ANEXO III

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Role of the High-Affinity Zinc Uptake *znuABC* System in *Salmonella enterica* Serovar Typhimurium Virulence

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The *Salmonella enterica* serovar Typhimurium *znuABC* genes encoding a high-affinity zinc uptake system and its regulatory *zur* gene have been cloned. *Salmonella* serovar Typhimurium *zur* and *znuC* knockout mutants have been constructed by marker exchange. The 50% lethal dose of the *znuC* mutant increased when either orally or intraperitoneally inoculated in BALB/c mice, while virulence of the *zur* mutant decreased only when mice were intraperitoneally challenged.

Zinc plays an important role in living organisms in which the functioning of many enzymes and structural proteins is involved. In *Escherichia coli*, two systems have been described for zinc uptake: a high-affinity system, which is made up of the products of the *znuABC* genes, and a low-affinity system depending on the *pitA* gene (2, 16). The first is negatively regulated by the Zur protein (17), whereas the second seems to be constitutively expressed and is also related to cellular inorganic phosphate transport (2). The *znuA*, *znuC*, and *znuB* operons (encoding a periplasmic-binding protein, an ATPase, and an integral membrane protein, respectively) are divergently oriented, and their transcriptional starting points are separated by 24 bp (17). The Zur protein belongs to the Fur family of metalloregulatory proteins, and in the presence of Zn^{2+}, it binds to a nearly perfect palindrome found in this region, blocking both *znuA* and *znuCB* transcription (17). If other genes under the Zur protein control exist, they remain to be elucidated.

The *zur* gene has also been functionally described in *Bacillus subtilis*, *Staphylococcus aureus*, and *Listeria monocytogenes* (5, 7, 13). Putative *E. coli*-like *zur* genes have also been identified in *Pseudomonas aeruginosa* (the *np20* gene) and *Vibrio cholerae* (the *iviXI* gene) through in vivo expression technology (4, 23). Analysis of unfinished sequences of different organisms has revealed the presence of similar *zur-znuABC* systems in different bacterial families such as *Enterobacteriaceae*, *Pseudomonadaceae*, *Neissereaceae*, and *Pasteurellaceae*, among others (17). Few and sometimes contradictory data on the importance of the zinc uptake systems in bacterial virulence are available. All of these data correspond to extracellular pathogens. Thus, it has been described that *Haemophilus influenzae znuA* and *P. aeruginosa np20* mutants are less virulent than wild-type strains (12, 23). On the other hand, virulence is not affected in an *S. aureus zur* mutant (13). Moreover, information about the role of zinc in intracellular pathogens does not exist. In this work, the importance of the *zur* and *znuC* genes in the virulence of the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium has been determined through the construction of *zur* and *znuC* knockout mutant derivatives.

Cloning of *Salmonella* serovar Typhimurium *zur* and *znuC* genes. To clone the *zur* and *znuC* genes, similarity searches were performed on the incomplete *Salmonella* serovar Typhimurium sequence genome database (http://www.genome.wustl.edu/gsc) from the Genome Sequencing Center of Washington University by using their homologs from *E. coli* as a probe. In this way, sequences corresponding to the *Salmonella* serovar Typhimurium *zur* and *znuABC* genes were identified. *Salmonella* serovar Typhimurium ATCC 14028 chromosomal DNA was used as a template to amplify the *zur* and *znuC* genes, as well as their surrounding regions, with oligonucleotide primers designed from the data obtained in the sequencing search (Table 1). These data pointed out that, as in *E. coli*, the *Salmonella* serovar Typhimurium *znuA* and *znuCB* genes are separated by a very short intergenic region (25 bp) and they are divergently transcribed.

PCR products of the expected size (943 bp and 1,447 bp containing the *zur* gene and the promoter of the *znuA* and *znuC* genes, respectively, as well as the encoding region of the former) were obtained. These fragments were cloned in pGEM-T, transformed in *E. coli* DH5α, and sequenced to confirm the presence of the desired gene. Similarity values existing between the *Salmonella* serovar Typhimurium and *E. coli zur* and *znuAC* genes were 85 and 95%, respectively.

