH5α was routinely used for plasmid cloning and recovery. Antibiotics were added, as required, at the following concentrations: kanamycin, 30 μg/ml; ampicillin, 100 to 200 μg/ml; chloramphenicol, 30 μg/ml; nalidixic acid, 15 μg/ml; and tetracycline, 10 μg/ml.

**DNA and genetic manipulations.** Standard methods were used for isolation of bacterial genomic DNA, DNA manipulation, and cloning (58). Restriction enzymes and DNA ligase used in this study were purchased from New England Biolabs, Invitrogen, or Amersham Pharmacia and used according to the suppliers' recommendations. Recombinant plasmids, PCR products, and restriction fragments were purified using plasmid miniprep, PCR cleanup, and gel extraction kits (Qiagen or Sigma) as recommended by the supplier. Transformation of *E. coli* strains was routinely done by using the calcium/manganese-based or electroshock method, as described previously (19).

**Presence of *sit* sequences among *E. coli* strains.** The presence of *sit* sequences among different *E. coli* strains was investigated by PCR amplification of segments of the *sitA* and *sitD* genes that span the length of the *sitABCD* system. The *sitA* primers (*sitA*-F [5'-CGCAGGGGGCACAACCTGAT-3'] and *sitA*-R [5'-CCCTGTACCAGCGTACTGG-3']) amplify a 663-bp segment of *sitA*, and the *sitD* primers (*sitD*-F [5'-CTGTGCGCTGCTGTGCGTC-3'] and *sitD*-R [5'-GCGTTGTGTCAGGAGTAC-3']) amplify a 570-bp segment of *sitD*. The specificity of each of the primers and the predicted amplification products were verified by comparative genomic/bioinformatic analysis against the *sit* genes from the *E. coli* and *Shigella* genomes or other nucleotide entries in the available databases, including GenBank (<http://www.ncbi.nlm.nih.gov/>) and coliBASE, an online database for comparative genomics of *E. coli* and related enterobacteria (<http://colibase.bham.ac.uk/>) (10). Crude DNA extracts of strains were prepared by alkaline lysis (48). The reactions were carried out using *Taq* DNA polymerase (New England Biolabs). A 5-μl volume of each bacterial cell lysate was added to a PCR mixture with a final volume of 25 μl containing 6.25 pmol of each primer, 5 nmol of each deoxynucleoside triphosphate, and 0.5 U of *Taq* polymerase in 1× buffer. 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 then an extension period of 72°C for 1 min. Specific amplification was confirmed using strains  $\chi$ 7122 and CFT073 as positive controls and *E. coli* K-12 MG1655, which lacks the *sit* genes, as a negative control.

**Construction of mutant derivatives of APEC strain  $\chi$ 7122 and single-copy complementation of the  $\Delta$ *sit* mutation.** The  $\Delta$ *sitABCD::tetAR* (QT205),  $\Delta$ *mntH::kan* (QT878), and  $\Delta$ *mntH*  $\Delta$ *sitABCD* (QT1239) derivatives of strain  $\chi$ 7122 are described elsewhere (56). QT1517 was generated by FLP-mediated excision of the  $\Delta$ *mntH::kan* allele from strain QT878 by using plasmid pCP20 (14). The  $\Delta$ *feoB::kan* allele from *E. coli* K-12 strain JWK3372\_1 (2) was used to introduce  $\Delta$ *feoB* mutations into APEC strains. Briefly, the  $\Delta$ *feoB::kan* allele was amplified from genomic DNA of strain JWK3372\_1 by using primers MfeoB1 (5'-TCTGGTCTCATGTCGCTGTC-3') and MfeoB2 (5'-GGTGGAACTCTGCTTTTTC-3') and was introduced into strains  $\chi$ 7122, QT205, and QT1517 by homologous recombination using the  $\lambda$  red recombinase method (14). Successful transfer of the  $\Delta$ *feoB::kan* mutation was confirmed by PCR, using primers flanking the *feoB* region. The  $\Delta$ *feoB::kan* derivatives of  $\chi$ 7122, QT205, and QT1517 were designated QT877, QT1240, and QT1539, respectively.

The  $\Delta$ *sitABCD* mutant strain QT205 was complemented by single-copy integration of plasmid pIJ93. pIJ93 was constructed by subcloning the XbaI-SalI fragment of pIJ28, containing the *sitABCD* operon, into the same sites of suicide vector pGP704. pIJ93 was conjugated from strain MGN-617 (16) to strain QT205. A strain that was resistant to ampicillin and found to contain a full-length copy of the *sit* genes, as confirmed by PCR, was designated QT770.

**Sensitivity of *E. coli* strains to reactive oxygen intermediate (ROI)-generating agents.** Sensitivity to oxidative stress was determined by an agar overlay diffusion method on LB and M9-glucose plates (1.5% agar) as described by Boyer et al. (4), with some modifications. Overnight cultures grown in LB broth were adjusted to an optical density at 600 nm ( $OD_{600}$ ) of 0.5. For tests on M9 medium, the overnight cultures were washed with M9-glucose prior to  $OD_{600}$  adjustment. One hundred microliters of each culture was suspended in molten top agar (0.5% agar) and poured over the agar plates. Filter paper disks (6-mm diameter; Becton Dickinson) were added to the surfaces of the solidified overlays, and 10  $\mu$ l of hydrogen peroxide (30%), paraquat (200 mM for LB and 40 mM for M9), plumbagin (53 mM), phenazine methosulfate (PMS) (15 mM), or phenazine ethosulfate (PES) (15 mM) was spotted onto the disks. The plates were then incubated overnight at 37°C, and following growth, the diameters of inhibition zones were measured.

**Experimental infection of chickens via the air sacs.** Two different infection models, a comparative single-strain infection model and a competitive coinfection model, were used to investigate the importance of different metal transporters for the virulence of APEC. Chickens used in these studies were White Leghorn specific-pathogen-free chickens obtained from either Charles River Spafas (now Charles River Laboratories [Franklin, CT]) or the Canadian Food Inspection Agency (Ottawa, Canada). For the single-strain infection model, groups of 3-week-old White Leghorn chickens were inoculated in the right thoracic air sac with 0.1 ml ( $10^7$  CFU) of a bacterial inoculum consisting of a diluted 24-h beef heart infusion broth culture of APEC strain  $\chi$ 7122 or an isogenic mutant derivative. For the coinfection experiments, strains were prepared as for the single-strain infections, and equal quantities ( $5 \times 10^6$  CFU) of each mutant strain and a virulent  $\Delta$ *lacZYA* derivative of strain  $\chi$ 7122 (QT51) were used as the inoculum. Use of the  $\Delta$ *lacZYA* derivative of strain  $\chi$ 7122 (QT51) in coinfections permitted a direct evaluation of the number of colonies of QT51 (Lac<sup>-</sup> colonies) compared to the metal transporter mutant (Lac<sup>+</sup> colonies) on each plate. For the coinfection studies, blood samples were collected aseptically from each chicken 6, 24, and 48 h following bacterial inoculation and were plated directly or diluted and then plated on MacConkey-lactose agar plates (Difco) supplemented with nalidixic acid (40  $\mu$ g/ml). All birds were euthanized at 48 h postinfection and then necropsied. For the single-strain infection experiments, gross mean lesion scores for the air sacs and combined lesion scores for the pericardium and liver were determined as described by Lamarche et al. (36). Organs were removed aseptically. The left lung, liver, and spleen of each animal were weighed, suspended in phosphate-buffered saline, and homogenized with an Omnimixer homogenizer. Dilutions of homogenates were plated onto MacConkey-lactose agar plates with appropriate antibiotics for bacterial quantification. Several randomly selected colonies per organ were verified by serotyping using O78-specific antiserum.

