Microbiology (2006), **152**, 745–758 DOI 10.1099/mic.0.28682-0

A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide

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Received 10 November 2005
Revised 24 November 2005
Accepted 29 November 2005

An operon encoding a member of the family of ATP-binding cassette (ABC) divalent metal ion transporters, homologous to *Salmonella enterica* SitABCD, has been identified in the avian pathogenic *Escherichia coli* (APEC) strain χ7122. The *sitABCD* genes were located on the virulence plasmid pAPEC-1, and were highly similar at the nucleotide level to the chromosomally encoded *sitABCD* genes present in *Shigella* spp. A cloned copy of *sitABCD* conferred increased growth upon a siderophore-deficient *E. coli* strain grown in nutrient broth supplemented with the chelator 2,2′-dipyridyl. Ion rescue demonstrated that Sit-mediated growth promotion of this strain was due to the transport of iron. SitABCD mediated increased transport of both iron and manganese as demonstrated by uptake of ^{55}Fe, ^{59}Fe or ^{54}Mn in *E. coli* K-12 strains deficient for the transport of iron (*aroB feoB*) and manganese (*mntH*) respectively. Isotope uptake and transport inhibition studies showed that in the iron transport deficient strain, SitABCD demonstrated a greater affinity for iron than for manganese, and SitABCD-mediated transport was higher for ferrous iron, whereas in the manganese transport deficient strain, SitABCD demonstrated greater affinity for manganese than for iron. Introduction of the APEC *sitABCD* genes into an *E. coli* K-12 *mntH* mutant also conferred increased resistance to the bactericidal effects of hydrogen peroxide. APEC strain χ7122 derivatives lacking either a functional SitABCD or a functional MntH transport system were as resistant to hydrogen peroxide as the wild-type strain, whereas a Δ*sit* Δ*mntH* double mutant was more sensitive to hydrogen peroxide. Overall, the results demonstrate that in *E. coli* SitABCD represents a manganese and iron transporter that, in combination with other ion transport systems, may contribute to acquisition of iron and manganese, and resistance to oxidative stress.

INTRODUCTION

Pathogenic *Escherichia coli* are divided into two major groups associated with either intestinal or extra-intestinal diseases (Johnson & Russo, 2002; Nataro & Kaper, 1998). The intestinal pathotypes cause diarrhoea in humans and animals, whereas pathotypes associated with urinary tract infections, neonatal meningitis and septicaemia have been collectively termed extra-intestinal pathogenic *E. coli* (ExPEC) (Johnson & Russo, 2002). Pathogenic *E. coli* strains are also classified according to the host species they infect (Gyles, 1994). Avian pathogenic *E. coli* (APEC) cause colibacillosis, a general term used to encompass a number of extra-intestinal infections of poultry, including respiratory infection (airsacculitis), septicaemia and cellulitis (Dho-Moulin & Fairbrother, 1999; La Ragione & Woodward, 2002). APEC strain χ7122 (O78 : K80 : H9) has been used as a model strain to study molecular mechanisms of APEC pathogenicity (Brown & Curtiss, 1996; Dozois *et al.*, 2003). χ7122 has several iron transport systems, including the aerobactin and salmochelin siderophore systems, which are encoded on a large virulence plasmid pAPEC-1 and are absent from non-pathogenic *E. coli* K-12. In addition, strain χ7122 possesses the chromosome-encoded enterobactin siderophore system and the Feo ferrous iron transporter. Siderophores mediate the acquisition of ferric (Fe^{3+}) iron by competing with the host iron-binding proteins. Other

Abbreviations: ABC, ATP-binding cassette; APEC, avian pathogenic *E. coli*; AT, annealing temperature; EDDA, ethylenediamine di-*o*-hydroxyphenylacetic acid; ExPEC, extra-intestinal pathogenic *E. coli*; LB, Luria–Bertani broth; NB, nutrient broth, NB-DIP, nutrient broth with 2,2′-dipyridyl.

The GenBank/EMBL/DDBJ accession numbers of the SitABCD-encoding DNA region of pAPEC-1 from strain χ7122, and the *sitA* gene from *E. coli* CFT073 sequenced from plasmid pIJ4, are AY598030 and DQ256074, respectively.

transporters such as FeoB transport ferrous (Fe^{2+}) iron directly from the environment (Kammler *et al.*, 1993).

In *E. coli* and most other bacteria iron is a cofactor of a number of essential metabolic enzymes (Andrews *et al.*, 2003). Importantly, iron plays a role in protection against oxidative damage, as it is a component of the *sodB*-encoded superoxide dismutase (FeSOD) and catalase enzymes which eliminate superoxide (O^{2-}) and H_2O_2 respectively. However, excess iron levels in bacterial cells contribute to oxidative damage through the generation of free radicals (Imlay, 2003).

The global regulator Fur, upon association with its co-repressor Fe^{2+}, represses transcription of genes encoding high-affinity iron transport systems and other proteins involved in iron metabolism, and thus tightly controls iron homeostasis of the bacterial cell (Andrews *et al.*, 2003; McHugh *et al.*, 2003). Iron metabolism is co-ordinately regulated with the oxidative stress response, and *fur* expression is positively regulated by the oxidative response regulators OxyR and SoxRS (Zheng *et al.*, 1999). In addition to regulating genes associated with iron metabolism or transport, Fur also regulates the expression of genes required for manganese transport (Kehres *et al.*, 2002b; Patzer & Hantke, 2001) and the manganese-dependent superoxide dismutase (MnSOD), SodA (Tardat & Touati, 1993). Hence, control of both manganese and iron transport and iron- and manganese-dependent defence against oxidative stress are coordinated.

Manganese contributes to protection against oxidative stress, is a cofactor for a number of enzymes in bacteria and other organisms (Kehres & Maguire, 2003), and can also contribute directly to the catalytic detoxification of reactive oxygen species (Horsburgh *et al.*, 2002). In the enterobacteria, two major types of manganese transporters have been identified: a proton-dependent Nramp-related transport system typified by MntH and an ATP-binding cassette (ABC) transporter typified by SitABCD (Cellier *et al.*, 2001; Kehres & Maguire, 2003). In addition to being regulated by iron levels and Fur, expression of these systems is also regulated by manganese levels and the regulator MntR (Kehres *et al.*, 2002a; Patzer & Hantke, 2001). In *E. coli* and *Salmonella enterica* MntH functions as a proton-dependent divalent cation transporter that is highly selective for Mn^{2+} (Kehres & Maguire, 2003). ABC transporters of divalent metal cations are widely distributed phylogenetically among bacteria (Claverys, 2001). They include SitABCD from *Sal. enterica* and YfeABCD from *Yersinia pestis*. SitABCD and YfeABCD were initially identified as Fe^{2+} transporters (Bearden *et al.*, 1998; Zhou *et al.*, 1999). However, it has since been demonstrated that these transporters can also mediate the transport of Mn^{2+} (Bearden & Perry, 1999; Kehres *et al.*, 2002b). A Sit homologue from *Shigella flexneri* 2a has been characterized (Runyen-Janecky *et al.*, 2003), although the capacity of SitABCD homologues from either *Shigella* or *E. coli* to transport iron or manganese has not been investigated.

We have identified a SitABCD homologue in APEC strain χ7122. Unlike other SitABCD and related transporters identified in the enterobacteria, which are encoded on the chromosome, the *sitABCD* genes in strain χ7122 are encoded on the colicin-V type plasmid pAPEC-1. In this study, we characterized SitABCD by investigating its capacity to transport manganese and iron in *E. coli* K-12 mutants deficient in the tranport of these cations. In addition, we determined the contribution of SitABCD to the resistance of *E. coli* strains to hydrogen peroxide.

METHODS

Bacterial strains, plasmids, media and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. In addition, clinical isolates from different sources were used. Archetypal ExPEC reference strains CFT073 (O6 : K2 : H1), CP9 (O4 : K54 : H5) and RS218 (O18 : K1 : H7) from human infections were kindly provided by Dr James R. Johnson (VA Medical Center, Minneapolis, MN, USA). Strain 536 (O6 : K15 : H31) was kindly provided by Professor Jorg Hacker (Universität Würzburg, Würzburg, Germany). Strain EB1 (O8 : K43) from a human wound infection was kindly provided by Dr Ben Otto (Vrije Universiteit, Amsterdam, The Netherlands). APEC strains MT78 (O2 : K1 : H5), MT458 (O78 : K80) and MT512 (O2 : K1 : H7) were kindly provided by Maryvonne Moulin-Schouleur (INRA, Tours, France). All strains were maintained in stock cultures at −80 °C in 25 % (v/v) glycerol following overnight culture in Luria–Bertani (LB) broth (10 g yeast extract, 5 g tryptone, and 10 g NaCl l^{-1}). Strains and clones were routinely grown in LB broth or on LB agar plates (15 g agar l^{-1}). *E. coli* strain DH5α was routinely used for plasmid cloning and recovery. Antibiotics were added as required at the following concentrations: ampicillin 100–200 μg ml^{-1}, kanamycin 30 μg ml^{-1}, chloramphenicol 30 μg ml^{-1}, nalidixic acid 15 μg ml^{-1} and tetracycline 10 μg ml^{-1}.

