UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI SCIENZE MATEMATICHE FISICHE E NATURALI

DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE E MOLECOLARE

XXII CICLO

"Characterization of the *Salmonella enterica* zinc import apparatus and of its relevance in the host-pathogen interaction".

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A.A. 2009/2010

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Summary

The ability of bacteria to colonize specific environments relies on their ability to obtain adequate supplies of the nutrients that are indispensable for their growth. Of particular relevance for human and animal health is to understand how bacterial pathogens face the problem of nutrient limitation in the infected host, in which several essential elements are not freely available for infectious microorganisms. In this respect, the recruitment of transition metals is a particularly challenging problem for bacterial pathogens, as these elements are usually present in forms that are not easily available for infectious microorganisms. For this reason, the sophisticated strategies adopted by pathogens to obtain iron have been the focus of intense investigations since a long time. Although the relevance of other metals in the host-pathogen interaction is usually considered as less important with respect to iron, evidences are accumulating that also the mechanisms ensuring the efficient uptake of zinc plays a critical role during bacterial infections.

Salmonella enterica, as well as many other Gram-negative bacteria, responds to zinc deficiency by producing the high affinity zinc uptake transporter ZnuABC. This complex belongs to the ABC transporters family and is constituted by three proteins: ZnuA, ZnuB and ZnuC. ZnuB is a membrane permease, ZnuC is the ATPase component, whereas ZnuA is a periplasmic metallochaperone which efficiently captures zinc in this cellular compartment and then delivers it to ZnuB. The expression of the *znuABC* operon is regulated by the metallated form of Zur, a dimeric protein which binds two zinc ions and thus represses *znuABC* transcription.

To investigate the relevance of zinc in host-pathogen interactions, we have constructed *Salmonella enterica* mutant strains in which the *znuA* gene, which encodes the periplasmic component of the ZnuABC high-affinity zinc transporter, was deleted. This mutation does not alter the ability of *Salmonella* to grow in rich media but drastically reduces its ability to multiply in media containing low levels of zinc (minimal medium) or in rich medium supplemented whit chelating agents (EDTA or TPEN). In agreement with this phenotype, ZnuA accumulates only in bacteria cultivated in environments poor in zinc. In spite of the nearly millimolar intracellular concentration of zinc, we have found that *znuA* is highly expressed in intracellular salmonellae recovered either from cultivated cells or from the spleens of infected mice. We have also observed that *znuA* mutants are impaired in their ability to grow in Caco-2 epithelial cells and

that bacteria starved for zinc display a decreased ability to multiply in phagocytes. Moreover, a dramatic reduction in the pathogenicity of the *znuA* mutants was observed in *Salmonella*-susceptible (BALB/c) or *Salmonella*-resistant (DBA-2) mice infected intraperitoneally or orally.

To better understand bacterial responses to zinc deficiency, we have also investigated the role of ZinT, a periplasmic protein with a putative role in zinc homeostasis, in *Salmonella*. We have found that *zinT* expression is regulated by Zur and parallels that of ZnuA and ZnuB. Despite ZinT contributes to *Salmonella* growth in media poor of zinc, disruption of *zinT* does not significantly affect virulence in mice. The role of ZinT became clear using strains expressing a mutated form of ZnuA lacking a characteristic histidine-rich domain. In fact, *Salmonella* strains producing this modified form of ZnuA exhibited a ZinT-dependent capability to import zinc either *in vitro* or in infected mice, suggesting that ZinT and the histidine-rich region of ZnuA have redundant function. The hypothesis that ZinT and ZnuA cooperate in the process of zinc recruitment is supported by the observation that they form a stable binary complex *in vitro*. Although, the presence of ZinT is not strictly required to ensure the functionality of the ZnuABC transporter, our data suggest that ZinT facilitates metal acquisition during severe zinc shortage.

In conclusion, this study establishes that there is a stringent control of zinc availability in eukaryotic tissues which is critically important to limit the ability of bacterial pathogens to multiply within the infected host and that functionality of the ZnuABC transporter is critical for maximizing zinc import in such a hostile environment. Moreover, we have shown that in *Salmonella* there is an additional protein (ZinT) which interacts with ZnuA and increases its ability to bind zinc. As ZnuABC is the only high affinity Zn(II) transporter in bacteria and there are no homologs in the mammalian hosts, these observations indicate that the mechanisms of zinc import could be a privileged target for novel antibacterial therapies.

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1. Introduction

1.1 *Salmonella* infections

Salmonella species are facultative intracellular Gram-negative bacteria causing a wide spectrum of diseases in humans and animals ranging from self-limiting gastroenteritis to life-threatening systemic infections. Although more than 2000 different *Salmonella* serotypes have been so far identified, only a limited number of *Salmonella enterica* serovars are recognized as important human or animal pathogens. The majority of these pathogenic serovars cause acute gastroenteritis characterized by a short incubation period and a predominance of intestinal over systemic symptoms. However, a small number of serotypes typically cause severe systemic disease characterized by septicaemia, fever and/or abortion. In any case, the type of disease caused by a specific *Salmonella* strain usually depends on the combination of the serotype and the host involved, so that a particular *Salmonella* serovar may cause a severe systemic disease in an animal species and be clinically asymptomatic in another species. This is very clear for host adapted strains such as S. enterica serovar Typhi or Pullorum, which cause systemic diseases only in humans and poultry, respectively, but also for serovars able to infect a broad range of different animals. For example, the *S. enterica* serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) cause diverse diseases in different animal species. In calves, S. Typhimurium (Petrie *et al*., 1977.) causes enterocolitis, and the animals can succumb to dehydration (Tsolis *et al*., 1999). In newly hatched chicks, serovars Enteritidis and Typhimurium cause systemic disease and diarrhea, whereas older chickens are asymptomatic carriers (Barrow *et al*., 1987; Barrow *et al*., 1987.). In immunocompetent humans, serovars Enteritidis and Typhimurium cause localized self-limiting enterocolitis, whereas systemic disease may develop in immunocompromised individuals (Tsolis *et al*., 1999.). Finally, serovars Enteritidis and Typhimurium cause a systemic typhoid feverlike disease in susceptible mouse strains, but no diarrhea. For this reason murine infection model with *Salmonella enterica* serovar Typhimurium have been useful for understanding the immune response to protect humans against S. Typhi. The mechanisms determining which type of disease is caused in which host by serovars Enteritidis and Typhimurium are still poorly understood.

Salmonella is typically acquired by the oral ingestion of contaminated food or water. After entering the small intestine, it traverses the intestinal mucous layer and evades killing by digestive enzymes, bile salts, secretory IgA, antimicrobical peptide and other innate immune in order to obtain access to the underlying epithelium (Haraga *et al*, 2008). *Salmonella* penetrates the intestinal barrier preferentially using the follicle associated epitelium overlying Peyer's patches and in particular exploits M cells (Jones *et al*, 1994), which are specialized epithelial cells that sample intestinal antigens through pinocytosis and transport them to lymphoid organs underlying Peyer's patches. M cells are typically characterised by sparse, irregular microvilli on their apical surface and by a basolateral cytoplasmic invagination that forms a pocket harbouring lymphocytes and occasional macrophages (Jepsona and Clarkb, 2001).

When S. Typhimurium comes in contact with these cells it can bypass surface receptors and manipulate the host cytoskeleton directly through the injection of an array of bacterial effector molecules into the cytoplasm of the infected host cells. The combined action of these effector proteins triggers more dramatic reorganization of the actin cytoskeleton, resulting in intense membrane ruffling and subsequent bacteria internalization. (Ly and Casanova, 2007).

Salmonella can penetrate also via enterocytes (Bolton *et al*, 1999 and Tam *et al*, 2008) or isolated lymphoid structure in witch are present M cells (Halle *et al*, 2006). Bacteria are present in this non-Peyer's patch lymphoid structure after oral infection of mice and thus it can be used for bacterial exit from the intestinal lumen (Mowat *et al*., 2003).

Moreover, dendritic cells residing in the intestinal lamina propria might provide another gateway into the organism. These cells have been shown to extend dendrites through the epithelia lining into the intestinal lumen, allowing the direct sampling of antigens as well as pathogens (Niess and Reinecker, 2006)

Bacterial translocation via villus epithelial cells themselves might also be a way for microbes to enter the lamina propria (Fig. 1.1). Although the epithelium overlying villi is less favorable to bacterial penetration than that overlying Peyer's patches, passage between epithelial cells, perhaps after bacteria-mediated destruction of epithelial layer integrity, could possibly occur (Tam *et al*, 2008).

Following passage through the epithelium of the Peyer's patch, virulent *Salmonella* strains enter the environment of the follicle dome, which is populated with host lymphocytes and macrophages. To move into deeper tissue, these bacteria must be able to avoid and/or survive the oxygen-

dependent and oxygen-independent killing mechanisms of professional phagocytes following internalization.(Jones and Falkow, 1996).

While bacteria which penetrate the intestinal epithelium via Peyer's patches or isolated lymphoid tissues land directly in a lymphoid organ, bacteria that enter the lamina propria must find their way to the mesenteric lymph node. *Salmonella* can reach the mesenteric lymph node via the lymph as free bacteria or be transported by cells, presumably dendritic cells (Tam *et al*, 2008).

Once the epithelial barrier has been breached, *Salmonella* serotypes that are associated with systemic illness are phagocytosed by intestinal macrophages. Internalized bacteria remain enclosed in a membrane-bound vacuole, referred to as the *Salmonella*-containing vacuole (SCV), which is modified by bacteria to prevent its maturation or fusion with lysosomal compartments. Bacterial directed maturation of the SCV leads to the formation of a protect intracellular niche that is permissive for bacterial replication (Ly and Casanova, 2007). Replication of pathogenic *Salmonella* into the host cells may lead to the death of these cells and to a subsequent release of bacteria, some of which may reach the blood via the thoracic duct. In addition pathogenic bacteria can also disseminate inside cells. These findings are supported by the observation that *Salmonella* is associated with phagocytes in the blood within minutes after oral infection (Worley *et al*, 2006). When *Salmonella* reaches the blood is disseminate in several additional tissue including the spleen, liver and bone marrow. In these tissues *Salmonella* is present inside dendritic cells, monocytes, macrophages and neutrophils (Tam *et al*, 2008).

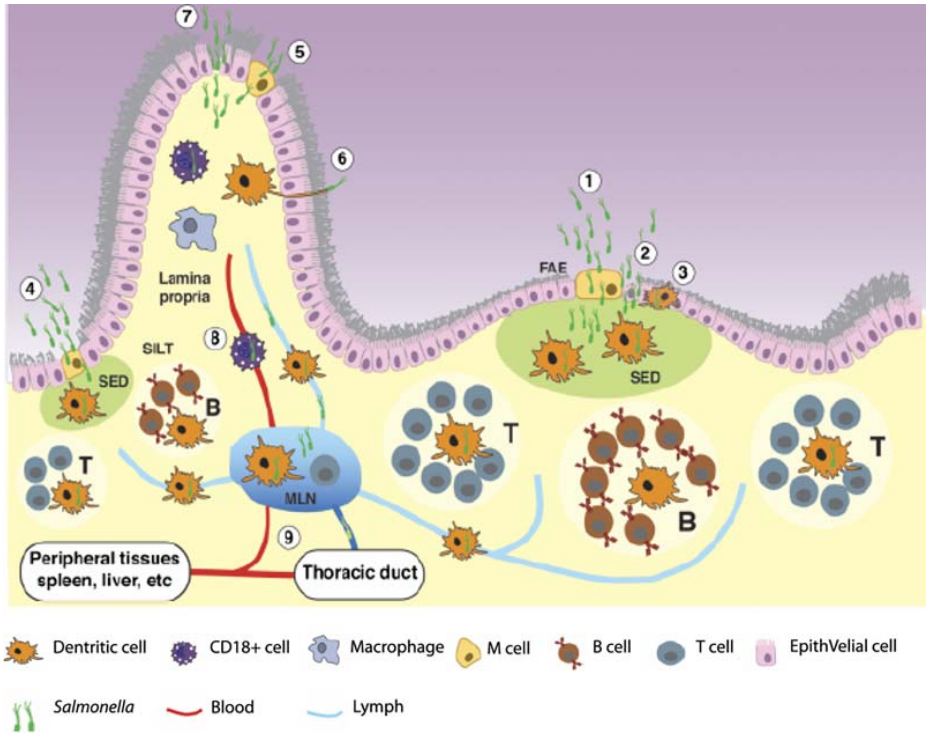


Figure 1.1: Entry and capture of orally acquired *Salmonella*. *Salmonella* can invade using different mechanisms, which are indicated with numbers. Within Peyer's patches (PP), *Salmonella* can traverse the intestinal barrier through: (1) M cells in the follicle-associated epithelium (FAE); (2) epithelial cells forming the FAE, in particular after bacteria compromise M cells and the intestinal barrier (Jones *et al*, 1994); (3) Dendritic cells (DCs), could capture *Salmonella* (Niess and Reinecker, 2006) . Once bacteria cross the FAE they can be captured by DCs located in the subepithelial dome (SED). DCs containing *Salmonella* can then initiate an adaptive immune response by stimulating T cells in the PP (marked T in the figure) or migrate to the mesenteric lymph node (MLN) to initiate adaptive immunity. In addition, *Salmonella* can cross the intestinal barrier through solitary intestinal lymphoid tissue (SILT) (Halle *et al*, 2007), probably in a similar manner as in PP, as represented in (4). At villi, *Salmonella* can enter in different ways: (5) through M cells; (6) captured by DCs extending dendrites; or (7) passing through or between compromised epithelial cells. After bacteria reach the lamina propria, they can access the MLN. Finally, bacteria may be able to reach the blood stream, presumably transported by CD181 phagocytes, as shown in (8). In addition to being transported to the MLN via lymph within DCs, it remains possible that free bacteria could be transported in lymph. (9) Bacteria can exit the MLN as free bacteria or possibly associated with cells and seed other tissues in the body (Adapted from Tam *et al*, 2008).

1.2 Salmonella strategies to evade host defences

The evolution of S. Typhimurium ability to resist against many of the host defence mechanism encountered in the lumen of the inflammed intestine has been driven by the acquisition of key virulence determinants through horizontal gene transfer (Baumler, 1997).

A remarkable feature of *S. enterica* in fact, is the presence of a large number of pathogenicity islands (PAI). PAI are distinct, relatively large chromosomal regions harbouring virulence genes that are present in pathogens but absent in benign relatives. PAI are characterized by a base composition different from the core genome and are often associated with transfer RNA (tRNA) genes and mobile genetic elements, like insertion sequence (IS) elements, transposons or phage genes.

The genome of *Salmonella* contains several of these clusters of genes, which are denominated *Salmonella* pathogenicity islands (SPIs). In general, *Salmonella* pathogenicity island 1 (SPI-1) encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory responses. In contrast, *Salmonella* pathogenicity island 2 (SPI-2) encodes genes essential for intracellular replication. Both SPI-1 and SPI-2, encode specialized devices for the delivery of virulence proteins into host cells, termed type III secretion systems (TTSSs) (Ohl and Miller, 2001). These systems appear to exert their function at different stages of the pathogenic cycle. While the SPI-1-encoded system is required to initiate intestinal infection, the type III secretion system encoded within SPI-2 appears to be required for the establishment of systemic infection (Zhoua and Galánb, 2001).

The SPI-1-encoded type III secretion system forms a needle-like complex that is responsible for the injection of bacterial effectors proteins into the host cell cytosol. Some of these effector proteins have been shown to be directly or indirectly involved in the regulation of the actin cytoskeleton dynamics (Ly and Casanova, 2007). For example, SipA promotes actin filament polymerization by reducing the monomer concentration necessary for filament assembly, enhancing the filament building activity of the host protein fimbrin and potentiating the nucleating activity of the C-terminal domain of SipC. SipC is a component of the bacterial translocon and contains two membrane-spanning domains (a 120-amino acid N-terminus and the 209-amino acid C-terminus) which extend into the host cytoplasm.

Moreover, the C-terminus portion promotes actin nucleation and effectors translocation (Ly and Casanova, 2007).

The *Salmonella* type III secretion system encoded by SPI-2, has been identified by virtue of its large impact on S. typhimurium virulence. In fact, the ability of S. typhimurium strains deficient in the SPI2-encoded T3SS to induce salmonellosis in mice is significantly decreased (Shea *et al*, 1996). SPI2 mutant strains attenuation is correlated with unability to proliferate within the organs of infected animals (Shea *et al*, 1996), and with reduced survival and proliferation inside cells.

Functional SPI-2 genes are clustered within six large transcriptional units. Thirty-one potential open reading frames on the SPI-2 region encode proteins that are directly involved in the assembly and regulation of the T3SS. The Ssa proteins are involved in the assembly of the syringe-like type III secretion injectisome. A set of nine Ssa proteins forms the injectisome core. Transport of some effectors through the injectisome is facilitated by formation of a complex between an effector and a chaperone encoded in SPI-2. For example, SscB, a protein encoded immediately upstream of sseF, acts as a chaperone for SseF (Waterman and Holden, 2003).

After phagocytosis, *Salmonella* undergoes extensive surface remodeling, as has been shown for the lipid A component of lipopolysaccharide (LPS) during growth within macrophages (Heithoff *et al*, 1999; Gibbons *et al*, 2005). Bacterial molecules that the host can recognize as indicators of infection, such as the SPI1 T3SS and flagellin, are repressed and the LPS structure is altered (Miao *et al*, 2006; Ernst *et al*, 2001). Some of the specific surface modifications include: decreasing the length of the O antigen, which is the repeating carbohydrate polymer of LPS; alterations to the number of acyl chains in the structure of the lipid A component of LPS; and changes in the protein content of the outer membrane, the inner membrane and the peptidoglycan layer (Guo *et al*, 1997; Gunn *et al*, 1998; Guo *et al*, 1998; Hilbert *et al*, 1999). Synthesis of enzymes that allow bacteria to cope with oxidative and nitrogenous radicals also occurs. Microarray studies have shown that up to 919 S. typhimurium genes are differentially regulated in response to the phagosomal environment, demonstrating that dramatic transcriptional and post-translational changes occur when *Salmonella* makes the transition from a nutrient-rich extracellular environment to the intracellular environment (Eriksson *et al*, 2003).

The observation that disruption of any single gene encoding for SPI2 T3SS effector protein reduces S. typhimurium virulence to a much lower extent than deletion of the entire SPI2 T3SS suggests that many effectors function cooperatively to exert their effects on the host cell. Furthermore, the

deletion or mutation of many effector genes has no virulence phenotype, suggesting that their functions might be redundant. Early studies of SPI2 T3SS effectors, which primarily focused on determining their subcellular localization in mammalian cells, revealed that they might have specific targeting sequences that direct their localization to endosomal compartments, the Golgi apparatus, the actin cytoskeleton and the microtubule network. These observations indicated that components of these host-cell structures were the intracellular targets of the SPI2 T3SS (Haraga *et al*, 2008).

1.3 Host response to Salmonella infection

In response to S. typhimurium, the intestinal epithelium promotes an intense inflammatory response consisting largely of the migration of polymorphonuclear leukocytes toward and ultimately across the epithelial monolayer into the intestinal lumen (Gewirts *et al*, 1999).

In addition, direct contact between bacteria and host cells can result in the production of neutrophil chemoattractants known as CXC chemokines. However, *in vivo* these mediators are substantially produced by epithelial cells located in the crypts and at the base of villi, areas of the epithelium that are not invaded by S. Typhimurium.

The first significant interactions between bacteria and the host occur at the lymphoid follicles of the intestine. (Jones, 1997).

Experiments performed to determine whether the presence of invasive S. typhimuhum in the murine intestine has an effect on the cellular composition of Peyer's patches showed that the presence of the invasive pathogen caused a number of different host responses in the Peyer's patches (Savidge *et al.* 1991). The total number of M cells increased in the follicle-associated epithelium as compared to uninfected mice, the average crypt depth lengthened, and the rate of enterocyte migration from the intestinal crypts increased. In addition, the numbers of CD4+ cells increased and CD8+ cells decreased. These results indicate that the damage elicited by invasive *Salmonella* induces a host response in the Peyer's patch tissue. Although the pathogen causes significant damage to the epithelium of lymphoid follicles at early points of infection, the findings of this study indicate that the host quickly replaces cells that have been damaged and/or destroyed. In addition, it is clear that the immune system is activated by and responds to the presence of *Salmonella* by activating both humoral and cellular immunity (Mastroeni *et al*. 1993).

Another major aspect of the antimicrobial innate immune response encountered during acute inflammation is the production of epithelial-derived antimicrobial proteins and peptides. An important cytokine for orchestrating this arm of the response is IL-22, which induces expression in epithelial cells of host defense effector molecules that seem to be directed against luminal bacteria (Figure 1.2 and Santos *et al*, 2008). During *Citrobacter rodentium* infection of mice, the IL-23/IL-22 axis is required for expression in the colonic mucosa of calprotectin, an antimicrobial protein mediating zinc deprivation, and RegIIIg (regenerating islet-derived 3 gamma), a bactericidal C-type lectin (Zheng *et al*, 2008). RegIIIg expression in the cecal mucosa is markedly increased by an IL-23 dependent mechanism during S. Typhimurium infection of streptomycin-pretreated mice (Godinez *et al*, 2009). Pretreatment of mice with streptomycin renders them susceptible to serovar Typhimurium colitis that closely resembles the inflammatory responses observed in the human colon and animal models for intestinal salmonellosis (Barthel *et al*, 2003). Secretion of RegIIIg into the intestinal lumen of wild type mice contributes to clearance of luminal bacteria, including *Listeria monocytogenes* and vancomycin-resistant *Enterococcus*, but this response is absent in mice deficient for myeloid differentiation primary response protein 88 (MyD88), an adaptor protein for all Toll Like Receptors except TLR3 (Brandl *et al*, 2007).
In vitro, IL-22 induces the expression in human colonic epithelial cells of inducible nitric oxide synthase (iNOS), mucin (MUC4) and lipocalin-2, an antimicrobial protein that prevents bacterial iron acquisition (Raffatellu *et al*, 2009). Whereas lipocalin-2 secretion is induced upon IL-22 stimulation, S. Typhimurium infection of colonic epithelial cell lines does not induce expression, suggesting that activation of this epithelial antimicrobial response requires paracrine IL-22 signaling rather than direct interaction of bacteria with enterocytes. *In vivo*, the epithelial cells of the ileal mucosa of rhesus macaques produce large quantities of lipocalin-2 in response to S. Typhimurium infection, resulting in accumulation of this antimicrobial in the intestinal lumen (Raffatellu *et al*, 2009). Other antimicrobial proteins and peptides whose transcripts are prominently induced in the intestinal mucosa during S. Typhimurium infection include iNOS, calprotectin, MUC4, dual oxidase 2 (Duox2) and bovine enteric b defensin (Godinez *et al*, 2008; Raffatellu *et al*, 2008; Raffatellu *et al*, 2007).

