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The ZupT transporter plays an important role in zinc homeostasis and contributes to *Salmonella enterica* virulence†

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Zinc is an essential metal for cellular homeostasis and function in both eukaryotes and prokaryotes. To acquire this essential nutrient, bacteria employ transporters characterized by different affinity for the metal. Several studies have investigated the role of the high affinity transporter ZnuABC in the bacterial response to zinc shortage, showing that this transporter has a key role in adapting bacteria to zinc starvation. In contrast, the role of the low affinity zinc importer ZupT has been the subject of limited investigations. Here we show that a *Salmonella* strain lacking ZupT is impaired in its ability to grow in metal devoid environments and that a *znuABC zupT* strain exhibits a severe growth defect in zinc devoid media, is hypersensitive to oxidative stress and contains reduced levels of intracellular free zinc. Moreover, we show that ZupT also plays a role in the ability of *S.* Typhimurium to colonize the host tissues. During systemic infections, the single *zupT* mutant strain was attenuated only in Nramp1$^{+/+}$ mice, but competition experiments between *znuABC* and *znuABC zupT* mutants revealed that ZupT contributes to metal uptake *in vivo* independently of the presence of a functional Nramp1 transporter. Altogether, the here reported results show that ZupT plays an important role in *Salmonella* zinc homeostasis, being involved in metal import both *in vitro* and in infected animals.

Introduction

Metals play essential roles in all organisms as structural or catalytic cofactors in a wide number of proteins. This concept has been highlighted by a recent survey of proteins with known 3D structure, which has revealed that almost half of all enzymes bind metals, with approximately 40% containing metals at their catalytic centers.[1] Among metals, zinc plays particularly important roles in cellular homeostasis and function. It has been estimated that zinc can selectively associate with about 5% of all bacterial proteins, including enzymes of all six functional classes.[2,3] Zinc is often the cofactor of choice for enzymatic reactions because of its ability to stabilize negative charges and to activate substrates due to its strong Lewis acid properties.[3] The binding of zinc to proteins is facilitated by its highly versatile coordination chemistry, as this metal can be ligated by nitrogen, oxygen and sulphur atoms and can assume different coordination numbers. This property, however, is potentially detrimental to cells because it favors the unspecific binding of zinc to polypeptides and free thiols. Therefore, the levels of intracellular zinc are tightly regulated and the pool of unbound metal is kept to a minimum.[1,4]

Because zinc is critical for many cellular functions, bacteria have evolved several mechanisms to respond to zinc deficiency, and acquire this metal from the host. In Gram-negative bacteria, the transcriptional regulator Zur controls the expression of a small number of genes required to adapt the cell to conditions of severe zinc paucity.[5] Under zinc-replete conditions, the zinc-containing form of Zur tightly binds to the promoter region of said genes, preventing their expression.[6] Conversely, when the intracellular zinc concentration falls below a critical threshold, the zinc-devoid form of Zur no longer represses transcription.

The number of Zur-regulated genes varies between different bacteria, but in all species they include the genes encoding for the different subunits of a high affinity zinc importer

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(ZnuABC in Gram-negative bacteria), as well as the genes encoding for one or more paralogs of zinc-containing ribosomal proteins.[7–10] ZnuABC significantly enhances the bacterial ability to recruit zinc under different environmental conditions characterized by low availability of this metal. At the same time, the substitution of zinc-containing ribosomal proteins with metal-independent homologues significantly reduces the zinc requirements of bacterial cells. In line with this change in ribosomal structure organization, bacteria grown in zinc-depleted media contain much less zinc than bacteria grown under zinc-replete conditions.[11]

Several studies have investigated the consequences of the inactivation of *znuABC* in different bacteria. Besides showing that this transporter is necessary to ensure bacterial growth *in vitro* in zinc-limiting environments, ZnuABC was found to play a critical role in bacterial pathogenesis, thus implying that zinc availability is limited within infected hosts.[12–22]

In previous studies, we investigated the role of ZnuABC during infection of mice with the Gram-negative pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). We found that *S.* Typhimurium strains lacking the *znuA* gene are significantly attenuated in mice in both systemic and gastrointestinal infections.[17,23,24] Deletion of the *znuA* gene, which encodes for the periplasmic component ZnuA, is sufficient to disrupt the function of the ZnuABC transporter, as no further attenuation is observed in mutant strains where the entire *znuABC* operon is deleted.[23,25] By producing the ZnuABC transporter, *S.* Typhimurium evades the antimicrobial activity of calprotectin, a zinc-sequestering protein released by neutrophils during infection in the intestinal lumen.[26] In this host environment, a strain lacking a functional ZnuABC transporter cannot compete with the microbiota and is killed by the inflammatory response.[26] As a matter of fact, the absence of *znuABC* also causes significant proteomic and ionomic alterations[11] and changes in the expression of putative virulence factors.[27]

While ZnuABC is exclusively produced under zinc starvation, in environments where zinc is more abundant metal uptake is ensured by other transporters with lower affinity for zinc and broader metal specificity.[28] The most important permease allowing entrance of zinc is thought to be ZupT, a membrane protein belonging to the ZIP (ZRT-, IRT-like Protein) protein family.[29] Studies on *Escherichia coli* ZupT have established that this transporter mediates the import of different metals, although a preference for zinc over manganese, copper and iron was shown.[30–32] In *E. coli*, the activity of ZupT is constitutive and it is not regulated in response to changes in metal availability.[31] Even so, ZupT activity is inhibited by ionophores, suggesting that the proton motive force drives ZupT-mediated metal-uptake.[32] This hypothesis was also supported by the observation that the phage shock protein (Psp) system, which is involved in the maintenance of the proton motive force, facilitates metal uptake through different importers, including ZupT.[33]