DNA and genetic manipulations. Standard methods were used for isolation of bacterial genomic DNA, DNA manipulation and cloning (Sambrook & Russell, 2001). Restriction enzymes and DNA ligase were purchased from New England Biolabs (NEB), Invitrogen or Amersham-Pharmacia and used according to the suppliers' recommendations. Native plasmids from clinical isolates were extracted and analysed as described by Kado & Liu (1981). Recombinant plasmids, PCR products and restriction fragments were purified using plasmid mini-prep, PCR clean-up and gel extraction kits (Qiagen or Sigma) as recommended by the supplier.

PCR and DNA hybridization. *Taq* DNA polymerase (NEB) was used for routine DNA amplifications (< 2 kb), and Elongase (InVitrogen) was used for longer high-fidelity amplifications and cloning of genes. For amplification of products of up to 2 kb, 10 μl of a bacterial whole-cell lysate was added to a PCR reaction mixture of a final volume of 25 μl containing 6·25 pmol of each primer, 5 nmol of each dNTP and 0·5 U of *Taq* polymerase in 1× buffer. The PCR conditions were as follows: 94 °C for 3 min; followed by annealing for 1 min as indicated, 72 °C for 1 min and 94 °C for 1 min for 25 cycles; and a final extension at 72 °C for 10 min. For Southern blots, plasmid extracts or digested genomic DNA were separated by agarose gel electrophoresis and transferred to nylon membranes. A 663 bp DNA fragment was amplified from *E. coli* χ7122 genomic DNA by using the *sitA* primer pair [CMD22, 5′-CCCTGTACCAGCGTACTGG-3′; and CMD23, 5′-CGCAGGGGG-CACAACTGAT-3′ with an annealing temperature (AT) of 54 °C] and was labelled by using the PCR DIG Labelling Mix (Roche).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Reference or source
Bacterial strains		
CFT073	Human uropathogenic *E. coli* O6 : H1 : K^+	Welch *et al.* (2002)
χ7122	APEC O78 : K80 : H9, *gyrA*, Nal^R	Provence & Curtiss (1992)
χ7274	χ7122 ΔpAPEC-1	Dozois *et al.* (2000)
QT205	χ7122 Δ*sitABCD*::*tetAR*, Tc^R	This study
QT878	χ7122 Δ*mntH*::*kan*, Km^R	This study
QT1239	QT205 Δ*mntH*::*kan*, Tc^R Km^R	This study
E. coli K-12		
1017	HB101 *ent*::Tn*5*, Km^R	Daskaleros *et al.* (1991)
χ289	W1485 F^- λ^- *glnV44*	Brown & Curtiss (1996)
DH5α	F^- λ^- ϕ80 Δ(*lacZYA–argF*) *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
H1771	MC4100 *aroB feoB7 fhuF*::λp*lac* Mu, Km^R	Kammler *et al.* (1993)
QT99	χ289 Δ*mntH*::*kan* Km^R	This study
Plasmids		
pABN1	Encodes aerobactin system, Ap^R	Bindereif & Neilands (1983)
pACYC184	p15A replicon, Cm^R Tc^R	Chang & Cohen (1978)
pCA6	Cosmid containing *sit* genes from *E. coli* χ7122	This study
pColV-K30	Native colicin V encoding reference plasmid	Gilson *et al.* (1987)
pIJ4	pTOPO-XL::*sitABCD* from CFT073	This study
pIJ5	pWSK29::*sitABCD* cloned *Hin*dIII fragment from pIJ4, Km^R	This study
pIJ28	8 kb *Hin*dIII fragment containing *sitABCD* from χ7122 cloned into pACYC184, Cm^R	This study
pIJ42	pACYC184::*mntH* cloned PCR product from χ7122, Cm^R	This study
pIJ43	*tetAR*(B) *Pst*I fragment cloned into *Pst*I sites of pIJ05, Km^R	This study
pIJ44	*Bss*HII fragment from pIJ43 cloned into *Asc*I sites of pMEG-375, Cm^R Ap^R	This study
pMEG-375	*sacRB mobRP4 ori*R6K, Cm^R Ap^R	Megan Health, St Louis, MO
pTOPO-XL	PCR cloning vector	Invitrogen
pWSK29	pSC101 replicon, multicloning site, Km^R	Wang & Kushner (1991)

*Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Tc, tetracycline.

Hybridization and detection of the hybridized fragments were performed using the Digoxigenin Detection Kit as recommended by the manufacturer (Roche).

Cloning of the *sitABCD* and *mntH* genes. A homologue of the SitABCD transporter of *Sal. enterica* was identified in APEC strain MT512 (O2 : K1) by DNA subtractive hybridization against the genome of the non-pathogenic avian *E. coli* strain EC79 (O2 : K–) (Schouler *et al.*, 2004). Genes encoding homologues of SitABCD are also present in the genomes of uropathogenic *E. coli* CFT073 (Welch *et al.*, 2002) and *Sh. flexneri* serotype 2a strains (Jin *et al.*, 2002; Wei *et al.*, 2003). Primers specific to *sitA* and *sitD* of *E. coli* and *Sh. flexneri* 2a were designed from the sequences available at GenBank. *sitA*-specific primers (CMD22, 5′-CCCTGTACCAGCGTACTGG-3′; and CMD23, 5′-CGCAGGGGGCACAACTGAT-3′ used at an AT of 54 °C) and *sitD*-specific primers (CMD41, 5′-GCGTTGTGTCAGGAGTACC-3′; and CMD42, 5′-CTGTGCGCTGCTGTCGGTC-3′ used at an AT of 52 °C) amplified fragments from the genomic DNA of strain χ7122. The *sit*-specific primer pairs were used to screen a cosmid bank containing DNA inserts that hybridized with *E. coli* χ7122 DNA that is absent from the genome of *E. coli* K-12 (Brown & Curtiss, 1996). A cosmid, pCA6, which was positive for PCR amplification using the *sitA* and *sitD* primer pairs was identified. A *Hin*dIII fragment containing the *sit* genes of χ7122 was subcloned from pCA6 into pACYC184, generating pIJ28.

The *sit* genes from strain CFT073 were amplified from genomic DNA by PCR using primers CMD20 (5′-AA**AAGCTT**AAGACGACAATCGTCGGA-3′; *Hin*dIII site underlined) and CMD21 (5′-TT**GAGCTC**GGAATAACGCTTACCGTGTAA-3′; *Sac*I site underlined) at an AT of 57 °C and Elongase DNA polymerase (Invitrogen) according to the manufacter's instructions. The amplification product was cloned directly into pTOPO-XL (InVitrogen), resulting in plasmid pIJ4. A segment of pIJ4 containing the *sitABCD* genes was obtained following digestion with *Hin*dIII and *Sac*I. This fragment was cloned into the *Hin*dIII and *Sac*I sites of pWSK29, resulting in plasmid pIJ5.

The *mntH* primer pair [CMD58, 5′-GGTT**AAGCTT**CCGTGCACATTCTATGTAA-3′; and CMD59, 5′-CT**AAGCTT**CGTAGGGCGGATAAGGCGTT-3′ (*Hin*dIII sites underlined) at an AT of 64 °C] was used to amplify and clone the *mntH* gene from genomic DNA of strain χ7122 by using the same strategy as above. Cloning of the *Hin*dIII-digested PCR product containing the *mntH* gene and promoter region into the *Hin*dIII site of pACYC184 generated pIJ42.

Construction of *sitABCD* and *mntH* mutant strains. In order to construct an isogenic Δ*sitABCD*::*tetAR*(B) mutant of strain χ7122 we used suicide-vector-based allele replacement. Plasmid pIJ5 containing the *sitABCD* genes from strain CFT073 was digested with *Pst*I, resulting in removal of a section of the *sitABCD* operon spanning from nucleotide 149 of *sitA* to nucleotide 25 of *sitD*. A *Pst*I

fragment bearing a *tetAR*(B) cassette (Dozois *et al.*, 2000) was cloned into the *Pst*I sites of *Pst*I-digested pIJ5, resulting in pIJ43. A *BssH*II fragment of pIJ43 encompassing the Δ*sitABCD*::*tetAR* construct was ligated to the compatible *Asc*I sites of suicide vector pMEG-375, resulting in plasmid pIJ44. pIJ44 was used to replace the *sitABCD* genes with a non-functional Δ*sitABCD*::*tetAR* region using *sacB*-mediated counterselection as described by Dozois *et al.* (2000). A derivative of χ7122, strain QT205, was confirmed as a double-crossover recombinant in which the *sitABCD* region had been replaced by Δ*sitABCD*::*tetAR* following homologous recombination.

mntH null mutants of *E. coli* K-12 strain χ289 and APEC strains χ7122 and QT205 were generated by the bacteriophage λ red recombinase-mediated mutagenesis method (Datsenko & Wanner, 2000). The *mntH*KO primer pair (CMD43, 5′-CTATGTTTTAGAGGCACAAGATGACGAACTATCGCGTGTAGGCTGGAGCTGCTTC-3′; and CMD44, 5′-TAGTGCCATATGCGACGCTCATTCAACTACAATCCCATATGAATATCCTCCTTAG-3′ at an AT of 65 °C) was used to generate a kanamycin resistance gene cassette containing a DNA fragment from the template plasmid pKD3. The PCR product was used to delete the *mntH* gene as described by Datsenko & Wanner (2000). PCR amplification using the *mntH* primer pair (CMD58 and CMD59), which was used to clone the *mntH* gene, confirmed the *mntH* deletion mutations in strains QT99 (χ289 Δ*mntH*), QT878 (χ7122 Δ*mntH*) and QT1239 (χ7122 Δ*mntH* Δ*sitABCD*).