**Bioinformatic analysis and comparison of *sit* sequences from different *E. coli* and *Shigella* strains.** The sequences and locations of *sit* gene clusters within the genomes of various *E. coli* and *Shigella* strains were identified from the available databases, including GenBank (<http://www.ncbi.nlm.nih.gov/>) and coliBASE. Sequence analyses, multiple sequence alignment by CLUSTALW, and generation of a phylogenetic tree were done using the MEGA3 software package (35;

<http://www.megasoftware.net>). The phylogenetic tree of the *sitABCD* gene clusters was constructed by using the neighbor-joining method (57) of pairwise comparison by maximum likelihood analysis using the Jukes-Cantor estimate (30) to calculate nucleotide substitution rates.

**Statistical analyses.** Statistical analyses were performed using the Prism 4.0b software package (GraphPad Software).

## RESULTS

**The *sit* genes are associated with prophage elements and conjugative plasmids.** A previous report indicated that depending on the *E. coli* strain, *sitABCD* genes were located either on pColV-type plasmids or on the bacterial chromosome in ExPEC or APEC, and certain strains contained both genomic and plasmid-carried copies of the *sit* genes (56). Analysis of the genome and nucleotide sequence databases indicated that *sit* sequences are present in ExPEC-UPEC strains CFT073, 536, and UTI89, APEC strain O1, enteroaggregative *E. coli* (EAEC) O42 (Fig. 1), and all of the currently available *Shigella* genomes but are absent from *E. coli* K-12 strains and the genomes of other pathogenic *E. coli* strains. *sit* gene clusters are also present on the plasmids of APEC O1(pAPEC-O1-ColBM), APEC O2(pAPEC-O2-ColV), and APEC  $\chi$ 7122 (pAPEC-1) (Fig. 1), and genomic and plasmid-carried copies of *sit* sequences are present in APEC O2:K5 strain IMT5155 (GenBank accession no. AM072350 and AM072351).

The *sit* gene clusters are present at four distinct chromosomal locations in the *E. coli-Shigella* genomes (Fig. 1A). Among the sequenced ExPEC-UPEC strains and APEC O1, the *sitABCD* genes are all part of genomic regions containing prophage sequences that are inserted at the *icd-ycgX* intergenic region, which is the *attB* attachment site for certain lambdoid bacteriophages. These prophage element-containing regions have identical junction borders to those in the *E. coli* K-12 genome and vary in size from 55 kb (CFT073) (67) to 47.3 kb (APEC O1) (28). In *E. coli* K-12 MG1655, this region harbors the e14 element, a vestige of a lambdoid prophage, which encompasses 15.4 kb (43). In EAEC strain O42, *Shigella sonnei*, and *Shigella dysenteriae*, the *sit* genes are part of 48.4-kb, 25.6-kb, and 19.9-kb regions, respectively, comprised mainly of phage elements, and are inserted at the *ydaO* (b1344)-*ynaF* (b1376) region of *E. coli* K-12 MG1655 (10). This region is the attachment site for Rac prophage and related prophage elements in *E. coli* K-12 and O157:H7 strains (8). In *S. flexneri* 2a strain 301, *sit* genes are in the middle of a 42-kb genomic island, *Shigella* island 19 (SI-19), located in a region that has undergone rearrangements compared to *E. coli* MG1655. SI-19 contains genes that are homologous to phage sequences and insertion sequence elements and is flanked by sequences corresponding to *ycjI* and *yebU* in *E. coli* K-12 MG1655 (26). In *S. flexneri* 2a strain 2457T, *sit* genes are in the middle of a 37-kb genomic island that is adjacent to *aspS*. Despite differences in the locations of the *sit* genes within the genomes of *S. flexneri* 2a strains 301 and 2457T, the lambdoid prophage sequences immediately flanking the *sit* genes in these two genomes are similar. Sequences flanking the *sit* genes on plasmids are similar to each other but are distinct from those adjacent to the *sit* genes present on the genomic islands of any of the *E. coli* or *Shigella* strains.

Comparison of the nucleotide sequences encoding SitABCD from *E. coli* and *Shigella* strains in a neighbor-joining tree

