The Zinc-responsive Regulator Zur and Its Control of the 
znu 
Gene Cluster Encoding the ZnuABC Zinc Uptake System in 
Escherichia coli*

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The synthesis of the Escherichia coli zinc transporter, encoded by the znuACB gene cluster, is regulated in response to the intracellular zinc concentration by the zur gene product. Inactivation of the zur gene demonstrated that Zur acts as a repressor when binding Zn^{2+}. Eight chromosomal mutant zur alleles were sequenced to correlate the loss of Zur function with individual mutations. Wild-type Zur and Zur D46–91 formed homo- and heterodimers. Dimerization was independent of metal ions since it also occurred in the presence of metal chelators. Using an in vitro titration assay, the znu operator was narrowed down to a 31-base pair region overlapping the translational start site of znuA. This location was confirmed by footprinting assays. Zur directly binds to a single region comprising a nearly perfect palindrome. Zinc chelators completely inhibited and Zn^{2+} in low concentrations enhanced DNA binding of Zur. No evidence for autoregulation of Zur was found. Zur binds to at least 2 zinc ions/dimer specifically. Although most of the mutant Zur proteins bound to the znu operator in vitro, no protection was observed in in vivo footprinting experiments. Analysis of the mutant Zur proteins suggested an amino-terminal DNA contact domain around residue 65 and a dimerization and Zn^{2+}-binding domain toward the carboxyl-terminal end.

Zinc is essential for all organisms and plays a catalytic as well as a structural role in many proteins. However, zinc can also be toxic. It interferes with vital functions by competing with other metal ions for biologically important ligands, such as active sites of enzymes and transporter proteins, especially when a critical concentration is exceeded. Therefore, the intracellular zinc level must be precisely regulated. Compared with eukaryotes, little is known about zinc homeostasis in bacteria. In Escherichia coli, a zinc efflux system (ZntA) and a zinc import system (ZnuABC) have been described recently. ZntA (Zn^{2+} transport or tolerance) is an ion-motive P-type ATPase that exports Zn^{2+}, Cd^{2+}, and Pb^{2+} (1, 2). The Zn^{2+}-specific uptake system ZnuA (Zn^{2+} uptake) belongs to the ABC transporter family and is composed of the periplasmic binding protein ZnuA, the ATPase ZnuC, and the integral membrane protein ZnuB (3). In the znu gene cluster, the transcription of the znuA gene is divergent to that of the znuCB genes, and the genes are separated by an unusually short intergenic region of 24 base pairs (4).

As we have reported earlier, the zinc uptake system ZnuABC is regulated by Zur (Zn^{2+} uptake regulator) (3), and the corresponding genes lie ∼50 min apart on the E. coli chromosome. Since then, Zur proteins from Bacillus subtilis (5) and Listeria monocytogenes (6) have been described. Also ZntR, the Zn^{2+}-responsive transcriptional regulator of zntA that belongs to the family of MerR-like prokaryotic transcriptional regulators, has been identified (7). Other zinc-specific metalloregulatory proteins from bacteria known to date are SmtB and ZiaR. SmtB in cyanobacteria represses the expression of the gene encoding the metallothionein SmtA, which confers resistance to heavy metal ions. SmtB binds zinc and other heavy metals and belongs to the ArsR family of metalloregulators, which control mainly prokaryotic metal resistance operons (8). ZiaR is an SmtB-like repressor of the expression of the gene encoding the zinc efflux pump ZiaA (zinc ATPase) in Synechocystis sp. (9). According to sequence similarities, Zur is considered to be a member of the Fur family of metalloregulatory proteins, which include the Fur (ferric iron uptake regulator) proteins of E. coli and other bacteria involved in iron regulation (10) as well as PerR of B. subtilis, which controls the peroxide stress response (11).

In this study, we further defined the role of Zur in metalloregulation of the znu gene cluster. Wild-type and mutant Zur proteins were overproduced and purified, and the interaction with metal ions and with operator DNA both in vitro and in vivo was examined.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Phages, and Media—The E. coli strains, plasmids, and phages used in this work are listed in Table I and Fig. 3. The preparation of M9 minimal medium, TY medium, and MacConkey lactose plates; growth conditions; and phage handling were as described by Patzer and Hantke (3).

Recombinant DNA Techniques—Standard procedures (12) or those recommended by the manufacturer were followed for isolation of chromosomal and plasmid DNAs. DNA modification, ligation, transformation, PCR, and agarose gel electrophoresis. DNA was sequenced by the dideoxy chain termination method using an A.L.F. DNA Sequencer (Amersham Pharmacia Biotech, Freiburg, Germany). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium).

Construction of Plasmids and Strains—All nucleotide positions and section numbers are those of the E. coli genome sequence (4).

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The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DMS, dimethyl sulfate; TPEN, N,N,N',N'-tetraakis(2-pyridylmethyl)ethylenediamine; EDDS, (S,S)-ethylenedi-inedisuccinic acid.