DNA sequencing and analysis of the *sitABCD*-encoding region. The *sitABCD* operon and flanking DNA regions were sequenced from plasmid pIJ28 and from PCR-amplified fragments of strain χ7122 DNA. Sequencing was achieved by generating derivatives of pIJ28 that contained the transposon Tn*5*seq1(Nag *et al.*, 1988). The Tn*5*seq1 sequence contains SP6 and T7 primers flanking each end and facilitates bidirectional sequencing. In addition, custom primers were used to complete the sequence. DNA sequencing was done at the Genome Québec facility (McGill University, Montréal, QC, Canada). Putative ORFs were identified using the ORF Finder program and similarity searches of the DNA sequence. Predicted ORFs were obtained using BLAST programs accessed from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Pairwise % identity/ % similarity of ORFs was determined using the Stretcher program available from the European Molecular Biology Open Software Suite (EMBOSS) (Rice *et al.*, 2000).

Siderophore production, growth assays and ion-rescue experiments. Chrome azurol S (CAS) agar plates (Schwyn & Neilands, 1987) were used to determine production of siderophores in the siderophore-negative *E. coli* strain 1017 containing cloned DNA from APEC strain χ7122. Growth curves of this construct were obtained as described by Gong *et al.* (2001). The growth assay medium was nutrient broth (NB) (Difco) supplemented with 0·5 % (w/v) NaCl, thiamine (1 μg ml^{-1}), histidine (22 μg ml^{-1}) and 70 μM of the chelator 2,2′-dipyridyl (Sigma). To minimize possible metal contamination, the medium was prepared in polypropylene bottles that were washed twice with distilled water, and bacterial cultures were grown in disposable plastic tubes. Bacterial growth was measured every hour by spectrophotometry (OD_{600}). For ion-rescue experiments we proceeded as for the growth assays, but the metal-restricted medium was supplemented with either 70 μM 2,2′-dipyridyl or 140 μM EDDA as chelator and 10 μM of either Mn^{2+}, Zn^{2+}, Fe^{2+} or Fe^{3+} as complementing ion. All experiments were performed in triplicate.

Transport of iron and manganese isotopes and isotope uptake inhibition experiments. Isotope uptake and isotope uptake inhibition assays were performed using ^{54}Mn, ^{59}Fe and ^{55}Fe isotopes purchased from Perkin Elmer. For iron uptake experiments, ferric iron ($^{55}FeCl_3$) was used. In addition, to determine ferrous iron uptake, the iron isotope was reduced to the ferrous state by addition of 100 mM ascorbate to the stock solution as described elsewhere (Kammler *et al.*, 1993). Iron and manganese uptake experiments were based on a protocol modified from Silver and Kralovic (Silver & Kralovic, 1969; Kehres *et al.*, 2002b). Briefly, the strains were grown overnight in dilute tryptone (DT) broth, comprising Bacto tryptone (4 g l^{-1}) (Difco) supplemented with 0·25 % (w/v) NaCl, 0·4 % (w/v) glucose, thiamine (1 μg ml^{-1}), histidine (22 μg ml^{-1}) and aromatic amino acids (tryptophan, phenylalanine and tyrosine) (20 μg ml^{-1} each) with appropriate antibiotics. Cultures were adjusted to an OD_{600} of 0·5, centrifuged at 1600 ***g***, and washed twice with equal volumes of room temperature DT broth (pH 7·0). In 2 ml Eppendorf tubes, 1 ml washed cells was centrifuged at 1600 ***g*** for 10 min and the bacterial pellets were suspended in DT medium containing isotope, ferric or ferrous ^{55}Fe or ferrous ^{59}Fe (to a final concentration of 100 nM) or ^{54}Mn (to a final concentration of 50 nM), and samples were left to stand for 3 min at room temperature. Samples were then centrifuged at 1600 ***g*** and cells were washed twice with isotope-free DT. A 2 ml volume of scintillation cocktail was added to the cells and scintillation was measured in a Wallac Microbeta Trilux scintillation counter equipped with an Eppendorf tube adaptor plate (Perkin Elmer). Samples were analysed on the channels 5–810 for ^{54}Mn, 5–980 for ^{59}Fe and 5–750 for ^{55}Fe. The scintillation cocktail Optiphase (Wallac) was purchased from Perkin Elmer. All experiments were done in triplicate. Values obtained were normalized with a positive control containing the isotope without cells and a negative control containing cells without isotope. For the isotope uptake inhibition, the samples were prepared as for the isotope uptake assays and ferrous ^{59}Fe, ferric ^{55}Fe or ^{54}Mn was used. The isotopes were mixed with from 0·01 μM to 100 μM of cold competing Fe^{2+}, Fe^{3+} or Mn^{2+} ions, and uptake was measured. All results were presented as a percentage of total isotope uptake determined in the absence of added cold ions.

Hydrogen peroxide sensitivity assay. Sensitivity of bacterial cultures to H_2O_2 was determined by using an agar overlay diffusion method as described by Boyer *et al.* (2002). Briefly, overnight-grown cultures were used to inoculate (1/100) fresh LB medium without antibiotics, and the resulting cultures were incubated until the OD_{600} was 0·5. Then 100 μl of each culture was mixed with 3 ml molten top agar and poured onto an LB agar plate. A 7-mm-diameter Whatman filter disk impregnated with 10 μl 30·4 % H_2O_2 was placed in the centre of the plate and plates were incubated overnight at 37 °C. The inhibition zone diameters were then measured.

Statistical analyses. Statistical analyses were performed using the Prism 4.0b software package (GraphPad Software). Statistically significant difference between two groups was established by unpaired *t*-test and comparison among three or more groups was done by one-way analysis of variance (ANOVA).

RESULTS

The *sit* genes are plasmid-encoded in APEC strain χ7122

APEC strain χ7122 contains a large plasmid, pAPEC-1, that encodes virulence factors including the aerobactin and salmochelin siderophore systems and the temperature-sensitive haemagglutinin (Tsh) autotransporter (Dozois *et al.*, 2000, 2003). Strain χ7274 is a pAPEC-1 cured attenuated derivative of strain χ7122 (Dozois *et al.*, 2000). Attempts to amplify *sit*-specific DNA by PCR in strain χ7274 were negative, suggesting that the *sitABCD* genes are located on plasmid pAPEC-1 of strain χ7122. This finding is in contrast

to *E. coli* CFT073 and *Sh. flexneri* 2a strains, which each contain one chromosomal copy of *sitABCD* (Jin *et al.*, 2002; Wei *et al.*, 2003; Welch *et al.*, 2002). Southern blotting of plasmid DNA using a *sitA*-specific probe demonstrated that pAPEC-1 of strain χ7122 contained the *sit* genes, whereas strain χ7274, which had lost pAPEC-1, did not hybridize with the *sitA*-specific probe (Fig. 1). Further, plasmids from three other APEC strains, *E. coli* strain EB1 isolated from a human wound abscess, and the prototype Colicin V plasmid pColV-K30 also hybridized to the *sitA* probe. By contrast, archetype ExPEC strains CFT073, CP9, RS218 and 536 each contained a chromosome-encoded copy of *sit* genes as demonstrated by hybridization of the *sitA*-specific probe to total genomic DNA digested with either *Hin*dIII or *Sal*I (Fig. 1). Further, digests from the total genomic DNA of strains MT78, MT458 and EB1 each demonstrated two DNA fragments that hybridized to the *sit* probe, suggesting that some *E. coli* strains contain a chromosomal as well as a plasmid-encoded copy of the *sit* genes.