The respective roles of ZupT and ZnuABC were also analyzed in an *E. coli* uropathogenic (UPEC) strain.[19] This study confirmed that ZupT mediates zinc uptake *in vitro* and showed that ZnuABC contributes to UPEC colonization of kidneys and bladders in CBA/J mice. Nonetheless, the *znuABC zupT* and the *znuABC* mutants showed a very similar ability to colonize host tissues, thereby suggesting that ZupT only modestly contributes to zinc uptake during UPEC infections. In contrast, the ZupT transporter is required for *S.* Typhimurium virulence in a typhoid model of infection in mice expressing the natural resistance-associated macrophage protein 1 (Nramp1) metal transporters.[33] However, it is not known whether the contribution of ZupT to *Salmonella* pathogenicity is related to the import of zinc and/or other metals. Here we set out to further investigate the role of ZupT in zinc homeostasis by analyzing its regulation during *in vitro* growth, as well as its contribution to pathogenesis in typhoid and gastrointestinal models of *S.* Typhimurium infection.

Experimental

Salmonella strains and growth conditions

The *S.* Typhimurium strains used in this work are listed in Table 1. Cultures were routinely grown aerobically in liquid Luria-Bertani broth (LB) or on LB agar plates at 37 °C. Agarose plates were prepared using molecular biology grade agarose (Vivantis) at 15 g l^{-1} in LB medium. Growth under zinc limiting conditions was achieved using either the Vogel–Bonner minimal medium E (VB-MM) (anhydrous $MgSO_4$ [0.04 g l^{-1}], citric acid [2 g l^{-1}], anhydrous K_2HPO_4 [10 g l^{-1}], NaH_4PO_4 [3.5 g l^{-1}], and glucose [2 g l^{-1}]), or the M9 minimal medium (M9-MM) (Na_2HPO_4 [7.52 g l^{-1}], KH_2PO_4 [3 g l^{-1}], NH_4Cl [1 g l^{-1}], NaCl [5 g l^{-1}], $MgSO_4{\cdot}7H_2O$ [1.23 g l^{-1}], $CaCl_2{\cdot}2H_2O$ [0.007 g l^{-1}], and glucose [0.2%]). To avoid zinc contaminations from glassware, minimal media were prepared in disposable plastic containers and sterilized by filtration in 500 ml Vacuum Filter–Storage Bottle Systems, 0.22 μm (Corning). The quality of each minimal medium batch was verified by monitoring the accumulation of ZnuA in strain SA140 (which is abolished by zinc at concentrations below 1 μM).[17] Antibiotics were used at the following concentration: ampicillin 100 mg l^{-1}, kanamycin 50 mg l^{-1} and chloramphenicol 30 mg l^{-1}.

Mutants construction

S. Typhimurium ATCC14028 *zupT* mutants (SA321 and AJP3, obtained respectively from A.B. and M.R. laboratories) were achieved following the one-step inactivation protocol,[34] using plasmid pKD4 as the kanamycin resistance cassette template. The insertions were confirmed by PCR with oligonucleotides annealing upstream the mutated allele and into the inserted antibiotic resistance cassette. The mutated alleles were then transduced by generalized transduction with phage P22 HT 105/1 *int-20*[35] into wild type and *znuABC*::*scar* (SA186) or *znuA*::*cam* (JZL3) strains to obtain, respectively, the *zupT* (SA321) and the *znuABC zupT* (SA327) or *znuA zupT* (AJP4) mutant strains.

Strain MC113 was obtained by transducing the *zinT*::3X Flag-kan allele[23] into the *znuABC*::*scar zupT*::*scar* mutant, previously obtained by electroporating plasmid pCP20 and harboring the FLP

Table 1 Strains used in this work

Strain	Relevant genotype or description	Source or ref.
DH5α	*E. coli* F-(80d *lacZ* M15) (*lacZYA-argF*)U169 *hsdR17*(r – m+) *recA1 endA1 relA1 deoR*	Lab collection
MA6926	Wild type *S.* Typhimurium	Lab collection
MA6926 (pKD46)	Wild type harboring plasmid pKD46	Lab collection
SA212	*ilvI*::Tn10dTac-*cat*::3xFLAG-*SCAR*	Lab collection
SA140	*znuA*::3xFLAG-*kan ilvI*::Tn10dTac-*cat*::3xFLAG-*kan*	17
SA186	*znuABC*::scar	25
SA321	*zupT*::*kan*	This work
SA327	*znuABC*::scar *zupT*::*kan*	This work
SA336	*sitABCD*::*kan mntH*::scar	Lab collection
SA337	*feoB*::scar *fepA/entF*::*kan*	Lab collection
SA340	*sitABCD*::*scar mntH*::*scar zupt*::*kan*	Lab collection
IR715	ATCC 14028, Nal^R derivative	49
JZL3	*znuA*::*cam*	26
AJP3	*zupT*::*kan*	This work
AJP4	*znuA*::*cam zupT*::*kan*	This work
PP127	*zur*::*kan*	23
PP134	*zinT*::3X Flag-*kan*	23
PP137	*zinT*::3X Flag-*kan znuA*::*cam*	23
MC113	*zinT*::3X Flag-*kan znuABC*::scar *zupT*::scar	This work
PP150	Wild type harboring plasmid pP_{zupT}-lacZ	This work
PP152	*znuABC*::scar harboring plasmid pP_{zupT}-lacZ	This work
PP153	*zur*::*kan* harboring plasmid pP_{zupT}-lacZ	This work

recombinase function,[34] in the SA327 strain. The mutant was selected for kanamycin resistance.

