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# Transcriptional Response of *Escherichia coli* to External Zinc

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**Transcriptional response of *Escherichia coli* to extracellular zinc was studied using DNA microarray and S1 mapping assays. Addition of external zinc induced the expression of zinc exporter ZntA and inhibited the expression of zinc importer ZnuC. In the continuous presence of zinc, ZnuC repression took place at lower zinc concentrations than ZntA induction. The microarray assay indicated that the addition of excess external zinc induces the expression of many genes that are organized in the regulon for cysteine biosynthesis, implying that cysteine plays a role in transient trapping of free zinc for maintenance of zinc homeostasis. Besides the RpoE regulon, other genes were also induced by zinc, suggesting that periplasmic proteins denatured by zinc induce the genes for protein repair. The microarray data of the newly identified zinc-responsive promoters were confirmed by S1 mapping.**

Zinc is one of the essential trace metal ions for living organisms, but excess zinc is toxic for cell growth. Based on a radioactive zinc-binding assay, we estimated that the total amount of zinc-binding proteins in *Escherichia coli* is as much as 3% of the total protein expressed in exponentially growing cells (15). The intracellular concentration of free zinc in *Escherichia coli*, however, stays constant at low levels in both rich and poor media (24). To maintain homeostasis for intracellular free zinc, a set of genes coding for zinc transporters and/or exporters and for detoxification of free zinc ions to nontoxic forms are induced upon exposure to excess zinc in the environment (2, 24).

*Escherichia coli* contains at least four zinc transport pumps, two zinc transporters (importers), ZnuABC (25) and ZupT (12), and two zinc exporters, ZntA (1, 28) and ZitB (11). The levels of these zinc transport proteins are considered to be regulated at the transcriptional level in a zinc-dependent manner. Two species of cytosolic transcription factor, ZntR and Zur, are involved in transcription regulation of the zinc response genes (3, 24). Transcription of the divergently transcribed *znuA* and *znuBC* genes is repressed by Zur when external zinc ions are high, leading to shutoff of zinc uptake. On the other hand, ZntR is an activator of *zntA* transcription for export of excess zinc. *E. coli* cells respond to external zinc ions at millimolar concentrations, but both Zur and ZntR respond in vitro to free zinc ions at femtomolar concentrations (24; K. Yamamoto and A. Ishihama, in preparation), suggesting that zinc ions within *E. coli* cells are present as complexes with proteins or zinc-chelating compounds but free zinc ions are maintained only at low concentrations.

In order to get insights into the response of *E. coli* to external zinc ions, we performed, in this study, microarray analysis of whole transcripts in the presence and absence of external

zinc. In addition to the known set of zinc-responsive genes for import and export of zinc through cell membranes, two groups of genes were found to be highly induced, a set of genes for the synthesis of cysteine and a set of genes under the control of the RpoE sigma factor. The microarray data were confirmed by S1 nuclease mapping of individual mRNAs.

## MATERIALS AND METHODS

**DNA microarray analysis.** *E. coli* strains used were W3110 type A (14) and KP7600 [W3110 (A) *lacI<sup>q</sup> lacZΔM15 galK2 galK22*] (17). Cells were grown at 37°C in Luria-Bertani (LB) medium with reciprocal shaking (120 rpm). The cell density was monitored by measuring the turbidity at 540 nm with a Klett-Summerson photometer. At the middle of exponential phase (50 to 60 Klett units), the culture was divided into two portions. Various concentrations of ZnCl<sub>2</sub> were added to one aliquot. Culture with or without ZnCl<sub>2</sub> addition was continued under the same conditions. After 5 min, cells were harvested and total RNAs were prepared with hot-phenol method, precipitated with ethanol, and dissolved in RNase-free H<sub>2</sub>O. After digestion with RNase-free DNase I (Takara), RNA was reextracted, precipitated with ethanol, dissolved in RNase-free water, and stored at –80°C until use. Preparation of the fluorescence-labeled cDNA, hybridization on DNA chips, scanning microarrays, and data analysis were performed according to the published procedure (23, 30) with slight modification (O. Ozoline, K. Yamamoto, and A. Ishihama, in preparation). The *E. coli* CHIPs (Takara) used were Takara Ver. 1 products. All fluorescent intensity data were statistically analyzed as described (23, 30; O. Ozoline, K. Yamamoto, and A. Ishihama, in preparation).

**S1 nuclease extension assay of mRNA.** The S1 nuclease protection assay of individual mRNA was carried out as described previously (30–32). Primers used are described in Table 1. Radiolabeling of primers was performed in vitro using 10 μCi [ $\gamma$ -<sup>32</sup>P]ATP (5,000 Ci/mmol) and T4 polynucleotide kinase (Toyobo), and labeled primers were purified by polyacrylamide gel electrophoresis. Total RNAs were purified from *E. coli* with or without prior treatment by zinc. Mixtures of one <sup>32</sup>P-end-labeled probe and total RNAs were incubated for 10 min at 75°C, followed by incubation at 37°C overnight for hybridization. The mixtures were digested with S1 nuclease (Takara) at 37°C for 10 min, and the remaining undigested products were extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis on gels containing 6 M urea.