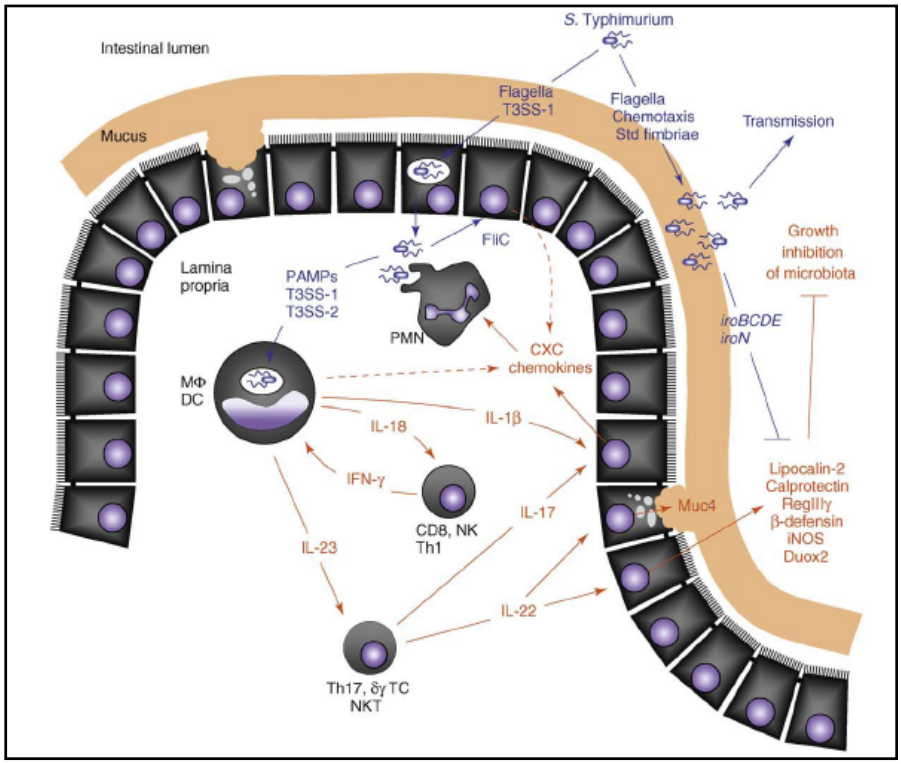


Figure 1.2: The inflammation-adapted pathogenic lifestyle of S. Typhimurium. The schematic shows host factors (red) and bacterial factors (blue) contributing to the development of acute intestinal inflammation within hours after S. Typhimurium infection. The bacterium initiates intestinal inflammation through direct interaction with host cells, resulting in a release of cytokines, such as IL-18 and IL-23. The cytokines IL-18 and IL-23 help to amplify responses in tissue by stimulating T cells to produce IFN-g (which activates macrophage antimicrobial activities), IL-17 (which orchestrates neutrophil barrier function) and IL-22 (which stimulates epithelial antimicrobial responses). The ability to grow on mucus carbohydrates and its resistance against antimicrobials (e.g. lipocalin-2 resistance mediated by the *iroBCDEN* genes) enable S. Typhimurium to benefit from the changes encountered in the inflamed intestine, resulting in its luminal outgrowth and enhanced transmission. CD8, CD8+ ab T cell; DC, dendritic cell; MF, macrophage; NK, natural killer cell; NKT, natural killer T cell; PAMPs, pathogen associated molecular patterns; PMN, neutrophil; dg TC, dg T cell; Th1, CD4+ ab memory type-1 T-helper cell; Th17, CD4+ ab memory type-17 T-helper cell (Adapted by Santos *et al*, 2008)

A variety of molecules in the mammalian intestine can impair growth and survival of microbes in this environment. One source of inhibitory molecules stems from many of the colonizing microbes themselves. Bacteriocins, including colicins, microcins, lantibiotics and others, are proteinaceous toxins that inhibit the growth of related bacteria (Baba *et al*, 1998). These molecules probably contribute to niche protection and inhibit colonization by potential pathogens. The other source of inhibitory molecules are products of the host, some of which are host defense molecules per se, whereas others have primary function in nutrient absorption. For example, bile salts and hydrolytic enzymes required for digestion are toxic for microbes and might contribute to controlling microbial proliferation and survival. Within the group of primary host defense molecules, epithelial cells make various peptides along the intestinal tract, some of which are constitutively expressed whereas others can be transcriptionally induced. In the small intestine, Paneth cells are the source of abundant quantities of antimicrobials in most mammals (Porter *et al*, 2002).. Among the most abundant and extensively studied antimicrobials of Paneth cells are the defensins. Constitutively expressed at high levels, defensins typically have membrane-targeted antimicrobial activity against a wide range of bacteria, some fungi and parasites. Transgenic and knockout mouse models have provided evidence that defensins contribute to the host defense against bacterial pathogens (Salzman *et al*, 2003; Wilson *et al*, 1999). Other secretory granule-associated constitutive antimicrobials produced by these cells are lysozyme and secretory phospholipase A2. Paneth cells also make some inducible antimicrobials, including RegIIIg, a C-type lectin with selective activity against Gram-positive bacteria (Cash *et al*, 2006; Brandl *et al*, 2007) In the colon, epithelial cells inducibly express a collection of antimicrobial peptides and proteins, including Resistin-like beta (RELM-b), RegIIIg, calprotectin, and b-defensins (Santos *et al*, 2009).

1.4 Infection and nutrient: the case of iron

The interaction between pathogenic bacteria and their host is determined by survival strategies on both sides, including competition for essential nutrients. In fact, deprivation of nutritive resource has an important role in host defences, but, during evolution, pathogenic bacteria have developed strategies to access specific nutrients from the host (Ratledge and Dover, 2000; Schaible and Kaufmann 2004; Schaible and Kaufmann, 2005).

An instructive example for nutritive host-pathogen competition is represented by the mutual requirement for iron.

Iron is an essential growth factor for bacteria and parasites but it is also required for the host metabolism and other important host functions. In the host, essentially all the available iron is bound to specific proteins, such as transferrin, lactoferrin and ferritin, or is complexed to haem within haemoproteins (Hentze *et al*, 2004; Kaplan *et al*, 2002).

Host cells respond directly to invasive pathogens by altering their iron status. For example, macrophages, which are the principal cells that sequester invading bacteria, produce key proteins that alter their own iron status, by sequestering iron in the intravacuolar compartments colonized by invading bacteria. Such proteins are the natural resistance-associated macrophage proteins, of which two related forms are known, Nramp1 and Nramp2 (Schaible and Kaufmann, 2005).

As iron is an essential element for its growth and survival, *Salmonella* has evolved several strategies to access mammalian iron resources. Through secretion of siderophores, *Salmonella* is able to bind ferric iron with high affinity. Iron-bound siderophores are then internalized by outer membrane receptors of *Salmonella*, such as IroN, FepA and Cir (Hantke *et al*., 2003; Rabsch *et al*., 2003). After binding to their specific receptors at the outer bacterial membrane, iron-bound siderophores are delivered to periplasmatic-binding proteins for shuttling through the periplasmatic space and finally taken up in the cytoplasm using ABC transporters, such as FepBCDG and iroC (Nairts *et al*, 2007). Moreover, S. typhimurium expresses another protein named Feo as a mechanism for ferrous iron uptake, which is important during conditions characterized by low oxygen tension (Hantke, 2001).

1.5 Zinc and infection

Zinc is an essential trace element which is required by all living cells as it plays key roles in a very large number of molecular processes. It is a component of more than 300 enzymes from all six major functional classes, where it has catalytic, structural, or regulatory roles. Zinc-dependent biological functions include DNA replication, RNA transcription, signal transduction, enzymatic catalysis, redox regulation, cell proliferation, cell differentiation, and apoptosis (Overbeck and Haase, 2008).

The importance of zinc for human health is demonstrated by the clinical manifestations associated with zinc deficiency, which comprise growth retardation, thymic atrophy, hypogonadism, infertility, dermatitis, delayed wound healing, alopecia, poor pregnancy outcomes, teratology, anorexia, diarrhea, and increased susceptibility to infectious diseases caused by bacterial, viral, and fungal pathogens. (Overback, 2008)

Study performed in a mouse model showed that after infection with *Salmonella enterica* serovar Typhimurium the concentration of zinc in the plasma decreases significantly, byincreases in the liver (Rishi *et al*, 2007). A parallel reduction of iron content in plasma, a critical aspect of the acute phase response to bacterial infections, has been observed in response to a wide range of bacterial pathogens. This redistribution of zinc between tissues is regulated by cytochines, in particular IL-6, which stimulates the removal of this metal from plasma. It has been shown that IL-6 induces the expression of the zinc transporter Zip 14, thereby increasing zinc uptake into hepatocytes (Liuzzi *et al*, 2005). IL-6 also upregulates the zinc binding protein metallothionein and increases cellular zinc in hepatocytes (Schroeder *et al*, 1990). Experiments with metallothionein knockout mice confirmed that during endotoxin-induced inflammation, metallothionein is required for zinc sequestration in the liver, leading to a significant reduction in plasma zinc levels (Philcox *et al*, 1995). The significance of the accumulation of hepatic zinc has not yet been explained but the most common thought is that it is useful for the synthesis of zinc-proteins of acute phase and the synthesis and maturation of immune cells. This hypothesis is supported by the observation that slight zinc deficiency causes a significant depression of the immune defences (Ibs and Rink, 2003).

In addition, zinc affects several aspects of monocytes signal transduction and secretion of pro-inflammatory cytokines. In fact, it has been shown that zinc supplementation has been shown to reduce the production of tumor

necrosis factor (TNF)-α and interleukin (IL)-1β in healthy human subjects (Ibs and Rink, 2003).

It is known that an increased hepatic concentration of zinc during infection is associated with beneficial responses, such as an increased hepatic synthesis of acute-phage globulins and an increased protection against hepatocellular damage from bacterial endotoxin. Zinc may protect against endotoxin-containing bacteria (e.g., S. typhimurium and E. coli) by stabilizing lysosomes or inhibiting lysosomal proteases (Bradley and Kluger, 1984) Among the immune cells that are affected by zinc deficiency, T lymphocytes seem to have the highest susceptibility. In fact,. zinc deficiency reduces the number of peripheral and thymic T cells, their proliferation in response to phytohemagglutinin, and the functions of helper and cytotoxic T cells, but also acts indirectly by reducing the levels of active serum thymulin (Overbeck *et al*, 2008). At the molecular level, zinc stimulates the autophosphorylation of the protein tyrosine kinase Lck by non-covalent interaction with the cytoplasmic tails of CD4 and CD8, leading to T cell activation. As a result, the delayed-type hypersensitivity reaction is usually reduced in zinc-deficient individuals (Salgueiro *et al*, 2000; Turner *et al*, 1990).

Moreover, other cells are also affected, leading to reduced antibody production and compromised function of cells of the innate immune system, such as natural killer cell activity, cytokine production by monocytes, and the chemotaxis and oxidative burst of neutrophil granulocytes (Ibs and Rink, 2003; Rink and Gabriel 2001).

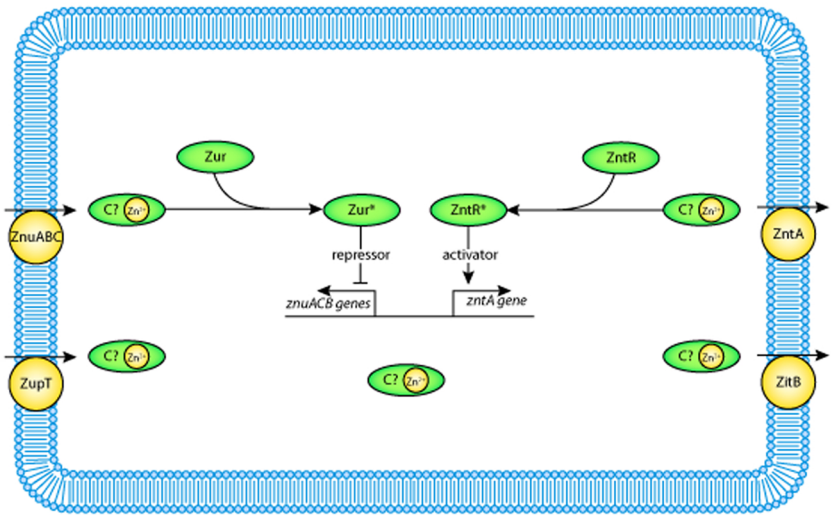
Another important aspect is the interaction between inflammation and zinc. Pro-inflammatory cytokines have a direct influence on zinc homeostasis. It has been shown that IL-6 induces the expression of the zinc transporter Zrt- and Irt-like protein ZIP14, thereby increasing zinc uptake into hepatocytes. (Overback *et al*, 2008)

1.6 Zinc homeostasis in bacteria

Although zinc plays an essential role in a large number of biological processes, it can be toxic at high concentrations, and, therefore, intracellular zinc levels must be carefully regulated.

Zinc homeostasis in bacteria is achieved by balancing export systems and uptake systems. In Gram-negative bacteria, as shown in figure 1.3, zinc ions are mainly transported into the cell via ZnuABC (an ABC-type transporter)

and ZupT (a zinc permease), whereas they are removed from the cell via ZntA (a P-type ATPase) and ZitB (a cation diffusion facilitator).



Schematic representations of E. coli zinc homeostasis system and the *in vitro* sub-processes 1.3: A schematic graph depicts the Zn2+ homeostasis system in *E. coli*. Extracellular Zn2+ enters the cytoplasm through ZnuABC and ZupT(Patzer and Hantke, 1998 Grass *et al*, 2002). In the presence of zinc, Zur binds to the znu operator and represses the transcription of znuACB gene cluster (Patzer and Hantke, 2000). Excess intracellular zinc ions are exported by ZntA and ZitB (Rensing *et al*, 1997; Chao and Fu, 2004; Grass *et al*, 2001). Intracellular zinc can bind with protein ZntR and convert it into a strong transcriptional activator of the *zntA* gene (Brocklehurst *et al*, 1999). The cytoplasmic zinc trafficking may involve chaperonelike proteins (Outten and Halloran, 2001). Abbreviations used in this graph are as follows: Zur* (active Zur); ZntR* (active ZntR); C? (zinc chaperone whose existence is still under debate) (Outten and Halloran, 2001), (Adapted by Cui *et al* 2008).

Whereas intracellular zinc is nearly millimolar, transcription of zinc uptake or efflux machinery are triggered by femtomolar zinc concentrations in vitro, i.e. six orders of magnitude less than one atom per cell. This is not consistent with a cytosolic pool of free zinc and suggests an extraordinary intracellular zinc-binding capacity. This evidence has led to hypothesize the presence of zinc chaperones in the cytoplasm, although the existence of this kind of proteins is still under debate (Outten and Halloran, 2001).

1.6.1 Export system

The ZntA protein from *E. coli* belongs to the family of P-type ATPases and confers resistance to cadmium and zinc (Rensing *et al*, 1997). P-type ATPases transport metal ions across membranes against a concentration gradient by utilizing the energy liberated from ATP hydrolysis.

Zinc efflux through ZntA is regulated by ZntR, a zinc-responsive MerR-like transcriptional regulator (Brocklehurst *et al*, 1999). The binding of zinc-bound ZntR to the promoter introduces conformational changes in the DNA, which apparently make the promoter a better substrate for RNA polymerase, thus strongly activating the transcription of the *zntA* gene and increasing the efflux of zinc from the cell.

Another system for zinc export is constituted by ZitB (Chao and Fu, 2004; Grass *et al*, 2001).This protein belongs to the Cation Diffusion Facilitator family (CDF), an ubiquitous family of metal transporters found in prokaryotes and eukaryotes (Paulsen *et al*, 1997).

An *E. coli* strain disrupted only in *zitB* did not exhibit decreased zinc tolerance, perhaps because its absence was balanced by the activity of ZntA. It is likely that ZitB contributes to zinc homeostasis at low concentrations of zinc, while ZntA is required for growth at higher and more toxic metal concentrations (Hantke, 2001).

1.6.2 Import system

ZupT is a low affinity zinc transporter and represents the first identified bacterial member of the ZIP (ZRT IRT-like Protein) family. To assess the physiological role of ZupT Grass and collaborators have generated an *E. coli* mutant strain devoid of the *zupT* gene and a strain deleted in both *zupT* and the *znuABC* operon. Whereas the deletion of *zupT* only slightly affected bacterial growth under zinc deficiency, the growth of the double mutant was severely inhibited by the presence of EDTA. This inhibition was more pronounced for the double mutant than for the mutant lacking *znuABC*. Addition of zinc but not of nickel, copper, or cadmium alleviated this inhibition, indicating that *zupT* is responsible for zinc uptake (Grass *et al*, 2002).

When bacteria grow in condition of zinc deprivation the metal is transporter into the cytoplasm via ZnuABC, that is the only high affinity zinc transporter in Gram-negative bacteria (Patzer and Hantke, 1998). This transporter belongs to the family of ABC transporter and like all the member

of this family, consists of three components: ZnuA, ZnuB and ZnuC. ZnuB is a membrane permease, ZnuC is the ATPase component of the transporter, whereas ZnuA is a periplasmic metallochaperone, which efficiently captures zinc in this cellular compartment and then delivers the metal to the transmembrane component of the transporter (Cui *et al*, 2008).

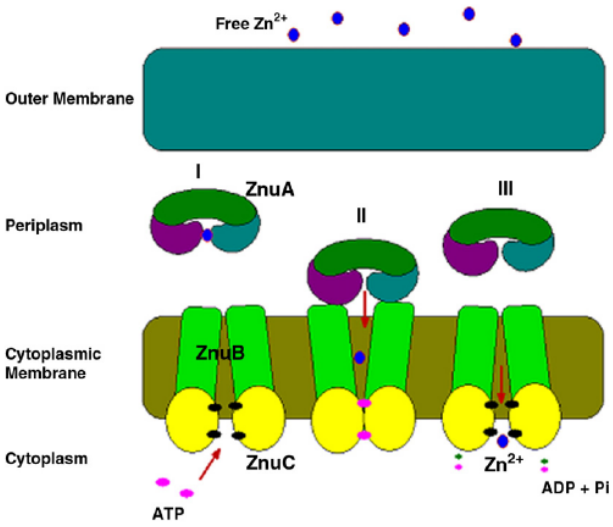


Figure 1.4: Schematic representation of ZnuABC transporter. ZnuA captures zinc in the periplasmic space (I) and then deliver it to ZnuB (II), the membran permease,that after ZnuC mediated ATP hydrolisis, transfer this metal into the cytoplasm (III) (Adapted from Chandra *et al*, 2007).

The genes encoding for these proteins are organized in a single operon in which the genes *znuA* and *znuCB* are transcribed divergently and the genes are separated by an unusually short intergenic region of 24 base pairs (Patzer anḍ Hantke, 1998). In addition, the coding region of *znuC* gene is partially overlapped to *znuB* gene. *znuA* is upstream *yebA*, a gene encoding a hypothetical zinc-dependent protease with unknown function.

The ZnuABC transporter was identified for the first time in *E. coli* (Patzer anḍ Hantke, 1998) and then also in *Salmonella* (Campoy *et al*, 2002).

It has been shown that an *E. coli* strain bearing mutations in the *znuABC* operon is unable to grow in a medium supplemented with the metal chelating agent EGTA at a 0.5 mM concentration, whereas the wild type is able to

grow. Supplementation of the medium with zinc restores the growth of the mutant strain, which, in presence of zinc, grows as well as wild type strain (Patzer and Hantke, 1998).

Investigations initially carried out in *E. coli* and then confirmed in other microorganisms have established that zinc homeostasis is finely controlled by the coordinated activity of import and export systems regulated by Zur and ZntR, two metalloproteins able to regulate gene transcription depending on their metallation state (Patzer and Hantke, 1998; Outten *et al*., 1999). Zur controls the expression of a few genes involved in bacterial response to zinc shortage, whereas ZntR regulates the expression of the zinc efflux pump ZntA. It is worth observing that, while the intracellular zinc concentration is rather constant and independent of the culture medium (close to 200 μM), both these regulators are able to respond to femtomolar variations in the intracellular concentration of free zinc (Outten and O'Halloran, 2001). These observations give emphasis to the dynamic nature of metal homeostasis and suggest that very small alterations in the intracellular zinc concentration may have a relevant influence on cellular physiology.

To identify the zinc-dependent regulator of the *znu* genes in *E. coli*, constitutive mutants were isolated and tested for complementation by a gene bank of *E. coli.* A complementing gene, *yjbK* of the *E. coli* genome, was identified and named *zur* (for zinc uptake regulation). The Zur protein shows 27% sequence identity with the iron regulator Fur. High affinity $^{65}Zn2+$ transport of the constitutive *zur* mutant is ten fold higher than that of the uninduced parental strain (Patzer and Hantke 1998).

Using an *in vivo* titration assay, the nucleotide binding site affording Zur regulation was narrowed down to a 31-bp region in the promoter region of *znuA* and *znuCB.* This location was confirmed by DNase I footprinting assays. Zinc chelators completely inhibit DNA binding of Zur, and addition of zinc in low concentrations enhances binding (Patzer and Hantke, 2000).

Zur protected a 23-bp palindrome GAAATGTTATAWTATAACATTTC on each strand of the *znu* operator upstream of the *znuA* gene (Patzer and Hantke, 2000). This footprint resembles that of typical DNA binding dimers, such as classical helix-turn-helix proteins. Analysis of the mutant Zur proteins suggested an aminoterminal DNA contact domain around residue 65 and a carboxy-terminal dimerization and zinc-binding domain. The repressing activity of the Zur protein is Zn2+ specific since addition of Cd2+, Hg2+, Pb2+, Mn2+, Fe2 +, and Cu2 + led to a derepression of the Znu transport system *in vivo* (Patzer and Hantke 2000).

In addition to the genes encoding for the proteins forming the ZnuABC transporter, Zur directly regulates one or more genes encoding paralogs of

ribosomal proteins (Panina *et al.*, 2003; Akanuma *et al.*, 2006; Shin *et al.*, 2007). The Zur-regulated ribosomal proteins lack a zinc-binding motif that is present in their paralogs, which are normally produced in zinc-replete conditions. The insertion of these proteins in ribosomes during zinc starvation likely facilitates growth by reducing the zinc requirements of bacterial cells.

Studied carried out in *Bacillus subtilis* have shown that there are two types of L31 protein, RpmE and YtiA. These proteins exchange in the ribosome in response to zinc concentration. During the growth in zinc repleted condition RpmE is present in the ribosome. Under this condition, the zinc containing Zur protein binds to the Zur box of the *ytiA* gene, thereby repressing its expression. Under condition of zinc starvation, RpmE would not contain the zinc ion and would thus no longer be stable in the cell. However, in the absence of zinc, Zur is unable to bind to the "Zur box," and derepression of ytiA occurs leading to YtiA incorporation in the ribosomes in placeof RpmE. . Since the newly synthesized YtiA has a higher affinity for the ribosome than RpmE, YtiA can be efficiently incorporated into the ribosome and actively displaces bound RpmE. RpmE thus released is then degraded by an unknown protease(s). On the other hand, under these conditions newly synthesized RpmE would not be able to bind zinc and would thus be unstable. In this model, zinc plays an important role in regulating the alternation between two types of L31 protein. Since ribosomes are highly abundant in the cell, this alternation may be virtually able to increase the concentration of zinc ions which are available for other zinc-binding proteins in the cell. Therefore, this regulatory system would contribute to the zinc homeostasis in the cell under zinc-deficient conditions, as proposed by Panina and collaborators (Panina *et al.*, 2003).

1.7 Role of ZnuABC in bacterial virulence

ZnuABC disruption reduces the ability of Gram-negative bacteria to grow in zinc-depeleted media. In addition, it has been shown that pathogenicity of some bacteria is dramatically affected by ZnuABC inactivation (Campoy *et al*, 2002; Chen *et al*, 2001; Garrido *et al*, 2003; Kim *et al*, 2003; Kim *et al*, 2004; Lewis *et al*, 1999; Lu *et al*, 1997, Yang *et al*, 2006).

The first study that has shown that ZnuA can play an important role in the infection process has been conducted on the bacterial pathogen *H. ducreyi* Lewis and coworkers demonstrated that mutation in the *znuA* gene causes a

significantly reduction of virulence in the temperature-dependent rabbit model. This decreased virulence was not observed when the *znuA* mutant was complemented with the wild-type *H. ducreyi znuA* gene provided in *trans* (Lewis *et al*, 1999).

A similar result was obtained for *P. multocida* where it was demonstrated the importance of znuA and znuBC genes in virulence in a mouse model of infection (Garrido *et al*, 2003).

In *B. abortus* it was shown that deletion of *znuA* causes a growth reduction in zinc depleted medium and failure to replicate in Hela cells and mouse bone marrow-derived macrophages. Transformation of a mutant strain with a plasmid containing *znuA* gene restored the growth in zinc-chelated medium and replication into Hela cells and macrophages.
No relevant differences in bacterial internalization and phagosome-lysosome fusion after uptake *in vivo* were detected, indicating that the ZnuABC zinc uptake systems has important roles for virulence and contributes to utilization of nutrients required for intracellular growth, but does not affect bacterial internalization or intracellular trafficking of *B. abortus* (Kim *et al*, 2004). In addition, Yang and collaborators using a *B. abortus znuA* mutant strain showed that its virulence in BALB/c mice was attenuated (Yang *et al*, 2006).
Studies carried out in S. typhimurium showed that the virulence of either orally or intraperitoneally inoculated *znuC* mutant strain is significally decreased with respect to the wild-type strain. (Campoy *et al*, 2002)
All these data, while referring to a limited number of bacterial species, show that ZnuABC is important not only for bacterial growth, but also for virulence of pathogenic bacteria.

1.8 ZnuA: periplasmic component of ZnuABC transporter

ZnuA is the periplasmic component of ZnuABC transporter, which efficiently captures zinc in the periplasmic space and then delivers the metal to the transmembrane component of the transporter (ZnuB).
This protein is homologous to other proteins involved in the transport of metal ions in both Gram-positive and Gram-negative bacteria (Claverys et al, 2001). Proteins homologous to *E. coli* ZnuA are for example ZnuA of *Sinechocystis 6803*, TroA of *Treponema pallidum* and PsaA of *Streptococcus pneumonia*
All these proteins belong to a large family of binding proteins that recognize either zinc, manganese, or iron as their substrate. Binding proteins of ABC

transporters has been grouped into eight clusters (Tam & Saier 1993), but since these metal-binding proteins has new characteristics, they were defined as the cluster 9 family of binding proteins by Dintilhac and coworkers (Dintilhac *et al*, 1997). This cluster comprises two metal-binding receptor families with primary specificities for zinc and manganese (Claverys, 2001).
Structural information is currently available for seven metal transporters of the cluster 9 family: the Zn2+-bound Mn-transporter PsaA (Lawrence *et al*., 1998) and AdcAII (Loisel *et al*., 2008) from *S. pneumoniae*, ZnuA from *E. coli* (ZnuA-Ec; Li & Jogl, 2007; Chandra *et al*., 2007, Yatsunyk *et al*, 2008), ZnuA from *Synechocystis* 6803 (ZnuA-Syn; Banerjee *et al*., 2003) and TroA from *Treponema pallidum* (Lee *et al*., 1999), the Mn2+-bound MntC from *Synechocystis* 6803 (Rukhman *et al*., 2005) and the more recent structurally characterized laminin binding protein (Lbp) from *S. pyogenes* (Linke *et al*., 2009).
The overall architecture of these proteins is similar to that of other periplasmic ligand binding proteins (PLBPs) from other ABC transporter systems. A substrate binding cleft is located between two domains connected by a flexible hinge region. PLBPs are proposed to function by a ‘‘Venus-fly-trap’’ mechanism with an open, solvent-accessible ligand-free state and a closed ligand-bound state that are in kinetic equilibrium. Binding of the substrate shifts the equilibrium toward the closed conformation (Liliya *et al*, 2008).
ZnuA has the same structure with a “C clamp” shape that is composed of two $(\beta/\alpha)_4$ domains related by a pseudo 2-fold symmetry (Banerjee *et al*, 2003).
The crystal structure from *Synechocystis* 6803 ZnuA (ZnuA_Syn) shows that the metal-binding site lies in the cleft between the two domains and is comprised of three histidine residues and one water molecule. The crystal structure from *E. coli* ZnuA (ZnuA_Ec) revealed that the zinc ion could be coordinated by three histidine residues (His78, His161, His225) and one glutamate (Glu77) (Li and Jogl, 2007; Yatsunyk *et al*, 2008). The metal coordination by Glu77 was unexpected based on sequence alignments of cluster 9 SBPs and previous structure determinations.
Least-squares superposition of the ZnuA_Ec metal-binding site with that of ZnuA_Syn illustrates that Glu77 occupies the fourth metal-coordination site. This residue is unique in the *E. coli* protein and replaces a hydrophobic residue (proline) in the ZnuA_Syn structure. Despite the unusual location of this residue, the coordinating oxygen atom is placed in a very similar position to the solvent water in ZnuA_Syn or to the oxygen of Asp279 in TroA or Asp295 in MntC (Li and Jogl, 2007). However, the Glu77 ligand

was not observed in a distinct crystal structure of the same proptein, suggesting that his role is less important than that of the three conserved histidine ligands (Chandra *et al*., 2007).
ZnuA has a highly charged and mobile loop that protrudes from the protein in the vicinity of the metal binding site. This region is not found in proteins that bind manganese
The function of this loop remained unknown but it was proposed to play a role in periplasmic metal acquisition (Banerjee *et al*, 2003), in the regulation of the ABC permease activity through zinc sensing (Wei *et al*, 2007), and/or in the ZnuA/ZnuB interaction (Claverys, 2001). There are limited experimental data to support these hypotheses, however. Notably, a number of Zn2+/Cd2+ P-type ATPases such as E. coli ZntA and Anabaena PCC sp. 7120 AztA (Liu *et al*, 2005) contain a similar loop, implying a common role for this motif in Zn2+ transport (Liliya *et al*, 2008).