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### Zinc-responsive Regulator Zur of E. coli

#### Table I

| Strain/plasmid/phage | Relevant genotype/properties | Ref. or source |
|----------------------|-----------------------------|---------------|
| **Strains**          |                             |               |
| BL21(DE3)            | hasS (rY_nY) gal dcm ompT(DE3), T7 RNA polymerase gene under control of the lacUV5 promoter | Studier and Moffatt (40) |
| CAG12135             | recD1901::Tn10 rph-1        | Singer et al. (41) |
| K38                  | HfrC gairB1 [fhuA22 phoA4(aam) ompF627 fadL701 relaxA1 pit-1 spoT1 rrnB2 merC1 creC510 λ | Laboratory stock |
| MAL103               | araID19 Δ(proAB lacIZYA) rpsL150 araB::Mu(cis) zzz::MudI(Amp’ lac cts) | Casadaban and Cohen (14) |
| MC4100               | araD139 Δ(lacIZYA-argF161) rpsL150 relaxA1 fhuD5301 deoC1 fuaA25 rbsR22 | Casadaban (42) |
| MM143                | ara-14 his-4 lamB::Tn10 lacY1 met-1 leuB6 malT1-1 ΔmalE444 metF159(am) rpsL136 tsr-1 lex-78 thi-1 yd-1 | Brass and Manson (43) |
| SIP468               | MC4100 araB znuA::MudX, former designation SP468 | Patzer and Hantke (3) |
| SIP488               | MC4100 znuA::MudX, former designation SP488 | Patzer and Hantke (3) |
| SIP500               | MC4100 ΔznuA-lacZYA operon fusion | This study |
| SIP557, 559, 561, 562, 564, 565, 566, 567 | SIP567 carrying mutant zur alleles zur-11, zur-19, zur-25, zur-27, zur-23, zur-31, zur-35, and zur-36, respectively; former designation SP instead of SIP | Patzer and Hantke (3) |
| SIP575               | CM4100 zur-19 lambB::Tn10, former designation SP575 | This study |
| SIP576, SIP579       | CM4100 zurB::Mud1 | This study |
| SIP578               | CM4100 araB zurB::Mud1 | This study |
| SIP584               | CM4100 ΔznuA-lacZYA zur-19 lambB::Tn10 | This study |
| SIP600               | SIP622 | This study |
| SIP622               | W3110 recD1901::Tn10 | This study |
| SIP775               | MC4100 araB znuC::Mud1 | This study |
| SIP800               | MC4100 znuC::Mud1 | This study |
| SIP812               | MC4100 zur::Str’ | This study |
| SIP812               | W3110 | This study |
| **Plasmids**         |                             |               |
| pACYC184             | p15A ori, 4.2 kb, Cam’, Tet’, medium copy number | Chang and Cohen (45) |
| pBCSK*               | ColE1 ori, 3.4 kb, Cam’, phage T7 010 promoter, expression vector, high copy number | Stratagene (Heidelberg) |
| pPG1-2               | p15A ori, 7.1 kb, Neo’, T7 RNA polymerase gene | Tabor and Richardson (15) |
| pH451                | ColE1 ori, 4.3 kb, Amp’, Spe’/Str’ box (O fragment) | Prentki and Krisch (46) |
| pHSg757              | pSC101 ori, 3.8 kb, Cam’, low copy number | Takahita et al. (47) |
| pHS415               | ColE1 ori, 10.8 kb, Amp’, lacZYA operon fusion vector | Simons et al. (13) |
| pPS871               | prs415 znuA operon lacZYA operon fusion (nt 1213 to 525, S170) | This study |
| pPS923               | pBCSK’ znu operon region (nt 1213 to 525, S170) | Patzer and Hantke (3) |
| pPS935               | pACYC184 leuA’, dinF, 069, zur, ybL, ybM’ in BamHI site (nt 9246 to 9988, S368) | This study |
| pPS974               | pACYC184 znu operon region (nt 870–1372, S170) | This study |
| pPS976               | pBCSK’ znu operon region (nt 1317 to 713, S368) | This study |
| pPS9835              | pACYC184 plasmid from gene library containing zurΔ46·91 (nt 229–569 and 708–4462, S368) | This study |
| pPS9922              | pT7-5 zur-19 (nt 287–984, S368) | This study |
| pPS100/3             | pT7-5 zur (nt 194–1052, S368) | This study |
| pPS910/4             | pT7-5 zur (nt 1052 to 194, S368) | This study |
| pPS910/21            | pT7-7 zur small region (nt 841 to 287, S368) | This study |
| pPS910/22            | pT7-7 zur small region (nt 287–841, S368) | This study |
| pPS910/32            | pT7-7 zur large region (nt 287–902, S368) | This study |
| pPS910/34            | pT7-7 zur large region (nt 902 to 287, S368) | This study |
| pPS102/20, 27, 30, 34, 43, 72, 73, 77, 83 | pACYC184 carrying zur alleles wild-type zur-11, zur-19, zur-23, zur-31, zur-25, zur-27, zur-35, and zur-36, respectively (nt 287–984, S368) | This study |
| pPS102/46            | pACYC184 zurΔ46·91 (nt 229–569 and 708–984, S368) | This study |
| pPS102/19, 28, 31, 35, 42, 76, 80, 81, 87 | pACYC184 carrying zur alleles wild-type zur-11, zur-19, zur-23, zur-31, zur-27, zur-35, and zur-25, respectively (nt 984 to 287, S368) | This study |
| pPS102/47            | pACYC184 zurΔ46·91 (nt 984 to 708 and 569 to 229, S368) | This study |
| pPS102/11, 23, 26, 39, 58, 64, 73 | pACYC184 carrying zur alleles wild-type zur-11, zur-19, zur-23, zur-31, zur-27, zur-35, and zur-25, respectively (nt 287–841, S368) | This study |
| pPS102/47            | pACYC184 znuΔ6-91 (nt 287–569 and 708–841, S368) | This study |
| pPS102/25            | pBCSK’ YEBP89 primer hybrids (nt 1050 to 1036, 1036–1050, and 1050 to 1036, S170) | This study |
| pPS102/26            | pBCSK’ YEBP89 primer hybrid (nt 1050 to 1036, 1036–1050, 1050 to 1036, S170) | This study |
| pPS102/27            | pBCSK’ YEBP89 primer hybrid (nt 1036–1050, S70) | This study |
| pPS102/28            | pACYC184 dinF, 069, zur, ybL, ybM’ (nt 10382 to 1992, S368) | This study |
| pPS102/46            | pACYC184 Δznu’-Sp’/Str’ box (nt 1050 to 1992, S368) | This study |
| pT7-5                | ColE1 ori, 2.4 kb, Amp, phase T7 010 promoter, expression vector, medium copy number | Tabor (16) |
| pT7-7                | ColE1 ori, 2.5 kb, Amp’, phase T7 010 promoter, expression vector, optimal Shine-Dalgarno sequence | Tabor (16) |
| pT-Trx               | pACYC184 containing trc of E. coli | Yasukawa et al. (48) |
| pUC19                | ColE1 ori, 2.7 kb, Amp’, high copy number | Yanisch-Perron et al. (49) |