Construction and phenotypic characterization of *zur* and *znuC* knockout mutants. To obtain *zur* and *znuC* knockout mutants, a 3.5-kb Cm^r cassette from the pHP45ΩCm plasmid was inserted in either the *Nsi*I or *Stu*I sites of these genes, which afterwards were ligated to the pGP704 suicide plasmid, giving rise to plasmids pUA925 and pUA926, respectively. These two plasmids were then introduced by biparental mating into a Rif^r derivative of *Salmonella* serovar Typhimurium

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TABLE 1. Oligonucleotide primers used in this work

Primer	Sequence[a]	Position
ZurA[b]	5′-GTGCCCCTCTCTACTGCCGC-3′	−395[f]
ZurB[b]	5′-CTCCGCTGCCATAATTCGCGG-3′	+548[f]
Zur1[c]	5′-*GGATCC*GTAACTCTTGCGTTGTGG-3′	+28[f]
Zur2[c]	5′-*GAATTC*TCGATATCAAGCTTAAGC-3′	−411[f]
ZnuC1[d,e]	5′-*GAATTC*GGCAAGTTGCGCAATGG-3′	−402[g]
ZnuC2[d]	5′-*GAATTC*GCTGTGCGCCATAATGC-3′	+1027[g]
ZnuC3[e]	5′-*GGATCC*GACACGTCAGAGAGGGAC-3′	+71[g]

[a] When present, added restriction sites are shown in italics.
[b] Primer used to obtain the 950-bp fragment containing the *S. enterica* serovar Typhimurium *zur* gene.
[c] Primer used to obtain the 439-bp fragment containing the *S. enterica* serovar Typhimurium *zur* promoter.
[d] Primer used to obtain the 1,450-bp fragment containing the *S. enterica* serovar Typhimurium *znuC* gene.
[e] Primer used to obtain the 474-bp fragment containing the *S. enterica* serovar Typhimurium *znuC* promoter.
[f] Position of the 5′ end of the oligonucleotide with respect to the translational starting point of the *S. enterica* serovar Typhimurium *zur* gene.
[g] Position of the 5′ end of the oligonucleotide with respect to the translational starting point of the *S. enterica* serovar Typhimurium *znuC* gene.

ATCC 14028 by using *E. coli* S17(λ*pir*) as the mobilizing strain (4), and the presence of the mutation was tested by both PCR (Fig. 1) and Southern analyses (data not shown) among the Cm^r Amp^s transconjugants obtained. Since it has been described that *Salmonella* serovar Typhimurium Rif^r strains may be avirulent (3), the *zur*::Cm and *znuC*::Cm constructions were transferred by P22*int7*(HT)-mediated transduction to wild-type *Salmonella* serovar Typhimurium ATCC 14028 cells. For each mutant, the presence of either the *zur*::Cm or the *znuC*::Cm construction was tested by PCR in eight of the Cm^r transductants obtained (data not shown). Likewise, absence of the P22*int7*(HT) prophage in transductants was determined by streaking the mutants onto green plates in which *Salmonella* serovar Typhimurium P22 lysogenic colonies were dark green whereas nonlysogenic cells formed light-colored colonies (6).

Salmonella serovar Typhimurium wild-type *zur* and *znuC* strains showed the same growth yield in Luria-Bertani (LB) broth (Table 2). This was probably due to the fact that the high concentration of zinc present in this medium (about 10 μM) enables cells to achieve the little quantity of this element required for bacterial growth (0.5 to 1 μM) through the low-affinity zinc uptake system, which is *pitA* dependent (14). The presence of the chelating agent EDTA dramatically decreased the growth of the *znuC* mutant, whereas *zur* and wild-type cells were practically not affected (Table 2). Addition of $ZnSO_4$ (1 mM) restored the wild-type growth yield of *znuC* cells in the presence of EDTA (Table 2), indicating that this growth defect was zinc specific. Furthermore, *Salmonella* serovar Typhimurium *znuC* cells containing plasmid pUA952 carrying the wild-type *znuCB* operon showed the same behavior as wild-type cells in the presence of EDTA (Table 2).

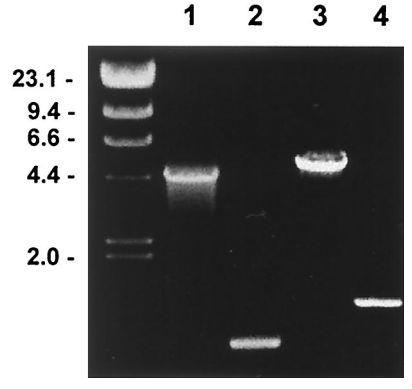

FIG. 1. Chromosomal DNAs from *Salmonella* serovar Typhimurium ATCC 14028 wild-type strain (lanes 2 and 4) and the *zur* (lane 1) and *znuC* (lane 3) mutants were subjected to PCR analysis with primer pairs ZurA-ZurB (lanes 1 and 2) and ZnuC1-ZnuC2 (lanes 3 and 4). Kilobase pairs are indicated on the left.