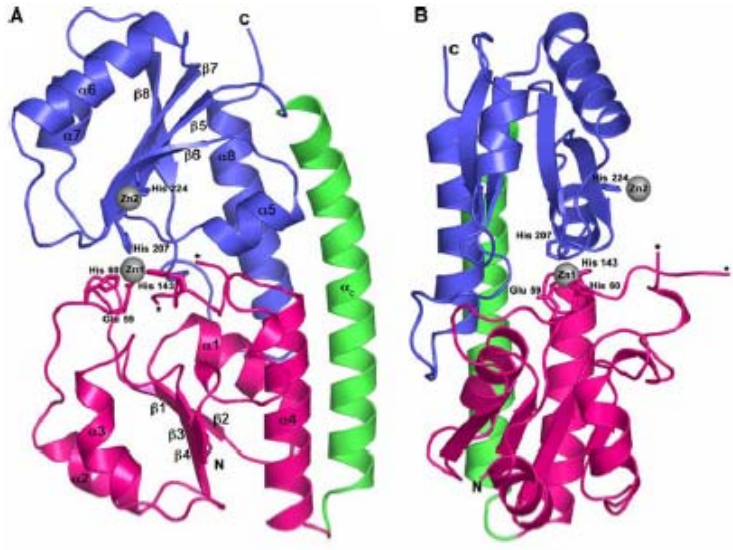


Figure 1.5: Crystal structure of zinc-bound ZnuA. **a)** Ribbon diagram of ZnuA showing the N-terminal domain in pink, the C-terminal domain in blue, and the connecting α-helix in green. The zinc ions are represented as gray spheres. The ends of the disordered His-rich loop are indicated by asterisks. **b**) Viewed approximately 90° around the vertical axis from the orientation in a (Adapted from Yatsunyk *et al*, 2008).

1.9 ZnuB and ZnuC proteins

The transmembrane component of ZnuABC transporter is codified by the *znuB* gene that after its activation led to a production of a 27.7 kDa protein. Due to the difficulties in purifying membrane proteins, there is no structural information about this protein. The only information available comes from studied carried out in *E. coli* in which the authors applied PhoA/GFP fusion approach to derive topology models for almost the entire E. coli inner membrane proteome (Daley *et al*, 2005). PhoA and GFP have opposite activity profiles: PhoA is active only in the periplasm of E. coli, whereas GFP is fluorescent only in the cytoplasm. When fused in parallel to the C terminus of a membrane protein, PhoA and GFP can accurately report on which side of the membrane the C terminus is located.
These studies revealed that the C-terminal domain of ZnuB is located in the cytoplasm (Daley *et al*, 2005).
The third component of the complex is ZnuC, this protein has a molecular weight of about 27.9 kDa. Very little is known about this protein, but its sequence is homologous to that of ATP-ase component of other ABC transporters.
The sequence of ZnuC contain the highly conserved Walker A and B consensus motifs for nucleotide binding and the 'LSGGQ' motif, the diagnostic signature sequence of ABC proteins.
Interestingly, the C-terminal region of ZnuC is characterized by a region rich in histidine and acidic amino acids. Therefore, it is tempting to speculate that the C-terminal region of ZnuC binds zinc and regulates the ATPase activity on the cytosolic side of the ABC transporter. At high cytosolic concentrations of zinc, metal could bind to the C-terminus of ZnuC and inhibit the import of further zinc (Wei *et al*, 2007).

1.10 ZinT: a periplasmic protein with a putative role in zinc import

Gram-negative bacteria have two compartments in which zinc is needed: the cytoplasm and the periplasm, in which several enzymes require zinc as a cofactor. Some representative examples from *E. coli* are: Cu,ZnSOD, PhoA, FtsK, YebA, YodA and ZnuA (Hantke, 2005).

Several of these enzymes seem to obtain their zinc cofactor in the periplasm and, therefore, under zinc-limiting conditions the availability of zinc for these enzymes, could be limited by the activity of the ZnuABC high-affinity zinc transporter.
This possibility is supported by studies carried out in our laboratory, which have demonstrated that periplasmic zinc-binding proteins effectively compete for metal binding when zinc availability is low (Berducci *et al*, 2004). The availability of zinc in the periplasmic space of *E. coli* has been investigated using a mutant Cu,Zn superoxide dismutase whose dimerization is triggered by zinc binding. This mutant enzyme accumulates in the monomeric form when wild type cells are grown in minimal medium, but assembles in the dimeric form when it is produced in the same medium by a mutant strain lacking the periplasmic zinc metallochaperone ZnuA (Berducci *et al*, 2004).
After this observation it was hypothesized that the presence of zinc-binding chaperones in the periplasm that might balance the activity of ZnuA by delivering zinc to apo-enzymes in need of their cofactor (Hantke, 2005). It was suggested that a putative candidate for this chaperone function in *E. coli* could be a protein named ZinT, which was previously identified as a cadmium-induced protein and therefore related to cadmium resistance.

Using a bioinformatics approach, upstream *zinT* gene was identified an additional putative Zur binding site (Panina et al., 2003).
Subsequent studies on *E. coli* have demonstrated that *zinT* is modulated in bacterial cells exposed to low pH (Birch *et al*, 2003; Kannan *et al*, 2008), to copper ions (Kershaw *et al*, 2007), to the transition metals chelator TPEN (Sigdel *et al*, 2006). Recent study show that the expression of *zinT* is induced in media containing very low levels of zinc and that the *zinT* gene is regulated by Zur (Graham *et al*, 2009).
ZinT crystal structure, which has been solved in the presence of different metal cofactors (cadmium, zinc or nickel) (David *et al*, 2002, David *et al*, 2003), revealed that two zinc binding site are present in the structure. One zinc ion is coordinated by His144 and His155, whereas the other interacts with His153, His193, and the caboxyl moiety of Glu189 (Fig. 1.6 B). Several water molecules lie in close contact with the zinc ions, but the authors are not able to give a precise description of the coordination geometry (David *et al*, 2003). The three-dimensional structure of YodA consists of two domains: an antiparallel, up-down β-barrel flanked by one α-helix (the "calyx" domain), and a helical domain that opens out at the side of the calyx β-barrel (the "helix" domain) (David *et al*, 2003).

Studied carried out on Enterohemorrhagic *E. coli* have shown that in this bacterial species a portion of YodA is secreted and that the strain lacking *zinT* is unable to adhere to Hela cells. Secretion of YodA by *E. coli* K-12 has not been observed, perhaps because the genes encoding the *E. coli* K-12 T2SS are usually transcriptionally silenced.
Although these investigations suggest that ZinT is involved in zinc homeostasis the exact function of this protein has not been elucidated.
Interestingly, ZinT structure (David *et al*, 2002, David *et al*, 2003) shows a very high homology to a domain of AdcA, a component of an ABC transporter involved in zinc acquisition in *Streptococcus pneumoniae* (Dintilhac *et al*, 1997, Panina *et al*, 2003). As the N-terminal portion of AdcA is homologous to ZnuA, this observation strongly suggests that ZinT could cooperate with ZnuA in zinc uptake within the periplasmic space.

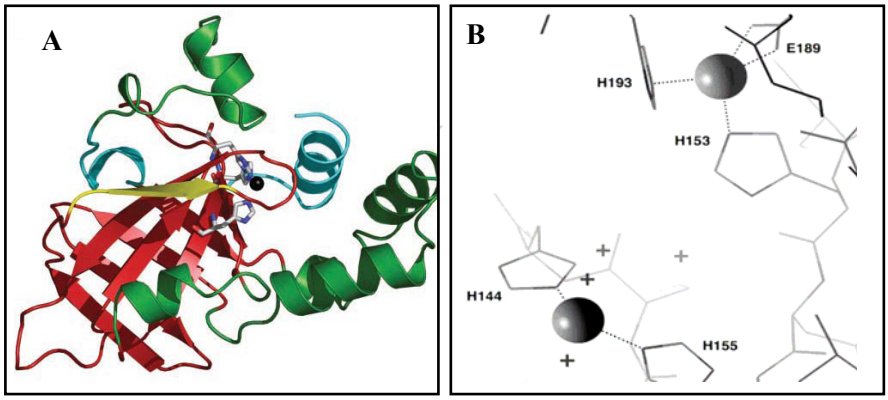


Figure. **A) Schematic views of the YodA molecule and its metal binding sites**. The "calyx" domain is in *brick red* (β-barrel) and *cyan* (helices), whereas the helical domain are colored in *green* (helices). The three histidines and the metal ion in the central metal-binding site are *highlighted*. **B) Detail of the metal-binding site in the zinc crystal forms**. (Adapted from David *et al*, 2003).

2 Aims of the project

The ability of bacteria to colonize specific environments relies on their ability to obtain adequate supplies of the nutrients that are indispensable for their growth. Of particular relevance for human and animal health is to understand how bacterial pathogens face the problem of nutrient limitation in the infected host, in which several essential elements are not freely available for infectious microorganisms. Between essential nutrients there are transition metals, which are used by all organisms as cofactors in a large number of proteins. It is well known that iron availability in eukaryotic tissues is carefully controlled by sequestration mechanisms involving proteins which remove iron from the cellular and extracellular compartments accessible to bacteria. Thus, pathogenic bacteria have evolved sophisticated strategies to acquire and utilize host iron, involving the production of molecules (siderophores, hemophores and membrane associated pumps) characterized by an extraordinary high iron affinity. The outcome of the competition for iron between the host cell and the microorganism is considered one of the most important factors which determine the ability of pathogens to multiply and cause the disease.

Although iron is traditionally considered the most important trace metal involved in the host-pathogen interaction, some recent studies have suggested that also the efficient uptake of other divalent metals plays a critical role in infection and has a major role in virulence. In particular, a few observations carried out in different bacterial pathogens have suggested that zinc is not freely available within the infected host. However, there is still some resistance in considering zinc availability as an element able to limit bacterial pathogenicity, because its concentration in all cells and in plasma is quite high.

In this context, the general purpose of this project has been to clarify the mechanisms of zinc import under conditions of metal deprivation in vitro and to analyze the importance of zinc uptake in bacterial infections. This problem has been investigated in *S.* Typhimurium, a very interesting model microorganism, which is genetically well characterized and can be easily manipulated to generate useful mutant strains. Moreover, this bacterium can readily multiply either in laboratory conditions or in cellular or animal models and is commonly considered an excellent tool to investigate the infectious processes mediated by facultative intracellular pathogens.

To investigate the importance of zinc in the host-pathogen interaction, we have investigated the contribution of ZnuABC, the unique high affinity zinc transporter present in Gram-negative bacteria, to *S.* Typhimurium survival and multiplication in zinc-poor media, within eukaryotic cells and in infected mice. Moreover, we have also investigated the role in zinc uptake and bacterial virulence of ZinT, a periplasmic protein with a putative role in metal homeostasis, and of the histidine-rich domain of ZnuA.

On the whole, the results reported in this study demonstrate that despite the apparent elevated concentration of zinc within the host tissues, the functionality of the ZnuABC is critical to ensure *Salmonella* ability to efficiently multiply within infected mice and to cause disease. In addition, we have shown that ZinT and the His-rich domain of ZnuA are two structurally distinct elements with apparently redundant roles, which enhance metal recruitment during conditions of severe zinc shortage.

These findings suggest an evident parallelism between the mechanisms of iron and zinc sequestration in the host-pathogen relationships and shed new light on the complex functions of zinc in vertebrate and bacterial physiology.

3 Materials and Metods

3.1 Materials

Antibiotics for bacteria growth were provided by Sigma, they have been sterilized by filtration and stored in aliquots at -20 °C. Antibiotics were used at the following concentration: kanamycin 50 μg/ml, chloramphenicol 30 μg/ml and ampicillin 100 μg/ml. Antibiotics for cell culture were provided by Eurobio and stored in aliquots at -20 °C.
Restriction enzymes, alkalin phosphatase and T4 DNA ligase were provided by New England Biolabs; the Taq DNA polimerase (Expand ^{TM}) was provided by Roche.

3.1.1 Bacterial strains

TABLE 3.1. Bacterial strains used in this work

***S. enterica* strains**	**Relevant genotype**	**Source/Tecnique** (*oligonucleotides* and template plasmid)
serovar Typhimurium		
MA6926	Wild type	L. Bossi
MA6926(pKD46)	wild type harbouring plasmid pKD46	L. Bossi
MA7223	ilvI3305::Tn10dTac-cat-43::3xFLAG-kan	Uzzau *et al*, 2001
MA7225	*ilvI3305*::Tn*10*dTac *cat*	Uzzau *et al*, 2001
SA123	*znuA*::*kan*	Lab collection
SA140	*znuA*::3xFLAG-*kan* *ilvI*::Tn10dTac-*cat*::3xFLAG-*kan*	Lab collection
SA150	*znuA*::*cam*	Lab collection
SA176	*znuA* ::scar †	Lab collection
SA182	*znuABC::kan*	Lab collection
SA229	*yebA::cam*	electroporation of fragment [*oli167-168* pKD3] in MA6926 pKD46; verified by PCR (*oli169/K1*) camR
SA233	*znuA*Δloop *yebA*::*cam*	see Materials and Methods section
SA287	*znuA*Δloop *yebA*::scar†	electroporation of pCP20 in SA233; camS
SA288	*znuA*::3xXFLAG-scar† *ilvI*::Tn10dTac-*cat*::3xFLAG-scar†	electroporation pCP20 in SA140; kanS
PP101	*znuB*::3xFLAG-*kan*	electroporation of fragment [*oli172-173* pSUB11] in MA6926 pKD46; verified by PCR (*oli136/K1*); kanR
PP116	*zinT*::*cam*	electroporation of fragment [*oli178-179* pKD3] in MA6926 pKD46; verified by PCR (*K3/oli181*) camR
PP118	*zinT*::*cam znuA*::*kan*	transduction P22(SA123) on PP116; kanR
PP120	znuA-3xFLAG-*kan zinT*::*cam*	transduction P22(SA140) on PP116; camR
PP125	*zinT*-scar†	electroporation of pCP20 in PP116; camS
PP126	*znuA*::3xFLAG-scar† *zinT*::scar†	electroporation of pCP20 in PP120; camS
PP127	*zur*::*kan*	electroporation of fragment [*oli184-185* pKD4] in MA6926 pKD46; verified by PCR (*oli177/K1*); kanR
PP128	*znuA*::3xFLAG-scar† *zinT*::scar† *ilvI*::Tn10dTac-*cat*::3xFLAG-*kan*	transduction P22(SA140) on PP126; camR
PP130	*zinT*::cam*znuA*[deltaloop]	transduction P22(PP116) on SA287;

	yebA::scar†	camR
PP131	*znuA*::3xFLAG-scar† *zur*::*kan* *ilvI*::Tn10dTac-*cat*::3xFLAG-scar†	transduction P22(PP127) on SA288; kanR
PP132	*zinT*::3xFLAG-scar *zur*::*kan*	transduction P22(PP127) on PP129; kanR
PP134	*zinT*::3xFLAG-*kan*	electroporation of fragment [*oli182-195* pSUB11] in MA6926 pKD46; verified by PCR (*oli180/K4*) kanR
PP137	*zinT*::3xFLAG-*kan znuA*::*cam*	transduction P22(PP134) on SA150; kanR
PP138	*zinT*::3xFLAG-scar†	electroporation pCP20 in PP134; kanS
PP141	*znuA*::3xFLAG-*kan* *zinT*::3xFLAG-scar†	transduction P22(SA140) on PP138; kanR
serovar Enteritidis		
LK5	Wild type	L. Bossi
SA157	*znuA*::*kan*	Lab collections

†The term "scar" refers to the DNA sequence remaining after excision of antibiotic-resistance cassette following homologous recombination between two flanking FRT mediated by a recombinase encoded by plasmid pCP20 (Datsenko *et al*, 2001).

3.1.2 Eukaryotic cell lines

Mouse macrophage cell lines J774 A.1 (ATCC # TIB-67) and TIB-63.
Human epithelial colorectal adenocarcinoma cells CaCo2.
Differentiated THP-1 monocytes.

3.1.3 Mice

Balb/c and DBA2 females.

3.1.4 Liquid culture medium for bacteria growth

Luria-Bertani Broth	
Bacto tryptone	10 g/l
yeast extract	5 g/l
NaCl	10 g/l

Minimal medium (E50X)

$MgSO_4$ anhydrous	0.04 g/l
citric acid	2 g/l
K_2HPO_4 anhydrous	10 g/l
$NaNH_4HPO_4 \cdot 4H_2O$	3.5 g/l
glucose	2 g/l

Dissolve in water, heating the solution, cool and then add a drop of chloroform.

Tris minimal medium

Tris-HCl pH 7.2	120 mM
K_2HPO_4	0.017 g/l
$MgCl_2$	2.03 g/l
NH_4Cl	1.06 g/l
$NaSO_4$	0.44 g/l
$CaCl_2$	0.06 g/l
NaCl	4.68 g/l
KCl	1.48 g/l
glucose	1.98 g/l

SOC

Mix in pairs 1:1 SOB 2X with salts 2X

SOB 2X		Salts 2X	
Bacto tryptone	40 g/l	$MgCl_2$	20 mM
yeast extract	10 g/l	$MgSO_4$	20 mM
NaCl	20 mM	glucose	0.8 %
KCl	5 mM		

P22 broth

LB	100 ml
E 50X	2 ml
glucose	1 ml
P22	0.1 (about 10^9 pfu/ml)

3.1.5 Solid culture medium for bacteria growth

Agar

agar	15 g/l

Dissolved in LB broth and then autoclaved for 20 minutes at 120 °C.

Soft agar

agar	7 g/l

Dissolved in LB broth and then autoclaved for 20 minutes at 120 °C.

Green Plates

glucose	7.6 g/l
Bacto tryptone	8.1 g/l
yeast extract	1 g/l
NaCl	5 g/l
Agar	15 g/l
Metyl blue	0.066 g/l
Azarin yellow GG	4.68 g/l
KCl	0.630 g/l

Mix in water and then autoclaved for 20 minutes at 120 °C.

3.1.6 Culture media for eukaryotic cell growth

Eagle's minimum essential medium (MEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum.
Dulbecco's modified Eagle's medium (D-MEM) low glucose supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum.
RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.5 g sodium bicarbonate and 10% fetal bovine serum.

3.1.7 Plasmids

Name	Features (reference)
pKD46	lambda red recombinase function (Datsenko, 2000)
pKD4	kanamycin resistance cassette template (Datsenxo, 2000)
pKD3	chloramphenicol resistance cassette template (Datsenko, 2000)
pCP20	FLP recombinase function (Datsenko, 2000)
pSUB11	3xFLAG-kanamycin resistance cassette template (Uzzau, 2001)
pSEZnuAEc	Plasmid constitutively expressing *E. coli* ZnuA (Berducci *et al*, 2004)
pEMBL18	Cloning vector (Dente *et al*, 1983)
p*znuA*	Plasmid expressing serovar Typhimurium ZnuA from its own promoter

3.1.8 Oligonucleotides

Name	Sequence
K1	CAGTCATAGCCGAATAGCCT
K3	AGCTCACCGTCTTTCATTGC
K4	CACTGCAAGCTACCTGCTTT
oli119	GGAAGCCTTTATGGAGAAGTCGGTCAGGAATATCCCTGATTGTAG GCTGGAGCTGCTTCG
oli120	CGCGCTATCTCTGGGGAGAGCCAAAGATGCATGTTATATTCATAT GAATATCCTCCTTAG
oli124	AAACCACGCGTACAAGCGTT
oli136	GTACGCGTGGTTTTAGGACT
oli163	GCAACTTGCCGATGTAAAACCGTTACTCATGAAGGGCGCGGGCGAATA

	TAACATGCATCT
oli167	AGACCTTCCGTGCGCGGCAATTTTGCTGTCAGAGGGTTAATGTAGGCT GGAGCTGCTTCG
oli168	GCCCTATGTTTACCACCCAGAATCCGCGCCAATCGTTAAACATATGAA TATCCTCCTTAG
oli169	TTTCGTCGTTACGACGCATC
oli172	GCTGCTGTTTATCTTCAGTATGATGAAAAAGCAGGCAAGCGACTACAA AGACCATGACGG
oli173	TTGAGGATGTGCTGGAGCCGTATCTGATTCAGCAAGGCTTCATATGAA TATCCTCCTTAG
oli177	TTCATGCCATTCGAGGTGCT
oli178	TTCTGGGAATGCTGTTGGTAAATAGTCCTGCCTTCGCGCATGTAGGCTG GAGCTGCTTCG
oli179	CCAGTTTTCCATCTCTTGCAACAATGCCTGCTGCGAGGTACATATGAAT ATCCTCCTTAG
oli180	CCGGATAGAGCATAGAGCTT
oli181	TGACACGAGTAATCAGGCGA
oli182	GTTAAAGGCGAATGAGGTCGTTGACGAAATGCTACATCATGACTACAA AGACCATGACGG
oli184	GACCACAACGCAAGAGTTACTGGCGCAAGCTGAAAAACTCTGTAGGCT GGAGCTGCTTCG
oli185	TTTTCTTCACCAGCACCGAGTGATCGTGACCACAGTTTCCCATATGAAT ATCCTCCTTAG
oli195	TTGTCACCAGCAGATCAATGTCGCTGTTTGGCTTCAGACCCATATG

3.1.9 Solutions

TBS 10X pH 8.0	Tris 500 mM NaCl 1.38 M KCl 27 mM
EB 10X pH 8.3	Tris 350 mM NaH_2PO_4 290 mM EDTA 5 mM
PBS 10X pH 8.3	NaCl 80 g/l KCl 2 g/l Na_2HPO_4 14.4 g/l KH_2PO_4 2.4 g/l
Tris-glycine 10X pH 8.3	Tris 250 mM Glycine 2.5 M
Acrylamide (30%)	Acrylamide 30% N',N'-metilbisacrylamide 0.8%
Resolving solution for SDS-PAGE	41.6% Acrylamide 30% 12.5% Tris-HCl 3M, pH 8.8 44.8% H_2O 1% SDS 10% 1% APS 10% 0.1% TEMED
Stacking solution for SDS-PAGE	3% Acrylamide 30% 25% Tris-HCl 0.5 M, pH 6.8 60% H_2O 1% SDS 10% 1% APS 10% 0.1% TEMED

Sample buffer for SDS-PAGE	SDS 4% Tris-HCl 125 mM, pH 6.8 β-mercaptoethanol 10% glycerol 20% Bromophenol blue 0.004%
Running buffer for SDS-PAGE	Tris-glycine 1X SDS 0.1 %
Blotting buffer	10% Tris-glycine 10X 20% methanol 70% H_2O
Blocking buffer	3% Non-fat Dry Milk (Biorad) 0.5% Tween-20 Dissolved in PBS

3.2 Methods

3.2.1 Mutants construction

All *Salmonella* knockout mutants and the 3xFLAG strains were obtained following the one step inactivation protocol described by Datsenko (Datsenko and Wanner, 2000) and the epitope tagging method described by Uzzau (Uzzau *et al*, 2001), respectively. The oligonucleotides and plasmids used for mutant's construction are listed in Table 2. Each new strain was confirmed by PCR with oligonucleotides annealing upstream or downstream the mutated allele and an internal primer annealing on the inserted antibiotic resistance cassette. The oligonucleotides used for the construction and the check of each new strain are specified in Table 3.1. Alleles were then transduced into a clean background by generalized transduction with phage P22 HT 105/1 *int-201* (Uzzau et al, 2002). In some cases the antibiotic resistance cassette was removed by the FLP recombinase transiently introduced by electroporation of plasmid pCP20 into the strain. The *znuA*Δloop mutant was obtained by the Datsenko and Wanner method (Datsenko and Wanner, 2000) modified as follows. First, an antibiotic resistance cassette was inserted into the *Salmonella* chromosome downstream the *znuA* gene by electroporating a PCR fragment obtained with oligonucleotides oli167/168 on pKD3 plasmid template. The chromosome of the resulting strain (*yebA*::*cam*, SA229) was then used as a template for a PCR with primers oli167/163, designed *ad hoc* to amplify the *znuA* region with a deletion in the His-rich loop (from nucleotide 411 to nucleotide 480 of the coding sequence) and the downstream antibiotic cassette. The obtained fragment was then electroporated into strain MA6926 pKD46 and recombinants were selected on chloramphenicol selective plates. The deletion of the His-rich loop of *znuA* was confirmed by nucleic acid sequencing of the mutant strain.