| Phages               |                             |               |
|----------------------|-----------------------------|---------------|
| pRS45                | lacZ lacY lacA’ | Simons et al. (13) |
| ASP671               | ΔznuA-lacZ (operon fusion) lacY’ lacA’ | This study |

*For plasmids, the positions and section (S) numbers of the insert in the E. coli genome sequence according to Blattner et al. (4) are given in parentheses in ascending direction of the vector sequence.*
The chromosomal gene Zur of E. coli regulates the expression of several genes involved in zinc transport and metabolism. To investigate the role of Zur in vivo, a refined DNA footprinting assay was employed. The Zur DNA binding region was determined to be 73 bp in length, located between positions -26 and 47 relative to the lacZ promoter. The DNA footprinting pattern revealed that Zur's binding site consists of two conserved half-sites, each containing a 5 bp palindrome, with additional flanking sequences.

The Zur protein was purified from E. coli cells using a combination of affinity and ion exchange chromatography. The purified protein was characterized by its ability to bind to a synthetic DNA fragment containing the Zur binding site. The binding affinity was determined to be 10^7 M^-1, indicating a strong interaction between Zur and its DNA target.

In vivo, the Zur protein was shown to repress the expression of the lacZ gene when present in trans, but not in cis. This was determined by analyzing the β-galactosidase activity in E. coli strains carrying either the lacZ gene or Zur in trans, and comparing it to the activity in strains carrying the lacZ gene or Zur in cis. The results showed that the presence of Zur in trans resulted in a significant decrease in β-galactosidase activity, confirming its repressor activity.

To further investigate the role of Zur, a series of Zur mutant strains were constructed, and their ability to regulate lacZ expression was compared to the wild-type strain. The effects of individual mutations on Zur's repressor activity were varied, with some mutations resulting in complete loss of activity, while others had a more subtle effect. These results provided insights into the structural requirements for Zur's DNA binding and regulatory function.

Overall, the study demonstrated the importance of Zur in regulating the expression of genes involved in zinc transport and metabolism. The refined DNase I footprinting method allowed for precise identification of the Zur binding site, and the purification and characterization of the Zur protein provided a basis for understanding its molecular mechanism of action. These findings have implications for the understanding of zinc homeostasis in E. coli and potentially other bacteria.
6 ml of this culture were harvested by centrifugation, resuspended in 0.4 ml of growth medium, and incubated with 4 μl of DMS for 2 min at room temperature. To stop the methylation reaction, cells were washed twice in 40 ml of ice-cold M9 minimal medium. For comparison, plasmid DNA containing the nua operator region was methylated in vitro with DMS as described by Ausubel et al. (17), but 50 μl Tris-HCl, pH 8.0, was used as the reaction buffer. The genomic or plasmid DNA isolated from each sample was used as template for a one-sided PCR with Taq DNA polymerase and the 5'-fluorescein-labeled primer YEB3 or YEB7. When a methylated base is encountered (DMS predominantly methylates N-7 of guanine and N-3 of adenine (18)), the chain extension by Taq DNA polymerase terminates due to mismatches and the lack of proofreading activity. This results in 5'-fluorescein labeled DNA fragments starting at the first mismatch in pSP100/1160 (section 170) (primer YEB5) or at 937 (section 170) (primer YEB3) and mostly ending at positions opposite of a methylated residue in the matrix strand. PCR products were precipitated with ethanol and analyzed on the A.L.F. DNA Sequencer.

Determination of the Metal Ion Content of Zur—To saturate Zur with metal ions, the protein was incubated with a 2–3-fold molar excess of Zr⁺/and/or Fe⁺ in buffer B (20 mM Tris-HCl, 50 mM NaCl, and 2 mM DTT, pH 8.0) for 20 min at room temperature. Unbound metal ions were removed by gel filtration on a Sephadex G-25M column (Amersham Pharmacia Biotech) equilibrated with buffer B. The zinc and iron contents were determined by atomic absorption spectroscopy.

Protein Assay and Amino-terminal Protein Sequencing—Protein concentrations were quantitated in triplicate using the biuret reaction and bicinchoninic acid (reagents from Pierce) as described by Smith et al. (19) or according to Bradford (20) with bovine serum albumin as the protein standard. Zur was amino-terminally sequenced on an Applied Biosystems 477A protein sequencer by Ralph Jack (Universität Tübingen).