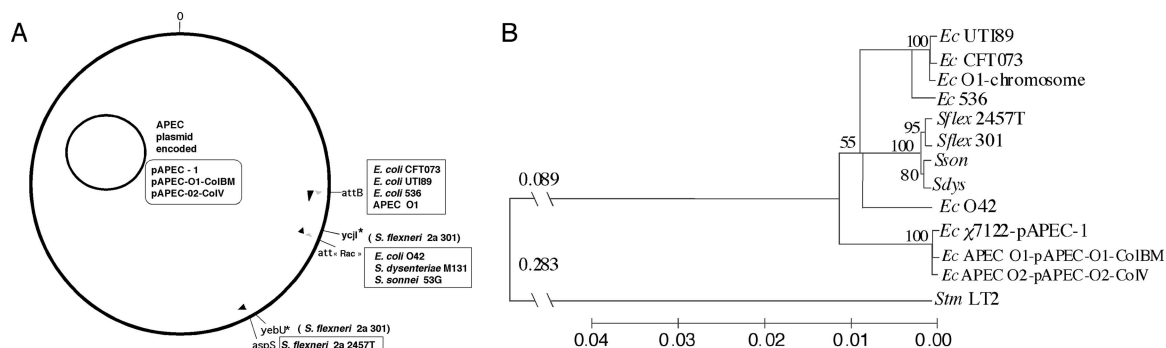


FIG. 1. (A) Map location of *sit* genes in *E. coli* and *Shigella* strains. The large circle represents the *E. coli* K-12 MG1655 genome as a comparative reference, with 0 indicating the start of the sequence. The insertion locations corresponding to *E. coli* K-12 are indicated on the outer perimeter. The inner black arrows represent the genomic islands and the orientation of the *sit* genes in different pathogenic strains. The outer gray arrows indicate corresponding e14 and Rac prophage elements located in the same regions in strain MG1655. The asterisks denote the *E. coli* K-12 corresponding genes flanking either side of SI-19, the genomic island encompassing the *sit* genes in *S. flexneri* 2a strain 301 (26). (B) Phylogenetic tree of the *sitABCD* genes from *E. coli* and *Shigella* spp., constructed using the neighbor-joining method with MEGA3 software (35). *Ec*, *Escherichia coli*; *Sflex*, *Shigella flexneri*; *Sson*, *Shigella sonnei* 53G; *Sdys*, *Shigella dysenteriae* M131. The full-length sequences spanning from the *sitA* start codon to the *sitD* stop codon were analyzed, and *sitABCD* genes from *Salmonella enterica* serovar Typhimurium LT2 were used as the outlier sequence. Distances were calculated using the Jukes and Cantor estimate, which assumes a uniform rate of nucleotide substitutions. Branch lengths are indicated on the scale or above branches that are off the scale. Bootstrap confidence levels are indicated adjacent to the nodes. *sit* sequence data were obtained from NCBI with GenBank accession numbers pAPEC-1 of *E. coli*  $\chi$ 7122 (56), AY545598 (*E. coli* APEC O2/pAPEC-O2-CoIV) (29), DQ381420 (*E. coli* APEC O1/pAPEC-O1-CoIBM) (27), NC\_004431 (*E. coli* CFT073) (67), NC\_008563 (*E. coli* APEC O1) (28), NC\_007946 (*E. coli* UTI89) (11), NC\_008253 (*E. coli* 536) (6), NC\_004337 (*S. flexneri* 2a 301) (26), NC\_004741 (*S. flexneri* 2a 2457T) (66), and NC\_003197 (*S. enterica* LT2) (42) or from *coli*BASE (10; <http://colibase.bham.ac.uk/>) for *E. coli* O42, *Shigella dysenteriae* M131, and *Shigella sonnei* 53G. Data available from *coli*BASE were produced by the sequencing group at the Sanger Institute ([http://www.sanger.ac.uk/Projects/Escherichia\\_Shigella/](http://www.sanger.ac.uk/Projects/Escherichia_Shigella/)).

demonstrated that *sit* gene clusters form three main groups, comprised of the ExPEC-APEC genomic *sit* sequences, the *Shigella sit* sequences, and the plasmid-carried APEC *sit* sequences (Fig. 1B). The *E. coli* O42 *sit* sequence branched separately but was most closely related to *Shigella* sequences. Phylogenetic analyses of each of the *sit* genes generated the same distinct groupings (data not shown). Overall, the *sit* sequences demonstrated nucleotide variability at 156 sites, which corresponded to a variability of 4.5% of the *sit* gene cluster. Sequences within each of the three main cluster groups demonstrated low variability (from 0.4% to 0.6% overall) within their respective clusters. In contrast, between cluster groups, variability increased two- to sixfold (from 1.2% to 2.5%). Taken together, these differences in genomic locations and nucleotide sequence variability among the *sit* genes from different *E. coli* and *Shigella* strains support the likelihood that *sit*

genes were acquired by these two closely related species via a number of distinct genetic events involving horizontal gene transfer mediated by phages, plasmids, or other mobile genetic elements.

**Distribution of *sit* genes among ECOR collection and *E. coli* clinical and fecal commensal isolates.** The presence and distinct localization of *sit* sequences among different *E. coli* and *Shigella* sp. strains prompted us to further investigate the distribution of *sit* sequences among a diverse population of strains, including ECOR collection, ExPEC, diarrheagenic *E. coli*, APEC, and avian fecal commensal *E. coli* strains. PCR analysis was performed using *sitA*- and *sitD*-specific primers which correspond to conserved regions in the *sitA* and *sitD* genes currently available from the sequence databases. For all strains tested, there was no discrepancy between results when using either the *sitA* or *sitD* primers. Among the 72 members of

TABLE 2. Distribution of *sit*-positive strains within phylogenetic groups among ECOR collection and *Escherichia coli* clinical isolates

Phylogenetic group	ECOR collection		Clinical isolate <sup>c</sup>			
	Total no. of strains	No. (%) of <i>sit</i> -positive strains <sup>a</sup> (%)	No. of isolates	No. (%) of <i>sit</i> -positive isolates	Diarrheagenic <i>E. coli</i>	
					No. of isolates	No. (%) of <i>sit</i> -positive isolates
A	25	5 (20)	6	6 (100)	12	1 (8.3)
B1	16	7 (43.8)	0	0	8	1 (12.5)
B2	15	15 (100) <sup>b</sup>	20	20 (100)	1	0
D	12	6 (50)	6	6 (100)	2	0
Other	4	1 (25)	NA	NA	NA	NA
Total	72	34 (47.2)	32	32 (100) <sup>d</sup>	23	2 (8.7)

<sup>a</sup> Positive PCR amplification using the *sitA* and *sitD* primer pairs.

<sup>b</sup> Among the ECOR collection strains, *sit* was significantly associated with group B2 compared to any other phylogenetic group ( $P < 0.0001$ ).

<sup>c</sup> NA, not applicable.

<sup>d</sup> *sit* was significantly associated with ExPEC strains compared to diarrheagenic *E. coli* or the ECOR collection strains ( $P < 0.0001$ ).

TABLE 3. Distribution of *sit* genes among APEC and avian commensal fecal isolates according to lethality class

Lethality class	APEC		Commensal fecal isolate	
	Total no. of isolates	No. (%) of <i>sit</i> -positive isolates <sup>a</sup>	Total no. of isolates	No. (%) of <i>sit</i> -positive isolates <sup>a</sup>
LC1 <sup>b</sup>	221	218 (99) <sup>c</sup>	1	0 (0)
LC2	38	31 (82)	12	5 (42)
LC3	38	26 (68)	19	9 (47)
Total	297	275 (93) <sup>d</sup>	32	14 (44)

<sup>a</sup> Positive PCR amplification using the *sitA* and *sitD* primer pairs.

<sup>b</sup> Lethality classes were defined as follows: LC1, 50% lethal dose (LD<sub>50</sub>) of <10<sup>8</sup> CFU; LC2, LD<sub>50</sub> of ≥10<sup>8</sup> CFU; LC3, not lethal at ≥10<sup>8</sup> CFU (16).

<sup>c</sup> *sit* sequences were significantly associated with APEC isolates from LC1 compared to APEC isolates from either LC2 or LC3 (*P* < 0.001).