3.2.2 Western blot analysis

To analyze the accumulation of ZinT, ZnuA and ZnuB, aliquots of bacterial cultures (approximately $5x10^8$ cells) were harvested, lysed by resuspending bacteria in sample buffer containing sodium dodecyl sulphate (SDS) and β-mercaptoethanol, and boiled for 8 min at 100 °C. Subsequently, proteins were run on 12% SDS-page gels and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). The epitope–flagged proteins were revealed by incubation of nitrocellulose membrane with an appropriate dilution of mouse anti-FLAG antibody (anti-FLAG M2, Sigma) and anti-mouse horseradish peroxidase-conjugated antibody (Bio-Rad), followed by the enhanced chemiluminescence reaction (ECL, Amersham).

3.2.3 Cloning of *S. enterica* serovar Typhimurium *znuA* gene

The *znuA* sequence was amplified by PCR from *S. enterica* serovar Typhimurium ATCC 14028 chromosome using oligonucleotides Sal*znuA*for and Sal*znuA*rev. The amplified DNA fragment contains 112 bp upstream of the GTG start codon, thus including the promoter region, and 21 bp downstream of the stop codon. The fragment obtained (about 1,100 bp) was digested with EcoRI and HindIII and cloned into vector pEMBL18, previously restricted with the same enzymes. The ligation mixture was used to transform commercial *E. coli* DH5 competent cells (Invitrogen), and transformants were selected on LB plates containing ampicillin. The *znuA*-containing plasmid (pP*znuA*) was then introduced by electroporation into serovar Typhimurium *znuA*-deleted strains SA123 and SA150.

3.2.4 Growth curves

Each strain was grown overnight in LB broth at 37 °C and then diluted 1:500 in fresh LB broth supplemented or not with appropriate concentration of EDTA and/or of metals. The absorbance at 600 nm was monitored every hour for 10 hours using a Perkin Elmer Lambda 9 spectrophotometer.

3.2.5 Cell cultures and in vitro infection studies

Eukaryotic cell lines were cultured at 37°C in humidified air with 5% CO_2. In the intracellular survival experiments, approximately 10^5cells/ml were infected with *S. enterica* serovar Typhimurium at a multiplicity of infection of 1:100 for 30 min, washed with phosphate-buffered saline (PBS), and supplemented with fresh medium containing gentamicin (100 g/ml) in order to kill extracellular bacteria (Elsinghorst *et al*, 1994). At 1, 3, 24, and 48 h post-infection, cells were washed twice with PBS and lysed with cold Triton X-100 solution (0.5% in PBS). Serial dilutions of the cellular lysates were plated on LB agar to determine the number of viable intracellular bacteria by CFU counts. In each experiment, the intracellular survival assay was carried out three times, and the intracellular viability data reported are the averages of at least three independent experiments. For expression studies of epitope-tagged ZnuA, the cells were lysed 24 h postinfection, and the lysates were harvested and prepared for Western blot analysis.

3.2.6 Measurements of intracellular labile zinc

Semi-confluent 25-cm2 flasks of Caco-2 (colonic epithelial cells) and differentiated THP-1 (human monocytes) were treated for 2 h at 37°C with 100 μM TPEN in order to deplete intracellular labile zinc or not treated with TPEN (Ho *et al*, 2004). The Zn-specific fluorophore Zinquin was added to each flask (25 μM for 30 min). Cells were washed extensively in PBS and lysed with PBS containing 0.5% Triton X-100; cellular debris was pelleted by centrifugation, and supernatants were collected for fluorometric analysis. Emission spectra of Zn(II)-Zinquin complexes were obtained with a Perkin-Elmer LS 50B spectrofluorometer (excitation wavelength of 364 nm). Fluorescence of the different samples was normalized according to the protein concentration determined by the method of Lowry *et al*. (Lowry *et al*, 1951), using bovine serum albumin as a standard.

3.2.7 Mouse infection studies

In the survival studies, groups of at least five BALB/c (*itys*) or DBA-2 (*ityr*) mice were utilized. Aliquots of bacterial cultures grown overnight in LB medium were diluted in sterile PBS (for intraperitoneal infections) or 10% sodium bicarbonate (for oral infections) at the desired concentrations. In experiments involving intraperitoneal infection, animals were infected with doses ranging from 10 to 6,250 CFU/mouse, while the doses used in oral infections were between 105 and 109 CFU/mouse. Mouse mortality was monitored daily. Kaplan-Meier analysis, carried out by GraphPad Prism 4, was used to determine the statistical significance of differences in survival of mice. *P* values of <0.05 were considered significant.
For the analysis of epitope-tagged ZnuA accumulation from bacteria colonizing the spleens of mice, BALB/c mice were infected with 2,000 CFU/mouse and sacrificed when they were terminally ill. The spleens were removed and homogenized.
The cellular extracts were prepared for SDS-polyacrylamide gel electrophoresis and Western blot analyses as previously described (Uzzau *et al*, 2002).

3.2.8 Competition assays in BALB/c mice

Overnight cultures of bacteria were diluted in PBS buffer to a final concentration of 10^4cells/ml and then mixed in pairs in a 1:1 ratio. 0.2 ml of each mixture was used to infect intraperitoneally female BALB/c mice of 10 weeks of age. Animals were sacrificed when exhibiting symptoms of terminal septic syndrome (4-5 days post infection). Bacteria recovered from spleens were plated for single colonies and then 200 colonies were picked on selective plates. The competitive index (CI) was calculated by the formula CI =output (Strain A/Strain B)/inoculum (Strain A/StrainB). Statistical differences between outputs and inputs were determined by the Student's t-test.

3.2.9 Cloning, expression and purification of ZnuA, ZinT and ZnuAΔloop

The *S.* Typhimurium *znuA* and *znuA*Δloop genes were amplified from chromosomal DNA extracted with ZRfungal/bacterial DNA Kit TM (Zymo Research) from wild type and SA233 strains respectively. In both cases the primers used were SalZnuAfor (5'-ATAGAATTCCGGGGCTCAATTCAAG-3') and SalZnuArev (5'-TTTAAGCTTAATCTCCTTTCAGGCAGCT-3') that amplify the coding sequences plus about 200 base pairs upstream of the start codon. The purified PCR products were digested with *Eco*RI and *Hind*III, ligated into the pEMBL-18 vector (Dente et al., 1983) obtaining plasmids p18PznuA and p18PznuAΔloop, which were introduced into *E. coli* DH5α cells. The sequences of the cloned DNA fragments were confirmed by nucleic acid sequencing. For the expression of recombinant proteins, cells were grown overnight in LB medium, harvested by centrifugation for 15 min at 8000 g, resuspended in 500 ml of isotonic solution and periplasmic proteins were released by osmotic shock, as already described (2). After a centrifugation at 13000 rpm for 20 min, the supernatant containing the periplasmic proteins was applied to a Ni-NTA column (Qiagen) pre-equilibrated with 50 mM Na-phosphate, 250 mM NaCl, pH 7.8 and eluted with a discontinuous gradient of 0-250 mM imidazole. ZnuA eluted with 20-40 mM imidazole. The protein was further purified by anionic exchange chromatography on a HiLoad^{Tm} 176 Q-Sepharose FPLC column (Pharmacia Biotech) pre-equilibrated with 20 mM Tris-HCl, pH 7.0 and eluted using a 0-400 mM NaCl linear gradient. The purified protein was 178 concentrated to 30 mg/ml in a buffer containing 20 mM Hepes, 10 mM NaCl, 5% glycerol pH 7.0 and stored at -20°C. Periplasmic extracts containing the ZnuAΔloop protein were initially purified by anionic exchange chromatography on a column equilibrated with 20 mM Tris-HCl, pH 7.0 and eluted using a 0-400 mM NaCl linear gradient. Subsequently, the protein was loaded on a cationic exchange HiLoad SP Sepharose column equilibrated with 20 mM Na-phosphate, pH 7.0 and eluted with a 0-400 mM NaCl linear gradient. A final chromatographic step was carried out on a HiLoad 26/10 Phenyl Sepharose HP column equilibrated with 30 mM Tris-HCl, 1.5 M $(NH_4)SO_4$, pH 7.0. Proteins were eluted with a 1.5-0 M $(NH_4)SO_4$, 30 mM Tris-HCl pH 7.0 linear gradient. The fractions containing the ZnuAΔloop protein were pooled and the protein resulted more than 98% pure, as judged by SDS-PAGE analysis. The purified protein was concentrated to 30 mg/ml in a buffer containing 20 mM

Hepes, 10 mM NaCl, 5% glycerol, pH 7.0 and stored at -20°C. The *S.* Typhimurium *zinT* gene was amplified from chromosomal DNA extracted from wild type strain utilizing primers zinT5 (5'TCCATGGATATTCATTTAAA-AAAACTGACAATG-3') and zinT2 (5'-ATCAAGCTTAATCAGACTTAA-TGATGTAGCAT-3'). The PCR product was digested with *Nco*I and *Hind*III, ligated into the pSE420 vector (Invitrogen) obtaining plasmid pSEzinT and then transformed into *E. coli* DH5α cells. The sequence of the whole cloned DNA fragment was verified by nucleic acid sequencing. Cells harbouring plasmid pSEzinT were grown in LB medium supplemented with 100 μg/ml ampicillin, and when the absorbance at 600 nm of the culture reached the value of 0.5, protein expression was induced overnight with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation for 15 min at 5000 rpm resuspended in 500 ml of isotonic solution and periplasmic proteins were released by osmotic shock, as already described (Ammendola *et al.*, 2008). Spheroplasts were separated from periplasmic proteins by centrifugation and the supernatant was applied to Ni-NTA column preequilibrated with 50 mM Na-phosphate, 250 mM NaCl pH 7.8 and eluted with a linear gradient of 0-500 mM imidazole. ZinT eluted at 250 mM imidazole. Fractions containing ZinT (>98% pure) were pooled, dialyzed against 20 mM Hepes, 20 mM NaCl, 5% glycerol, pH 7.0, concentrated to 30 mg/ml and stored at -20 °C.

3.2.10 Preparation of apo-ZinT and apo-ZnuA

Metal-free ZnuA and ZinT were prepared by extensive dialysis against 50 mM sodium acetate buffer, 2 mM EDTA, pH 5.5. The proteins were subsequently dialyzed twice against 50 mM sodium acetate, 0.1 M NaCl, pH 5.5 to remove excess EDTA. Finally, the proteins were dialyzed against 20 mM Hepes, 100 mM NaCl, pH 7.0 to carry out gel filtration experiments. The metal content of the apo-proteins was evaluated by atomic absorption using a Perkin Elmer spectrometer Analyst 300 equipped with the graphite furnace HGA-800. The zinc content of the demetallated proteins was below the 5 %.

3.2.11 Analysis of ZinT and ZnuA interaction with a gel filtration column

To analyze the possible formation of a complex between ZnuA and ZinT, the two proteins were mixed in a 2:1 (ZnuA:ZinT) w/w ratio, corresponding to a 1.4:1 molar ratio, to favour complex formation. After 90 min incubation at room temperature, proteins were injected onto a High Load 16/60 Superdex 75 gel filtration FPLC column (Amersham Biosciences) equilibrated with 20 mM Hepes, 100 mM NaCl, pH. 7.0. Elution was carried out at room temperature. Fractions of 1 ml were collected and analyzed by SDS-PAGE.
To minimize metal contamination during apo-protein interaction, all solutions were treated with Chelex-100 (Biorad) and the gel filtration column and FPLC apparatus were extensively washed with 1 mM EDTA.

4 Results and Discussions

4.1 *znuA* is required for *Salmonella enterica* growth in poor zinc environments.

A few investigation carried out in recent years in different bacteria have suggested an important role for the ZnuABC zinc uptake system for growth in environments poor in zinc and for virulence of gram-negative pathogens (Campoy *et al*, 2002; Chen *et al*, 2001; Garrido *et al*, 2003; Kim *et al*, 2003; Kim *et al*, 2004; Lewis *et al*, 1999; Lu *et al*, 1997, Yang *et al*, 2006). These studies, however, have not analyzed the molecular bases of the contribution of ZnuABC to bacterial virulence. To clarify the role of this high-affinity metal uptake system in bacterial physiology and of zinc availability in host-pathogen interactions, we have investigated the role of the ZnuABC transporter in *S. enterica*. *znuA* deletion mutants of *Salmonella* serovar Typhimurium strain ATCC 14028 and serovar Enteritidis strain LK5 were produced as described in Materials and Methods. Figure 1 shows that the absence of the *znuA* gene does not alter the ability of the bacteria to grow in rich media (LB) but significantly affects serovar Typhimurium growth in a minimal medium (MM) where the zinc concentration is below the micromolar range (Outten *et al*, 2001). Similar results were obtained with serovar Enteritidis (data not shown).

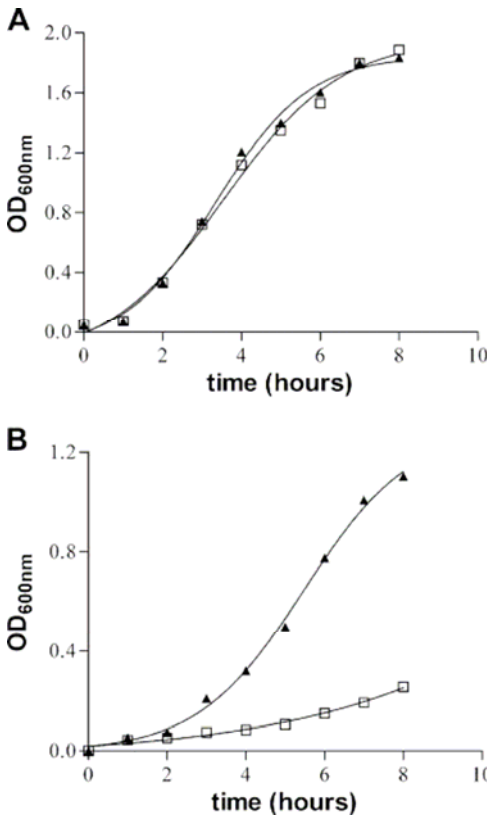


Figure 4.1. Growth curves of *S. enterica* serovar Typhimurium in synthetic media. Wild-type serovar Typhimurium (triangles) and *znuA::kan* SA123 mutant (squares) were grown in LB medium (A) and minimal medium (B). Overnight cultures of both strains grown in LB were diluted 1:500 in fresh LB medium or MM, and the optical density at 600 nm (OD_{600}nm) was registered every hour during exponential growth

To analyze the role of ZnuABC for bacterial growth in metal-depleted media, we have also investigated the ability of *Salmonella* to grow in rich medium (LB) supplemented with the chelating agent EDTA. Table 4.1 shows that after a 24-h incubation at 37°C, the ability of the wild-type strain to form colonies on LB plates is not affected by the presence of up to 2 mM EDTA. In contrast, growth of *znuA* mutant strains SA123 and SA157 was heavily affected by the presence of this chelating agent. EDTA is a rather

unspecific chelating agent, able to sequester zinc and several other divalent metals. Therefore, similar experiments were carried out in the presence of TPEN, a chelating agent with significant zinc specificity and low affinity for Mg2+ and Ca2+. Also, in the presence of TPEN, the growth of the *znuA* mutant strain proved to be severely impaired compared to the wild-type strain (Table 4.1). The significant ability of TPEN to permeate membranes likely explains its higher toxicity with respect to EDTA.

Table 4.1: Growth of *S. enterica* serovar Typhimurium on LB plates

Chelating agent and metal added	Growt of *S. enterica* serovar Typhimurium ATCC14028 strains [a]		
	WT	SA123	SA123(pP*znuA*)
EDTA			
0	+	+	+
0.2 mM	+	+/-	+
0.5 mM	+	-	+
1 mM	+	-	+
2 mM	+	-	+
TPEN			
0	+	+	+
0.01 mM	+	+/-	+
0.02 mM	+	+/-	+
0.08 mM	+	-	+
0.15 mM	+/-	-	+/-

a. Bacteria were grown overnight in LB medium and then streaked on LB plates containing the indicated amounts of EDTA and TPEN. WT, wild type. Symbols: +, growth; -, no growth; +/-, weak growth.

The specificity of the observed phenotype is confirmed by the complementation with plasmid pPznuA, which produces serovar Typhimurium ZnuA under control of its own promoter, or with plasmid pSEZnuAEc, which constitutively produces *E. coli* ZnuA (Berducci *et al*, 2004). However, it should be noted that, irrespective of the concentration of the chelating agent, growth of the complemented strains on LB plates was

slower than that of wild-type *Salmonella.* As SDS-polyacrylamide gel electrophoresis analysis of *Salmonella* extracts revealed that ZnuA is the most abundant protein in bacteria containing pPznuA or pSEZnuAEc (data not shown), we believe that the growth disadvantage of the complemented strains should be attributed to toxic effects of protein overproduction. These results were confirmed using the SA150 and SA176 mutants instead of SA123 (data not shown), showing that the growth defect does not depend on the kanamycin cassette insertion into the chromosome or on the specific *S. enterica* serovar analyzed. In fact, we have analyzed also the ability of S. Enteritidis *znuA* mutant strain to grow in LB plates containing variable amounts of EDTA (Table 4.2). These results demonstrate that the growth of both *S. enterica* serovar Typhimurium and serovar Enteritidis strains lacking the periplasmic component of the ZnuABC transporter is drastically impaired in media containing low levels of zinc or chelating agents able to sequester divalent ions. The observed effect of EDTA on the growth of *Salmonella znuA* mutants is evidently due to a lack of capability to recruit zinc, since introducing a plasmid expressing recombinant ZnuA (Table 4.1 and 4.2) or supplementing the medium with zinc (data not shown) restores microbial growth.

Table 4.2: Growth of *S. enterica* serovar Enteritidis on LB plates

EDTA concentration	**S. Enteritidis LK5**		
	WT	SA157	SA157 pSEZnuAEc
0	+	+	+
0,2 mM	+	-	+
0,5 mM	+	-	+
1 mM	+	-	+
2 mM	+	-	+

a. Bacteria were grown overnight in LB medium and then streaked on LB plates containing the indicated amounts of EDTA and TPEN. WT, wild type. Symbols: +, growth; -, no growth; +/-, weak growth.

Furthermore, the critical relevance of the ZnuABC transporter for bacterial growth under zinc limiting conditions was also outlined by an *in*

vitro competition assay. The wild-type strain and *znuA* mutant strain (SA123) of serovar Typhimurium were grown separately and then mixed at an approximate 1:1 ratio before inoculation in LB medium or MM. After 24 h of growth at 37°C, bacteria were serially diluted and plated onto LB plates.

The exact numbers of bacteria of each strain in the inoculum and in the cultures grown for 24 h were calculated by replica plating of cells on LB plates with or without kanamycin. The competitive index (CI) was calculated as follows: (percentage of strain A recovered/percentage of strain B recovered)/(percentage of strain A inoculated/percentage of strain B inoculated). The CI of each set of assays was analyzed statistically by using the Student's t test. Three experiments were done for each strain on each medium. The median CI for bacteria grown on LB was 1.22, and the median CI for bacteria grown on MM was 0.031. The P values were 0.547 and 0.005 for bacteria grown on LB and bacteria grown on MM, respectively. Such results indicate that there is no selective pressure in LB medium, whereas the wild-type strain outcompetes the *znuA::kan* mutant when bacteria are grown under zinc-limiting conditions (MM). All together, these results suggest that an adequate zinc supply is crucial for bacterial survival and multiplication in vitro and that a functional ZnuA protein is required for efficient zinc uptake in environments where this metal is scarcely available.

4.2 Expression of *znuA* is up-regulated when zinc is scarcely available.

Using a strain bearing an epitope-tagged copy of the chromosomal *znuA* gene, we have been able to indirectly examine expression of the gene in bacteria cultivated in different environments. Zinc-dependent accumulation of ZnuA was monitored by introducing a 3xFLAG epitope-encoding tail at the 3' end of the chromosomal copy of the *znuA* gene, thus obtaining production of a transporter tagged at its C-terminus. The SA140 *znuA* epitope-tagged strain displayed a growth rate comparable to that of the wild type either in minimal medium or in LB supplemented with EDTA, thus indicating that the ZnuA-flagged protein is fully functional (data not shown). The concomitant presence of the constitutively transcribed epitope-tagged *cat* allele, moved by general transduction from strain MA7223 (Uzzau *et al*, 2001), allowed us to easily analyze *znuA* expression by Western blotting. Strain SA140 (ilvI3305::Tn10dTac-*cat*-43::3xFLAG-kan *znuA*::3xFLAG-kan) was grown overnight in LB medium (Fig. 4.2A) and

minimal medium (Fig. 4.2B) supplemented with increasing amounts of $ZnSO_4$ or the divalent ion chelators EDTA. As shown in Fig. 4.2, ZnuA accumulation was negligible in the presence of $ZnSO_4$ amounts greater than 0.1 μM, while *znuA* was induced in media containing a lower zinc concentration, achieved by growing the strain either in minimal medium or in LB medium supplemented with EDTA at concentrations ranging from 0.4 mM to 1.5 mM. These findings corroborate the hypothesis that ZnuA is necessary to optimize zinc uptake under metal shortage. To further investigate ZnuA metal specificity, the SA140 strain was grown in the presence of different chelating agents. As shown in Fig. 4.2C, ZnuA accumulated at high levels in bacteria grown in LB medium containing EDTA or TPEN, but not in the presence of the iron chelator 2,2'-bipyridyl. The concentration of 2,2'-bipyridyl used in this experiment was sufficient to induce high-level accumulation of IroB and SodA, two well-characterized fur-dependent iron-responsive genes (Bjarnason *et al*, 2003; Compan *et al*, 1993; data not shown). Finally, the effect of different transition metals on ZnuA accumulation was analyzed in bacteria grown in minimal medium (Fig. 4.2D). ZnuA accumulation was drastically repressed by the addition of zinc to bacteria, whereas copper, iron, and manganese had no effect, thus demonstrating the high metal selectivity of ZnuA.

These experiments have shown that ZnuA is not expressed in a complex media, such as LB broth, where zinc concentration is elevated (Outten *et al*, 2001). In contrast, the epitope-tagged form of ZnuA accumulates under all the conditions in which the *znuA* mutants exhibit a growth defect, i.e., in minimal medium or in LB supplemented with EDTA.

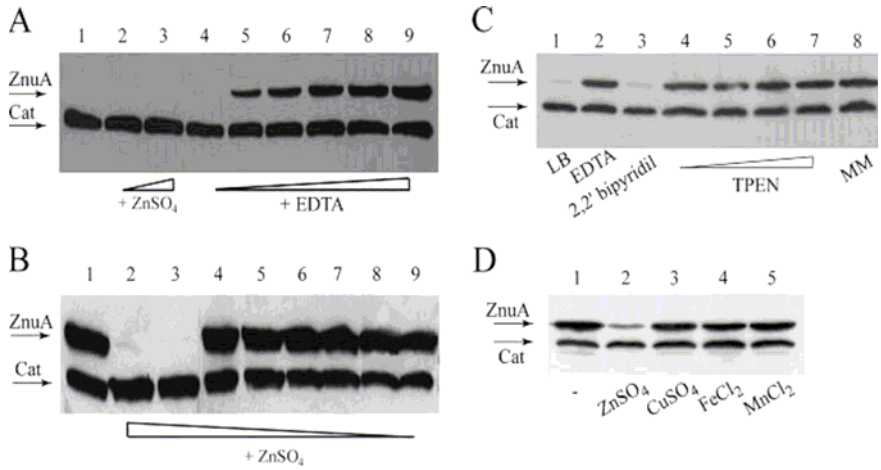


Figure 4.2. Zinc-dependent ZnuA accumulation in *S. enterica* serovar Typhimurium (SA140). (A) Bacteria were grown in LB medium (lane 1) supplemented with 5 μM (lane 2) and 10 μM (lane 3) ZnSO4 and in LB supplemented with EDTA at the following concentrations: 0.2 mM (lane 4), 0.4 mM (lane 5), 0.6 mM (lane 6), 0.8 mM (lane 7), 1 mM (lane 8), and 1.5 mM (lane 9). (B) Bacteria were grown in minimal medium (lane 1) or in minimal medium supplemented with various concentrations of ZnSO4. Minimal medium was supplemented with the following concentrations of ZnSO4: 1 μM (lane 2), 0.5 μM (lane 3), 0.1 μM (lane 4), 0.05 μM (lane 5), 0.01 μM (lane 6), 0.005 μM (lane 7), 0.001 μM (lane 8), and 0.0005 μM (lane 9). (C) Bacteria were grown in LB medium (lane 1) supplemented with 1 mM EDTA (lane 2), 0.2 mM 2,2'-bipyridyl (lane 3), and TPEN at the following concentrations: 0.05 mM (lane 4), 0.1 mM (lane 5), 0.15 mM (lane 6), and 0.2 mM (lane 7). The bacteria in lane 8 were grown in minimal medium. (D) Bacteria were grown in minimal medium. When the cultures reached an optical density at 600 nm of 0.5, the medium was supplemented with 3 μM ZnSO4 (lane 2), 3 μM CuSO4 (lane 3), 3 μM FeCl2 (lane 4), and 3 μM MnCl2 (lane 5), and bacteria were grown for 3 hours before harvesting. Lane 1 shows ZnuA accumulation in bacteria grown in standard minimal medium.

