A new role for Zinc limitation in bacterial pathogenicity: modulation of α-hemolysin from uropathogenic Escherichia coli

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Metal limitation is a common situation during infection and can have profound effects on the pathogen’s success. In this report, we examine the role of zinc limitation in the expression of a virulence factor in uropathogenic Escherichia coli. The pyelonephritis isolate J96 carries two hlyCABD operons that encode the RTX toxin α-hemolysin. While the coding regions of both operons are largely conserved, the upstream sequences, including the promoters, are unrelated. We show here that the two hlyCABD operons are differently regulated. The hlyII operon is efficiently silenced in the presence of zinc and highly expressed when zinc is limited. In contrast, the hlyI operon does not respond to zinc limitation. Genetic studies reveal that zinc-responsive regulation of the hlyII operon is controlled by the Zur metalloregulatory protein. A Zur binding site was identified in the promoter sequence of the hlyII operon, and we observe direct binding of Zur to this promoter region. Moreover, we find that Zur regulation of the hlyII operon modulates the ability of E. coli J96 to induce a cytotoxic response in host cell lines in culture. Our report constitutes the first description of the involvement of the zinc-sensing protein Zur in directly modulating the expression of a virulence factor in bacteria.
Uropathogenic *Escherichia coli* (UPEC) is the main causal agent of community-acquired urinary tract infections. In order to colonize its host, it expresses a battery of virulence factors including adhesins, siderophores, flagella, and toxins. One of these factors is the pore-forming toxin α-hemolysin, whose expression induces tissue damage and more severe infections. Approximately 40–50% of UPEC isolates are able to produce α-hemolysin. Remarkably, a recent study in mice linked zinc concentrations to the pathogenic effect of α-hemolysin during gastrointestinal disorders, showing that treatment with zinc protects against α-hemolysin-induced intestinal barrier dysfunction.

α-hemolysin is encoded in the polycistronic operon hlyCABD, which contains four open reading frames (ORFs). The hlyC gene codes for an acyltransferase required for the activation of pro-α-hemolysin. Pro-α-hemolysin is encoded by hlyA, while the hlyB and hlyD genes encode two components of the secretory system that transports α-hemolysin out of the cells. The hlyCABD operon in *E. coli* is tightly regulated and strongly affected by several environmental and physiological signals. A number of regulators, including RfaH, CpxRA and H-NS, modulate α-hemolysin expression. In hemolytic UPEC isolates from humans, the hlyCABD operon is present in the chromosome within pathogenicity islands, together with genes coding for other virulence factors. Although most hemolytic UPEC strains carry a single copy of the operon, the presence of two functional copies of the hlyCABD operon has been detected in two pyelonephritis isolates, J96 and 536.

In this article, we show that the two hemolytic operons present in the chromosome of *E. coli* J96 are differentially regulated. The hlyI operon does not respond to zinc limiting conditions whereas the hlyII operon is silenced in the presence of zinc in a Zur-dependent manner. A highly conserved Zur binding site was found in the promoter sequence of the hlyII operon. Direct binding of Zur to this promoter was confirmed by electrophoretic mobility shift assay (EMSA). Although a role of Zur-mediated regulation in bacterial pathogenicity has previously been suggested, our research represents the first description of the involvement of zinc and the zinc-sensing transcription factor Zur in regulating the expression of a virulence factor in bacteria directly.

**Results**

**The two hemolytic operons of J96 are differentially expressed.** Two UPEC isolates, J96 and 536, extensively used as model organisms in pathogenicity studies, have two functional hlyCABD operons (designated I and II) coding for α-hemolysin (HlyA) and another factor, respectively. The full sequences of the two operons from 536 and the hlyCABD operon (hlyI) from J96 are known; however, only a fragment of the hlyCABD operon (hlyI) from J96, carrying part of the hlyC gene (224 bp) and its upstream sequence, is available. While the ORFs of the sequenced operons are almost identical, the hlyC upstream sequences carrying the promoter and the regulatory motifs diverge extensively between operons (Fig. 1a). Consistent with a previous report, pairwise alignments showed two different types of sequence upstream of hlyC and indicated that the hlyI operon is identical to the hlyI operon and that hlyI is identical to hlyII. The hlyI operon was the first hlyCABD operon cloned from human isolates and used to substantiate the contribution of HlyA to UPEC virulence.

J96 derivative mutant strains carrying partial deletions of either hlyI or hlyII were obtained by gene replacement. The hemolytic phenotype of the mutant derivatives was monitored on Columbia Blood Agar (CBA) plates (Fig. 1b). The mutant J96ΔII (∆hlyI) carries only the hlyI operon, displays a phenotype identical to the J96 strain. However, the J96ΔI (∆hlyII), carrying only the hlyII operon, exhibits a very thin hemolytic halo, suggesting that very little HlyA is produced from hlyII operon under optimal conditions for HlyA detection. The levels of secreted HlyA by J96 and its derivatives J96ΔI and J96ΔII were determined in bacterial cultures grown in LB, with no NaCl added at 37 °C up to late-logarithmic phase. SDS-PAGE separation of secreted protein extracts from J96 revealed a single band at 110 kDa, corresponding to HlyA. Consistent with the hemolytic phenotype, the amount of HlyA secreted by the J96ΔII strain (hlyII operon) was very similar to the amount detected in the extract from the wild-type strain, whereas the amount of HlyA secreted by the J96ΔI strain (hlyI operon) was much lower (Fig. 1c). These results suggest that in the assessed experimental conditions, the hlyII operon is the main contributor to the HlyA production and the hlyI operon is somehow silenced. The extracts analyzed in Fig. 1c were obtained from cultures grown in LB, however, the described differential expression of the two hly operons of J96 does not depend on the external osmolarity. Secreted protein extracts from cultures grown in LB (5 g l⁻¹ of NaCl) were also analyzed. Although expression of HlyA was lower in all the strains, hlyII expressed most of the HlyA, while expression of HlyA from the hlyI operon was barely detected (Fig. S1).