<sup>d</sup> *sit* sequences were significantly associated with APEC isolates compared to commensal fecal isolates (*P* < 0.001).

the ECOR collection, which have been arranged into four phylogenetic groups (A, B1, B2, and D) on the basis of multilocus enzyme electrophoresis (22), the presence of *sit* sequences was significantly associated with phylogenetic group B2 compared to the other groups (*P* < 0.0001) (Table 2). All B2 group strains from the ECOR collection were *sit* positive, whereas in the other phylogenetic groups 50% or fewer were *sit* positive. In addition, all 32 human ExPEC strains tested were *sit* positive, whereas only 2 of 23 diarrheagenic *E. coli* isolates contained *sit* sequences (Table 2). The presence of *sit* sequences was also investigated in APEC and avian fecal commensal isolates (Table 3). Among 297 APEC strains classified according to their lethality for 1-day-old chicks, *sit* sequences were more common in the highly virulent APEC (class 1 lethality) strains (99%) than in the less virulent strains (81% for class 2 and 68% for class 3) (*P* < 0.001). In addition, *sit* sequences were highly associated with APEC compared to avian fecal commensal isolates (*P* < 0.001). For APEC isolates, 275/297 (93%) isolates were *sit* positive, whereas only 14/32 (44%) avian fecal isolates were *sit* positive. Overall, the results demonstrate that *sit* sequences are highly prevalent in *E. coli* strains associated with extraintestinal infections compared to diarrheagenic *E. coli* and commensal fecal isolates.

**SitABCD contributes to virulence of APEC during infection of chickens.** Since *sit* sequences were highly associated with *E. coli* isolates from extraintestinal infections in poultry, we investigated the importance of *sit* genes for the virulence of APEC O78 strain  $\chi$ 7122 in chickens. We first compared the virulence potential of the  $\Delta$ *sitABCD* mutant strain QT205 with that of its isogenic wild-type parent strain,  $\chi$ 7122, in a single-strain infection model. Compared to wild-type parent strain  $\chi$ 7122, strain QT205 ( $\Delta$ *sitABCD*) was attenuated and demonstrated significantly reduced bacterial numbers in the lungs and livers of infected chickens (Fig. 2a and b). QT205 demonstrated a mean 1-log reduction in colonization of the spleens; however, the difference was not significant (*P* = 0.0545) (Fig. 2c). The *sit*-complemented derivative of QT205 (QT770) regained virulence to levels comparable to that of the wild-type parent strain,  $\chi$ 7122 (Fig. 2). Despite the decreased persistence of QT205 in chickens, this strain caused gross lesions of airsacculitis and pericarditis/perihepatitis that were similar to those caused by the wild-type parent (data not shown).

We also investigated the role of the Feo ferrous iron transporter and the MntH manganese transporter in the virulence of APEC strain  $\chi$ 7122. In single-strain infection experiments, in contrast to strain QT205 ( $\Delta$ *sitABCD*), strains QT877 ( $\Delta$ *feoB*) and QT878 ( $\Delta$ *mntH*) persisted in all tissues at levels similar to that of wild-type APEC strain  $\chi$ 7122. Thus, in the presence of a functional SitABCD system, FeoB or MntH did not appear to be of major importance for the virulence of APEC  $\chi$ 7122 in chickens. In addition, in the single-strain infection model, mutant strains QT1239 ( $\Delta$ *sitABCD*  $\Delta$ *mntH*) and QT1240 ( $\Delta$ *sitABCD*  $\Delta$ *feoB*) were not any more attenuated than the  $\Delta$ *sitABCD* single mutant QT205 (data not shown). The mutant derivatives lacking functional Sit, MntH, and/or Feo transporters demonstrated no appreciable difference in growth rate compared to the wild-type parent when cultured in either rich (Luria broth) or minimal (M9-glucose) medium (data not shown). Therefore, the reduced colonization of tissues by the *sit* and other attenuated mutants during infection of chickens was most likely due to a decreased capacity to survive in vivo, not a global reduction in fitness or growth.

Compared to single-strain infection models, competitive

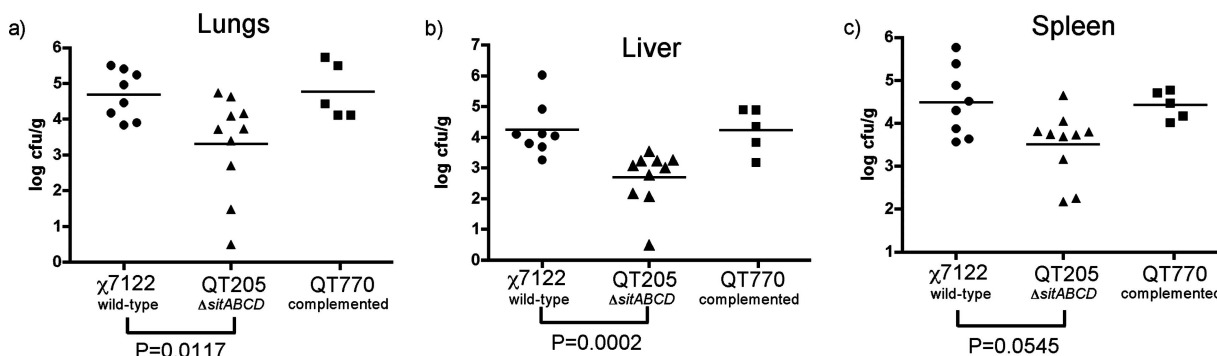


FIG. 2. Colonization of extraintestinal organs of chickens by APEC strain  $\chi$ 7122 and derivatives in the single-strain infection model. Data are presented as log CFU/gram of tissue. Each data point represents a tissue sample from an individual infected chicken at 48 h postinfection. Organs sampled were the lungs (a), liver (b), and spleen (c). Strains tested were wild-type APEC strain  $\chi$ 7122 (●), QT205 ( $\Delta$ *sitABCD*) (▲), and QT770 (QT205:*sitABCD*) (■). *P* values for comparative differences in colonization by QT205 and the wild-type strain, as determined by the Mann-Whitney test, are indicated below the *x* axes.