Growth analyses

Overnight cultures grown in LB were washed with PBS and normalized to an optical density at 600 nm of 1.0. Bacterial suspensions were serially diluted 1 : 10 and 5 μl of each dilution were plated onto appropriate LB agarose plates. Growth was imaged after an incubation of 24 h at 37 °C.

To analyse bacterial growth in liquid media, strains were grown overnight in VB-MM at 37 °C and then diluted 1 : 500 in the same medium supplemented or not with the appropriate concentration of metals. Aliquots of 300 μl of these dilutions were inoculated in 96-well plates (Becton–Dickinson) and incubated at 37 °C with shaking. Bacterial growth was monitored at 595 nm every hour for 15 hours using a microtiter-plate reader (Sunrise™, Tecan). Assays were performed in triplicate and each strain was tested in three independent experiments.

Western blot analyses

ZinT accumulation was analyzed in *Salmonella* strains expressing an epitope-tagged protein, containing a 3X FLAG epitope at its C-terminus, as previously described.[23] The amount of bacteria corresponding to the optical density of 0.25 at 600 nm was loaded in each lane. Strain SA212, constitutively expressing a 3xFLAG epitope-tagged chloramphenicol acetyl transferase protein (CAT), was used as a control for protein loading. All the strains used for these experiments exhibited an identical correspondence between the optical density at 600 nm and the number of colony forming units in VB-MM (with or without zinc supplementation) as well as an identical content of soluble proteins (as evaluated by quantifying proteins using the method of Lowry), thus suggesting that the mutations have no evident effects on cell size and intracellular protein content. Proteins tagged with the 3XFLAG epitope were revealed by incubation of nitrocellulose filters with a mouse anti-FLAG antibody (anti-FLAG M2, Sigma) and an anti-mouse horseradish peroxidase-conjugated antibody (Bio-Rad), followed by the enhanced chemiluminescence reaction (ECL, Amersham).

Mouse infections and competition assays

Experiments with mice performed either at the Italian Istituto Superiore di Sanità (ISS) or at the University of California were carried out according to the respective national regulations. All experiments were previously approved by the ISS Ethical Committee and the Institutional Animal Care and Use Committee at the University of California, Irvine and carried out under the supervision of certified veterinarians. We used C57BL/6J (Taconic farms), BALB/c and DBA/2 (Charles River, Calco, Italy) female mice of 8–12 weeks of age. Mice were fed a commercial diet and water was provided *ad libitum*. All mice were acclimatized for a minimum of 1 week prior to experimentation.

Intraperitoneal mouse infections and competition assays were performed as previously described.[17] Bacteria recovered from spleens were plated for single colonies, and then 200 colonies were picked on selective plates. The competitive index (CI) was calculated using the formula CI = output (strain A/strain B)/input (strain A/strain B). Statistical differences between outputs and inputs were determined by Student's *t* test. Oral infections were carried out in streptomycin-pretreated animals, as previously described.[26]

Cloning the *zupT* promoter region and β-galactosidase activity assay

A 218 bp fragment located upstream the *zupT* coding sequence, including the GTG start codon, was amplified by PCR from *S.* Typhimurium chromosomal DNA (extracted using the ZR fungal/bacterial DNA Kit™, Zymo Research) using oligonucleotides P*zupT*pMC For (CAGAATTCATAATCGTTATCGTCCAGCA) and

P*zupT*pMC Rev (GCGGATCCATCCATTACTCCTTATCAAT). The purified fragment (DNA Clean and Concentration™, Zymo Research) was then digested with the restriction enzymes EcoRI and BamHI (New England Biolabs) and cloned into plasmid pMC1403,[36] obtaining plasmid pMCP$_{zupT}$-*lacZ*. The nucleotide sequence of the DNA insert was verified by sequencing (Genechron). Subsequently, the plasmid was extracted from *E. coli* (HiYeld RBC™ Plasmid Mini Kit) and electroporated into *S.* Typhimurium strains MA6926 (wild type), SA186 (*znuABC*::scar) and PP127 (*zur*::*kan*) obtaining, respectively, strains PP150, PP152 and PP153 (see Table 1). β-galactosidase activity of the resulting strains was measured as previously described.[37]

H_2O_2 sensitivity assay

S. Typhimurium strains were grown for 6 hours in LB medium and then diluted 1 : 1000 in M9-MM with or without the addition of zinc. After growing the strains overnight (at 37 °C with aeration) cultures were diluted to 10^6 bacteria ml^{-1} in sterile PBS (time 0) and 0.5 mM H_2O_2 was added. After 1 hour incubation at 37 °C with aeration, 1000 units of catalase (Boehringer) were added to each sample. At time 0 and 1 hour bacteria were plated on agar plates for counting, after suitable dilutions. The percentage of viable bacteria was calculated by assuming bacterial viability at time 0 as 100% survival. The assay was performed in duplicate and the reported results are the average of three independent experiments.