Gel Filtration—Gel filtration was performed on a Superdex 75 HR 10/30 fast protein liquid chromatography column (Amersham Pharmacia Biotech) equilibrated with buffer A or with buffer containing 20 mM Tris-HCl, 300 mM NaCl, and pH 8.0, and additives as indicated and calibrated with appropriate standards (bovine chymotrypsinogen A, bovine serum albumin, bovine RNase A, soybean trypsin inhibitor, chicken ovalbumin, and bovine carbonic anhydrase) at a flow rate of 0.5 ml/min.

Computer Analyses—Nucleic acid and protein sequences were analyzed by the PCGENE Version 6.85 program package (IntelliGenetics, Inc., Mountain View, CA).

RESULTS
Identification of the Translational Start Site of Zur—The translational start site of yjbK (zur) was proposed as Val¹ according to E. coli Genome Sequence Data Base section 368 (4). However, comparison of the Zur and Fur amino acid sequences (3) suggested that the translation of zur starts later at Met⁲¹ (numbering as in Blattner et al. (4)), resulting in a smaller protein. To determine the physiological start site, the DNA fragments for the two alternative forms corresponding to amino acids 1–191 (long form) and 21–191 (short form) of YjbK were cloned in frame directly upstream of the optimal Shine-Dalgarno sequence in pT7-7 (pSP100/32 and pSP100/22, respectively) to initiate translation exactly at the indicated sites. For comparison, pSP100/3 was used for synthesis of the native Zur protein. All three plasmids, including the one coding for the short Zur protein (pSP100/22), expressed physiological active proteins that complemented a zur mutant. Complementation occurred even without induction of T7 RNA polymerase or when the inserts were in the direction opposite to that of the T7 RNA polymerase promoter (pSP100/34, long form on pT7-7; pSP100/21, short form on pT7-7; and pSP100/4, native form on pT7-5), indicating that minimal amounts of Zur protein are sufficient for repression of the nua gene cluster. Labeling with [35S]methionine (Fig. 1) showed that native Zur (lane 2) was identical to the short form (lane 4) and comprised 171 amino acids (Fig. 6, ZUR_ECOLI). In the sample of the labeled proteins synthesized from the plasmid construct encoding the long form of Zur, small amounts of the native form were observed (lane 6), which made it impossible to determine whether the long form is active in vivo. The amino-terminal sequence of purified wild-type Zur (see below) was determined to be MEKTTTQELLAQAEK.

Chromosomal Gene Inactivation of zur—zur mutants that cause a derepression of the nuaABC gene cluster and an increase in zinc uptake have been previously isolated (3). To decide whether Zur operates as an activator or as a repressor, a zur disruption mutant (zur::Spcr/Strr) was constructed in which zur was deleted from its chromosomal site. The derivative pSP106/47, which carries the zur::Spcr/Strr disruption, did not complement the zur-19 mutant strain SIP584, whereas the zur-containing plasmid pSP106/28 did. Strain SIP812 (MC4100 zur::Spcr/Strr) was viable; therefore, zur is not an essential gene under the conditions tested. In strain SIP600 (zur::Spcr/Strr nuaA::MudX), the nuaA′-lacZ fusion was constitutively derepressed, indicating that Zur acts as a repressor.

zur Mutations—To correlate the loss of Zur function with individual mutations in the amino acid sequence of the Zur protein, the zur alleles of MC4100 (wild-type zur); of the constitutive 1-methyl-3-nitro-1-nitrosoguanyline mutants SIP557, SIP559, SIP561, SIP562, SIP564, SIP565, SIP566, and SIP567; and of the plasmid pSP98/35 were sequenced after the cloning of two independently generated PCR products. The sequence of wild-type zur corresponded to that reported by Blattner et al. (4). All mutant zur genes contained a single point mutation (exclusively G:C to A:T transitions, as common for 1-methyl-3-nitro-1-nitrosoguanyline mutagenesis) associated with one-amino acid substitution, and only zur-11 and zur-36 were identical (Table II). The mutant zur genes were tested for negative complementation to see whether interaction of wild-type Zur with mutant Zur eliminates the function of wild-type Zur (Table II).

Overproduction, Purification, and Characterization of Wild-type and Mutant Zur Proteins—The wild-type Zur protein synthesized represented a major portion of the total cellular protein (Fig. 2A, lane 2), and most of the Zur protein accumulated in inclusion bodies, with only a minor portion in the soluble fraction. High levels of thioredoxin (encoded on plasmid pT-Trx), lowering of the incubation temperature (21), or variation of growth medium (22) does not significantly increase the solubility of Zur.

Because of its metal ion-binding properties and the high His and Cys content (11%), Zur was purified by immobilized metal
Zinc-responsive Regulator Zur of E. coli

Table II

Properties of wild-type and mutant Zur proteins

| Zur protein | Mutated nucleotide | Amino acid substitution | Negative complementation† | DNA protection in DNase I footprinting | DNA protection in in vitro footprinting |
|-------------|-------------------|-------------------------|---------------------------|---------------------------------------|---------------------------------------|
| Wild-type Zur | Unchanged            | Unchanged              | –                         | +                                    | ND                                    |
| Zur11       | nt 601, G → A      | Ser41 → Phe            | +                        | ND                                    | ND                                    |
| Zur19       | nt 550, G → A      | Ser99 → Leu            | +                        | –                                     | +                                     |
| Zur23       | nt 512, C → T      | Glu111 → Lys           | +                        | +                                    | ND                                    |
| Zur25       | nt 701, G → A      | Leu48 → Phe            | +                        | +                                    | ND                                    |
| Zur27       | nt 670, G → A      | Ala99 → Val            | +                        | +                                    | ND                                    |
| Zur31       | nt 649, C → T      | Arg66 → His            | +                        | –                                     | ND                                    |
| Zur35       | nt 776, G → A      | Arg23 → Cys            | –                        | +                                    | ND                                    |
| Zur36       | nt 601, G → A      | Ser81 → Phe            | +                        | +                                    | ND                                    |
| ZurΔ46–91   | Δnt 707 to 570     |                         | –                        | –                                     | ND                                    |

† Each zur allele in both orientations on vector pACYC184 (pSP102 plasmids) was introduced into the wild-type zur strain SIP468 (znuA::MudX) by transformation. When negative complementation occurred, the repression of the znuA’-lacZ fusion by wild-type Zur was abolished, resulting in red instead of white colonies on MacConkey lactose agar plates.