**A mutation in zur, encoding the zinc uptake regulator, causes derepression of the hlyII operon.** To identify putative regulators of the hlyII operon responsible for its low expression under the assessed conditions, random mutagenesis using a gentamycin resistance mariner transposon was performed on strain JFV3, a J96 derivative that carries a chromosomal hlyII::lacZ reporter fusion. Mutants were selected according to their Lac phenotype on LB agar plates with X-gal, to detect hlyII::lacZ expression. The clone #13 was selected for its deep blue colour compared to the parental strain, which indicates increased expression of hlyII::lacZ (Fig. 2a). Quantitative expression studies showed that the selected Gm₄ mutant causes a drastic derepression (47-fold) of the hlyII operon transcriptional expression (Fig. 2b). Using primers for the mariner transposon, the nucleotide sequence adjacent to the insertion was determined in clone #13. The transposon was inserted within the zur gene, in codon 142 of 172.

The zur gene encodes the Zur protein (zinc uptake regulator), a transcriptional repressor that binds to DNA in the presence of zinc and thereby represses specific genes. To corroborate the association of Zur with the observed derepression, a new zur mutant was generated in J96 by gene replacement. The sequence of the zur gene between positions 43 and 381 bp (position 1 corresponds to the first nucleotide of the first codon) was deleted, and a Cm₄ cassette was inserted. The zur::Cm₄ allele causes the same effect on hlyII transcriptional expression as the Gm₄ allele obtained by random mutagenesis both in late-logarithmic phase (Fig. 2b) and in mid-logarithmic phase (Fig. S2) of bacterial growth. The effect of the zur mutation on hlyII expression was also determined by...
measuring the amount of the secreted HlyA in culture supernatants of Zur proficient and deficient derivatives of J96, J96ΔI and J96ΔII strains. Both Coomassie-staining and immunodetection using specific HlyA antibodies showed a clear increase in the amount of HlyA in the presence of the Δzur mutation in the two strains carrying the hlyII operon (J96 and J96ΔI) (Fig. 2c). By contrast, derepression of the hlyI operon (J96ΔII strain) was not detected in the zur mutant (Fig. 2c and Fig. S1). Due to the low expression level of the hlyI operon in LB, HlyA immunodetection in J96ΔII extracts was performed on 20-fold concentrated samples. Furthermore, the severity of the hemolytic phenotype correlates with the amount of HlyA in each strain. The strain carrying only the operon hlyII showed minimal hemolysis in the presence of an intact zur gene and a higher hemolytic phenotype when the zur gene was mutated. Strains carrying only the hlyI operon showed the same phenotype, regardless of the presence or absence of an intact zur gene (Fig. 2d). These results clearly indicate that Zur specifically regulates the hlyII operon. Zur-mediated regulation of hlyII transcription was confirmed by trans-complementation experiments (Fig. 2b). The strong derepression of the hlyII operon observed in the zur mutant was fully complemented when a pBR322 derived plasmid containing the J96 zur gene with its own promoter was introduced in the mutant strain. Our findings on the Zur-mediated regulation of the hlyII operon of J96 are the first description of a regulatory factor that modulates differently the two hemolytic operons present in J96.

Hemolysin has a role in cell toxicity and urothelial damage during infection. In order to determine the impact of Zur-mediated regulation of α-hemolysin on bacterial pathogenicity, we assessed the ability of different derivatives of the J96 strain to induce hemoglobin release after infection of blood cell suspensions and cell detachment after infection of bladder epithelial cell monolayers. A slight increase in hemolytic activity was detected after infection of the Δzur derivatives of J96 and J96ΔI as compared to the zur+ counterparts (Fig. 3A). Remarkably, the zur mutation caused a very drastic effect when blood cell suspensions were infected with derivatives of the J96ΔI strain. Hemolytic activity was more than 12-fold higher in the Δzur mutant as compared to wild-type strain, consistent with the described derepression of the hlyI operon in strains lacking Zur. As a control, J96 derivative strains lacking both hlyI and hlyII operons showed very low hemolysis in either zur+ or Δzur background. These results clearly demonstrate that the effect observed was caused by α-hemolysin.

Next, we performed infection assays of bladder epithelial cells (T24 monolayers) using the same bacterial strains (Fig. 3B). After 3.5 hours of infection with the J96ΔI strain, the monolayers were intact, showing a percentage of attached cells similar to the uninfected monolayers. In contrast, when monolayers were infected with the Δzur derivative of J96ΔI less than 20% of the cells remained attached after 3.5 hours. Again, the
The effect can be attributed to the production of \( \alpha \)-hemolysin since cell attachment did not decrease after infection with J96 \( \Delta I \Delta II \Delta zur \). The specific effect of \( \alpha \)-hemolysin production on cell monolayers could be detected 2 hours post-infection as cells became round-shaped when infected with strain J96 \( \Delta zur \) (Fig. 3C), an \( \alpha \)-hemolysin-induced phenotype previously described 30. Both wild-type and \( \Delta zur \) derivatives of the J96 and J96 \( \Delta II \) strains, carrying the Zur-independent hly I operon, disrupt cell adhesion at different extents (Fig. 3B). In line with the aforementioned expression studies on the hly II operon, the \( \Delta zur \) mutation triggers virulence-associated features such as hemoglobin release and disruption of epithelial cell adhesion.