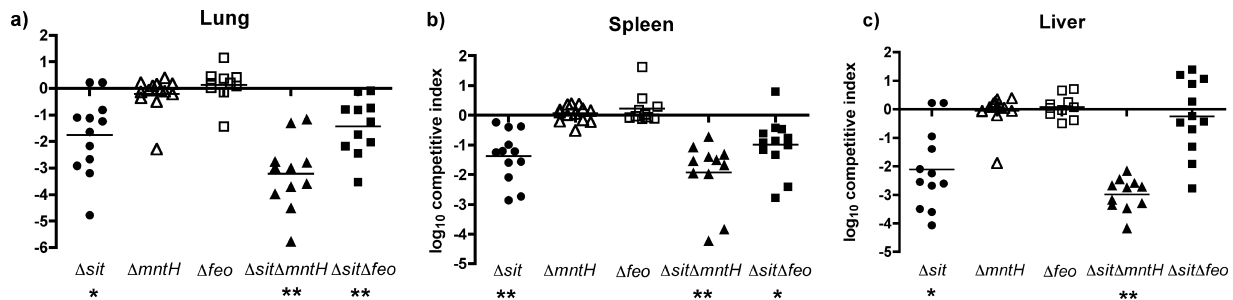


FIG. 3. Comparative colonization of chicken tissues by APEC  $\chi$ 7122 derivatives lacking the Sit, MntH, or Feo metal transporter and virulent  $\chi$ 7122  $\Delta$ lac derivative strain QT51. A competitive coinfection model was used in which QT51 and different metal transporter mutants were inoculated simultaneously. At 48 h postinfection, tissues were sampled, and results are presented as the  $\log_{10}$  CI. The CI represents the relative numbers of the two test strains from the tissues sampled (the output ratio) compared to the initial numbers of the strains in the inoculum (input ratio). Negative CI values indicate a decreased capacity for the mutant to compete with the virulent test strain (QT51). Horizontal bars indicate the mean  $\log_{10}$  CI values. Each data point represents a sample from an individual chicken. Ten to 12 chickens were used for each infection group. Organs sampled were the lungs (a), spleen (b), and liver (c). Strains tested were QT205 ( $\Delta$ sitABCD) (●), QT878 ( $\Delta$ mntH) (△), QT877 ( $\Delta$ feoB) (□), QT1239 ( $\Delta$ sitABCD  $\Delta$ mntH) (▲), and QT1240 ( $\Delta$ sitABCD  $\Delta$ feoB) (■). Statistically significant decreases in CI values are indicated with asterisks (\*,  $P < 0.005$ ; \*\*,  $P < 0.001$ ), as determined by the Wilcoxon matched-pair test.

coinfection models using virulent strains and isogenic mutants can demonstrate more sensitivity to differences in colonization or virulence. Because single-strain infections in chickens did not show any attenuation for strains QT877 ( $\Delta$ feoB) and QT878 ( $\Delta$ mntH), and since no differences in attenuation between strain QT205 ( $\Delta$ sitABCD) and the double mutants QT1239 ( $\Delta$ sitABCD  $\Delta$ mntH) and QT1240 ( $\Delta$ sitABCD  $\Delta$ feoB) were observed in single-strain infections, we tested these strains in a competitive coinfection model. For this model, we used APEC strain QT51, a  $\Delta$ lacZYA derivative of strain  $\chi$ 7122, as the competitor strain. QT51 was shown to be as virulent as the wild-type parent strain  $\chi$ 7122 in both single-strain infection and coinfection experiments (data not shown). The use of a virulent Lac-negative wild-type derivative permitted us to directly compare the levels of the Lac-positive mutant derivative and the virulent Lac-negative competitor strain from the same biological samples by differential counts on MacConkey-lactose agar plates.

In coinfection experiments, strain QT205 ( $\Delta$ sitABCD) was clearly attenuated and showed a significantly reduced competitive index (CI) compared to that of competitor strain QT51, with a mean 50-fold decrease in the lung (Fig. 3a) and a mean

126-fold decrease in the liver (Fig. 3b). These results were consistent with the results observed for the single-strain infection experiments. In addition, QT205 was also significantly reduced, by a mean of 25-fold, in the spleen (Fig. 3c). During the course of the infection, no significant differences between QT51 and QT205 were apparent in the blood at 6 h and 24 h postinfection (Fig. 4). However, QT205 was significantly reduced, by a mean of 3.6-fold, in the blood by 48 h postinfection. Unlike strain QT205, strains QT877 ( $\Delta$ feoB) and QT878 ( $\Delta$ mntH) were present at similar levels to those of strain QT51 in all tissues and blood (Fig. 3 and 4). These results were consistent with the lack of attenuation of these strains in the single-strain infection model.

In coinfections using the double mutants, strain QT1239 ( $\Delta$ sitABCD  $\Delta$ mntH) was the most attenuated strain (Fig. 3 and 4). QT1239 demonstrated a significantly reduced CI in the blood at all times, with mean decreases of 2.2-fold, 16.2-fold, and 10-fold compared to the competitor strain at 6 h, 24 h, and 48 h, respectively. QT1239 also demonstrated a mean 1,400-fold reduction in the lungs, 954-fold reduction in the liver, and 83-fold reduction in the spleen compared to strain QT51. In contrast, strain QT1240 ( $\Delta$ sitABCD  $\Delta$ feoB) demonstrated sig-

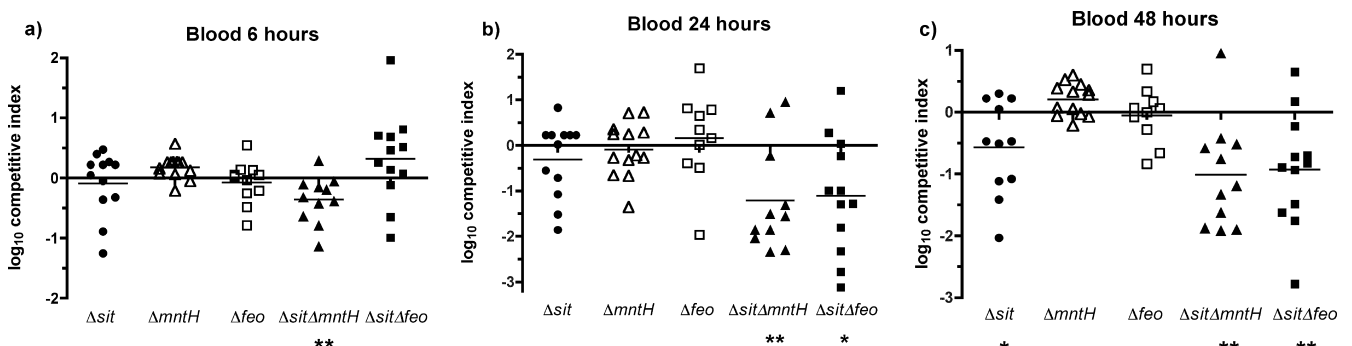


FIG. 4. Comparative persistence of APEC  $\chi$ 7122 derivatives lacking the Sit, MntH, or Feo metal transporter and virulent  $\chi$ 7122  $\Delta$ lac derivative strain QT51 in the blood of infected chickens. Experiments were conducted using a coinfection model as described in the legend to Fig. 3. Blood was sampled at 6 h (a), 24 h (b), and 48 h (c) postinfection. Strains tested were QT205 ( $\Delta$ sitABCD) (●), QT878 ( $\Delta$ mntH) (△), QT877 ( $\Delta$ feoB) (□), QT1239 ( $\Delta$ sitABCD  $\Delta$ mntH) (▲), and QT1240 ( $\Delta$ sitABCD  $\Delta$ feoB) (■). Statistically significant decreases in CI values, as determined by the Wilcoxon matched-pair test, are indicated with asterisks (\*,  $P < 0.005$ ; \*\*,  $P < 0.001$ ).