To further analyze *Salmonella* serovar Typhimurium *zur* and *znuC* mutants, the expression pattern of the *zur* and *znuC* genes in either the presence or absence of zinc was studied through transcriptional fusions with the promoterless *lacZ* gene contained in the broad-host range and low-copy-number pHRP309 plasmid to obtain the pUA927 and pUA928 plasmids. Both plasmids were afterwards introduced into the wild-type, *zur*::Cm, and *znu*::Cm strains. Figure 2 shows how the expression of *znuC* was higher in the *zur*::Cm cells than in both the wild-type and *znuC*::Cm cells, indicating that, as happens in *E. coli*, expression of the *Salmonella* serovar Typhimurium *znuC* gene is negatively controlled by the product of the *zur* gene. As expected, expression of the *znuC* gene in the wild-type strain was higher in the presence of EDTA than in its absence. Addition of $ZnSO_4$ to cells growing in the presence of EDTA repressed *znuC* transcription again (Fig. 2). It is worth noting that *znuC* transcription is higher in the *znuC*::Cm mutant than in the wild-type strain. This could be attributed to a lower level of intracellular zinc being present in the *znuC* mutant, which would trigger the expression of the *zur* network to which the *znuC* gene belongs. Although *zur* transcription is under its own control in *S. aureus* and *L. monocytogenes* (5, 13), *Salmonella* serovar Typhimurium *zur* gene transcription, as in *E. coli* (17), is not self regulated since expression of the *zur-lacZ* fusion is the same in wild-type cells as in *zur*::Cm cells (Fig. 2).

Recently, it has been reported that Fur protein can bind zinc (1). For this reason, and to determine if any relationship exists between the *Salmonella* serovar Typhimurium *fur* and *zur* networks, the expression of these two genes in both the *zur* and *fur* mutants was studied. Results obtained indicate that expression of the *fur* gene was not affected by the presence of a mutation in the *zur* gene (data not shown). In agreement with this, no increase in siderophore production was detected in *Salmonella* serovar Typhimurium *zur* cells on specific growth plates (19) (data not shown). Likewise, *zur* and *znuC* transcription was not enhanced in a *fur* mutant with respect to wild-type cells (data not shown).

Animal virulence and survival of *zur* and *znuC* mutants in cell cultures. To determine the 50% lethal dose (LD_{50}) of the *zur* and *znuC* strains, groups of four 6- to 8-week-old BALB/c female mice were inoculated either orally or intraperitoneally with seriated dilutions of the desired *Salmonella* serovar Typhimurium strain. Mortality was recorded up to 28 days postinfection, and the LD_{50} value was calculated as reported (18). Table 3 indicates that, contrary to what happens when the regulatory protein of the iron uptake system (encoded by the *fur* gene) is mutagenized (8), *zur* cells showed the same level of virulence as the wild-type strain when orally inoculated. This

TABLE 2. Comparative growth of the *S. enterica* serovar Typhimurium *znuC* mutant in the presence and absence of the chelating agent EDTA

Strain	Absorbance of culture grown in[a]:			
	LB medium	LB + EDTA (1 mM)	LB + EDTA (1.5 mM)	LB + EDTA (1.5 mM) + $ZnSO_4$ (1 mM)
Wild type				
ATCC 14028	2.1	1.9	1.8	2
ATCC 14028 (pUA952 [ZnuCB$^+$])	2	1.8	1.8	2.1
ZnuC mutant				
UA1778	2	0.27	0.2	2
UA1778 (pUA952 [ZnuCB$^+$])	2.1	1.9	1.8	2.1

[a] Overnight LB cultures of each *S. enterica* serovar Typhimurium strain presenting the same growth yield (an A_{550} of about 2) were diluted 1:50 into the indicated culture medium and incubated at 37°C, and A_{550} was measured after 16 h of growth. For all cases, data presented are the means of three independent experiments (each in triplicate), and the single standard deviation of any value was never greater than 10%.

attenuation of the orally inoculated *fur* mutant has been attributed to its higher sensitivity against acid pH (8), whereas the pH behavior of *zur* cells is like that of wild-type cells (data not shown). Another significant difference existing between *fur* and *zur* cells is that *recA fur* double mutants are only viable in the absence of oxygen (22) whereas *recA zur* strains support fully aerobic growth (data not shown).