Expression of the hly II operon responds to the presence of zinc. In the presence of zinc, the transcriptional regulator Zur forms a complex that binds to DNA, causing transcriptional repression 8. We hypothesized that the hly II operon should be repressed by the presence of zinc in a Zur-dependent manner. To test this hypothesis, transcriptional expression of the hly II operon was monitored in cultures of strain JFV3 grown in either LB depleted from zinc (M-LB) or the same medium replenished with 1 \( \mu M \) and 10 \( \mu M \) ZnCl\(_2\). In the zur\(^{+}\) strain, hly II transcriptional expression is high in the absence of zinc and severely drops in medium replenished with this metal ion (Fig. 4a). Remarkably, the response of hly II expression to zinc was dose-dependent. Furthermore, zinc-mediated regulation is strictly dependent on the presence of Zur, since no repression is observed in a zur mutant strain by the presence of zinc. The growth kinetics of the JFV3 strain was not altered by the absence or presence of different zinc concentrations (Fig. 4b). The effect of zinc on hly II transcriptional expression was corroborated by monitoring the level of secreted HlyA. In cultures of the strain J96\( \Delta I \), a high amount of secreted HlyA was detected in the absence of zinc, whereas the HlyA amount was very low in cultures grown in the presence of ZnCl\(_2\) (10 \( \mu M \)) (Fig. 4c). The amount of secreted HlyA from hly II operon was also monitored in both the absence and presence of zinc. Consistent with the fact that hly II operon expression was not altered by the zur mutation, the expression of hly II operon was not affected by the presence of zinc. In cultures of the J96 strain, carrying both hly I and hly II operons, a slight effect on HlyA production by the presence of zinc was detected.

Zur directly represses hly II expression by binding to its promoter. The effect of sudden addition of ZnCl\(_2\) (10 \( \mu M \)) on hly II transcriptional expression was monitored in cultures of strains JFV3 and its \( \Delta zur \)
derivative grown in M-LB medium up to an OD 600 nm of 0.3. Bacterial growth was not substantially affected by adding ZnCl₂ (10 μM) to the zinc-free medium cultures (Fig. 5a). The ZnCl₂ exposure caused a rapid decrease of the hlyII transcriptional expression in the Zur proficient strain, as compared to cultures where no ZnCl₂ was added (Fig. 5b). After 15 minutes, a drop in hlyII expression was already detected, falling to 53% of the levels measured in cultures grown with no ZnCl₂ addition. Expression progressively declined to 20% after one hour exposition to ZnCl₂. In agreement with all previous results, exposure to ZnCl₂ during one hour did not significantly alter the expression of the hlyII operon in a zur mutant strain. As a control, the level of hlyII operon expression was very low in cultures grown in M-LB replenished with ZnCl₂ from the start.

Figure 3. Effect of zur mutation in UPEC virulence-associated phenotypes. (A) Hemolysis after 1 hour infection of sheep blood cell suspensions with J96, JFV21 (J96ΔΙ), JFV16 (J96ΔΙΙ) and EV64 (J96ΔΙΔΙΙ) strains and their otherwise isogenic zur mutants (EV27, EV34, EV38 and EV65). The release of haemoglobin was monitored by OD ₅₄₅ nm. (B) T24 cell monolayer detachment activity. Percentage of remaining attached T24 cells, measured as OD ₅₉₀ nm after 3.5 h post-infection with the same strains as in (A) Mean values with standard deviation from three independent experiments (A) and three biological replicates (B) are plotted. *P < 0.05 **P < 0.0001 n.s.: non-significant, t test with p-values adjusted by Bonferroni’s method (C) Phase contrast microscopy of T24 bladder epithelial cell monolayers infected for 2 hours with the indicated strains. Scale bar, 0.1 mm.