4.3 ZnuA contributes to *Salmonella* multiplication within eukaryotic cells and accumulates in intracellular environments.

In order to verify whether *znuA* contributes to *Salmonella* multiplication within eukaryotic cells we have compared the invasiveness and intracellular

survival of the wild type and *znuA* mutant of *S. enterica* serovar Typhimurium in different cell lines, including J774 and TIB-63 macrophages, differentiated THP-1 monocytes, and epithelial Caco-2 cells. We have observed that bacterial entry and multiplication in cultured macrophages or monocytes (J774, TIB-63, and THP-1) were only marginally affected by the lack of *znuA* (Fig. 4.3A and data not shown), in good agreement with a previous study carried out with a *znuC* mutant (Campoy *et al*, 2002). However, the invasive efficiency and bacterial survival in the earliest hours post-infection were somewhat affected by precultivation of the strains in zinc depleted medium (LB containing 1 mM EDTA), as shown in Fig. 4.3B. A slightly more consistent decrease in bacterial multiplication was observed in epithelial cells (Fig. 4.3C), even if both wild-type and *znuA* strain were grown in rich medium.

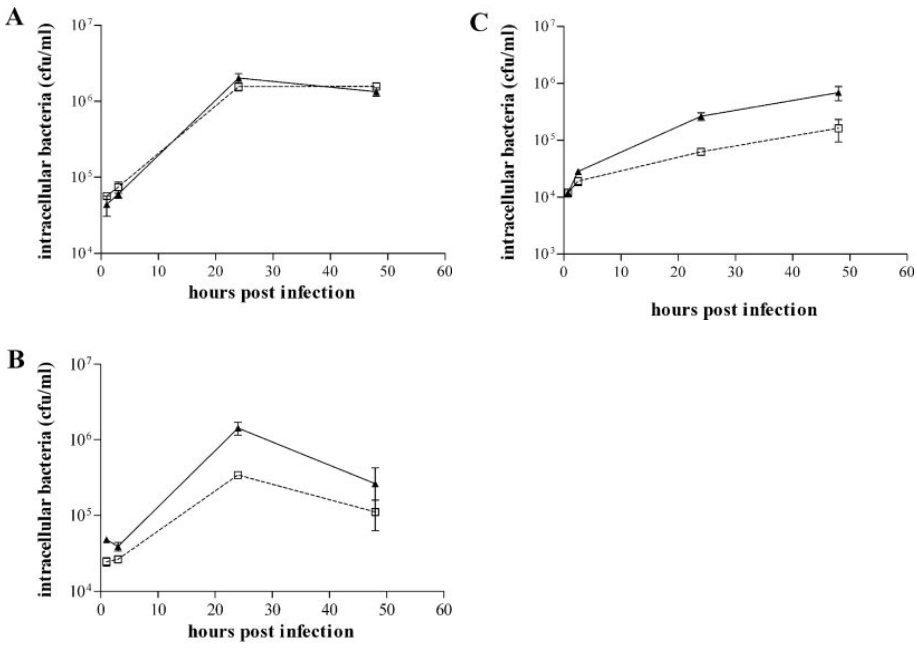


Figure 4.3: Intracellular growth of *Salmonella* strains. Intracellular growth of the *znuA* mutant strain SA123 (squares) compared to wild type serovar Typhimurium (triangles) in differentiated THP-1 human monocytes (A and B) and Caco-2 colon epithelial cells (C). Prior to infection, bacteria were grown in LB medium (A and C) or in LB supplemented with 1mM EDTA (B). The reported CFU/ml values are the means standard deviations (error bars) of at least three independent experiments.

These experimental results suggest that the intracellular pool of labile zinc could vary within different cell lines. To prove this hypothesis, THP-1 and Caco-2 cells were incubated with the zinc-specific fluorophore Zinquin, whose fluorescent signal greatly increases upon binding of zinc (Ho *et al*, 2004; Zalewski *et al*, 2006). For a control, cells were incubated with TPEN before the addition of Zinquin to remove the labile intracellular concentration of zinc (Ho *et al*, 2004).

Figure 4.4 shows that Zinquin fluorescence from either Caco-2 or THP-1 cells pretreated with TPEN was very low. Zinquin fluorescence from Caco-2 cells was modestly affected by preincubation with TPEN, suggesting that most of the intracellular zinc is stably bound by intracellular proteins and is not available for *Salmonella* growth. In contrast, Zinquin fluorescence from THP-1 cells was significantly higher than that obtained from cells incubated with TPEN, indicating that these cells contain significant amounts of labile zinc.

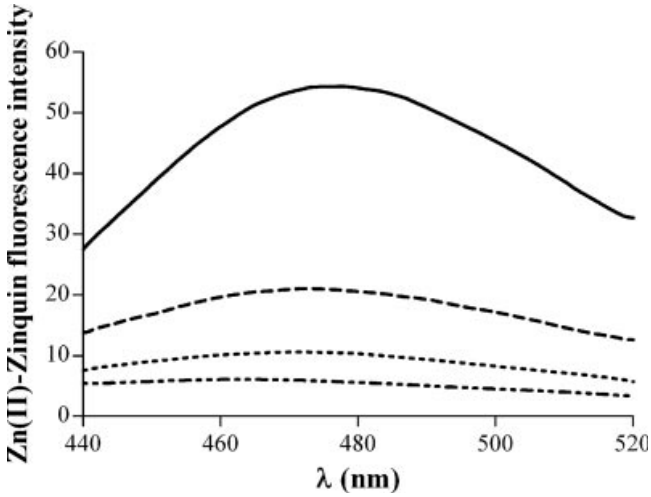


Figure 4.4: Zn(II)-Zinquin-dependent fluorescence of THP-1 and Caco-2 cells. Emission spectra of Zn(II)-Zinquin complexes from cellular lysates of differentiated THP-1 cells (solid black line), THP-1 cells precultivated with 100 μM TPEN (dashed line), Caco-2 cells (dotted line), and Caco-2 cells precultivated with 100 μM TPEN (dotted-dashed line). Zn(II)-Zinquin fluorescence intensity on the y axis is shown in arbitrary florescence units normalized to the protein concentration of the samples.

To better understand the role of ZnuABC transporter during *Salmonella* infection of eukaryotic cells, the ZnuA-tagged strain was used to analyze the intracellular expression of *znuA* in bacteria growing within cultured cells or within macrophages recovered from the spleens of infected mice.

ZnuA accumulation has been analyzed in J774, THP-1, and Caco-2 cells in comparison with its accumulation in LB and minimal media (Fig. 4.5A). In all cell types, the enzyme accumulates at levels significantly higher than in bacteria grown in LB medium. *znuA* expression was also analyzed in bacteria extracted from the spleens of mice infected with *S. enterica* serovar Typhimurium SA140 strain (Fig. 4.5B). Also, in this case, the periplasmic zinc transporter, as well as the other virulence factors SodCI (Cu,Zn superoxide dismutase I), is clearly induced (Uzzau *et al*, 2002).

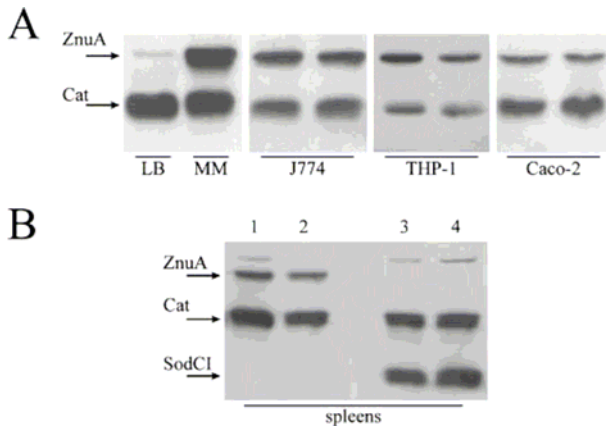


Figure 4.5. ZnuA accumulation in intracellular salmonellae. (A) ZnuA accumulation in bacteria grown in rich medium (LB) and under zinclimiting conditions (MM) compared to ZnuA accumulation in bacteria extracted from infected macrophages (J774), differentiated monocytes (THP-1), and colon epithelial cells (Caco-2). Each gel shows the results from two independent experiments. (B) Accumulation of ZnuA in bacteria harvested from spleen homogenates of BALB/c mice infected with strain SA140 (*znuA*::3xFLAG *cat*::3xFLAG) (lanes 1 and 2) or strain MA7225 (*sodCI*::3xFLAG *cat*::3xFLAG) (lanes 3 and 4). Each lane shows the epitope-flagged proteins recovered from a different mouse.

Therefore, the observation that ZnuA accumulates at significant levels in intracellular *Salmonella* strongly suggests that within host cells bacteria are starved for zinc, in spite of the very high total zinc concentration in

eukaryotic cells (between 0.1 and 0.5 mM [Brown *et al*, 1998; Outten *et al*, 2001]). This apparent contradiction may have different explanations. One possibility is that most of the zinc present inside the eukaryotic cells could be accumulated in vesicular sites (Cousins *et al*, 2006), which are not accessible to bacteria. Alternatively, it is possible that nearly all the intracellular zinc is tightly bound to proteins and is not easily available to invading bacteria. This last hypothesis is appealing, and it is consistent with previous studies of the *E. coli* metalloproteins regulating the uptake and efflux of zinc (Outten *et al*, 2001). In fact, whereas the intracellular concentration of zinc in *E. coli* is in the millimolar range, Zur, the protein regulating the transcriptional activity of *znuABC*, and ZntR, the protein regulating the expression of the ZntA efflux pump, show femtomolar sensitivity to zinc, indicating that there is not a consistent pool of free zinc within the cell (Outten *et al*, 2001). Recent studies have also shown that ZntR and Zur are highly sensitive to extracellular zinc variations (Yamamoto *et al*, 2005) and that the half-life of ZntR is enhanced when intracellular zinc levels are high (Pruteanu *et al*, 2007), thus emphasizing the dynamic nature of intracellular zinc (Chivers *et al*, 2007).

4.4 A functional ZnuABC transporter is essential for *Salmonella* pathogenicity.

Even if the survival of a *znuA* mutant is only slightly affected compared to the wild-type strain in a cellular *in vitro* model, we decided to analyze whether the lack of a functional high-affinity zinc transporter could influence *Salmonella* pathogenicity in a mouse model. Since activation of different virulence factors, such as those encoded by *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2), rely on different environmental conditions (Hueffer and Galan, 2004), oral or parenteral deliveries of wild-type *S. enterica* serovar Typhimurium and strain SA123 were performed either in susceptible (BALB/c) or resistant (DBA-2) mice.

The most important characterized differences between resistant and susceptible mice is that predicted protein sequence analysis of natural resistance-associated macrophage protein 1 (NRAMP1) between *Ityr* (DBA-2) and *Itys* (BALB/c) strains revealed a single mutation resulting in a glycine to aspartic acid substitution at position 169 (Malo *et al*, 1994), which results in a complete lack of function of NRAMP1 in susceptible mice (Vidal *et al*, 1996).

The survival of mice, monitored after infections, is depicted in Fig. 4.6 and 4.7. Infection of mice with wild type serovar Typhimurium induced high mortality rates, irrespective of the delivery route or the genetic background of the recipient mice. In contrast, strain SA123 showed a marked degree of attenuation under all the condition tested. In particular, when SA123 was injected by the intraperitoneal route either in BALB/c or DBA-2 mice, the mortality curves at each dose were significantly different ($P \leq 0.01$) from the mortality curves observed in mice injected with different doses of the wild-type strain. When strain SA123 was injected by the oral route in BALB/c mice, the mortality curves at the highest doses were significantly different ($P \leq 0.01$) from the mortality curves observed in mice injected with similar doses of the wild-type strain. Finally, when strain SA123 was injected by the oral route in DBA-2 mice, the mortality curves at each dose were not significantly different ($P > 0.05$) from the mortality curves observed in mice injected with similar doses of the wild-type strain, even if the difference between the mortality rates of mice injected with the highest dose was somewhat significant. Similar results were obtained in experiments carried out with the SA150 or SA176 strain (data not shown), thus confirming that the introduction of a kanamycin resistance cassette within the *znuA* mutant strain does not contribute to the observed phenotype.

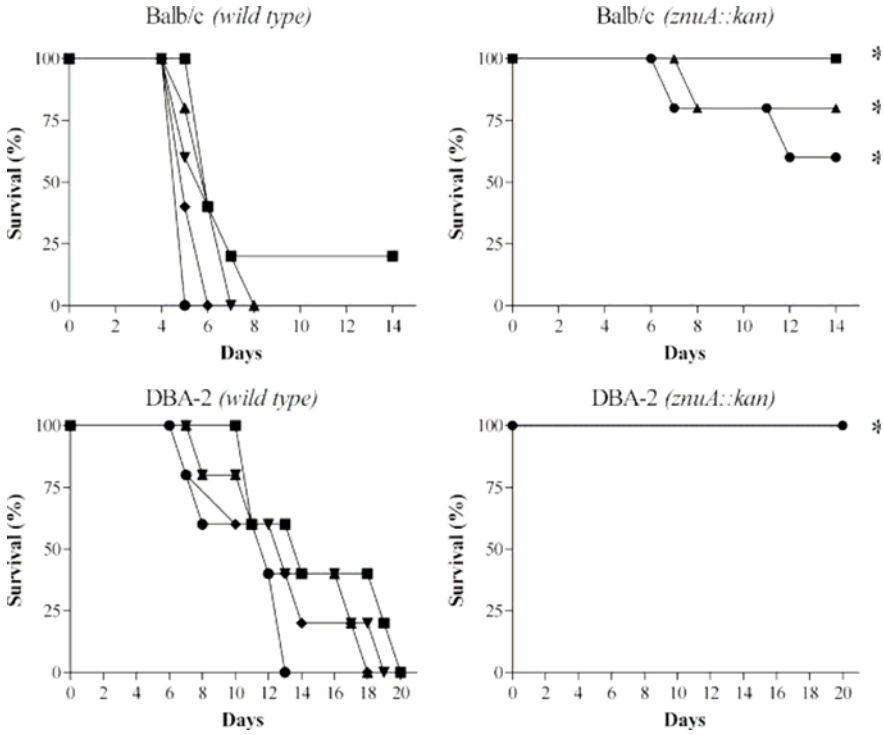


Figure 4.6. Survival of BALB/c and DBA-2 mice infected intraperitoneally with different doses of *S. enterica* serovar Typhimurium wild-type and SA123 strains. Infection doses were 10 CFU/mouse (■), 50 CFU/mouse (▲), 250 CFU/mouse (▼), 1,250 CFU/mouse (♦), and 6,250 CFU/mouse (●). Asterisks indicate mortality curves showing significant differences between the wild-type and mutant strains.

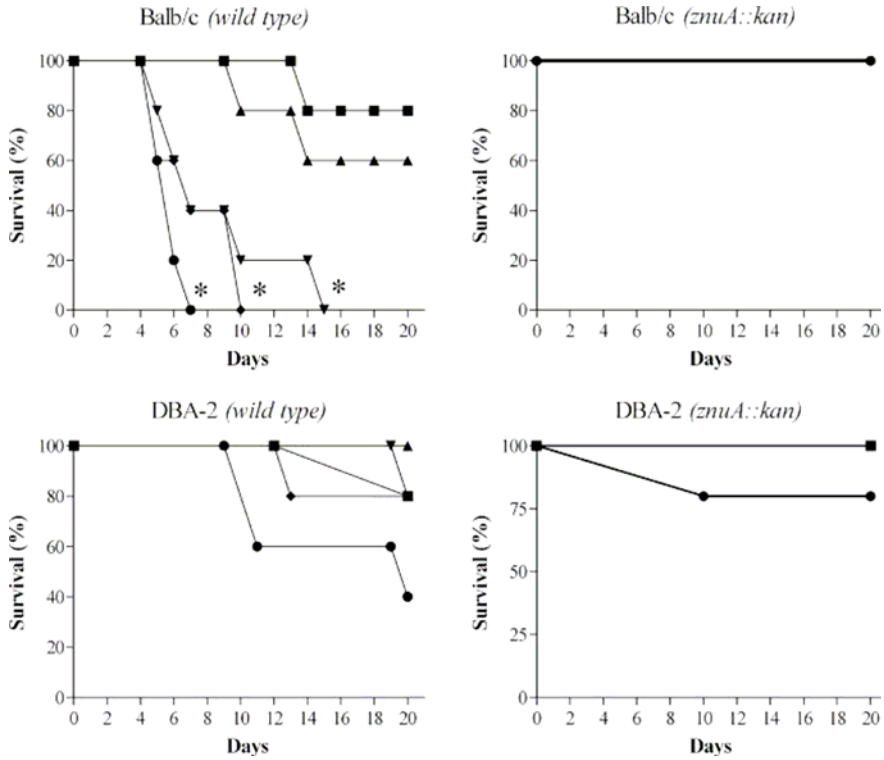


Figure 4.7. Survival of BALB/c and DBA-2 mice infected orally with different doses of *S. enterica* serovar Typhimurium wild-type and SA123 strains. Infection doses were 105 CFU/mouse (■), 106 CFU/mouse (▲), 107CFU/mouse (▼), 108 CFU/mouse (♦), and 109 CFU/mouse (●). Asterisks indicate mortality curves showing significant differences between the wild-type and mutant strains.

Attempts to restore the virulence of the *znuA* mutant by complementation with multicopy plasmids expressing *znuA* were unsuccessful, likely due to toxicity of the overproduced protein. In fact, the possibility that the lack of complementation could be due to plasmid loss was excluded by competition experiments carried out in BALB/c mice. In fact, when mice were intraperitoneally inoculated with a mixture of bacteria containing equal amounts of the SA150 and SA123 strains, no differences in spleen colonization between the two strains was observed in animals sacrificed 4 days after the infection. In contrast, when the experiment was carried out with a mixture of strain SA150 transformed with plasmid pPznuA and strain

SA123, only kanamycin-resistant bacteria were identified in the spleens of infected mice (data not shown). We hypothesize that the high level accumulation of ZnuA in cells containing multiple copies of the *znuA* gene could be due to titration of the intracellular pool of Zur, the transcriptional repressor of *znuABC*.

In order to verify whether the transporter contributes to the virulence of other *Salmonella* serotypes, serovar Enteritidis LK5 SA157 mutant strain was used in similar experiments. The degree of attenuation displayed by this mutant strain was comparable to that obtained with serovar Typhimurium SA123 strain (data not shown).

4.5 *znuA* and *zinT* distribution in eubacteria.

To better understand the mechanism of zinc import in gram negative bacteria and in particular in *S.* Typhimurium, we have analyzed a possible role of ZinT protein in *Salmonella* zinc homeostasis. Although the most recent investigations suggest that ZinT is involved in zinc homeostasis (Graham, *et al*; 2009; Kershaw *et al*, 2007; Sigdel *et al*, 2006), the exact function of this protein has not been elucidated. A previous study has shown that ZinT is present in some bacterial species as an isolated protein, whereas in other bacteria it is a domain of the AdcA protein involved in zinc uptake (Panina *et al*., 2003). The amino acids alignment reported in figure 4.8 shows that the C-terminal portion of AdcA from different bacteria displays high homology with ZinT (53,4% amino acids identity between the overlap region of *S. pneumoniae* AdcA and mature *Salmonella* ZinT), whereas its N-terminal portion is similar to ZnuA (26% identity amino acids identity between the overlap region of the *S. pneumoniae* protein and mature *Salmonella* ZnuA). The functional and structural homology between ZnuA and AdcA is confirmed by two additional features: the conservation of the three histidine residues which coordinate the zinc ion in the crystal structures of *E. coli* (Chandra *et al*., 2007; Li and Jogl, 2007; Yatsunyk *et al*., 2008) and *Synechocystis 6803* (Banerjee *et al*., 2003; Wei *et al*., 2007) ZnuA and the presence in the same sequence position of a charged loop rich in acidic and histidine residues (His-rich loop), typical of proteins involved in the transport of zinc (Banerjee *et al*., 2003; Claverys, 2001).

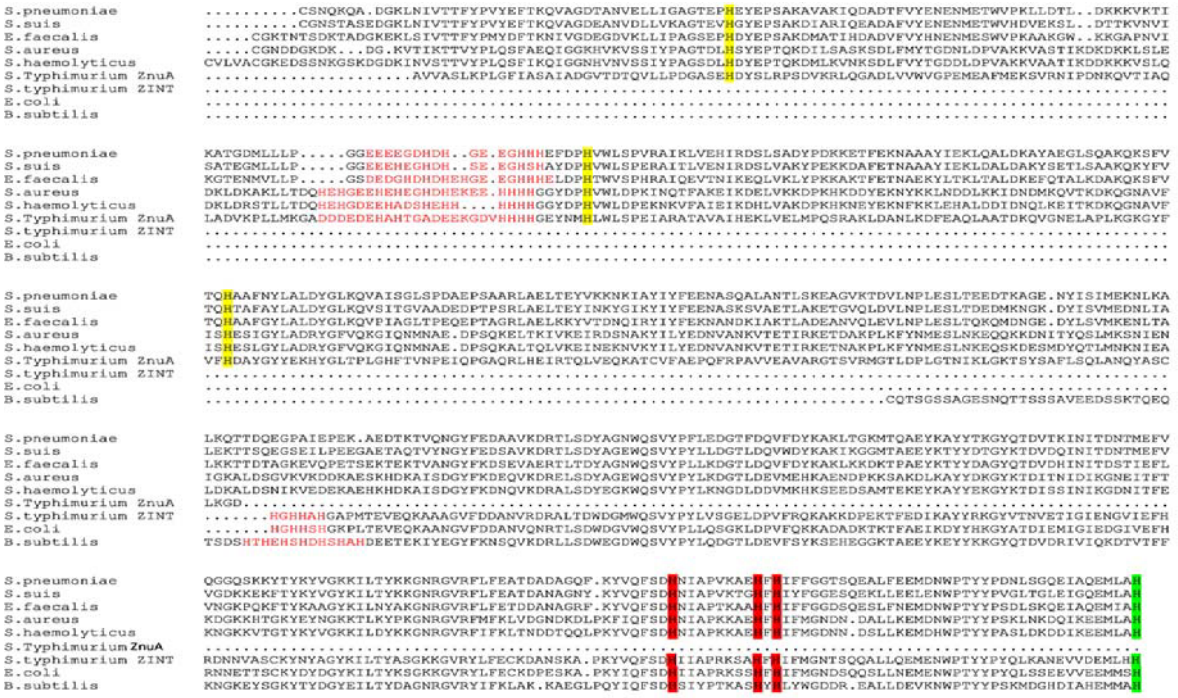


Figure 1. Sequence alignment of AdcA, ZnuA and ZinT: Sequence alignment of the mature (without the signal peptide) AdcA from *S. pneumoniae*, *S. suis*, *E. faecalis*, *S. aureus*, *S. haemoliticus*; ZnuA from *S.* Typhimurium and ZinT from *S.* Typhimurium *E. coli*, *B. subtilis*. Residues involved in zinc binding in ZnuA and ZinT are highlighted in yellow and red respectively. A strictly conserved histidine residue present at the C-terminus of ZinT is shown in green. The protein sequences corresponding to the His-rich loop of ZnuA and AdcA and the N-terminal His-rich region of ZinT have been put in evidence.

Interestingly, the three residues involved in zinc binding in ZinT are strictly conserved in the AdcA proteins, as well as a C-terminal histidine residue which is likely involved in metal binding (David *et al*., 2003). However, it should be noted that ZinT differs from the C-terminal domain of AdcA for the presence of an N-terminal histidine-rich domain. The observations that in some bacterial species ZinT is fused to a ZnuA-like protein involved in zinc transport and that *zinT* and *znuA* are likely coregulated by Zur (Panina *et al*., 2003, Graham *et al*., 2009), strongly suggest that ZinT could participate to the ZnuABC mediated zinc uptake process.