## RESULTS

**Influence of external zinc on cell growth and zinc response gene transcription.** As an attempt to get insight into the

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TABLE 1. Oligonucleotides used in this study

Primer <sup>a</sup>	Sequence (5' → 3')	Purpose
ZNTAF-1	GCTGTTTATCAGTAACTTIG	<i>zntA</i> promoter
ZNTAR-1*	ATTGAGGGGCTTTCTTGCCG	<i>zntA</i> promoter
ZNUCF-1	GGCGGATAATGCTGCGAAAA	<i>znuC</i> promoter
ZNUCR-1*	TCAAAATTTTTCCAGGTTTA	<i>znuC</i> promoter
CYSSPF	CGTGATTGGTGTGGAAAAGTAAACCATTTTTG	<i>cysS</i> promoter
CYSSPR*	ATCGTAAACGGTGATTCCACACACGTACAT	<i>cysS</i> promoter
CYSZPF	TCTTTACGGCTGGTCCAGAAACCATGTACC	<i>cysZ</i> promoter
CYSZPR-1*	CGTGGGGCAGATGTGAATGATGAAACCATA	<i>cysZ</i> promoter
CYSKPF	GTGGGTTCGATTGCTATCGCGATAAACACGC	<i>cysK</i> promoter
CYSKPR*	TACCGATGCGATTCCAGGCGAACAGCGGCG	<i>cysK</i> promoter
CYSPPF	TGTGTACGCTCCGGTTTTCTCCGCGCTGTTT	<i>cysP</i> promoter
CYSPPR*	CGCGGGAGACGTCATAAGAACTGTTTCAGCA	<i>cysP</i> promoter
CYSDPF	GTACGGTGGCGCAATGCGGAAAACATAGTG	<i>cysD</i> promoter
CYSDPR*	GAGAATTCTGCCGCCACCTCGCGAATAATG	<i>cysD</i> promoter
CYSJPF	CGTGGTGGACATCATAATTTCTCTACAGGA	<i>cysJ</i> promoter
CYSJPR*	GGGAGTTAAATCGGTCGTGGCCGCTGAAG	<i>cysJ</i> promoter
NIRBPF	TGTTAGGTTTTCGTCAGCGTCACCGTCAGC	<i>nirB</i> promoter
NIRBPR*	AAACGGTAATATCAAAGTTGGCCGCATCAG	<i>nirB</i> promoter
CYSEPF	ATTGGTCAGGTGGTGGAAAGGCTACCGCAAT	<i>cysE</i> promoter
CYSEPR*	GCGTGGTAAAAACTGGCCAGCATTGGCTCA	<i>cysE</i> promoter
METBPF	GGGTACTGACCGTAAACCCGCATAGTTTAC	<i>metB</i> promoter
METBPR*	CGGTGGGACAACGCAACCATACTGTTTCGTC	<i>metB</i> promoter
FKPAPF	ATGAGCGGTACCGTGACGTTTCAGTTCTTC	<i>fkpA</i> promoter
FKPAPR*	GTGGTCGCCAGCAGCGTTACTTTAAACAGT	<i>fkpA</i> promoter
HTRAPF	CGGTATGACCGACCTCTATCGGTGGGATGA	<i>htrA</i> promoter
HTRAPR-2*	TGCTGGGCTGTCGTTGCTGAAGAAGTCTCA	<i>htrA</i> promoter
RPOEPF	TTAATTTACTAAACATGGTTTGGTCAGCAT	<i>rpoE</i> promoter
RPOEPR*	GGTCCGTTAACTGCTCGTCCATCCGAGGTA	<i>rpoE</i> promoter
RPOHPF	GCGGAAGTGGCACAGGTTTTTCGGAACGAAG	<i>rpoH</i> promoter
RPOHPR*	ATCGGCCACGCGTTAGCTGCCGATGTAG	<i>rpoH</i> promoter
YGGNPF	GAAGAACATCAGGCGATTGTGCGTAAGTGG	<i>yggN</i> promoter
YGGNPR*	GCGGCGTGACGCTGCACTGGTAGTCGGCGT	<i>yggN</i> promoter

<sup>a</sup> <sup>32</sup>P-labeled primers used for generation of radiolabeled probes are marked with an asterisk.