Results

zupT expression is increased under zinc limiting conditions

Whereas it has been reported that in plants a ZIP transporter may be induced in roots in response to zinc or iron deficiency,[38] *zupT* is constitutively expressed in *E. coli*.[31] To determine whether zinc deficiency influences the expression of *zupT* in *S.* Typhimurium, we analyzed *zupT* expression in the wild type strain (PP150) and in a *znuABC* mutant strain (PP152), either under zinc-limiting conditions or in zinc-replete media, using a transcriptional fusion between the *zupT* promoter and the *lacZ* reporter gene. As shown in Fig. 1, basal levels of *zupT* expression can be measured in both the wild type (black bars) and *znuABC* (grey bars) strains cultured in rich medium (LB). Transcription of the *zupT* promoter was not affected by the addition of 1 mM zinc *per se*. However, *zupT* expression increased upon treatment with the metal chelator EDTA, and it was restored to basal levels by the addition of zinc to the medium. Notably, EDTA-mediated induction of *zupT* was more pronounced in the *znuABC* background, supporting the hypothesis that *zupT* expression may be modulated by zinc availability. Moreover, a slight decrease in *zupT* transcriptional activity was observed in bacteria grown in a defined medium (VB-MM) supplemented with zinc (Fig. 1), both in the wild type and in the *znuABC* background.

As Zur is the main transcriptional regulator governing bacterial responses to zinc shortage, the observation that *zupT* expression is partly modulated by zinc prompted us to investigate

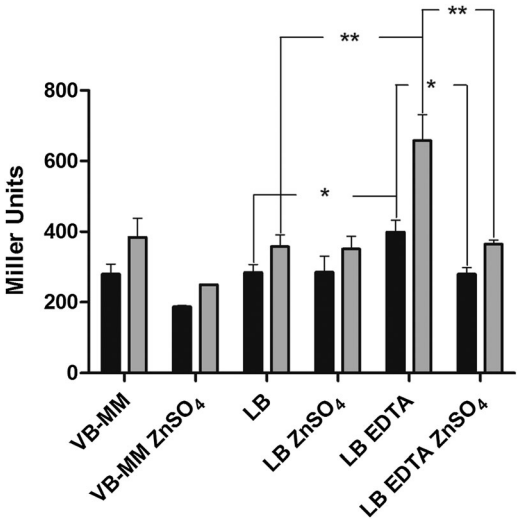

Fig. 1 Transcriptional analysis of the *S.* Typhimurium *zupT* promoter in bacteria grown overnight in VB-MM or in LB, in the presence or absence of 0.01 mM $ZnSO_4$ (VB-MM) or in LB containing 1 mM $ZnSO_4$ or 1 mM EDTA or both. Black and grey bars represent the wild type strain (PP150) and the *znuABC* strain (PP152) respectively. Each experiment was repeated at least twice and samples were always assayed in triplicate. *P* values: *$p < 0.05$; **$p < 0.01$.

whether Zur regulates the expression of *zupT*. A nucleotide sequence showing 62% homology to the *Salmonella* Zur-box upstream of the *znuABC* and *zinT* genes[23] was also identified within the *zupT* promoter (about 100 bp upstream the start codon) (Fig. S1, ESI†). In contrast, consistent with the hypothesis that *zupT* is constitutively expressed in *E. coli*, this putative consensus sequence was found to be absent in the *E. coli zupT* promoter.[31]

To test whether *S.* Typhimurium *zupT* is regulated by Zur, we electroporated plasmid pMCP$_{zupT}$-*lacZ* in a strain lacking *zur* (PP127), obtaining the strain PP153 (pMCP$_{zupT}$-*lacZ zur*). β-galactosidase assays were carried out on strains PP150, PP152 and PP153 grown under zinc limiting conditions (VB-MM or LB supplemented with EDTA). As β-galactosidase activity was similar in PP150 and PP153 (data not shown), these results suggested that *zupT* expression is not regulated by Zur.

Contribution of ZupT to zinc uptake during growth of *S.* Typhimurium

To characterize the role of ZupT in the process of zinc acquisition, the growth of strains lacking *zupT* (SA321), *znuABC* (SA186) or both transporters (SA327) was compared to that of the wild type strain under zinc limiting conditions, both in semi-solid and in liquid media.

We have previously shown that the growth of *S.* Typhimurium strains lacking a functional ZnuABC transporter is inhibited in agar plates containing EDTA or TPEN.[17,23] A potential problem with these experiments is that chelating agents have a broad metal specificity, thus some of the observed phenotypes could be only indirectly related to zinc uptake. We have more recently observed that the growth of strains lacking genes involved in

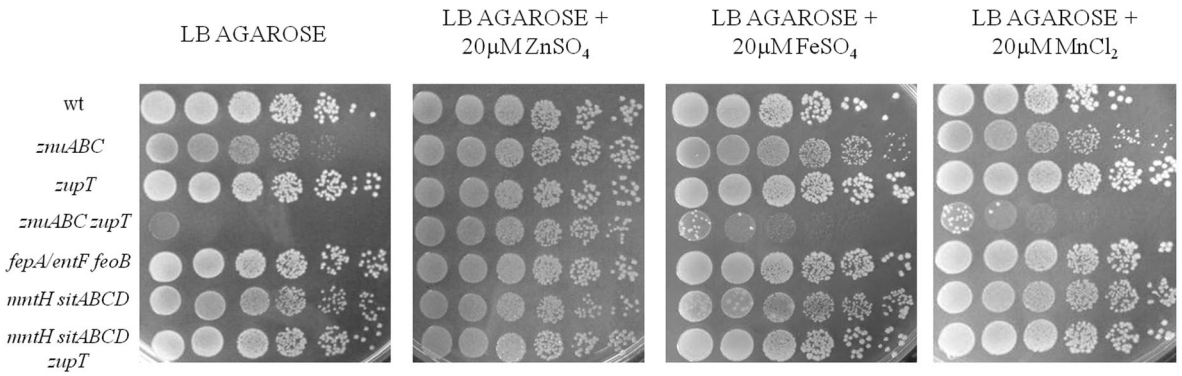

Fig. 2 Growth of *S.* Typhimurium on LB-agarose plates. Plates were prepared with standard LB or with LB supplemented with zinc, iron or manganese.