An approximately tenfold increase in the LD_{50} of the *zur* mutant when mice were intraperitoneally challenged was found (Table 3). The reason for this decrease in the virulence of *Salmonella* serovar Typhimurium *zur* cells is still unknown, but it could be related to the fact that expression of the *fliAZ* transcriptional unit is lower in this mutant than in the wild-type strain (data not shown) and it has been recently demonstrated that the *fliZ* gene product is necessary for the virulence of *Salmonella* serovar Typhimurium cells (10).

The virulence of either orally or intraperitoneally inoculated *znuC* cells is decreased much more in comparison with that of both the wild-type and *zur* cells (Table 3). To confirm this attenuation of the *znuC* mutant, competitive experiments were carried out. Then, wild-type and *znuC* strains were grown separately and mixed in an approximate 1/1 ratio before animal injection. The total dose of mixed population inoculated was 2×10^3 CFU per animal, and the concentration of both strains was checked by plating seriated dilutions of the bacterial suspensions onto LB medium before mixing. Samples of blood were taken by heart puncture immediately after the death of the mouse (4 to 5 days generally after inoculation), and the concentration of bacterial cells was determined by plating dilution series onto LB medium. The percentage of each strain was calculated afterwards by replica plating of cells recovered from the animal on LB plates supplemented with chloramphenicol at 34 μg/ml.

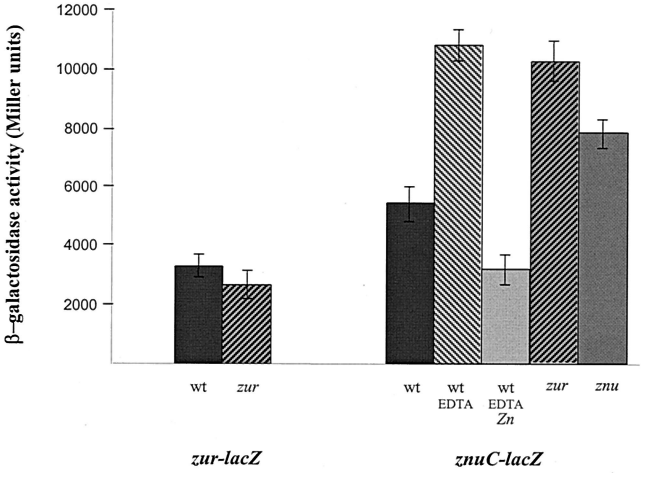

FIG. 2. Expression of *zur* and *znuC* genes in wild-type (wt), Zur$^-$, and ZnuC$^-$ strains of *Salmonella* serovar Typhimurium determined as β-galactosidase activity through either *zur-lacZ* or *znuC-lacZ* fusions. Transcription of the *znuC* gene in wild-type cells in the presence of EDTA with or without $ZnSO_4$ (*Zn*) is also shown. Concentrations of EDTA and $ZnSO_4$ used were 1.5 and 1 mM, respectively. β-Galactosidase was measured in mid-logarithmic phase cultures growing in LB medium as described previously (6). Data presented are the averages of three independent experiments with duplicate samples.

Data obtained indicate that *znuC* cells were almost fully excluded by the wild-type cells in these competition assays (Table 4). This exclusion did not exist when mixed cultures were carried out in LB medium (Table 4), indicating that the selective advantage of the wild type over the *znuC* mutant is specifically due to the environmental and growth conditions of bacterial cells during animal infection and must be attributed to the low concentration of free zinc existing in mammalian tissues (15). The poor availability of zinc could interfere in the growth of the *Salmonella* serovar Typhimurium *znuC* mutant in mice because it was necessary for either general bacterial cellular processes or for the activity of specific proteins induced during animal infection.