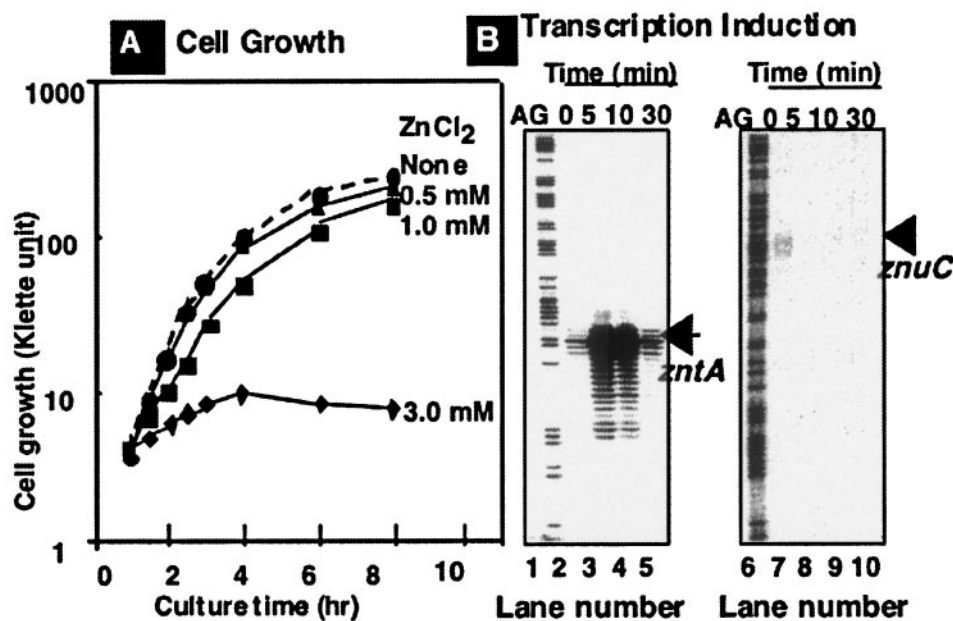


FIG. 1. Response of wild-type *E. coli* W3110 to external zinc. [A] *E. coli* W3110 culture grown overnight at 37°C in LB medium was 100-fold diluted with fresh LB medium with 0 mM (●), 0.5 mM (▲), 1.0 mM (■), and 3.0 mM (◆) of ZnCl<sub>2</sub>, and incubated at 37°C with reciprocal shaking. Cell growth was monitored by measuring turbidity with a Klett-Summerson photometer. [B] *E. coli* W3110 was grown in the absence of ZnCl<sub>2</sub> until exponential phase, and then ZnCl<sub>2</sub> was added at a final concentration of 0.5 mM. Before (lanes 2 and 7) and at 5 min (lanes 3 and 8), 10 min (lanes 4 and 9), and 30 min (lanes 5 and 10) after addition of ZnCl<sub>2</sub>, cells were harvested and total RNA was isolated with the hot-phenol method (30, 32). The S1 nuclease protection assay was carried out with the *zntA* (lanes 1 to 5) or *znuC* (lanes 6 to 10) promoter probe as described in Materials and Methods. <sup>32</sup>P-labeled probes were prepared by PCR. Lanes 1 and 6 indicate Maxam-Gilbert AG sequencing reactions. Arrows on the left were the bands protected against S1 nuclease.