zinc uptake is decreased in LB plates containing 1.5% pure agarose in place of agar, without the need for adding chelating agents. When we prepared LB plates with 1.5% pure agarose, we observed that this polysaccharide has an intrinsic ability to sequester zinc, but not other biologically relevant metals such as iron and manganese. Specifically, the growth of *S.* Typhimurium strains lacking *znuABC* was slightly inhibited in these plates (Fig. 2). Although the *zupT* and the wild type strains displayed a comparable growth on agarose plates, the growth of the *znuABC zupT* double mutant was nearly completely abolished, suggesting that these two transporters have synergistic roles. Noticeably, the growth of the *znuABC zupT* strain was rescued to wild type levels by the addition of zinc, but not of manganese or iron, to LB-agarose plates. In contrast, the growth of a *mntH sitABCD* mutant strain lacking the two major manganese transporters and of a *fepA/entF feoB* mutant, deleted of the most important inorganic iron uptake systems, was not impaired in agarose plates, although these strains show clearly impaired growth in VB-MM (Fig. S2, ESI†).

The role of ZupT in enhancing *S.* Typhimurium growth under zinc limiting conditions was also analyzed in liquid VB-MM (Fig. 3). Deletion of *zupT* had a modest, but clearly discernible, effect on *S.* Typhimurium growth in this medium, whereas the absence of *znuABC* resulted in a much more severe growth defect, as previously shown.[17,23] Remarkably, the *znuABC zupT* mutant was hardly able to grow in this medium. When VB-MM was supplemented with 3 μM zinc, a metal concentration largely adequate to repress *znuA* expression,[11,17] the growth of all mutant strains was restored to levels comparable to those of the wild type strain (Fig. 3, panel B). Nonetheless, we observed slight but significant differences in the lag and exponential phases of growth among the wild type and *znuABC* mutant strains. It is likely that such growth retardation of mutant strains is due to an alteration in the rate of zinc entry within cells due to the absence of zinc transporters. Manganese or iron were not able to rescue the growth of the *znuABC zupT* mutant strain in VB-MM (Fig. S3, ESI†).

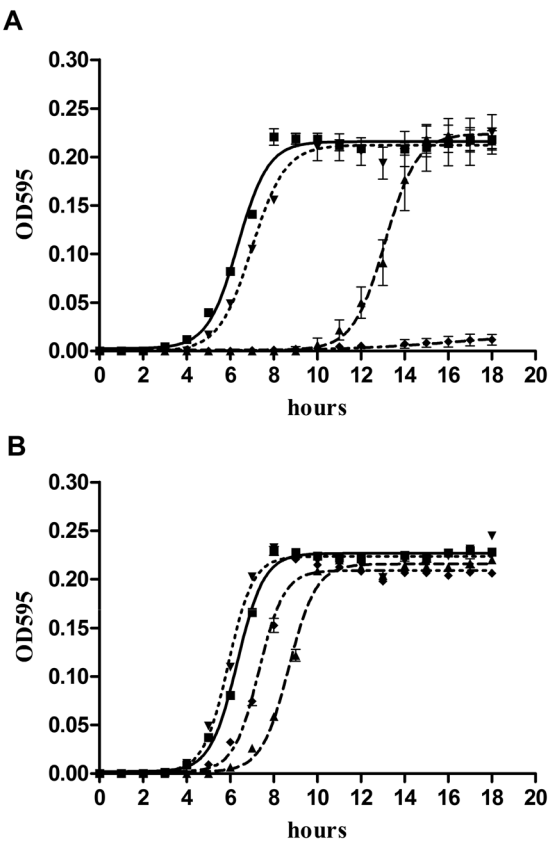

Fig. 3 Growth curves of *S.* Typhimurium wild type (■), *znuABC* (▲), *zupT* (▼), *znuABCzupT* (◆). Strains were grown in VB-MM alone (panel A) and VB-MM supplemented with 3 μM zinc (panel B). OD_{595}, optical density at 595 nm.

Lack of ZupT determines changes in intracellular zinc concentration

Previous studies have shown that *zinT* and *znuABC* are coregulated by Zur and that ZinT accumulation is strongly induced by EDTA and repressed by zinc.[23] The addition of 0.5 μM $ZnSO_4$ to the culture medium causes the complete abrogation of ZinT accumulation in a wild type *Salmonella* strain, but this expression profile is changed in a strain lacking the *znuA* gene due to a reduced intracellular zinc influx. In line with the finding that *znuA* and *znuABC* mutant strains have similar phenotypes,[23] we demonstrated the same *zinT* expression pattern in strains lacking either *znuA* alone or the whole *znuABC* operon. In this work, we set out to compare ZinT accumulation in the *znuA*