Characterization of the SitABCD-encoding region of strain χ7122

A 10 094 bp section of pAPEC-1 encompassing the *sit* genes was sequenced (Fig. 2, Table 2). The predicted SitABCD proteins of strain χ7122 ($SitABCD_{E.\ coli\ \chi7122}$) exhibited the highest identity/similarity to SitABCD of *Sh. flexneri* 2a strain SA100 (Table 2). None of the genomic regions flanking *sit* genes from *E. coli* or *Shigella* strains identified in the DNA databases exhibited identity with the regions flanking the *sitABCD* genes from pAPEC-1. On pAPEC-1, a complete IS*1* element is located 5′ of the *sit* operon. The 5′ and 3′ regions adjacent to the *sit* genes are identical to a contiguous segment of *E. coli* plasmid p1658/97. Identity to the segment of p1658/97 in the 5′ region of the *sit* genes includes the IS*1* element and ends exactly at the end of the IS*1* left repeat (position 1273 of the *sit* region) and the identity to p1658/97 sequence resumes 3′ of the *sit* genes. The 3′ region adjacent to *sitD* contains a 288 bp sequence exhibiting 93 % identity to a Tn*1000*-like transposase-encoding sequence, and this segment overlaps ORF1 described from p1658/97 (Fig. 2). Other ORFs 3′ to the *sit* genes share identity at the protein level with part of a putative enolase, Eno-2, from *Pseudomonas syringae*, CrcB from *Nitrosomonas europaea*, ShiF and IucA (Table 2). ShiF is an ORF that is part of the aerobactin siderophore encoding pathogenicity islands present in *Sh. flexneri* 2a (Moss *et al.*, 1999; Vokes *et al.*, 1999) and *E. coli* CFT073 (Welch *et al.*, 2002). The first gene of the aerobactin operon, *iucA*, is adjacent to *shiF* on pAPEC-1 (Fig. 2). When cosmid pCA6 or its subclone pIJ28 (*sitABCD*) was transferred to the *E. coli* siderophore-negative K-12 strain 1017, pCA6 conferred siderophore production upon this strain, whereas pIJ28 was negative for the production of siderophores. PCR analysis demonstrated that in addition to the *sit* genes, pCA6 also contained genes encoding aerobactin siderophore synthesis

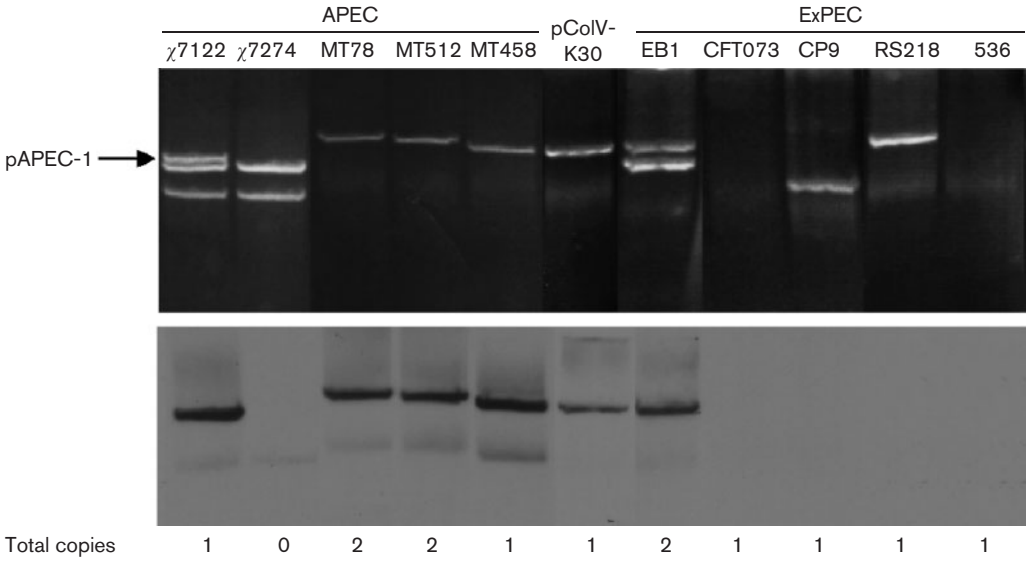

Fig. 1. Localization of *sit* genes to either large plasmids or the chromosomes of *E. coli* strains. The source of the strains as either APEC or ExPEC from human infections is indicated at the top. Strains corresponding to each plasmid extract are indicated. Plasmid pColV-K30 is the colicin V reference plasmid that was extracted from an *E. coli* K-12 strain. Strain χ7274 is a derivative of APEC strain χ7122 which has lost the pAPEC-1 plasmid. Upper panel: visualization of native plasmids present in *E. coli* strains by ethidium bromide staining. Plasmid pAPEC-1 of APEC strain χ7122 is indicated with an arrow on the left. Lower panel: Southern hybridization of the same plasmid samples as above using a *sitA*-specific probe. Numbers at the bottom of the figure indicate the total number of copies that hybridized with the *sit*-specific probe on Southern blot of total genomic DNA digested with *Hin*dIII or *Sal*I.

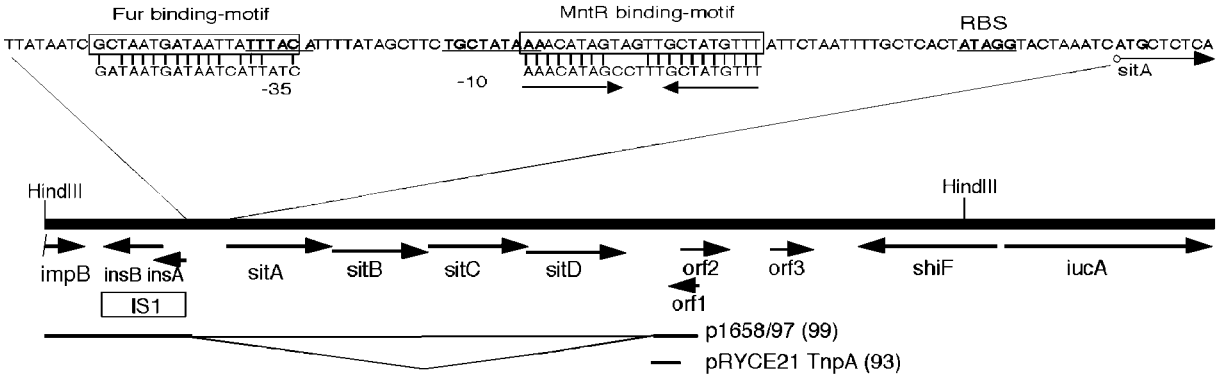

Fig. 2. Organization and analysis of the *sitABCD*-encompassing region from plasmid pAPEC-1 of APEC strain χ7122. The DNA fragment which was cloned to generate plasmid pIJ28 is indicated by the *Hin*dIII sites. Solid arrows below the sequence length indicate predicted ORFs. Horizontal lines below the sequence length represent plasmid DNA sequences sharing high identity with the corresponding regions. The percentage nucleotide identities of the regions are indicated in parentheses. The triangle indicates a specific gap in the sequence of plasmid p1658/97 corresponding to the region that encodes *sitABCD* on pAPEC-1. GenBank accession numbers for p1658/97 and pRYCE21 TnpA are AF550679 and AY598759 respectively. The sequence upstream of the *sit* genes contains motifs that correspond to binding sites for the Fur and MntR regulatory proteins. The putative Fur- and MntR-binding sites are indicated as boxed regions. Matches to the consensus binding motifs are illustrated with vertical lines. The inverted repeat motif in the MntR-binding sequence is illustrated with arrows pointing in opposite directions. The putative −35 and −10 regions are shaded and underlined, and a putative ribosome-binding site (RBS) is indicated. The start of the *sitA* gene is indicated with an open-circled arrow, and the ATG start codon is indicated in bold. Consensus sequences for regulatory proteins Fur and MntR are derived from de Lorenzo *et al.* (1987) and Patzer & Hantke (2001) respectively.

(*iucABC*) and uptake (*iutA*) (data not shown). These results are in accordance with the sequencing data, which indicate that the *shiF* and *iucA* genes are adjacent to the *sit* operon (Fig. 2, Table 2).

The promoter region of $sitABCD_{E.\ coli\ \chi7122}$ contains a potential operator sequence for the binding of the iron(II)-responsive Fur regulatory protein (Fig. 2). In addition, a region highly similar to the binding site of the

Table 2. Summary of ORFs within the SitABCD encoding region of pAPEC-1 and homologies to other sequences

ORF or sequence	Position (bp)*	Product length (aa)	GC content (mol%)	Homology (%identity/%similarity)	Function	Region	Accession no.†
ImpB	3>341	112	50	ImpB (100) *E. coli* plasmid p1658/97	UV protection component	9>341	AAO49619
InsB	521<1024	167	54	InsB (100) *E. coli* plasmid p1658/97	IS*1* transposase component	Full-length	AAO49620
InsA	943<1218	91	53	InsA (100) *E. coli* plasmid p1658/97	IS*1* transposase component	Full-length	AAO49621
SitA	1574>2488	304	48	SitA (98) *Sh. flexneri* 2a	Periplasmic binding protein	Full-length	AAM95574
SitB	2488>3315	275	52	SitB (98/99) *Sh. flexneri* 2a	ATP-binding component	Full-length	AAM95575
SitC	3312>4169	285	52	SitC (99) *Sh. flexneri* 2a	Inner membrane component	Full-length	AAM95576
SitD	4166>5023	285	49	SitD (96/98) *Sh. flexneri* 2a	Inner membrane component	Full-length	AAM95577
ORF1	5391<5654	87	49	Hypothetical (100) *E. coli* plasmid p1658/97	Unknown	5391<5654	AAO49622
ORF2	5492>5926	144	47	Eno-2 (69/85) *Pseudomonas syringae*	Putative enolase	5514>5921	NP_794367
ORF3	6268>6648	126	42	CrcB (52/72) *Nitrosomonas europaea*	Putative role in chromosome condensation	Full-length	CAD85615
ShiF	7029>8221	397	56	ShiF (89/93) *Sh. flexneri* 2a *E. coli* CFT073	Putative permease	Full-length	AAN82076
IucA	8357>10084	575	54	IucA (98/98) *E. coli* pColV-K30	Aerobactin synthesis	Full-length	X76100

*Numbers correspond to nucleotides in GenBank accession number AY598030.
†GenBank accession number.

manganese-responsive regulator protein MntR was identified (Fig. 2). The potential Fur- and MntR-binding sites both overlap the predicted −10 to −35 region, suggesting that both iron and manganese levels are likely to influence regulation of *sit* gene expression. The predicted Fur- and MntR-binding regions were derived from the consensus sequences described by de Lorenzo *et al.* (1987) and Patzer & Hantke (2001) respectively, and are also conserved in P*sitABCD* of *E. coli* CFT073 and in *Sh. flexneri* strains (data not shown; Runyen-Janecky *et al.*, 2003).