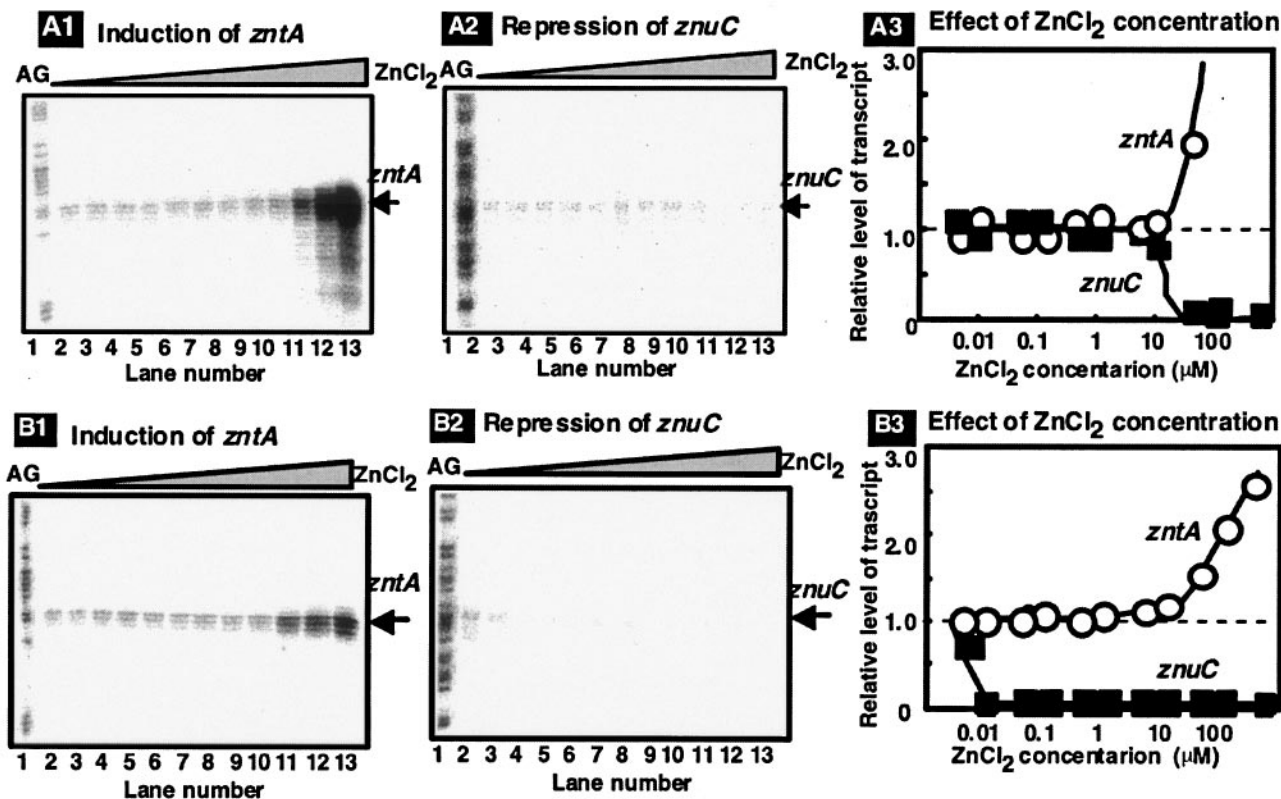


FIG. 2. Zinc-induced activation of *zntA* transcription and repression of *znuC* transcription. [A] *E. coli* W3110 was grown in the absence of ZnCl<sub>2</sub>. At the exponential phase of cell growth, various concentrations of ZnCl<sub>2</sub> were added and after 5 min, total RNAs were prepared and subjected to S1 nuclease mapping using <sup>32</sup>P-labeled *zntA* [A1] or *znuC* [A2] probes. [A3] The amounts of *zntA* (○) or *znuC* (■) RNA relative to those in the absence of ZnCl<sub>2</sub> addition were measured. [B] ZnCl<sub>2</sub> was added from the beginning of cell culture. The levels of *zntA* [B1] and *znuC* [B2] RNA and the relative levels of these RNAs relative to those in the absence of zinc addition [B3] were determined as in A.

genomewide response of *E. coli* to environmental changes, we have analyzed its transcriptional response to external metals. Previously we published the regulatory networks for response to magnesium (19) and copper (30). Along this line we describe in this report the initial survey of the *E. coli* response to external zinc. Up to the present, only the minimum set of zinc-responsive genes have been identified (1, 11, 12, 25, 28), which are all involved in import or export of zinc ions. Here we first analyzed the influence of various concentrations of external zinc on cell growth and transcription of the previously identified zinc-responsive genes. When wild-type *E. coli* W3110 was grown at 37°C in LB medium in the continuous presence of various concentrations of zinc, cell growth was significantly reduced above 1 mM ZnCl<sub>2</sub>, but not at 0.5 mM, as measured by the culture turbidity (Fig. 1A), indicating that *E. coli* is capable of maintaining homeostasis of internal free zinc at least up to 0.5 mM external zinc.

Next we measured the possible influence of the sudden addition of 0.5 mM ZnCl<sub>2</sub> on transcription of two typical zinc-responsive genes, *zntA*, encoding the zinc exporter, and *znuC*, encoding a subunit of the zinc importer. When 0.5 mM ZnCl<sub>2</sub> was added to the *E. coli* KP7600 (W3110 derivative) culture at the middle of the exponential phase (50 to 60 units as measured with a Klett-Summerson photometer), the induction of *zntA* transcription was detected by S1 mapping of *zntA* mRNA

even at an early time (5 min) after zinc addition (Fig. 1B). After 30 min, the level of *zntA* transcript decreased to the preshift level prior to zinc treatment. On the other hand, *znuC* transcripts disappeared or markedly decreased at 5 min after zinc addition (Fig. 1B), and even after 30 min, *znuC* transcripts were hardly detected. These results together indicated that *E. coli* monitors increases in the environmental zinc level and quickly induces or represses the zinc-responsive genes. Moreover, differential regulation of the zinc importer and exporter genes was indicated in such a way that during the recovery after sudden exposure to external zinc, the induction of zinc exporter (*ZntA*) was turned off prior to the induction of the zinc importer (*ZnuC*).

**Differential response of the zinc-responsive genes to external zinc.** To confirm the differential response of the zinc importer and exporter genes to external zinc, we next determined the induction of *zntA* transcription and the repression of *znuC* transcription in the continuous presence of various concentrations of ZnCl<sub>2</sub>. When the *E. coli* culture was exposed to ZnCl<sub>2</sub> for a short period (as in Fig. 1B), both *zntA* induction (Fig. 2-A1) and *znuC* repression (Fig. 2-A2) were observed at ZnCl<sub>2</sub> concentrations above 10 μM (Fig. 2-A3). On the other hand, upon exposure to zinc for a prolonged time (ZnCl<sub>2</sub> was added from the beginning of cell culture), the induction of *ZntA* was observed essentially at the same zinc concentration, above 10



$\mu\text{M}$  (Fig. 2-B1), but the maximum repression of *znuC* was detected at  $\text{ZnCl}_2$  concentrations as low as  $0.01 \mu\text{M}$  (Fig. 2-B2 and 2-B3). This finding indicates that in the continuous presence of zinc, repression of synthesis of the zinc importer (*ZnuC*) takes place at a lower concentration of external zinc than that needed for induction of the zinc exporter (*ZntA*).

The induction of *zntA* transcription requires the *ZntR* transcription activator, while *znuC* transcription is repressed by the *Zur* transcription repressor (3, 25). This was confirmed by microarray assays using *E. coli* mutants lacking the *ZntR* or *Zur* transcription factors (K. Yamamoto, T. Miki, and A. Ishihama, in preparation). Thus, two different transcription factors appear to respond to different concentrations of external zinc.

**Profiling of genome transcription in response to external zinc.** Based on a combination of proteome analysis and radioactive zinc blotting assays, we estimated that the total number of zinc-binding protein species in *E. coli* is as much as 3% of the total proteins expressed in the exponential growth phase (15). Some of these zinc-binding proteins were considered to increase after zinc shock and play roles in trapping excess free zinc. To identify the entire set of zinc-responsive genes, we then performed the DNA microarray assay using the *E. coli* DNA chip (Takara, Japan).