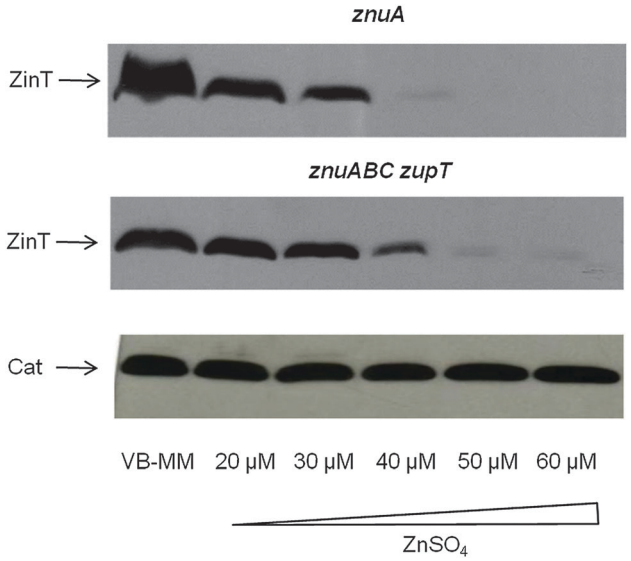

Fig. 4 ZinT::3xFLAG accumulation in the *znuA* deleted strain (PP137, upper panel) and in the *znuABC zupT* deleted strain (MC113, intermediate panel) grown in VB-MM alone or supplemented with $ZnSO_4$ as indicated. The lower panel shows the accumulation of CAT-3xFLAG in the control strain SA212.

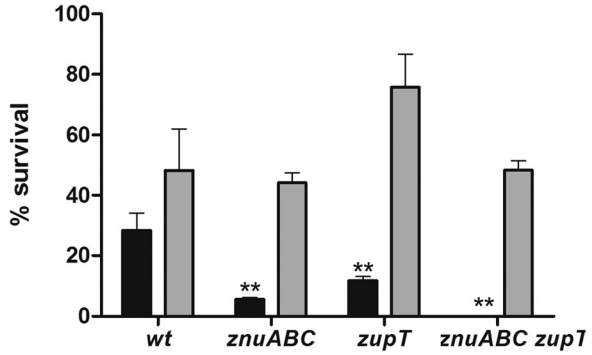

Fig. 5 Zinc import and hydrogen peroxide resistance. *S.* Typhimurium wild-type strains, SA186 (*znuABC*), SA321 (*zupT*) and SA327 (*znuABC zupT*) were grown in M9-MM with (gray bars) or without (black bars) the addition of 0.01 mM $ZnSO_4$ and exposed to H_2O_2 for one hour. Samples were assayed in triplicate and the graph shows the average of survival for each strain (percent with respect to H_2O_2 unexposed bacteria). *P* values: $^{**}p < 0.01$.

mutant and in the *znuABC zupT* mutant strains, as a means to indirectly infer whether the lack of *zupT* causes a decrease in the intracellular zinc content. Consistent with these early studies, ZinT accumulation was abolished when the *znuA* mutant was grown in a defined medium (VB-MM) containing approximately 40 μM of zinc sulfate (Fig. 4). Conversely, at this zinc concentration *zinT* was clearly expressed in the *znuABC zupT* strain and low protein levels were detected even in bacteria grown in the presence of 60 μM zinc (Fig. 4). As *zinT* expression is under the direct control of Zur, the main transcriptional regulator activated in response to zinc deprivation, the observed differences in the ZinT accumulation pattern in the two mutant strains strongly support the hypothesis that the absence of *zupT* further reduces the ability of *Salmonella enterica* to import zinc from the environment.

ZupT enhances *S.* Typhimurium resistance to H_2O_2

Previous studies carried out in uropathogenic *E. coli*,[19] *Corynebacterium diphtheriae*,[39] *Bacillus subtilis*,[40] and *Lactococcus lactis*[41] have suggested that zinc uptake mediates bacterial resistance to oxidative stress and that inactivation of zinc transporters enhances the susceptibility of these microorganisms to exogenous H_2O_2-mediated killing. These observations may be at least partially explained by the requirement of zinc as a cofactor in proteins involved in the response to reactive oxygen species, such as PerR[42] and SodCI/SodCII[43] and by the role of this metal in protecting protein thiols from oxidation.[40,41] We thus set out to determine whether ZupT enhances *S.* Typhimurium resistance to H_2O_2-mediated killing. To this end, we monitored the survival of *S.* Typhimurium wild type or mutant strains grown overnight in M9-MM with or without zinc supplementation, after 1 hour incubation with 0.5 mM H_2O_2 (Fig. 5). In comparison to *S.* Typhimurium wild type, all mutant strains showed increased susceptibility to H_2O_2, although the *zupT* mutant strain was less susceptible to H_2O_2 than the *znuABC* mutant. Moreover, the absence of both *znuABC* and *zupT* rendered *S.* Typhimurium hypersensitive to hydrogen peroxide. The enhanced sensitivity of mutant strains to H_2O_2 is dependent on their reduced ability to import zinc, as the growth of the same strains in a defined medium supplemented with zinc re-established resistance to oxidative damage comparable to that of the wild type strain. These results indicate that ZupT also cooperates with ZnuABC in conferring resistance to oxidative stress.

ZupT contributes to *Salmonella* virulence in mice

To assess the contribution of the low affinity zinc transporter ZupT to *Salmonella* virulence, we carried out *in vivo* competition assays between *S.* Typhimurium wild type and mutant strains in different mouse models of infection. First, we tested whether ZupT contributes to *S.* Typhimurium systemic infection by using the typhoid mouse model. Intraperitoneal infections were performed both in *Salmonella*-resistant DBA-2 mice ($Nramp1^{+/+}$) and in the highly susceptible BALB/c mice ($Nramp1^{-/-}$). As recently observed,[33] when infections were carried out in mice expressing a functional Nramp1 protein, the *zupT* mutant was significantly outcompeted by the wild type strain in the spleens of infected mice (Fig. 6A). Moreover, we found that a strain lacking both *znuABC* and *zupT* was significantly disadvantaged with respect to the single mutant *znuABC*.