nificant, 2.2-fold and 5.6-fold reductions in the blood at 24 h and 48 h, respectively. QT1240 was also reduced 26.8-fold in the lungs and 6.8-fold in the spleen but was not significantly reduced in the liver (Fig. 3). Strain QT1539 ( $\Delta feoB \Delta mntH$ ) did not demonstrate any significant decrease compared to strain QT51 in either the blood or tissues (data not shown). Comparison of the mean CIs for strains QT205 ( $\Delta sitABCD$ ), QT1239 ( $\Delta sitABCD \Delta mntH$ ), and QT1240 ( $\Delta sitABCD \Delta feoB$ ) demonstrated no significant differences between the groups in the blood and spleen. In the lung, QT1239 ( $\Delta sitABCD \Delta mntH$ ) was significantly more attenuated in the coinfection model than was QT205 ( $\Delta sitABCD$ ) ( $P = 0.0178$ ). In contrast, QT205 ( $\Delta sitABCD$ ) was significantly more attenuated than QT1240 ( $\Delta sitABCD \Delta feoB$ ) ( $P = 0.0061$ ) in the liver. Taken together, these results demonstrate that, individually, the Sit system is most important for competitive survival and persistence compared to the Feo and MntH transporters. In addition, a cumulative loss of Sit and MntH transporters globally resulted in more marked attenuation than that with the loss of only the Sit transporter. In contrast, a cumulative loss of the Sit and Feo systems compared to the individual loss of Sit actually resulted in reduced persistence in the blood but increased colonization of the liver.

#### Sensitivity of *E. coli* strains to ROI-generating compounds.

The sensitivities of APEC strain  $\chi 7122$  and its mutant derivatives to ROI-generating compounds was assessed on both rich (LB) and minimal (M9-glucose) media (Fig. 5). On LB medium, only the  $\Delta mntH \Delta sitABCD$  (QT1239) strain was more sensitive than the wild-type parent strain to the ROI-generating compounds  $H_2O_2$  ( $P = 0.002$ ) and plumbagin ( $P = 0.027$ ). On minimal medium, QT1239 was more sensitive to  $H_2O_2$  ( $P = 0.015$ ), plumbagin ( $P = 0.004$ ), and paraquat ( $P = 0.004$ ). On minimal medium, the  $\Delta mntH$  mutant QT878 was more sensitive to  $H_2O_2$  ( $P = 0.012$ ) and paraquat ( $P < 0.001$ ) than the APEC wild-type parent but was less sensitive to these products than the  $\Delta mntH \Delta sitABCD$  derivative. In addition, on minimal medium, the  $\Delta feoB \Delta sitABCD$  strain (QT1539) was also somewhat more sensitive to  $H_2O_2$  ( $P = 0.007$ ) than the wild-type parent. In contrast, the  $\Delta sitABCD$  mutant QT205 did not demonstrate any increased sensitivity to ROI-generating products compared to the wild-type parent (data not shown).

APEC strain  $\chi 7122$  was intrinsically more resistant to all of the ROI-generating compounds tested than the K-12 control strain following growth on either LB or minimal medium ( $P < 0.05$ ) (Fig. 5). In addition, the APEC metal transporter mutants that were more sensitive to certain ROI-generating compounds were always resistant to PMS and PES. Compared to the other ROI-generating compounds, PMS and PES specifically generated a superoxide stress response, as the  $\Delta soxS$  K-12 strain, but not the  $\Delta oxyR$  derivative, demonstrated sensitivity to these compounds (Fig. 5). Taken together, these results demonstrate that the MntH and SitABCD divalent metal transporters synergistically contributed to increased resistance to ROIs that generate either a  $H_2O_2$ -specific or a mixed  $H_2O_2$  and superoxide stress response. In contrast to the in vivo studies with chickens, in which the Sit transporter was the only system studied that individually contributed to virulence, in vitro only the MntH transporter was shown to individually contribute to resistance to oxidative stress.

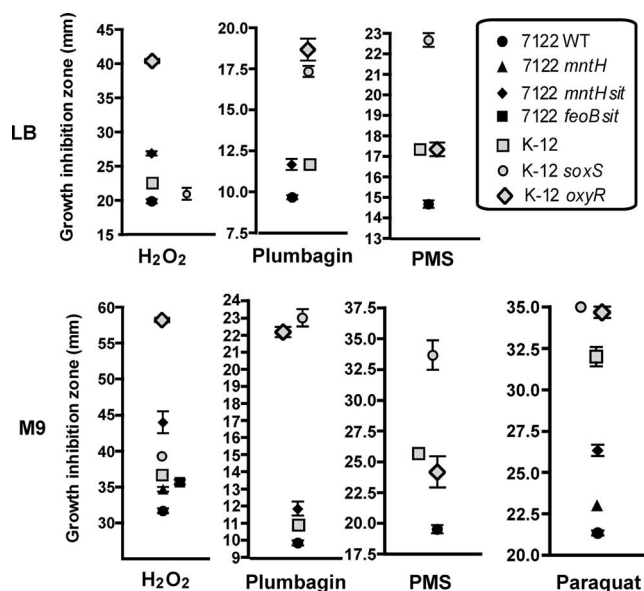


FIG. 5. Sensitivities of wild-type APEC strain  $\chi 7122$  and isogenic metal transport mutants to ROI-generating compounds. Strains were grown on either LB agar or M9 agar, and tests were performed as described in Materials and Methods. ROI-generating compounds tested were 30% hydrogen peroxide ( $H_2O_2$ ), 53 mM plumbagin, 15 mM PMS or PES, and 40 mM paraquat. Paraquat was ineffective against all strains grown on LB medium, even at higher concentrations (up to 200 mM). Sensitivity to PES was similar to sensitivity to PMS (not shown). The results represent the means of replicate experiments for a minimum of three samples. Vertical bars represent the standard errors of the means. Only wild-type (WT) strain  $\chi 7122$  and its mutant derivatives that demonstrated a significant increase ( $P < 0.05$ ) in sensitivity to ROI-generating products are indicated. APEC strains indicated in the legend are  $\chi 7122$  (7122 WT; ●), QT878 (7122 *mntH*; ▲), QT1239 (7122 *mntH sit*; ◆), and QT1240 (7122 *feoB sit*; ■). *E. coli* K-12 strains BW25113 (K-12 ■), JW3933 (K-12 *oxyR*; ◇), and JW4023 (K-12 *soxS*; ○) were used as comparative controls. For all conditions tested, K-12 strain BW25113 was significantly more sensitive ( $P < 0.05$ ) to ROI-generating compounds than was APEC strain  $\chi 7122$ .

## DISCUSSION

In this report, we investigated the individual and combined roles of the SitABCD, MntH, and FeoB transporters in the virulence of APEC strain  $\chi 7122$ . These transport systems are mainly involved in the import of manganese and/or ferrous iron. MntH is a proton-dependent NRAMP-related transporter that is highly selective for manganese (33, 41), FeoB is a GTPase that functions in ferrous iron transport (20, 31), and SitABCD (YfeABCD in *Yersinia* spp.) is an ABC transporter that mediates uptake of manganese and iron (3, 33, 56).