*E. coli* W3110 was grown to the exponential phase, and then treated for a short period with  $0.5 \text{ mM ZnCl}_2$  (the minimum concentration required for maximum induction of *zntA* and maximum repression of *znuC*). Since the microarray assay should detect the genes which are affected, directly and indirectly, by the addition of zinc, samples were prepared at an early time (less than 5 min) after addition of  $\text{ZnCl}_2$  for detection of the genes directly induced by the addition of zinc. Total RNAs were prepared from both zinc-treated and untreated cultures, and subjected to the microarray assay under the conditions described in Materials and Methods. In brief, cDNAs were synthesized using Cy3- or Cy5-labeled nucleotide substrates and avian myeloblastosis virus reverse transcriptase. One cDNA sample was labeled with Cy3 and the other with Cy5. Two cDNA samples were mixed and hybridized with the whole set of *E. coli* genes spotted on a single slide glass.

Here we analyzed two independent cultures. In the first experiment, we labeled untreated sample with Cy3 and the zinc-treated sample with Cy5. The labels were reversed in the second experiment. The fluorescent intensity of each gene spot was measured and statistically analyzed for normalization. Table 2 shows the list of genes which are activated or repressed more than twofold after zinc addition. A total of 26 genes were up-regulated and three genes were down-regulated. As expected, the *zntA* transcript was included in the up-regulated gene group, while the *znuC* transcript was a member of the down-regulated genes. *E. coli* contains the second zinc importer *ZupT* and the second exporter *ZitB*, which were identified by genetic analyses using *znuABC* and *zntA* mutants, respectively (11, 12). Our microarray data, however, showed that the expression of *zupT* and *zitB* was not significantly affected under the conditions employed (5 min after addition of  $0.5 \text{ mM ZnCl}_2$  at the exponential growth phase) (data not shown).

**Induction of the genes for cysteine biosynthesis.** Surprisingly, 9 out of the total 26 up-regulated genes (more than twofold) encode the enzymes which are involved in cysteine

TABLE 2. Zinc-responsive genes in *Escherichia coli* W3110<sup>a</sup>

Class and gene	Function
<b>Upregulated genes</b>	
<i>cysA</i>	Sulfate/thiosulfate transport
<i>cysD</i>	Sulfate adenylyltransferase small subunit
<i>cysH</i>	Adenylylsulfate reductase
<i>cysI</i>	Sulfite reductase beta subunit
<i>cysJ</i>	Sulfite reductase alpha subunit
<i>cysK</i>	Cysteine synthase A
<i>cysN</i>	Sulfate adenylyltransferase large subunit
<i>cysP</i>	Thiosulfate-binding protein
<i>cysU</i>	Sulfate transport system permease
<i>htrA/degP</i>	Periplasmic protease
<i>hyfG</i>	Hydrogenase 4 subunit
<i>nhaR</i>	Transcriptional activator
<i>rpIV</i>	50S ribosomal protein L22
<i>rpsS</i>	30S ribosomal protein S19
<i>zntA</i>	Zinc exporter (P-type ATPase)
<i>zipA</i>	Cell division protein for FtsZ ring formation
<i>yabP</i>	Hypothetical protein
<i>yacH</i>	Putative membrane protein
<i>ybaK</i>	Suppressor of <i>ushA</i> transcription
<i>ybbA</i>	Putative ABC transporter
<i>ybiK</i>	Putative L-asparaginase
<i>yedV</i>	Putative sensor histidine kinase
<i>yeeE</i>	Putative transport system permease
<i>yfgD</i>	Putative oxidoreductase
<i>yhhP/sirA</i>	Putative protein involved in sigma S stability
<i>yhjJ</i>	Homologue of paptidase family M16
<b>Downregulated genes</b>	
<i>moaC/paaZ</i>	Molybdopterin biosynthesis
<i>nmpC</i>	Outer membrane protein
<i>znuC</i>	High-affinity zinc uptake system

<sup>a</sup> Genes induced upon sudden exposure to  $\text{ZnCl}_2$  were identified by microarray assays using an *E. coli* DNA chip (for details, see text). Genes activated or repressed more than twofold at 5 min after zinc addition are listed.

synthesis (Table 2). A total of about 70 genes are involved in the pathway for cysteine biosynthesis, and most of these genes besides the nine highly up-regulated genes were included in the group of up-regulated genes, albeit at lower levels. To confirm the zinc induction of cysteine synthesis genes, we next performed S1 nuclease assays for each of the up-regulated genes. Seventy genes for cysteine biosynthesis are organized into nine transcription units on the *E. coli* genome, each being under the control of the *cysSp*, *cysZp*, *cysKp*, *cysPp*, *cysDp*, *cysJp*, *nirBp*, *cysEp*, or *metBp* promoter (Fig. 3B). Two promoters, *cysSp* and *cysEp*, were newly identified in this study, while six promoters, *cysKp*, *cysPp*, *cysDp*, *cysJp*, *nirBp*, and *metBp*, have been reported by others (5, 13, 16, 18, 26). In good agreement with the DNA microarray results, the S1 assay showed that at least six promoters, *cysKp*, *cysPp*, *cysDp*, *cysJp*, *nirBp*, and *cysEp*, were significantly induced by zinc (Table 1 and Fig. 3A). Under the experimental conditions employed, however, transcription of *cysZ* was not detected with or without zinc addition (data not shown).