To corroborate this hypothesis we have analyzed the distribution of ZinT, ZnuA and AdcA in available bacterial genomes (see Appendix). ZnuA belongs to a specific cluster of the large family of bacterial periplasmic ligand binding proteins (PLBPs), which comprises proteins involved in manganese or zinc transport (Claverys, 2001). Zinc transporters differ from PLBPs able to bind manganese for the presence of a His-rich loop and for a different arrangement of metal ligands (Banerjee *et al*., 2003). Although PLBPs are present in the vast majority of bacterial genomes, we have classified as ZnuA proteins only those variants showing a His-rich domain. Table 1S shows that AdcA proteins are present in *Streptococci* and in a few other Gram-positive bacteria including *Enterococcus faecalis*, *Staphylococcus aureus* and some, but not all, *Bacillus species*. Several of these proteins possess an N-terminal leader sequence containing a canonical lipo-box motif (LXYC), indicating that the protein is anchored to the bacterial membrane through lipidation of an N-terminal cysteine residue. A typical AdcA protein is present also in *Campylobacter fetus*, which is a Gram-negative bacterium. Proteins showing high sequence homology with ZnuA can be identified in a larger number of bacteria including several Gram-negative species and some Gram-positive bacteria (*Clostridia*, *Listeriacee*, *Streptococci*, *Corynebacterium diphtheriae* and *Bacillus subtilis*). A PLBP with typical ZnuA features can be identified in *M. smegmatis*, but not in other mycobacteria. The isolated ZinT domain shows a more limited distribution, as it can be identified in *Proteobacteria* of the gamma subdivision (including *E. coli*, *S. enterica*, *Shigella dysenteriae* and *Klebsiella pneumoniae*) and in few other bacterial species. Interestingly, ZinT may be present or absent in closely related species, suggesting that this protein could be useful for colonization of specific ecological niches. For example, a typical ZinT protein can be identified in the genome of *Yersinia*

enterocolitica, but not in that of *Yersinia pestis*. On the whole, this analysis shows that several bacteria possessing the ZnuABC system do not contain ZinT. However, bacterial species lacking a ZnuA homologue do not have a ZinT protein, whereas occurrence of ZinT is always associated to the presence of ZnuA. This observation supports the hypothesis that the role of ZinT is related to that of the ZnuA.

4.6 ZinT is not involved in cadmium resistance.

zinT was initially identified as a cadmium induced gene (Ferianc *et al*, 1998) and, for this reason, it was postulated that *zinT* could be involved in bacterial response to this toxic metal (Puškárová *et al*, 2002) .

To verify whether ZinT is involved in cadmium resistance, we have constructed an epitope tagged mutant by introducing a 3xFLAG sequence at the 3' end of the chromosomal copy of the *zinT* coding sequence. The PP134 strain (*zinT*::3xFLAG) shows a growth rate similar to that of the wild type strain (data not show). In line with previous investigations carried out with *E. coli*, ZinT accumulates in *S.* Typhimurium grown in LB medium supplemented with 0.5 mM cadmium acetate (Fig. 4.9A). However, also ZnuA and ZnuB, the periplasmic and the transmembrane component of the ZnuABC zinc transporter, respectively, show a comparable increase in protein accumulation in bacteria cultivated in presence of cadmium (Fig. 4.9 B and C, respectively). This finding suggests that cadmium induces the expression of all Zur-regulated proteins participating to zinc homeostasis.

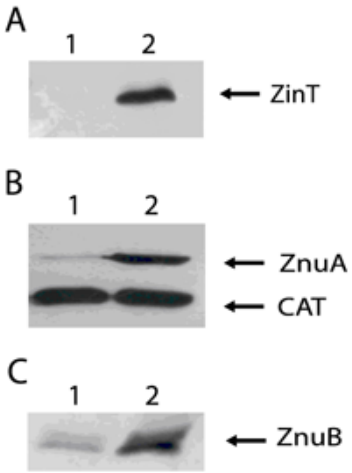


Figure 4.9: Effect of cadmium on ZinT, ZnuA and ZnuB accumulation. Western Blots of bacterial lysates from strains PP134 (zinT::3xFLAG-kan) (Panel A), SA140 (znuA::3xFLAG-kan ilvI::Tn10dTac-cat::3xFLAG-kan) (Panel B) and PP101 (znuB::3xFLAG-kan) (Panel C) grown in LB medium (lane 1) and LB supplemented with 0.5 mM cadmium acetate (lane 2). In Panel A, the accumulation of ZnuA is shown in comparison with an internal standard (Cat::3xFLAG). It was not possible to use this protein as an internal standard to follow ZinT and ZnuB accumulation, as migration of these proteins overlapped with that of Cat.

To better evaluate whether the induction of *zinT* plays a role in cadmium resistance, we have analyzed the ability of a *zinT* mutant strain to grow in LB plates containing variable amounts of cadmium.

As shown in Table 4.2, the *zinT* mutant strain exhibited cadmium susceptibility comparable to that of the wild type strain, thus confirming recent observations showing that ZinT does not contribute to bacterial growth/survival in the presence of this toxic metal (Kershaw *et al*, 2007; Graham *et al*, 2009). The reduced ability of a *znuA* mutant strain to grow in presence of cadmium confirmed that this metal interferes with zinc homeostasis.

Table 4.2: *Salmonella* growth on LB plates

Chelating agent and metal added	Growt of *S. enterica* serovar Typhimurium ATCC14028 strains [a]		
	WT	SA123	PP116
EDTA			
0	+	+	+
0.02 mM	+	+	+
0.06 mM	+	+/-	+
0.1 mM	+	-	+
0.5 mM	+	-	+
1 mM	+	-	+/-
1.2 mM	+	-	-
1.4 mM	+	-	-
Cadmium			
0	+	+	+
0.06 mM	+	+	+
0.1 mM	+	+	+
0.5 mM	+	+/-	+
0.6 mM	+/-	-	+/-
0.7 mM	-	-	-
TPEN			
0	+	+	+
0.005 mM	+	+	+
0.025 mM	+	-	+
0.05 mM	+	-	-
0.5 mM	-	-	-

a. Bacteria were grown overnight in LB medium and then streaked on LB plates containing the indicated amounts of EDTA, TPEN and Cadmium acetate. WT, wild type. Symbols: +, growth; -, no growth; +/-, weak growth.

These experiments demonstrate that ZinT has no role in bacterial resistance to cadmium toxicity. In fact, although *zinT* is strongly induced by this metal, deletion of the gene does not impair bacterial growth in cadmium-containing media. We have also observed that cadmium induces the

accumulation of ZinT as well as of other proteins involved in zinc transport, i.e. ZnuA and ZnuB. The mechanisms responsible for cadmium toxicity are not completely understood, but can be largely explained by the ability of cadmium to deplete cells from intracellular glutathione, to react with the sulphydryl groups of proteins and to compete with other metals for the binding to metalloproteins (Binet *et al*, 2003; Valko *et al*., 2005). The here reported results add new insights to understand the molecular basis of cadmium toxicity in bacteria. In fact, we have observed that cadmium induces the expression of the Zur-regulated genes in bacteria growing in a zinc replete medium (LB), suggesting that cadmium alters zinc homeostasis in bacteria. Additional studies are required to understand whether this is due to a general ability of cadmium to substitute the proper metal cofactor in zinc-containing proteins or to a specific effect on Zur (which, upon cadmium binding, might adopt an altered conformation unable to bind to DNA) or on the functionality of the ZnuABC transporter. However, it is worth nothing that cadmium binds with high efficiency to metal sites characterized by cysteine ligands, such as metallothioneins (Henkel and Krebs, 2004) or zinc-dependent transcription factors (Hartwig *et al*, 2001) and that X-ray absorption studies have shown that zinc binding to Zur involves different cysteine residues (Outten *et al*., 2001). This last observation suggests that Zur could be a privileged target for cadmium ions.

4.7 *zinT* is induced under zinc starvation and belongs to the Zur-regulated operon.

In the previously reported experiments we have shown that ZnuA accumulation is induced by metal chelating agents (EDTA or TPEN) and repressed by the addition of zinc (Fig. 4.2 and Fig. 4.10A). To verify if also ZinT accumulation is modulated by zinc availability, we have grown strain PP134 in presence of 0.5 mM EDTA with or without equimolar amounts of zinc. As shown in figure 4.10, ZinT accumulation is strongly induced by EDTA, but is repressed by zinc (Fig.4.10C). The inhibition of ZinT accumulation is specific for zinc, as manganese, iron and copper have no effect (Fig. 4.10E). To verify that *zinT* and *znuA* are co-regulated by the transcriptional factor Zur, we have transduced the *znuA*::3xFLAG and the *zinT*::3xFLAG alleles in a *zur* deleted strain, obtaining the PP131 and PP132 strains, respectively. The accumulation of ZinT and ZnuA in these strains was completely deregulated and insensitive either to EDTA or to zinc

supplementation (Fig. 4.10D). This observation confirms that transcription of *zinT* is under control of Zur, as recently observed in *E. coli* (Kershaw *et al*, 2007; Graham *et al*, 2009).

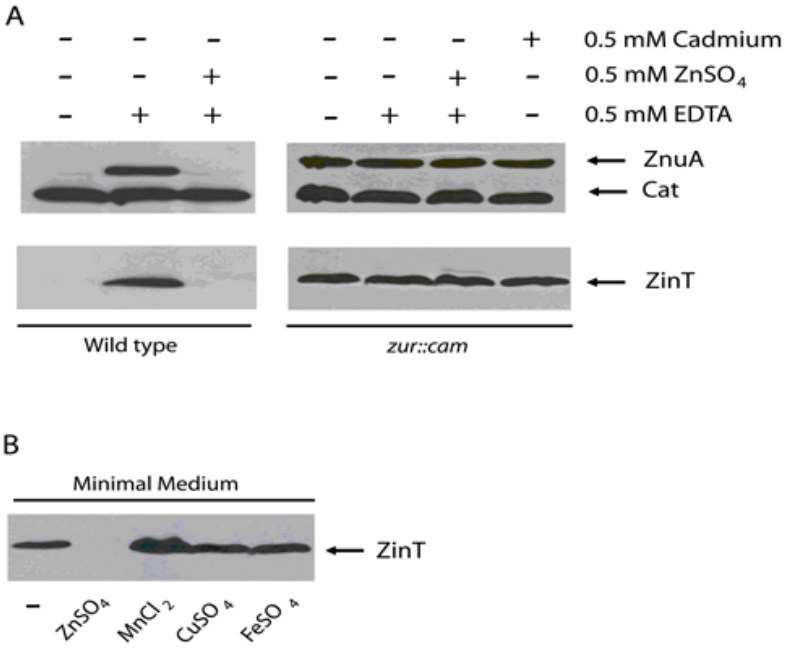


Figure 4.10. ZinT and ZnuA accumulation in wild type and in Zur deleted strains. Western Blots of bacterial lysates. In panel A strains SA140 (*znuA*::3xFLAG-kan ilvI::Tn10dTac-cat::3xFLAGkan), PP134 (*zinT*::3xFLAG-kan), PP131 (*znuA*::3xFLAG-scar *zur::kan* ilvI::Tn10dTac cat::3xFLAG-scar) and PP132 (*zinT*::3xFLAG-scar *zur::kan*) were grown in LB medium alone or supplemented with cadmium acetate, EDTA or $ZnSO_4$ as indicated. Panel B shows ZinT accumulation in strain PP134 (*zinT*::3xFLAG-kan) grown in minimal medium supplemented with 5 μM $ZnSO_4$, 5 μM $MnCl_2$, 5 μM $CuSO_4$ or 5 μM $FeSO_4$.

To better analyze the relationships between ZinT/ZnuA accumulation and zinc availability, we have constructed a double epitope tagged strain (*znuA*::3xFLAG *zinT*::3xFLAG, PP141). When this strain was grown in a zinc depleted medium (minimal medium) both ZnuA and ZinT accumulated at high levels (Fig. 4.11). However, the two proteins exhibit a slightly

different response to zinc availability. In fact, the results reported in the figure show that the addition of 0.5 μM $ZnSO_4$ to the culture medium causes the complete abrogation of ZinT accumulation, but not that of ZnuA (Fig. 4.11A), which show a low level of accumulation also at higher zinc concentrations.

In agreement with this observation, ZinT and ZnuA are maximally expressed in bacteria cultivated in LB medium supplemented with 0.5 mM EDTA, but accumulation of ZnuA is observed at EDTA concentrations lower than those required to induce accumulation of ZinT (Fig. 4.11B). Moreover, ZinT accumulation in a strain lacking the *znuA* gene (PP137) is not inhibited by the addition of zinc (Fig. 5A). In contrast, ZnuA accumulation in a strain lacking the *zinT* gene (PP128) is comparable to that observed in the wild type strain (Fig. 5B).

These experiments show that *znuA* is induced at higher zinc concentration than those required to activate *zinT* transcription, indicating that ZnuA is a protein involved in the frontline response to zinc deficiency, whereas ZinT participated to the bacterial response to more severe zinc deficiency. Moreover, the observation that ZinT accumulation can not be repressed by the external supply of zinc suggests that ZnuA plays a role in zinc import within the cell that can not be substituted by ZinT.

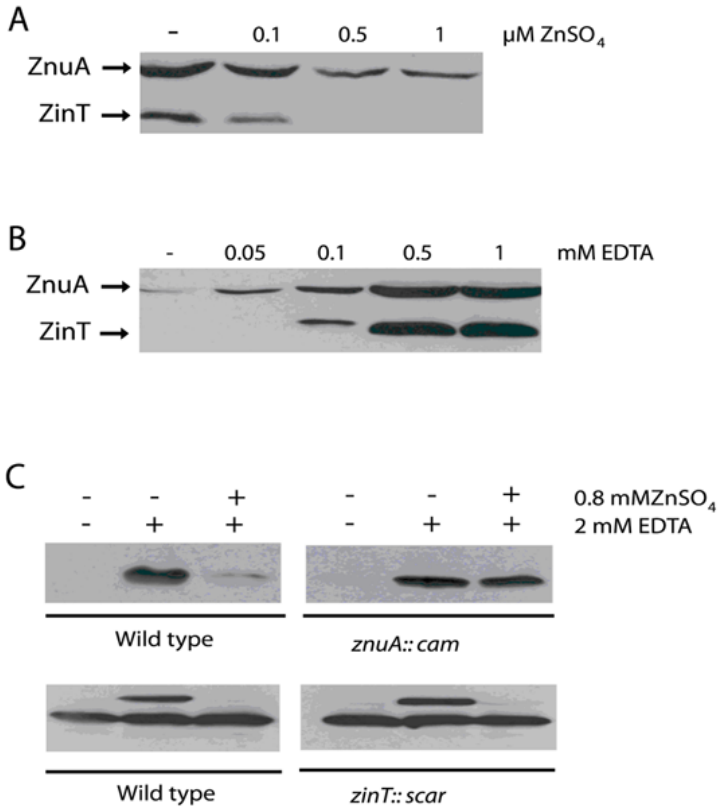


Figure 4.11: Differential accumulation of ZinT and ZnuA in zinc repleted and zinc deficient conditions. Western blots of bacterial lysates. Panel A: strain PP141 (*znuA*::3xFLAG-*kan zinT*::3xFLAG-scar) was grown in minimal medium alone or supplemented with increasing amounts of ZnSO4 as indicated. Panel B: the same strain was grown in LB medium alone or supplemented with increasing amounts of EDTA as indicated. Panel C shows zinc dependent accumulation of ZinT in a wild type background (strain PP134) and in a *znuA* deleted strain PP137 (*zinT*::3xFLAG-*kan znuA*::*cam*) and accumulation of ZnuA in a wild type background (strain PP140) and in a *zinT* deleted strain PP128 (*znuA*::3xFLAG-scar *zinT*::scar *ilvI*::Tn10dTac *cat*::3xFLAG-*kan*).

4.8 Consequences of *zinT* deletion on *Salmonella* growth

In agreement with the above reported expression studies, table 4.2 shows that growth on LB agar plates of a *zinT* mutant strain is inhibited by divalent metals chelators such as EDTA and TPEN. The growth impairment due to *zinT* deletion is lower than that observed for a *znuA* mutant strain, confirming the prominent role of ZnuA in zinc homeostasis.

This observation was confirmed by the analysis of the growth curves of wild type and *znuA* and *zinT* mutant strains in presence of EDTA (Fig. 5). In fact, whereas the growth of all the strains tested is nearly identical in standard LB medium, in presence of 2 mM EDTA the growth of the *zinT* mutant strain is impaired compared to the wild type strain. The growth defect, however, is lower than that exhibited by the strain lacking *znuA*. Zinc, but not iron or manganese, supplementation to the growth medium completely abolished this phenotype (data not shown), indicating that it is specifically due to the zinc sequestration ability of EDTA and not to EDTA-induced shortage of other metals. Interestingly, the growth of the double mutant *zinT-znuA* is comparable to that of the *znuA* mutant strain, confirming that ZinT can not substitute ZnuA and transfer the metal to ZnuB during the process of zinc import within the cytoplasm.

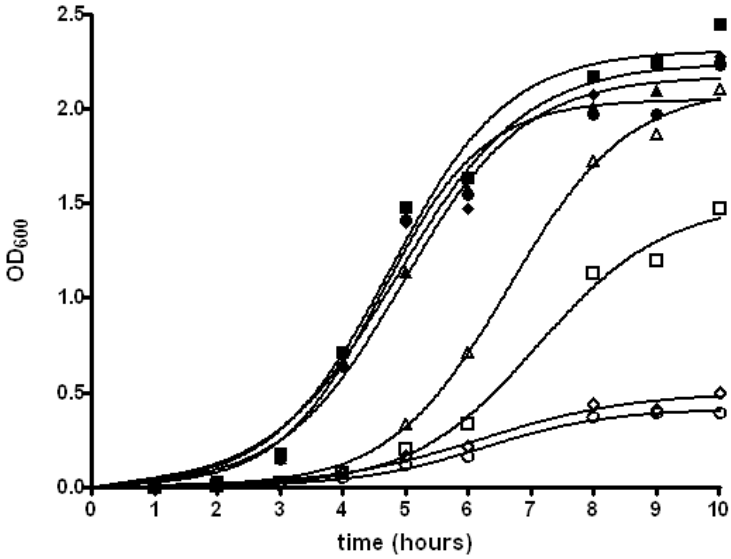


Figure 4.12: Growth curves of *S.* Typhimurium mutants: Growth curves of the wild-type (triangles), *zinT::cam* (squares), *znuA::kan* (circles) and *zinT::cam-znuA::kan* (diamonds) strains. Each strain was grown in LB medium (closed symbols) and LB medium supplemented with 2 mM EDTA (open symbols).

4.9 ZinT role in *Salmonella* pathogenicity.

The above reported experiments have established that disruption of *znuA* dramatically decreases *Salmonella* pathogenicity (Fig 4.13). To evaluate whether *zinT* may have a role during bacterial infections we have compared the contribution of *zinT* and *znuA* to *Salmonella* ability to colonize host tissues by carrying out competition experiments in BALB/c mice.

For each experiment the bacterial strains were grown over night in LB medium and then mixed at an approximate 1:1 ratio. These mixtures were used to infect mice by intraperitoneal injection. When exhibiting symptoms of terminal sepsis, animals were sacrificed (4-5 days post infection) and the bacteria were recovered from the spleens (output). The exact numbers of bacteria of each strain in the inoculum and in the output were calculated by replica plating of bacteria cells on LB plates with or without specific

antibiotic. The CI of each set of assays was analyzed statistically by using Student's t test.

This approach confirmed the relevance of ZnuA in *Salmonella* infections (Table 4.3). In fact, the ability of the strain lacking *zinT* (PP116) to colonize the spleen of infected mice was comparable to that of the wild type strain, whereas the *zinT* mutant (PP116) outcompeted the double mutant *zinT-znuA* (PP118). In addition, we did not observe differences in spleen colonization between *znuA* (SA123) and the *znuABC* (SA182) mutant strains. Quite surprisingly a *zinT-znuABC* (PP119) mutant strain was favored with respect to the *znuABC* (SA182) mutant strain, suggesting a detrimental role of ZinT in the absence of ZnuABC. These experiments suggest that ZinT facilitates zinc transport through the ZnuABC system, but that it has a dispensable role during mice infections. Moreover, the observation that the disruption of *zinT* does not attenuate mutant strains lacking a functional ZnuABC transporter (SA123 or SA182) provides further support to the hypothesis that ZinT is not involved in a ZnuABC-independent mechanism of zinc transport.

Table 4.3: Competition assays with *zinT*, *znuA* and *znuAdeltaloop* mutant strains

Strain A (relevant genotype)	**Strain B** (relevant genotype)	Median CI [a]	No. of mice [b]	P [c]
MA6826 (wild type)	SA123 (*znuA::kan*)	>200[d]	5	<0.001
MA6826 (wild type)	PP116 (*zinT::cam*)	1.202	4	NS
SA123 (*znuA::kan*)	SA182 (*znuABC::kan*)	1.021	10	NS
PP116 (*zinT::cam*)	PP118 (*zinT::cam znuA::kan*)	>200[d]	5	<0.001
SA123 (*znuA::kan*)	PP118 (*zinT::cam znuA::kan*)	1.041	5	NS
SA182(*znuABC::kan*)	PP119 (*zinT::cam znuABC::kan*)	0.394	5	0.037
SA229 (*yebA::cam*)	ATCC14028 (wild type)	0.850	5	NS
PP125 (*zinT*-scar)	SA233 (*znuA*Δloop *yebA::cam*)	1.522	5	NS
SA229 (*yebA::cam*)	SA233 (*znuA*Δloop *yebA::cam*)	1.062	4	NS
PP125 (*zinT*-scar)	PP130 (*zinT::cam znuA*Δloop *yebA::cam*)	1.820	10	0.006
PP118 (*zinT::cam* znuA::kan)	PP130 (zinT::cam *znuA*Δloop *yebA::cam*)	0.006	5	<0.001

a. Competitive index= output (Strain A/Strain B)/inoculum (Strain A/Strain B).
b. Experiments were performed whit BALB/c mice inoculated by the intraperitoneal route.
c. Statistical differences between output and inocula (the P-values) were determined by the Student t-test. NS, not significant.
d. Only colonies of one of the two strains were isolated during the selection procedure. However, a small number of bacteria of the outcompeted strains were present in the spleens of infected mice, as demonstrated by plating the concentrated recovered bacteria on appropriate selective plates. No attempts were carried out to evaluate the exact ratio between the two strains.

Additional experiments, however, shed more light on the role of ZinT in the mechanism of zinc uptake. The ZnuA protein possesses a charged flexible loop rich in histidines and acidic residues, whose function has not yet been clarified (His-rich loop). It has been hypothesized that the role of this loop could be to increase the zinc sequestering ability of ZnuA in environments poor of this metal (Berducci *et al*., 2004; Desrosiers *et al*., 2007), whereas other Authors have suggested that it could be a sensor of periplasmic zinc concentration (Wei *et al*., 2007).

To investigate the role of this His-rich region, we have constructed a *znuA* mutant strain producing a ZnuA protein devoid of this loop (SA233). As shown in figure 4.14, the growth of this mutant strain is not impaired in LB medium containing EDTA, indicating that ZnuA can mediate zinc transport also in the absence of the His-rich region. However, a severe growth defect was observed in a mutant strain expressing such mutated form of ZnuA and unable to produce ZinT. The growth of this mutant, in fact, was comparable to that of the strain lacking *znuA*. Similar results were obtained in competition experiments. The data reported in table 4.3 show that deletion of *yebA*, which was required for the construction of the *znuA*Δloop mutant strain, has no effect on *Salmonella* virulence. Similarly, the *znuA*Δloop mutant strain was not significantly attenuated in comparison to the wild type strain, although a slightly higher number of wild type bacteria were recovered from the spleens of infected mice. In agreement, there was not a significant difference in the spleen colonization ability of a *zinT* mutant strain (PP125) and of the *znuA*Δloop strain (SA233). However, the *Salmonella* strain lacking *zinT* and expressing the mutated form of *znuA* (PP130) was attenuated with respect to the strain unable to produce ZinT (PP125). This strain maintained some ability to transport zinc through the ZnuB channel, as it was advantaged over strain PP118, which does not express *znuA*.

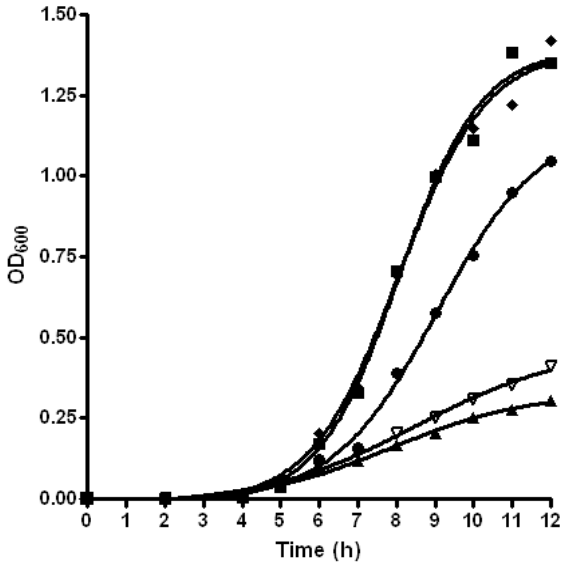


Figure 4.14: Growth curves of *znuA*Δloop mutant strain. Growth curves of the wild-type (triangles), *zinT*::cam (squares), znuA::kan (circles), *znuA*Δloop-*yebA*::cam (diamonds) and *znuA*Δloop-*yebA*::cam-*zinT*::scar (inverted triangles). Each strain was grown in LB medium supplemented with 2 mM EDTA.

These experiments suggest that ZinT and the His-rich domain of ZnuA are two independent elements which participate to the ZnuABC-mediated zinc transport by playing an overlapping role in facilitating zinc recruitment by ZnuA. Although the simultaneous presence of ZinT and of the His-rich domain is apparently not indispensable to ensure the functionality of the transporter under the conditions investigated in this work, disruption of each one of the two elements disclose a role for the other one in enhancing the efficiency of zinc uptake.