The $sitABCD_{E.\ coli\ \chi7122}$ genes are most similar to those of *Sh. flexneri* 2a strains 301 (Jin *et al.*, 2002) and SA100 (Runyen-Janecky *et al.*, 2003), which are identical to each other. The 3450 bp region encompassing $sitABCD_{E.\ coli\ \chi7122}$ contains 69 nucleotide differences with $sitABCD_{Sh.\ flexneri\ 301}$ and 92 differences with $sitABCD_{E.\ coli\ CFT073}$, whereas $sitABCD_{E.\ coli\ CFT073}$ demonstrates 62 differences with $sitABCD_{Sh.\ flexneri\ 301}$. The *sitA* gene from the complete genome sequence of strain CFT073 contains six deletions that result in a predicted truncated SitA precursor protein of 285 amino acids, compared to the predicted 304 residue products of *E. coli* χ7122 and *Shigella* strains. Because of the discrepancies between *sitA* sequences among strains we verified the sequence of the *sitA* gene of strain CFT073 from clone pIJ4 (Table 1). The DNA sequence we obtained lacked the six deletions that are present in the sequence submitted for *sitA* from the CFT073 genome (GenBank accession no. AE016759) and encoded a predicted 304 amino acid precursor protein, but was otherwise identical.

The characterized systems currently identified in other bacteria that are most similar to SitABCD of *E. coli* χ7122 are SitABCD of *Sal. enterica* serovars Typhimurium and Typhi and YfeABCD of *Y. pestis*. The *Sal. enterica sitA* gene product (GenBank accession no. AAD41065) encodes the predicted periplasmic cation-binding protein and exhibits a percentage identity/similarity of 70/82 to $SitA_{E.\ coli\ \chi7122}$, whereas YfeA from *Y. pestis* (GenBank accession no. CAC47605) demonstrates a percentage identity/similarity of 64/77 to $SitA_{E.\ coli\ \chi7122}$.

SitABCD confers Fur-regulated repression upon an *aroB feoB* mutant of *E. coli* K-12 grown on iron-replete medium

To further investigate the role of SitABCD in the transport of iron in *E. coli*, we used *E. coli* K-12 H1771 as an indicator strain to determine *fur*-regulated control of iron acquisition on MacConkey-lactose agar supplemented with either 50 μM $FeSO_4$ or 50 μM 2,2′-dipyridyl. Strain H1771 lacks both siderophore-mediated (Fe^{3+}) and FeoB-mediated (Fe^{2+}) transport systems and contains a Fur-regulated *fhuF*::λ*plac*Mu53 fusion. Due to a reduced ability to obtain iron, the Fur-regulated *fhuF-lacZYA* fusion in this strain remains derepressed even on MacConkey-lactose plates containing added iron (Kammler *et al.*, 1993). Introduction of pIJ28 to strain H1771 resulted in repression of *fhuF-lacZYA* expression and production of white colonies on MacConkey-lactose containing 50 μM Fe^{2+}, whereas strain H1771 produced red colonies on MacConkey-lactose containing either 50 μM Fe^{2+} or 50 μM 2,2′-dipyridyl. These results suggest that SitABCD conferred increased iron transport ability upon strain H1771 in iron-supplemented medium and this resulted in the formation of Fur–Fe^{2+} complexes and repression of the *fhuF-lacZYA* fusion.

SitABCD promotes the growth of an *E. coli* K-12 enterobactin-deficient strain

The capacity of *sit* genes to promote the growth of the siderophore-negative strain 1017 in NB containing 75 μM of the chelator 2,2′-dipyridyl (NB-DIP) was assessed. The growth of strain 1017 that contained the *sit* genes (pIJ28), the aerobactin-encoding genes (pABN1), or a vector control (pACYC184) in NB-DIP was determined at hourly intervals. Strain 1017 containing pIJ28 ($sitABCD_{E.\ coli\ \chi7122}$) grew well and at a rate similar to that of strain 1017 containing pABN1 (aerobactin system). By contrast, strain 1017 transformed with pACYC184 (vector control for plasmid pIJ28) exhibited poor growth (Fig. 3). Introduction of pIJ5 ($sitABCD_{E.\ coli\ CFT073}$) complemented the growth defect of strain 1017 as effectively as did pIJ28 (data not shown). These results indicate that introduction of the SitABCD system alone effectively complements the growth defect of strain 1017 in NB-DIP.

SitABCD promotes growth of strain 1017 by compensating for an iron deficit

To determine if the limited growth of strain 1017 in NB-DIP was due primarily to iron starvation and whether iron acquisition by SitABCD compensated for this poor growth, we investigated the effect of addition of metal cations to the growth of strain 1017 with or without $sitABCD_{E.\ coli\ \chi7122}$. Ferrous or ferric iron, manganese or zinc was added to NB supplemented with either 70 μM 2,2′ dipyridyl or 140 μM

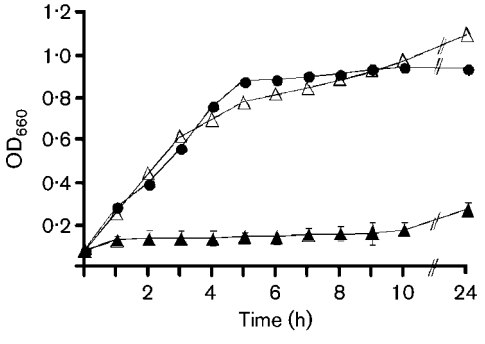

Fig. 3. Growth of *E. coli* K-12 strain 1017 (*ent*) containing plasmids pACYC184 (▲), pIJ28 (●) or pABN1 (△) in NB medium containing 75 μM 2,2′-dipyridyl at 37 °C. pACYC184 is the vector for pIJ28 and serves as a negative control. pIJ28 encodes the *sitABCD* genes. pABN1 encodes the aerobactin gene cluster and serves as a positive control. Results represent means ± SEM for three independent experiments.

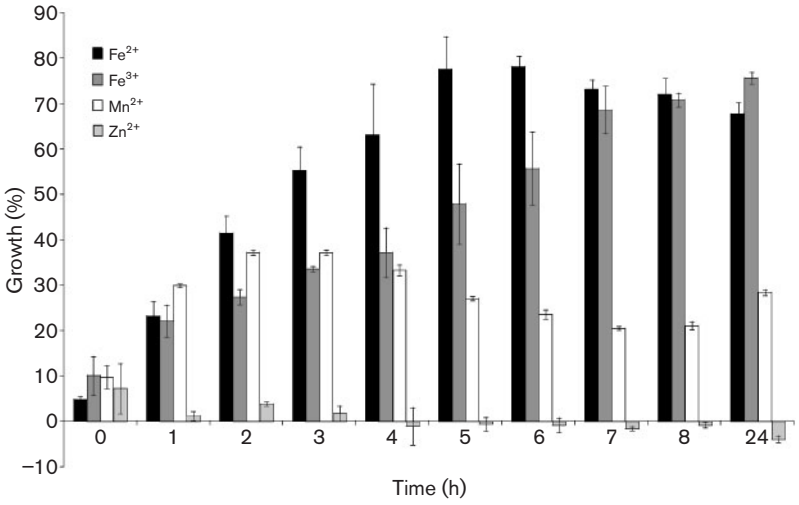

Fig. 4. Growth rescue of the siderophore-deficient strain 1017 (*ent*), carrying pIJ28 (*sitABCD*), in NB medium supplemented with 70 μM 2,2′-dipyridyl and 10 μM of either Fe^{2+}, Fe^{3+}, Mn^{2+} or Zn^{2+}. Growth is indicated as a percentage of the equivalent growth of the strain grown in NB medium without chelator.

EDDA, and growth of strain 1017(pACYC184) was determined. Following overnight culture in either NB-DIP or NB-EDDA only ferrous or ferric iron could complement the growth deficit of strain 1017(pACYC184) in a significant manner (80 %), whereas manganese complemented the growth defect only partially (40 %) and zinc did not improve growth (data not shown). The effect of addition of metals on the growth (measured at hourly intervals) of strain 1017 containing pIJ28 ($sitABCD_{E.\ coli\ \chi7122}$) in NB-DIP is presented in Fig. 4; this clearly demonstrates the effect on growth of addition of iron compared to manganese or zinc. Further, introduction of the Sit system to strain 1017 increased growth to 80 % in NB-DIP supplemented with iron, as compared to NB without chelator, within 6 h, whereas it took strain 1017(pACYC184) over 10 h to reach a similar growth level (data not shown). These results suggest that SitABCD contributes to improved growth of strain 1017 by compensating for an iron deficit.