These findings together suggest that the sudden exposure of *E. coli* to excess zinc in the environment induces a quick increase in the intracellular level of cysteine, which may play a role in transient trapping of excess free zinc prior to export of excess zinc (Fig. 3C). Increased expression of the genes for cysteine biosynthesis was also observed as a response of *E. coli*

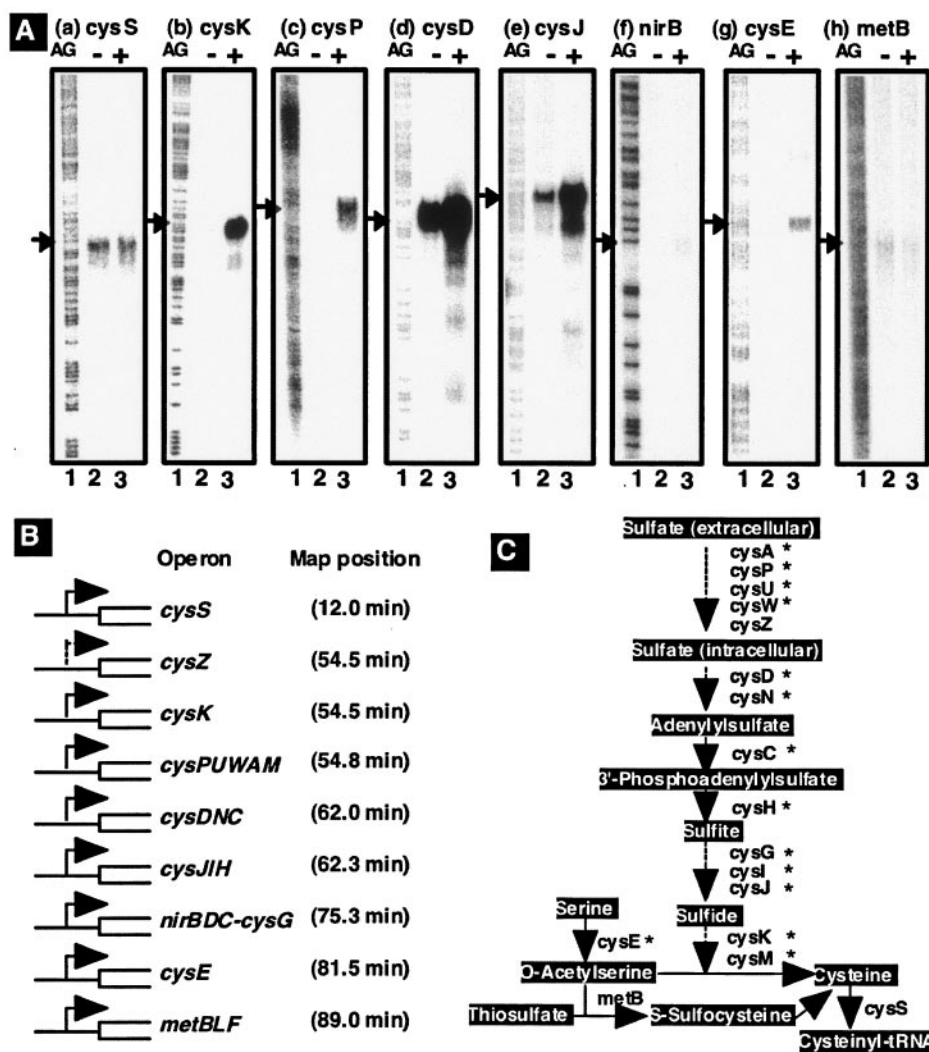


FIG. 3. Zinc-induced activation of the genes for cysteine biosynthesis. [A] *E. coli* W3110 was grown in LB in the absence of ZnCl<sub>2</sub> until exponential phase and then divided into two equal aliquots. ZnCl<sub>2</sub> was added to one aliquot at a final concentration of 0.5 mM. After 5 min incubation with (lane 2) and without (lane 3) ZnCl<sub>2</sub>, cells were harvested and total RNA was isolated with the hot-phenol method as described for Fig. 1. The S1 nuclease protection assay was carried out with the *cysS* (a), *cysK* (b), *cysP* (c), *cysD* (d), *cysJ* (e), *nirB* (f), *cysE* (g), and *metB* (h) promoter probes as described in Materials and Methods. Lane 1, Maxam-Gilbert AG sequencing reactions. Arrows on the left were the bands protected against S1 nuclease. [B] A total of 70 genes for cysteine biosynthesis are organized into nine transcriptional units. The first gene of each operon is shown, together with the location of the gene on the *E. coli* genome on the right. The bold arrows indicate zinc-inducible promoters. The dashed arrow indicates an unidentified promoter for the *cysZ* operon. [C] The genes activated after addition of external zinc include a number of the genes involved in the synthesis of cysteine. The activated genes, shown by stars, are indicated along the pathway of cysteine biosynthesis.

to a sudden increase in external copper (30). Taken together, we speculate that some heavy metals induce a transient increase in the intracellular concentration of cysteine for transient trapping of free metal ions.