The scattered distribution of *zinT* in eubacteria expressing *znuA*, the observation that ZnuABC is critical for successful infection in several bacteria lacking *zinT*, including *H. ducreyi* (Lewis *et al*., 1999), *B. abortus* (Yang *et al*., 2006) and *C. jejuni* (Davis *et al*., 2009) and the results showing that deletion of *zinT* does not attenuate *S*. Typhimurium and only marginally decreases bacterial growth in presence of very high concentrations of chelating agents, all together indicate that ZinT has a role in zinc transport which is significantly less important than that of ZnuA. In support of this

view, we have observed slight differences in the regulation of *zinT* and *znuA*, suggesting that transcription of *znuA* occurs at zinc concentrations higher than those required to activate *zinT* expression. In fact, ZinT accumulation is completely repressed in bacteria growing in a minimal medium containing 0.5 μM $ZnSO_4$ or in LB medium supplemented with 0.05 mM EDTA, whereas under the same conditions ZnuA is partially induced (Fig. 4). Such a flexible response to zinc deficiency may provide an explanation for the production of two separate proteins, instead of a single one (AdcA) as in some Gram-positive bacteria. It should be noted that this finding is in apparent contrast with previous transcriptomics studies showing a greater increase in mRNA levels of *zinT* with respect to *znuA* in response to TPEN (Sigdel *et al*., 2006) or to zinc deficiency (Graham *et al*., 2009). However, both the studies were carried out using defined media containing low levels of zinc and that Graham and coworkers (Graham *et al*., 2009) used a medium containing high EDTA concentrations. We believe that the media used in these works provided conditions sufficient to induce a significant basal expression of *znuA*, thus explaining the much greater mRNA induction of *zinT* upon further depletion of zinc.

4.10 ZinT and ZnuA form a binary complex in vitro

The above reported experiments showing that ZnuA and ZinT functionally interact in the mechanism of ZnuABC-mediated zinc import and the observation that ZinT can be found only in bacteria expressing ZnuA homologues, either as a fused or as an independent protein, suggest that ZinT might physically interact with ZnuA. To prove this possibility we have analyzed the ability of these two proteins to form a complex in vitro. ZinT and ZnuA were cloned, expressed and purified as described in materials and methods. The two proteins were mixed together, incubated for 90 min at room temperature and then loaded on a gel filtration column. Figure 4.15 shows a comparison of the elution of the individual proteins and of the ZnuA:ZinT mixture. ZinT eluted from the column with an apparent molecular weight of 22.3 kDa (peak centered at 75 ml, Fig. 4.15A), whereas ZnuA eluted with an apparent molecular weight of 34.9 kDa (peak centered at 67 ml, Fig. 4.15B). When the mixture of ZinT and ZnuA was applied to the column, the elution profiles of the two proteins significantly changed. In fact, in this case maximal ZinT and ZnuA concentration was found in

fraction 63 (Fig. 4.15C), indicating that, due to formation of a binary complex, the apparent molecular weight of the two proteins was shifted to values significantly higher than those of the single proteins. The elution profile of the complex was rather broad, possibly due to incomplete involvement of the two proteins in complex formation and/or to partial complex dissociation during the elution. However, when the proteins recovered from fraction 63 were concentrated and subjected to a new gel filtration chromatography, the largest part of ZnuA and ZinT still coeluted with a high molecular weight, indicating that the complex between the two proteins is very stable (data not shown).

The elution profile of ZinT was not altered when the protein was preincubated with bovine serum albumin or Cu,Zn superoxide dismutase (Fig. 4.15 F and G, respectively), thus indicating that the interaction of ZinT and ZnuA is highly specific.

To verify if the histidine rich region of ZnuA is involved in the formation of the complex between ZinT and ZnuA, similar experiments were carried outusing the ZnuAΔloop mutant protein. As show in figure 4.15E, when these proteins were coincubated they eluted in the same fractions with apparent molecular weights higher than those of isolated ZnuAΔloop (4.15D) and ZinT (4.15A). In line with functional studies, this observation demonstrates that the His-rich region of ZnuA is not required for the formation of a ZinT /ZnuA complex *in vitro*.

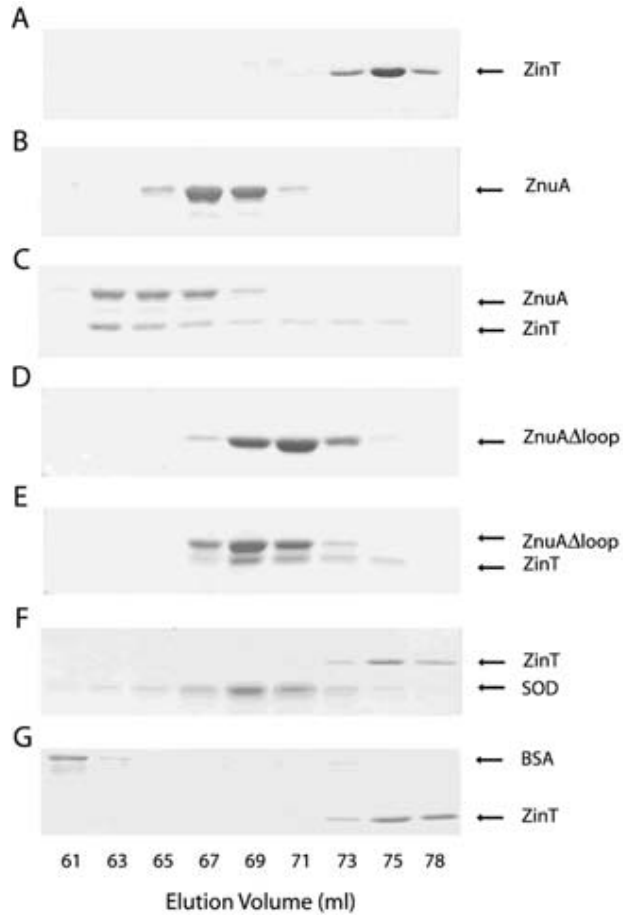


Figure 4.15: Analysis of ZinT and ZnuA interaction by gel filtration chromatography: SDS-PAGE analysis of *Salmonella* ZinT and ZnuA eluted from a HiLoadTM 16/60 SuperdexTM 75 gel filtration FPLC column, calibrated with bovine serum albumin (67000 Da), ovoalbumin (43000 Da), chymotrypsinogen A (25000 Da) and ribonuclease A (13700 Da). The samples loaded on the column were collected in 1 ml fractions. The gel shows the proteins contained in the 61-78 ml fractions, as indicated at the bottom of the figure. Panels correspond to: A) isolated ZinT; B) isolated ZnuA; C) mixture of ZnuA and ZinT; D) mixture of apo-ZnuA and apo-ZinT; E) mixture of apo-ZnuA and apo-ZinT reconstituted with an equimolar amount of zinc; F) isolated ZnuA_loop; G) mixture of ZnuA_loop and ZinT; H) mixture of Cu,ZnSOD and ZinT; I) mixture of BSA and ZinT.

To better analyze the ability of ZnuA and ZinT to form a complex *in vitro* we have also verified whether the presence of metal is necessary for the protein interaction. For this reason we have produced a metal-free ZinT and ZnuA (apoZinT and apoZnuA) proteins as described in materials and methods.

ApoZinT and apoZnuA were mixed, incubated at room temperature for 90 minutes and then loaded on a gel filtration column. The column was first washed with a buffer containing EDTA, to remove trace of metals, and then equilibrated with a chelex-treated buffer. Figure 4.16 shows the elution profiles of the apo-proteins mixtures. .The two apo-proteins were not able to form a stable complex (Fig 4.16A) However, the ability of ZnuA and ZinT to stably interact was completely restored when the two proteins were individually reconstituted with an equimolar amount of zinc before protein incubation (Fig. 4.16D). The addition of zinc to a single protein (ZnuA or ZinT) was not sufficient to observe the coelution of the two proteins from the gel filtration column (figure 4.16 panel B and C respectively). These results suggest that the binding of zinc induces structural rearrangements in ZnuA and ZinT which are necessary for the formation of a stable complex between the two proteins.

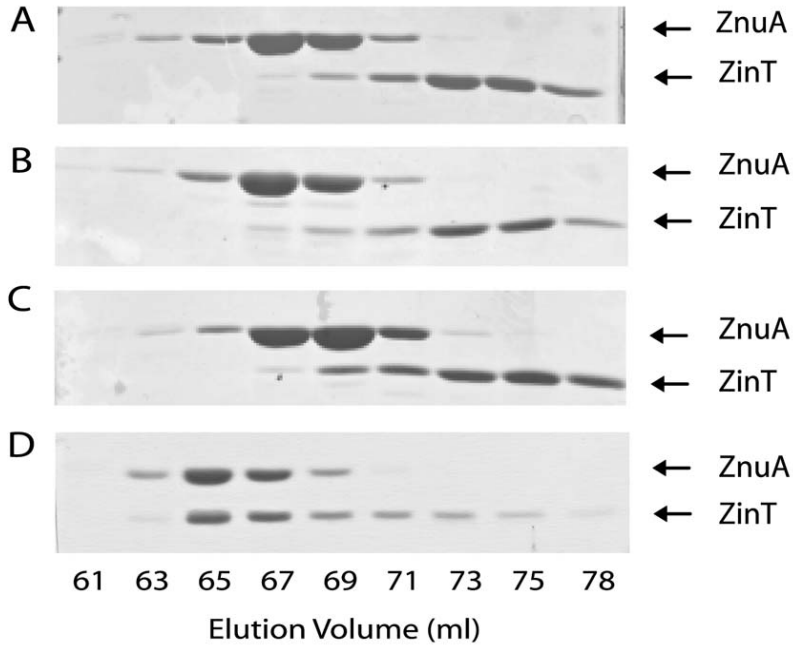


Figure 4.16: Analysis of apoZinT and apoZnuA interaction by gel filtration chromatography: -PAGE analysis of *Salmonella* ZinT and ZnuA eluted from a HiLoadTM 16/60 SuperdexTM 75 gel filtration FPLC column The gel shows the proteins contained in the 61-78 ml fractions, as indicated at the bottom of the figure. Panels correspond to: A) mixture of apoZinT and apoZnuA; B) mixture of apoZnuA and apozinT reconstituted with an equimolar amount of zinc; C) mixture of apoZinT and apoZnuA reconstituted with an equimolar amount of zinc; D) mixture of apo-ZnuA and apo-ZinT both reconstituted with an equimolar amount of zinc.

5 CONCLUSIONS

In recent years studies carried out in different bacteria have suggested an important role for the ZnuABC zinc uptake system for virulence of gram-negative pathogens (Campoy et al, 2002; Chen et al, 2001; Garrido et al, 2003; Kim et al, 2003; Kim et al, 2004; Lewis et al, 1999; Lu et al, 1997, Yang et al, 2006).

To clarify the role of this high-affinity metal uptake system in bacterial physiology and of metal availability in host-parasite interactions, we have constructed *Salmonella enterica* mutant strains in which the *znuA* gene, which encodes the periplasmic component of the ZnuABC high-affinity zinc transporter, was deleted. This mutation does not alter the ability of *Salmonella* to grow in rich media but drastically reduces its ability to multiply in media deprived of zinc. In agreement with this phenotype, ZnuA accumulates only in bacteria cultivated in environments poor in zinc.

In spite of the nearly millimolar intracellular concentration of zinc, we have found that *znuA* is highly expressed in intracellular *Salmonellae* recovered either from cultivated cells or from the spleens of infected mice. We have also observed that *znuA* mutants are impaired in their ability to grow in Caco-2 epithelial cells and that bacteria starved for zinc display decreased ability to multiply in phagocytes (THP-1 cells). By measuring the intracellular pool of labile zinc with a zinc-specific fluorophore (Zinquin) we have found that the impairment of *znuA* mutant growth is due to the different metal quota available for intracellular bacteria in different cell types.

A dramatic reduction in the pathogenicity of the *znuA* mutants was observed in *Salmonella*-susceptible (BALB/c) or *Salmonella*-resistant (DBA-2) mice infected intraperitoneally or orally. This study shows that the amount of free metals available for bacterial growth within the infected animal is limited, despite the apparent elevated concentration of free metals within cells and in plasma and suggests that *Salmonella* exploits the ZnuABC zinc transporter to maximize zinc availability in such conditions.

To better understand *Salmonella* responses to zinc deficiency, we have also investigated the role of ZinT and the Histidine-rich domain of ZnuA. We have found that *zinT* expression is regulated by Zur and parallels that of ZnuA. Despite ZinT contributes to *Salmonella* growth in media poor of zinc, disruption of *zinT* does not significantly affect virulence in mice, the role of ZinT became clear using strains expressing a mutated form of ZnuA lacking

a characteristic histidine-rich domain. In fact, *Salmonella* strains producing this modified form of ZnuA exhibited a ZinT-dependent capability to import zinc either *in vitro* or in infected mice, suggesting that ZinT and the histidine-rich region of ZnuA have redundant function. The hypothesis that ZinT and ZnuA cooperate in the process of zinc recruitment is supported by the observation that they form a stable binary complex *in vitro*. Although, the presence of ZinT is not strictly required to ensure the functionality of the ZnuABC transporter, our data suggest that ZinT facilitates metal acquisition during severe zinc shortage.

We propose that when *Salmonella* grows under conditions of moderate zinc deficiency (I) the expression of ZnuA is sufficient to ensure the adequate zinc recruitment. Under conditions of severe zinc deprivation (II) both ZnuA and ZinT are produced. The two proteins can independently bind zinc and then form a complex which enhances ZnuA capability to recruit zinc within the periplasmic space (figure 5.1). Further investigations will be required to clarify the details of metal trafficking between the ZnuA/ZinT complex and from the complex to the membrane permease ZnuB.

In conclusion, this study establishes that there is a stringent control of zinc availability in eukaryotic tissues which is critically important to limit the ability of bacterial pathogens to multiply within the infected host and that functionality of the ZnuABC transporter is critical for maximizing zinc import in such a hostile environment. As ZnuABC is the only high affinity Zn(II) transporter in bacteria and there are no homologs in the mammalian hosts, these observations indicate that the mechanisms of zinc import could be a privileged target for novel antibacterial therapies

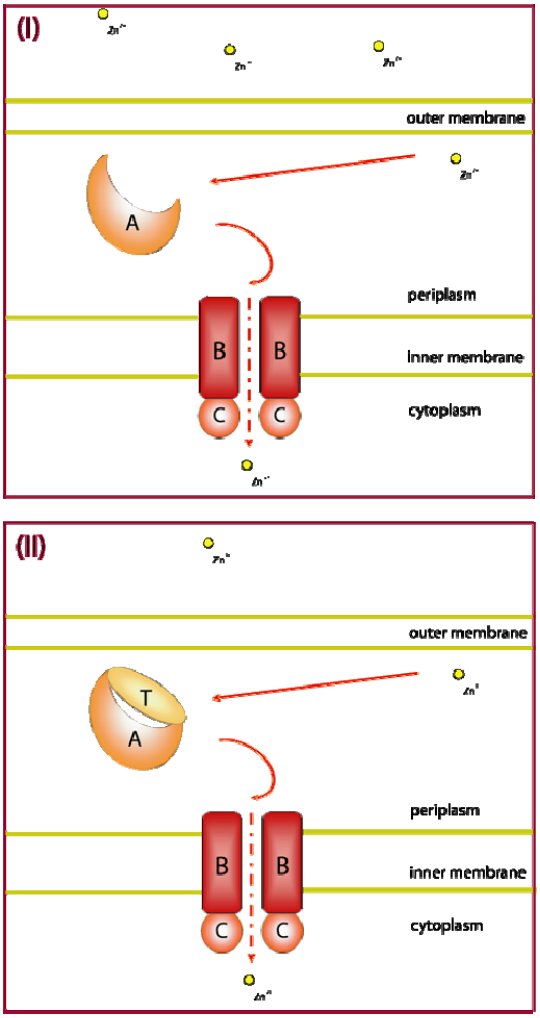


Figure 5.3: Proposed role for ZinT in ZnuABC-mediated zinc uptake. (I) In bacteria lacking ZinT or in *Salmonella* growing in conditions of moderate zinc depletion, ZnuA binds zinc in the periplasmic space and then delivers the metal to ZnuB, which, in turn, transfers zinc into the cytoplasm. **(II)** When *Salmonella* or other bacteria expressing zinT grow under severe zinc depleted conditions, both ZnuA and ZinT are produced. The two proteins can independently bind zinc and then form a complex. Zinc is then transferred from the complex to ZnuB. This picture does not take into consideration the possible alternatives in the pathways of zinc transfer from ZinT to ZnuB (see text). A: ZnuA; B: ZnuB; C: ZnuC; T: ZinT

6 References

Alpuche-Aranda, C.M., Racoosin, E.L., Swanson, J.A., Miller, S.I. (1994). *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. J Exp Med 179, 601-608.

Ammendola, S., Pasquali, P., Pacello, F., Rotilio, G., Castor, M., Libby, S.J., Figueroa-Bossi, N., Bossi, L., Fang, F.C., and Battistoni, A. (2008). Regulatory and structural differences in the Cu,Zn-superoxide dismutases of *Salmonella enterica* and their significance for virulence. J. Biol. Chem. 283, 13688-13699.

Baba, T., and Schneewind, O. (1998) Instruments of microbial warfare: bacteriocin synthesis, toxicity and immunity. Trends Microbiol. 6, 66–71.

Barrow, P.A., Huggins, M.B., Lovell, M.A., and Simpson, J.M. (1987). Observations on the pathogenesis of experimental *Salmonella* typhimurium infection in chickens. Res Vet Sci. 42, 194-199.

Barrow, P.A., Tucker, J.F., and Simpson, J.M. (1987). Inhibition of colonization of the chicken alimentary tract with *Salmonella* Typhimurium Gram-negative facultatively anaerobic bacteria. Epidemiol. Infect. 98, 311–322.

Barthel, M., Hapfelmeier, S., Quintanilla-Martinez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Russmann, H., and Hardt, W.D. **(**2003). Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect. Immun. 71, 2839–2858.

Baumler, A.J., (1997). The record of horizontal gene transfer in *Salmonella*. Trends Microbiol 5, 318-322.

Binet, M.R., Ma, R., McLeod, C.W., and Poole, R.K. **(**2003). Detection and characterization of zinc- and cadmium-binding proteins in *Escherichia coli* by gel electrophoresis and laser ablation-inductively coupled plasma-mass spectrometry. Anal. Biochem. 318, 30-38.

Birch, R. M., O'Byrne, C., Booth, I.R., and Cash, P. (2003). Enrichment of *Escherichia coli* proteins by column chromatography on reactive dye columns. Proteomics 3, 764–776.

Bjarnason, J., Southward, C.M., and Surette, M.G. **(**2003). Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar Typhimurium by high-throughput screening of a random promoter library. J. Bacteriol. 185, 4973–4982.

Bolton, A.J., Osborne, M.P., Wallis, T.S., and Stephen, J. (1999). Interaction of Salmonella choleraesuis, *Salmonella* dublin and *Salmonella* typhimurium with porcine and bovine terminal ileum in vivo. Microbiology 145, 2431-2441.

Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R.P., Pamer, E.G. (2007). MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J. Exp. Med. 204, 1891–1900.

Brocklehurst, K.R., Hobman, J.L., Lawley, B., Blank, L., Marshall, S.J., Brown, N.L., Morby, A.P. (1999). ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. Mol Microbiol. 31, 893-902.

Brown, K.H. (1998). Effect of infections on plasma zinc concentration and implications for zinc status assessment in low-income countries. Am. J. Clin. Nutr. 68, 425S–429S.

Campoy, S., Jara, M., Busquets, N., Pe´rez de Rozas, A.M., Badiola, I., and Barbé, J. **(**2002). Role of the high-affinity zinc uptake *znuABC* system in *Salmonella enterica* serovar Typhimurium virulence. Infect. Immun. 70, 4721–4725.

Cash, H.L. et al. (2006) Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 313, 1126–1130.

Chandra, B.R., Yogavel, M., and Sharma, A. (2007). Structural analysis of ABC-family periplasmic zinc binding protein provides new insights into mechanism of ligand uptake and release. J. Mol. Biol. 367, 970-987.

Chao, Y., and Fu, D. (2004). Kinetic study of the antiport mechanism of an *Escherichia coli* zinc transporter, ZitB. J Biol Chem. 279, 12043-12050.

Chen, C.Y., Stephan, A., and Morse, C. (2001). Identification and characterization of a high-affinity zinc uptake system in *Neisseria gonorrhoeae*. FEMS Microbiol. Lett. 202, 67–71.

Chivers, P.T. **(**2007). A galvanizing story—protein stability and zinc homeostasis. J. Bacteriol. 189: 2953–2954.

Cirillo, D.M., Valdivia, R.H., Monack, D.M., and Falkow, S. (1998). Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. 30, 175–188.

Claverys, J.P. **(**2001). A new family of high-affinity ABC manganese and zinc permeases. Res Microbiol. 152, 231-243.

Compan, I., and Touati, D. **(**1993). Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J. Bacteriol. 175, 1687–1696.

Cousins, R.J., Liuzzi, J.P., and Lichten, L.A. (2006). Mammalian zinc transport, trafficking, and signals. J. Biol Chem. 281, 24085–24089.

Daley, D.O., Rapp, M., Granseth, E., Mele'n, K., Drew, D., and von Heijne, G. (2005). Global Topology Analysis of the *Escherichia coli* Inner Membrane Proteome. Science 308, 1321-1323.

Datsenko, K.A., and Wanner, B.L. **(**2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97, 6640–6645.

David, G., Blondeau, K., Renouard M., and Lewit-Bentley, A. (2002). Crystallization and preliminary analysis of *Escherichia coli* YodA. Acta Crystallogr. D Biol. Crystallogr. 58, 1243–1245.

David, G., Blondeau, K., Schiltz, M., Penel S., and Lewit-Bentley, A. (2003). YodA from *Escherichia coli* is a metal-binding, lipocalin-like protein. J. Biol. Chem**.** 278, 43728–43735.

Dente, L., Cesareni, G., and Cortese, R. (1983). pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. 11, 1645–1655.

Dintilhac, A., Alloing, G., Granadel C., and Claverys, J.P. (1997). Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol Microbiol**.** 25, 727-739.

Elsinghorst, E.A. **(**1994). Measurement of invasion by gentamicin resistance. Methods Enzymol 236, 405–420.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol**.** 47, 103–118.

Ernst, R.K., Guina, T., and Miller, S.I. (2001). *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect. 3, 1327–1334.

Ferianc, P., Farewell, A., and Nystrŏm, T. (1998). The cadmium-stress stimulon of *Escherichia coli* K-12. Microbiology 144, 1045-1050.

Garrido, M. E., Bosch, M., Medina, R., Llagostera, M., Pe´rez de Rozas, A.M., Badiola, I., and Barbe, J. **(**2003). The high-affinity zinc-uptake system znuABC is under control of the iron-uptake regulator (*fur*) gene in the animal pathogen *Pasteurella multocida*. FEMS Microbiol. Lett. 221, 31–37.

Gibbons, H.S., Kalb, S.R., Cotter, R.J., and Raetz, C.R. (2005). Role of Mg2+ and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. Mol. Microbiol**.** 55, 425–440.

Godinez, I., Haneda, T., Raffatellu, M., George, M.D., Paixão, T.A., Rolán, H.G., Santos, R.L., Dandekar, S., Tsolis, R.M., and Bäumler, A.J. (2008) T cells help to amplify inflammatory responses induced by

Salmonella enterica serotype Typhimurium in the intestinal mucosa. Infect. Immun. 76, 2008–2017.

Godinez, I., Raffatellu, M., Chu, H., Paixão, T.A., Haneda, T., Santos, R.L., Bevins, C.L., Tsolis, R.M., and Bäumler, A.J. (2009) Interleukin-23 orchestrates mucosal responses to *Salmonella* enterica serotype Typhimurium in the intestine. Infect. Immun. 77, 387–398.

Graham, A.I., Hunt, S., Stokes, S.L., Bramall, N., Bunch, J., Cox, A.G., McLeod, C.W., and Poole R.K.**.** (2009). Severe Zinc Depletion of *Escherichia coli*: roles for high affinity zinc binding by zint, zinc transport and zinc-independent proteins. J Biol Chem. 284, 18377-18389.

Grass, G., Fan, B., Rosen, B.P., Franke, S., Nies, D.H., Rensing, C. (2001). ZitB (YbgR), a Member of the Cation Diffusion Facilitator Family, Is an Additional Zinc Transporter in *Escherichia coli*. J Bacteriol. 183, 4664-4667.

Grass, G., Wong, M.D., Rosen, B.P., Smith, R.L., Rensing, C. (2002). ZupT is a Zn(II) uptake system in *Escherichia coli.* J Bacteriol. 184,864-866.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S.I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. 27, 1171–1182.

Guo, L, Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., and Miller, SI. (1997). Regulation of lipid A modifications by *Salmonella* typhimurium virulence genes phoP/phoQ. Science 276, 250–253.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., and Miller, S.I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95, 189–198.

Halle S., Bumann, D., Herbrand, H., Willer, Y. Dähne, S., Förster, R., and Pabst, O. (2007). Solitary Intestinal Lymphoid Tissue Provides a Productive Port of Entry for *Salmonella enterica* Serovar Typhimurium. Infection and Immunity 75, 1577–1585.

Hartwig, A. (2001). Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxid. Redox Signal 3, 625-634.

Heithoff, D.M, Conner, C.P., Hentschel, U., Govantes, F., Hanna, P.C., Mahan, M.J. (1999). Coordinate intracellular expression of *Salmonella* genes induced during infection. J. Bacteriol. 181, 799–807.

Henkel, G. and Krebs, B. (2004). Metallothioneins: zinc, cadmium, mercury, and copper thiolates and selenolates mimicking protein active site features--structural aspects and biological implications. Chem. Rev. 104, 801-824.