Figure 4. The expression of the hlyII operon of J96 responds to the external levels of zinc. (a) Transcriptional expression from the hlyII promoter in cultures of the strains JFV3 and its zur::CmR mutant derivative (EV46) grown in M-LB and M-LB replenished with either 1 or 10 μM ZnCl₂. Culture samples were taken at late-log growth phase (OD ₆₀₀ nm of 1.0). β-galactosidase activity (expressed in Miller units) was determined from three independent cultures; mean values with standard deviation are plotted. *P < 0.05, ANOVA with Tukey’s multiple comparisons test (b) Growth curves of JFV3 strain cultured in M-LB and in M-LB replenished with 10 μM ZnCl₂. Growth was monitored by measuring OD ₆₀₀ nm from two independent cultures; mean values are plotted. (c) Detection of the α-hemolysin in secreted protein extracts from cultures of J96, JFV21 (J96ΔΙ) and JFV16 (J96ΔΙΙ) strains and their otherwise isogenic zur mutants (EV27, EV34 and EV38) grown in M-LB and M-LB replenished with 10 μM ZnCl₂. Full-length gel images are shown in Fig. S8.
The fast response to ZnCl₂ of hlyII transcriptional expression suggested that Zur directly represses the hlyII promoter. Zur regulates transcription by cooperative binding of two dimers to a well-characterized DNA sequence located in the vicinity of the promoter of Zur-regulated genes. Using the consensus sequence reported for the E. coli Zur box, the putative Zur binding site was scrutinized, revealing a putative Zur binding site (Fig. 6a and b). The bases of the Zur box involved in establishing hydrogen-bonds with Zur have been identified and are indicated in Fig. 6b. A dashed line represents the symmetry axis. Below are depicted the positions of Zur binding sites in promoters of znuABC, zinT, rpmE2, and hlyII. Zur boxes were drawn to scale in respect of transcription start site. (d) Electrophoretic mobility shift assay. Titration of a Cy5 labeled 50-bp hlyII promoter fragment carrying the putative Zur binding site was carried out in the presence of excess salmon sperm DNA which serves as a non-specific competitor. Samples contain 60 pM DNA, plus 0, 15, 30, 50, 100, 150, 200, 300, 350, 900 pM Zur (calculated as dimer), respectively. Samples were resolved on a 10% polyacrylamide gel. Both gel and electrophoresis buffers contain 50 µM ZnSO₄. Full-length gel image is shown in Fig. S9.
A transcriptional start for the hly\textsubscript{II} operon was previously proposed based on primer extension analyses on RNA samples from K12 strains carrying the hly\textsubscript{II} operon in a multicopy plasmid\textsuperscript{35}. In our study, 5' RACE with RNA samples from J96 was performed and a hly\textsubscript{II} operon transcription start was defined 9 nucleotides upstream of the previously described +1 position. This transcriptional start site is properly positioned to a putative promoter identified with the Neural Network Promoter Prediction software (Fig. 6a and c). Consequently, the putative Zur binding site is located at positions −25 to −6. Zur binding would overlap the −10 sequence, blocking the binding of RNA polymerase and repressing hly\textsubscript{II} transcription. The relative position of the putative Zur binding site in the hly\textsubscript{II} operon and other genes of the E. coli Zur regulon was compared after searching the Zur consensus sequence described\textsuperscript{31} within the upstream regions of Zur-regulated genes\textsuperscript{8}. The relative location of the putative Zur binding site in the hly\textsubscript{II} operon turned out to resemble the position of other members of the Zur regulon (Fig. 6b). For instance, the location of the Zur box in znuC and zinT is −22 to −3 and −23 to −4, respectively.

The ability of Zur to bind to the hly\textsubscript{II} promoter sequences in vitro was assessed using EMSA. The Zur protein used in this assay is derived from the E. coli strain MG1655, which shares 97.7% identity and 100% similarity with the Zur protein from J96. The results clearly demonstrate that Zur binds the hly\textsubscript{II} promoter in vitro (Fig. 6d). Remarkably, E. coli Zur binds to the hly\textsubscript{II} operon even more tightly than it does to the E. coli MG1655 Zur promoter region for the L31p promoter (0.025 ± 0.01 × 10\textsuperscript{−18} M\textsuperscript{2}), which corresponds to the highest affinity of any of the operator sites evaluated\textsuperscript{31}. While the binding is too tight to calculate a reliable dissociation constant under these experimental conditions, the Zur concentration at half maximal binding (100 nM Zur dimer) allow us to estimate an upper limit for this dissociation constant, i.e. <0.02 × 10\textsuperscript{−18} M\textsuperscript{2}. Together with the in vivo studies on gene expression, these data reveal that Zur likely represses transcription of the hly\textsubscript{II} operon directly and confirm that the hly\textsubscript{II} operon is part of the Zur regulon in the UPEC strain J96. Interestingly, the putative Zur binding site was also found in the hly\textsubscript{II} operon from the UPEC strain 536 that is equivalent to hly\textsubscript{II} from J96. In 536 two mismatches from the consensus sequence were found: the mismatch already found in J96 hly\textsubscript{II} and a second base which does not directly interact with Zur (in position 13, a G instead of A or T is found). The upstream intergenic sequence of the hly\textsubscript{II} operon of J96 was also analyzed for a Zur binding site and, as expected, none was found. No Zur binding sites were found in upstream intergenic regions of hly operons from other UPEC isolates such as CFT073, UTI89 and F11. Pairwise alignments showed that these operons and their promoters are J96 hly-like. Accordingly, the Δzur mutation in CFT073 does not cause an increase of HlyA production and hemolysis after infection of blood cells (Fig. S4).

**Discussion**

The presence of two hlyCABD operons in the UPEC isolates J96 and 536, with more than 7 Kb DNA sequence sharing 99% identity, is intriguing considering that the presence of large homologous DNA sequences in a chromosome may cause genome instability and result in detrimental effects on bacterial fitness. Interestingly, the sequences upstream of the hlyC gene from both operons differ greatly, indicating that their regulatory sequences are unrelated and suggesting the existence of different mechanisms of regulation. Indeed, in this report we demonstrate that the hly\textsubscript{II} operon is silenced by the Zur regulator allowing expression only under zinc limiting conditions. In contrast, the operon hly\textsubscript{I} does not respond either to zinc or this regulator (Zur). Interestingly, most hlyCABD operons from human isolates described in databases showed homology to the Zur-insensitive operon hly\textsubscript{I}, whereas the hly\textsubscript{II} operon was only detected in two pyelonephritis isolates, 536 and J96, and in the environmental isolate MRE600. Recent genomic and phylogenetic studies revealed that MRE600 is an E. coli strain with distinctive genomic properties and more similar to *Shigella* than to other *E. coli* strains\textsuperscript{43}.

One may hypothesize that expressing hemolysin under a wide variety of environmental conditions, including zinc limitation, may be beneficial to ensure success during the process of host colonization. In this scenario, the versatility provided by having two operons that respond to different conditions may constitute an adaptive advantage that outweighs the detrimental effect in fitness caused by genome instability due to the coexistence of large and highly homologous DNA sequences.