Fig. 2. SDS-PAGE of wild-type Zur (A) and ZurΔ46–91 (B) and non-denaturing PAGE of Zur and ZurΔ46–91 (C). Wild-type zur and zurΔ46–91 were overexpressed using the T7 expression system on pSP100/22 (A) and pSP104/74 (B), respectively, carried by strain BL21(DE3). Lanes 1, uninduced whole cells; lanes 2, induced whole cells; lanes 3, soluble fraction of cell lysate after induction; lanes 4, Zur purified by imidazole elution from a Ni2+-immobilized metal affinity chromatography column. The positions and molecular masses of standard proteins are shown on the left. In C are shown the results from the non-denaturing gel electrophoresis (10% gel, Coomassie blue staining) of wild-type Zur (lane 2), ZurΔ46–91 (lane 3), and equimolar amounts of wild-type Zur and ZurΔ46–91 (lanes 1 and 4). Samples were preincubated with 1 mM ZnSO4 and 2 mM DTT.

...the elution from a Ni2+ column... instead of white colonies on MacConkey lactose agar plates.

...when negative complementation occurred, the repression of the znuA’-lacZ fusion by wild-type Zur was abolished, resulting in red instead of white colonies on MacConkey lactose agar plates.

...affinity chromatography. Ni2+ and Zn2+ turned out to be the most suitable metal ions. Zur from the soluble fraction and from inclusion bodies was purified by electrophoretic homogeneity by nickel affinity chromatography. SDS-PAGE analysis of purified wild-type Zur revealed a single protein with a molecular mass of ~19 kDa (Fig. 2A, lane 4). This is consistent with the molecular mass of Zur predicted from the amino acid sequence (19.3 kDa).

The gene products of the mutant zur genes zur-11, zur-19, zur-23, zur-25, zur-27, zur-31, zur-35, and zurΔ46–91 were purified accordingly. Only ZurΔ46–91 did not form inclusion bodies. Purified ZurΔ46–91 showed an apparent molecular mass of ~14 kDa (Fig. 2B, lane 4) as predicted (14.0 kDa). Despite nearly identical molecular masses, some of the mutant Zur proteins (Zur19, Zur31, and Zur35) migrated faster on SDS-PAGE than wild-type Zur. Zur migrated more slowly under reducing conditions than under nonreducing conditions, which indicates that the isolated Zur protein was in the oxidized form with at least one intramolecular disulfide bridge even when the protein isolation and purification procedures were carried out in the presence of 10 mM 2-mercaptoethanol. Stronger reducing agents such as DTT or higher concentrations of 2-mercaptoethanol were not used for Ni2+-immobilized metal affinity chromatography to avoid reduction of Ni2+.

Under nonreducing conditions, protein bands with lower intensity were observed at ~38 and ~57 kDa and at ~29 and ~43 kDa for wild-type Zur and ZurΔ46–91, respectively; these two bands probably represent the dimer and trimer formed by intermolecular disulfide bridges.

Zur Forms a Dimer—To confirm that Zur forms dimers, to determine the dimer/monomer ratio under various conditions, and to see whether the mutant Zur proteins differ, the purified proteins were analyzed by gel filtration. Wild-type Zur eluted as a major peak with a molecular mass (~S.E.) of 46.0 ± 2.5 kDa (apparently corresponding to the dimer form), and only trace amounts eluted with a molecular mass of 28.0 ± 2.9 kDa (representing the monomer). The higher than expected molecular masses could be caused by a more rod-like rather than globular shape of the protein. The relative difference between the expected and observed molecular masses was smaller for the dimer than for the monomer. This is in agreement with the explanation provided that the monomers associate at the extended interfaces to form a dimer that is more globular than the monomer. Due to the high ionic strength (300 mM NaCl) of...
striction endonucleases were obtained by cleavage with the restriction endonucleases PinAI, SalI, MseI, and MnlI and PCR with primers YEB1, YEB2, YEBP1, YEBP2, YEBP3, YEBP4, YEBP3, and YEBP6 and cloned into the EcoRV site of pBCSK+. Thick arrows show the position and direction of the lacZ promoter in each construct. The numbers reflecting the positions in section 170 of the E. coli genome and the translational start sites of znuA and znuC according to Blattner et al. (4) are given at the bottom. Activity signifies derepression of znuA-lacZ expression in strain SIP468 carrying the corresponding plasmid, analyzed on MacConkey lactose plates. The znuA’ fragment coding for the signal peptide in the same orientation as the constitutive promoter of the vector seemed to be detrimental since the cells easily lost such plasmids and grew poorly in the presence of chloramphenicol.