In contrast, both the wild type and the *zupT* mutant strains colonized the spleens of intraperitoneally-infected BALB/c mice to similar levels (Fig. 6B). Nonetheless, in mixed infections the *znuABC* strain outcompeted the *znuABC zupT* double mutant, revealing that ZupT contributes to metal uptake during *S.* Typhimurium infection also in $Nramp1^{-/-}$ mice.

To further evaluate the role of ZupT, competition assays were also carried out in the streptomycin-pretreated mouse colitis model of *S.* Typhimurium infection. C57BL/6 mice

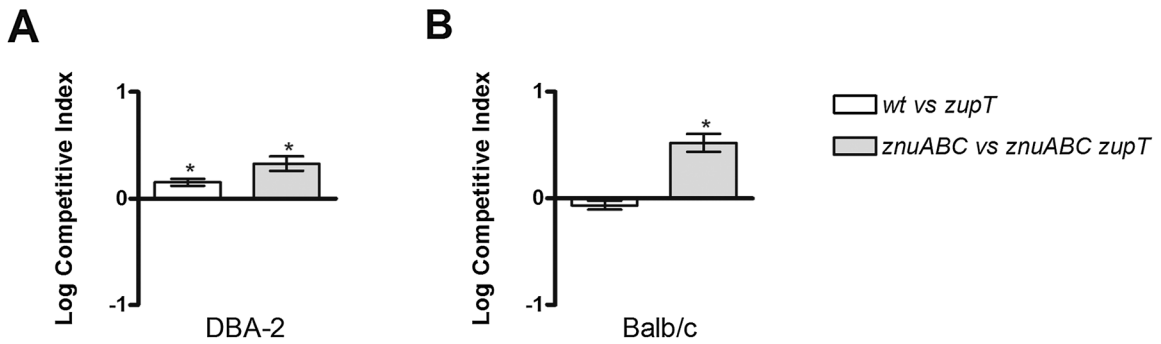

Fig. 6 Competition assay in intraperitoneally infected mice. Bacteria were recovered from the spleens of Nramp$^{+/+}$ (DBA-2, panel A) and Nramp$^{-/-}$ (Balb/C, panel B) mice infected with mixed inocula (wild type *vs. zupT* and *znuABC vs. znuABCzupT*, see legend). *P* values: *$p < 0.05$.

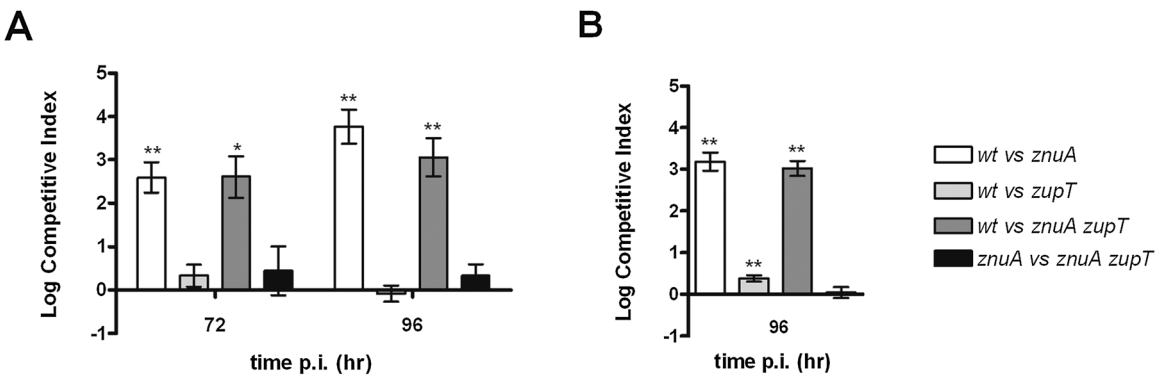

Fig. 7 Competition assay in orally infected mice. Competitive index in cecal contents (panel A) and in the spleens (panel B) of C57BL6 mice infected with mixed inocula (wild type *vs. znuA*; wild type *vs. zupT*; wild type *vs. znuA zupT*; *znuA vs. znuA zupT*; see legend). *P* values: *$p < 0.05$; **$p < 0.01$.

pretreated with streptomycin were orally infected with different mixtures of strains and the ability of these bacteria to colonize the cecum and the spleen was evaluated at 72 and 96 hours post infection. In line with previous studies,[26] the *S.* Typhimurium mutant strains *znuA* and *znuA zupT* were poorly able to colonize the gut of infected mice. In contrast, the wild type and the *zupT* strains were equally able to colonize the cecum at 96 h, whereas the *znuA zupT* strain showed only a small reduction in the ability to colonize this intestinal tract with respect to the *znuA* mutant (Fig. 7A). Moreover, we found that inactivation of *zupT* did not alter the ability of *S.* Typhimurium to colonize the Peyer's patches (data not shown), but was associated with a small but significant reduction in the number of bacteria recovered from the spleen and the mesenteric lymph nodes of orally infected mice (Fig. 7B). Taken together, these data further underline that ZnuABC plays a crucial role in the recruitment of Zn(II) in the infected host, but also suggest that ZupT contributes to zinc acquisition during *Salmonella* infection.