Previous studies suggest that zinc may play a relevant role during host colonization since zinc uptake systems are important for fitness in different pathogens, including UPEC\textsuperscript{34}. However, the exact role of the Zur protein in the control of virulence factors has not yet been elucidated\textsuperscript{40}. In *S. aureus*, Zur does not have an apparent role in pathogenicity, as concluded from assays using an in vivo infection model\textsuperscript{40}. In *M. tuberculosis* and *S. suis*, Zur regulates genes coding for proteins belonging to the early secretory antigen target and metalloproteases, respectively, although the role of those proteins in virulence is unclear\textsuperscript{33,38}. In *A. baumannii*, the zur mutant exhibits a defect in dissemination in a pneumonia mouse model, and RNA-seq assays showed that several genes encoding secreted proteins and putative virulence factors were upregulated in the mutant strain\textsuperscript{33}. Our study is the first description of a direct Zur-mediated regulation of the expression of a virulence factor, UPEC’s α-hemolysin. More precisely, Zur represses the transcriptional expression of the hly\textsubscript{II} operon of J96, which consequently is only expressed under zinc limiting conditions. This finding raises thought-provoking questions. Is α-hemolysin expression a mechanism used by the bacterium to release or mobilize zinc from cells? If so, this may facilitate growth of bacterial pathogens in spite of host-induced zinc limitation. In this scenario, hly\textsubscript{II} expression would be acting as a bacterial factor involved in zinc homeostasis. Is the response of the J96 hly\textsubscript{II} operon to zinc, and therefore the acquisition of the Zur box, required for proper sensing of specific environments? If so, the presence of hly\textsubscript{II} may facilitate efficient colonization of sites within the host.

As exemplified by iron, metal limitation is a common situation during host-pathogen interactions\textsuperscript{1}. Zinc concentration in most human tissues is extremely low\textsuperscript{39}. Within the cytosol of human cells available zinc is particularly scarce, due to its sequestration by metallothioneins\textsuperscript{40}. Furthermore, during infection phagocytes and epithelial cells express other zinc-chelating proteins, such as calprotectin\textsuperscript{41–43}. In the case of UPEC, which invades the urinary tract, it inevitably faces zinc-limiting conditions since zinc concentration in human urine
was reported to range between 0.17 and 0.5 ppm (2.6 to 7.6 μM) in healthy subjects, being one of the human corporeal fluids with lowest zinc concentration reported69,44,45. Furthermore, UPEC probably encounters zinc starvation during intracellular growth, where bioavailable zinc is limited due to sequestration by host proteins like metallothioneins.

On the other hand, zinc concentration in the intestine, the reservoir of UPEC, is considerably high, due to the predominant fecal excretion of this metal69. One may speculate that during intestinal transit hlyA expression would be repressed by Zur, and therefore very little HlyA would be produced from this operon. Interestingly, during acute gut inflammation, calprotectin released by neutrophils sequesters zinc and, during this zinc-limiting conditions, Salmonella enterica Typhimurium grows better than gut commensal bacteria due to increased expression of the Zur-regulated ZnuABC transporter1. Moreover, anomalously high expression of hlyA by the UPEC isolate 536 in the intestine has been detected during active ulcerative colitis, which produces acute inflammation69. Therefore, it is reasonable to hypothesize that intestinal zinc limitation due to inflammation could promote the production of HlyA by UPEC strains residing in the gut and carrying hemolytic operons responsive to zinc, thus producing further inflammatory lesions in ulcerative colitis. A recent report described zinc treatment to be efficient against α-hemolysin-induced intestinal leakage in mice colonized by the pyelonephritis isolate 53614. It is known that zinc is an important factor for intestinal barrier integrity48. Although the authors showed that zinc has a protective effect on α-hemolysin-mediated damage per se, our data indicate that zinc may have an additional protective effect by promoting downregulation of α-hemolysin expression from the 536 strain, which contains a homolog of the Zur-regulated operon here reported. Our study describes the direct involvement of Zur in the control of virulence expression and suggests that zinc limitation may play a pivotal signaling role during the infectious process.

Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains used in this work, shown in Table 1, are all derived from the pathogenic isolates J96 and CFT073. All strains were grown routinely in LB (Lennox broth, tryptone 10 g l−1, yeast extract 5 g l−1 and NaCl 5 g l−1) at 37°C with aeration. LB without NaCl and with 10 g l−1 of NaCl was used and denoted LB0 and LB10, respectively. In most experiments, bacterial cultures were grown up to an OD600 nm of 1.0, denoted late-log phase of growth. The zinc-free medium (M-LB) was prepared as indicated49. Briefly, LB medium was treated with 50 μg ml−1 of chelating resin Chelex 100 (Bio-Rad) one hour at room temperature while stirring. After removing the resin by filtration (0.2 μm), trace elements were replenished by adding 1 μM FeCl3, 1 μM CaCl2, 20 μM MgCl2 and 1 ml l−1 SL-7 trace element solution (without zinc). Finally, 20 μM TPEN (N,N,N′,N′-Tetraakis(2-pyridylmethyl)ethylenediamine, Sigma-Aldrich) was added to further chelate zinc. Replenishment solutions were prepared out of concentrated stock solutions and sterilized by filtration using 0.2 μm filters when necessary. SL-7 trace element solution without zinc has the following composition: 10 ml 25% HCl, 1.5 g FeCl2·4H2O, 190 mg CoCl2·6H2O, 100 mg MnCl2·4H2O, 63 mg H2BO3, 36 mg Na2MoO4·2H2O, 24 mg NiCl2·6H2O, 17 mg CuCl2·2H2O and deionized water to a final volume of 1 L. In order to minimize contamination by zinc, all cultures with M-LB were performed in 5 ml culture medium using 50 ml conic centrifuge plastic tubes. Any other used material was also made of plastic and handled so as to avoid metal contamination. To monitor hemolytic phenotype Columbia Blood Agar plates (Scharlau) were used.