Iron plays a number of vital functions in bacterial cells and is a cofactor of numerous enzymes (1). Importantly, iron plays a role in protection against oxidative stress, as it is a component of the SodB superoxide dismutase (SOD) and catalase enzymes, which eliminate superoxide ( $O_2^-$ ) and  $H_2O_2$ , respectively. However, excess iron can also contribute to oxidative damage through the generation of free radicals (1). Due to the limited availability of iron in the host, pathogenic bacteria have acquired numerous iron transport systems, some of which are major virulence factors (5). Bacteria obtain ferric iron from the host via either siderophore systems, low-molecular-weight

chelators that solubilize iron, or host iron transport protein receptors, whereas ferrous iron is transported by FeoB and SitABCD-like systems (5). Manganese is also a cofactor for a number of bacterial enzymes, contributes to protection against oxidative stress (33), and can contribute directly to the detoxification of ROIs (23). In *E. coli* and other enterobacteria, two main systems, MntH and SitABCD, have been identified as being involved in manganese transport (33, 41, 56). Since iron and manganese are critical for bacterial tolerance to oxidative stress as well as other metabolic functions, SitABCD, MntH, and FeoB could potentially promote bacterial survival during infection by facilitating the transport of these trace metals and by countering oxidative stress during infection of the host.

FeoB and MntH orthologs are widespread among a diverse population of bacteria (20, 49), and these systems are conserved among *E. coli* strains, as they are present in all the currently sequenced *E. coli* and *Shigella* genomes. Unlike *feoB* and *mntH*, *sit* genes are present in only a subset of *E. coli* strains. *sit* genes were predominant among ExPEC and APEC isolates and *E. coli* strains belonging to phylogenetic group B2 but were absent from *E. coli* K-12 and most diarrheagenic *E. coli* strains, except for enteroaggregative *E. coli* strain O42 (Fig. 1A; Tables 2 and 3). Among avian *E. coli* strains, we determined that *sit* genes were highly associated with the virulence of APEC for 1-day-old chicks (Table 3). This is the first report describing an association of *sit* genes with the virulence of APEC. In addition, *sit* genes were significantly associated with APEC (93%) compared to avian fecal commensal isolates (44%) (Table 3). Similarly, Rodriguez-Seik et al. (51) detected *sit* in 86.4% of APEC strains, compared to 42.7% of fecal commensal isolates from poultry. Overall, *sit* sequences are clearly prevalent among ExPEC and APEC strains compared to intestinal commensal *E. coli* strains.

In some *E. coli* strains, two or more copies of the *sit* genes are present, with one copy being carried on a plasmid and another copy being chromosomally carried (17, 27–29). Recently, Ewers et al. (17) also detected the specific presence of episomal and chromosomal copies of *sit* by PCR using specific primers. They detected 31.6% prevalence of *sit* among APEC strains by using chromosome-specific primers and 73.2% prevalence of *sit* among APEC strains by using episome-specific primers. These results suggest that APEC strains more commonly contain a plasmid-carried copy of *sit* than chromosomally carried *sit* genes. Chromosomal copies of *sit* genes are associated with different phage elements (Fig. 1A). Hence, in certain strains, *sitABCD* represents another virulence factor that may have been acquired via bacteriophages, which are common contributors to bacterial diversification and adaptability (8, 46). It is tempting to speculate that recombination or integration events within a single strain may have led to the duplication of *sit* genes. However, comparative sequence analysis demonstrates higher identity among *sit* sequences located in genomic islands from different strains than between genomic island- and plasmid-carried *sit* sequences from the same strain (Fig. 1B). These findings favor the likelihood that *sit* genes may have been acquired independently from different sources by distinct events resulting in the incorporation of these genes into either plasmids or prophage-associated genomic islands in the genomes of *Shigella* and ExPEC.

The increased association of *sit* genes with ExPEC and

APEC compared to other *E. coli* strains suggested a possible role for this transporter in virulence during extraintestinal infection. In UPEC strain CFT073, *sit* genes were upregulated in the urines of mice during urinary tract infection or in vitro following growth in human urine (63). Also, in *Shigella flexneri* 2a, the expression of *sit* genes was upregulated during infection of cells (39, 54). This further suggests that SitABCD may contribute to metal transport in vivo and to virulence during extraintestinal infections. In both the single-strain infection and competitive infection models, the APEC  $\Delta$ *sitABCD* mutant was clearly attenuated and less able to colonize the tissues and persist in the blood of infected chickens (Fig. 2 to 4). In contrast, the single-strain infection and coinfection results demonstrated that in the presence of a functional SitABCD transporter, the inactivation of FeoB or MntH did not reduce the virulence of APEC strain  $\chi$ 7122. In addition, the combined loss of *feoB* and *mntH* did not reduce virulence. Hence, in contrast to the MntH and FeoB transporters, the Sit transporter contributed significantly to the survival of APEC in extraintestinal tissues. These results indicate that the divalent-ion transporter SitABCD imparts a selective advantage compared to the MntH or FeoB transporter in vivo. The infection model used for these studies was the air sac inoculation model (15, 16), which has been shown to result in a more uniform rate of infection of extraintestinal tissues and blood than that obtained with the more natural aerosol route of infection (18, 64). However, by using the air sac route in the single-strain infection model, despite the significantly decreased survival of the  $\Delta$ *sitABCD* mutant in chickens, this strain caused gross lesions of airsacculitis and pericarditis/perihepatitis that were similar to those caused by the wild-type parent. This is likely because the attenuated strains remained in the tissues at levels of 3 log or above at 48 h postinfection in most birds. Such bacterial levels are likely to be sufficient to still elicit a strong inflammatory response and the generation of gross lesions. It is possible that the use of the aerosol route in single-strain infection experiments could potentially demonstrate differences in pathology of colibacillosis between strains that were not observed via the air sac route, as the aerosol route requires APEC to initially colonize and proliferate in the upper respiratory tract and then subsequently invade deeper tissues.

The use of the competitive infection model provided increased sensitivity compared to that of the single-strain infections. This was most evident in analyses of mutants that had lost both SitABCD and MntH divalent manganese transport functions or SitABCD and FeoB divalent iron transport functions (Fig. 3 and 4). No significant difference at any site was observed in the single-strain infections by comparing these double mutants to the mutants which lacked the Sit transporter alone. This is likely due to the combination of competitive pressure between strains, in addition to pressures due to immune defenses and nutritional limitations within the host, in the competitive infection model (52, 65).