**Induction of the RpoE regulon.** The microarray data showed more than fourfold induction by zinc of the *htrA* gene encoding a periplasmic protease, which is involved in degradation of damaged proteins (Table 2). The *htrA* gene is transcribed by RNA polymerase containing the RpoE sigma factor (reviewed in reference 21). Including *htrA*, a total of 26 genes are known to be transcribed by RNA polymerase holoenzyme containing RpoE sigma (7). In addition to *htrA*, four genes of the RpoE regulon, *fkpA*, *rseA*, *rseC*, and *yggN*, were 1.5- to 2.0-fold up-regulated by zinc (see Table 3). We then carried out detailed

analysis of transcriptional response to zinc of some RpoE regulon genes, including not only *htrA*, *fkpA*, and *yggN* but also two well-characterized sigma genes, *rpoE* and *rpoH*. S1 nuclease experiments showed two bands for *fkpA*, *htrA*, and *yggN*; three bands for *rpoE*; and five bands for *rpoH* (Fig. 4A). The location of two *fkpA* promoters, upstream p1 and downstream p2, agreed with those previously identified by Danese and Silhavy (6). *fkpA*p1 was induced markedly by zinc [Fig. 4A(a)]. This p1 promoter was found to contain the conserved promoter -10 and -35 sequences for RpoE sigma (Fig. 4B) as noted by Dartigalongue et al. (7) (note that the promoter naming is reversed in this report).

Detection of a long undigested probe of the *htrA* promoter [p1 in Fig. 4A(b)] is consistent with the location of its known

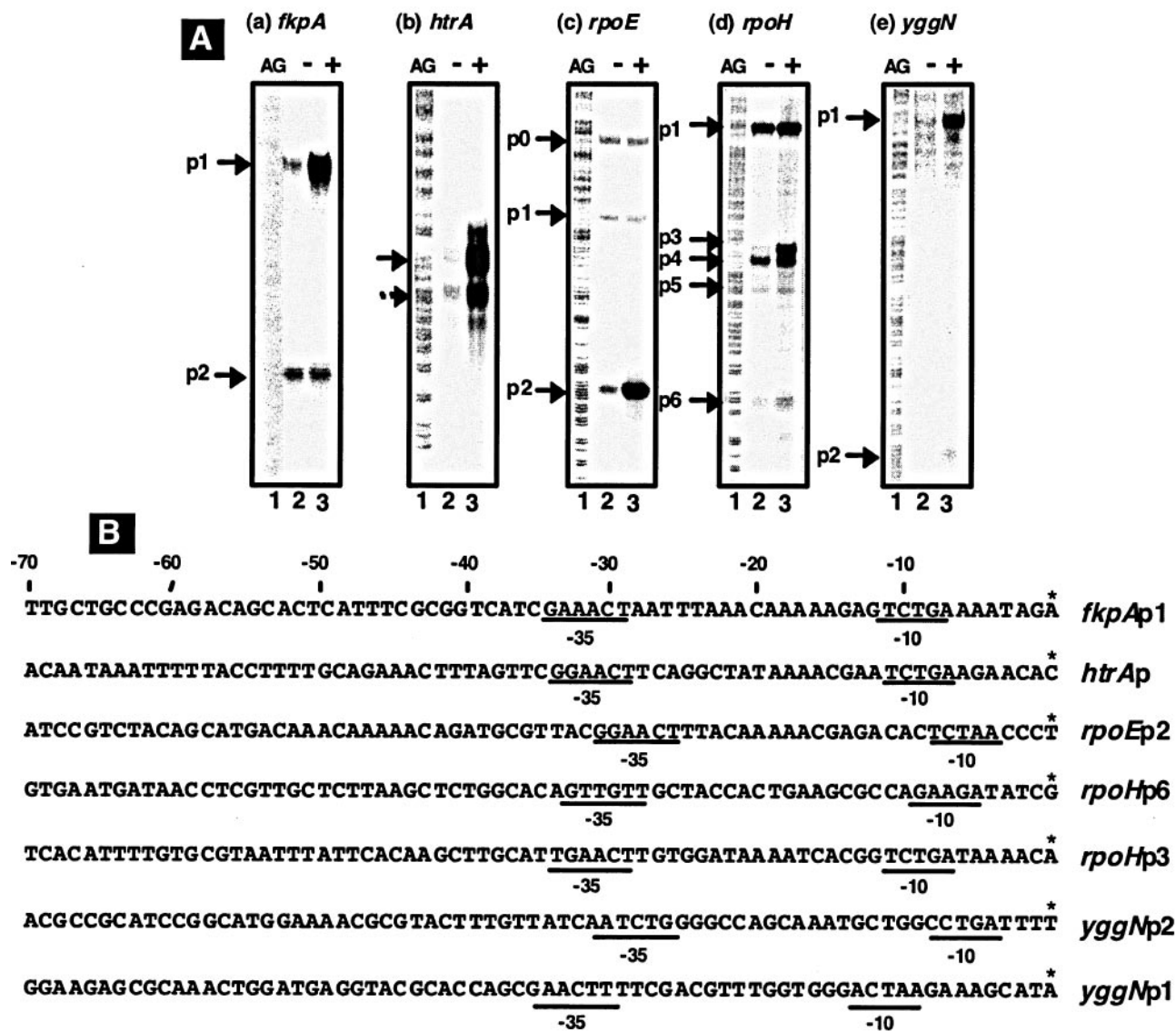


FIG. 4. Zinc-induced activation of the RpoE-dependent genes. [A] *E. coli* W3110 was grown in the absence of  $ZnCl_2$  until exponential phase, and then treated with 0.5 mM  $ZnCl_2$  as in Fig. 3A. After 5 min culture with (lane 2) and without (lane 3)  $ZnCl_2$ , cells were harvested and total RNA was isolated with the hot-phenol method as described for Fig. 1. The S1 nuclease protection assay was carried out with the *fkpA* (a), *htrA* (b), *rpoE* (c), *rpoH* (d), and *yggN* (e) promoter probes as described in Materials and Methods. Lane 1, Maxam-Gilbert AG sequencing reactions. Arrows on the left were promoters which were detected as the bands protected against S1 nuclease. [B] RpoE-dependent and zinc-inducible promoter sequences. The underlines show the conserved promoter -10 and -35 sequences recognized by RpoE (7).