The behavior of the *Salmonella* serovar Typhimurium *zur* and *znuC* strains in cell cultures was also analyzed. The PK15 epithelial pig cell line (ATCC CCL-33) and the RAW264.7 mouse macrophage line (ATCC TIB-71) were used in invasion and intracellular survival experiments, respectively. Macrophages and epithelial cells were both grown at 37°C in 5% CO_2 in either Dulbecco's minimal Eagle's medium (Sigma) or minimal essential Eagle's medium (Sigma), respectively. Macrophage or epithelial lines (90% confluent growth) were infected (multiplicity of infection, 50:1) with *Salmonella* serovar Typhimurium *zur* or *znuC* cells grown in LB medium either without agitation to stationary phase or with agitation to mid-log phase, respectively. Infection of both cell cultures was allowed for 20 min. After internalization, the supernatant containing extracellular bacteria was removed. Mammalian cells were then washed with phosphate-buffered saline, and after the addition

TABLE 3. Virulence in BALB/c mice and infecting ability of *S. enterica* serovar Typhimurium *zur* and *znuC* mutants

Strain	LD_{50}[a] after inoculation		Invasion of epithelial cells[b]	Survival in macrophage cells[c]
	oral	i.p.		
ATCC 14028 (wt)[d]	5.47×10^5	9	5.95 ± 1.79	1.00 ± 0.05
UA1776 (Zur^-)	5.57×10^5	104	6.06 ± 1.22	1.12 ± 0.35
UA1778 ($ZnuC^-$)	$>3.25 \times 10^7$	1,110	5.85 ± 1.51	1.25 ± 0.59
UA1779 (Fur^-)	$>1.83 \times 10^7$	115	5.04 ± 0.44	1.09 ± 0.42

[a] The LD_{50} value was determined as reported previously (18). In all cases, the single standard deviation of any value was never greater than 10%. i.p., intraperitoneal.
[b] Percentage of bacteria (10^{-4}) added that were amikacin resistant, after infecting epithelial cells for 20 min. Values represent the mean ± the standard deviation from two experiments with duplicate samples.
[c] Ratio between the number of viable intracellular bacteria at 24 and 4 h postinfection. Values represent the mean ± the standard deviation from at least two experiments with triplicate samples.
[d] wt, wild type.

of fresh culture medium, incubation was allowed in the presence of amikacin (100 μg/ml) to kill extracellular bacteria remaining in the wells after washing (21). From this moment, samples were periodically taken and cells were lysed with phosphate-buffered saline containing 1% Triton X-100. The number of viable *Salmonella* serovar Typhimurium cells in the lysates was determined by plating serial dilutions on LB solid medium.

No relevant differences in intracellular survival or invasion rates were detected for the *zur* and *znuC* mutants in macrophages and epithelial cells with respect to those for the wild-type strain (Table 3). Similar behavior has been reported for *Salmonella* serovar Typhimurium *fur* cells (8). In concordance with this, it has been shown that production of the siderophore aerobactin in *Shigella dysenteriae* is important for extracellular multiplication but not for the intracellular stages of infection (9). Furthermore, mutants of *Salmonella* serovar Typhimurium in the *mgtA* or *mgtCB* genes involved in magnesium transport are not affected in their invasion efficiency or short-term survival within eukaryotic cells (20). This has been attributed to a very high extracellular magnesium concentration added to the eukaryotic cell growth medium which would enable *Salmonella* serovar Typhimurium cells to acquire the intracellular concentration required through a low-affinity system (20). A similar phenomenon to that described for magnesium may be responsible for the behavior of the *znuC* mutant in cell cultures. Likewise, it has also been suggested that invasive pathogens such as *Salmonella* serovar Typhimurium do not need high-affinity iron uptake systems once they are inside host cells (11).

In conclusion, our findings concerning virulence of the *Salmonella* serovar Typhimurium *znuC* mutant indicate that the *znuABC* system must be the principal one by which zinc uptake takes place in this bacterial species during mouse infection and give support to previous suggestions about the protective role that zinc-chelating systems of mammalian organisms may play against bacterial pathogens (16).

TABLE 4. Competition assay of *S. enterica* serovar Typhimurium *zur* and *znuC* mutants with wild-type strain in mixed cultures[a]

Mutant	CI	
	Mouse	In vitro
UA1776 (Zur^-)	0.54	0.75
UA1778 ($ZnuC^-$)	$<8.17 \times 10^{-5}$	1.33
UA1779 (Fur^-)	0.86	1.01

[a] Competitive indices (CIs) were calculated as described in the text and are defined as the output ratio (mutant/wild type) divided by the input ratio (mutant/wild type). Mouse CI is the average of the CIs determined from three mice. In vitro CI is the average from three independent cultures. In all cases, the single standard deviation of any value was never greater than 10%.

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