The contribution of Sit to the virulence of APEC may be due in large part to its function as an efficient manganese transporter (56) and to the importance of manganese for coping with oxidative stress. Cellular accumulation of manganese contributes to protection against oxidative stress by direct catalytic scavenging of ROIs and can also lead to increased activity of bacterial manganese-dependent SOD (MnSOD) (23). In *E.*

*coli*, MnSOD, encoded by *sodA*, is an inducible SOD that responds to increases in oxidative stress, and MnSOD more effectively protected *E. coli* against paraquat and H<sub>2</sub>O<sub>2</sub> than did FeSOD, encoded by *sodB* (7). An efficient capacity to accumulate Mn by APEC via the Sit and MntH transporters is therefore likely to contribute to survival in host extraintestinal sites. The infection results demonstrated that loss of MntH alone had no discernible effect on virulence in the chicken, whereas loss of Sit alone did reduce virulence. Conversely, MntH alone contributed to protection of APEC against ROIs, whereas Sit alone had no significant contribution to resistance to oxidative stress in vitro (Fig. 5). This may be explained by differences in environmental conditions, which may affect the activity of each of these transport systems. MntH transports metals via a proton-dependent gradient and, as such, functions best under acidic conditions (32). In contrast, Sit-mediated transport is most efficient under alkaline conditions (32, 56). The pH in chicken blood and tissues is maintained at approximately 7.4, a pH at which Sit efficiently mediates Mn transport (4, 56). It is also possible that the ATPase-mediated Sit ABC transporter may be more effective at tolerating stresses within the host that could impede the proton gradient required for proper functioning of MntH-mediated transport. Although Sit alone appeared to be more important than MntH in vivo, it is clear that the combined loss of the Sit and MntH transporters resulted in the greatest attenuation, particularly in the lung, and in greater sensitivity to a number of ROI-generating compounds. Taken together, these results support a synergistic role for Sit and MntH to transport manganese and contribute to a greater overall resistance to oxidative stress and persistence during infection.

Loss of Sit and MntH rendered the APEC strain more susceptible to a number of ROI-generating compounds, particularly on minimal medium (Fig. 5). Manganese transporters in a number of bacteria have been shown to contribute to resistance to H<sub>2</sub>O<sub>2</sub> and superoxide (23). As observed for *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* 2a *mntH sit* mutants (4, 53), the APEC  $\Delta mntH \Delta sitABCD$  mutant was more sensitive to H<sub>2</sub>O<sub>2</sub> (Fig. 5) (56). In addition, the APEC  $\Delta mntH \Delta sitABCD$  mutant was also more sensitive to certain redox cycling agents, including plumbagin and paraquat. These compounds generate superoxide radicals, but superoxide can be converted readily into H<sub>2</sub>O<sub>2</sub> by SODs within bacterial cells. The use of  $\Delta oxyR$  and  $\Delta soxS$  K-12 control strains, which were both more sensitive to plumbagin and paraquat, also suggested that subsequent generation of H<sub>2</sub>O<sub>2</sub> occurred following exposure to these compounds. In contrast, neither APEC  $\Delta mntH \Delta sitABCD$  nor the  $\Delta oxyR$  control was more sensitive to phenazines (PMS and PES) than the relevant parent strain, although these compounds more effectively killed the  $\Delta soxS$  control. Differences in sensitivity to redox cycling agents may therefore be due to their mechanisms of action for generation of oxidative stress or possibly other toxic effects. In *E. coli*, the response to oxidative stress generated by PMS differs considerably from that generated by paraquat or plumbagin, with PMS generating a high level of catalase activity and little increase in SOD activity, whereas paraquat and plumbagin induce increases in both SOD and catalase activities (21, 60).

Although the Sit transporter from APEC strain  $\chi$ 7122 was

shown to mediate uptake of iron in an iron transport-deficient *E. coli* K-12 mutant (56), it is unlikely that iron transport by SitABCD is a key feature of APEC virulence. Strain  $\chi$ 7122 uses three confirmed siderophore systems (aerobactin, salmochelins, and enterobactin) in addition to the Feo and SitABCD transporters. Elimination of the aerobactin and salmochelin siderophore systems resulted in a nearly complete loss of persistence of APEC strain  $\chi$ 7122 in chickens (15), suggesting that the remaining iron transport systems (FeoB, SitABCD, and enterobactin) were unable to meet the iron transport requirements for survival of APEC during extraintestinal infection. This is not surprising, since FeoB and SitABCD function mainly as ferrous iron transporters (20, 56), and iron present in extraintestinal tissues is predominantly ferric iron associated with host ferro-proteins. Furthermore, although enterobactin is a highly efficient siderophore in vitro, it competes poorly as a siderophore in host tissues (1, 5). The finding that the cumulative loss of the Sit and Feo ferrous iron transporters did not result in greater attenuation than that of the  $\Delta sitABCD$  mutant is in further support of a limited requirement of these systems for iron acquisition in extraintestinal tissues (Fig. 3). However, the combined loss of Sit and Feo resulted in a decreased capacity to persist in the blood (Fig. 4). It is possible that in the blood, where iron availability is minimal, the ferrous transport functions of Feo and Sit in combination with efficient ferric siderophore systems could provide a modest competitive edge. Furthermore, the combined loss of Feo and Sit also increased the sensitivity to hydrogen peroxide (Fig. 5), suggesting the cumulative importance of these two transporters under certain conditions. In contrast, combined loss of Sit and Feo actually resulted in a significant regain in colonization of the liver compared to that of the  $\Delta sitABCD$  mutant in the competitive coinfection model (Fig. 3c). The liver functions in iron storage and recycling. During infection, increased cell death and tissue necrosis in the liver may lead to localized tissue anoxia and increased availability of ferrous iron at lesion sites. Under anaerobic conditions and with increased availability of ferrous iron, the Feo transporter may therefore be deleterious. In support of this, compared to its wild-type parent, an *E. coli* K-12 *feo* mutant grown anaerobically was more resistant to H<sub>2</sub>O<sub>2</sub>-mediated killing than its isogenic parent strain (34).

In APEC, the *sit* genes are commonly located on large conjugative (ColV or ColBM) plasmids (17, 27, 29, 56, 59). The roles of such plasmids in various APEC or pathogenic *E. coli* strains have been established in a number of reports (16, 18, 24, 61, 62, 64), although specific plasmid-carried genes contributing to APEC virulence are less well characterized. The Sit transporter, along with the aerobactin and salmochelin siderophores (15) and Tsh (16), represents another plasmid-encoded virulence factor of APEC strain  $\chi$ 7122. Compared with the divalent manganese or iron transporter MntH or FeoB, the importance of SitABCD iron and manganese transport for virulence of APEC is preponderant. Hence, in addition to providing an advantage for intracellular survival for *Salmonella enterica* and *Shigella flexneri* 2a, in APEC Sit appears to provide an adaptive advantage during extraintestinal survival. The reduction in virulence together with the increased sensitivity to ROI-generating compounds observed for some of the APEC metal transport mutants suggests that reduced survival during infection could possibly be due to increased killing by phago-

cytes or the products they may liberate into the extracellular environment. Although APEC strain  $\chi$ 7122 is refractory to internalization by avian phagocytes, APEC strains belonging to other serogroups, such as O2 or O1, are more readily internalized and can survive within phagocytes (44). In future studies, it will be interesting to determine if Sit contributes to increased survival of certain APEC strains within host cells and its potential role in the virulence of ExPEC in human extra-intestinal infections.

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