transcription start sites (7). In addition a fast migrating band 5 nucleotides shorter in length [p1' in Fig. 4A(b)] was detected, which might be a degradation product of *htrA* mRNA because both *htrA* p1 and p1' transcripts increased in parallel after zinc addition but p1' lacks the RpoE promoter sequence. In the case of *yggN*, one major band [p1 in Fig. 4A(e)] and a minor band [p2 in Fig. 4A(e)] were detected, both being activated by zinc and both containing the consensus -10 and -35 sequences for RpoE (Fig. 4B).

Both the *rpoH* and *rpoE* genes carry multiple promoters, of which at least one promoter for each gene is known to be under the control of RpoE. S1 mapping indicates that *rpoEp1* and *rpoEp2* were 21 nucleotides downstream and 4 nucleotides

upstream, respectively, from the sites previously identified (27). Besides these two promoters, a new upstream promoter was detected for the *rpoE* gene [p0 in Fig. 4A(c)]. However, zinc activated only the *rpoEp2* promoter, which is known to be under the control of RpoE sigma. The gene for RpoH sigma has been reported to be transcribed from six promoters (27), of which *rpoHp2* is transcribed by RNA polymerase containing RpoE. The locations of three promoters, *rpoHp1*, *rpoHp3*, and *rpoHp4*, were the same as identified previously (9, 10), whereas the initiation sites of *rpoHp5* and *rpoHp6* transcription were 2 nucleotides upstream and 14 nucleotides downstream, respectively, from the reported sites (22). Previously one promoter, *rpoHp3*, was identified to be under the direct control of RpoE.



TABLE 3. RpoE-dependent zinc response genes<sup>a</sup>

Gene	Function	Induction level (fold)
<i>fkpA</i>	Peptidyl prolyl isomerase	1.5–2.0
<i>htrA/degP</i>	Periplasmic protease	>4.0
<i>rseA</i>	Regulator for RpoE	1.5–2.0
<i>rseC</i>	Regulator for RpoE	1.5–2.0
<i>yggN/ecfF</i>	Putative periplasmic protein	1.5–2.0

<sup>a</sup> Genes activated more than 1.5-fold at 5 min after zinc addition were identified by microarray assays, of which the RpoE-dependent genes are listed. Note that other RpoE-dependent genes are also activated, albeit at lower levels.

Our S1 assay, however, indicates significant induction of two promoters, *rpoHp3* and *rpoHp6*, after exposure to zinc [Fig. 4A(d)]. Accordingly the consensus –10 and –35 sequences were identified not only for the *rpoHp3* but also the *rpoHp6* promoter (Fig. 4B). These observations suggest that zinc provides a signal for the induction of the RpoE regulon.

## DISCUSSION

After microarray analysis of the whole set of mRNAs after addition of zinc to *E. coli* cultures, transcription of two groups of genes was found to be significantly stimulated (see Table 2); one group of these genes was for the synthesis of cysteine and another group of genes was organized in the RpoE regulon (see Table 3 and Fig. 3 and 4). Bacteria are directly exposed to frequent changes in the level of external metals, but are believed to maintain homeostasis of intracellular free metals, which are often toxic for cell growth. Upon sudden exposure of *E. coli* to external zinc, the intracellular levels of most zinc-binding proteins do not increase instantly as measured by proteome analysis (data not shown). The induction of the set of genes for cysteine synthesis suggests that cysteine plays a role in transient capture of excess free zinc prior to export through the zinc pump.

Transcription stimulation by zinc was also observed for many members organized in the RpoE regulon (see Table 3 and Fig. 4). Zinc homeostasis is maintained inside *E. coli* cells, but proteins integrated into the cell wall and surface membrane are unavoidably exposed to external metals. Interaction of cell surface proteins with excess metals may lead to denaturation. The abnormal forms of proteins are recognized by refolding systems for repair or protease systems for degradation. The induction of the *rpoE* regulon by zinc agrees well with the fact that the genes encoding the proteins for repair or degradation of abnormal proteins are under the control of RpoE sigma (7). Under normal growth conditions, unused RpoE sigma is stored as a membrane-bound form with anti-RpoE sigma (8, 20). Thus, the anchoring target of RpoE sigma on cell membranes plays a role in sensing zinc level in environment. The CpxRS two-component system is also involved in induction of several enzymes for protein folding (29). Exposure of *E. coli* to excess zinc, however, does not induce the CpxRS regulon genes (data not shown), implying that CpxRS sense a different signal(s) or condition(s) for induction of a protein-folding system.

Brocklehurst and Morby (4) performed microarray assay for *E. coli* strain TG1, which adapted to survive in the continuous presence of external zinc, for identification of the genes in-

involved in iron tolerance. The sets of increased and decreased genes are, however, different from the sets of up- and down-regulated genes in *E. coli* W3110 which was exposed to zinc for a short period (Tables 2 and 3).

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