SitABCD of *E. coli* χ7122 mediates transport of iron and manganese

Since $sitABCD_{E.\ coli\ \chi7122}$ conferred restored growth and Fur-mediated gene regulation upon iron transport deficient *E. coli* mutants, and since homologues of this system are characterized manganese and iron transporters (Bearden & Perry, 1999; Kehres *et al.*, 2002b), we investigated the capacity of $SitABCD_{E.\ coli\ \chi7122}$ to transport iron and manganese. SitABCD mediated the transport of ferrous iron and manganese under the conditions used for the assay (see Table 3). Under non-reducing conditions, in which iron is predominantly in the ferric state, the mean transport of iron by H1771 containing pIJ28 ($sitABCD_{E.\ coli\ \chi7122}$) was not statistically different ($P=0{\cdot}111$): a mean increase of 1597 ± 784 c.p.m. compared to the control strain H1771-(pACYC184) (Table 3). By contrast, when iron was treated with 100 mM ascorbate, to reduce it to the ferrous state, plasmid pIJ28 ($sitABCD_{E.\ coli\ \chi7122}$) conferred a significant increase in iron uptake: a mean increase of 3008 c.p.m. compared to H1771(pACYC184) ($P=0{\cdot}001$) (Table 3). Despite the increase in iron transport mediated by SitABCD in strain H1771, the level of iron uptake observed was considerably less than transport by the Aro^+ Feo^+ *E. coli* K-12 strain χ289, which was 3·6-fold higher and 2·6-fold higher than that of H1771(pIJ28) under non-reduced and reduced conditions respectively.

Table 3. Sit-mediated metal isotope uptake in *mntH* and *aro feo E. coli* K-12 strains

Strain	Radioactivity (c.p.m.)*
	$^{54}Mn^{2+}$
QT99 (*mntH*)/pACYC184	291 ± 25
QT99 (*mntH*)/pIJ28 (pACYC184::*sitABCD*)	923 ± 139†
QT99 (*mntH*)/pIJ42 (pACYC184::*mntH*)	1399 ± 206†
χ289 (K-12 WT)/pACYC184	474 ± 27†
	$^{55}Fe^{3+}$ **(non-reduced)**
H1771 (*aro feo*)/pACYC184	4388 ± 359
H1771 (*aro feo*)/pIJ28 (pACYC184::*sitABCD*)	5985 ± 697‡
χ289 (K-12 WT)/pACYC184	21371 ± 500§
	$^{55}Fe^{2+}$ **(reduced)**
H1771 (*aro feo*)/pACYC184	5205 ± 134
H1771 (*aro feo*)/pIJ28 (pACYC184::*sitABCD*)	8213 ± 314§
χ289 (K-12 WT)/pACYC184	21022 ± 2009§

*Means ± SEM ($n=3$).

†Significant difference compared to *mntH* strain QT99 ($P\leqslant0{\cdot}01$).

‡No significant difference compared to *aro feo* strain H1771 ($P=0{\cdot}11$).

§Significant difference compared to H1771 *aro feo* mutant ($P\leqslant0{\cdot}001$).

Manganese uptake in the *mntH* mutant strain QT99 containing the vector control pACYC184 was significantly reduced ($P=0{\cdot}008$) compared to that of the isogenic parent strain χ289(pACYC184), which exhibited a 1·63-fold higher mean uptake. Introduction of pIJ28 (*sitABCD*) to the *mntH* mutant strain QT99, conferred a statistically significant ($P=0{\cdot}01$) 3·2-fold increase in uptake of $^{54}Mn^{2+}$ compared to the vector control. Complementation of QT99 with plasmid pIJ42 (*mntH*) resulted in $^{54}Mn^{2+}$ uptake 4·8-fold higher than that seen with QT99 containing pACYC184 ($P=0{\cdot}006$) (Table 3). Hence, *sitABCD* cloned on vector pACYC184 effectively increased manganese transport by an *mntH* mutant K-12 strain, but not as effectively as reintroduction of a functional *mntH* allele on the same vector. Taken together, the results demonstrate that a cloned copy of the *sitABCD* genes from APEC strain χ7122 is able to complement mutants impaired in either iron or manganese transport function, and suggest that $SitABCD_{E.\ coli\ \chi7122}$ mediates transport of both ferrous iron and manganese under the conditions used for the assays.

SitABCD affinities for iron and manganese are dependent on the strain background

To investigate the relative affinity of SitABCD from APEC strain χ7122 for iron or manganese we conducted isotope transport inhibition assays by addition of competing cold ions to iron or manganese isotopes during transport assays. Ferrous ^{59}Fe or ferric ^{55}Fe uptake by SitABCD was investigated in the iron transport deficient strain H1771 (*aro feo*), and ^{54}Mn uptake by SitABCD was investigated in the manganese transport deficient strain QT99 (*mntH*) in the presence of increasing concentrations of Fe^{2+}, Fe^{3+} or Mn^{2+} (Fig. 5). The apparent affinity or inhibition constants were determined as the concentration of cold cation required to inhibit 50 % of the isotope uptake. In strain H1771, the apparent affinity for Fe^{2+} was 0·9 μM and for Fe^{3+} it was 3·2 μM. For strain QT99 the apparent affinity for Mn^{2+} was 4 μM. For strain H1771, $^{59}Fe^{2+}$ uptake was more inhibited by Fe^{3+} (2·5 μM) than by Mn^{2+} (5 μM); in the same strain $^{55}Fe^{3+}$ uptake was more inhibited by Fe^{2+} (0·7 μM) and was less inhibited by Mn^{2+} (20 μM). In QT99 $^{54}Mn^{2+}$ uptake was better inhibited by Fe^{2+} (7 μM) and only poorly inhibited by Fe^{3+} (13 μM). Strains QT99 and H1771 differ in their native functional iron or manganese uptake systems. Strain QT99 is deficient in manganese transport, whereas H1771 is deficient in iron transport. Thus, the observed differences in SitABCD substrate specificity in these two strains are likely due to intrinsic differences in manganese and iron transport.

SitABCD confers resistance to H_2O_2

To determine whether the SitABCD transporter contributes to resistance to H_2O_2, we introduced pIJ28 (*sitABCD*) into strain QT99, the *mntH* null mutant strain of *E. coli* K-12. Strain QT99 was more sensitive to H_2O_2 than the isogenic parent strain χ289, whereas introduction of either plasmid pIJ28 (*sitABCD*) or pIJ42 (*mntH*) restored resistance to

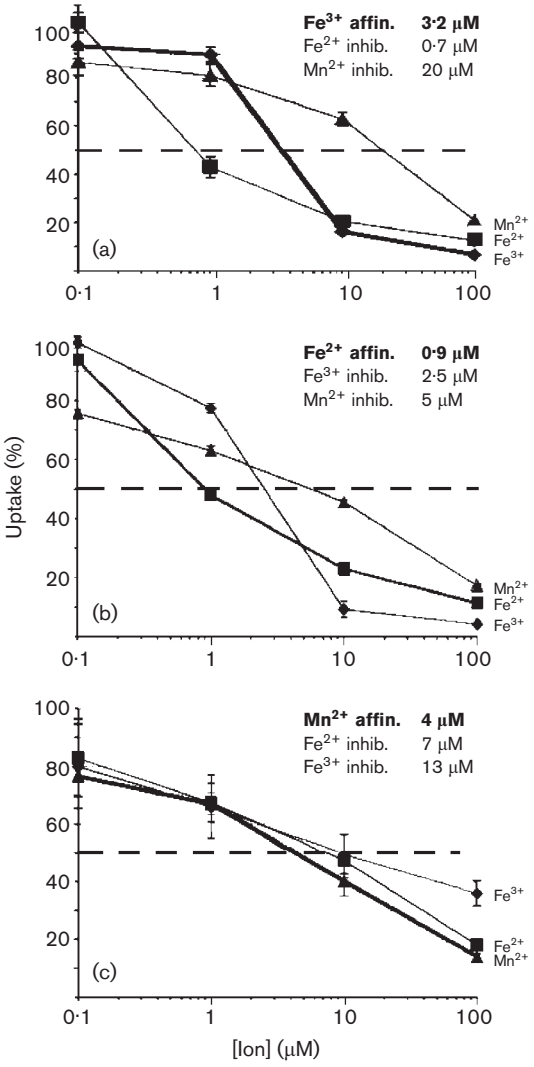

Fig. 5. Uptake inhibition for $^{55}Fe^{3+}$ (a) and $^{59}Fe^{2+}$ (b) in iron transport deficient *E. coli* K-12 strain H1771 (*aro feo*) carrying plasmid pIJ28 (*sitABCD*), and for $^{54}Mn^{2+}$ (c) in manganese transport deficient *E. coli* K-12 strain QT99 (*mntH*) carrying pIJ28 (*sitABCD*). Affinities for each ion are indicated in bold text and with thick lines in each of the graphs.