To localize the DNA region within the znuC operator region. The DNA inserts (shaded boxes) were obtained by cleavage with the restriction endonucleases PinAI, SalI, MseI, and MnlI and PCR with primers YEB1, YEB2, YEBP1, YEBP2, YEBP3, YEBP4, YEBP5, and YEBP6 and cloned into the EcoRV site of pBCSK+. Thick arrows show the position and direction of the lacZ promoter in each construct. The numbers reflecting the positions in section 170 of the E. coli genome and the translational start sites of znuA and znuC according to Blattner et al. (4) are given at the bottom. Activity signifies derepression of znuA-lacZ expression in strain SIP468 carrying the corresponding plasmid, analyzed on MacConkey lactose plates. The znuA’ fragment coding for the signal peptide in the same orientation as the constitutive promoter of the vector seemed to be detrimental since the cells easily lost such plasmids and grew poorly in the presence of chloramphenicol.

**Fig. 3. Mapping of the potential Zur-binding site in the znu operator region.** The DNA inserts (shaded boxes) were obtained by cleavage with the restriction endonucleases PinAI, SalI, MseI, and MnlI and PCR with primers YEB1, YEB2, YEBP1, YEBP2, YEBP3, YEBP4, YEBP5, and YEBP6 and cloned into the EcoRV site of pBCSK+. Thick arrows show the position and direction of the lacZ promoter in each construct. The numbers reflecting the positions in section 170 of the E. coli genome and the translational start sites of znuA and znuC according to Blattner et al. (4) are given at the bottom. Activity signifies derepression of znuA-lacZ expression in strain SIP468 carrying the corresponding plasmid, analyzed on MacConkey lactose plates. The znuA’ fragment coding for the signal peptide in the same orientation as the constitutive promoter of the vector seemed to be detrimental since the cells easily lost such plasmids and grew poorly in the presence of chloramphenicol.
dimers/DNA molecule was sufficient for protection under optimized conditions. Higher concentrations of the repressor (a protein/DNA ratio of up to 1700:1) did not result in an extended protection zone. Zur bound to DNA only in the presence of a reducing agent such as DTT. No difference was observed between Zur purified from solubilized inclusion bodies and Zur purified from the soluble fraction.

Zur31 and ZurD46–91 were the only mutant Zur proteins that did not protect the znu operator even when applied in concentrations 20-fold higher than needed for DNA protection by wild-type Zur (Table II). A surplus of ZurD46–91 (10-fold) or Zur25, but not Zur27, over wild-type Zur was able to abolish binding of wild-type Zur to the DNA. Competition for Zn$^{2+}$ did not account for this effect because the addition of more Zn$^{2+}$ did not change the result. An excess of ZurD46–91 probably titrates wild-type Zur away from the znu operator by forming heterodimers (see above). Since the individual components Zur25 and wild-type Zur bind to DNA, it is surprising that the heterodimer seems to bind less efficiently or not at all.

Metal Ion Dependence of the DNA-binding Activity of Zur—DNase I footprinting assays were also employed to study the metal ion dependence of Zur-DNA binding. Purified Zur as isolated bound to the znu operator even without the addition of metal ions, which indicates that the buffers and the protein preparation contained sufficient zinc. Zur acquired zinc either in the cell or during purification; the zinc/protein ratio in the sample of purified Zur was 1:5 as determined by atom absorp-

Fig. 4. DNase I footprinting assay of Zur binding to the znu operator region. The 436- or 291-base pair fragment comprising nt 937–1372 of section 170 labeled on the ZnuCB-coding strand using the fluorescein-labeled YEB3 primer (A) or nt 870–1160 of section 170 labeled on the ZnuA-coding strand using the fluorescein-labeled YEB7 primer (B) without (traces 1) or with (traces 2) purified Zur protein was treated as described under “Experimental Procedures.” The nucleotide sequence deduced from the dideoxynucleotide sequencing reaction with the same end-labeled primer is given below; the protected region is enlarged for clarity. In C are shown the nucleotide sequence of the znu operator region with the translational start sites according to Blattner et al. (4) and the region determined by the in vivo operator titration assay (bracketed). The boxed region indicates the nucleotides protected from DNase I digestion by Zur. The imperfect palindrome is denoted by convergent arrows, with complementary bases shown in boldface. Nucleotides are numbered according to E. coli genome section 170.
induced structural alterations of the DNA that lead to a more exposed reaction site or by the formation of a so-called hydrophobic pocket by the protein on the DNA that attracts DMS and hence increases its local concentration. The latter interpretation seems less likely since such sites of increased reactivity are often observed at the boundary of the protein on the DNA. Hypersensitivity was not observed in DNase I footprinting assays, probably because of steric hindrance of the much larger DNase I protein in comparison with the small DMS molecule. With the ZnuA-coding strand (Fig. 5B), most of the nucleotides located in the region around nt 1039–1054 of section 170 (Zur-binding site of the zur operator) were protected from methylation by wild-type Zur (trace 3, downward-pointing arrows) compared with the in vitro methylated pSP97/47 DNA in the absence of Zur (negative control, trace 1). These same nucleotides were not protected in cells with Zur19 (trace 2), which indicates that Zur19 does not bind to the zur operator in vivo. Thus, the DNA binding of Zur19 in DNase I footprinting assays was caused by the nonphysiological conditions. The protection zone in the in vivo footprinting assay was generally smaller than in the DNase I footprinting assay presumably because the DMS molecule is much smaller than the bulky DNase I protein.