When needed, antibiotics were added to culture media at the indicated concentrations: kanamycin (50 μg ml−1), ampicillin (50 μg ml−1), chloramphenicol (25 μg ml−1) and gentamycin (10 μg ml−1). X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) was added to the medium at 40 μg ml−1.

Genetic techniques. The primers used in this work are listed in Table S1 (supplementary material). The strain J96Δlac is a ΔlacZ derivative of J96 strain obtained as previously described50. The strain JFV16 (J96ΔII), carrying a hlyA deletion from codon 20 to 434 was constructed using the primers HlyA-P1 and HlyA-P2 and the method described by Datsenko and Wanner51. Strain JFV21 (J96ΔI), carrying a deletion from upstream of hlyC

| Strain     | Genotype                      |
|------------|-------------------------------|
| J96        | Pathogenic isolate            |
| JFV23      | J96 Δlac                      |
| EV27       | J96 Δzar::CmR                 |
| JFV16      | J96 ΔhlyA                      |
| EV34       | JFV16 Δzar::CmR               |
| JFV3       | JFV23 hlyA::lacZ KmR          |
| Clone #13  | JFV3 zar::CmR                 |
| EV46       | JFV3 Δzar::CmR                |
| JFV21      | J96 ΔhlyA                     |
| EV38       | JFV21 Δzar::CmR               |
| EV64       | J96 ΔhlyA ΔhlyE::CmR          |
| EV65       | J96 ΔhlyA ΔhlyE::CmR Δzar::KmR |
| CFT073     | Pathogenic isolate            |
| DJ1        | CFT073 Δzar::CmR              |

Table 1. Strains used in this work.
to the codon 422 of hlyA was constructed using the same methodology and the primers Hly1-P1 and Hly1-P2-2. Strain EV64 (J96Δ1ΔII) was obtained using the same methodology and the primers Hly1-P1 and Hly1-P2-2 on the JFV16 strain. Similarly, zur mutants were generated using primers ZurP1 and ZurP2.2 in J96 and ZurP1.2 and ZurP2.1 in CFT073. The strain JFV3 carries a promoter less lacZ gene linked to a kanamycin resistance cassette inserted in the hlyA gene of the hlyT operon. The lacZ Km cassette from plasmid pKG137 was inserted into the hlyA gene of JFV16 following the method described previously. The zur gene of J96 together with the intergenic region located upstream of the zur ORF was PCR-amplified using the primers Zur-BamHI and Zur-EcoRI. The PCR-amplified fragment was cloned in pBR322 resulting the plasmid pBRzur.

SDS-PAGE and Western immunoblotting analyses. The standard SDS-PAGE procedure was used to monitor HlyA production. Gels were routinely stained with Coomassie blue. For immunoblotting, proteins were transferred to PVDF membranes and detected with the monoclonal anti-α-hemolysin H1053 and a horse-radish peroxidase-conjugate antibody (Promega) using the ECL Plus Western Blotting Detection System (GE Healthcare). Gels were analyzed on a Chemidoc System (BioRad) equipped with the QuantityOne® Software.

β-Galactosidase assay. β-Galactosidase activity was monitored by the method described earlier. Data shown are mean values and standard deviation of duplicate determinations from three independent experiments.

Assay of hemolytic activity. Quantitative hemolytic assay was performed essentially as previously described. Briefly, a 10% defibrinated sheep blood suspension was prepared in 0.9% NaCl containing 10 mM CaCl2, centrifuged and resuspended in the same volume of the same solution. The wash step was repeated three times in order to eliminate debris from broken cells. Bacterial cultures were grown in LB at 37 °C to an OD600nm of 1.0, centrifuged to discard supernatant and the bacterial pellet resuspended in the same volume of LB. In 96-well microtiter plates 50 µl of blood suspension was mixed with an equal amount of a 1/125 dilution of the bacterial cell suspension, centrifuged (400 g, 10 min, 4 °C) and incubated for 60 min at 37 °C. Thereafter, 100 µl of ice-cold 0.9% NaCl was added and the microtiter plate was centrifuged (400 g, 10 min, 4 °C). A 100 µl aliquot of the supernatant was removed to another plate and the release of haemoglobin measured spectrophotometrically at 545 nm. In all plates non-infected wells were used as controls and the resulting OD545nm from those controls was subtracted to the value obtained for the different samples. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three independent experiments. Control experiments using blood suspension prepared in 0.9% NaCl, in the absence of CaCl2, were performed. Consistent with the fact that the activity of hemolysin is calcium-dependent no hemoglobin release was detected. LB10 was used since regular LB (5 g/L NaCl) causes unspecific release of haemoglobin due to osmotic pressure alterations.

T24 cell culture assays. T24 bladder epithelial cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Bacterial cultures were grown in LB at 37 °C. Infection assays were performed in 24-well plates containing RPMI-10% FBS where 10 μL of bacterial growth (OD600nm of 0.5) were added to a monolayer of T24 cells. After infection, the cells were incubated at 37 °C, 5% CO2 for 2 h for microscopy analysis and for 3.5 h for measurements of cell detachment. The T24 monolayers were visualized after 2.5 h with an Axiovert 40 C inverted optical microscope (Carl Zeiss) and images were captured with EOS 1000D Canon camera at 25X increase. After 3.5 h infection, the cells were washed three times with ice-cold PBS, fixed in 10% formalin for 15 min at room temperature and stained in 0.1% Crystal Violet, 0.2% ethanol for 10 min at room temperature. After 2 washes with water, the cells were allowed to dry at room temperature and then lysed in 2% sodium dodecyl sulphate (SDS) for 30 min. The absorbance was quantified by spectrophotometry at 590 nm as an indicator of crystal violet staining. The data is given as percentage of attached cells. For each experiment the OD590nm of non-infected samples was given arbitrarily the value 100%. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates.