H_2O_2 which was similar to that of wild-type strain χ289 (Fig. 6a). Analysis of variance of the means indicated that the growth inhibition zones generated by H_2O_2 were significantly different among the *mntH* mutant strain and the mutant complemented with either *sitABCD* or *mntH* ($P=0{\cdot}0312$). By contrast, differences in growth inhibition zones were not significant among the wild-type strain and QT99 complemented with either pIJ28 (*sitABCD*) or pIJ42 (*mntH*) ($P=0{\cdot}0788$). The wild-type pathogenic *E. coli* strain χ7122 did not demonstrate a significant difference in resistance to H_2O_2 when compared to wild-type K-12 strain χ289 (Fig. 6b). In addition, strains QT205 (χ7122 Δ*sitABCD*) and QT878 (χ7122 Δ*mntH*) were as resistant to H_2O_2 as wild-type APEC strain χ7122. However, the Δ*sitABCD* Δ*mntH* of

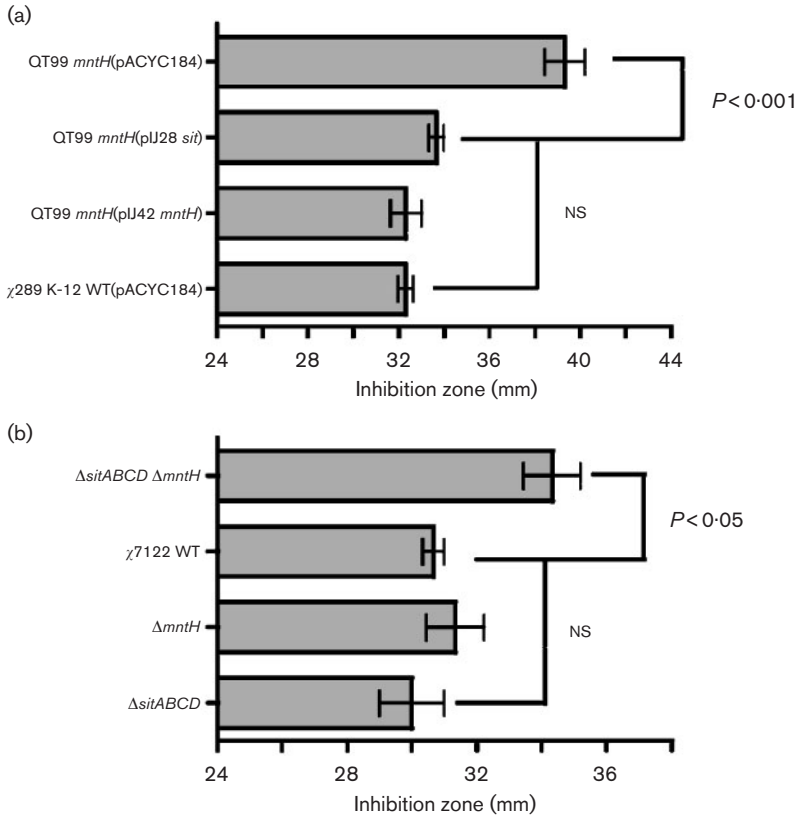

Fig. 6. Resistance to H_2O_2. *E. coli* K-12 strains (a) or APEC strain χ7122 and derivatives (b) with or without functional MntH and/or SitABCD transport systems were seeded onto agar plates and subjected to H_2O_2 placed on sterile filter disks. Differences in resistance were assessed from the diameter of the inhibition zone of a culture following overnight growth. APEC strains used were wild-type (WT) χ7122 and isogenic mutants QT205 (Δ*sitABCD*), QT878 (Δ*mntH*) and QT1239 (Δ*sitABCD* Δ*mntH*).

strain χ7122 (QT1278) was more sensitive to H_2O_2 than APEC strain χ7122 (Fig. 6b). These results indicate that *sitABCD* contributes to resistance to H_2O_2 in *E. coli* K-12 that lacks a functional MntH transporter. However, deletion of *sitABCD* genes from APEC strain χ7122 does not cause an appreciable difference in resistance to H_2O_2, and the loss of both the SitABCD and MntH systems is necessary to render APEC strain χ7122 sensitive to H_2O_2.

DISCUSSION

Presence of *sitABCD* genes on the plasmids and genomes of *E. coli* and *Shigella* spp.

We have identified a homologue of the SitABCD transport system that is encoded by an operon located on the colicin V-type plasmid pAPEC-1 of APEC strain χ7122. In addition, we demonstrated that *sitABCD* is also encoded by large plasmids in other pathogenic *E. coli* strains and that *sit* genes are also present on the reference colicin V plasmid pColV-K30, as well as on the genomes of certain strains (Fig. 1). Previous reports have described *sit* homologues that are located on the genomes of *Sh. flexneri* 2a strains (Jin *et al.*, 2002; Runyen-Janecky *et al.*, 2003; Wei *et al.*, 2003) and ExPEC strain CFT073 (Welch *et al.*, 2002). Similarly, operons of the related systems such as $sitABCD_{Sal.\ enterica}$ (Zhou *et al.*, 1999) and *yfeABCD* in *Y. pestis* (Bearden *et al.*, 1998) are located on the chromosome. The presence of plasmid-located *sit* sequences in other *E. coli* strains has also been reported (Rodriguez-Siek *et al.*, 2005; Chouler *et al.*, 2004). The SitABCD system represents yet another metal-sequestering system that is encoded by genes located on ColV-type plasmids as well as the chromosomes of certain *E. coli* and *Shigella* strains. Other known metal transporters present on ColV-type or other conjugative plasmids in *E. coli* include the aerobactin and salmochelin siderophore systems encoded by the *iucABCDiutA* genes and *iroBCDEN* genes respectively (Dozois *et al.*, 2003; Sorsa *et al.*, 2003; Warner *et al.*, 1981; Waters & Crosa, 1991). Thus ColV-type plasmids commonly carry genes encoding transporters important for the acquisition of metallic cations, particularly iron. In addition, both the aerobactin- and salmochelin-encoding operons are localized on the chromosomes of certain pathogenic *E. coli* or *Shigella* strains (Dobrindt *et al.*, 2001; Moss *et al.*, 1999; Vokes *et al.*, 1999; Waters & Crosa, 1991), further attesting to the presence of these systems on either plasmids or pathogenicity islands.

Iron and manganese transport

In this report we have demonstrated the capacity of *sitABCD* from an APEC strain to function as a manganese and iron transporter. By using *E. coli* K-12 strains deficient in either transport of iron (*ent* or *aroB feoB*) or manganese (*mntH*), we investigated whether a cloned copy of *sitABCD* from APEC strain χ7122 could compensate for mutations in these transport systems. Introduction of the *sitABCD* genes resulted in a significant regain in growth of the *ent*

mutant strain 1017 in NB containing chelators (either 2,2′-dipyridyl or EDDA). The growth increase due to the presence of SitABCD was as marked as that seen after introduction of the aerobactin siderophore system (Fig. 3). Ion-rescue experiments demonstrated that only iron was able to complement the growth defect of strain 1017 (*ent*) to a high level (80 % of the growth yield in medium without chelator) and that in the presence of pIJ28 (*sitABCD*) growth was faster (6 h to reach 80 % growth yield, compared to more than 10 h in the absence of *sitABCD*). Addition of manganese partially restored the growth of strain 1017 (*ent*) (40 % maximum growth increase) whereas zinc did not contribute to any discernible growth increase. These results suggest that lack of iron and not manganese or zinc was largely responsible for the growth deficit of strain 1017. This conclusion is reinforced by the fact that aerobactin, an iron-specific siderophore, was equally able to compensate the growth of strain 1017 in NB-DIP (Fig. 3). Hence, increased growth of the *E. coli* K-12 *ent* mutant strain 1017 containing *sitABCD* was likely due to a greater capacity to obtain iron and suggests a role for SitABCD$_{E.\ coli\ \chi7122}$ as an iron transporter. These findings are in accordance with previous results demonstrating that SitABCD homologues from other bacterial species may function as iron transporters (Bearden & Perry, 1999; Runyen-Janecky *et al.*, 2003; Zhou *et al.*, 1999).

In the *aroB feoB* mutant strain H1771, *sitABCD* conferred a significant increase in uptake of ^{55}Fe and ^{59}Fe under reduced conditions, suggesting it may function as a ferrous iron transporter (Table 3). However, the levels of iron uptake observed in strain H1771 containing *sitABCD*$_{E.\ coli\ \chi7122}$ were significantly lower when compared to the *E. coli* K-12 control strain containing the functional endogenous iron transport systems, FeoB and enterobactin (Table 3). Taken together, these results suggest that, under our assay conditions, despite demonstrating a lower efficiency in iron uptake compared to the endogenous systems present in *E. coli* K-12, SitABCD is nevertheless able to compensate for iron transport deficiencies in *E. coli* K-12 and confer sufficient iron acquisition for restoration of growth under iron-limiting conditions.