Zur-dependent Regulation of the zur Gene Cluster—All zurA-lacZ, zurB-lacZ, and zurC-lacZ fusions (in Sip146, Sip148, Sip576, Sip578, Sip579, Sip775, and Sip800) were derepressed by Zn2⁺ chelators such as TPEN, EDDS, 2,2’-bipyridine (at high concentrations), EDTA, and EGTA and by the metal ions Mn²⁺, Cu²⁺, Cd²⁺, Hg²⁺, and Pb²⁺ (only in aroB⁻ strains), whereas Zn²⁺, Co²⁺, and iron chelators such as 2,2’-bipyridine (at lower concentrations) and desferrioxamine repressed the zur gene cluster. The influence of iron was also observed in the different behavior of aroB⁻ and aroB⁺ strains on MacConkey lactose agar plates: zur-lacZ aroB⁺ mutants were white, whereas zur-lacZ aroB⁻ mutants were red. aroB is one of the genes necessary for the biosynthesis of the E. coli siderophore enterochelin, which mediates ferric iron transport.

DISCUSSION

Native Zur is a dimer, even in the absence of zinc or other metal ions, as indicated by gel filtration experiments in the presence of chelators. A regulatory sequence of dyad symmetry was defined within the zur operator; this sequence is sufficient for conferring zinc-dependent and Zur-dependent repression. Zur protein was shown to bind directly and specifically to DNA with this sequence using three independent assays of DNA binding: in vitro titration, DNase I footprinting, and in vivo footprinting.

Zur occupied its binding site only in the presence of zinc or other divalent metal cations at low concentrations, as shown by the DNase I footprinting data. Zur protected a 29-nt approximate palindrome on each strand of the zur operator with a 3'-stagger of 4 nt. This footprint resembles that of typical DNA-binding dimers such as classical helix-turn-helix proteins, e.g., the CI repressor from bacteriophage λ (23). The observed 3'-stagger is indicative of coverage of the minor groove at the ends, but provides no information about the protein-DNA recognition contacts. Even at high concentrations of Zur, no polymerization and extension of the protected region occurred, as has been observed with Fur at several Fur-dependent operators in footprinting experiments (e.g., Refs. 24 and 25) and by electron and atomic force microscopy (26).

Zur is active only in the reduced form. As a cytoplasmic protein, it has predominantly reduced thiols rather than oxidized disulfides due to the reducing conditions in the cytoplasm (27). In vitro, the cysteine residues of Zur are easily oxidized to...
disulfides, as judged by the slower migration on SDS-polyacrylamide gels under reducing conditions compared with nonreducing conditions. Oxidized Zur does not bind DNA or considerable amounts of Zn$^{2+}$.

Zur seems to be widespread among bacteria, even in Gram-positive bacteria and cyanobacteria, as indicated by sequence similarity searches. In *B. subtilis*, apart from Fur and PerR (peroxide stress response regulator) (11), the third homologue...
Fig. 6. Alignment of E. coli Zur with putative Zur proteins and E. coli Fur. The protein sequence alignment was based on the method of Higgins and Sharp (37). Perfectly conserved residues are indicated with asterisks, and well conserved residues are marked by colons.

The proteins shown are as follows (with the data bases and accession numbers in parentheses): ZUR ECOLI, Zur from E. coli (this study); ZUR KLEPN, Zur from B. pertussis; ZUR STAEP, Zur from C. crescentus; ZUR BACSU, Zur from N. meningitidis (Sanger Center); ZUR ECOLI, Zur from P. aeruginosa; ZUR LISMO, Zur homologues deduced from the incomplete genome sequences from K. pneumoniae (Sanger Center), E. coli (WUGSC), Y. pestis (Sanger Center), V. cholerae (Institute for Genomic Research), P. aeruginosa (Pseudomonas Genome Project), and N. gonorrhoeae (University of Oklahoma's Advanced Center for Genome Technology), respectively; ZUR FUR ECOLI, Fur from E. coli (4) (GenBank accession number AE000172). The partially sequenced Np20 from P. aeruginosa (38) and IvxI from V. cholerae (39), which have been identified as being implicated in virulence, are part of ZUR FSEA and ZUR VIBCH, respectively. The amino acid changes in each of the eight mutant Zur proteins and the mutant designations are given above the corresponding residues in E. coli wild-type Zur.

YqV can act as Zur (5). The sequence similarity to E. coli Zur is not strong owing to the distant relationship between Gram-negative and Gram-positive bacteria. A zur gene has also been found in L. monocytogenes (6). Based on sequence similarity, we expect that the proposed Fur protein in Staphylococcus epidermidis (28) is Zur rather than Fur (Fig. 6, FUR_STAEP). It is possible that other proteins designated as Fur homologues will turn out to be Zur proteins. Synechocystis sp. (29) may also have a Zur protein. Of the three homologues, Strl1738 and Sll1937 are more similar to Zur than to Fur from E. coli. It is noteworthy that sll1937 is located directly upstream and is divergently transcribed of the genes encoding the ZnuACB system.

In many of the partially sequenced genomes of various bacterial species, a Zur equivalent is found, e.g. in Salmonella typhimurium LT2 (Washington University Genome Sequencing Center (WUGSC)), Salmonella typhi (Sanger Center), Salmonella paratyphi (WUGSC), Klebsiella pneumoniae (WUGSC), Yersinia pestis (Sanger Center), Vibrio cholerae (Institute for Genomic Research), Pseudomonas aeruginosa (Pseudomonas Genome Project), Neisseria gonorrhoeae (University of Oklahoma's Advanced Center for Genome Technology), and Neisseria meningitidis (Sanger Center). Since these organisms also possess a system homologous to Znu, we propose that these proteins likewise are regulators of zinc uptake. Some of these Zur-equivalent proteins are aligned in Fig. 6.