Discussion

Bacterial pathogens have evolved several mechanisms to acquire essential metals even when they are limited by the host or in the environment. In this work, we investigated the contribution of the permease ZupT to zinc uptake in *S.* Typhimurium, showing that this transporter contributes to metal import both *in vitro* and during infection of mice. We found that strains lacking the *zupT* gene have a growth defect in a defined minimal medium poor of zinc (Fig. 3). Even though the observed growth disadvantage with respect to the wild type strains is rather small, it was consistently observed in many different experimental settings and was always reverted by the addition of zinc to the culture medium. These observations suggest that, under conditions of severe zinc limitation, the reduced zinc acquisition derived from the absence of ZupT cannot be fully compensated by the strong induction of *znuABC*.[17] The requirement for two distinct zinc importers to ensure maximal efficiency in zinc recruitment is further underlined by the comparison of the growth rate of the *znuABC* and the *znuABC zupT* mutant strains in VB-MM or in agarose-LB plates. Under these conditions, the growth of a strain lacking both ZupT and ZnuABC is barely detectable, unless zinc is added to the culture medium. Moreover, addition of other metals like manganese (Fig. 2 and Fig. S3, ESI†) or iron (Fig. 2), which are known to be the substrate of ZupT, does not rescue the growth defect of this mutant. Therefore, although bacteria possess several low affinity zinc importers,[28] the severe growth defect of the *znuABC zupT* mutant strain in the absence of abundant environmental zinc suggests that *Salmonella* does not encode for other efficient zinc importers besides ZnuABC and ZupT.

The growth defect of a mutant strain lacking both ZnuABC and ZupT was particularly evident on agarose-LB plates.

The ability of polysaccharides (*i.e.* pectin and other oligosaccharides) to chelate transition metals from aqueous solutions has been known for a long time.[44,45] For example, it is well known that algae produce cell wall exopolysaccharides that protect them from heavy metal toxicity.[46] As a matter of fact, polysaccharides are investigated as an inexpensive and effective tool for the removal of toxic metals from contaminated materials.[47] Despite the chemical bases of zinc sequestration by agarose require further investigations, we propose that the use of agarose-LB plates represents a potential alternative to chelating agents for the analysis of the mechanisms of response to zinc limitation also in other bacterial strains.

Two other observations highlight the relevance of ZupT in zinc homeostasis. Unlike the high affinity zinc uptake transporter ZnuABC, which is produced exclusively under conditions of severe zinc limitation,[17] ZupT is only moderately induced by metal shortage and is constitutively expressed in media containing abundant levels of metals. However, using ZinT as a reporter of available zinc inside the cell, we have also shown that in the absence of ZupT, the Zur-mediated response to zinc shortage is repressed at higher concentrations of environmental zinc. Taken together, the observations reported in this study indicate that ZupT has a central role in zinc homeostasis in *Salmonella*, either by ensuring zinc uptake under conditions of moderate zinc requirements or by participating in metal transport upon severe zinc starvation, a condition characterized by the induction of the Zur-regulated operon.

Our results also indicate that ZupT plays a role in promoting *S.* Typhimurium colonization of host tissues. In line with a recent study,[33] we have found that a *zupT* mutant strain of *S.* Typhimurium is attenuated in Nramp1$^{+/+}$, but not in Nramp1$^{-/-}$ mice. However, it is difficult to directly correlate these observations to the ability of *Salmonella* to recruit zinc *in vivo*. Nramp1 is a macrophage metal transporter likely showing significantly higher affinity for iron and manganese than for zinc.[48] As ZupT may also transport iron and manganese in addition to zinc, it is possible that the reduced ability of the *zupT* mutant to colonize the spleen of Nramp1$^{+/+}$ mice is related to its reduced ability to acquire these metals in the *Salmonella*-containing vacuole. Nonetheless, our findings that the *znuABC zupT* double mutant is less able to colonize the spleen of intraperitoneally infected mice than the *znuABC* mutant strain suggest that ZupT is active during infections and likely contributes to zinc uptake. The involvement of ZupT in the ability of *Salmonella* to colonize the host was further confirmed by oral infections in streptomycin pre-treated mice. In fact, although ZnuABC is much more important than ZupT for gut colonization (Fig. 7A), we observed that disruption of *zupT* significantly affects the ability of *Salmonella* to infect the spleen (Fig. 7B) or the mesenteric lymph nodes (data not shown), thus suggesting that the ability to successfully colonize these organs requires redundant mechanisms to resist metal starvation. In addition, it is worth noting that mutant strains lacking *zupT* show reduced resistance to oxidative stress and that a *znuABC zupT* is hypersensitive to hydrogen peroxide (Fig. 5). It is likely that this feature contributes to the *in vivo* attenuation of *Salmonella* mutant strains defective in zinc homeostasis.

Most of the studies on the importance of zinc in host–pathogen interaction have been focused on the ZnuABC transporter. Undoubtedly the ZnuABC transporter plays a key role in the ability of bacteria to rapidly adapt to conditions of severe zinc paucity, such as those encountered in the infected host. Inactivation of ZnuABC determines phenotypic effects which are much more marked than those observed in *zupT* mutant strains and this likely explain why relatively few studies have focused on the role of ZupT in metal homeostasis. However, this study suggests that the contribution of the ZupT permease to the acquisition of zinc is much more significant than previously thought. In fact, the results presented here show that ZupT remarkably contributes to zinc acquisition under conditions of severe zinc shortage either *in vitro* or *in vivo* and suggest that the induction of ZnuABC is not sufficient to completely compensate for the reduction in metal uptake that is consequent to *zupT* inactivation. These observations demonstrate that ZupT is critical in the process of acquisition of zinc in *Salmonella* (and, most likely, in the majority of Gram-negative bacteria), where it likely represents a central route of zinc entry, whereas ZnuABC is the necessary complement to the basal activity of ZupT to adapt bacteria to conditions of zinc limitation.

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