Hentze, M.W., Muckenthaler, M.U., and Andrews, N.C. (2004). Balancing acts: molecular control of mammalian iron metabolism. Cell 117, 285–297.

Hilbert, F., Garcia-del Portillo, F., and Groisman, E.A. (1999). A periplasmic d-alanyl-d-alanine dipeptidase in the Gram-negative bacterium *Salmonella enterica*. J. Bacteriol. 181, 2158–2165.

Ho, L.H., Ruffin, R.E., Murgia, C., Li, L., Krilis, S.A., and Zalewski, P.D. (2004). Labile zinc and zinc transporter ZnT4 in mast cell granules: role in regulation of caspase activation and NF-kappaB translocation. J. Immunol. 172, 7750–7760.

Hueck, C. J. (1998). Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. Microbiology and Molecular Biology Reviews 62, 379–433.

Hueffer, K., and Gala´n, J.E. (2004). *Salmonella*-induced macrophage death: multiple mechanisms, different outcomes. Cell. Microbiol. 6, 1019-1025.

Ibs, K.H., and Rink, L. (2003). Zinc altered immune functions. J. Nutr.133, 1452S-1456S.

Jepsona, M. A., and Clarkb, M. A.. (2001). The role of M cells in *Salmonella* infection. Microbes and Infection 3, 1183−1190.

Jones B.D. (1997) Host responses to pathogenic *Salmonella* infection. Genes Dev. 11, 679-687.

Jones, B.D., Ghori, N., and Falkow, S. (1994). *Salmonella* typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med. 180, 15–23.

Kannan, G., Wilks, J.C., Fitzgerald, D.M., Jones, B.D., Bondurant, S.S., and Slonczewski J.L.. (2008). Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. BMC Microbiol. 8, 37.

Kaplan, J. (2002). Mechanisms of cellular iron acquisition: another iron in the fire. Cell 111, 603–606.

Kershaw, C.J., Brown, N.L. and Hobman, J.L.. (2007). Zinc dependence of zinT (yodA) mutants and binding of zinc, cadmium and mercury by ZinT. Biochem. Biophys. Res. Commun. 364, 66–71.

Kim, S., Watanabe, K., Shirahata, T., and Watarai, M. (2004). Zinc uptake system (*znuA* locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. J. Vet. Med. Sci. 66, 1059–1063.

Kim, S., Watarai, M., Kondo, Y., Erdenebaatar, J., Makino, S., and Shirahata, T. (2003). Isolation and characterization of mini-Tn*5*Km2 insertion mutants of *Brucella abortus* deficient in internalization and intracellular growth in HeLa cells. Infect. Immun. 71, 3020–3027.

Lee, Y.H., Deka1, R.K., Norgard, M.V., Radolf J.D., and Hasemann, C.A. (1999). *Treponema pallidum* TroA is a periplasmic zinc-binding protein with a helical backbone. Nature structural biology 6, 628-633.

Lewis, D.A., Klesney-Tait, J., Lumbley, S.R., Ward, C.K., Latimer, J.L., Ison, C.A., and Hansen, E.J.. **(**1999). Identification of the *znuA*-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect. Immun. 67, 5060–5068.

Li, H., and Jogl, G. (2007). Crystal Structure of the Zinc-binding Transport Protein ZnuA from *Escherichia coli* Reveals an Unexpected Variation in Metal Coordination . J. Mol. Biol. 368, 1358–1366.

Linke, C., Caradoc-Davies, T.T., Young, P.G., Proft, T., and Baker, E.N. (2009). The Laminin-Binding Protein Lbp from *Streptococcus pyogenes* is a Zinc Receptor. Journal of Bacteriology 191, 5814–5823.

Liu, T., Golden, J.W., and Giedroc, D.P. (2005). A zinc(II)/lead(II)/cadmium(II)-inducible operon from the cyanobacterium *Anabaena* is regulated by AztR, an α3N ArsR/SmtB metalloregulator. Biochemistry. 44, 8673–8683.

Liuzzi, J.P., Lichten, L.A., Rivera, S., Blanchard, R.K., Aydemir, T.B., Knutson, M.D., Ganz, T. and Cousins, R.J. (2005): Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc. Natl. Acad. Sci. USA 102, 6843–6848.

Lowry, H.O., Rosebrough, N.J., Farr, L.A., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lu, D., B. Boyd, and C. A. Lingwood. 1997. Identification of the key protein for zinc uptake in *Haemophilus influenzae*. J. Biol. Chem. 272, 29033–29038.

Malo, D., Vogan, K., Vidal, S., Hu, J., Cellier, M., Schurr, E., Fuks, A., Bumstead, N., Morgan, K., and Gros, P. (1994). Haplotype mapping and sequence analysis of the mouse Nramp gene predict susceptibility to infection with intracellular parasites. Genomics 23, 51–61.

Miao, E.A., Alpuche-Aranda, C.M., Dors, M, Clark, A.E., Bader, M.W., Miller, S.I, Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1β via Ipaf. Nature Immunol. 7, 569–575.

Mowat AM. (2003) Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol. 3, 331–341.

Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S.M., Fritsche, G., and Weiss, G. (2007). The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular *Salmonella* typhimurium. Cellular Microbiology 9, 2126–2140.

Niess, J.H., and Reinecker, H.C. (2006). Dendritic cells in the recognition of intestinal microbiota. Cell Microbiol. 8, 558–564.

Ochman, H., Soncini, F. C., Solomon, F. and Groisman, E.A. (1996). Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. USA 93, 7800–7804.

Ohl, M.E., and Miller, S.I. (2001) SALMONELLA: AModel for Bacterial Pathogenesis. Annu. Rev. Med. 52, 259–274.

Outten, C.E., and O'Halloran, T.V. **(**2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292, 2488–2492.

Overbeck, S., Rink, L., and Haase, H. (2008). Modulating the immune response by oral zinc supplementation: a single approach for multiple diseases. Arch. Immunol. Ther. Exp. 56, 15–30.

Panina, E.M., Mironov, A.A., and Gelfand, M.S.. (2003). Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci. 100, 9912-9917.

Patzer, S.I., and Hantke, K. (1998). The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol. 28, 1199-1210.

Patzer, S.I., and Hantke, K. (2000). The zinc-responsive regulator Zur and its control of the znu gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J Biol Chem. 275, 24321-24332.

Paulsen, I.T., and Saier, M.J. **(**1997). A novel family of ubiquitous heavy metal ion transport proteins. J. Membr. Biol. 156, 99–103.

Pekarek, R.S., Kluge, R.M., DuPont, H.L., Wannemacher, R.W., Hornlck R.B., Bostlan, K.A., and. Belsel, W.R. (1975). Serum Zinc, Iron, and Copper Concentrations during Typhoid Fever in Man: Effect of Chioramphenicol Therapy Clin. Chem. 21, 528-532.

Petrie, L., Selman, I.E., Grindlay, M., Thompson H., (1977). Salmonellosis in young calves due to *Salmonella* enteritidis. Vet. Rec. 101, 398–402.

Philcox, J.C., Coyle, P., Michalska, A., Choo, K.H., and Rofe, A.M. (1995). Endotoxin-induced inflammation does not cause hepatic zinc accumulation in mice lacking metallothionein gene expression. Biochem J. 308, 543–546.

Porter, E.M, Bevins, C.L., Ghosh, D., Ganz, T. (2002) The multifaceted Paneth cell. Cell Mol. Life Sci 59, 156–170.

Pruteanu, M., Neher, S.B., and Baker, T.A. **(**2007). Ligand-controlled proteolysis of the *Escherichia coli* transcriptional regulator ZntR. J. Bacteriol. 189, 3017–3025

Puškárová, A., Ferianc, P., Kormanec, J., Homerová, D., Farewell, A., and Nyström, T. (2002). Regulation of *yodA* encoding a novel cadmium-induced protein in *Escherichia coli***.** A. Microbiology 148, 3801–3811.

Raffatellu,M., Santos, R.L., Chessa, D., Wilson, R.P., Winter, S.E., Rossetti C.A., Lawhon, S.D., Chu, H., Lau, T., Bevins, C.L., Adams, L.G., Bäumler, A.J. (2007) The capsule encoding the via B locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infect. Immun. 75, 4342–4350

Raffatellu, M Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, Godinez I, Sankaran S, Paixao TA, Gordon MA, Kolls JK, Dandekar S, Bäumler AJ. (2008) Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. Nat. Med. 14, 421–428.

Raffatellu, M., George, M.D., Akiyama, Y., Hornsby, M.J., Nuccio, S.P., Paixao, T.A., Butler, B.P., Chu, H., Santos, R.L., Berger, T., Mak, T.W., Tsolis, R.M., Bevins, C.L., Solnick, J.V., Dandekar, S., and Bäumler, A.J. (2009) Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. Cell Host Microbe 5, 476–486.

Ratledge, C., and Dover, L.G. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol 54, 881-941.

Rensing, C., Mitra, B., and Rosen, B.P. (1997). The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci USA. 94, 14326-14331.

Rink, L., and Gabriel, P. (2001): Extracellular and immunological actions of zinc. Biometals 14, 367–383.

Rukhman, V., Anati, R., Melamed-Frank, M., and Adir, N. (2005). The MntC crystal structure suggests that import of Mn2+ in cyanobacteria is redox controlled. J. Mol. Biol. 348, 961–969.

Salgueiro, M.J., Zubillaga, M., Lysionek, A., Cremaschi, G., Goldman, C.G., Caro, R., De Paoli, T., Hager, A., Weill, R., and Boccio, J. (2000). Zinc status and immune system relationship. Biol. Trace Elem. Res. 76, 193–205.

Salzman, N.H. Ghosh D, Huttner KM, Paterson Y, Bevins CL. (2003) Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature 422, 522–526

Schaible, U.M., and Kaufmann, S.H.E., (2004). Iron and microbial infection. Nat. Rev. Microbiol 2, 946-953.

Schaible, U.M., and Kaufmann, S.H.E., (2005). A nutritive view on the host-pathogen interplay. Trends in Microbiology 13, 373-380.

Schroeder, J.J., and Cousins, R.J. (1990). Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc. Natl.Acad. Sci. USA 87, 3137–3141.

Shea, J. E., Beuzon, C. R., Gleeson, C., Mundy, R., and Holden, D.W. (1999). Influence of the *Salmonella* typhimurium Pathogenicity Island 2 type III secretion system on bacterial growth in the mouse. Infect. Immun. 67, 213–219.

Sigdel, T.K., Easton, J.A., and Crowder, M.W. **(**2006). Transcriptional Response of *Escherichia coli* to TPEN. J. Bacteriol**.** 18, 6709–6713.

Tocco-Bradley, R., and Kluger, M.J. (1984). Zinc Concentration and Survival in Rats Infected with *Salmonella* Typhimurium. Infection And Immunity 45, 332-338.

Tsolis, R.M., Adams, L.G., Ficht, T.A. and Bäumler, A.J. (1999). Contribution of *Salmonella* typhimurium virulence factors to diarrheal disease in calves. Infect. Immun. 67, 4879–4885.

Tsolis, R.M., Kingsley, R.A., Townsend, S.M., Ficht, T.A., Adams, L.G., and Bäumler, A.J. (1999). Of mice, calves, and men. Comparison of the mouse typhoid model with other *Salmonella* infections. Adv Exp Med Biol. 473, 261-274.

Turner, J.M., Brodsky, M.H., Irving, B.A., Levin, S.D., Perlmutter, R.M., and Littman, D.R. (1990). Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell 60, 755–765.

Uzzau, S., Bossi, L., and Figueroa-Bossi, N. **(**2002). Differential accumulation of *Salmonella* [Cu, Zn] superoxide dismutases SodCI and SodCII in intracellular bacteria: correlation with their relative contribution to pathogenicity. Mol. Microbiol. 46, 147–156.

Uzzau, S., Figueroa-Bossi, N., Rubino, S., and Bossi, L. **(**2001). Epitope tagging of chromosomal genes in *Salmonella*. Proc. Natl. Acad. Sci. USA 98, 15264–15269.

Valko, M., Morris, H., and Cronin, M.T. **(**2005). Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161-1208.

Vidal, S.M., Pinner, E., Lepage, P., Gauthier, S., and Gros, P. **(**1996). Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains. J. Immunol. 157, 3559–3568.

Waterman, S.R., and Holden, D.W. (2003). Cellular Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Microbiology 5, 501–511.

Wilson, C.L. Ouellette AJ, Satchell DP, Ayabe T, López-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC. (1999) Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 286, 113–117.

Worley, M.J., Nieman, G.S., Geddes, K., and Heffron, F. (2006). *Salmonella* typhimurium disseminates within its host by manipulating the motility of infected cells. Proc Natl Acad Sci USA 103, 17915-17920.

Yamamoto, K., and Ishihama, A. 2005. Transcriptional response of *Escherichia coli* to external zinc. J. Bacteriol. 187, 6333–6340.

Yang, X., Becker, T., Walters, N., and Pascual, D.W. (2006). Deletion of *znuA* virulence factor attenuates Brucella abortus and confers protection against wild-type challenge. Infect. Immun. 74, 3874–3879.

Yatsunyk, L.A., Easton, J.A., Kim, L.R., Sugarbaker, S.A, Bennett, B., Breece, R.M., Vorontsov, I.I., Tierney, D.L., Crowder, M.W., and Rosenzweig, A.C. **(**2008). Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*. J. Biol. Inorg. Chem. 13, 271-288.

Zalewski, P., Truong-Tran, A., Lincoln, S., Ward, D., Shankar, A., Coyle, P., Jayaram, L., Copley, A., Grosser, D., Murgia, C., Lang, C., and Ruffin, R. (2006). Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media. BioTechniques 40, 509–520.

Zheng, Y., Valdez, P.A., Danilenko, D.M., Hu, Y., Sa, S.M., Gong, Q., Abbas, A.R., Modrusan, Z., Ghilardi, N., de Sauvage, F.J., and Ouyang, W. (2008) Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat. Med. 14, 282–289.

Zhoua, D., and Galánb, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes and Infection 3, 1293−1298.

7. Appendix

Table 7.1 Distribution of ZnuABC, ZinT and AdcA in eubacteria

Bacterial Phyla	Bacterial species	Protein				
		AdcA	**ZnuA**	**ZinT**	**ZnuB**	**ZnuC**
Actinobacteria	*Bifidobacterium animalis*	YES	-	-	YES	YES
	Corynebacterium diphtheriae		YES	N0	YES	YES
	Mycobacterium avium		N0	N0		
	Mycobacterium bovis		N0	N0		
	Mycobacterium leprae		N0	N0		
	Mycobacterium smegmatis str.		YES*	N0	YES	YES
	Mycobacterium tuberculosis		N0	N0		
	Streptomyces coelicolor		YES	N0	YES	YES
	Streptomyces griseus		YES	N0	YES	YES
Bacteroidetes/Chlorobi	*Bacteroides fragilis*		YES*	N0	SEP	SEP
	Bacteroides thetaiotaomicron		YES*	N0	SEP	SEP
	Bacteroides vulgatus		YES *	N0	SEP	SEP
	Chlorobaculum parvum		YES	N0	YES	YES
	Porphyromonas gingivalis		YES*	N0	SEP	SEP
Chlamydiae	*Chlamydia trachomatis*		NO	N0		
	Chlamydophila pneumoniae		NO	N0		
Cyanobacteria	*Anabaena variabilis*		YES	N0	SEP	YES
	Cyanothece sp. ATCC 51142		YES	N0	YES	YES
	Nostoc punctiforme PCC 73102		YES	N0	YES	YES
	Synechococcus elongatus		YES	N0	YES	YES
	Synechocystis sp. PCC 6803		YES	N0	YES	YES

	Thermosynechococcus elongatus BP-1		YES	N0	SEP	YES
Bacillales	*Bacillus anthracis*	YES	-	-	SEP	SEP
	Bacillus cereus	YES	-	-	SEP	SEP
	Bacillus subtilis		YES	YES	YES	YES
	Geobacillus sp. WCH70		YES	N0	SEP	SEP
	Listeria innocua Clip11262		YES	N0	YES	YES
	Listeria monocytogenes		YES	N0	YES	YES
	Oceanobacillus iheyensis HTE831		YES**	YES	SEP	SEP
	Staphylococcus aureus	YES	-	-	SEP	SEP
	Staphylococcus epidermidis		YES	NO	SEP	SEP
	Staphylococcus haemolyticus JCSC1435	YES	-	-	SEP	SEP
Clostridia	*Clostridium acetobutylicum ATCC 824*		YES	N0	SEP	SEP
	Clostridium botulinum		YES	N0	YES	YES
	Clostridium difficile		YES	N0	SEP	SEP
	Clostridium perfringens		YES	N0	SEP	SEP
	Clostridium tetani E88		YES	N0	YES	YES
Lactobacillales	*Enterococcus faecalis*	YES	-	-	YES	YES
	Lactobacillus acidophilus		NO	N0		
	Lactobacillus brevis		NO	N0		
	Lactobacillus casei		NO	N0		
	Lactococcus lactis	YES***			YES	YES
	Streptococcus agalactiae	YES	-	-	SEP	SEP
	Streptococcus equi	YES	-	-	SEP	SEP
	Streptococcus mutans UA159	YES	-	-	SEP	SEP
	Streptococcus pneumoniae	YES	-	-	SEP	SEP
	Streptococcus pyogenes	YES	-	-	SEP	SEP

	Streptococcus suis	YES		-	SEP	SEP
Mollicutes	*Mycoplasma agalactiae PG2*		NO	NO		
	Mycoplasma genitalium G37		NO	NO		
	Mycoplasma pneumoniae M129		NO	NO		
Proteobacteria						
alpha subdivision	*Brucella abortus*		YES	NO	YES	YES
	Brucella melitensis		YES	NO	YES	YES
	Brucella suis		YES	NO	YES	YES
	Caulobacter sp. K31		NO	NO		
	Methylobacterium extorquens		YES	NO	FUSED	
	Nitrobacter hamburgensis X14		YES	NO	YES	YES
	Paracoccus denitrificans PD1222		YES	NO	YES	YES
	Rhodobacter sphaeroides		YES	YES	YES	YES
	Rhodospirillum rubrum ATCC 11170		YES	NO	YES	YES
Rhizobiaceae	*Agrobacterium tumefaciens*		YES	YES	YES	YES
	Rhizobium leguminosarum		YES	NO	YES	YES
Rickettsiales	*Ehrlichia canis str. Jake*		NO	NO		
	Rickettsia rickettsii		NO	NO		
beta subdivision						
Bordetella	*Bordetella avium 197N*		YES	NO	YES	YES
	Bordetella bronchiseptica RB50		YES	NO	YES	YES
Burkholderiaceae	*Burkholderia cenocepacia*		NO	NO		
	Burkholderia mallei		NO	NO		
	Burkholderia multivorans		NO	NO		
	Burkholderia pseudomallei		NO	NO		

	Burkholderia thailandensis		NO	NO		
	Ralstonia metallidurans		NO	NO		
Neisseriaceae	*Neisseria gonorrhoeae*		YES	NO	YES	YES
	Neisseria meningitidis		YES	NO	YES	YES
delta subdivision	*Desulfovibrio desulfuricans*		YES	NO	YES	YES
	Geobacter metallireducens GS-15		YES	NO	YES	YES
	Geobacter sulfurreducens PCA		YES	NO	YES	YES
	Myxococcus xanthus DK 1622		NO	NO		
epsilon subdivision	*Campylobacter fetus*	YES	-	-	YES	YES
	Campylobacter jejuni		YES	NO	YES	YES
	Helicobacter pylori		NO	NO		
gamma subdivision	*Citrobacter koseri ATCC BAA-895*		YES	YES	YES	YES
	Escherichia coli		YES	YES	YES	YES
	Klebsiella pneumoniae		YES	YES	YES	YES
	Proteus mirabilis		YES	YES	YES	YES
	Salmonella enterica		YES	YES	YES	YES
	Shigella dysenteriae		YES	YES	YES	YES
	Shigella flexneri		YES	YES	YES	YES
	Yersinia enterocolitica subsp. enterocolitica 8081		YES	YES	YES	YES
	Yersinia pestis		YES	NO	YES	YES
	Yersinia pseudotuberculosis		YES	NO	YES	YES
others	*Francisella tularensis*		NO	NO		
	Legionella pneumophila		NO	NO		
	Shewanella baltica		YES	NO	YES	YES
Pasteurellaceae	*Actinobacillus pleuropneumoniae*		YES	NO	YES	YES

	Haemophilus ducreyi 35000HP		YES	NO	YES	YES
	Haemophilus influenzae		YES	NO	YES	YES
	Pasteurella multocida subsp. multocida str. Pm70		YES	NO	YES	YES
Pseudomonadaceae	*Azotobacter vinelandii DJ*		YES	NO	YES	YES
	Pseudomonas aeruginosa		YES	NO	YES	YES
	Pseudomonas fluorescens		YES	NO	YES	YES
	Pseudomonas putida		YES	NO	YES	YES
	Pseudomonas syringae group genomosp. 3		YES	NO	YES	YES
Vibrionaceae	*Photobacterium profundum*		YES	NO	YES	YES
	Vibrio cholerae		YES	NO	YES	YES
	Vibrio fischeri		YES	NO	YES	YES
	Vibrio harveyi		YES	NO	YES	YES
Xanthomonadaceae	*Stenotrophomonas maltophilia*		NO	NO		
	Xanthomonas campestris		NO	NO		
	Xylella fastidiosa		NO	NO		
Spirochaetales	*Borrelia burgdorferi*		NO	NO		
	Leptospira biflexa		NO	NO		
	Treponema pallidum		YES	NO	YES	YES
	Treponema Denticola		YES	YES	YES	YES

*These proteins exhibit mutations in same of the histidine residues involved in zinc coordination.
**This protein shows a C-terminal extension, showing homology with the N-terminal portion of ZinT, but lacks several residues involved in zinc binding and therefore can not be considered a member of the AdcA family.
***This is a truncated form of AdcA, lacking the N-terminal portion of the protein.
The term SEP indicates that the genes encoding ZnuB and ZnuC are not clustered with *znuA*, but are located in a different chromosomal position.

Legend to Table 7.1:

ZnuA belongs to a specific cluster of the large family of bacterial periplasmic ligand binding proteins (PLBPs), which comprises proteins involved in manganese or zinc transport (11). Zinc transporters differ from PLBPs able to bind manganese in the presence of a His-rich loop and in a different arrangement of metal ligands (5). Although PLBPs are present in the vast majority of bacterial genomes, we have classified as ZnuA proteins only those variants showing a His-rich domain. We have included within the list of presumed ZnuA proteins a few variants showing substitutions in one of the histidine residues identified as zinc ligands (5). AdcA proteins are present in Streptococci and in a few other Gram-positive bacteria including *Enterococcus faecalis*, *Staphylococcus aureus* and some, but not all, *Bacillu*s species. Several of these proteins possess an N-terminal leader sequence containing a canonical lipobox motif (LXYC), indicating that the protein is anchored to the bacterial membrane through lipidation of an N-terminal cysteine residue. A typical AdcA protein is present also in *Campylobacter fetus*, which is a Gram-negative bacterium.

Proteins showing high sequence homology with ZnuA can be identified in a larger number of bacteria including several Gram-negative species and some Gram-positive bacteria (Clostridia, Listeriacee, Streptococci, *Corynebacterium diphtheriae* and *Bacillus subtilis*). A PLBP with typical ZnuA features can be identified in *M. smegmatis*, but not in other mycobacteria.

The isolated ZinT domain shows a more limited distribution, as it can be identified in Proteobacteria of the gamma subdivision (including *E. coli*, *S. enterica*, *Shigella dysenteriae* and *Klebsiella pneumoniae*) and in few other bacterial species. Interestingly, ZinT may be present or absent in closely related species, suggesting that this protein could be useful for colonization of specific ecological niches. For example, a typical ZinT protein can be identified in the genome of *Yersinia enterocolitica*, but not in that of *Yersinia pestis*.

We have not attempted to identify *znuB* and *znuC* genes in bacteria lacking ZnuA or AdcA, because, due to the lack of specific structural information concerning ZnuB and ZnuC, their identification is less straightforward than for ZnuA. However, it should be noted that all bacteria possessing a ZnuA protein posses genes encoding proteins showing high homology to ZnuB and ZnuC. In most Gram-negative bacteria, both the permease and the ATP-binding subunit of the transporter are localized close to z*nuA*, although the specific orientation of the genes may show species-specific variations. In contrast, in several Gram-positive bacteria the *znuB* and *znuC* genes are located in a different genetic context with respect to *znuA* or *adcA*. Whereas *znuB* and *znuC* are usually clustered together, in *Anabaena variabilis* and *Thermosynechococcus elongatus znuC* is close to *znuA*, whereas *znuB* is localized in a different chromosomal position. A peculiar case is represented by *Methylobacterium extorquens* where ZnuB and ZnuC are fused in a single polypeptide chain.

The data reported in this thesis are published in the following papers:

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., **Petrarca, P.**, Rotilio, G., and Battistoni, A. (2007) High-Affinity Zn^{2+} Uptake System ZnuABC Is Required for Bacterial Zinc Homeostasis in Intracellular Environments and Contributes to the Virulence of Salmonella enterica.. Infection and Immunity 75, 5867-5876.

Petrarca, P., Ammendola, S., Pasquali, P. and Battistoni, A.(2010). The Zur-regulated ZinT protein is an auxiliary component of the high affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage. Journal of Bacteriology , published on line and ahead of print.