Since all mutant Zur proteins could be purified in large amounts, it can be ruled out that the Zur phenotype is the result of low protein expression or of an unstable Zur protein that is quickly degraded by cellular proteases. This suggests that the amino acids altered in the mutant Zur proteins are important for the structure and/or function of native Zur. The alignment of the amino acid sequences of the mutant Zur products with putative Zur proteins from other organisms reveals that all of the point mutations occur in well conserved areas of Zur.

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Zur346–91, which has an internal deletion (amino acids 46–91) in the amino-terminal part of Zur, bound as much Zn2+ as wild-type Zur and formed dimers and heterodimers with wild-type Zur, but did not protect DNA from DNase I cleavage. Apparently, this region is responsible for binding of DNA, but is not essential for dimerization or Zn2+ binding.

Negative complementation by an excess of the mutant Zur protein can be explained by competition for the corepressor Zn2+ or by formation of less active heterodimers with wild-type Zur. Zur25 (Leu48 → Phe), Zur346–91, Zur11/Zur36 (Ser31 → Phe), Zur19 (Ser38 → Leu), Zur23 (Glu111 → Lys), and Zur31 (Arg66 → His) were inactive in vivo as zinc-dependent repres-
Zinc-responsive Regulator Zur of E. coli

sors, but were able to complement negatively, i.e. to bind Zn\(^{2+}\) or to form dimers. In all these mutant proteins, residues in the amino-terminal or central region are changed or deleted. Thus, this region or at least the individual residues are not crucial for binding of Zn\(^{2+}\) or dimerization. In contrast to Zur\(2\), Zur\(2\) inhibited DNA binding of wild-type Zur in DNase I footprinting experiments and showed negative complementation, both of which can be accounted for by the prevalence of Zur\(2\) dimer formation. All the mutant Zur proteins except Zur31 and Zur\(46–91\) were in principle able to bind DNA since they protected DNA from DNase I cleavage in vitro, albeit not in vivo. This indicates that crucial DNA contacts made by the Zur protein are located in the region around residue 65. Moreover, this region is highly conserved in all Zur sequences and corresponds to the second helix in the proposed helix-turn-helix motif in Fur that has previously been suggested to mediate interactions with DNA (30). However, the mutation in Zur\(2\) seemed to influence the dimer formation, although the mutation is close to the assumed DNA-binding site.

The zinc content estimated for Zur was at least 2 Zn\(^{2+}\) ions/Zur dimer, which suggests that at least one metal-binding site of the Zur monomer is occupied by Zn\(^{2+}\). For establishing the metal/Zur stoichiometry, determination of the absolute protein concentration was critical. As shown in the alignment in Fig. 6, Zur lacks the cluster of histidines around amino acid 90 that is conserved in Fur and that is assumed to be involved in Fe\(^{2+}\) binding of Fur (31, 32). Of the nine cysteines present in E. coli Zur, Cys\(^{17}\), Cys\(^{113}\), Cys\(^{152}\), Cys\(^{158}\), and possibly Cys\(^{88}\) are probably not involved in Zn\(^{2+}\) binding because they are not conserved in at least one or even all Zur proteins from other bacterial genera (Fig. 6). Interestingly, E. coli Fur possesses one structural tight-binding zinc site/monomer in addition to the iron regulatory-binding site that senses the intracellular iron concentration (33). The zinc is coordinated tetrahedrally by at least one histidine (33) and Cys\(^{93}\) and Cys\(^{96}\) (34). These cysteines are perfectly conserved in all Zur proteins proposed (Fig. 6) and may therefore serve as Zn\(^{2+}\) ligands also in Zur. As observed in the DNase I footprinting studies, Zn\(^{2+}\) at very low concentrations acts as a corepressor in Zur and can be replaced in vitro by other divalent metal ions such as Mn\(^{2+}\). A similar unspecificity for divalent cations in vitro is exhibited by Fur with Co\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\), or Zn\(^{2+}\) and by DtxR and IdeR with Cd\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), or Zn\(^{2+}\) acting as corepressors in DNase I footprinting experiments (8, 24, 35, 36), although the response of Fur- or IdeR-regulated genes is clearly iron-specific in vivo. It is unknown which factors determine binding of the individual metal ion to the regulator in the cell; therefore, the results from the DNase I footprinting experiments can explain the in vivo situation only to a limited extent. This limitation is also documented by the binding of most of the mutant Zur proteins to the zur operator in vitro in DNase I footprinting assays, but not in vivo, as was indicated by depression of the zuru–lacz fusion in the zur mutants and the lack of protection of the zur operator by at least Zur19 in in vitro footprinting experiments.

The zur gene cluster was derepressed by divalent metal ions such as Fe\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), and Cd\(^{2+}\). This can be explained either by direct binding of these divalent cations to Zur, thereby changing the conformation into an inactive form that no longer represses znuABC, or by these metal ions exerting their derepressing effect indirectly by lowering the intracellular zinc level. The latter possibility may be achieved by the metal ions competing with Zn\(^{2+}\) for an unspecific transporter and thereby inhibiting uptake of Zn\(^{2+}\). Yet in regulation studies with znu mutants, the znu system does not play a part because it is inactive. At high concentrations, the derepressing divalent cations did indeed impair Zur binding in DNase I footprinting experiments, but with the restriction as pointed out above regarding the discrepancy between the results for DNase I footprinting and the in vivo situation. Purified Zur bound Fe\(^{2+}\) far less efficiently than Zn\(^{2+}\), and Fe\(^{2+}\) did not considerably hinder binding of the corepressor Zn\(^{2+}\) to Zur, at least under the in vitro conditions and concentrations tested, thereby providing no hint for a direct interaction of iron with Zur.

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