Random mutagenesis with pBT20 plasmid. Mutagenesis experiments were performed using the mariner-based transposon system carried on plasmid pBT20. Bacterial suspensions of the donor (E. coli S17- λpir containing pBT20) and the recipient (JFV3) were recovered from overnight plates and resuspended in LB. These suspensions were adjusted to OD600nm of 40 and 20, respectively, and mixed in a 1:1 ratio. Aliquots of 50 µl were spotted on a LB agar plate and incubated at 37 °C for 6 h. The mating mixtures were subsequently recovered in LB and cultured on LB agar plates containing kanamycin, gentamicin, and X-gal at 37 °C. Clones depicting darker blue color were selected, and hlyT transcriptional expression was monitored by β-galactosidase quantification. Clone #13 genomic DNA was extracted using the Blood & Cell Culture DNA Midi kit (Qiagen) with the Genome DNA Buffer Set (Qiagen) and sequenced using the primer 526.

5′RACE assay. The 5′ end of the hlyT operon transcript was determined using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Total RNA was isolated from cultures of the J96 strain grown in LB at 37 °C up to an OD600nm of 1.0. Retrotranscription was done using the oligo hlyIIBamIII, specific for the hlyT operon. Next, a polyC tail was added to the 3′ end of the cDNA by using terminal deoxynucleotidyl transferase. Two rounds of PCR amplification were performed using specific primers for the hlyT operon, hlyII-GSP2 and hlyII-GSP3, and primers provided by the commercial kit. The final PCR product was sequenced and sequenced.

Electrophoretic Mobility Shift Assay. Zur binding to the Zur box of the J96 hlyT operon was assessed as previously reported. A fluorescence-labeled 50-bp fragment of the hlyT promoter containing the putative Zur binding site was prepared by annealing two single strand DNA oligos. An oligo with a 5′ end Cy5 label (5′-5Cy5/ TCTTTATTATTGTGTGTTATGTGTATAACATATAAAAGATGTTGTTT-3′) and its reversed complement

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**Note:** The text is a direct transcription of the scientific report content, formatted for readability. Each section is clearly outlined, and the experimental methodology is described in a coherent and logical sequence, adhering to scientific writing standards. This representation allows for easy comprehension and analysis by a pure text model.
sequence with no labels (5′-AAACAAATCTTTTATAGTTGTAATAACACAACAATAAATAAGA-3′) were ordered from Integrated DNA Technologies (IDT). To make double stranded DNA, oligos were dissolved in the annealing buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.5), incubated at 95 °C for 5 min and slowly cooled to room temperature. 60 pm of DNA and various concentrations of the wild-type Zur protein (from E. coli MG1655) was mixed in the binding buffer (10 mM Tris, 10 mM NaCl, pH 8.0) containing 2 mM MgCl₂, 1 mM CaCl₂, 166 mM KCl, 100 mM L-potassium glutamic acid, 100 μg/mL bovine serum albumin (BSA), 2 μg/mL sonicated salmon sperm DNA, 5 mM DTT, 50 μM ZnSO₄, 5% glycerol. DNA-protein mixtures were incubated at room temperature shielded from light for 30 minutes. The purity of the Zur protein used is shown in Fig S5. In all Zur/DNA binding assays, protein samples were equilibrated with operator DNA with addition of excess non-specific competitor DNA (i.e. salmon sperm DNA). This allows direct monitoring of specific protein-DNA interactions. Samples were then resolved in a 10% polyacrylamide (37.5:1 acrylamide:bisacrylamide) gel. Both gel and electrophoresis buffers contain 89 mM Tris (pH 8.0), 89 mM boric acid and 50 μM ZnSO₄. Gels were run at 10 V/cm for 60–70 minutes and visualized by Typhoon 9400 imager (GE Healthcare).

Statistical analysis. Analysis was carried out using R software. Unpaired t tests with p-values adjusted by Bonferroni's method for multiple comparisons were used to make comparisons between two from a data set grouped by several groups (i.e. cell detachment and cytokinesis data). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used for comparison of a data set formed by more than two groups (i.e. complementation assay). Two-way ANOVA was used to compare a data set with two variables (i.e. hly₅₁ expression in several genotypes under different zinc concentrations). A value of P < 0.05 was considered statistically significant.