Introduction of the *sitABCD*$_{E.\ coli\ \chi7122}$ genes to an *E. coli* K-12 *mntH* mutant effectively restored manganese transport. In fact, when cloned copies of either *sitABCD*$_{E.\ coli\ \chi7122}$ or *mntH* were introduced into the *mntH* mutant, manganese transport levels were greater than that of the wild-type parent (Table 3). This increased transport is most likely due to a plasmid copy number effect. In our studies, the first ^{54}Mn uptake assay was conducted at pH 7·0. In subsequent ^{54}Mn uptake assays done under acid to alkaline conditions ranging from pH 5 to pH 9, we observed that *mntH* mutant strain QT99 complemented with *mntH* (pIJ42) more effectively transported manganese under acid conditions (pH 5–6), whereas complementation with *sitABCD*$_{E.\ coli\ \chi7122}$ (pIJ28) demonstrated increased manganese uptake at alkaline pH (pH 8–9) (M. Sabri & C. M. Dozois, unpublished results). These results were similar to those observed for *Sal. enterica*, which demonstrated that SitABCD$_{Sal.\ enterica}$ mediated ^{54}Mn uptake most effectively at alkaline pH (Kehres *et al.*, 2002b). SitABCD$_{Sal.\ enterica}$ has also been shown to be more specific for transport of manganese than iron (Kehres & Maguire, 2003). The YfeABC from *Y. pestis* was shown to mediate both manganese and iron uptake, although no preferential uptake of either of these metals was established (Bearden & Perry, 1999).

SitABCD and related systems are members of the Mn-Zn-Fe transport family (TC 3.A.1.15) based on bioinformatics analysis (Claverys 2001; Saier, 1999), and these tranporters have been shown to mediate uptake of iron or manganese in *Y. pestis* (Bearden & Perry, 1999) and manganese in *Sal. enterica* serovar Typhimurium (Kehres & Maguire, 2003). For SitABCD$_{Sh.\ flexneri}$ no isotope uptake or isotope uptake inhibition experiments were performed, although the authors demonstrated an iron- and manganese-dependent regulation of *sitABCD*$_{Sh.\ flexneri}$ (Runyen-Janecky *et al.*, 2003). To determine if SitABCD exhibits a preference for uptake of iron or manganese we conducted isotope uptake and inhibition assays using ferric iron, ferrous iron or manganese. Since zinc did not demonstrate any growth rescue to strain 1017 pIJ28 (*sitABCD*) in NB containing chelators it was not tested for ion inhibition. Results indicated that in a strain impaired for the transport of iron, SitABCD$_{E.\ coli\ \chi7122}$ demonstrated a higher affinity for iron than for manganese and confirmed that SitABCD displayed a higher affinity for ferrous iron than for ferric iron (Fig. 5). By contrast, when uptake and inhibition experiments were done in a strain impaired for manganese transport, SitABCD$_{E.\ coli\ \chi7122}$ transported manganese with higher affinity than iron. The differences observed in ion transport affinities between the two strains are possibly due to the influence of other ion transport systems present in these strains which may affect the availability and/or uptake of competing cold ions. In the iron transport impaired strain H1771 (*aro feo*), the MntH manganese transporter is functional and may compete with the Sit system for uptake of Mn^{2+} ions, which could reduce the levels of cold Mn^{2+} ions available for the ^{55}Fe or ^{59}Fe uptake inhibition, thus resulting in lower apparent inhibition constants for manganese. Similarly in the manganese transport impaired strain QT99 (*mntH*), functional enterobactin and Feo transport systems may have influenced the apparent inhibition constants for Fe^{2+} and Fe^{3+}. It is noteworthy that previous SitABCD$_{Sal.\ enterica}$ transport affinity studies by Kehres & Maguire (2003) were done in strains which had functional siderophore and Feo systems, which may have led to the consequent finding that SitABCD$_{Sal.\ enterica}$ exhibited a considerably lower affinity for iron than for manganese. Ion transport studies with YfeABC$_{Y.\ pestis}$ used strains that contained functional MntH and Feo homologues (Bearden & Perry, 1999). It is therefore likely that some discrepancies observed in the transport specificity of Sit and related transporters that have been reported may be at least partially explained by differences in strain backgrounds as well as

potential functional differences in the metal tranporters themselves.

The apparent affinities of uptake for Fe^{2+}, Fe^{3+} and Mn^{2+} indicated that under our assay conditions SitABCD$_{E.\ coli\ \chi7122}$ transported ferrous iron with the highest affinity (0·9 μM), followed by ferric iron (3·2 μM) and manganese (4 μM). In this regard, we believe our studies are the first to demonstrate a preference for ferrous iron uptake by a SitABCD transport system. This observation was determined by comparison of Sit-mediated ^{55}Fe uptake under reducing (Fe^{2+}) versus non-reducing (Fe^{3+}) conditions (Table 3) as well as by comparison of the uptake of $^{59}Fe^{2+}$ and $^{55}Fe^{3+}$ isotopes and inhibition with cold Fe^{2+} or Fe^{3+} ions (Fig. 5). The role of SitABCD as both an iron and manganese transporter is further supported by the improved growth under conditions of iron deficit and H_2O_2 resistance phenotypes conferred upon *E. coli* K-12 strains that were deficient in iron or manganese transport respectively.

Protection against oxidative stress and resistance to H_2O_2

The ability to obtain manganese in bacteria contributes to detoxification of free radicals and protection against oxidative damage from agents such as H_2O_2 (Horsburgh *et al.*, 2002; Kehres & Maguire, 2003). In our studies, increased sensitivity to H_2O_2 correlated with a decreased capacity to transport manganese. The *E. coli* K-12 *mntH* mutant was more sensitive to H_2O_2 than its wild-type parent strain, and complementation of the *mntH* mutant with either *sitABCD* or *mntH* restored resistance to H_2O_2 (Fig. 6a). Therefore *sitABCD* was shown to effectively compensate for the loss of MntH by restoring transport of manganese as well as resistance to H_2O_2 in a K-12 *E. coli* strain. By contrast, in APEC strain χ7122, deletion of either *sitABCD* or *mntH* alone did not render it more sensitive to H_2O_2 (Fig. 6b). This suggested that the presence of either MntH or SitABCD sufficed for maintaining resistance to H_2O_2 in the absence of the other transporter. In support of this, the double Δ*sitABCD* Δ*mntH* mutant derivative of strain χ7122 (QT1239) demonstrated a significant loss of resistance to H_2O_2 toxicity. These results suggest that under our assay conditions either SitABCD- or MntH-mediated manganese import systems were sufficient for maintaining a similar level of H_2O_2 resistance and that each of the two systems is able to compensate for the loss of function of the other transporter in the APEC strain. These findings also support a potential combined role for the SitABCD and MntH transporters for the virulence of APEC and perhaps other ExPEC.

Role of SitABCD in virulence

Inactivation of *sitABCD* alone in APEC strain χ7122 had no appreciable effect on its sensitivity to H_2O_2 (Fig. 6). In line with this, following infection with the Δ*sitABCD* mutant QT205, chickens developed lesions of airsacculitis, pericarditis and perihepatitis that were as severe as those observed in birds infected with the wild-type pathogenic parent strain χ7122 (M. Caza, R. Curtiss III & C. M. Dozois, unpublished results). In the extra-intestinal tissues of infected chickens a combination of iron transport systems is needed for full virulence, and the APEC *sit* derivative QT205 retains functional siderophores and other iron and manganese transporters such as FeoB and MntH. Taken together, these results suggest that other iron and/or manganese transport systems present in APEC strain χ7122 may compensate for the loss of a functional Sit transport system and provide sufficient transport for growth in iron-restricted medium and resistance to H_2O_2. Recently, by using signature-tagged mutagenesis (STM), the Sit tranporter was identified as a potential virulence factor in an APEC O2 strain in experimentally infected chickens (Li *et al.*, 2005). Since STM-based studies involve co-infections with pools of mutants, in such studies it is not possible to establish if specific mutants are less able to generate lesions during infection. The potential contribution of SitABCD to the virulence of APEC strains may also differ among different APEC strains or serogroups since the pathogenic mechanisms of different strains have been shown to differ. For instance an APEC O2 strain demonstrated uptake and survival within avian phagocytes, whereas APEC O78 strain χ7122 has been shown to avoid phagocytosis by avian heterophils or macrophages (Mellata *et al.*, 2003). In *E. coli*, SitABCD-encoding genes are associated with clinical strains isolated from extra-intestinal infections from poultry and human urinary tract infections (Rodriguez-Siek *et al.*, 2005; Schouler *et al.*, 2004). Recently, transcriptome analysis of uropathogenic *E. coli* strain CFT073 indicated that *sit* genes were highly upregulated in the urine of infected mice (Snyder *et al.*, 2004), further supporting a potential role for the Sit system during infection. Further virulence studies will be required to assess the individual and cumulative roles of SitABCD and other iron and manganese transport systems for the virulence of APEC and other pathogenic *E. coli* strains.

ACKNOWLEDGEMENTS

We thank Jorg Hacker, Klaus Hantke, James R. Johnson, Maryvonne Moulin-Schouleur, Ben Otto and Robert D. Perry for kindly supplying *E. coli* strains. We acknowledge Sébastien Houle for technical assistance and France Daigle for critical reading of the manuscript. Funding for this research was provided by the Natural Sciences and Engineering Council (NSERC) of Canada, the Canada Foundation for Innovation (CFI) and a Canada Research Chair (CRC). S. L. was the recipient of a 'Fonds de la recherche en santé Québec' (FRSQ) scholarship. M. S. was the recipient of a Fondation Armand-Frappier scholarship.

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