Data availability. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

References
1. Porcheron, G. & Dozois, C. M. Interplay between iron homeostasis and virulence: Fur and RyhB as major regulators of bacterial pathogenicity. Vet. Microbiol. 179, 2-14 (2015).
2. Wen, Y. T. et al. Differential secretomics of Streptococcus pyogenes reveals a novel peroxide regulator (PerR)-regulated extracellular virulence factor mitogen factor 3 (MF3). Mol. Cell. Proteomics 10(M110), 007013 (2011).
3. Liu, J. L. et al. Zinc sequestration by the neutrophil protein calprotectin enhances Salmonella growth in the inflamed gut. Cell Host Microbe 11, 227–239 (2012).
4. Ma, L., Terwilliger, A. & Maresco, A. W. Iron and zinc exploitation during bacterial pathogenesis. Metallomics 7, 1541–1554 (2015).
5. Hantke, K. Bacterial zinc uptake and regulators. Curr. Opin. Microbiol. 8, 196–202 (2005).
6. Palmer, L. D. & Skaar, E. P. Transition Metals and Virulence in Bacteria.
7. Madan, S. et al. Molecular cloning and physical characterization of a chromosomal hemolysin from Escherichia coli. Infect Immun 62, 309–316 (1994).
8. Patzer, S. I. & Hantke, K. The Zinc-responsive Regulator Zur and Its Control of the znu Gene Cluster Encoding the ZnuABC Zinc Uptake System in Escherichia coli. Infect Immun 74, 2432–24332 (2006).
9. Panina, E. M., Mironov, A. A. & Gelfand, M. S. Comparative genomics of bacterial zinc regulators: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc. Natl. Acad. Sci. USA 100, 9912–9917 (2003).
10. Outtten, C. E. & O’Halloran, T. V. Fetmotolormal Sensitivity of Metallothionein Proteins Controlling Zinc Homeostasis. Science (80-.). 292, 2488–2492 (2001).
11. Terlizzi, M. E., Gribaudo, G. & Maffei, M. E. UroPathogenic Escherichia coli (UPEC) Infections: Virulence Factors, Bladder Responses, Antibiotic, and Non-antibiotic Antimicrobial Strategies. Front. Microbiol. 8 (2017).
12. Ristow, L. C. & Welch, R. A. Hemolysin of uropathogenic Escherichia coli: A cloak or a dagger? Biochim. Biophys. Acta 1858, 538–545 (2016).
13. Móbily, H. L. et al. Plyneurotrophic Escherichia coli and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect Immun 58, 1281–1289 (1990).
14. Wiegandt, S. et al. Zinc treatment is efficient against Escherichia coli α-hemolysin-induced intestinal leakage in mice. Sci. Rep. 7, 45649 (2017).
15. Welch, R. A. Pore-forming cytolysins of gram-negative bacteria. Mol Microbiol 5, 521–528 (1991).
16. Stanley, P. M. et al. Environmental regulation of alpha-haemolysin expression in Escherichia coli. Mol. Microbiol Mol Biol Rev 45649 (2017).
17. Nakamura, H. et al. α-Hemolysin expression alters the course of acute and persistent urinary tract infection. Proc. Natl. Acad. Sci. USA 112, 871–880 (2015).
18. Morote, J. M. et al. Expression of the hemolysin operon in Escherichia coli is modulated by a nucleoid-protein complex that includes the proteins Hha and H-S. Mol Gen Genet 267, 349–358 (2000).
19. Mounir, M. et al. Environmental regulation of alpha-haemolysin expression in Escherichia coli. Microb Pathog 16, 249–259 (1994).
20. Dobrin, D. et al. Toxin genes on pathogenicity islands: impact for microbial evolution. Int. J. Med. Microbiol. 290, 307–311 (2000).
21. Nagy, G. et al. Loss of regulatory protein RhH attenuates virulence of uropathogenic Escherichia coli. Infect Immun 70, 4406–4413 (2002).
22. Nagamata, K. et al. Dysregulation of Escherichia coli α-hemolysin expression alters the course of acute and persistent urinary tract infection. Proc. Natl. Acad. Sci. USA 112, 871–880 (2015).
23. Morose, J. M. et al. Expression of the hemolysin operon in Escherichia coli is modulated by a nucleoid-protein complex that includes the proteins Hha and H-S. Mol Gen Genet 263, 349–358 (2000).
24. Mounir, M. et al. Environmental regulation of alpha-hemolysin expression in Escherichia coli. Microb Pathog 16, 249–259 (1994).
25. Dobrin, D. et al. Toxin genes on pathogenicity islands: impact for microbial evolution. Int. J. Med. Microbiol. 290, 307–311 (2000).
26. Nagy, G. et al. Both alpha-hemolysin determinants contribute to full virulence of uropathogenic Escherichia coli strain 536.
27. Mortensen, B. L., Rathi, S., Chazin, W. J. & Skaar, E. P. Acinetobacter baumannii Response to Host-Mediated Zinc Limitation Requires the Transcriptional Regulator Zur. J. Bacteriol. 196, 2616–2626 (2014).
28. Welch, R. A., Hull, R. & Falkow, S. Molecular cloning and physical characterization of a chromosomal hemolysin from Escherichia coli. Infect Immun. 42, 178–186 (1983).
29. Welch, R. A., Dellingler, E. P., Minshew, B. & Falkow, S. Hemolysin contributes to virulence of extraintestinal E. coli infections. Nature 294, 665–667 (1981).
30. Balsalobre, C. et al. Release of the type I secreted alpha-hemolysin via outer membrane vesicles from Escherichia coli. Mol Microbiol 59, 99–112 (2006).
31. Patzer, S. I. & Hantke, K. The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli. Mol. Microbiol. 28, 1199–1210 (1998).
32. Skals, M., Jorgensen, N. R., Leipziger, J. & Praetorius, H. A. Alpha-hemolysin from Escherichia coli uses endogenous amplification through P2X receptor activation to induce hemolysis. Proc. Natl. Acad. Sci. USA 106, 4030–4035 (2009).
33. Dhakal, B. K. & Mulvey, M. A. The UPEC pore-forming toxin α-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways. Cell Host Microbe 11, 58–69 (2012).
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Author Contributions

All authors conceived and designed the study. E.V., M.S., J.F.-V., S.W., D.J., E.G. and C.B. performed the investigations. C.B. and E.V. wrote the original draft of the manuscript text and prepared the figures. All authors reviewed the manuscript.

Additional Information

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